Histopathology of Human Ischemic Retinopathies

A dissertation presented by

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in fulfilment of the requirements for the degree of

Doctor of Philosophy

Supervised by Professor Marcus Fruttiger

Institute of Ophthalmology,
University College London

October 2020
Declaration

I, Qian Yang, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

I have clearly stated the contribution of others to my thesis, including experiment design, technical assistance, statistical assistance, and any original research work used or reported in my thesis. The content of my thesis is the work I have carried out since the commencement of my PhD degree and does not include a substantial part of work that has been quantified for any other degree in any university or institution.
Acknowledgements

First and foremost, I would like to express my sincerest gratitude to Professor Marcus Fruttiger for his patience, guidance and support throughout my PhD. Importantly, I would like to thank Santen Pharmaceutical Co., Ltd. for generously funding this project.

Many thanks also go to Dr. Sasha Woods for technical help with ISH and sending probes for the ROP project and Dr. Marina Yasvoina for her initial induction into lab techniques. I am grateful for Meaghan, Yanja and Liv being my desk mate and helping with imaging and tissue processing. Thanks to our collaborator in Australia, Meidong Zhu for kindly sending a good number of diabetic eyes which made the DR project more possible.

I would like to give my sincere thanks to my secondary supervisor Professor Adnan Tufail for his support and feedback. Also thank clinicians at Moorfields Eye Hospital, especially Dr. Cathy Egan, Dr. Abraham Olvera and Dr. Tjebo Heeren for constructive feedback on my work.

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Last but not least, I could never thank my family enough for understanding, encouraging and persistently supporting me to do what I wanted to pursue.
Abstract

Retinal ischemia is a key feature in sight threatening eye diseases such as retinopathy of prematurity (ROP) or diabetic retinopathy (DR). Whilst the longterm consequences of ROP and DR are well described, our understanding of the early pathobiological events is much less clear. In particular cellular changes during the early stages of these disease are poorly studied so far. Most of our current insights about the pathobiological events are derived from animal models, with little confirmation in humans. The aim of this thesis is therefore to fill this gap by carefully characterizing vascular features and cellular damage in post-mortem tissue from patients with early stages of ROP and DR.

To better understand the early cellular events in ROP, post-mortem eyes from postnatal, premature infants were collected. Different vascular phenotypes could be distinguished in whole mount retinal vasculature stains. Differences in branching profiles and capillary free zone morphology implicated different levels of oxygen exposure in the infants studied. Furthermore, characterizing a hyperplastic ridge, distal to the edge of the growing vascular plexus, revealed a correlation between the retinal astrocyte marker PAX2 and increased expression of vascular endothelial growth factor (VEGF). This suggests that retinal astrocytes make an important, but so far overlooked, contribution to the pathology in ROP.

To better understand early stages of DR, eyes from diabetic donors without diagnosed DR were collected. Whole mount imaging revealed an indistinguishable retinal vasculature phenotype compared to controls, confirming the absence of DR. However, detailed quantification of vessel profiles on retinal cross sections demonstrated a 5-fold increase in acellular (and presumed non-perfused) capillaries (7-fold in the deeper plexuses) in retinas from diabetics without DR. Interestingly,
localized capillary dropout of individual capillaries in the deeper plexuses did not correlated with a reduction of cells in the vicinity of the non-perfused capillaries. Instead, there was a panretinal loss of cells in the inner nuclear layer (INL) in diabetic retina, suggesting an ischemia independent mechanism for INL cell loss in diabetic retina.
Impact Statement

This thesis established a method which enables researchers to perform experiments on cross sections which can be traced back to original whole mount images. Visualization of retinal vasculature on whole mount resembles clinical ophthalmic imaging technologies and gives an overview of the most up to date vasculature profile before the death of the donor. With this method, different pathological phenotypes can be classified in the absence of clinical imaging records.

Despite mounting clinical imaging studies on the density of retinal vasculature in living diabetic patients, no equivalent work has been done on human post-mortem retina. To our knowledge, this is the first study reporting the following findings: 1) detailed characterisation of retinal vasculature on whole mounts and evaluation of retinal astrocytes on cross sections from retinæ of premature infants at differential postnatal stages. 2) Detailed quantification of capillary dropout at different retinal plexuses in human diabetic post-mortem retinæ. 3) detailed analysis of cell loss in relation to vessel damage in human diabetic post-mortem retinæ.

Studies from this thesis will be published in peer reviewed journals in the field of ROP and DR. A combination of poster and oral presentations have been given at international conferences, for instance Association for Research in Vision and Ophthalmology (ARVO). This will broaden the impact of the current study and allow for multidisciplinary communication with other researchers.

This project is part of the translational pipeline and is sponsored by Santen Pharmaceutical Co., Ltd. Results generated from this thesis will broaden our knowledge in underlying mechanisms of ROP and DR and will provide insights for the development of better treatment and improved quality of life for these patients.
Statement of Others’ Contribution

All data analysis, figure production and writing in this thesis was done entirely by Qian Yang. The work in the chapters of this thesis was performed primarily by Qian Yang with normal supervisorial input from Marcus Fruttiger. Qian Yang’s contribution to each chapter was described as below:

Chapter 1: This chapter was planned, researched and written by Qian Yang.

Chapter 2: This chapter was conceptualized by Qian Yang and Marcus Fruttiger. Experiments were done by Qian Yang at Shenzhen Eye Hospital, Shenzhen, China. Data production and analysis were entirely performed by Qian Yang.

Chapter 3: Dr. Abraham Olvera and Qian Yang contributed equally to the literature review presented in Table 5, Chapter 3. Contribution from others to the chapter has been shown in the table below.

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<td>Marina Yasvoina</td>
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<td>Meaghan O'Neill</td>
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Chapter 4: This chapter was conceptualized by Qian Yang and Marcus Fruttiger. Experiments, data production and analysis were done entirely by Qian Yang.
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<th>Description</th>
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<td>AGE</td>
<td>advanced glycation end-product</td>
</tr>
<tr>
<td>AQP4</td>
<td>aquaporin 4</td>
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<tr>
<td>BCVA</td>
<td>best corrected visual acuity</td>
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<tr>
<td>BM</td>
<td>basement membrane</td>
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<tr>
<td>BRB</td>
<td>blood retina barrier</td>
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<tr>
<td>CBC</td>
<td>cone bipolar cell</td>
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<tr>
<td>CC</td>
<td>choroidal capillary</td>
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<td>CFZ</td>
<td>capillary free zone</td>
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<td>central nervous system</td>
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<td>CRALBP</td>
<td>cellular retinaldehyde-binding protein</td>
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<td>CRYAA</td>
<td>crystallin alpha A</td>
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<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
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<tr>
<td>DVP</td>
<td>deeper vascular complex</td>
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<tr>
<td>DFD</td>
<td>optic disc to fovea distance</td>
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<tr>
<td>DME</td>
<td>diabetic macular edema</td>
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<tr>
<td>DMI</td>
<td>diabetic macular ischemia</td>
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<td>DR</td>
<td>diabetic retinopathy</td>
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<td>ERG</td>
<td>electroretinogram</td>
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<tr>
<td>ETDRS</td>
<td>Early Treatment Diabetic Retinopathy Study</td>
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<td>fluorescein angiography</td>
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<td>inner limiting membrane</td>
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<td>inner nuclear layer</td>
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<td>IPL</td>
<td>inner plexiform layer</td>
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<td>IRMA</td>
<td>intraretinal microvascular abnormality</td>
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<td>mfERG</td>
<td>multifocal electroretinogram</td>
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<td>NND</td>
<td>nearest neighbour distance</td>
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<td>non-proliferative diabetic retinopathy</td>
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<td>outer limiting membrane</td>
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<td>outer nuclear layer</td>
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<td>PDGFRb</td>
<td>platelet-derived growth factor receptor beta</td>
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<td>protein kinase C alpha</td>
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<td>rob bipolar cell</td>
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<td>retinal ganglion cell</td>
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<td>retinal pigment epithelium</td>
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<tr>
<td>SVC</td>
<td>superficial vascular complex</td>
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<tr>
<td>SpO2</td>
<td>oxygen saturation</td>
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<tr>
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<td>type 1 diabetes mellitus</td>
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<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
</tr>
<tr>
<td>UEA</td>
<td>Ulex Europaeus Agglutinin I</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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Chapter 1.

General Introduction
1.1 The Retina

‘ut imago est animi voltus sic indices oculi.’

[the face is a picture of the mind as the eyes are its interpreter.]

This quote from Orator by the Roman philosopher Marcus Tullius Cicero (106-43 BC) is the first to reveal the connection between the eye and brain (Knowles, 2006). Numerous scientific lines of evidence since then support this concept. Developmentally, the retina extends from the embryonic diencephalon, thus is considered an extension of the central nervous system (CNS) (London et al., 2013). Anatomically, the optic disc bundles retinal ganglion axons and sends visual information to higher visual centres in the brain for further analysis. Although the neuroretina is only around 200 to 400 μm thick, every single retinal neuron is processing an unimaginable amount of visual information whilst you are reading this sentence (Grimes et al., 2010).

1.1.1 Organization of the Retina

Nomenclature used to describe the central retina can be confusing, as different terms have been assigned for the same region anatomically and clinically (summarized in Table 1). Corresponding regions are illustrated in Figure 1A.

### Table 1. Definition of clinical and anatomical terms in ophthalmology.

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<tr>
<td>Macula</td>
<td>Circle, diameter of 5.5 mm</td>
<td>Over one layer of ganglion cells</td>
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<tr>
<td>Fovea</td>
<td>Circle, diameter of 1.5 mm</td>
<td>Presence of xanthophyll pigments</td>
</tr>
<tr>
<td>Foveola</td>
<td>Circle, diameter of 0.35 mm</td>
<td>Capillary free and cones only</td>
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Figure 1. Regional specification of the human retina.

A, Photograph showing the position and size of optic disc, macula and fovea of a human retina using anatomical terms. B, Thickness of adult human retina at different eccentricity. The retina is the thickest in the parafovea, where there are the most nuclei in the GCL and a dense capillary network (thick arrows) in the INL. In the foveola, only cone photoreceptors and a few RGCs are present, cone nuclei form a single layer along the OLM (arrowheads) in the para- and peri-fovea. The number of dark-staining rods (long arrow) increases towards the periphery. Peripheral retina is marked by only one layer of RGCs.

Figure modified from https://en.wikipedia.org/wiki/Foveola and Provis et al. (2005).
In the human retina, most colour and useful vision attributes to the macula, which occupies less than 5% of the whole retinal area (Provis et al., 2005). The term “macula lutea” derives from the presence of xanthophyll pigments lutein and zeaxanthin. Locating in the centre of the macula is a small pit called the foveola (Figure 1A), the foveola is completely devoid of vascular networks allowing uninterrupted light transmission to photoreceptors. Although the foveola only occupies 0.02% of the entire retina (Hendrickson, 2005), it has the highest visual resolution thanks to the highest density of cone photoreceptors and less scattering of light by the inner retina.

The fovea and macula are structurally different from the peripheral retina (Figure 1B). At seven month of gestation, the lateral displacement of nuclei in the inner retina starts and gives rise to the perspective foveola, which is only comprised of cones and a few retinal ganglion cells (Hendrickson, 2005; Penfold et al., 2005; Bringmann et al., 2018). Consequently, photoreceptor axons elongate and shift lateral to maintain synaptic connection with bipolar cells (further discussed in Section 1.1.2.1). The layer created by the lateral displacement of the inner retina is called the fibre layer of Henle’s (FH), which is only present in the macula (Bringmann et al., 2018). Due to the absence of FH and thinner GCL and INL, the peripheral retina is thinner (~ 200 μm) than the macula, which can reach over 300 μm in thickness.

1.1.2 Retinal Cells

The earliest description of the retina layers dates back to the early 20th century. Using the Golgi stain, Santiago Ramón y Cajal deciphered that cells in the retina are arranged in a highly ordered, laminated manner (Figure 2). Neurons and glial cells comprise most of the retina. Microglial cells spread sporadically in the inner retina and retinal pigment epithelium lie in between the retina and the Bruch’s membrane.
Figure 2. Santiago Ramón Cajal’s drawing of “Cells in the retina of the eye”.

The ten layers identified by Cajal are currently termed as: A – RPE; B – Photoreceptors; C – OLM; D – ONL; E – OPL; F – INL; G – IPL; H – GCL; I – NFL; J – ILM.

Figure adapted from https://www.nytimes.com/2018/01/18/arts/design/brain-neuroscience-santiago-ramon-y-cajal-grey-gallery.html
1.1.2.1 Neurons

Photoreceptor

Rods (Figure 2b) and cones (Figure 2c) are the photoreceptors in the mammalian retina. There is only one type of rod and three types of cones present (Kolb, 2005a). Rods are highly sensitive to light thus responsible for scotopic vision (night vision). Blue (short wavelength/S), green (medium wavelength/M) and red (long wavelength/L) cones are responsible for the broad spectrum trichromatic vision that humans have in daylight (Molday et al., 2015).

Despite differences in function, rods and cones share similarities in structure. Both are highly compartmentalized in a similar manner and can be divided into five sections: outer segments (OS), connecting cilium (CC), inner segments (IS), nuclear region and synaptic region (Molday et al., 2015). Outer segments contain stacked membranous disks, which are fully packed with photo pigments and related proteins that mediate the initial steps of phototransduction. The connecting cilium connects OS with IS, allowing the essential protein trafficking across two compartments. The IS is the centre of energy and biosynthesis in photoreceptors, it contains an abundant number of organelles, especially mitochondria, endoplasmic reticulum and Golgi complex. Photoreceptors terminate at the synaptic region by forming synapses with bipolar cells and other neurons in the IPL (Kolb, 2005a; Molday et al., 2015).

The first step of visual perception, phototransduction, takes place at the photoreceptor OS. It begins when a photon is absorbed by a photoreactive chromophore, which induces the isomerization of photopigment (Purves et al., 2001). This photoisomerization converts the 11-cis-retina chromophore to all-trans-retinol, which is transported to the RPE to be converted back to 11-cis-retinal and recycled to photoreceptor OS (Lamb et al., 2004), thus completing the visual cycle. Conformational change of the conjugate leads to a cascade of intracellular signalling.
through G-coupled receptors. This process enormously amplifies the original signal (Purves et al., 2001). Consequence of the signalling cascade is the closing of related ion channels, which hyperpolarize the OS membrane and eventually reduce the release of the neurotransmitter glutamate (Purves et al., 2001; Molday et al., 2015).

**Bipolar Cell**

Change in glutamate release from excited photoreceptor leads to signal induction in retinal bipolar cells (Figure 2h), which can be classified as rod or cone bipolar cells according to which photoreceptor they receive major inputs from. It has been estimated that there are 10 types of cone bipolar cells (CBCs) and only one type of rod bipolar cell (RBC) in the primate retina (Boycott et al., 1991). Bipolar cells shape the main ON and OFF visual pathways in the neuroretina.

Under light-adapted condition, RBCs and ON CBCs depolarize as a result of activated metabotropic glutamate receptor (Vardi et al., 2000), which consequently opens transient receptor potential cation channels (Morgans et al., 2009); while OFF CBCs hyperpolarize in response to light through synaptic ionotropic glutamate receptors.

Several markers for retinal bipolar cells have been suggested. RBCs are specifically immunoreactive for protein kinase C alpha (PKCo), which labels the cell membrane of RBCs (de Souza et al., 2016). ON BC can be labelled with Islet-1 and Go-α. All RBCs expressing PKCα immunostained also for Islet-1, but Islet-1 is not expressed by all ON CBCs that are labelled by Go-α (Haverkamp et al., 2003). Guanine nucleotide-binding protein β3 (GNB3) is another candidate marker for ON CBCs, which also labels entire cones (Ritchey et al., 2010; Cuenca et al., 2018). OFF CBCs are widely immunoreactive for glutamate transporter 1 (GLT1) (Haverkamp et al., 2003), other markers such as recoverin (Milam et al., 1993), calbindin and CD15 (Puthussery et al., 2013) have been shown to label a subpopulation of OFF CBCs.
Lateral Interneuron

The radial glutamatergic visual signals are shaped by two lateral inhibitory networks at different synaptic levels. Horizontal and amacrine cells are the key interneurons forming these networks with their cell bodies residing at the INL and projecting to synapses in OPL and IPL, respectively.

Horizontal cells (Figure 2g) contribute to the antagonistic surround of the photoreceptor receptive fields (Baylor et al., 1971). This centre-surround receptive field is a fundamental property for high spatial acuity (Diamond, 2017). Mammalian retina constitutes two to three types of horizontal cells (Kolb, 1974; Boycott et al., 1978), they make synaptic contact with bipolar cells and photoreceptors, which are believed to provide feedforward and feedback inhibition, respectively (Dowling et al., 1966). Three morphologically distinct horizontal cells have been identified in human retina. H1 horizontal cells mainly interact with L/M cone terminals, but also expand axons widely to contact rods; whereas H2 and H3 subtype are specialized to contact S and L/M cones, respectively (Ahnelt et al., 1994). Antibodies against parvalbumin (Nag et al., 1996) and calbindin (Nag et al., 1996; Haverkamp et al., 2003) have been used to detect human horizontal cells, however some suggested that calbindin positive horizontal cells are restricted to the H2 subtype, while parvalbumin is a pan-horizontal cell marker (Wässle et al., 1989).

Amacrine cells (Figure 2i) share some functional similarities with horizontal cells. Despite providing feedback to neurons that drive them\textsuperscript{22}, amacrine cells also make feedforward modulation to GCL and lateral inhibition to neighbouring amacrine cells (Diamond, 2017). The name “amacrine” was coined by Santiago Ramón y Cajal, meaning “without long fibre” (Diamond, 2017). This name pictures the morphological feature of most amacrine cells. Computing synaptic outputs on the same dendrite that also receiving inputs enables amacrine cells to perform multiple lines of early
processing in parallel (Diamond, 2017). Yet large field amacrine cells have been reported with their long processes sending outputs (Kolb, 2005b; Manookin et al., 2015). In fact, amacrine cells are the most diverse neurons in the retina, with over 24 subtypes being identified in human (Kolb et al., 1992). They are marked by their immunoreactivity to inhibitory neurotransmitters GABA or glycine. GABAergic amacrine cells commonly place their cell bodies in the GCL and present long axons in the IPL (de Souza et al., 2012); while glycinergic amacrine cells demonstrate small and brushy dendritic trees (Marc et al., 1985; Menger et al., 1998). Other markers were also used to identify subsets of amacrine cells based on specific neuropeptides they use. For instance, cholinergic amacrine cells can be immunostained with anti-choline acetyltransferase (ChAT) antibody (Rodieck et al., 1992), the enzyme that synthesize the neurotransmitter acetylcholine. Mixed amacrine cells can also be labelled with calbindin (Eliasieh et al., 2007), calretinin (Lee et al., 2016) and parvalbumin (Hendrickson et al., 2007).

**Retinal Ganglion Cell**

RGC, the retina’s projection neuron is the final station of the neuroretina to relay information to higher visual centre. Cell bodies of RGC sit in the GCL, while their axons run along the NFL. Unlike neurons in the brain, the RGCs are not myelinated within the retina. But they are, from the lamina cribrosa outwards.

**1.1.2.2 Retinal glial cell**

Retinal glial cells can be subdivided into astrocytes, Müller cells and microglial cells. Retinal astrocytes are restricted to NFL and are crucial for the development of retinal vasculature, Müller cells expand throughout the retina and perform structural and metabolic support to retinal neurons, whereas microglial cells represent resident immune cell population of the CNS.
Müller Cell

Müller cells (Figure 2n) are the dominant glial cells in the retina, representing 90% of all retinal glia (De Melo Reis et al., 2008). Their cell bodies reside at the INL with projections spanning throughout the retina, from ILM to OLM. In this way, they form a radial scaffold which is essential to stabilize retina structure during development (Willbold et al., 1995).

Müller cells are vital for neural function (Reichenbach et al., 1993). By extending processes enclosing synapses at two plexiform layers (Robinson et al., 1990), Müller cells remove excessive neurotransmitter from the extracellular space to prevent excitotoxicity (Matsui et al., 1999). They possess powerful mechanisms to take up extracellular neurotransmitters, such as glutamate (Bringmann et al., 2013). After being taken up by Müller cells, glutamate is converted to glutamine by glutamine synthetase (GS), which is specifically found in Müller cells (Linser et al., 1979). Inhibition of GS immediately impairs the light response of the retina (Barnett et al., 2000). Glutamine in Müller cells is then either transported intracellularly to mitochondria for hydrolysis (Takatsuna et al., 1994) or is released to neighbouring neurons to aid the synthesis of glutamate (Pow et al., 1996), thus completing the glutamine-glutamate cycle.

Müller cells are also responsible for spatial buffering of extracellular potassium ions (K\(^+\)) and associated water flux. Müller cells are equipped with K\(^+\) channels. Their endfeet are endowed with inward-rectifying potassium channels (Wolfe et al., 1986; Nilius et al., 1988), especially Kir4.1 (Kofuji et al., 2000), which siphons K\(^+\) released by depolarized neurons (Ishii et al., 1997; Tada et al., 1998; Kofuji et al., 2000). Upon uptake by Müller cells, K\(^+\) is shunted through the endfoot processes to the vitreous humour (Newman et al., 1984; Karwoski et al., 1989). Impaired extracellular K\(^+\) regulation is found in diseased retina, where Müller cells membrane potentials are
also affected (Francke et al., 1997). Osmolyte changes will lead to osmotic water flux along the gradient, which is largely regulated by aquaporins (AQP), the selective channels for water molecules. Various types of aquaporins are expressed in the retina (Tenckhoff et al., 2005), amongst all, AQP1 and AQP4 are the most abundantly expressed types (Stamer et al., 1997). In particular, AQP4 is enriched in Müller cell processes and endfeet (Nagelhus et al., 1998; Amann et al., 2016). Given the physical coenrichment of AQP4 and Kir4.1 in Müller cells (Nagelhus et al., 1999), it is suggested that they are also functionally associated to control K\(^+\) concentration together during light responses (Goodyear et al., 2009).

Müller cells actively regulate dilation and constriction of blood vessel in an activity dependent manner, which involves the release of K\(^+\) from endfeet (Reichenbach et al., 2010). Glia evoked vasodilation and vasoconstriction are also mediated by the synthesis of arachidonic acid metabolites EET and 20-HETE, which are closely associated with influx of Ca\(^{2+}\) into the glia (Ye et al., 2002; Metea, 2006). Dilation of retinal capillaries is found to be followed by spontaneous calcium signalling in Müller cells that they are in direct contact (Biesecker et al., 2016). Under pathological conditions, such as diabetes, redistribution of Kir4.1 in Müller cells (Pannicke et al., 2006), faster breakdown of EET and 20-HETE (Hu et al., 2017), and altered calcium signalling (Guerrero-hernandez et al., 2014) are likely to impair control of Müller cells on retinal blood vessels, therefore may induce and/or exacerbate hypoxia.

Akin to brain astrocytes, retinal Müller cells become reactive in response to insult and/or injury, this process is known as reactive gliosis (Sofroniew, 2005), which is marked by upregulation of GFAP (Lewis et al., 2003), thus GFAP has been a widely used marker for Müller cells reactivity. In rodent diabetes models, reactive Müller cells has been widely reported (Barber et al., 2000; Li et al., 2002; Kur et al., 2016). However, this does not seem to be the case in human, where Müller cells in diabetic
retinae without DR seldom upregulate GFAP (unpublished results), although debate still exists (Mizutani et al., 1998). In some diseased retinae, such as proliferative vitreoretinopathy (Eastlake et al., 2016), Müller cells do dramatically upregulate GFAP and become reactive. Müller cells also collaborate with microglia to exert inflammatory function. A recent finding highlighted Müller cells as a primary inflammation sensor and initiator, which recruit microglia to site through CD40 receptor (Portillo et al., 2017).

Equivalent to the blood brain barrier in the brain, the retina has a blood retina barrier (BRB) which protects retinal neurons from direct exposure to circulating cells and molecules in the blood. Tight junctions between endothelial cells limit the diffusion of small molecules from the blood stream (Janzer et al., 1987; Tout et al., 1993).

Astrocyte

Many functional similarities are shared between astrocytes and Müller cells. They both have a role in glycogen storage (Pfeiffer-Guglielmi et al., 2005), ion homeostasis and maintaining the BRB (Ridet et al., 1997), as has been discussed previously. Astrocytes are restricted to the ganglion cell layer and NFL (Stone et al., 1995). Their processes wrap closely around blood vessels to form the inner blood-retina barrier with the superficial vasculature and also expand horizontally in the NFL to form glial investment for the retinal ganglion axons (Stone et al., 1995).

Another important function of retinal astrocytes is driving retinal vascularization during development. They originate from a precursor lineage at the optic nerve head, which expresses the transcription factor Pax2 (Mi et al., 1999; Chu et al., 2001). Some of the astrocytes will then migrate outwards to the periphery and form a mesh network on the inner surface of the retina (Chu et al., 2001). Migrating astrocytes can be identified by platelet derived growth factor receptor alpha (PDGFRA), which is expressed days prior to their invasion into the retina (Mudhar et al., 1993). Moreover,
platelet derived growth factor alpha (PDGFA), the ligand for PDGFRA, is expressed by RGCs (Fruttiger et al., 2000), and is believed to trigger the invasion and migration of the retinal astrocytes during development (Mudhar et al., 1993).

The retinal vasculature is hugely dependent on the distribution of astrocytes. In animals with avascular retina, such as possum, retinal astrocytes are absent (Stone et al., 1987). However, in horse’s retina, which is poorly vascularised, astrocytes only appear in a small vascularised region near the optic disc (Schnitzer, 1987); similarly, in primates, astrocytes exist throughout the retina, except from the avascular fovea centralis (Schnitzer, 1987). This peculiar distribution of astrocytes supports the idea that they enter the retina along with endothelial cells and guide vasculature formation by releasing vascular endothelial growth factor (VEGF) (Watanabe et al., 1988; Stone, Itin, et al., 1995). As the vascular network expands, astrocytes start to mature and upregulate glial fibrillary acidic protein (GFAP) while downregulating VEGF (West et al., 2005). Therefore, in adult human retina, all astrocytes are positive for GFAP and negative for Pax2 as the vasculature has been fully developed, except for some double positive astrocytes in a small region around optic nerve head (Chu et al., 2001).

1.1.2.3 Retinal Immune Cells

Microglial Cells

Histological characterization of microglial cells (Figure 2o) has identified them as a distinct cell type from neurons, vascular cells and other glia in the CNS (Thanos et al., 1996). In the human retina, microglial cells exist widely in the inner retina from the NFL to the INL (Provis et al., 1995). Yet under pathological conditions, such as retinitis pigmentosa, they can invade the ONL (Gupta et al., 2003). Microglia can be categorized into two types according to ontogeny. One originates from microglial precursors entering the retina before vascularization (Diaz-Araya et al., 1995), these cells develop to ramified parenchyma microglia and are immunoreactive for CD45
(pan-leukocyte marker), MHC class I and II but lack macrophage antigens (Provis et al., 1995). The other type of microglia are positive for macrophage antigens CD68, S22, and also CD45, MHC class I and II (Penfold et al., 1991). This type of microglia enters the developing retina via the optic disc (Diaz-Araya et al., 1995) and closely associate with retinal vasculature. They then differentiate into perivascular and para-vascular microglia (Chen et al., 2002).

The concept that microglial cells are a double-sided sword has been widely recognized (Karlstetter et al., 2015). On the one hand, under healthy condition, microglial cells help maintaining tissue integrity, on the other hand they can trigger destructive responses in various retinal diseases (Thanos, 1992; Zhao et al., 2015). Microglia can present ranges of phenotypes thus cannot be tagged “quiescent” or “detrimental” simply based on morphology. Furthermore, studies have shown that activated microglia are neurotoxic and contribute to retinal neovascularization (Fischer et al., 2011) and age related retina diseases (Xu et al., 2009). Such activation has also been reported in post-mortem human retinae with diabetic retinopathy (Zeng et al., 2008).

Professional Immune Cells

Recruitment of monocytes and/or macrophages is also important in various eye diseases, for instance uveitis, in which the resident microglial cells are essential in initiating the infiltration (Okunuki et al., 2019). In experimental retinal vein occlusion studies, infiltrating macrophages were shown to protect the vein endothelial cells against apoptosis (Roubeix et al., 2019). These professional immune cells were also found to recruited under pathological conditions, such as diabetic retinopathy (Zeng et al., 2008).

1.1.2.4 Retinal Pigment Epithelium

RPE (Figure 2a) is a monolayer of cobble-stone shaped cells that form a
boundary between choroidal capillaries (CC) and the neuroretina. RPE is highly polarized, with the apical side forming long microvilli and establishing direct interaction with photoreceptor OS; while the basolateral side facing the Bruch’s membrane functions to separate the RPE from the fenestrated CC (Strauss, 2005).

RPE contains a large amount of melanin pigments, which absorbs lights passing through the neuroretina thus protecting against photo-oxidation and reducing scatter (Strauss, 2005). In addition to its role in aiding the completion of the visual cycle (see page 24), RPE also facilitates daily shedding of photoreceptor OS. Shedding takes place in a diurnal manner (Young, 1960). Shed OSs are directly engulfed by RPE microvilli and degraded by phagolysosomes (Nguyen-Legros et al., 2000).

1.1.3 Retinal Vasculature

The retina is one of the most metabolically active tissue in the body. Cells in the retina are constantly under high oxygen demand. Therefore, two different circulation systems are in place to sustain the retina: the choroidal vasculature to feed the outer one-third of the retina, comprised mainly by photoreceptors; and the retinal vasculature to supply the inner retina (Eshaq et al., 2014).

1.1.3.1 Development of human retinal vasculature

In the human retina, the primary plexus starts to emerge from an existing capillary ring at the optic nerve head around 14-16 weeks gestational age (GA) (Gariano, 2003; Selvam et al., 2017). It penetrates through the NFL/RGCL and spreads towards the periphery in four lobes, each of which represents the territory of a quadratic artery later in the mature retina (Provis, 2001). As the vasculature continues to expand, the nasal superior and inferior lobes fuse along the horizontal meridian, whereas the temporal superior and inferior lobes skirt around the incipient presumptive fovea and
meet at the horizontal axis temporal to the fovea around 25 GA (Provis, 2001). This avascularised area defines the foveal avascular zone (FAZ). Interestingly, the incipient FAZ is devoid of astrocytes (Provis et al., 2000), which fits with the point addressed before that the pattern of astrocytes determines the vasculature.

The deeper plexus starts to develop by angiogenic sprouting from veins of the primary plexus at 25-26 GA (Hughes et al., 2000). Sprouting commences in the vicinity of ONH, where the deeper plexus penetrates down perpendicularly into the INL and forms two laminar layers on either boundary of INL (Gariano et al., 1994). Notably, the development of deeper plexuses is independent of astrocytes. Instead, it is triggered by a transient expression of VEGF in the soma of the neuroglia in the INL (Stone, Itin, et al., 1995). However, in contrast to the primary plexus, little attention has been paid to study the mechanism by which the deeper plexus is induced and guided during development; thus, our understanding of this event is very limited.

By the time the infant reaches full term (40 GA), vascularization by the primary plexus is complete (Gariano et al., 1994; Provis, 2001); yet the deeper plexuses only covers the central ¾ of the retina, as also seen in the adult (Gariano et al., 1994; Provis, 2001). However, under some pathological conditions, the vascularization of the retina can be hugely impeded. For instance, peripheral retinal avascularity is seen in patients with familial exudative vitreoretinopathy (FEVR), an inherited retinal diseased caused by gene mutations in NDP or FZD4 (Kondo et al., 2003; Chamney et al., 2011). At present, there is no clinical data demonstrating the disruption of deeper plexuses in these patients (Gilmour, 2015). But there is evidence that it might be missing in FEVR eyes (Enyedi et al., 1991); similarly, model systems mimicking FEVR by mutating genes that has been validated in cohort studies have revealed the absence of deeper plexus in the retina (Xu et al., 2004; Xia et al., 2008; Beck et al., 2017).
1.1.3.2 Arrangement of vascular plexuses in adult human retina

There are up to four plexuses of vasculature in the retina (Figure 3). The radial peripapillary capillary plexus (RPCP) runs within the NFL (Snodderly et al., 1992; Chan et al., 2012; Tan et al., 2012), which functions to supply the densely packed axons in the peripapillary region (Alterman et al., 1968). The superficial vascular plexus (SVP) is composed of larger arteries, veins and capillaries which mainly supplies the GCL and NFL. There are two deeper capillary networks that originally branched from the SVP, namely the intermedia capillary plexus (INL) and the deep capillary plexus (DCP). During development, the DCP sprouts vertically from SVP down to the lower INL border, ICP then sprouts from the DCP framework to the IPL/INL interface (Usui et al., 2015).

To visualize the ocular vasculature, fluorescein angiography (FA) has been for a long time the dominant approach. It captures the fluorescent signal from intravenously injected dye in the retinal and choroidal vasculature. Currently, most retinal diseases are defined by FA patterns. For instance, BRB breakdown can be easily visualised as extravasated fluorescein leakage. However, ICP and DCP are beyond the detection scope of FA due to blocked fluorescent signal (Weinhaus et al., 1995; Spaide, Klancnik, et al., 2015).

In recent years, optical coherence tomography angiography (OCTA) emerged as a novel imaging technique for the retinal vasculature. It detects blood flow even in terminal capillaries and provides a cross-sectional view of the retina. This technology has been extensively reviewed by Spaide et al., (2018). Unlike FA, OCTA uses motion as an internal contrast, thus injection of dyes is not required. In addition, OCTA can be used for en face visualization of ocular vasculature. Auto segmentation can also be applied to subdivide the retinal vasculature into SCP and DCP according to retinal layers (Figure 3). However, attempts to auto segment have been hindered by flow
projection artefacts (Spaide, Fujimoto, *et al.*, 2015; Spaide *et al.*, 2017). These artefacts originate from the blood flow in the SCP being projected onto the DCP and generating shadows, both of which may be interpreted as flow by the algorithm (Campbell *et al.*, 2017). Because of the same technical obstacle, *en face* images are often not properly segmented (Zhang *et al.*, 2016).

Campbell *et al.* (2017) therefore proposed a new standard nomenclature for retinal vascular networks. They grouped vascular plexus RPCP and SVP into the superficial vascular complex (SVC), ICL and DCP into deep vascular complex (DVC) (*Figure 3*), these criteria will be used in this thesis.

![Human retinal vasculature in the macula.](image)

**Figure 3. Human retinal vasculature in the macula.**

An illustration of the retinal vascular plexuses (labelled on right) on top of a histological section showing anatomic layers (labelled on left) of the human retina. Campbell *et al.* (2017) proposed that two major vascular plexuses can be distinguished: the superficial (SVC) and the deeper vascular complexes (DVC) as shown on the right, while current segmentation criteria divide the SCP and DCP using the IPL/INL interface.

Figure adapted from Campbell *et al.* (2017).
1.1.4 Metabolism of the Retina

1.1.4.1 Warburg Effect

The retina has a considerably high energy demand to keep neurons in an excitable state for neurotransmission and maintain normal cell function. The retina uses energy in a similar way to cancer cells, which is known as the Warburg effect (Ng et al., 2015).

In general, the metabolism of glucose is initiated by glycolysis, a stepwise conversion of one mol glucose to two mols of pyruvate. During this process two mols of adenosine triphosphate (ATP), the energy currency, are produced. Following glycolysis, in presence of oxygen, pyruvate is usually modified and enters the mitochondrial tricarboxylic acid (TCA) cycle to generate 18 mols ATP per mol of pyruvate; this process is called oxidative phosphorylation (OXPHOS). When oxygen is scarce, cells will convert pyruvate into lactate. This will allow glycolysis to continue while maintaining a minimum production of ATP. However, in cancer cells and proliferating cells, the majority of pyruvate (85%) is converted to lactate despite the presence of oxygen, while the rest is metabolized through the OXPHOS. This unusual metabolism is known as aerobic glycolysis or Warburg effect (Heiden et al., 2009; Ng et al., 2015).

Several lines of evidence support this special metabolism in the retina. Noell demonstrated that the retinal function resisted to the effect of anoxia but was highly susceptible to the inhibition of glycolysis despite of normal OXPHOS function (Noell, 1951). In addition, in isolated rat retinae, electrical activity was maintained in anoxic condition, but was affected dramatically when glucose supply was reduced (Winkler, 1972). Furthermore, inducing hyperoxia in the retina had minimal effect on glucose consumption and lactate production (Wang et al., 1997). These substantiated the favor of using Warburg effect to metabolize glucose in the retina.
1.1.4.2 Metabolism at Different Retinal Layers

The inner and outer retina has very distinct metabolic features, with the Warburg effect more prominent in the outer retina, especially in photoreceptors (Wang et al., 1997a). In the pig retina, the ratio of glucose being metabolized via glycolysis and OXPHOS is 1:3 in the inner retina, but is 3:1 in the outer retina (Wang et al., 1997b, 1997a). This finding is in accordance with the different oxygen consumption in vascularized mammalian retina (Yu et al., 2001).

Despite of the favor in metabolizing glucose via OXPHOS, the inner retina is in extremely low oxygen tension and anoxia. In addition, the entire inner retina only consumes 6%, up to 17%, oxygen of that in the outer retina (Yu et al., 2001). Neurons in the inner retina, such as RGCs, are suggested to be very susceptible to hypoxia. Elevated vitreous glucose during induced retinal ischemia had a protective role on RGCs (Casson et al., 2004; Holman et al., 2010). Such effect is possibly due to the ATP produced from glycolytic processes (Han et al., 2013). Müller cells metabolize most glucose (99%) via aerobic glycolysis and the rest via OXPHOS in culture (Winkler et al., 2000). Therefore, Müller cells also have low reliance on the oxygen levels.

Although the adult mammalian retina is not proliferative, it has heavy biosynthesis requirements owing to the fast turnover of photoreceptor outer segments in the outer retina (Casson et al., 2013). A study found that the rhodopsin is constantly shedding and renewing in the mouse, rat and frog retinas (Young, 1967). Such constant turnover creates similar metabolic demands comparable to proliferative tissue.
1.1.5 Gliovascular Unit and Glymphatic System

Retinal gliovascular units are based on functional coupling between retinal glial cells and neurons, which together regulate local blood flow to meet metabolic demands (Duh et al., 2017). The functionality of neurovascular coupling in the gliovascular unit can be shown by the flicker-induced response of the retinal vasculature (Riva et al., 2005). Prior to the appearance of vascular abnormalities, this response of retinal arteries and veins has been attenuated, and decreases progressively with DR severity (Lim et al., 2014). It has therefore been proposed that flicker-induced vasodilation may be used as a predictive marker in early DR (Nguyen et al., 2009).

The retinal gliovascular unit can be easily assessed by transmission electron microscope and/or immunohistochemistry. Figure 4 illustrates the major components of the unit. The vascular part consists of endothelial cells (purple) that are surrounded by pericytes (green). Both lay down an extracellular matrix, commonly referred to as basement membrane (BM, yellow), that is rich in collagen IV. Glial cells endfeet (blue) complete the vascular unit by sending processes around the BM. Retinal astrocytes mainly project to the SVC, and to a lesser extent, the DVC. Whilst Müller cells are responsible for the DVC.

An intact gliovascular unit is critical for normal retinal function. Disruption in the unit has been shown in retinal diseases such as DME, where downregulation of AQP4 is suggested to contribute to the development of oedema (Daruich et al., 2018). A better understanding of the function and pathology of the gliovascular unit is important for the development of preventative and therapeutic strategies.

Similar to the brain, the retina does not have a conventional lymphatic system to clear excessive water and metabolic waste. Due to the presence of inter-endothelial
junction and the absence of AQP1 expression by endothelial cells (Motulsky et al., 2010; Nakada, 2015), water and water soluble material cannot be transported freely across vessels (Cunha-Vaz, 2017). Although AQP4 is expressed by brain astrocytes and retinal glial cell endfeet around blood vessels, it does not allow fluid to cross the BRB, but only to move between interstitial matrix and paravascular spaces (Daruich et al., 2018). Such paravascular drainage is suggested as the glymphatic pathway (Daruich et al., 2018).

In the central retina, the glymphatic pathway is formed between Müller cells and vessels, with drainage being maintained by AQP4. However, expression of AQP4 is downregulated in the fovea of a human diabetic retina (Daruich et al., 2018), and even more significantly reduced in DME fovea (Behar-Cohen et al., 2018). Similarly, in animal models of diabetes, AQP4 (Iandiev et al., 2007) and Kir4.1 (Pannicke et al., 2006) were found redistributed in the retina. Since Kir4.1 is the primary type of potassium channel expressed by the Müller cells to regulate water/ion homeostasis for the DCP, mislocalisation of this protein may reduce perivascular concentration of potassium and eventually lead to poor drainage in the DCP (Longden et al., 2016; Daruich et al., 2018). Clinical observation correlated cysts in DME to areas of reduced flow (vascular void) in the DCP (Mané et al., 2016; Spaide, 2016). These observations may serve as a reasonable explanation for fluid accumulation in the DME that is nonresponsive to either anti-VEGF or anti-inflammatory treatments. However, the existence of the glymphatic system remains controversial and further investigation is required.
Figure 4. Retinal gliovascular unit.

Transmission electron microscopy image highlighted to show major components of the retinal gliovascular unit. Endothelial cells (E, purple) form the lumen (L, pink), through which the blood flows. Basement membrane (BM, yellow) wraps several layers around endothelial cells, in between which lie pericytes (P, green). The retinal vascular unit is also intimately associated with glial endfeet (G, blue).

Figure modified from Powner (2011)
1.2 Retinopathy of Prematurity

Retinopathy of prematurity (ROP) is a vision threatening disease caused by disrupted vasculature growth owing to altered oxygen environment at birth compared to in utero. In the 1940s, the use of oxygen supplementation rescued premature infants, but also laid the stage for a ROP epidemic (Smith et al., 2016). Against this background, limiting oxygen therapy, even to infants with respiratory distress, successfully reduced the occurrence of ROP; however, this was only achieved at the expenses of increased overall mortality rate of premature infants (Avery et al., 1960; Bolton et al., 1974).

A number of large and randomized studies, for instance the BOOST II trial (Stenson et al., 2011, 2013), SUPPORT trial (Finer et al., 2010; Carlo et al., 2015) and the Canadian Oxygen trial (Schmidt et al., 2013), compared the incidence of ROP and survival rates of premature infants (<28 weeks gestational age) supplied with high (90-95%) or low (85-89%) SpO2 oxygen therapy. Results from these trials were analysed in a meta-analysis study, which concluded that the low oxygen decreased the occurrence of severe ROP but was accompanied by a higher mortality; in contrast, the high oxygen group provoked more severe ROP but lowered overall mortality (Saugstad et al., 2013). Based on these results, current European guidelines recommend treating these premature babies with higher SpO2 (90-95%) despite of inducing more ROP cases (Sweet et al., 2019). Even now, seven decades from the initial discovery of ROP, there is no definitive answer as to how to minimize the occurrence of severe ROP without compromising the survival rate.

1.2.1 Clinical Classification

ROP pathology classification distinguishes three zones and five stages. Each of the three zones are concentric circles centring on the optic disc with different radii.
The radius of zone I is twice as optic nerve – maculae distance. Zone II extends peripherally beyond zone I with a radius of the distance from optic disc to the nasal ora serrata. Vascularization extending beyond zone I and II is classified as zone III (Hartnett et al., 2012).

Stages are used to classify severity of ROP. Stage 1 is characterized by the development of a demarcation line, which separates the avascular region from the vascularized region (Figure 5A, arrows). This demarcation line then becomes a ridge, which is elevated and extends above the plane of the retina. The appearance of the ridge marks ROP stage 2 (Figure 5B, arrows). In fact, these early stage lesions are mild and will regress in most cases (Hellström et al., 2013). However, when left untreated and progressed to stage 3 ROP, it is almost unstoppable. Stage 3 is characterised by extraretinal fibrovascular proliferation, thickened ridge and occasional vasoproliferation (Figure 5C). Late stages ROP feature irreversible retinal damage – partial (stage 4, Figure 5D) or total (stage 5) retinal detachment, which, in most cases, can lead to vision loss (An international committee for the classification of retinopathy of Prematurity, 2005).

In addition to original classification, plus disease can also present. This includes, but is not limited to, increased tortuosity and dilation of posterior pole vessels (Figure 5E), which is believed ominous of progressive disease (Hellström et al., 2013).
Figure 5. RetCam and FA images showing ROP at different stages.

**A**, Stage 1 ROP is characterized by the demarcation line (black arrows); **B**, Stage 2 ROP is characterized by the ridge (black arrows); **C**, stage 3 ROP shows thickened ridge (black arrows) with vasoproliferation (white arrow); **D**, Stage 4 ROP shows partial retinal detachment, white arrows point to lase scars. **E**, Fluorescein angiography image of ROP shows tortuous vessels (arrows) and arrested growth of developing vasculature (arrowhead).

Figure **A**, **B** and **D** are adapted from Shah (2016), **C** from (Hartnett *et al.*, 2012). Figure **E** courtesy of Dr. Yuhang Yang, Shenzhen Eye Hospital, China.
1.2.2 Oxygen Induced Retinopathy Model Systems

In the 1950s, a few studies proposed a role of oxygen supplementation in the development of ROP (previously known as retrolental fibroplasia) (Campbell, 1951; Lanman et al., 1954; Kinsey, 1956). This hypothesis was validated by studies from Ashton et al. (1954), in which kittens were exposed to high oxygen and were found to develop vaso-obliteration (Phase I) after a few hours, this was followed by a hypoxia-mediated vasoproliferation (Phase II) on returning to normoxic condition. This set up the foundation of the two-phase hypothesis of ROP, which has been refined and also applies to human ROP (Hartnett et al., 2012). However, studying cell interactions and signalling events in human ROP eyes is virtually impossible. As rodents only complete retinal vascularization postnatally, they serve as useful model systems to study abnormal angiogenesis and molecular mechanisms in response to varying oxygen levels during development. Although OIR models resemble preterm infants in a way that they have incomplete retinal vasculature at birth, they were delivered in a natural and appropriate developmental stage of the species.

Current rodent OIR models are facing several limitations. Firstly, the mouse OIR model exposes seven-day old mice to high oxygen (75%) for five days. This was shown to increase partial pressure of arterial oxygen level (PaO₂) to 500 mm Hg (Penn et al., 1995). However, in real life scenario in human infants, the median PaO₂ was shown to be 100 mm Hg for ROP infants born at extreme low gestational age (GA) on day 1, and less than 0.3% participants exceeded 500 mm Hg in the first three days from birth (Hauspurg et al., 2011). Secondly, the mouse OIR causes vaso-obliteration (Figure 6A) and subsequent neovascularization (Figure 6B), which does not resemble human ROP cases. Lastly, OIR mice are exposed to constant hyperoxia, whereas oxygen levels fluctuate heavily in premature babies. There is a rat OIR model that recreates oxygen level fluctuations by exposing pups to 10% and...
50% oxygen which fluctuates every 24h. This results in a delayed retinal vasculature
development (Figure 6C) followed by vasoproliferation (Figure 6D), which mimics
ROP. Further investigation and application using rat OIR model have been somehow
hindered by difficulties to edit the rat genome. Despite of abovementioned limits,
these OIR models have considerably broadened our understanding of ROP,
especially the vaso-proliferative stage of the disease.
Figure 6. Retinal vasculature changes in rodent OIR model.

In mouse OIR model, pups are exposed in hyperoxia condition from P7 to P12, this cause acute vaso-oblitration at p12 (A) followed by vasoproliferation (B).

Rat OIR model using fluctuating level of oxygen (between 10% and 50%) every 24h for 14 days. This model presented delayed vasculature development at p14 (C). Neovascularization at the vascular/avascular peripheral junction then develops after returning to normoxia (D).

Figures modified from Hartnett and Penn (2012).
1.2.3 Pathogenesis of ROP

1.2.3.1 Birthweight and gestational age

Birthweight and GA are factors reflecting the level of immaturity and low values increases the risk for ROP (Darlow et al., 2005). Low GA means longer exposure of the infant to fluctuated oxygen level when the vasculature is not yet mature, thus more likely to develop ROP (Hellström et al., 2013). Furthermore, low birthweight for a gestational age is usually a readout of in utero restriction. Some suggested that being small for a preterm GA increases the chances of getting ROP (Palmer et al., 1991; Bardin et al., 1997; Dhaliwal et al., 2009), whereas others did not find a clear association (Fortes Filho et al., 2009). Therefore, further work is required to evaluate the importance of in utero restriction in contributing to ROP development.

1.2.3.2 Oxygen Supplementation

Oxygen therapy is shown to be a vital risk factor of ROP (Campbell, 1951; Lanman et al., 1954) and still remains the major one after years of studies (Anderson et al., 2004; Askie et al., 2011). An experimental study showed that exposing mice to 100% hyperoxia for around 1h results in elevated pO$_2$ in retinal arteries, veins and capillaries by around 3 times (Esipova et al., 2018), which can be highly inductive for vessel growth arrest. Therefore the oxygen supplemental treatment must be well controlled in order to balance the need for adequate oxygen delivered to the lung and the risk of ROP (Liegl et al., 2016).

An important study from Castillo et al. (2008) revealed the link between pulse oximetry SpO$_2$ and arterial oxygen level. They showed that babies breathing supplementary oxygen targeting a SpO$_2$ value between 85-93% maintained arterial oxygen levels within 40-80 mm Hg for 86% of the time. However, when the SpO$_2$ target was elevated to 93% or higher, their arterial oxygen level was around 100 mm Hg on average and remained greater than 80 mm Hg for 60% of the time. An old
report from Flynn et al. (1992) also shows that PaO$_2$ greater than 80 mmHg is already sufficient to inhibit normal vascularization and cause damage to the immature retinal capillaries.

Although in the absence of oxygen therapy, premature babies still experience hyperoxic insults immediately after birth. In utero, the PaO$_2$ is around 50 mmHg while that of the ambient air is 160 mmHg (Nicolaides et al., 1989). The induction of oxygen supplementation is only to exaggerate such difference. However, ROP also happens in premature infants not ventilated with high oxygen supplementation and severe ROP occurs in 25-45% infants with threshold ROP at birth (The STOP-ROP Multicenter Study Group, 2000; McGregor et al., 2002) suggests that high oxygen supplementation is neither sufficient nor necessary for ROP development, and there are other factors driving the pathogenesis of ROP.

1.2.3.3 VEGF

The transcription of VEGF is strongly regulated by oxygen tension; where high oxygen (hyperoxia) suppresses VEGF expression and low oxygen (hypoxia) induces it (Claxton et al., 2003; West et al., 2005; Fruttiger, 2007). During development, the avascular regions of the retina is highly hypoxic thus high in VEGF (West et al., 2005). In situ hybridization reveals that VEGF in the avascular retina is mainly expressed by cells at the INL (Stone et al., 1995).

Hypoxia in the avascular retina mainly occurs in the inner retina. In a mouse model which contains a retinal degeneration mutation and where the retina is half the thickness of normal retina, the retina did not become hypoxic in the OIR model because it was thin enough to be supplied by the choroid vasculature. Interestingly, these mice did not develop neovascularization. This means that hypoxia, triggered by metabolic demand of retinal cells, is the main driver of neovascularisation (Scott et al., 2014). Ablating the avascular retina by laser to reduce oxygen consumption and
metabolic need has long been one of the main treatment strategies for ischemic retinopathies (Landers et al., 1982).

VEGF is best known for its key role in angiogenesis (Stone et al., 1995). VEGF gradients promote tip endothelial cells to form filopodia and to sprout (Gerhardt et al., 2003; Eilken et al., 2010) via a complex interplay between the Notch and TGF beta signalling pathways (Wacker et al., 2011). Detailed signalling network have been comprehensively reviewed by Selvam et al. (2017) thus will not be further described here. Disturbed VEGF gradients during vascular development can lead to pathological angiogenesis. Intraocular injection of anti-VEGF agents, such as VEGF trap, into P4 mice leads to less filopodia and reduced sprouting (Rojo Arias et al., 2019), meaning that tip cells mobility is affected. Similarly stimulating the developing vasculature with excessively high concentrations of VEGF, will also cause arrested angiogenesis (Pontes-Quero et al., 2019).

High VEGF at Phase II of ROP progression (mentioned in Section 1.2.2), featured by neovascularization, is suggested to be one of the main driving factors of the disease (Hellström et al., 2013). Tortuous vessels, a feature of plus disease frequently observed in ROP infants, is believed to attributed to high VEGF levels in the vitreous humour (Baum, 1971; Scott et al., 2014). In addition, elevated concentration of VEGF protein in other retinal neovascular diseases, such as DR, has been found in the aqueous and vitreous humour (Aiello et al., 1994; Wu et al., 2017).

1.2.3.4 Retinal Astrocytes

Although the migration of developing vessel is tightly regulated by oxygen and VEGF, the pattern of retinal vasculature is dependent on the template created by retinal astrocytes (Fruttiger et al., 1996; Fruttiger, 2007).

Pax2 is a transcription factor expressed by the retinal astrocyte lineage emerging
from the optic nerve during development, these cells will later differentiate into retinal astrocytes (Chu et al., 2001). It is strongly expressed by immature astrocyte precursors anterior to the developing vasculature; whereas the mature and stellated retinal astrocytes, which are those in close contact with vasculature, strongly express GFAP in addition to Pax2 (Chan-Ling et al., 2004).

Another marker distinguishing the retinal astrocyte lineage from those residing in the optic nerve and CNS is the platelet derived growth factor receptor alpha (PDGFRα), which labels all retinal astrocytes in the developing mouse retina (Fruttiger, 2002, 2007). The ligand of PDGFRα, PDGFA, is expressed by RGCs cell bodies (Fruttiger et al., 2000). Transgenic mice overexpressing PDGFA in RGCs have over-proliferative astrocyte meshwork and proportionally denser vascular networks (Fruttiger et al., 1996). In contrast, ablating PDGFRα in astrocytes completely suppresses, if not at all, retinal vasculature expansion (Tao et al., 2016). These lines of evidence suggest that the pre-existing astrocyte framework formed by immature astrocyte precursors is determinant for the future vascular network.

During development, VEGF is strongly expressed at the NFL by astrocytes (Pierce et al., 1996; West et al., 2005) and acts as the mitogen for the developing endothelial cells. However, deleting VEGF in retinal astrocytes does not halt the growth of retinal vasculature at all (Scott et al., 2010), meaning that VEGF released by retinal astrocytes during development is dispensable, possibly it can be compensated by cells in the INL, which also releases a considerable amount of VEGF (Stone et al., 1995). Previous histological examination on ROP tissues has revealed an accumulation of spindle-shaped nuclei anterior to the arrested developing vasculature (Foos, 1975), most of these cells were later shown to be Pax2+ astrocyte precursors (Sun et al., 2010). However, the mechanism behind this hyperplasia still remains unclear and needs to be elucidated.
1.2.3.5 Fibrosis

The initial name of ROP, “retrolental fibroplasia” (Terry, 1942), reveals that this disease is characterised by the fibrosis behind the lens of the eye. However, we now know that this initial description of ROP corresponds to current advanced ROP (stage 4 and beyond), during which stages the ROP eye presents fibrovascular tissue proliferation, but its mechanism remains unknown.

Hu and colleagues (2012) reported cases with recurrent fibrosis after anti-VEGF monotherapy, which has been a lasting concern in ROP management. They pointed out that although both laser and IVR can reduce ROP progression, IVR is specially associated with risk of fibrotic contraction (Lyu et al., 2019). CTGF is one of the key regulators of fibrosis and is correlated with the degree of fibrosis in vitreoretinal diseases (Kuiper et al., 2006). Therefore, some investigators have proposed that anti-VEGF treatment disrupts the CTGF-VEGF balance in the eye, as a sharp decline in VEGF can cause a temporary shift to CTGF induced fibrosis (Kuiper et al., 2008). In addition to ROP, the importance of fibrosis has been reported in other ocular diseases such as proliferative vitreoretinopathy (PVR). He et al. (2008) proposed that CTGF is a major mediator for fibrosis by showing that CTGF+ stromal cells in human PVR membrane are also immunoreactive to cytokeratin, a marker for fibrotic cells.

1.2.4 Current Treatment for ROP

Current treatment for ROP is not preventative but aims to deal with the vasoproliferation phase using laser (Liegl et al., 2016). The rationale of using laser to treat ROP is based on the hypothesis that neurons in the avascular region are metabolically active and are under high demand for oxygen, both of which are triggering the neovascularization in ROP during phase II, therefore, ablating cells in the avascular regions can rescue such hypoxic insult (Landers et al., 1982; Scott et
al., 2014). The CRYO-ROP group treated premature infants with ROP at stage 3 in one eye, with the other eye being a natural control. Results revealed a beneficial effect of reducing the incidence of blindness by 17% after 10 years (Cryotherapy for Retinopathy of Prematurity Cooperative Group, 2001). Depending on the need for treatment, the ETROP group have further classified ROP into type 1 (requires treatment) and 2 (to follow up), based on the assessment of plus disease (Good et al., 2003; Hellström et al., 2013). This group have also pointed out that early treatment is essential in reducing risks for blindness (Good et al., 2001).
1.3 Diabetic Retinopathy

Diabetes mellitus (DM) is a chronic metabolic disease and has become a growing crisis for human health. DM can be broadly classified into two types, based on the presence (type 1, abbreviated as T1DM) or absence (type 2, abbreviated as T2DM) of deficient insulin production by pancreatic \( \beta \)-cells (American Diabetes Association, 2018). T2DM patients account for 93.6% of total DM cases (Ahlqvist et al., 2018). As of 2017, the estimated incidence of diabetic patients in the United Kingdom is over 4 million, which represents a national incidence of 6.6% (Diabetes UK, 2018). Worse situations have been reported in developing countries. China, for instance, overall prevalence of diabetes has reached almost 11% in 2013 (Wang et al., 2017).

High incidence of DM increases the risk of diabetes complications. Being one of the most common complication, Diabetic retinopathy (DR) affects over 35% of diabetic patients, and 7.24% of them have sight threatening DR (Yau et al., 2012). Benefiting from improvements in diabetes management during the last three decades, the rate of progression to proliferative DR (PDR) has been greatly slowed down. Back in the 1980s, one-year progression from non-proliferative DR (NPDR) to PDR was 52% (Early Treatment Diabetic Retinopathy Study, 1991), whereas current four-year progression rate to PDR from baseline DR is around 11% (Wong et al., 2009; Jones et al., 2012; Medeiros et al., 2015).

1.3.1 Disease Staging and Clinical Manifestation

DR can be broadly categorized into NPDR and PDR stage. So far, classification and severity staging of DR are based on ophthalmoscopically visible signs and ranked stepwise from no retinopathy to PDR. There are four DR classification standards in use, although they differ in nomenclature for different DR stages, considerable overlaps are shared, as reviewed in Table 2.
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<tr>
<td>R0 none</td>
<td>R0 none</td>
<td>No apparent retinopathy</td>
<td>10 none</td>
<td>Microaneurysms and other characteristics absent</td>
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<td>R1 background</td>
<td>R1 mild background</td>
<td>Mild NPDR</td>
<td>20 microaneurysms only</td>
<td>Microaneurysms definite, other characteristics absent</td>
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<td>35 mild NPDR</td>
<td>One or more of the following:</td>
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<td>1. Venous loops ≥ D/1; 2. SE, IRMA, or VB = Q;</td>
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<td>3. Retinal haemorrhages present; 4. HE ≥ D/1; (5) SE ≥ D/1</td>
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<td>R2 preproliferative</td>
<td>R2 moderate BDR</td>
<td>Moderate NPDR</td>
<td>43 moderate NPDR</td>
<td>H/Ma = M/4-5 – S/1 or IRMA = D/1-3 (not both)</td>
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<td>47 moderately severe NPDR</td>
<td>Both L43 characteristics and/or one (only) of the following: 1. IRMA = D/5; (2) H/Ma = S/2-3; (3) VB = D/1</td>
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<td>R3 severe BDR</td>
<td>R3 severe BDR</td>
<td>Severe NPDR</td>
<td>53 severe NPDR</td>
<td>One or more of the following: (1) ≥ 2 of the 3 L47 characteristics; (2) H/Ma ≥ S/4-5; (3) IRMA ≥ M/1; (4) VB ≥ D/2-3</td>
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<td>R3 proliferative</td>
<td>R4 PDR</td>
<td>PDR</td>
<td>61 mild PDR</td>
<td>FPD or FPE present with NVD and NVE absent; or NVE = D</td>
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<td>65 moderate PDR</td>
<td>Either of the following: (1) NVE ≥ M/1 or NVD = D; and VH and PRH = A or Q; (2) VH or PRH = D and NVE &lt; M/1 and NVD absent</td>
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<td>71 high risk PDR</td>
<td>Any of the following: (1) VH or PRH ≥ M/1; (2) NVE ≥ M/1 and VH or PRH ≥ D/1; (3) NVD = 2 and VH or PRH ≥ D/1; (4) NVD ≥ M and VH or PRH ≥ D/1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>81, 85 advanced PDR</td>
<td>NVD = cannot grade, or NVD &lt; D and NVE = cannot grade in ≥ 1 field and absent in all others; and retinal detachment at centre of macula ≤ D, or VH = VS in fields 1 and 2.</td>
</tr>
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Abbreviations: ETDRS, Early Treatment Diabetic Retinopathy Study; AAO, American Academy of Ophthalmology; NSC, National Screening Committee; SDRGS, Scottish Diabetic Retinopathy Grading Scheme; NPDR, Non-proliferative diabetic retinopathy; BDR, background diabetic retinopathy; PDR, proliferative diabetic retinopathy.
retinopathy. HE, hard exudates, SE, soft exudates, IRMA, intraretinal microvascular abnormalities, NPDR, non-proliferative DR, VB, venous beading, (continuing) (continued) H/Ma, haemorrhages/microaneurysms, PDR, proliferative DR, NVE, new vessels elsewhere (> 1 DO from disc), NVD, new vessels disc (within 1 DO of disc margin), FPD, fibrous proliferations disc, FPE, fibrous proliferations elsewhere, VH, vitreous haemorrhage, PRH, preretinal haemorrhage.

*Severity categories for characteristics graded in multiple fields are of the form of “maximum severity/extent”, where maximum severity can be absent (A), questionable (Q), definitely present (D), moderate (M), severe (S), or very severe (VS), and extent is the number of fields at that severity level.

Table summarized from ETDRS (Early Treatment Diabetic Retinopathy Study, 1991) and Diabetic Retinopathy Guidelines (Royal College of Ophthalmologists, 2013).
1.3.1.1 Early DR

Depending on the number and severity of microvascular abnormality in the retina, NPDR can be subdivided into mild, moderate and severe stages. These abnormalities include, but are not limited to: microaneurysms, intraretinal microvascular abnormality (IRMA), venous beading, cotton wool spots and hard exudates.

Early DR is marked by the presence of microaneurysms (Figure 7A and B), which persist in more advanced NPDR (Early Treatment Diabetic Retinopathy Study, 1991). Microaneurysms appear as small saccular outpouchings of the vessel wall. They occur at high incidence in diabetic retina (N Ashton, 1963), most are found in the INL (Stitt et al., 1995; Moore et al., 1999), i.e. originating from ICP and/or DCP. Various cellular components have been described for retinal microaneurysms, such as accumulated red blood cells (Bloodworth Jr. et al., 1965; Stitt et al., 1995) and inflammatory cells (Stitt et al., 1995). Some researchers have suggested that microaneurysms are a form of early neovascularization (G et al., 1976; Aguilar et al., 2003). Pericyte loss and capillary dropout are also related closely with microaneurysms (Ashton, 1963; Stitt et al., 1995). The ratio of leaky microaneurysms versus non-leaky ones has been proposed as an indicator of progression to PDR (Early Treatment Diabetic Retinopathy Study, 1991).

IRMAs (Figure 7C) are believed to be remnant capillaries in areas of vessel occlusion (Kummer et al., 2014). They appear tortuous in shape and dilated compared to normal capillary network. Some have speculated IRMAs to be an early form of neovascularization, however, unlike neovascularization, IRMAs are not leaky under fluorescein angiography. IRMAs are also found abutting cotton wool spots (Figure 7E) (Brownlee et al., 2016), however the reason for that is not yet clear. Several studies have highlighted the significance of IRMA in predicting retinopathy progression (Early Treatment Diabetic Retinopathy Study, 1991; Lee et al., 2017).
Beaded veins have sausage-shaped dilation (Figure 7D), but the pathophysiology is not fully understood. Presence of venous beading is suggested to be closely linked to local ischemia. It is believed to be a powerful indicator of progression to PDR (Early Treatment Diabetic Retinopathy Study, 1991).

Cotton wool spots (or soft exudates) (Figure 7E, arrows) are opaque swellings in the inner retina. They appear as grey or white areas with feathery borders under ophthalmoscopy. Focal retinal arteriole occlusion is believed to be the major cause. Although cotton wool spots are asymptomatic in most cases (Schmidt, 2008), visual defects may occur: small and localized scotomas at the site of cotton wool spots due to regional hypoxia (Bek et al., 1991); while a large arcuate scotoma spanning through the cotton wool spot is more likely to be caused by disrupted signal transmission in axon bundles as a consequence of hypoperfusion (McLeod, 2005; Alencar et al., 2007; Chui et al., 2009). Although they are often seen in the retina and indicate poorly perfused areas, they are not predictive of retinopathy progression (Early Treatment Diabetic Retinopathy Study, 1991).

Hard exudates (Figure 7F) are an accumulation of lipid deposits, appearing with sharply defined edges. They occur mainly around leaky capillaries and/or microaneurysms. High blood cholesterol levels are believed to increase the incidence and severity of hard exudates (Klein et al., 1991; EY et al., 1996). But these are not DR specific phenotypes, they can occur in patients with no diabetes and/or no vascular abnormalities. So far, no literature has reported that hard exudates are associated with DR development and/or progression.
Figure 7. Showcase of DR characteristics from fundus photographs.

A mildest (A) and severe (B) standard for haemorrhages (arrowheads) and microaneurysm (arrows). ETDRS defines any red spot over 125 μm in its longest axis as haemorrhage, unless it presents features such as smooth margins, round shape and central light reflex that is suggestive of microaneurysm. C, more severe standard for IRMAs, which are visible in all quadrants (arrows). Inset shows a zoom-in image of IRMA that is superior and temporal to the macula (arrowheads). D, more severe standard for beaded veins (arrows). In this example, most vein branches, despite their sizes, presents signs of venous beading. E, less severe standards for cotton wool spots (arrows) and IRMA (arrowheads), inset presents closer look of each features. F, severe standard for hard exudates (arrows), they can be arranged as individual dots, patches or partial or complete ring shapes. G, severe new vessels at the disc (NVD). All new vessels within one-disc distance from the disc margin are recognized as NVD, in this case, it occupies an area about 1.5 times that of the disc. H, severe standard for new vessels elsewhere (NVE) (arrowheads) with dilated tips present (arrows).

1.3.1.2 Sight threatening complications

The Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) reported that over 70% diabetic patients developed DR after 10 years of diagnosis (Klein et al., 1995). Based on a population screening study in Liverpool, T1DM patients are at higher risks of DR, especially advanced DR, than T2DM patients at baseline (Younis et al., 2002), possibly attributable to longer diabetes duration.

As mentioned before, NPDR is usually not sight threatening, however, when patients progress to PDR, diabetic macular edema (DME) and/or diabetic macular ischemia (DMI), their vision is under considerable threat. And usually, these complications are usually not mutually exclusive.

Proliferative Diabetic Retinopathy

The presence of new vessels discriminates PDR from NPDR. Such neovascularization is believed to be a consequence of extensive vascular dropout. Depending on location, they can be described as new vessels at the disc (NVD, Figure 7G) or new vessels elsewhere (NVE, Figure 7H). The former usually arise from veins either on the disc or within one-disc diameter. Neovascularization can be distinguished from normal vessels in that they often, if not always, loop back, whereas normal vessels always taper to an end. It has been suggested that any vessel loop should be regarded as a sign of neovascularization (Royal College of Ophthalmologists, 2013). NVE occurs more peripheral than NVD and can sometimes be confused with IRMAs. However, IRMAs tend to occur within areas of extensive vascular dropout, whereas NVE present at the border separating normally and poorly perfused regions. Furthermore, PDR is often, if not always, accompanied with haemorrhage due to damage in the BRB. This can be visualized under FA, and leakage is often seen around neovascularization but not around IRMAs.
Diabetic Macular Oedema

Oedema can occur in two fashions, cytotoxic and vasogenic (Michinaga et al., 2015); or both at the same time. The former is attributed to a disturbed ion balance intra-extracellularly, leading to cell swelling as a result of an inflow of sodium ions and fluid into the cells. This is particularly observed in CNS ischemia (Kahle et al., 2009). The latter is characterized by the breakdown of BRB and/or tight junctions, leading to extracellular accumulation of fluids. In the brain, astrocytes are believed responsible for cerebral oedema (Stokum et al., 2015; Lafrenaye et al., 2019). Similarly, the main glial cells in the retina, Müller cells, are the main player in retinal oedema (Reichenbach et al., 2007). In both cases, pharmacological modification of ion channels and fluid clearance functions may be an effective way of alleviating the oedema and consequent neuronal damages (Bringmann et al., 2005; Lafrenaye et al., 2019).

In the USA, around 1 in every 25 individuals aged over 40 is suffering from DME (Varma et al., 2014). This complication is characterized by thickening of the macula due to abnormal accumulation of fluid in intraretinal cavities, which is commonly referred to as “cysts”; or in the subretinal spaces (Daruich et al., 2018). Visual acuity of DME patient depends mainly on the degree to which the retinal structure has been damaged by oedema. However, the degree of structural disorganization of inner (Sun et al., 2015) and/or outer retina (Otani et al., 2010) may serve as a better predictors of visual loss than retinal thickness (Otani et al., 2010; Sun et al., 2015).

Diabetic Macular Ischemia

DMI is featured as abnormal enlargement of the foveal avascular zone (FAZ), and/or non-perfusion in the macula (Bresnick et al., 1984; Early Treatment Diabetic Retinopathy Study Research Group et al., 1991a; Sim et al., 2013). It is most prevalent in PDR (77.2%) and clinically significant macular oedema (69.4%) (Sim et
Before the emerging OCTA technique, DMI has been ignored in DR epidemiology studies due to the requirement of fluorescein angiography (FA) for assessment (Klein et al., 1992; Varma et al., 2004). Up to now, the underlying mechanism for DMI remains unsolved. Although the NFL was reported to be thinner in DMI patients (Sim et al., 2014), distribution of NFL axons was not interfered in corresponding ischemic region (Sim et al., 2013). Currently there are no effective treatments for DMI, vascular stem cell therapy remains as a potential candidate (Stitt et al., 2011). Interestingly, several small trials pointed out that the presence of DMI as a complication of DME might reduce treatment benefit for the latter (Jonas et al., 2005; Chung et al., 2008), yet such a negative effect failed to replicate in large studies such as ETDRS (Early Treatment Diabetic Retinopathy Study Research Group, 1995) and RESTOR (Mitchell et al., 2011).

1.3.2 Vascular Dysfunction in DR

As aforementioned, current staging criteria for DR is largely, if not entirely, based on assessing the retinal vasculature. Components of the vasculature, endothelial cells, mural cells and basement membrane have all been shown to be altered to some extent in the diabetic environment.

1.3.2.1 Vascular dropout

Vessel dropout can result in ghost vessels, acellular capillaries, or string vessels. This topic has been reviewed extensively by Brown (2010). It is characterised by the appearance of an empty basement membrane shell that lacks inner endothelial lining. Acellular capillaries exist widely in normal human CNS tissues at all ages (Challa et al., 2002), and with increased incidence in pathological conditions such as Alzheimer’s disease (Challa et al., 2004), Parkinson disease (Yang et al., 2015) and ischemia (Lugo-hernandez et al., 2017). A recent study correlating post-mortem
vasculature with FA images from the same patient has revealed that acellular blood vessels are incapable to carry blood flow (Powner et al., 2016), therefore the presence of acellular capillaries can be regarded as an indicator of focal ischemia. Various factors have been suggested to cause endothelial dropout in diabetes, such as hyperglycaemia (Steinle, 2012) and leukostasis (Kim et al., 2005), these factors will be further discussed later in Section 1.3.3.

Two types of mural cells have been classified in the retina, namely smooth muscle cells and pericytes. The former are mainly found around major arterioles and venules, while the latter predominate in terminal arterioles, capillaries and postcapillary venules (Kornfield et al., 2014; Hartmann et al., 2015; Thalgott et al., 2015; Smyth et al., 2018). Pericytes have been extensively studied in diabetes, as they were believed to be the first vascular component affected during early diabetes. Electron microscopy images have revealed that pericytes are embedded within layers of basement membranes (Powner et al., 2011), while their processes can span across several endothelial cells (Trost et al., 2016), which indicates their role in mediating functional coupling of neighbouring endothelial cells (Von Tell et al., 2006). In the absence of pericytes, capillaries are leaky due to the failure of forming interendothelial junctions (Brown, 2010). The density of pericytes varies across tissues, with CNS, particularly the retina, containing the highest pericyte density (Tilton et al., 1985; Frank et al., 1987, 1990) with pericytes covering over 85% of capillary surface in the human retina (Frank et al., 1990).

Loss of pericytes used to be recognized as a primary hallmark of DR (Shepro et al., 1993; Beltramo et al., 2013), but this concept is facing serious challenge. In a monkey model of ischemic DR, Irvine et al. have demonstrated that capillary endothelial cells are preferentially damaged in DR, pericytes loss only occurred as a secondary event (Irvine et al., 1981). In experimental diabetes models, increased
apoptosis of retinal pericytes has been widely reported (Puro, 2007; S. S. Roy et al., 2011; Park et al., 2014), however this remains controversial in human post-mortem diabetic tissue (Li et al., 1997; Nguyen et al., 2018). The effect of mild pericytes loss has also been questioned, as neighbouring pericytes are capable of compensating such loss by extending their processes (Berthiaume et al., 2018). In rodent models, pericytes are usually detected using antibodies against their surface antigens, platelet-derived growth factor receptor beta (PDGFRb), chondroitin sulphate proteoglycan 4 (CSPG4 / NG2) and/or aminopeptidases A and N (CD13) (He et al., 2016; Smyth et al., 2018; Vanlandewijck et al., 2018). In depth study of human retinal pericytes has been hindered greatly by unsuccessful validation of above mentioned pericyte markers in post-mortem human retina.

Basement membrane (BM) thickening is believed to be a fundamental alteration in diabetes and has been widely reported diabetic rodent models (Kozak et al., 1986; Evans et al., 2000), dogs (Stitt et al., 1994) and human (Street et al., 1949; To et al., 2013). In addition to increased synthesis of essential BMs proteins during diabetes (Evans et al., 2000; Tsilibary, 2003), compositional change of BMs has also been identified (To et al., 2013). Major components of BMs are collagen IV, laminin and heparan sulphate proteoglycan (Roy et al., 2010). Fibronectin and tenascin, which were not expressed in the ILM BMs in normal retina, showed up in ILM, large blood vessels and aneurysms in diabetic eyes (To et al., 2013). However, the detrimental effects of thickened retinal BMs during diabetes and whether their prevalence correlates to risk of DR development remained elusive.

1.3.2.2 Consequences of ischemia

One consequence of vascular dropout is local ischemia/hypoxia, this can lead to localized VEGF upregulation (Weis et al., 2005). VEGF plays a crucial role in the development of neovascularization, which is a hallmark of PDR. Numerous studies
have identified significantly elevated VEGF concentration in the vitreous fluid of PDR patients in comparison to controls (Aiello et al., 1994; Burgos et al., 1997; Sydorova et al., 2005; Baharivand et al., 2012). Higher serum VEGF levels in PDR have also been reported (Sydorova et al., 2005; Zehetner et al., 2013). However, others have failed to find as significant difference between DR and non-diabetic patients (Burgos et al., 1997). It has been suggested that a positive correlation exist between vitreous and serum VEGF level (Baharivand et al., 2012), while others found none (Burgos et al., 1997). Therefore, serum VEGF level does not always reflect that in the eye/vitreous fluid. Factors such as glycaemic control (Zehetner et al., 2013), medication and stage of nephropathy (Baharivand et al., 2012) might also alter systemic VEGF concentration. In this sense, proteomics analysis of vitreous fluid would be a better approach to access protein alteration in PDR. Surprisingly, vitreous VEGF fell outside of detection limit of mass spectrometry in several studies (Skeie et al., 2012; Loukovaara et al., 2015; Balaiya et al., 2017), likely due to still low picogram levels even in PDR (Loukovaara et al., 2013; Zehetner et al., 2013).

VEGF levels are also upregulated in vitreous fluid of DME patients (Funatsu et al., 2005; Nguyen et al., 2006), which is not usually marked by ischemia. VEGF plays a crucial role in the development of DME, as intraocular injections of anti-VEGF agent significantly reduce macular thickness and improve vision in a subset of, but not all, DME patients (Nguyen et al., 2006; Agarwal et al., 2015). However, suboptimal response to anti-VEGF treatment suggested that VEGF is not the only contributing factor for the manifestation of DME. Secondary effects, such as inflammation and permeability changes, which happen because of VEGF upregulation might not be sufficiently restrained by anti-VEGF treatments alone.

Ultrastructural studies revealed that endothelial cells exposed to VEGF allow small particles to pass through by the formation of fenestration (Roberts et al., 1995)
or caveolae (Esser et al., 1998). In human diabetic retinas, plasmalemma vesicle associated protein (PLVAP, also known as PAL-E) was detected on retinal endothelia (Schlingemann et al., 1999). PLVAP was extensively expressed on fenestrated vessels (such as the CC) but not intact BRB endothelia (Schlingemann et al., 1999). Notably, PLVAP expression correlates largely with fibronectin and IgG staining, which indicates increased permeability and/or leakage (Schlingemann et al., 1999; Wisniewska-Kruk et al., 2016).

Leakage of larger particles, such as red blood cells, is more likely due to disrupted inter-endothelial junctions (Weis et al., 2004). VEGF can increase barrier permeability by inducing phosphorylation of tight junction proteins (Antonetti et al., 1999) and/or affecting their assembly (Wang et al., 2001). When this happens, serum and plasma protein accumulate in the extracellular space, which lead to detrimental tissue swelling (Weis et al., 2005). On the site of broken vascular barrier, activated platelets are found to adhere to exposed BMs, possibly acting as a plug to reduce vessel patency (Weis et al., 2004). On the other hand, activated platelet also act as sinks of VEGF (Salgado et al., 2001). Accumulation of platelets may occlude the original leaky vessel and further aggravate local ischemia (Weis et al., 2005). Increased platelet aggregation has been observed in vivo in experimental diabetic rodent models (Yamashiro et al., 2003). Proinflammatory molecules mediate the extravasation of leukocytes on the site of platelet activation and the adherence of leukocytes to the vasculature. Recently, adherent leukocytes were shown to trigger endothelial cell apoptosis (Joussen et al., 2003), while injured endothelial cells have been shown to be procoagulant by nature (Bombelli et al., 1997), which in turn enhances leukostasis and accelerates death of endothelial cells.

In experimental diabetic non-human primates, higher numbers of leukocytes were found adjacent to capillary occlusion compared to non-diabetics (Kim et al.,
Moreover, occluding leukocytes have been observed upstream of capillary closure in the DCP (Schröder et al., 1991). Yet, whether occluding leukocytes are initial causative of vascular dropout remains unknown.

1.3.3 Molecular Mechanisms of DR

1.3.3.1 Hyperglycaemia

Hyperglycaemia remains the most widely recognised risk factor for DR. In the Diabetes Control and Complications Trial (DCCT), T1DM patients in the intensive therapy group were treated with three or more daily insulin injections, aimed to achieve normoglycemia; whereas patients in the conventional treatment group were treated to maintain asymptomatic glucose level (The Diabetes Control and Complications Trial Research Group, 1993; Nathan, 2014). Significantly reduced risk of DR development and progression was reported in the intensively controlled group compared to conventional group (The Diabetes Control and Complications Trial Research Group, 1993). A four-year follow-up study involved the same cohort revealed that less patients (10%) in the intensive treatment group had DR worsening than the other (The Diabetes Control and Complications Trial Research Group, 2000). In the long term, strict blood glucose control by intensive therapy had lowered DR progression by 56% in ten years (White et al., 2010). This line of evidence demonstrated that DR development and/or progression could be postponed by well controlled blood glucose levels (Nathan, 2014). In contrast, the 50-year Joslin Medallist study found no association between glycaemic control and reduced microvascular complications in T1DM patients in a bigger study (Keenan et al., 2007). More recent statistical evaluation on DCCT studies have demonstrated that glucose exposure only explains approximately 11% of DR risk (Lachin et al., 2008) and the majority of the risk was accredited to hyperglycaemia independent factors.
Chronic hyperglycaemia was suggested to contribute to DR development via two major pathways. Firstly, the activation of the polyol pathway, which functions to metabolize glucose to sorbitol and eventually fructose (Tarr et al., 2010). The enzyme that facilitates glucose conversion to sorbitol is aldose reductase, whose activity was found to be significantly higher in DR patients than those without (Reddy et al., 2008; Aldebasi et al., 2013). Sorbitol accumulates inside the cell and causes various damage (Tarr et al., 2010). A preclinical study presented a promising role of using aldose reductase inhibitors in preventing microaneurysms and pericytes loss in diabetic animals (Kato et al., 2003). However, subsequent clinical trials have failed to draw significant benefit (Sorbinil Retinopathy Trial Research Group, 1990; Oates, 2008).

Another concerning consequence of hyperglycaemia is the production of advanced glycation end-products (AGEs), which is formed via the Maillard reaction between glucose and certain amino acids. AGEs have been widely reported to be toxic to retinal pericytes and endothelial cells (Chibber et al., 1997; Chen et al., 2006). Accumulation of AGEs in endothelial cells stimulated the production of VEGF, which is a major mediator of neovascularization observed in advanced DR as discussed before (Yamagishi et al., 1997, 2002; Lu et al., 1998; Stitt et al., 2000). Furthermore, inhibiting AGE in experimental models was effective in preventing the development of retinopathy (Hammes et al., 1991; Stitt et al., 2002; Luo et al., 2012). The anti-AGE drug Pimagedine was evaluated in a phase I clinical trial primarily for diabetic nephropathy, but retinopathy status was also monitored by fundus photography. Results showed a beneficial effect of pimagedine in preventing DR progression (Bolton et al., 2004). However, side effects on the immune system and kidney has limited further clinical evaluation (Goh et al., 2008).

1.3.3.2 Inflammation
Emerging evidence has brought up the idea that inflammation is one of the pivotal initiating factors in DR pathogenesis (Joussen et al., 2004; Tang et al., 2011). More specifically, leucocytes are proposed to play a central role by regulating the survival of endothelial cells (Adamis, 2002). In an experimental diabetic model, leucocytes adhered to endothelial cells via adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1). This process is known as leukostasis. ICAM-1, a major adhesion molecule, has been demonstrated to cause endothelial cell death and eventually ischemia (Joussen et al., 2001). These processes can be further enhanced and exacerbated by AGE and its receptors, located on endothelial cells (Schmidt et al., 1995). Moreover, ICAM-1 and VEGF were proposed to work together towards BRB breakdown. Blocking either factors has shown to prevent BRB breakdown (Aiello et al., 1995; Adamis et al., 1996; Qaum et al., 2001; Joussen et al., 2002). However, potential flaw of the leukostasis hypothesis is that, once adhering to the vessel wall, leucocytes will migrate para- or trans-endothelially (Ley et al., 2007), thus only has limited impact on endothelial cells themselves. Therefore, to what extent does leukostasis contribute to the development of DR remains unclear.

Retinal glial cells are proposed to be the major sensor of tissue injury. Müller cells produce higher amounts of inflammatory mediators such as ICAM-1 and nitric oxide (NO) in response to increased glucose concentrations (Du et al., 2011). Microglial activation was also observed in early DR animal models and human eyes (Rungger-Brändle et al., 2000a; Zeng et al., 2008). Upon activation, microglia release pro-inflammatory factors, among which interleukin-1-beta (IL-1β) and tumour necrosis factor-alpha (TNFα) are considered as the most important. Elevated vitreous level of the two are observed in PDR patients (Kocak et al., 2010). An animal knock-out model proved that lacking the IL-1β receptor can prevent a loss of capillaries in diabetic eyes (Vincent et al., 2007). Additionally, IL-1β is known to activate NF-κB, which is a
transcription factor for a large number of inflammation-related genes (Tang et al., 2011). TNFα was shown to increase vascular permeability directly by reducing the expression of tight junction proteins (Aveleira et al., 2010). Intravitreal injection of TNFα induced loss of retinal ganglion cells (Kitaoka et al., 2006).

In animal studies, the nonsteroidal anti-inflammatory drug aspirin prevented acellular capillaries and ischemia in diabetic dogs (Kern et al., 2001). However, aspirin failed to draw any benefits for early DR patients in a clinical trial (Early Treatment Diabetic Retinopathy Study Research Group et al., 1991b). To date, all clinical trials testing corticosteroids aim at treating DME. Intravitreal injection of triamcinolone acetonide (TA) is efficient in reducing hard exudate compared with sham group after a three-month treatment period (Larsson et al., 2009). Nevertheless, visual acuity benefit brought by TA was indistinguishable and impersistent (Gillies et al., 2009; Yilmaz et al., 2009). Moreover, in the long term, TA was inferior to laser treatment in visual acuity improvement, mainly because of the development of cataracts (Diabetic Retinopathy Clinical Research Network, 2008). Concerning short action duration of steroids, intravitreal implants have been designed to achieve a longer duration and sustained release. A three-year study using TA inserts for DME patients revealed that a greater percentage of patients in the TA group had sustained visual improvement over a three-year period. Interestingly, those with at least three-year DME history at baseline seemed to benefit the most from TA inserts (Campochiaro et al., 2012). Another anti-inflammatory corticosteroid implant, dexamethasone, was also tested in DME patients in comparison with the anti-VEGF agent, bevacizumab. The results demonstrated an improvement in visual acuity in both groups at a similar rate, but gradual vision loss was noted in dexamethasone treated patients due to the development of cataracts (Gillies et al., 2014). Taken together, the use of corticosteroid is widely associated with cataract formation, which
hugely affects vision acuity.

### 1.3.3.3 Neurodegeneration

There is strong evidence suggesting that diabetes causes neural and vascular apoptosis, and DR may therefore be regarded as a chronic neurodegenerative disease (reviewed by Barber et al., 2011). Some suggested that retinal dysfunction can be identified at early DR (Feit-Leichman et al., 2005), when microvascular anomalies are not detected (Fortune et al., 1999; Han et al., 2004). This may be attributed to neuron dysfunction and/or cell loss in the neuroretina. Significantly higher numbers of apoptotic cells in the retina whole mount have been identified in diabetic human eyes than in nondiabetics (Barber et al., 1998). RGCs and INL neurons seemed to be the first affected under diabetic conditions. In early diabetes, loss of neuron cell bodies, 10% nuclei in the GCL and 14% in the INL, was detected after inducing diabetes with streptozotocin (STZ), whereas no change could be identified in the ONL. Cell loss in the GCL and INL was accompanied by 22% reduction in retinal thickness of IPL (Barber et al., 1998), which may indicate loss of synapses between lateral inhibitory neurons and RGCs. Another study showed more severe loss of GCL (20%) and INL (50%) cells in STZ-induced diabetic mice after 4 months compared with matched controls (Zeng et al., 2000). Amongst neurons in the INL, amacrine cells appeared to be most vulnerable to diabetes, cumulative loss of which has been reported in diabetic mice (Gastinger et al., 2006). In addition to neuron loss, reduction in RGCs axons has also been identified in diabetic model system (Scott et al., 1986) and human cases (Chihara et al., 1993; Skarf et al., 1997; Carpineto et al., 2016). Progressive loss of RGCs cell bodies and axons have been observed especially in patients with moderate to severe DR (Ng et al., 2016).

These observations solidified the hypothesis that neurodegeneration persist during early diabetes, which is not detectable by clinical imaging tools. However, the
underlying mechanism has not been fully explored. One possibility being that cells in the neuroretina tend to overexpress various proapoptotic markers under diabetic conditions (Abu El-Asrar et al., 2004). This will lead to an imbalance between proapoptotic and survival factors in the retina (Valverde et al., 2013). In human post-mortem studies, major proapoptotic mediators, such as Bim, were upregulated while survival signals remained unchanged (Abu El-Asrar et al., 2004).

Neuroprotective factors are suggested to protect neurons from degeneration induced by various threats (Simó et al., 2014). One of the main neuroprotective factors in the retina, somatostatin (SST), was found to be significantly reduced in early DR neuroretina and RPE in comparison with healthy subjects (Carrasco et al., 2007). Moreover, SST was lowered within the vitreous fluid in patients with advanced DR pathologies compared to control patients, however this was not observed in early stage DR eyes (Carrasco et al., 2007; Simó et al., 2007).

1.3.4 Current Treatment for DR

Currently, only sight threatening DR is treated. PDR is routinely treated with laser photocoagulation. It is suggested that oxygen supply is improved after treatment because the laser burns regionally destroy tissue (Stefánsson, 2001), in particular photoreceptors, which are the major oxygen consuming cell type (Scott et al., 2014). The exact mechanism remains unclear, but it is reasonable to speculate that better oxygenation improves hypoxia and reduces VEGF levels, which is critical to prevent further neovascularization (Evans et al., 2014). Depending on the site of neovascularization, peripheral photocoagulation (PRP) includes hundreds of laser burns in a wide area in the periphery, while focal laser photocoagulation (FLP) applies a small number of burns to individual abnormal vessels or small neovascularization regions in the central retina (Evans et al., 2014).
DME used to be similarly treated with FLP at the macular region, which aims to reduce oedema by slowing fluid leakage. However, FLP has been largely replaced or is used along with anti-VEGF intravitreal injections and/or corticosterone implants. As VEGF plays an important role in inducing BRB breakdown, intravitreal injections of anti-VEGF agents will ideally reduce retinal oedema. Currently three anti-VEGF agents, ranibizumab (Lucentis), aflibercept (Eylea) and bevacizumab (Avastin) have been approved by the FDA for DME treatment (Powers et al., 2017). The first two drugs are also FDA approved for first-line management for DR.

Corticosteroids are mainly used as anti-inflammatory agents to treat DME, they also facilitate re-establishment of the BRB (Tamura et al., 2005). Fluocinolone acetonide, triamcinolone acetonide and dexamethasone are the most used agents. Although eyes treated with these corticosteroids agents have improved BCAV, such benefits were lost when treatment was discontinued (Sutter et al., 2004; Gillies et al., 2009; Pearson et al., 2011). A more concerning fact was that the use of corticosteroids hugely increases the incidence of high IoP and the risk of developing cataract (Diabetic Retinopathy Clinical Research Network, 2008; Campochiaro et al., 2012; Boyer et al., 2014; Gillies et al., 2014).

Besides the abovementioned standard and sophisticated treatments for DR and/or DME, there are have been a number of clinical trials targeting several major DR mechanisms which had produced promising preclinical effects. Most of these trials are reviewed in Appendix A.

As an analogue of somatostatin (SST), an anti-angiogenic agent, octreotide was shown to improve leakage in PDR eyes (McCombe et al., 1991) and laser treated persistent PDR eyes (Boehm et al., 2001). In addition, it reduced the incidence of progression from NPDR to PDR (Grant et al., 2000). However, such beneficial effect was limited in mild DR (Kirkegaard et al., 1990). The mechanism which somatostatin...
and/or octreotide act on is not yet clear. Current body of evidence suggests that they are involved in a number of signalling pathways, including but not limited to, growth hormone (Murray et al., 2004), anti-angiogenesis (Grant et al., 1993) and neuroprotective factors (Hernández et al., 2014), which is discussed briefly in Section 1.3.3.3. In the context of neurodegeneration, a more recent large EUROCONDOR trial (EudraCT no. 2012-001200-38) assessing SST eye drops on early DR patients suggested that it is effective in arresting DR progression in patients presented with some extent of neurodegeneration as revealed by mfERG (EUROCONDOR, 2016).

Attention and efforts have been paid to PKC pathways, which are activated under hyperglycaemic conditions, and believed to signal via the VEGF pathway (Suzuma et al., 2002). Two major clinical studies investigating the efficacy of PKCβ inhibitor, ruboxistaurin, in treating early DR (The PKC-DRS Study Group, 2006) and DME (The PKC-DMES Study Group, 2007) failed to draw any significance of the treatment.

Last but not least, the kallikrein-kinin system (KKS) is a new emerging therapeutic tactic. A few lines of evidence demonstrated its role in DR/DME development. A proteomics study revealed key KKS players in the vitreous sample of advanced DR patients (Kim et al., 2007; Gao et al., 2008). Importantly, activating the KKS pathway can induce vascular permeability via a non-VEGF signalling pathway (Kita et al., 2015). As the efficacy of anti-VEGF treatment is limited in DME, KKS might be a sensible answer to one of the main VEGF-independent pathways in DME (Murakami, 2015). In addition, the main receptors in the KKS, bradykinin B1 (B1R) and B2 (B2R) were shown expressed in human retina and relevant cell lineages (Ma et al., 1996). The expression of B1R was increased in diabetic rats’ retina (Abdouh et al., 2008; Pouliot et al., 2012), especially in vessels at early stages in experimentally induced diabetes (Abdouh et al., 2003). Yet no post-mortem study has shown whether diabetes/DR effects these receptors in the human retina in a similar way.
Thesis Aims

It has been three decades since the original comprehensive classification of ROP and DR. Extensive efforts have been made trying to replicate both diseases in model systems, which, without doubt, has broadened our knowledge about the potential underlying disease mechanisms. However, efforts put in studying model systems have only provided limited insights into clinical practice. Because, after all, ROP and DR is a disease of homo sapiens. Only by studying human tissue, can we validate human disease mechanisms.

Therefore, the aim of this thesis was to investigate vascular and cellular damage in detail in two of the most studied ischemic retinopathies, i.e. ROP and DR, by taking full advantage of the human post-mortem tissues available to us.
Chapter 2.

Influence of Postnatal Oxygen Disruption on Retina
2.1 Introduction

ROP was first described by Terry in the early 1940s as "retrolental fibroplasia" (Terry, 1942), which refers to current end-stage ROP with retinal detachment. It was not until a decade after its initial discovery, that the pathogenesis was suggested to relate to the use of oxygen therapy for premature babies (Campbell, 1951; Lanman et al., 1954). That was when OIR model systems evolved and much attention was paid to understand pathogenesis in these animal models (Ashton et al., 1954). Potential underlying mechanisms have been reviewed in Section 1.2.3 and will not be discussed in further detail here.

However, OIR animal studies in the last seven decades have only provided limited insights into the treatment of human ROP. One of the main reasons is that these model systems do not fully replicate features of human ROP at different stages (Figure 5 and Figure 6). That is to say, only when we understand more of human ROP, will we be able to treat ROP in human effectively.

Our lack of knowledge on human ROP attributes to the lack of relevant tissues available. Since its initial discovery, there have been a very limited number of studies investigating the histology of ROP. In the late 20th century, Robert Foos carried out a series of studies characterising the histological features of human ROP tissues (Foos, 1975, 1985, 1987, 1992), where he first described a hypercellular region at the leading edge of the growing vascular plexus, which he termed as the "ridge" (Foos, 1975). The demarcation line is suggested to be an early consequence of thickened line of growing vasculature (Foos, 1992). Regions anterior and posterior to the demarcation line is thus referred to as "vanguard" and "rearguard", respectively (Foos, 1975). All of these features are hallmarks of current ROP classification standards (see Section 1.2.1).
More recently, Sun et al. (2010) focused on characterising cells at the ridge. They revealed that most cells are PAX2+ astrocytes and some scattered K67+ cells. However, the poor morphology of the tissue hindered further quantitative analysis. More recently, Fernandez et al. (2017) reported on the VEGF expression using immunohistochemistry in anti-VEGF treated and non-treated ROP eyes. They showed that anti-VEGF injection successfully reduced the level of VEGF protein in ROP retina compared with non-treated disease eyes and highlighted a strong expression of VEGF at the vanguard.

However, little is known about what the phenotype is in early disease, i.e. right after oxygen treatment and before the development of ROP, and before it progresses to a more advanced stage. In addition, direct evidence showing the relationship between astrocytes and the vasculature is missing, which is likely to be important because the expansion of vessels is directly mediated by the astrocytes (Fruttiger et al., 1996). To name a few, there are many unresolved questions in the histopathology of human ROP and addressing them could be of great help in understating the driving mechanisms.

Therefore, in this study, a few post-mortem eyes were collected from premature infants who received oxygen treatment and died at different postnatal ages. We performed a detailed characterisation of the retinal vasculature from retinal whole mounts and quantified changes in oxygen related vascular morphometric parameters across tissues. In addition, attention was also paid to how the number of cells at the vanguard changed with distance from the distal vessel. Finally, VEGF related gene expression was assessed using in situ hybridization.
2.2 Materials and Methods

Experiments on eyes collected in China were performed at Shenzhen Eye Hospital, China, during 13th August – 12th December 2019.

2.2.1 Ethical Approval

In this chapter, eyes from premature babies aged from 23- to 26-week GA were collected in Shenzhen, China, between the period of October 2017 to August 2018. Full compliance was adhered, and all required ethical approval was obtained.

To apply for ethical approval, these tissues received full consent for donation to scientific research from the parents within 12 hours of death of the donor. Moreover, this study, entitled “Histopathological study on human post-mortem premature and ROP eyes” (“早产儿视网膜病变组织学研究”), has received ethical approval to use the above-mentioned eyes of premature infants for this study. This approval is granted by the Ethical Committee at Shenzhen Eye Hospital on 5th August 2019 (code: 20190717-02). The final ethical approval document is shown in Appendix B. In preparation for the ethics application, the research proposal was written by Qian Yang and Mingmin Yang. Two ethical committee meetings were organized for this approval.

2.2.2 Tissue Collection and Storage

Four pairs of eyes from premature infants were collected after obtaining formal signed consent from parents within 12h of death. The eyes were immediately fixed in 4% PFA and stored at 4°C until analysis. Information of these premature infants are listed in Table 3.

In addition, one eye from a 21-week GA aborted foetus was collected from UCL Institute of Child Health, Human Developmental Biology Resource (project code: 200455). Experiment on this eye was performed at UCL Institute of Ophthalmology.
In the UK, it was almost impossible to collect tissues older than 21-week GA, and the availability was very limited. Although this eye was not strictly age-matched to the ones collected in China, the fact that it was not exposed to varying level of oxygen postnatally made it an good example to demonstrate the migration of PAX2+ astrocytes during normal retinal development.

### Table 3. Donor information of infant eyes used in current study.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Gender</th>
<th>GA / week + d</th>
<th>Duration to death / d</th>
<th>BW / g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>Male</td>
<td>26 + 1</td>
<td>5</td>
<td>880</td>
</tr>
<tr>
<td>Case 2</td>
<td>Male</td>
<td>25 + 0</td>
<td>3</td>
<td>580</td>
</tr>
<tr>
<td>Case 3</td>
<td>Male</td>
<td>24 + 3</td>
<td>59</td>
<td>780</td>
</tr>
<tr>
<td>Case 4</td>
<td>Male</td>
<td>23 + 6</td>
<td>66</td>
<td>390</td>
</tr>
</tbody>
</table>

Abbreviation: GA, gestational age; BW, birthweight.

#### 2.2.3 Tissue Processing

##### 2.2.3.1 Dissection Strategy

Before dissection, ocular tissues were washed in RNase free PBS and the lens was removed. Eyes were cut open by making four incisions from the conjunctiva to the optic nerve via superior, lateral, medial and inferior rectus muscles, as illustrated in Figure 8, where greens dashed lines show the sites of incisions.

##### 2.2.4 Processing OCT Embedded Tissues

Although paraffin embedded tissues retain good morphology, the size and orientation of the tissue can be greatly limited. Therefore, in this chapter, tissues were embedded and cryosectioned. Briefly, retinal tissues were washed in PBS three times before cryoprotected in 30% (w/v) sucrose in PBS overnight at 4°C. The lobe of interest was cut off and embedded in optimal cutting temperature compound (OCT, Agar Scientific, UK) at -80°C for 20 minutes until the medium solidifies and stored at
-80°C until use. Before sectioning, tissues were warmed up at -20°C for 10 minutes and sectioned at 15µm on a cryostat (Leica, CM1850). Cryosections were collected using Superfrost™ Plus Slides (Thermo Scientific, USA) and air dried at room temperature before stored at -80°C until analysis.

![Diagram of Ocular Tissues](image)

**Figure 8. Illustration of Dissection Strategies of Ocular Tissues**

Lateral and anterior view of the right eye to demonstrate the incisions (green dashed line). Briefly, four cuts from the optic nerve to conjunctiva between the four rectus muscles (superior, inferior, lateral and medial) were made, thus the eyeball could be opened up enabling visualisation of the back of the eye.

### 2.2.5 Immunostaining

#### 2.2.5.1 Whole Mount Staining of Retinal Vasculature

After dissection, eyes were blocked with whole mount blocking buffer (1% FBS, 3% Triton X-100, 0.5% Tween 20 and 0.2% sodium azide in 2X PBS) overnight at 4°C. The next day, the tissue was washed in PBS three times and incubated in rhodamine labelled Ulex Europaeus Agglutinin I (UEA, RL-1062, Vector Laboratories) at 1:500 dilution overnight at 4°C. During imaging, if necessary, RNAseZap (AM9780, Invitrogen, UK) cleaned staples were used to pin the tissue on to a 1.5% agarose gel solidified in a standard petri dish. Tissues were submerged in DEPC treated PBS throughout the imaging process.
2.2.5.2 Immunohistochemistry on Frozen Sections

To prevent tissue detachment during experiment, slides were air dried for 5 minutes and dry baked at 45°C in an oven for 30 minutes. OCT was removed by submerging sections in PBS for 5 minutes. Cryosections were then incubated in immuno blocking buffer (1% bovine serum albumin, 0.3% triton X-100 in PBS) for 1 hour followed by overnight incubation in primary antibody at 4°C.

Antigen retrieval with sodium citrate-EDTA solution (P0086, Beyotime, China) was performed for staining with anti-KI67 and -pax2 antibodies. Slides were submerged into the solution for 10 minutes, during which the temperature was kept between 70-80°C. Slides were then washed gently in three changes of PBS and proceeded to blocking. Primary antibodies used in this chapter were shown below in **Table 4**. (Other antibodies tested, but were not presented, in this thesis are listed in **Table 9**). The next day, slides were washed in PBS and subsequent for 1-hour incubation in secondary antibody at 1:400 and counterstained with hoechst diluent at 1:50,000 for 5 minutes and mounted with mounting media (Fluoro-Gel, Electron Microscopy Sciences, USA).

**Table 4. List of primary antibodies used in Chapter 2.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalogue number</th>
<th>Dilution</th>
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<tr>
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<td>Abcam</td>
<td>ab36595</td>
<td>1:500</td>
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<tr>
<td>CD68</td>
<td>Santa Cruz</td>
<td>sc-20060</td>
<td>1:500</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>Chemicon</td>
<td>AB769</td>
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<tr>
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<td>BioRad</td>
<td>2150-0140</td>
<td>1:500</td>
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<tr>
<td>GFAP</td>
<td>Sigma</td>
<td>C9205</td>
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</tr>
<tr>
<td>MHC-II</td>
<td>Abcam</td>
<td>ab55152</td>
<td>1:250</td>
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<td>Iba1</td>
<td>WAKO</td>
<td>019-19741</td>
<td>1:2000</td>
</tr>
<tr>
<td>KI67</td>
<td>DAKO</td>
<td>GA626</td>
<td>1:200</td>
</tr>
<tr>
<td>PAX2</td>
<td>Novus</td>
<td>H00005076-M01</td>
<td>1:200</td>
</tr>
</tbody>
</table>
2.2.6 In Situ Hybridization

All solutions and equipment used during this protocol were kept Rnase-free by using DEPC (Diethyl pyrocarbonate, Sigma) treated and autoclaved water and PBS throughout the entire procedure.

Chromogenic in situ hybridization was performed with the commercially available ViewRNA™ Tissue Assay (Thermo Fisher Scientific, UK). Slides were air dried for 5 minutes and then dehydrated sequentially in 50%, 75% and 100% ethanol, 10 minutes each and dry baked at 60°C for 1 hour. On-section digestion was performed using protease at 1:100 dilution for 5 minutes at RT and fixed in NBT (10% formaldehyde in PBS) for 5 minutes at RT. Slides were washed in PBS twice before hybridized with probe of interest overnight at 40°C.

The next day, slides were washed in washing buffer (provided in the ViewRNA™ Kit) three times and incubated in Pre-Amplifier diluent at 1:100 dilution for 1 hour at 40°C. After being washed with washing buffer three times, slides were incubated in Amplifier diluent at 1:100 dilution for 30 minutes at 40°C. Subsequently, slides were washed three times in washing buffer and incubated in Label Probe diluent at 1:500 dilution for 1 hour at 40°C and washed three times in washing buffer. Slides were incubated in Tris-NaCl buffer (pH 9.0) at RT for 10 minutes, colour was developed with staining buffer (10% 1M MgCl₂, 0.35% BCIP, 0.54% NBT and 0.2% Tween in 10% PVA) overnight at 40°C. Once desired colour has developed, slides were washed with Tris-NaCl buffer (pH 9.0) on microplate shaker for 30 minutes, mounted with glycerol and sealed with nail polish.

2.2.7 Microscopy

Olympus Axioskope stereo fluorescence microscope (MVX10, Tokyo, Japan) was used for imaging retinal whole mounts and Nikon Eclipse TI-U microscope
equipped with a motorized stage (Prior, HLD117NN/E) was used to image slides. Nikon NIS-Elements V4.50 (Nikon) was used to merge images automatically. Nikon A1 microscope with 63X objective lens was used for confocal imaging.

2.2.8 Branching Analysis of Retinal Arteries

For each measurement, five major arteries per donor were analysed. For the length of the side branch, the distance from branching point from the main artery stem to its next branching point was measured. The width of CFZ was defined as the distance between the main artery and its nearest parallel capillary, over 60 CFZ was measured from each donor. The number of branches per unit length analysis was performed by firstly measure the number of side branches between two branching points along an artery and then divided by the length of that artery segment in calibrated millimetre. To measure the fraction of area being covered by vasculature, twenty 1×1mm² areas were selected randomly per sample, default threshold was used to generate binary images.

2.2.9 Morphometric Analysis of Capillary Free Zones

Capillary free zone is a readout of the level of oxygenation in the blood during development. It refers the enclosed space formed by a main artery, parallel capillary networks and its neighbouring branches. Figure 16A demonstrates how CFZ was measured on a lobe of the ROP tissue (case 4).

For each donor, four to five major arteries were selected and capillary free zones around it were traced manually with FIJI (NIH, RRID: SCR_002285). “Area” and “Shape description” was selected under the “Set Measurement” setting in FIJI software. The following three parameters were displayed for each measurement:

1. “Area” measures the area of selected region in calibrated square millimetre.
2. “Circularity” describes the roundness of the selected region, calculated as:

\[
4\pi \frac{[\text{Area}]}{[\text{Perimeter}]^2}
\]

With value “1.0” indicates a perfect circle.

3. “Aspect Ratio” defines the shape of the region’s fitted ellipse, calculated as [Major Axis] / [Minor Axis] of the fitted ellipse, with value “1.0” indicates a perfect circle.

2.2.10 Statistical Analysis

Datasets are assessed for normal distribution using Shapiro-Wilk test with a significance level of 0.05. If datasets were normally distributed, two tailed Student t-test is performed to compare the means of two groups. Otherwise, nonparametric Mann-Whitney test is used to compare the median and distribution of two datasets.

One-way ANOVA test is used to compare the means of more than two groups. Dunnett’s post hoc analysis is used to compare the mean of control and other groups; Tukey’s post hoc analysis is used compare the mean between any two groups. P values in ANOVA test are adjusted. P value below 0.05 is considered statistically significant. GraphPad Prism (version 8.0, GraphPad Software, San Diego, California, USA) is used for statistical test.
2.3 Results

2.3.1 Altered Retinal Vasculature Network upon Disputed Oxygenation

2.3.1.1 Case 1: A presumptive hyperoxic retina

This donor eye came from an 880-gram male baby born at 26 weeks GA and treated with oxygen for respiratory distress syndrome. The baby was diagnosed of intrauterine pneumonia and neonatal jaundice and died 5 days after birth.

Staining the retinal whole mounts with rhodamine conjugated UEA revealed the endothelial cells of the vasculature. To reveal the situation in a normal developing retina, I used the eye of an aborted baby at 21 weeks’ GA, who was not exposed to fluctuated level of oxygen during/after birth. UEA stained retinal whole mount of this donor presented a normal developing retinal vasculature (Figure 9A). Case 1 appeared to have a typical hyperoxic vascular network, with overly dilated capillary network and large capillary free zones around arteries (arrowheads) (Figure 9B). However, central vessel obliteration, a common pathological feature of OIR rodent models (Figure 6A), was not seen in this case (Appendix C).
2.3.1.2 Case 2: A presumptive hypoxic retina

This eye was donated by a male baby weighted 580 grams and born at 25 weeks’ GA. He received oxygen treatment for respiratory distress syndrome at birth. The baby was diagnosed pneumorrhagia and died 3 days after birth.

Case 2 had lung bleeding after birth, and it was reasonable for us to speculate that deliver of oxygen by the circulating system was reduced, leading to hypoxia in the retina. Under brightfield, an opaque scar mark was seen (Figure 9C), which extend beyond the developing vasculature (Figure 9D). We think this is most likely caused by extensive proliferation of glial cells. The vascular network in this case was overly dense (Figure 9D) compared to the normal retina (Figure 9A), which is in line with our speculation that this eye was severely hypoxic before the baby died. A magnified image outlines the margin of scar (Figure 9E), which overlays well with what can be seen in UEA staining (Figure 9G).
Figure 9. Retinal vasculature of presumptive hyperoxic and hypoxic eyes.

A, A donated eye from a baby aborted at 21 weeks' GA. This normal retina presented developing vasculature that is not exposed to fluctuated oxygen level after delivery. Case 1 presented a considerably dilated vasculature network (B). In contrast, in case 2, the outline of the vascular network could be seen under brightfield (C), which was overly dense as revealed by UEA staining (D). E and F were magnified images of squared region in C and D, respectively. Green dot lines delineated the wedge-shaped gliosis scar marks under brightfield I, which was also presented vaguely in the whole mount image (F). Merging the two revealed a perfect overlay (G). Scale bar 500 μm (A-D) and 100 μm (E-G).

Please see Appendix C and D for full whole mount image of case 1 and 2, respectively.
The developing vascular network is dynamic and undergoes changes such as vascular remodelling. The donors of case 1 and 2 both died within a few days after birth and/or receiving oxygen treatment, thus any vascular malformation may be regarded as response to short-term exposure to disrupted level of oxygen. To reveal the pre-existing vasculatures on whole mounts, I stained the retinal whole mounts using an antibody raised against collagen IV.

In case 1, the presumptive hyperoxic retina, endothelial cell distribution (the “current” vasculature) was sparse (Figure 10A”), whereas collagen IV unravelled a much denser, pre-existing network (Figure 10A’), compared to the normal retina (Figure 9A). This suggests that a considerable number of immature and thin developing capillaries must have been obliterated in response to hyperoxia (Figure 10A-A”, asterisks), but their basement membranes were not yet degraded. Some residual endothelial cells at the front of the developing network could be seen as discontinuous UEA+ dots (Figure 10A and A”, long arrows).

The collagen IV staining of case 2 (Figure 10B’) showed that vessels were thicker than that in case 1 (Figure 10A’), despite loss of endothelial cells (Figure 10B-B”, asterisks). Another conspicuous feature of case 2 was the appearance of hyper-reflective UEA dots/cells in the vessels (Figure 10B and B”, short arrows) The origin of this phenomenon is unknown, but it is possible that the dots are inflammatory cells. In addition, string vessels lacking endothelial lining were also identified bridging two vascular arms (Figure 10B and B’, arrowheads).

Collectively, these results suggest that case 1 was a presumptive hyperoxic retina, marked by loss of capillaries and a modified retinal vasculature topology consistent with hyperoxia exposure. On the contrary, the retinal vascular network presented in case 2 was presumptive hypoxic, characterised by the overly dense and thick capillary bed.
Figure 10. Collagen IV and UEA co-staining shows pre-existing vascular network.

A, Collagen IV staining revealed a much denser, pre-existing vascular network in case 1 (A'); however only some vessels in these network survived (A''), which resulted in discontinuous UEA+ fragments in the front of the developing vasculature (arrows). Small capillaries were obliterated (asterisks).

B, Collagen IV staining revealed a dense vasculature network (B'), diameters of capillaries was wider than that in case 1. Some inter-connecting string vessels (B', arrowheads) were present, which are devoid of endothelial lining (B'', arrowheads)). Note the double layered capillary networks in this case (B'). There were also some hyper-reflective dots residing on/at the vessels (B and B'', short arrows). Scale bar 500 μm in all figures.
2.3.1.3 Case 3: A presumptive early ROP retina

This donor eye came from a 780-gram twin male baby born at 24 weeks plus 3 days GA, who received oxygen treatment for respiratory distress syndrome at birth. This baby was diagnosed of gastrointestinal perforation, intraventricular haemorrhage, necrotizing enterocolitis, neonatal jaundice and sepsis. He died 59 days after birth. It is worth mentioning that this donor was assessed with indirect ophthalmoscopy 5 days before death and revealed no ROP. At the same time, his twin was diagnosed with zone 2 stage 2 ROP in both eyes, which progressed to zone 1 stage 3 with plus diseases in 5 days.

During histological examination I found that the superior retina presented early ROP featured fan-like vascular tufts (Figure 11A and B). This region showed evident hyperaemia, together with moderately elevated and dilated vessels (Figure 11A and B, arrowheads) posterior to the arteriovenous shunt, which was recorded in clinical report (Garoon et al., 1980). Intriguingly, the avascular vanguard region anterior to the vessel shunt appeared opalescent (Figure 11A, asterisks) which presented with a cribriform architecture, marked by many little “holes”. Extending even more anteriorly, these opaque marks acquired a filament-like shape (Figure 11A, long arrows). This area will be referred as “early ROP region” in the following text. In contrast, the inferior part of the retina presented relatively healthy vasculature (Figure 11C and Appendix E), this will be referred as “normal region” from now on.

Taken together, these results presented a premature case, which was initially diagnosed to not have ROP, but appeared to have an early ROP phenotype in our histological analysis. The opalescent and cribriform feature anterior to the developing vasculature were likely to be a consequence of gliosis.
Figure 11. Retinal vasculature of a presumptive early ROP eye.

Brightfield image (A) and whole mount staining using rhodamine labelled UEA (B) showing the early ROP region of case 3. Note that vascular tufts under brightfield (A). Cribriform pattern in the vanguard (asterisks) and extending wedges (arrows) also could be seen in brightfield images (A). Vessel dilation could be visualized in the UEA stained whole mount (B, arrowheads). Nasal side of the retina was relatively normal (C), which presented no identifiable pathological vasculatures. Scale bar 500 μm in all figures.

Please see Appendix E for full whole mount image of this case.
2.3.1.4 Case 4: A diagnosed AP-ROP retina

The ROP eyes were donated by a 390-gram twin male infant delivered at 23 weeks and 6 days GA. He was treated with oxygen for respiratory distress syndrome at birth. This baby was diagnosed with gastrointestinal perforation, gastrointestinal haemorrhage, intraventricular haemorrhage, neonatal jaundice and ventilator-associated pneumonia. He died 65 days after birth.

Eight days before death, i.e. 57 days after birth, this baby was diagnosed with aggressive-posterior ROP (AP-ROP) by a retina specialist at Shenzhen Eye Hospital. RetCam images were acquired on diagnosis (Figure 12), which showed multiple sites of haemorrhages (asterisk) and the appearance of the ridge (long arrows) in both eyes. Tortuous vessels also appeared to suggest the presence of plus disease (Figure 12).

This donor was then treated with ranibizumab (Lucentis; Novartis, Basel, Switzerland) the next day after diagnosis. A dose of 0.3 mg (in 0.03 mL) ranibizumab was injected intravitreally under topical anaesthesia. After one week of receiving the treatment, the donor died of multiple organ failure.

His twin survived but was diagnosed of zone 2 stage 1 ROP in the right eye and zone 2 stage 2 ROP in the left eye at 71 days after birth. No treatment was undertaken for this infant. The latest follow up showed that this twin developed zone 2 stage 1 ROP in both eyes after one month.

The right eye was used for histological examination in this study. Sites of haemorrhages (asterisks) and clear demarcation lines (long arrows) could be visualized under brightfield imaging (Figure 13A). UEA revealed the whole retinal vasculature, which helped identifying the main veins (blue lines) and arteries (red lines) in this retina (Figure 13B). All of these features can be matched to RetCam
images acquired previously (Figure 13C). Intriguingly, it appears that the tortuosity of retinal vessels, namely vessels v1, v2 and a1 in Figure 13C, is less pronounced in the histology, compared to the in vivo imaging, which may be linked to the anti-VEGF treatment (corresponding vessels in Figure 13B).

Looking at a magnified image of the whole mount, haemorrhage appeared as diffuse staining anterior to the advancing vasculature (Figure 14A, C and E, asterisks). An arterio-venous anastomose (AVA) presented right next to an extensive haemorrhage site, with the vein outlined in blue (v) and artery in red (a) (Figure 14A). Clinical FA image also recorded a similar structure in a different ROP infant (B, vessels outlined in a similar manner). Similar to the abovementioned early ROP case, this ROP retina presents fan-like vessel fingers posterior to the haemorrhage, as also commonly seen clinically (Figure 14D). Dilation of the small vessel at the fan was also noted (Figure 14A and C, arrowheads).

However, unlike case 3, this ROP retina did not show arteriovenous shunt; instead, there were protruding vessels anterior to the vascular tufts. Advancing vasculature in this eye was pathological to different extents (Appendix F). The inferior part of the retina (Figure 14E) had a milder phenotype with less haemorrhage (asterisks) but thickening of veins was also visible (arrowheads). Interestingly, sprouting endothelial tip cells appeared at the distal vasculature (long arrows), which was not observed in other three cases. In addition, UEA staining revealed a developed fovea (Figure 14F). It is worthy to note that the central vasculature appeared normal despite of pathological vessel morphologies in the periphery (Figure 14F and Appendix F).
Figure 12. RetCam images of the ROP retinae.

RetCam images were acquired one day before anti-VEGF (Lucentis) treatment, eight days before death. The right eye presents a more severe phenotype than the left eye. Diagnosis of AP-ROP was made mainly based on haemorrhages, blurred edge and tortuous vessels. Arrows point to the ridge, asterisk showing sites of haemorrhage.

Original RetCam images courtesy of Dr. Guoming Zhang and Dr. Mingmin Yang, Shenzhen Eye Hospital, China.
Figure 13. Matching histological and clinical images of the ROP retinae.

A clear ridge (long arrows) and sites of haemorrhages (asterisk) can be recognised in images acquired under brightfield (A), these can be matched to corresponding features seen in the clinical RetCam images (C, asterisks and long arrows). Major arteries (a, red lines) and veins (v, blue lines) revealed from the UEA stained whole mount (B) can be matched accordingly to those on the RetCam images (C, red and blue lines).
Figure 14. UEA stained whole mount image of the ROP retinae.

The retinal vasculature presented arterio-venous anastomose (AVA), defined as direct connection of small arteries (a, red lines) and veins (v, blue lines) (A). B showed a clinical presentation of AVA during FA examination of a different infant. C provided a different view of the fan-like structured vasculature, which is also commonly seen in clinical FA images (D). Region shown in E was marked by less haemorrhages but evident sprouting endothelium tips (long arrows). Note the sites of haemorrhage anterior to the developing vasculature (asterisks) and dilated small vessels (arrowheads) (A-C and E). F revealed the developed fovea in this case. Scale bar 500 μm (A, C, E and F).

Figure B and D courtesy of Dr. Yuhang Yang, Shenzhen Eye Hospital, China.

Please see Appendix F for full whole mount image of this ROP case.
2.3.2 Quantitative Evaluation of Retinal Vasculature

2.3.2.1 Branching profile of retinal arteries

As described in previous sections, all four cases showed a clearly altered vascular network to differential degrees. To investigate how common vascular parameters are altered, I performed quantitative evaluations on these networks.

Oxygen is a regulator of vessel growth, acting through signalling molecules such as VEGF. Since arteries have high oxygen tension, vascular networks around it are sensitive to changes in oxygenation levels. Length of artery side branches (Figure 15A, green lines) and width of the capillary free zone (CFZ) (Figure 15A, purple lines) can therefore be considered as readouts of oxygen levels in the artery. Quantitative results showed that case 1 has statistically significant longer side branches (Figure 15B) (202.50 ± 109.00 vs 87.59 ± 34.06, case 1 vs case 2) and wider CFZ (Figure 15C) (183.70 ± 94.50 vs 97.91 ± 24.12, case 1 vs case 2) than the hypoxic case 2 (p < 0.0001 for both tests). This is probably because, in a high oxygen atmosphere, more oxygen directly dissolves into the plasma (haemoglobin independent) and diffuses further into tissues surrounding arteries, which suppresses expression of VEGF, thus preventing endothelial cell survival and growth close to arteries. Furthermore, in the early ROP case 3 and the ROP case 4, there did not seem to be a big difference in branch length (132.30 ± 62.26 vs 149.20 ± 67.22, case 3 vs case 4, p = 0.012) or CFZ width (114.8 ± 34.54 vs 110.10 ± 23.36, case 3 vs case 4, p = 0.788) (Figure 15B and C). This is possibly because they were at older postnatal stages, meaning that their retinal vessels were more mature and less susceptible to increased oxygenation.

The number of artery branches in a unit distance also reflects the level of oxygen in the tissue, since endothelial cells survival, proliferation and differentiation is strongly regulated by oxygen via the VEGF pathway during development. Results
presented here show that the presumptive hypoxic case 2 (3.99 ± 1.64) had almost
double the number of branches per 1mm artery than in the presumptive hyperoxic
case 1 (2.16 ± 1.30, p < 0.0001) (Figure 15D); whereas difference between the two
older tissues was not significant (3.04 ± 1.66 vs 2.88 ± 2.07, case 3 vs case 4, p =
0.326) (Figure 15D).

Vascular area fraction measures the proportion of the area covered by
vasculature and is a common parameter evaluated in vascular studies to reveal the
level of perfusion (Rust et al., 2019). Random one mm² regions were sampled across
the retina (Figure 15E), images were then binarized with default threshold (Figure
15E’), data showed the fraction of red regions per 1 mm². As expected, results
showed a two-fold higher fraction of vascularised area per unit square in case 2
(hypoxic) (24.22 ± 5.55) than case 1 (11.01 ± 4.04, p < 0.0001). Whereas in older
cases, such difference was not significant (15.09 ± 3.72 vs 17.49 ± 6.11, case 3 vs
case 4, p = 0.121) (Figure 15F).

Taken together, these data indicate that during early postnatal stages of
premature babies, due to their immaturity, retinal vessels are susceptible to be
influenced by altered oxygen levels. However, this effect was not as evident in 2-
month older babies, which is suggestive of a self-rescuing ability of the retina during
development. This is also implied by clinical reports, which state that some ROP
infants can naturally recover and revascularize the peripheral retina without the need
for any treatment (Garoon et al., 1980).
Figure 15. Branching and area fraction analysis of retinal vasculature.

A, Representative image of the ROP retina showing vascular parameters assessed. Quantitative evaluation of length of side branches from arteries (B), width of capillary free zone (C), number of branches per unit length (D) and vascular area fraction (F). E illustrated image processing with ImageJ (Fiji). E’ is an overlay with binarized image (red). Scale bar 1mm (A) and 0.5mm (E and E’). Violin plot shows data distribution, quarterlies (thin lines), and median values (thick lines). Ns, not significant, * P < 0.05, *** P < 0.001.
2.3.2.2 Morphometric analysis of capillary free zone

As mentioned before, CFZ surrounding the arteries are formed due to high oxygen levels during development (Henking et al., 1967), which suppresses the expression of VEGF and consequent growth of endothelial cells. Therefore, CFZ is an important indicator of the level of oxygen circulating in the artery.

Figure 16A shows an example of identifying the CFZ in the vascular plexus (green outlined regions), from which three parameters were measured. First, the CFZ area appeared to be significantly larger by almost 4 times in the presumptive hyperoxic case 1 (0.16 ± 0.15) than in hypoxic case 2 (0.04 ± 0.03, p < 0.0001). The ROP case 4 also presented a slightly larger CFZ than the early ROP case 3 (0.12 ± 0.07 vs 0.77 ± 0.06, p < 0.0001) (Figure 16B). Second, the shape of normal CFZs tends to be rectangular rather than round. Measuring the circularity of CFZs can therefore also be a readout of vascular plexus abnormalities. In this study, this measurement revealed that the CFZ was morphologically rounder (value closer to 1) in hyperoxic case 1 than in hypoxic case 2 (0.54 ± 0.13 vs 0.48 ± 0.13, case 1 vs case 2, p < 0.0001). Early ROP case 3 appeared to have slight more circular CFZs than the ROP case 4 (0.48± 0.16 vs 0.39 ± 0.18, case 3 vs case 4, p < 0.0001) (Figure 16C). The third parameter, the aspect ratio of the CFZ, fits the CFZ into the best-fit ellipse, from which the ratio of its long axis over short axis is calculated, i.e. we can think of it as a width to hight ratio. An aspect ratio of 1 indicates a round CFZ. This analysis showed no significant difference between early (2.28 ± 1.00 vs 2.47 ± 1.02, case 1 vs case 2, p = 0.089) or late case pairs (2.81 ± 1.43 vs 3.01 ± 1.54, case 3 vs case 4, p = 0.255) (Figure 16D).

In conclusion, my morphometric analysis suggests that the CFZs in the hyperoxic retina were considerably enlarged (4 times) compared to hypoxic retina, and in ROP they were doubled compared to early ROP. Morphology wise, a declining trend of
CFZ circularity and increasing elongation was observed from case 1 to 4.

Figure 16. Morphometric analysis of the capillary free zone.

A, Representative image showing the measurement of capillary free zones (green outline areas) around arteries of the ROP eye. Scale bar 1 mm. The area (B), circularity (C) and aspect ratio (D) of the sampled CFZ is further characterised using ImageJ. Bar and whisker show mean ± s.d. Statistical significance is tested by Mann-Whitney test (B-D). ns denotes not significant, *** P < 0.001.
2.3.3 Characterising Cells at the Vanguard

2.3.3.1 Astrocytes

After having qualitatively and quantitatively characterized the retinal vasculature topology in detail, I wanted to further explore the potential mechanisms causing the abnormal vascular features. As previously mentioned, the presence of a pre-existing astrocyte framework is essential for retinal vessel development (Stone et al., 1987). An overly dense astrocyte network (in transgenic mice overexpressing PDGFA) creates a proportionally denser vasculature (Fruttiger et al., 1996). Earlier histological studies in humans have also shown that the vanguard anterior to the developing vasculature is composed of densely compacted spindle-shaped cells (Foos, 1975), which were recently shown to be mostly astrocytes (Sun et al., 2010). However, it remains unanswered as to how much exactly do astrocytes account for the cells in the vanguard? How far do they migrate to the periphery at different ages? And are there any other cells types that reside in the ridge? Therefore, I decided to carefully characterize cells at the vanguard in our cases.

GFAP is one of the standard marker for astrocytes, and specifically labels mature astrocytes in the developing human retina (Chu et al., 2001). GFAP staining on the retina whole mount of the ROP case 4 revealed that the GFAP+ filaments were confined posteriorly to the developing vasculature (Appendix G, Figure 31). This agreed with what we saw in the early postnatal case 1 and 2 (not shown). However, later in the development, in case 3 (Figure 17A), GFAP+ astrocytic processes extended beyond the vessel shunts in the early ROP region of case 3 (Figure 17A′-A″, long arrows) and anteriorly to the most distal vessel in the ROP case 4 (Figure 17B-B″, long arrows). Note that in case 4, due to the extensive haemorrhage, red blood cells, that do not have nuclei, are autoflorescent under the red channel (Figure 17B″, short arrows).
PAX2 is commonly used as a marker for astrocyte precursor lineage merging from the optic nerve head, from which precursor the retinal astrocytes are developed (Chu et al., 2001). During normal retinal vasculature development (Figure 17C), PAX2 staining on a cross section of an eye obtained from the aborted infant revealed that the PAX2+ astrocytes (Figure 17C’) normally migrate around 50 μm ahead of the distal developing vasculature (Figure 17C’’). Magnified confocal image from case 1 (Figure 17D-D’) showed the distribution of PAX2+ astrocyte precursors in the NFL and GCL. In the early ROP regions of case 3 (Figure 17E), we found hyperplasia in the vanguard, most of which were PAX2+ astrocyte precursors (Figure 17E’). With regards to the ROP case 4, a thin layer of continuously spread of PAX2+ precursors was found in the well vascularised central retina (Figure 17F-F’); whereas at the avascular and hyperplastic ridge (Figure 17G-G’) and the vanguard (Figure 17H-H’), there was extensive accumulation of PAX+ astrocyte precursors.

I also quantified the percentage of PAX2+ astrocyte precursors at the vanguard (Figure 17I). In early postnatal case, the fraction was around 60% for both case 1 (58.81%) and 2 (62.2%). This proportion was elevated in older cases. In the normal and early ROP region of case 3, PAX2+ precursors accounted to 70.11 ± 5.53% and 77.79 ± 4.80% of the vanguard cells, respectively. Such intra-tissue difference was not statistically significant (p = 0.228). A similar fraction was seen in the ROP case (78.86 ± 4.70%). One-way ANOVA test revealed no difference in the fraction of PAX2+ astrocyte precursors in the later postnatal tissues (p = 0.274).

To summarise, results presented here show that most cells in the vanguard are PAX2+ and GFAP-, they comprise about 60% in the vanguard tissue. At later postnatal stages, as astrocytes continued to migrate and proliferate, the PAX2+ and GFAP- astrocyte precursors accounted for a higher fraction (about 80%) at the vanguard.
GFAP staining on cross sections of early ROP region in case 3 (A) and ROP case 4 (B) showed mature astrocytes, especially their processes (long arrows). These GFAP positive astrocytic filaments extended only slightly beyond the distal vasculature (A” and B”). In the case 4, there were some red blood cells in the tissue due to haemorrhage in this region, these cells were Hoechst negative (no nuclei) but autofluorescent in the red channel (B’-B”, show arrows).

PAX2 staining showed immature astrocytes in the developing vasculature. C, A cross section from the eye of an aborted baby at 21 weeks GA showed that PAX2 positive astrocytes (C’) migrate anterior to the vasculature (C”). Staining on cross sections from case 1 (D) and 2 (similar, not shown) revealed a slightly thicker layer composed of PAX2 positive astrocytes in the NFL (D’).

The hyperplastic vanguard further thickened in older tissue, such as case 3 (E). In the ROP case, PAX2 positive astrocytes spread sparsely in the well vascularized central regions (F). The vanguard of the ROP tissue was mostly, if not all, composed of PAX2+ immature astrocytes (G), in the avascularised far peripheral retinal region, there was also a considerable number of immature astrocytes (H). Scale bar 50 μm in all figures.

The proportion of the pax2+ immature astrocytes was further quantified by sampling the fraction of these cells from random 200 μm regions in the NFL and GCL (I). Results were presented as mean ± s.d. Statistical significance was tested by Student’s t test and one-way ANOVA with Tukey’s post hoc comparison (I). ns denotes not significant and *P < 0.05
2.3.3.2 Proliferating cells

Anti-KI67 antibody was used to label the nuclei of proliferating cells. Tissues at early postnatal life had a short vanguard and only a few proliferating cells anterior to the developing vasculature (Figure 18A and B, arrowheads). KI67+ cells were also found in the neuroblast layer in case 2 (Figure 18B-B', short arrows). Intriguingly, in the early ROP region of case 3, clusters of KI67+ cells were found to be closely associated with the congested end vessel, which resembles a renal glomerulus on cross section (Figure 18C-C', long arrows). Magnified imaging showed colocalization of KI67+ and spindle-shaped nuclei (Figure 18C, arrowheads), the latter was validated in the previous section to be PAX2+ GFAP- astrocyte precursors (Section 2.3.3.1). However, these proliferating cells were not seen at the NFL of well vascularized region in all cases, Figure 18D-D' is an representative from the ROP case. Furthermore, there were only a few KI67+ cells distributed sparsely at the ridge of the ROP case (Figure 18E-E', arrowheads). At the far vanguard, similar spindle-shaped KI67+ cells were found (Figure 18F-F', arrowheads).

In order to quantitatively demonstrate the trend of KI67+ cell changing with distance, the total number of nuclei and KI67+ cells was counted in every 200 μm bin from the most distal vessel (Figure 18G, curly arrow) to where the vanguard ends. Results showed hyperplasia at the vanguard (Figure 18H, grey box and blue line), which is likely to correspond to the cribriform-like opalescent region identified previously Figure 11A). In addition, the number of KI67+ cells also increased in this region to some extent ($18.50 \pm 6.66$ in the grey box and $11.63 \pm 5.93$ outside the box, $p = 0.064$) (Figure 18H, green line).

In summary, results suggest that a fraction of the previously identified astrocyte precursors are proliferative and accumulate at the hyperplastic region, but the proliferating cells only account for 10-20% of total cells throughout the vanguard.
Figure 18. KI67+ proliferating cells at the vanguard.

Immunohistochemistry with antibody raised against KI67 marks proliferating cells on cross sections. In case 1 (A) and 2 (B), anterior to the distal vessel, there were only a few KI67+ cells at NFL (A' and B', arrowheads). Note the proliferating cells in the neuroblast cell layer (B-B', short arrows). These KI67+ cells at the vanguard were also observed in the early ROP region of case 3 (C, arrowheads). In addition, the distal fan-like vasculature seems to be closely associated with a group of KI67+ cells (C, long arrows). In the ROP case, there was no KI67+ cells in the well vascularized region (D-D'). Surprisingly, only a few KI67+ cells were seen at the ridge (E-E', arrowhead) and in the far periphery (F-F', arrowheads). The UEA and KI67 non-specific background in E' is due to haemorrhage in this region.

G and H, the change of KI67 positive proliferating cells with distance in the early ROP region of case 3. Grey box highlights the manifest hyperplasia. Total number of nuclei in the NFL and GCL, as well as KI67 positive cells (G'), are quantified in every 200 μm bin from the from most distal vessel (G, curly arrow). Scale bar 50 μm (A-C), 25 μm (D-G) and 100 μm (H).
2.3.3.3 Inflammatory cells

Immunohistochemistry with anti-Iba1 antibody was used to identify resident microglial cells, which presented with an amoeboid shape around distal developing vessels in early postnatal tissues (Figure 19A-A’’ and B-B’’, arrowheads). In the early ROP region of case 3, ramified Iba1+ microglial cells were found to be closely associated with the choroidal (Figure 19C-C’, short arrows) and shunted retinal vasculature (Figure 19C-C’’, long arrows). It is worth to point out that Iba1+ cell bodies and processes were found in proximity to the vascular tufts (Figure 19C-C’’, arrowheads), which, as characterised before, also contains GFAP+ astrocytic projections (Figure 17A).

The macrophage marker, CD68, was only identified in the ROP retina (Figure 19D, long arrows) and choroid (Figure 19D, short arrows). Macrophage projections were co-labelled with Iba and CD68 (Figure 19E’ and E’’, arrowheads), and were in spatial proximity of the developing vasculature (Figure 19E’’’, arrowheads). Quantifying changes in the number of these two inflammatory cell types in the ROP retina showed that half of Iba1+ microglial cells at the vanguard were presumed phagocytic (CD68+), both of which peaked at around 1500 μm anterior to the distal vessel (Figure 19F). In addition, other common markers for microglial cells were also investigated. CD14, a mononuclear macrophage marker, is also used to identify macrophages, and was only found around choroidal vasculature (Figure 19G-G’). MHC-II is a marker for a class of antigen presenting microglial cells in the retina. It located sparsely throughout the tissue with no clear association with the developing vasculature at the ridge (Figure 19H-H’).

Collectively, these results present a clear association of Iba1+ retinal microglial with the developing vasculature in all premature eyes, and additional recruitment of CD68+ macrophage in relation to obvious tissue damage in the ROP retina.
Figure 19. Inflammatory cells at the vanguard.

Immunohistochemistry using antibody raised against Iba1 was used to identify all retinal microglial cells. In case 1 (A-A'') and 2 (B-B''), microglial cells present an amoeboïd shape. Only a few microglial cells can be seen at the vanguard and they reside closely to the developing vasculature (arrowheads). In the early ROP region of case 3 (C-C''), a large number of microglia were present. They were closely associated with the distal vasculature (long arrows), some also show up in the near vanguard (arrowheads). Iba1+ microglial cells were also present in the choroid (short arrows).

In the ROP case (D), CD68+ phagocytic microglial cells were seen in the retina (long arrows) and choroid (short arrows). Magnified image showed the colocalization of Iba1 and CD68 in some microglial cells (E', arrowheads), and these cells were spatially associated to the developing vasculature (E''', arrowheads). Quantifying the two types of retinal microglial cells the in cross section of the ROP retina showed their accumulation anterior to the distal vasculature (F). Bin size is 250 μm. Distance at 0 refers to the location of the most distal UEA+ vasculature.

Other markers of inflammatory cells, CD14 (G-G') and MHC-II (H-H') did not show as prominent relation to the vasculature. Scale bar 50 μm in all figures.
2.3.4 VEGF Related Gene Expression in the ROP Retina

Having characterised the cellular compositions at the vanguard of eyes from premature cases using immunohistochemistry, I then wondered how gene expression changes across the retina in these cases, especially hypoxia related ones. However, due to technical difficulties with \textit{in situ} hybridization, I was only able to show results on the ROP case. Consecutive sections were used to examine changes in expression of \textit{VEGF}, \textit{FLT1}, \textit{KDR} and \textit{PAX2} (\textbf{Figure 20}).

\textit{PAX2} was extensively expressed by astrocyte precursors (\textbf{Figure 20A}, hollow arrowhead), but mature astrocytes in the central retina only expressed \textit{PAX2} at a much lower extent (\textbf{Figure 20A}, arrows and insert). In the figure, strong \textit{PAX2} expression starts right at the hyperplastic area in the rearguard and ends about 2.5 mm anterior to the dashed line (\textbf{Figure 20A}, hollow arrowhead), which is spatially close to where high \textit{VEGF} expression finishes in the periphery (\textbf{Figure 20B}, hollow arrowhead). The length of the \textit{PAX2} positive area ahead of the vessels in the aborted eyes considerably shorter (\textbf{Appendix H, Figure 32B}). Furthermore, coinciding \textit{PAX2} and \textit{VEGF} expression from rearguard to the periphery at the NFL suggests that continuous high level of \textit{VEGF} was likely to be released by \textit{PAX2}+ astrocyte precursors.

In addition, \textit{VEGF} receptors, \textit{FLT1} and \textit{KDR}, were highly expressed at the INL by Müller cells at INL and by endothelial cells at NFL (\textbf{Figure 20C and D}, long arrows and inserts) in the vascularised area. In the avascular far periphery, \textit{FLT1} and \textit{KDR} were mainly expressed at the INL (\textbf{Figure 20B-D}, filled arrowheads).

Taken together, results presented here suggest that the \textit{PAX2}+ astrocyte precursors continue to migrate anteriorly to the arrested developing vasculature and release high levels of \textit{VEGF}.
Figure 20. The expression of PAX2, VEGF and its receptors in ROP retina.

Chromogenic *in situ* hybridization using probes against human PAX2, VEGF, FLT1 and KDR genes. Insert demonstrate a zoom-in image of the solid line box in the panorama image. 

PAX2+ astrocytes (A, hollow arrowhead) migrate ahead from where the distal developing vessel finishes (dashed line). Insert also demonstrate low level expression on PAX2 in the vascularize region (A, insert). PAX2+ astrocytes stop at an approximate location of where the high-level expression of VEGF ends (B, hollow arrowhead). Note the strong VEGF expression at the ridge (B). VEGF and its receptors are expressed in the INL throughout the retina (B-D, inserts). FLT1 and KDR are also expressed by the endothelial cells in the vascularized region (C and D, long arrows) and strongly at INL in the avascular region (C and D, filled arrowheads).

Consecutive sections were used. Figures were aligned at the vanguard. Dashed line shows the approximate location of the most distal vessel. Scale bar 500 μm in panorama and 100 μm in inserts.
2.4 Discussion

In this study, we were very fortunate to have access to eyes from premature babies with varying phenotypes. My study presents discoveries in the following three aspects: 1) Qualitative and quantitative characterisation of retinal vasculature topology in post-mortem ROP eyes. 2) Changes in retinal astrocytes and microglial cells regarding to their spatial relation to vessels. 3) \textit{In situ} hybridisation showing changes in gene expression in the ROP retina. These three points will be discussed in detail in the subsequent subsections.

2.4.1 Postnatal Oxygen Treatment Alters Retinal Vasculature

2.4.1.1 Short-term Effect within One-Week Postnatal Life

Case 1 and 2 died within five days after birth, during which time they were under oxygen supplementation. Therefore, these two cases reflect more of “short-term” effect of exposure to high oxygen.

The presumed hyperoxic retina (case 1) showed features of massive capillaries obliteration (\textbf{Figure 10A-A’’}). Similarly, collapse of capillaries has also been described in kitten and premature infants subjected to high oxygen (Ashton, 1966). This can also be recreated in mice (Smith \textit{et al.}, 1994; Claxton \textit{et al.}, 2003). However, in the classic OIR model (\textbf{Figure 6}), capillaries in the centre of the retina are more susceptible to obliteration; whereas in human, the developing vasculature is affected throughout (\textbf{Appendix C}). This could be attributed to the differences in timepoints at which the eye switches from being supported by the hyaloid vasculature to the retinal vasculature. In mice this happens in the first postnatal week, whereas in humans, hyaloid vessels start to regress \textit{in utero} around 13 weeks GA and have completely regressed just before birth (Zhu \textit{et al.}, 2000). This means that in our presumed hyperoxic case 1, where the donor was born at 26 weeks GA, hyaloid vessels had
already started to regress.

In comparison to case 1, case 2 presented an opposite vascular phenotype, which was overly dense, and we postulated it to be hypoxic given the known medical history. *In vitro* experiment exposing mouse embryonic bodies to hypoxia results in formation of a dense capillary-like mesh (Nilsson *et al.*, 2004; Claxton *et al.*, 2005) similar to what we observed in this case (**Figure 9D**).

The morphometric analysis we performed validated that the presumed hyperoxic case 1 had wider CFZ, less branches and less areas covered by vasculature than the presumed hypoxic case 2, which is in accordance with observations made of relevant model systems (Claxton *et al.*, 2003, 2005).

**2.4.1.2 Prolonged Effect after Two-Month Postnatal Life**

Case 3 and 4, collected at later postnatal life, may have gone through a similar phenotype at a previous stage as case 1. However, because the babies survived for two months longer, their central retinal vasculature seems to have normalised (**Appendix E and F**). It is known that the vasculature can remodel during development, but the detailed mechanism is unclear. An early report suggested the role of regulatory T cells in repairing pathological vasculopathy in OIR mice models (Deliyanti *et al.*, 2017). A more recent report also revealed the involvement of innate immune systems, in particular, neutrophils, in repairing or remodelling vasculature following damage in OIR model systems (Binet *et al.*, 2020). However, this concept has not been validated in human postmortem tissues.

The region in case 3 with early ROP features is characterised by the fan-like vascular tufts, which appear to be the precursors of arteriovenous shunts and neovascular tufts. In some cases these tufts will regress and eventually vascularize the peripheral retina in a normal manner (Garoon *et al.*, 1980). No sign of
angiogenesis could be visualized at these tufts in case 3 (Figure 11B). In contrast, actively sprouting vessels could be observed at the distal edge of the vasculature in the active ROP case (Figure 14E). It has been revealed that the growth of vascular sprouts is dependent on a gradient of VEGF acting on VEGFR2 (also known as KDR) on endothelial cells (Gerhardt et al., 2003). Our in situ hybridization results confirmed the expression of KDR by Müller cells, but not so much by endothelial cells (Figure 20D), whereas FLT1 clearly labelled both cell types (Figure 20C). Similar KDR expression was also observed from my previous in situ hybridization (Appendix H, Figure 32C). Reasons for lower KDR expression in vasculature during retina development could be that the section happened to go through a vasculature-thin region; or the staining on Müller cells developed so fast and intense that I stopped the colour reaction too early before the vasculature staining developed; or that KDR-related regulation comes later in the development than FLT1. In either case, this experiment will need repeating to validate my findings.

It is worth mentioning that we could not observe in any of our cases the deeper plexus of the retinal vasculature, which should start developing from 21-week GA in human. It may be the case that the development of the deeper vasculature was delayed due to the exposure to high oxygen. Absence of the deeper plexus agrees with results from others on retinae from kittens exposed to hyperoxia (Ashton et al., 1954) and OIR mice (Stahl et al., 2010).

2.4.2 Cells at the Vanguard

2.4.2.1 Spindle-shaped cells are astrocyte precursors

Ashton (1966) postulated that spindle-shaped cells ahead of the growing vasculature are mesenchymal cells. However, Foos (1992) argued that spindle cells are negative for desmin, GFAP or factor VIII, suggesting a different identity of these
cells other than mesenchymal cells or endothelial cells. In addition, Fruttiger (2002) presented that in mice, cells anterior to developing retinal vasculature are entirely, if not all, PDGFRα+ retinal astrocytes. My current study provides another line of evidence, showing that most spindle-shaped cells are PAX2 positive and therefore retinal astrocyte precursors (Figure 17).

2.4.2.2 Genesis of Astrocyte Precursor Hyperplasia

With regard to hypercellularity at the vanguard initially reported by others (Foos, 1975; Sun et al., 2010) and replicated in the current study (Figure 18), a key study from West et al. (2005) provided insights into the possible genesis of this feature. They suggested that the avascular areas are highly hypoxic and high in VEGF, this will stimulate proliferation of astrocytes whilst inhibiting their maturation. As retinal astrocytes migrate into the avascular region, they develop a template for endothelial cells to grow upon. Reciprocally, the developing vessels exert a negative feedback on the proliferation of retinal astrocytes whilst inducing their maturation, thus limiting the number of astrocytes (West et al., 2005).

Under pathological conditions, however, oxygen treatment given to premature babies will create a larger non-hypoxic area around vessels, especially arteries (Claxton et al., 2003; West et al., 2005). Although hyperoxia does not significantly affect the speed of astrocyte migration (Zhang et al., 1999), they receive less negative feedback from the arrested vasculature. My study underpins this model two-fold. Firstly, GFAP+ mature astrocytes were never seen far away from the developing vasculature in the current thesis (Figure 17) nor in other studies (Fruttiger, 2002; Gerhardt et al., 2003). Because they need the feedback from the vasculature to maturate (West et al., 2005). Secondly, the opaque cribriform patterned gliosis in the vanguard I observed, which was also reported by Foos (1985), could be explained by a lack of a differentiation signal from the blood vessels (Figure 11A). However, based
on this hypothesis, astrocytes precursors at far periphery should proliferate even more extensively than those closer to the vasculature. Yet I observed the hyperplasia to be most severe between 500 μm to 1500 μm from the distal vasculature, and then decreasing gradually towards the periphery (Figure 18G-H). Therefore, a so far unknown factor is also likely to contribute to the retinal astrocyte hyperplasia.

Interestingly, this hyperplasia of astrocyte precursors had never been replicated in the classic OIR models (Bucher et al., 2013; Lajko et al., 2016; Duan et al., 2019). However, continuous exposure of mouse pups to 65% oxygen from P0 for P7 appears to reproduce astrogliosis at least partially (McMenamin et al., 2016). Unpublished data from our group has also confirmed this finding. More recently, a preprint from (Perelli et al., 2019) showed that exposing mouse pups from P0 to P4 can induce hypercellularity of Sox9+ astrocytes at P8. However, to what extent this neonatal OIR model relates to human ROP currently remains unknown and detailed characterisation is still needed.

2.4.2.3 Elongated Astrocyte-Endothelium Margin

The astrocyte-endothelium margin is significantly elongated in eyes of premature infants. For instance, case 2 is marked by the opacified band extending slightly anterior to the developing vasculature (Figure 9E-G). Foos (1975) recorded similar observations, where he viewed it as a consequence of the proliferative response of cells in the vanguard region, which we know know are the astrocyte precursors. Chu et al. (2001) showed that GFAP+ astrocytes preceded the developing vasculature by a small margin of less than 50 μm; while this margin is 6 times longer in case 2 (around 300 μm) and 40 times longer in case 3 (around 2 mm) (Figure 11A).

This phenomenon could occur as a result of either accelerated astrocyte migration or halted vasculature development. My observation suggestion that the former is unlikely, because during normal development, expansion of the astrocyte
precursors (PAX2+) will completely cover the entire retina around by 28 weeks’ GA (Chu et al., 2001). However, in our late postnatal tissues (case 3 and 4) (which reached the approximate age of 35 weeks GA at death), the PAX2+ astrocytes still did not reach the pars caeca (Figure 20), meaning that migration of the astrocytes was slower than normal. In contrast, exposing mouse pups to hyperoxia may slow the expansion of the vasculature but not that of astrocytes (Zhang et al., 1999).

Last but not least, astrocytes endfeet wrap closely around mature and immature vasculature during development and has been suggested that they contribute to the formation of the blood-retina barrier (Tout et al., 1993). In cell culture, astrocytes also induce barrier properties in CNS endothelial cells (Kuchler-Bopp et al., 1999). Thus, the extensive haemorrhage observed in our ROP case may indicate blood-retina barrier breakdown (Figure 14).

2.4.2.4 Inflammatory Cells

A few types of inflammatory cells were investigated in the current study. Resident retinal microglial cells are part of normal vasculature development (Diaz-Araya, Provis, and Penfold, 1995; Diaz-Araya et al., 1995). The majority of which present a stellate morphology, known as the “resting state” in the retina. However, they can become amoeboid shape when activated (Li et al., 2015). Furthermore, enlarged soma size is commonly reported under pathological conditions, such as mice received optic nerve crush (Davis et al., 2017), OIR models (Fischer et al., 2011) and in various human retinal diseases (Gupta et al., 2003). In my samples, most, if not all, cases I observed Iba1+ microglial cells were in amoeboid shape (Figure 19). However, these cells will need to be carefully measured and compared to those in eyes of aborted babies; but this plan has been hugely hindered by the COVID19 pandemic.

With regards to CD68+ phagocytotic microglial cells, they are known to be involved in normal retinal vasculature development (Penfold et al., 1990). A panretinal
increase of CD68+ microglial cells was reported in human DR post-mortem retinal tissues (Zeng et al., 2008). And in the current study, we also found an elevated number of CD68+ cells especially around the leaking distal vasculature in the ROP case (Figure 19D-F), this suggests a role of activated inflammatory cells in the vessel tufts and/or leakage, which needs to be investigated carefully in the future.

Furthermore, CD14+ phagocytes have been suggested to be associated with pathology in human geographical atrophy tissues (Eandi et al., 2016). But in our samples, CD14+ inflammatory cells did not seem to be directly associated with retinal vasculature abnormalities (Figure 19G-G').

Finally, fibrosis is considered to be an important contributor to late-staged ROP (Hu et al., 2012), however, there is little information regarding to what extent it contributes at earlier stages. Immunostaining CK18, a marker for fibrotic tissues, did not stain any cells in our retinal tissues (not shown), nor did we see obvious fibrotic tissues during dissection. This suggests a limited role of fibrosis in early ROP.

2.4.3 Limitations

Due to ethical reasons, the medical history of donors examined in this study is very limited. As it is known that high oxygen levels used in therapy are a huge risk for ROP, staffs at Shenzhen hospital are currently working on retrogradely tracing relevant information, this includes, but is not limited to, blood oxygen level and SpO₂ of oxygen supplementation. This will help us understand the different phenotypes we observed in the current study. For instance, we speculate that case 2 is hypoxia due to pneumorrhagia, this will need to be validated with relevant clinical information revealing blood oxygen saturation levels.

There were unexpected technical issues with in situ hybridization, which I spent almost two month troubleshooting but still was not able to resolve. Due to the amount
of work and analysis to be completed within a limited timeframe, and also the need to come back to the UK to finish up my PhD, I only managed to get to work on the ROP tissue in the first couple of runs. I plan to work on troubleshooting the kit after submitting my thesis and then to perform further in situ hybridisation experiments with a number of other probes on these tissues to further characterise changes in gene expression.
2.5 Summary

In this study, I characterised the whole mount retinal vasculature from premature infants at early and later postnatal stages. My results demonstrate phenotypes that can be readily interpreted as consequences of altered oxygen levels in the retina. Furthermore, I have characterised the so far neglected phenomenon of gliosis in human ROP, which is likely an important pathogenic element that deserves further investigation.
Chapter 3.

Vessel Loss in Human Diabetic Retinae

Some results reported in this chapter have been presented at:


3.1 Introduction

The discovery of nonperfused retinal capillaries in diabetic retinal tissues dates back to over half century, when shunt capillaries were observed in the retina of diabetic donors (Cogan et al., 1963). These vessels are also known as string vessels, acellular capillaries or ghost vessels, and have been reviewed extensively by Brown (2010). These acellular capillaries are not exclusive to diabetes, but they appear at very low incidence in the retina and brain of healthy donors and also in those with degenerative diseases such as Alzheimer’s (Challa et al., 2004). They have also been widely reported in model systems mimicking type 1 (Barber et al., 2005; Feit-Leichman et al., 2005; Park et al., 2014) and type 2 diabetes (Midena et al., 2009; Toh et al., 2019), and those presenting diabetes-like phenotypes (Enge et al., 2002; Ruberte et al., 2004; Zheng et al., 2007). Many studies on model systems have used trypsin digestion to isolate retinal vasculature beds, in which acellular capillaries are marked by the absence of nuclei inside the vessel wall. However, this method can be technically challenging and can result in tissue loss if not performed properly (Chou et al., 2013).

Clinically, fluorescein angiography (FA) has been a routine modality to check for leaky retinal vessels. Yet studies on monkey and human retinæ revealed that only the superficial, but not deep, capillary plexuses are visible in FA (Weinhaus et al., 1995; Spaide, Klancnik, et al., 2015). Study using adaptive optics have also found ghost and/or abnormal vessels in NPDR eyes (Burns et al., 2014). Recently, optical coherence tomography angiography (OCTA) has emerged and provided unprecedented opportunity to visualize retinal blood flow in a depth resolved manner. Retinal microvascular abnormalities, for instance capillary dropout, dilated capillary loops and tortuous capillary branches, can all be observed with OCTA (Choi et al., 2017). Moreover, Couturier et al. (2015) have demonstrated inferiority of FA in
visualizing nonperfusion compared to OCTA.

Since 2015, mounting clinical studies started comparing vascular nonperfusion in different vascular plexuses in control and diabetic patients with or without DR. Most, if not all, of these studies detected reduced capillary density at early DR. However, it remained contentious regarding which retinal plexus is the first and more severely affected. In diabetics without DR, some investigators reported more prominent nonperfusion at the DCP than the SCP (Scarinci et al., 2018; Simonett et al., 2017; Carnevali et al., 2017); some observed a more severe reduction in vessel density in the SCP (Ting et al., 2017), whilst others found comparable dropout incidence in both plexuses (Dimitrova et al., 2017; Cao et al., 2018). Other vascular abnormalities observed in diabetic patients have also been documented, Vujosevic et al. (2019) identified capillary loss and presence of tortuous capillaries in over half of diabetic cases, especially in the DCP. In cases of DR, Couturier et al. (2015) found SCP capillary nonperfusion in all DR patients, yet only one third presented nonperfusion in DCP. Kaizu et al. (2017) identified focal capillary dropout in the DCP and an inversely correlated flow density with disease severity. Moreover, Scarinci et al. (2015) proposed that macular capillary nonperfusion at the level of DCP is coupled with outer retinal structural disruption.

However, there have been few concerning challenges that OCTA is facing. Firstly, current studies used built-in algorithms to segment retinal capillary plexuses, which varies considerably across devices (Table 5). Although measurements tend to be consistent for inter- and intra-visit with the same device (You et al., 2017), different algorithm can result in inconsistent slabs used for segmentation, which can confound analysis (Spaide et al., 2017; Corvi et al., 2018). As Magrath et al. (2017) documented, there can be significant variations between different machine measuring the FAZ and VD on the same group of patients, and direct comparisons across machines should
be considered with caution.

Secondly, Spaide and Curcio (2017) compared OCTA images with post-mortem histology from the same patient only to find that none of the widely used OCTA algorithms followed anatomic layers correctly. Therefore, the SCP and DCP measurements in the reported studies are likely to be contaminated by artifacts and anatomically incorrect. In fact, some studies did not exclude DR patients with DME, which brought further difficulties to accurately delineate plexuses (Lavia et al., 2019). Furthermore, Bonnin and colleagues (2015) reported alterations in the convergency of DCP signals from superimposition of the SVC in two-thirds cases they examined. A recent study using projection resolved OCTA seemed to offer a potential solution to correct projection artefacts (Binotti et al., 2019). As shown by their study, the difference in vessel density between SCP and DCP was obscured without correction but was sharpened considerably after correction for projection artefacts. This implies that some OCTA measurements, such as vessel density, at the DCP are confounded.

Thirdly, some investigators have raised the concern that diabetic patients have slower blood flow which might fall below lower detection limit (0.3 mm/s) of OCTA (Tokayer et al., 2013). However, normal human retinal capillary flow ranges from 0.25 – 3.28 mm/s (Riva et al., 1980; Flower et al., 2008), and although slower perifoveal flow in diabetic retinae was detected, it ought to be still be well above the detecting threshold by OCTA (Funatsu et al., 2006). Notably, OCTA does not image non-perfused vessels or some capillaries that are transiently no-flow. Last but not least, the routine OCTA screening area is limited to a 3 X 3 mm perifoveal region around the FAZ. Some may extend that to 6 X 6 mm, which is still not large enough to represent what happens in the periphery. Thus, we performed a histological study with the aim to overcome these limits and to allow for sensitive detection of vascular loss throughout the retina in eyes with early diabetic retinopathy.
Table 5. Clinical OCTA studies investigating vascular nonperfusion in DR.

<table>
<thead>
<tr>
<th>Participants (eyes)</th>
<th>Scan size (mm x mm)</th>
<th>OCTA device</th>
<th>Segmentation criteria</th>
<th>Key findings</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Nonperfusion in DCP &gt; SCP (11 papers)</strong></td>
<td></td>
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<tr>
<td>84 T2DM any DR, 34 controls</td>
<td>3 × 3</td>
<td>RTVue-XR Avanti (Optovue, USA)</td>
<td>SCP: ILM TO 110μm above RPE DCP: 110μm above RPE to RPE</td>
<td>VAD and VLD are notably lower only in DCP between mild NPDR and moderate-severe NPDR/PDR.</td>
<td>(Samara et al., 2017)</td>
</tr>
<tr>
<td>25 T1DM NDR, 25 controls</td>
<td>3 × 3</td>
<td>Cirrus 5000 (Carl Zeiss Meditec, Germany)</td>
<td>Not specified</td>
<td>Decreased DCP density in NDR eyes, no difference in SCP or CC density.</td>
<td>(Carnevali et al., 2017)</td>
</tr>
<tr>
<td>47 T1/2DM DR, 29 controls</td>
<td>3 × 3 and 6 × 6</td>
<td>RTVue-XR Avanti (Optovue, USA)</td>
<td>SCP: 3 to 15μm from ILM DCP: 15 to 70μm from ILM</td>
<td>FD is significantly reduced in DCP in NDR compared to SCP.</td>
<td>(Kaizu et al., 2017)</td>
</tr>
<tr>
<td>28 T1DM NDR or mild DR, 23 controls</td>
<td>3 × 3</td>
<td>RTVue-XR Avanti (Optovue, USA)</td>
<td>SCP: ILM to IPL DCP: IPL to OPL</td>
<td>Reduction in parafoveal DCP density in T1DM patients with no or mild signs of DR.</td>
<td>(Simonett et al., 2017)</td>
</tr>
<tr>
<td>33 T2DM NDR, 29 controls</td>
<td>3 × 3</td>
<td>RTVue-XR Avanti (Optovue, USA)</td>
<td>SCP: 3 to 15μm from ILM DCP: 15 to 70μm from ILM</td>
<td>Statistically significant reduction in SCP and DCP VD in NDR compared to control, especially in DCP.</td>
<td>(Dimitrova et al., 2017)</td>
</tr>
<tr>
<td>102 T1/2DM DR, 62 controls</td>
<td>6 × 6</td>
<td>RTVue-XR Avanti (Optovue, USA)</td>
<td>SCP: ILM to IPL/INL DCP: IPL/INL to OPL/ONL</td>
<td>Reduction in perfusion indices was significantly pronounced in DCP than SCP in the perifovea.</td>
<td>(Sambhav et al., 2017)</td>
</tr>
<tr>
<td>20 T1DM NDR, 23 controls</td>
<td>3 × 3</td>
<td>AngioVue OCTA (Optovue, USA)</td>
<td>SCP: ILM to IPL/INL DCP: IPL/INL to OPL/ONL</td>
<td>Reduced vessel density in the DCP in NDR.</td>
<td>(Scarinci et al., 2018)</td>
</tr>
<tr>
<td>71 T2DM NDR, 67 controls</td>
<td>6 × 6</td>
<td>RTVue-XR Avanti (Optovue, USA)</td>
<td>SCP: 3 to 15μm from ILM DCP: 15 to 70μm from ILM</td>
<td>Reduced vessel density in SCP and DCP in T2DM NDR patients, in which DCP is affected more. No difference in FAZ area.</td>
<td>(Cao et al., 2018)</td>
</tr>
<tr>
<td>Study Details</td>
<td>Imaging Details</td>
<td>Vascular Details 1</td>
<td>Vascular Details 2</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>------------------------------------------------------------------------------</td>
<td>-----------------</td>
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<td>-----------------------------------------------------------------------------------</td>
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<td></td>
</tr>
<tr>
<td>22 T1D DR, 12 controls</td>
<td>3 × 3 and 6 × 6</td>
<td>RTVue-XR Avanti (Optovue, USA) SCP: 3μm below ILM to IPL</td>
<td>VD is reduced in diabetic eyes with lower visual acuity than in those with normal visual acuity in all vascular plexuses. Visual acuity is associated with degree of capillary loss in the DCP.</td>
<td>(Dupas et al., 2018)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ICP: IPL to 9μm above OPL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DCP: 19μm below IN/OPL to 9μm below OPL/ONL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>102 varying DR, 30 NDR, 42 controls</td>
<td>3 × 3</td>
<td>DRI OCT Triton plus (Topcon, Japan) SCP: 2.6μm below ILM to 15.6μm below IPL/INL</td>
<td>FAZ area and perimeter correlate positively with DR severity. Decreasing trend of FAZ CI at DCP. Retinal microvasculature changes in DCP preceded that in SCP.</td>
<td>(Kim et al., 2018)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DCP: 15.6μm below IPL/INL junction to 70.2μm below IPL/INL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 T1/2DM NDR, 30 controls</td>
<td>3 × 3</td>
<td>DRI OCT Triton plus (Topcon, Italy) SCP: ILM to 15.6μm above IPL/INL</td>
<td>Almost all NDR patients presented parafoveal capillary loss, with higher incidence in the DVC.</td>
<td>(Vujosevic et al., 2019)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DCP: 15.6μm above to 70.2μm below IPL/INL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nonperfusion in SCP &gt; DCP (4 papers)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 T1/2DM DR, 22 controls</td>
<td>3 × 3</td>
<td>DRI OCT Triton (Topcon Corp., Japan) SCP: ILM to IPL/INL</td>
<td>Significantly reduced SCP VD in mild and moderate NPDR compared to controls; differences in DCP were not statistically significant.</td>
<td>(Al-Sheikh et al., 2016)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DCP: IPL/INL to INL/OPL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>92 any DR, 44 NDR, 44 controls</td>
<td>3 × 3</td>
<td>RTVue-XR Avanti (Optovue, USA) SCP: 3μm below ILM to 25μm above IPL</td>
<td>In all three plexuses, with worsening DR, VD decreases while PAN increases.</td>
<td>(Onishi et al., 2018)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ICP: IPL/INL to 30μm below IPL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DCP: 15μm slab below INL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>86 any DR, 44 controls</td>
<td>3 × 3</td>
<td>RTVue-XR Avanti (Optovue, USA) SCP: 3μm below ILM to 15μm below the IPL</td>
<td>PAN and AFI is positively and negatively related to DR severity, respectively. DCP VD correlates strongly with DR severity.</td>
<td>(Nesper et al., 2017)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DCP: 15 to 70μm below IPL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>84 any DR, 14 controls</td>
<td>3 x 3</td>
<td>Cirrus SD-OCT (Carl Zeiss Meditec, USA)</td>
<td>SCP: ILM to 110μm above RPE DCP: 110μm above RPE to RPE</td>
<td>Statistically significant reduction in SD, VD and FD, and increase in VDI, between healthy and moderate-severe NPDR and PDR in both plexuses.</td>
<td>(Kim et al., 2016)</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------</td>
<td>----------------------------------------</td>
<td>-------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Did not compare between plexuses (4 papers)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56 varying DR, 21 controls</td>
<td>3 x 3 and 6 x 6</td>
<td>RTVue-XR Avanti (Optovue, USA)</td>
<td>SCP: ILM to IPL/INL DCP: IPL/INL to OPL/ONL</td>
<td>CPD values significantly lower in nearly all layers of all DR groups compared with control.</td>
<td>(Agemy et al., 2015)</td>
</tr>
<tr>
<td>81 T2DM DR, 19 T2DM NDR</td>
<td>3 x 3</td>
<td>Swept-source OCTA (Topcon Corp., Japan)</td>
<td>SCP: 3 to 15μm from ILM DCP: 15 to 70μm from ILM</td>
<td>FD in both SCP and DCP is positively related to DR severity. No comparisons made between plexuses.</td>
<td>(Ting et al., 2017)</td>
</tr>
<tr>
<td>17 T1DM with severe NPDR or PDR, 17 controls</td>
<td>3 x 3</td>
<td>RTVue-XR Avanti (Optovue, USA)</td>
<td>SCP: ILM to 9μm above IPL/INL ICP: 9μm above IPL/INL to 6μm below INL/OPL DCP: 6μm below INL/OPL to 9μm below OPL/ONL.</td>
<td>VD decreases significantly with DR severity in all three vascular plexuses. Inner retinal thickness correlated with VD in the SCP, but outer retinal thickness does not correlate with DVC VD.</td>
<td>(Lavia et al., 2019)</td>
</tr>
<tr>
<td>24 T1DM NDR, 24 controls</td>
<td>Not specified</td>
<td>RTVue-XR Avanti (Optovue, USA)</td>
<td>SCP: 3μm from ILM to IPL/INL ICP: IPL/INL to 20μm below IPL/INL DCP: 20μm below IPL/INL to 15μm below OPL/ONL.</td>
<td>Attenuation of both SCP and DCP in the T1DM group compared with the control group in para and perifoveal regions.</td>
<td>(Sousa et al., 2019)</td>
</tr>
</tbody>
</table>

Anatomical abbreviations: SCP, superficial capillary plexus; DCP, deep capillary plexus; CC, choriocapillaris; FAZ, foveal avascular zone; DR, diabetic retinopathy; NDR, diabetic with no DR; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; T1/2DM, type 1 or 2 diabetes mellitus; ILM, inner limiting membrane; IPL/INL, inner plexiform layer / inner nuclear layer interface; INL/OPL, inner nuclear layer / outer plexiform layer interface; OPL/ONL, outer plexiform layer / outer nuclear layer interface.
Parameter abbreviations: VD, vessel density; PAN, percent area of nonperfusion; AFI, adjusted flow index; CPD, capillary perfusion density; FD, flow density; VAD, vessel area density; VLD, vessel length density; VDI, vessel diameter index.

Qian and Dr. Abraham Olvera contributed equally to this table.
3.2 Materials and Methods

3.2.1 Literature Review

Ninety-five original studies containing keywords of “retinal vascular plexus” AND “diabetic retinopathy” were identified in PubMed. Titles and abstracts were reviewed, and publications were included in the review following the predefined criteria: (1) written in English, (2) OCTA studies, (3) studied on human, (4) patients with DR as study group, (5) a control group of patients with no DM, (6) results displayed measurements for retinal SVC and DVC separately.

3.2.2 Donor Tissue Information

A total of 14 human post-mortem eyes were obtained from Moorfields Eye Bank (London, UK) and Lions NSW Eye Bank (Sydney, Australia), as shown in Table 6. Eyes with fixation delay of less than 24 hours were processed with priority for our investigation. Quality control on cross sections was performed for every eye analysed, and only those with intact retinal structure were included for further investigations. UEA-stained whole mount staining images of these eyes are shown in Appendix I, Figure 33.

3.2.3 Tissue Processing

3.2.3.1 Dissection Strategy

The lens was removed, and the peripheral ocular tissue was cut away. In order to capture as many vascular lesions as possible, we first check for vascular lesions in the half globe, if none were identified, the whole mount was then dissected down to at least 6 X 5 cm by size (naso-temporal X superior-inferior axis), comprising an area covering the standard seven ETDRS fields. This is usually a rectangle of 5 cm by length and 2 cm by width covering the mid-periphery of the retinae.
3.2.3.2 Tissue Embedding

PFA-fixed, paraffin-embedded tissue was used in this study. After obtaining magnified whole mount images (described in Section 3.2.4.1 later), ocular tissues were washed in PBS overnight, dehydrated through graded alcohol and left in paraffin wax to sink, this was automatically processed by a LEICA TP1020 tissue processor (Leica, UK). Naso-temporal sections were cut at 6 μm using an Anglia AS200 sledge microtome (Cambridge, England) and then floated on a 50-55°C bath in distilled water. A few drops of 100% ethanol were added to remove creases. Sections were then collected with Leica BOND™ Plus Slides (Leica, USA) and drained vertically before airdrying on a 48°C heating plate.

Table 6. Donor tissue and fixation information.

<table>
<thead>
<tr>
<th>Group</th>
<th>Donor</th>
<th>Age</th>
<th>Gender</th>
<th>DM type</th>
<th>Cause of death</th>
<th>Fixation delay (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1</td>
<td>69</td>
<td>M</td>
<td>None</td>
<td>mesothelioma</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>73</td>
<td>F</td>
<td>None</td>
<td>end stage COPD</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>67</td>
<td>F</td>
<td>None</td>
<td>breast cancer</td>
<td>22.5</td>
</tr>
<tr>
<td>Diabetic no dropout</td>
<td>4</td>
<td>76</td>
<td>M</td>
<td>II</td>
<td>metastatic gastric cancer</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>87</td>
<td>M</td>
<td>II</td>
<td>lung cancer</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>62</td>
<td>M</td>
<td>II</td>
<td>malignancy</td>
<td>24.5</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>72</td>
<td>M</td>
<td>unknown</td>
<td>lung adenocarcinoma</td>
<td>20</td>
</tr>
<tr>
<td>Diabetic with dropout</td>
<td>8</td>
<td>55</td>
<td>M</td>
<td>I</td>
<td>N/A (found unresponsive)</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>87</td>
<td>M</td>
<td>II</td>
<td>myocardial infarction</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>74</td>
<td>F</td>
<td>II</td>
<td>cardiac arrest</td>
<td>8.75</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>79</td>
<td>F</td>
<td>II</td>
<td>metastatic ovarian cancer</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>66</td>
<td>M</td>
<td>II</td>
<td>respiratory failure</td>
<td>26.5</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>61</td>
<td>F</td>
<td>II</td>
<td>metastatic adenocarcinoma</td>
<td>17</td>
</tr>
<tr>
<td>DR</td>
<td>14</td>
<td>64</td>
<td>F</td>
<td>unknown</td>
<td>myocardial infarction</td>
<td>57</td>
</tr>
</tbody>
</table>

Abbreviation: I: Type I diabetes mellitus; II, Type II diabetes mellitus; COPD, chronic obstructive pulmonary disease; N/A, not applicable
3.2.4 Immunohistochemistry

3.2.4.1 Immunohistochemistry of Retinal Whole Mounts

Methods used in this chapter have been described in Section 2.2.5.1.

3.2.4.2 Immunohistochemistry of FFPE Sections

Staining was performed as previously described (Powner et al., 2016). In brief, slides were washed in xylene twice, 10 minutes each, then rehydrated sequentially in 100%, 70%, 50% ethanol for 10 minutes each and finally washed with distilled water. Slides were submerged in antigen retrieval buffer (90% glycerol, 10% 10Mm sodium citrate pH 6), then gradually heated to boiling using a generic microwave. These were then left at room temperature to cool down for 25 minutes and washed with distilled water.

After washing, slides were then placed horizontally in a humidified container and blocked with immuno block (1% BSA, 0.5% Triton and 0.2% sodium azide in PBS) for 1 hour at room temperature, followed by incubation in 1:500 anti-collagen IV antibody (AB769, Chemicon) at 4°C overnight. After washing three times with PBS the next day, slides were incubated in secondary antibody at 1:500 dilution (A11055, Invitrogen) for 1 hour at room temperature. Slides were then washed with PBS before counterstaining in 1:10,000 Hoechst diluents (H33258, Sigma-Aldrich, St Louis, MO) for 5 minutes and then mounted with mounting medium containing mowiol 4-88 (Calbiochem), glycerol and 0.2M Tris pH 8.5, and stored in dark storage folders. Other antibodies tested, but were not presented, in this thesis are listed in Table 9.

3.2.4.3 Quenching autofluorescence from RPE

To eliminate the autofluorescence and or/lipofuscin from RPE cells, sequential application of potassium permanganate and oxalic acid were applied on the section as previously reported (Mishima et al., 1999). This was performed after cooling down
from antigen retrieval mentioned in Section 3.2.4.2. Briefly, 1% potassium permanganate (KMnO4) was applied to sections for 2 minutes after antigen retrieval, followed by washing with distilled water and 2 minutes in 1% oxalic acid and a washing in distilled water before proceeding with blocking as described in Section 3.2.4.2.

3.2.5 Microscopy

Retinal whole mounts stained with rhodamine labelled UEA (see Section 3.2.4.1) were imaged using an Olympus SZX16 stereoscope (Tokyo, Japan) with 1X objective lens to generate an overview of the vasculature. Then a higher magnified image was generated using 3.2X objective lens for a detailed view. Zeiss Axioskop 2 Upright Fluorescence Microscope (Carl Zeiss Microscopy LLC, USA) or Invitrogen™ EVOS™ FL Auto 2 (Thermo Fisher, UK) with 10X objective lens was used to visualize capillaries on cross sections.

3.2.6 Image Processing and Analysis

Panorama images were generated with Adobe Photoshop CS6 (AdobeSystems, Inc.). For retinal whole mounts, panorama images were generated with the built-in “Load Files into Stacks” and “Auto-Blend Layers” functions. Stacks of each region were stitched together manually. In cross sections, panorama images were generated using the built-in automated “Panorama” function. Manual alignment and adjustment were performed when needed.

3.2.7 Incidence of Vessel Dropout

In this chapter, I sampled across the entire tissue by assessing three consecutive sections from every hundredth section. These sections were stained with Rhodamine labelled UEA and antibody raised against collagen IV to reveal endothelial cells and the vascular basement membrane, respectively.
Retinal capillaries were firstly counted and assigned to a vasculature complex on FITC and DAPI channel showing basement membrane and nuclei, respectively. Endothelial lining was then identified independently on a single TRITC channel. An empty basement membrane without internal endothelial lining was considered as vessel loss, or ghost vessel (Powner et al., 2016). Normal and ghost vessels in the different vascular plexuses were counted manually with Adobe Photoshop CS6 (AdobeSystems, Inc.). Incidence of capillary dropout was expressed as the percentage of ghost vessels over total vessels overall or within the same plexus. Plexuses segmentation criteria followed what has been proposed in Campbell et al. (2017), also shown in Figure 3. Regions where the retinal vasculature plexuses cannot be distinguished, for instance, around the optic nerve head, were excluded from analysis.

3.2.8 Statistical Analysis

Statistical analysis used in this chapter have been described in Section 2.2.10.

Because of the limited availability of postmortem DR tissue, we had only one cases that is diagnosed with DR. It served as a positive control in this chapter, and the one sample t-test was used to compare values from the DR sample with mean values of other groups of interest.

With regards to linear regression, Pearson correlation coefficient was calculated to assess potential relationship between datasets. Linear relationship was assumed when the slope of the best fit model was significantly different from zero. All data are presented as mean ± standard deviation unless otherwise stated.
3.3 Results

3.3.1 Visualizing Retinal Vasculature

In order to gain an overview of vascular damage in the retina, we firstly stained the retinal whole mounts with Ulex Europaeus Agglutinin I (UEA) for endothelial cells and then screened for vascular abnormalities. All UEA-stained whole mount images were presented in Appendix I, Figure 33.

Figure 21A is a representative sample of a whole mount image from a type II diabetic patient. The optic disc and the sclera were surgically removed before collection. Similar to *en face* OCTA imaging, the FAZ (Figure 21B, yellow dashed line) and the foveola (Figure 21B, yellow dotted line) can be delineated on whole mount images. The peripapillary capillary plexus (RPCP), which is known to supply the peripapillary layer, rich in axons from optic disc to the macula, can also be clearly observed (Figure 21B, short arrows). In addition, the perivascular capillary free zone (Figure 21B-D, long arrows), appearing as a "gap" between arteries (Figure 21C, a) and neighbouring parallel capillaries, was also clearly observed. During retinal vasculature development, oxygen diffusing from arteries, prevents the growth of nearby capillaries. In contrast, oxygen concentration in veins (Figure 21C, v) is lower and perivascular avascular zones are not frequently observed around veins.

Interestingly, despite well perfused vasculature in the macula (Figure 21C), areas of capillary dropout were detected in the peripheral retina (Figure 21D, arrowheads), which would by standard FA or OCTA normally not be identified because these nonperfused regions located outside of the standard 3 X 3 mm, or even 6 X 6 mm, screening area of OCTA.
Figure 21. Visualizing human retinal vasculature on whole mount.

A, A retina whole mount stained with UEA revealed endothelial cells. B, C and D were zoomed-in images of boxes shown in A. Arrowheads denoted regions of vessel loss. Long arrows pointed to the perivascular zone. Short arrows showed the radial peripapillary capillary plexus (RPCP). Yellow dashed and dotted line delineated the FAZ and the foveola, respectively. * indicates artefacts generated during dissection. a, artery; v, vein. Scale bar 1 mm.
3.3.2 Vessel Loss Increases with DR Severity

As showcased in Figure 21, normal vascularization in the macula did not guarantee the no capillary dropout in the periphery. Clinical evidence suggested a predictive role of peripheral vascular lesion, i.e. those outside the standard ETDRS 7 standard field, in 4-year DR progression (Silva et al., 2015). In fact, most diabetic retinae we screened did not present identifiable vascular damage (Figure 33); instead, their retinal vasculature appeared very similar to non-diabetic controls, and specialised ophthalmologists were not able to confidently discriminate between the two (62.5% accuracy). We thus worked on developing a method which made measuring every single vessel loss possible. A previous study from our group showed that nonperfused vessels preserved their basement membrane despite losing the endothelial lining (Powner et al., 2016). As demonstrated in Figure 22A, normal vessels possess a thin inner lining of endothelial cells (Figure 22B-B’’, arrow), which acellular capillaries lack; instead, they appeared as hollow sheaths of basement membrane (Figure 22B-B’’, arrowheads). This anatomical difference between normal and nonperfused vessel provides a useful and valuable readout to assess capillary dropout in post-mortem tissues.

Eyes were assessed systematically by examining three consecutive sections from every hundredth section for capillary dropout. Results in Figure 22C showed that vessel dropout occurs at a low incidence in control eyes (1.06 ± 0.14%). Overall, diabetic retinae presented a moderately, but significantly, higher incidence of vascular dropout than controls (3.82% vs 0.98%, p < 0.05). Note that these diabetic retinae did not present vasculature abnormalities that could be identified on whole mounts.

Interestingly, we noticed two subgroups in the diabetic group, with distinguishable incidence of vessel loss. The “diabetic no dropout” subgroup presented level of capillary dropout (1.69 ± 0.37%) below the overall mean of the whole diabetic group.
(3.82 ± 1.89%, Figure 22C, dashed line); whereas the “diabetic with dropout” subgroup, has notably higher incidence (5.23 ± 0.57%). As assessed by one-way ANOVA test with post hoc analysis, the means of the control and the two diabetic subgroups were significantly different from one another (p < 0.001). Whilst the diabetic no dropout subgroup appeared to have a slightly elevated degree of vessel loss compared to controls (p = 0.04), there was clearly more vessel loss in the diabetic with dropout subgroup (p < 0.0001 when compared to control or diabetic no dropout subgroup). It was not surprising to find that the incidence of vessel loss was considerably increased in DR eyes (29.83%), indicating substantial vascular damage in advanced DR (p < 0.001 compared to any other groups). Although there was a possibility that vessel loss increases with age. As reported previously, aging can cause changes in the basement membrane of the vasculature (Powner et al., 2011). But in this study, we did not find significant differences between control (69.7 ± 3.1 years old) and all diabetic donors (73.9 ± 11.3 years old, p = 0.55), nor between two diabetic subgroups (diabetic no vs with dropout, 74.3 ± 10.3 vs 70.3 ± 11.9 year-old, p = 0.61). Therefore, increased vessel loss in diabetic retinae did not appear to be simply consequential of aging.

To investigate whether acellularity results in morphological change of capillaries, I measured the inner diameter of normal and acellular capillaries. Data were normalized to size of RPE nuclei of each donor to correct for tissue shrinkage during processing. Results suggested that capillaries become narrower after endothelial cells loss in all groups (Figure 22D). Although a mild dilation of normal capillaries in the diabetic with dropout subgroup was observed (1.46-fold, p = 0.0051), the diameter of the acellular capillaries remained comparative to control (p = 0.72). In addition, the DR eye presented an almost two-fold dilation in both acellular (1.67-fold, p = 0.457) and normal capillaries (1.85-fold, p = 0.0046) compared to control.
Figure 22. Appearance of acellular capillaries in human diabetic retinas.

A. Immunohistochemistry showing UEA labelled endothelial cells and collagen IV stained basement membrane from a region with acellular capillary of donor 10 (diabetic with dropout subgroup). Higher magnification of the boxed area in A is shown in B-B''. Internal lining of endothelial cells was present in normal capillary (B'', arrow), but not in dropouts (B'', arrowheads). Scale bar 50 μm. C, Overall capillary loss in control, subgroups of diabetic, and DR eyes. Diabetic retinae presented a mildly, but statistically significant, higher incidence than controls. DR retina presented an overwhelmingly higher dropout incidence than any other groups. The dashed line showed average capillary loss incidence in the diabetic group as a whole (3.82%). At least 13,500 vessels were assessed for each donor. D, Capillaries became narrower after losing endothelial cells. Slight increase in the size of normal capillaries was seen in the diabetic with dropout subgroup, but the size of acellular capillaries was consistent amongst non-DR groups. At least 15 capillaries were measured from each donor, no less than 52 capillaries were measured for each group.

N = 3 (control), 4 (diabetic no dropout), 6 (diabetic with dropout) and 1 (DR). Results were presented as mean ± s.d. Statistical significance are tested by one-way ANOVA with post hoc Dunnett’s multiple comparison with controls (C and D). * P < 0.05, ** P < 0.005, *** P < 0.001.

(Contribution from other people to this chapter has been stated in Page 8)
3.3.3 Vessel Loss is More Severe in DVC

We showed that a subgroup of diabetic retinas with no obvious vascular lesions on the whole mount had a moderate increase in capillary dropout in our examination on cross sections. It is worth noting that only the superficial vasculature was visible in the whole mount image. Couturier et al. (2015) reported a rarefaction of capillary nonperfusion in the SVC of DR patients, whereas over 30% of them presented nonperfusion in the DVC, which suggested that vascular anomalies can be present in the DVC before detectable lesion shows up in the SVC. In addition Spaide and Curcio (2017) pointed out that current vasculature segmenting algorithms are facing challenge to automatically and correctly differentiate superficial from deeper vascular plexus, which could result in inconsistent findings among studies (reviewed in Table 5). Thus, in this histopathological study, we segmented the retinal vasculature on cross sections based on a well-characterised and widely-accepted criteria (Figure 3), this enables detection of single acellular capillaries whilst guarantee accuracy of segmentation.

In all donors examined, we found that vessel loss in the DVC was never lower than that in the SVC (Figure 23A). On average, differences in inter-plexus vessel loss was not significant in control (1.38% vs 0.75%, p = 0.07) or diabetic no dropout subgroup (2.02% vs 1.36%, p = 0.08), but was significant in diabetic with dropout subgroup (7.77% vs 2.67%, p < 0.0001) and DR (15.28% vs 44.38%, p < 0.001) (Figure 23B). Inter-group differences of vessel loss were not significant between control and diabetic no dropout subgroup (p = 0.13 in SVC, p = 0.07 in DVC). But there was a significant increase in the diabetic with dropout subgroup (p < 0.001 in both plexuses), with a steeper increase observed in the DVC. In both plexuses, vessel loss in DR was always overwhelmingly higher (p < 0.001 compared to any other group at any plexuses) (Figure 23C).
3.3.4 DVC Vessel Loss Positively Correlates to That in the SVC

Since we found that vessel loss in the DVC is always higher than in the SVC, we asked whether there was a potential correlation between them. Figure 23D reveals that vessel loss in DVC is positively, and linearly, associated with that in the SVC ($R^2 = 0.972$, $p < 0.0001$).

We could identify a cluster formed by controls and the diabetic no dropout subgroup at the bottom left corner, indicating low level of capillary dropout in both plexuses, from which the diabetic with dropout subgroup forms a clearly separate cluster. Further away at the top right corner is the DR eye, with a considerably higher level of vessel loss in both plexuses. Most, if not all, data were within the 99% confidence interval area (Figure 23D, red region) from the best fit model (Figure 23D, solid line), which has the following formula:

$$Y = 2.94X - 0.84$$

This means with every 1% increase of vessel loss at the SVC, loss in the DVC will increase by almost 3%.

Attempts have also been made to investigate whether vessel loss happens randomly or following a specific pattern that is geographically dependent. To this purpose, vessel loss at both plexuses was plotted against its distance to the optic nerve head-fovea axis. No linear relationship was found between either plexus and eccentricity in control or diabetic retinas. However, a slight linear relationship was spotted between DVC dropout and eccentricity of the DR case ($R^2 = 0.889$, $p = 0.001$, not shown). This suggested that capillary dropout occurred randomly in control and non-DR diabetic retinas but was increased in peripheral region of the DR eye.
Figure 23. Vessel loss at different retinal vascular plexuses.

**A and B**, Intragroup differences in vessel loss of SVC and DVC. Plotting vessel loss from each donor revealed that SVC loss was never higher than that of DVC in all cases examined (A). On average, only diabetic with dropout subgroup and DR group had higher incidence of vessel loss (B). (At least 9,000 vessels were examined for each plexus for each donor.)

**C**, Intergroup differences in vessel loss of SVC and DVC. Control and diabetic no dropout subgroup showed no inter-plexus difference, which was significant in diabetic with dropout subgroup and DR, with DVC showing considerably elevated proportion of vessel loss.

**D**, A linear regression model plotting vessel loss in the DVC against SVC in all donors and revealed a strong linear relationship between ($R^2 = 0.972$, $p < 0.0001$). Control and diabetic no dropout subgroup formed a clearly separate cluster from the diabetic with dropout subgroup. DR locates at a distinct region that is further away for the rest groups.

$N = 3$ (control), 4 (diabetic no dropout), 6 (diabetic with dropout) and 1 (DR). Box and whisker show the mean ± s.d. Statistical significance was tested by two-tailed Student’s t-test (A) or one-way ANOVA with post hoc Dunnett’s analysis (C). ns denotes not significant, * $P < 0.05$, *** $P < 0.001$.

Abbreviations: LOI, line of identity; SVC, superficial vascular complex; DVC, deeper vascular complex; DR, diabetic retinopathy.

(Contribution from other people to this chapter has been stated in Page 8)
3.4 Discussion

To the best of my knowledge, this is the first histological study systematically investigating vessel loss in different plexuses in human post-mortem retinae. I found a 5-fold increase in the incidence of capillary dropout in a subset of the diabetic group compared to controls. Similarly, an OCTA study from de Carlo et al. (2015) reported that nonperfusion were five times more likely to be found in diabetic eyes with no DR compared to controls.

3.4.1 Capillary Dilation in the Diabetic no DR eyes

An acellular capillary is a sign of local nonperfusion. An old clinicopathological study demonstrated that acellular capillaries are unable to carry blood flow (Kohner et al., 1970), and a more recent study from Powner et al. (2016) concurred with this view. Sandison (1928) observed that the endothelial cells retract into parental vessels after a day of no circulation, in fact, the first thing the authors noticed after circulation stopped was the appearance of acellular capillaries. Acellular vessels were recognised as an early form of string vessels, i.e. they have lost their endothelial lining but have not yet collapsed into a thin strand. In this study, I measured the diameter of the capillaries in the DVC to investigate in histopathological aspect whether vessels became narrower after losing their endothelial cells. My results suggested that they did (Figure 22D).

Another interesting finding was the dilated normal DVC capillaries in the diabetic with dropout subgroup, whereas the diameter of acellular capillaries remained consistent amongst non-DR groups. There was a lack of consistence in experimental diabetic studies using rodent models. Those findings either oppose to ours (Yoon et al., 2016) or showed no change in capillary size between control and diabetic mice (Liu et al., 2017). This can be explained by species difference and/or that rodent models are mimicking very early diabetes, as they rarely develop the type of vascular
lesions commonly observed in DR patients. Some clinical studies suggest that retinal vessels become wider with DR severity. An OCTA study from Kim et al. (2016) identified a correlation between capillary diameter index and DR severity. Fundus photography studies are in support of this by demonstrating that widening of central venules, but not arteries, is associated with high DR incidence (Klein et al., 2012; Roy et al., 2011). More intriguingly, increased retinal capillary diameter was also found in clinically study using AOSLO during early DR (Burns et al., 2014). It is therefore speculated that the widening of normal capillaries in diabetic with dropout subgroup and DR we observed can be a consequence of DR development and deterioration.

In addition, we noticed a two-fold increase in both capillary diameter of DR compared to the controls. Although it was known that this DR donor had received laser treatment for her retinopathy, and it could be claimed that laser burns destroy any tissue, including endothelial cells inside normal capillaries which could possibly increase the value of acellular capillaries, this still does not explain why normal capillaries are also dilated to a similar extent.

It is noteworthy that although measurements for vessel diameters can be performed with OCTA or FA, these instruments tend to measure the central arteries and/or veins, which ranges from 31 to over 100 μm (Drobnjak et al., 2017; Ghasemi Falavarjani et al., 2017). Although OCTA has poor ability to detect small capillaries in the DCP (Balaratnasingam et al., 2018) and these vessel calibre measurements may not be accurate (Ghasemi Falavarjani, et al., 2017). If lesions and anomalies were to happen first in the retinal microvasculature, measuring changes in the large vessels will not be a sensitive readout. Therefore, my measurements focused on the capillaries in the deep retina, ranging from 0.56 to 8.81 μm (before normalization). This was accomplished with a precision and accuracy that is hard for OCTA to achieve. In addition, clinical studies measures the outer diameter of the vessel, i.e. including the thickness of basement membrane (Ghasemi Falavarjani et al., 2017);
while in the current study, I only measured the lumen. Given that basement membrane become thicker with age (Powner et al., 2011) and in diabetes animal models (Hainsworth et al., 2002; Tsilibrary, 2003; Lee et al., 2010), excluding basement membrane would be essential to avoid confounding factors.

It was speculated that retinal vasculature dilation may be indicative of hypoxia (Stefansson et al., 1983; Curtis et al., 2009). In support of this was the finding from de Jong et al. (2008) that retinal venular diameters negatively correlate to the level of oxygen saturation in lower arterioles. Inflammation was also suggested to affect vein size in diabetic patients, as higher levels of inflammatory molecules have been found to be associated with larger diameters of retinal venules (Klein, 2006). Impaired flicker induced vasodilation in diabetic patients was believed to indicate endothelial dysfunction. However, in most reports, the change in vessel size fluctuated between 0.6 to 4.5% (Mandecka et al., 2007; Nguyen et al., 2009), which is even a milder change than using different clinical imaging methods (10% difference between using FA and OCTA) (Ghasemi Falavarjani, et al., 2017), and makes one wonder whether such subtle change can make difference at all.

Another possibility causing the dilated capillaries is pericytes dropout, one of the key features consistently reported in histological study on diabetic/DR eyes (Stitt et al., 1995; Beltramo et al., 2013). Several lines of evidence suggested pericytes-related functional impairments during early diabetes. For instance, pericytes are known for its role in controlling blood flow of capillaries (Hall et al., 2014). Vasomotor function, the contraction and relaxation cycle of the capillary in response to neighbouring stimulus, usually regulated by pericytes, was notably declined during the early phase of experimentally induced diabetes (Ivanova et al., 2017). Concurrent with this finding was a 20% reduction in pericytes density, which is in line with histological findings from human post-mortem retina. Pericytes dropout will also lose
the focal contract of them on vessels, especially in peripheral capillaries. Attempts have been made to stain pericytes with several antibodies against PDGFRB and/or NG2. However, none of them worked on paraffin embedded tissues no matter what antigen retrieval was used (Table 9). Instead, one antibody worked well on OCT embedded frozen cross sections, yet all diabetic related tissues used in this chapter are embedded and sectioned in paraffin, which hindered further investigation on pericytes.

3.4.2 The significance of DVC is underexplored

One of the main findings of this study was a more pronounced trend of vessel loss in DVC than SVC, which is in line with most OCTA studies reviewed in Table 5 and the OCTA finding that poor perfusion in the DVC precedes changes in the SVC as DR progresses (Sambhav et al., 2017).

Various studies have shown vascular lesions favour the DVC. A histopathological study by Moore et al. (1999) found a higher incidence of microaneurysms within the INL, i.e. DVC. A similar observation was reported by Stitt et al. (1995) using transmission electron microscopy. Clinical imaging studies using OCTA identified more microaneurysms and nonperfusion at DCP than the SVC (Couturier et al., 2015; Hasegawa et al., 2016). Moreover, Rodrigues et al. (2019) presented that the parafoveal vessel density in the DCP is the parameter most robustly associated with ETDRS level.

There are two main possible contributing factors to the fragility of DVC upon nonperfusion. Firstly, DVC are solely regulated by Müller glia, whereas SVC receive regulation from both types of retinal glia. In the present study we have shown that the distribution of AQP4 in the Müller glia endfeet are altered; yet there are other aspects of the functional alteration that is unable to be revealed simply by immunohistochemistry. Numerous animal studies have revealed that Müller glia are
functionally disrupted upon induction of diabetes (Francke et al., 1997; Pannicke et al., 2006; Guerrero-hernandez et al., 2014). Therefore, the DVC is more likely to be affected when its sole regulator, Müller glia, is disrupted.

Secondly, the DVC can be viewed as the most distal vascular plexus in the retinal vascular network, which is reflected in diabetic vascular complications. For instance, peripheral artery disease, 30% of which are diabetic, can result in foot ulceration, or even amputation, due to vessel nonperfusion in the lower extremities (Setacci et al., 2009). Moreover, Veves et al. (1998) found impaired endothelial-dependent and independent vasodilation in diabetic patients with disposed foot ulceration. Another example is the deep vein thrombosis, which is 1.5 times more likely to develop in diabetic patients than in those without (Piazza et al., 2012). Nonperfusion in these patients is mainly caused by the formation of thrombosis in the vein (Shabani Varaki et al., 2018). Wondering whether it is the same case in diabetic retina, attempts have also been made by myself using several antibodies raised against thrombin on diabetic sections upstream of acellular capillaries, however no specific and localized signals were detected. Nevertheless, thrombosis is not exclusive to diabetic retinæ, it has been observed in many other non-diabetic post-mortem retinæ (Ashton, 1963). In addition, leukostasis can also cause blockage of the vessel and is widely reported in diabetic model systems (Kim et al., 2005) and human post-mortem studies (Schröder et al., 1991). Indeed, narrower capillaries of lower vascular extremities may compound the process of vessel occlusion, however, to what degree such occluding events may contribute to the vessel loss remains uncertain.

Another interesting finding in this study was that vessel loss in the DVC associated strongly with that in the SVC. Not only do DCP capillary vortexes locate underneath SVC venules, they also drain into the latter. Bonnin et al. (2015) observed that in case where there is nonperfusion in the SVC, no capillary perfusion persisted.
in the underlying DCP. They also suggested that single SVC vessels receive drainage from multiple DVC capillaries, therefore it is reasonable to speculate that anastomotic organization of the DVC mitigates the effect of a few nonperfused capillaries (Bonnin et al., 2015). With the linear correlation of vessel loss between DVC and SVC, we found that the DVC is three times more sensitive, which, in this sense, indicates that a considerable amount of vessel loss at DVC is required to drive detectable changes in the SVC. This offers a possible explanation as to why capillary dropout at SVC is not seen in our diabetic retinae despite of that detected at the DVC.

This is the first study investigating the correlation of vessel loss at different retinal plexuses, although similar attempts have been made in OCTA studies. Lavia et al. (2019) found a strong linear relationship of vessel density at ICP against that in the DCP. In addition, they have also identified that SVC vessel density increases with ILM to IPL thickness, while similar correlation was not found for DVC and INL thickness. Nevertheless, there does not seem to be a difference in INL thickness between controls and diabetic patients with no DR (Tavares Ferreira et al., 2016).

3.4.3 Limitations

This study is underpowered due to a limited number of samples investigated. However, this is a necessary compromise due to our selection criteria ensuring good tissue quality and the number of eyes received within the limited timeframe during which the current study was conducted.

Another common concern for post-mortem study is fixation delay. In practice, the interval between death of the donor and tissue fixation is often long. As autolysis starts immediately after death, this process will affect protein antigenicity leading to changes of qualitative and/or quantitative results revealed by immunohistochemistry (Scudamore et al., 2011). The rate of autolysis can be affected by various factors such as intrinsic metabolic rate and ambient temperature (Scudamore et al., 2011). It
appeared that prolonged post-mortem fixation delay is one of the major determinants for antigenicity. Some reported that the intensity of the immunohistochemistry staining of oestrogen receptors is diminished by long fixation delay (Khoury et al., 2009), whereas others failed to see as significant difference (Apple et al., 2011). We performed quality control for all tissues available to us and three tissues with signs of tissues damage of suspiciously low staining quality were excluded from the study. For all retinal tissues analysed, no difference in staining quality and/or intensity was noticed throughout the study.

There is a chance that the vessel loss incidence in this study is underestimated. As only collagen IV positive staining with an identifiable tubular structure were considered as “vessels”, i.e. acellular capillaries at early stage of string formation, to avoid false positive counts. However, it is worth mentioning that I did see some intensely stained collagen IV positive dots in diabetic tissues, especially in the diabetic with dropout subgroups (not shown). If those collagen IV positive dots I observed were actual string vessels, this would suggest that degenerated vessels in the diabetic tissues are neglected and underestimated in this study. Yet at this stage, immunostaining alone is not enough to determine whether these dots were actual remnants of collapsed string vessels or whether they could also be artefacts, such as extracellular matrix protein deposition in disease eyes, or unwashed secondary antibodies.

In this study, only limited medical history from the donors was obtained. It would have been of great help if we could have obtained more detailed clinical information, for instance, the duration and management of the donors’ diabetes. Whilst the DCCT study addressed the importance of blood sugar control in lowering DR progression rate (The Diabetes Control and Complications Trial Research Group, 1993, 2000), the Joslin 50-year Medallist study failed to show as significant correlation of blood
glucose level to DR; instead, CEL and pentosidine appeared to be predictive of diabetes related complications (Sun et al., 2011). Attempts have been made by me to extract protein from FFPE tissue on sections aiming to compare the level of AGE or AGE derived products in different groups. However, protein extracted from the tissue was not of good quality, which is was a huge obstacle to process further. This is a common issue in over-fixed tissues (Sadick et al., 2017).

Recently, Ahlqvist and colleagues (2018) proposed a new stratification standard for diabetes based on six main variables, i.e. glutamate decarboxylase antibodies, BMI, age at diagnosis of diabetes, HbA1c, β-cell function and insulin resistance. They defined five different subgroups with significantly different clinical characteristics. Notably, the subgroup featured with the highest blood glucose level, the worst β-cell function and medium-high level of insulin resistance had the highest risk of developing DR. It would have been interesting to see whether our diabetic with dropout subgroup falls into this high-risk subgroup had we had the relevant clinical information.
3.5 Summary

In this study, I systematically sampled human post-mortem retinae and provided histopathological evidence showing that 1) vessel loss increased with DR severity and was more severe in the DVC than in the SCV; 2) normal DVC capillaries in the diabetic with dropout subgroup were dilate by around 50%; 3) there was a strong linear relationship of vessel loss in the DVC against the SVC. Collectively, our results suggest that microvascular lesions deeper in the retina occur at very early stages of DR.
Chapter 4.

Consequence of Deep Capillary Dropout

Some results reported in this chapter have been presented at:

4.1 Introduction

There has been an on-going debate as to which comes first in DR, vascular lesion or neurodegeneration (Jonsson et al., 2016; Simó et al., 2018). Each hypothesis is supported by a few lines of evidence. People argue that during early DR, vascular pathologies exist, such as microaneurysms and loss of pericytes, before significant change in BCVA is noticed; whereas others propose that certain ERG readouts are impaired before vascular lesion can be identified in diabetic patients or relevant model systems. Both school of thoughts are based on the assumption that the clinical assessment for vascular lesion/abnormality is sensitive and accurate. However, this does not seem to be always the case.

In Chapter 3, I have developed a sensitive method to detect single events of capillary dropout in human post-mortem retinal cross sections. Despite the fact that most diabetic retinal vasculatures appeared hardly distinguishable from controls in my analysis, I was still able to identify a moderate but significant increased level of dropout in the DVC, which is usually difficult to reveal by OCTA or FA during clinical examinations. Therefore, vascular damage in the DVC has been largely overlooked in the clinic, which, as shown by my own results, is likely an early vascular pathology in DR.

However, despite the vascular pathology in the DVC, it is known that early DR patients are relatively asymptomatic and central visual acuity is not affected (Adams et al., 2012; Gardner et al., 2017). Nevertheless, subtle changes in functional readouts have been described. For instance, a reduced ERG b-wave amplitude was found in both, experimental diabetic models and in human diabetic patients (Yamamoto et al., 1996). Bipolar cells and horizontal cells were believed to be the main contributors to the generation of b-waves (Stockton et al., 1989). More importantly, prolonged b-wave onset was reproduced in many studies in diabetic
patients with no clinical DR (Bresnick et al., 1987; Shirao et al., 1998; Fortune et al., 1999; Abdelkader, 2013). Interestingly, localized functional deficits revealed by prolonged implicit time, but not amplitude, were found to be spatially correlated to sites of retinal vasculopathy in NPDR patients (Fortune et al., 1999). In addition, the delay in implicit time exaggerated with the severity of DR. Moreover, regional variations in multifocal ERGs were predictive of future vascular lesions (Han et al., 2004).

Oscillatory potentials (OPs) are another important element in the ERG waveform. The exact cellular origin of OPs is not yet known, but was speculated to arise from the bipolar cells at INL (Heynen et al., 1985). OPs are sensitive to regions of ischemia, in patients with PDR, the amplitude of OPs decreased progressively with increased severity (Algvere et al., 1974). For this reason, it was suggested to be a useful parameter to predict the risk of DR development (Frost-Larsen et al., 1980; Simonsen, 1980). In early DR, it was reported that altered OPs and prolonged peak latency could be found in DM patients event without DR (Shirao et al., 1998), indicating the possibility of subclinical functional deficit in the inner retina.

These lines of evidence suggest that there is subclinical functional deficit likely originating from bipolar cells and/or horizontal cells at INL, which precedes clinically vascular lesions in diabetic patients. However, there is a lack of evidence from human histological studies, validating the loss of INL cells in diabetic retinal tissues. Therefore, in this study, I took the opportunity to assess localized neurodegeneration in the INL around normal and nonperfused deeper capillaries in human post-mortem retinal tissues.
4.2 Materials and Methods

4.2.1 Immunohistochemistry

Immunohistochemistry on FFPE sections were performed as previously described (Section 3.2.4.2). Primary antibodies used in this chapter are shown in Table 7. Other antibodies tested, but were not presented, in this thesis are listed in Table 9.

Table 7. List of primary antibodies used in Chapter 4.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalogue number</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquaporin-4</td>
<td>Novus</td>
<td>NBP187679</td>
<td>1:300</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>Chemicon</td>
<td>AB769</td>
<td>1:500</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>BioRad</td>
<td>2150-0140</td>
<td>1:500</td>
</tr>
<tr>
<td>CRALBP</td>
<td>Thermo Fisher</td>
<td>MA1-813</td>
<td>1:300</td>
</tr>
<tr>
<td>Crystallin αA (CRYAA)</td>
<td>Novus</td>
<td>NB120-5595</td>
<td>1:1000</td>
</tr>
<tr>
<td>GFAP</td>
<td>Sigma</td>
<td>C9205</td>
<td>1:500</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>Millipore</td>
<td>MAB302</td>
<td>1:300</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>Swant</td>
<td>235</td>
<td>1:500</td>
</tr>
<tr>
<td>PKCα</td>
<td>Santa Cruz</td>
<td>sc-17769</td>
<td>1:500</td>
</tr>
</tbody>
</table>

All secondary antibodies were from Invitrogen (UK) and used at 1:400 dilution unless otherwise stated.

4.2.2 Microscopy

Slides were first imaged with Zeiss Axioskop 2 Upright Fluorescence Microscope (Carl Zeiss Microscopy LLC, USA) with 10X objective lens to generate panorama, as described in Section 3.2.6. Regions of interest were then imaged with Zeiss LSM 700 with 40X objective lens. For consistency, six z-stacks with interval of 1 μm were taken for each region of interest.
4.2.3 Nearest Neighbour Distance of DVC

To reveal the spacing profile of capillaries in the DVC, the nearest neighbour distance (NND) was measured, which has been a widely used method to quantify cell and capillary spacing profiles (Egginton et al., 2010; Zhang et al., 2018). In my analysis I measured the distance to its nearest neighbour of each capillary residing in the INL on cross sections. This was performed manually using Fiji (NIH, RRID: SCR_002285) as demonstrated in Figure 24. For each donor, NND was measured on every hundredth sections, at least five sections were examined, resulting in at least 1000 measurements for each donor.

In order to correct for possible tissue shrinkage during processing, the height of the RPE nuclei were measured and used as a reference. At least 15 measurement were performed per donor. NND values of each donor were normalized to its reference accordingly. Data were presented as fold change to the reference.

![Figure 24. Demonstrating the measurement of NND.](image)

Images were acquired using a Zeiss Axioskope (with 10X objective lens). Collagen IV (green) staining was used to visualize the capillary network, whilst UEA (red) staining was used to reveal the existence of endothelial cells. The distance between each capillary and its nearest neighbour (lines) was measured manually in FIJI software.

4.2.4 INL Cell Loss within Zone of Influence

On cross sections, a circle was drawn from the basement membrane of each
capillary with the radius of half of the mean NND. Taking into account the upper and lower boundaries of INL, nuclei were counted in a circle around each DCP capillary (Figure 29A). LSM700 laser scanning confocal microscopes (Zeiss) with 40X objective lens was used to visualize immunolabelled inner nuclear layer neurons. Five Z-stacks, of 1-μm step wise, was scanned in each field analysed. The number of nuclei was counted manually with Photoshop CS6 (AdobeSystems, Inc.).

Random distribution simulation of the same sample size, mean and standard deviation was generated using Microsoft Excel. Data were plotted using Origin (OriginLab, Northampton, MA) and/or GraphPad Prism (version 8.0, GraphPad Software, San Diego, California, USA).

4.2.5 Statistical Analysis

Statistical analysis used in this chapter have been described in Section 2.2.10 and Section 3.2.7.
4.3 Results

4.3.1 Gliovascular Units in Control and Diabetic Retina

The gliovascular unit is composed of endothelial cells, pericytes and glial endfeet (from astrocytes and/or Müller cells). It provides a crucial functional coupling for the retinal vasculature, which is known to be disrupted in some retinal diseases such as DME and cystoid macular edema, both of which are sight threatening (Spaide, 2016; Daruich et al., 2018)

I firstly assessed whether gliovascular units were altered in human diabetic retina compared to control using a panel of markers for retinal glial cells. In this chapter, I focuses particularly on no DR diabetic retina. The DR retina has been carefully characterised in a previous study from our group (Yasvoina et al., 2017), thus will not be further investigated in the current study.

Figure 25 presents staining of a no DR diabetic from the diabetic with dropout subgroup (donor 10) and an age-matched control (donor 2). Müller cells were visualized with AQP4 (Figure 25A-B') and CRALBP (Figure 25C-D') and GS (Figure 25E-F') immunostaining. AQP4 was extensively expressed by the endfeet at the gliovascular units around vessels in the control retina (Figure 25A-A'). However, in the diabetic retinas, there was a universal upregulation of AQP4, spanning from OLM to ILM. But the staining intensity was slightly reduced at the gliovascular units (Figure 25B-B'). On the other hand, CRALBP and GS revealed cell bodies as well as processes around capillaries and endfeet reaching ILM and OLM. Staining pattern of these two markers did not vary hugely between control and diabetic retina (Figure 25C-F').

The other type of retinal glial cell, astrocytes, is important in contributing to the formation of the gliovascular unit at the NFL. GFAP positive astrocytes were found at
the NFL, especially surrounding vessels and occasionally projecting to the DVC (Figure 25G-G'). The same staining pattern was also seen in the diabetic retina (Figure 25H-H'), with no signs of gliosis, as reported by others (Mizutani et al., 1998).

Some heat shock stress proteins, such as CRYAA, were reported to be upregulated in diabetic and aged retinæ (Kase et al., 2011). Previous study from our group reported a similar result in a human DR retina (Yasvoina et al., 2017). In the current study, I found that CRYAA can also be a Müller cells marker, which especially labels processes in general, but not endfeet around vessels (Figure 25I-I'). This staining pattern or intensity was not obviously altered in the diabetic retina (Figure 25J-J').

To conclude, despite of a slight pan-retina upregulation of AQP4 in the diabetic retina, other markers did not reveal obvious changes in the retinal glial cells, especially at gliovascular units.
Figure 25. Retinal glial cells in control and diabetic human retina.

Immunohistochemistry using antibodies against AQP4 (A-B), CRALBP (C-D), GS (E-F), GFAP (G-H) and CRYAA (I-J) shows retinal glial cells in an age-matched control (donor 2: A, C, E, G and I) and a diabetic retina from the diabetic with dropout subgroup (donor 10: B, D, F, H and J). Markers of interest were also shown separately in black and white on the right of each merge image. Scale bar 50 μm.
4.3.2 Gliovascular Units in the Diabetic with Dropout Subgroup

As I have shown in the previous chapter, there is a higher level of capillary dropout in diabetic retina, yet little is known about whether gliovascular unit around these capillary dropouts are affected. Therefore, I chose the five most used retinal glial markers and compared their appearance around normal and dropout capillaries in the retina from the diabetic with dropout subgroup as defined in the previous chapter.

The retina is known to express a variety of different types of aquaporin channels, which belong to a water channel family that regulates water flux in and out of the cell (Schey et al., 2014). Amongst all, aquaporin 4 (AQP4) is found to be the most highly expressed at the endfeet of glial cells (Nagelhus et al., 1999). My results agreed with these findings, as I found high levels of AQP4 positive structures around vessels (Figure 26A). Although AQP4 staining could still be seen around nonperfused capillaries (Figure 26B-B’’, arrowheads), the staining intensity was clearly lower than that around normal capillaries (Figure 26B-B’’, arrow) in sections from the diabetic with dropout subgroup.

Cellular retinaldehyde–binding protein (CRALBP) is marker for Müller glia but has a different cellular distribution than AQP4. CRALBP positive staining was seen faintly around vessels but more intensely in the soma of Müller cells and their processes in the OPL (Figure 26C and D). CRALBP expression around Müller cell endfeet was visible in both normal (Figure 26D’’, arrows) and acellular capillaries (Figure 26D’’’, arrowheads). Yet no staining differences could be confidently drawn between cases and controls. Notably, CRALBP was also expressed abundantly by RPE, which is associated with their role in aiding the completion of visual cycle (Figure 26C).

Similarly, glutamine synthetase (GS) was highly expressed by glial endfeet at both ILM and OLM, and processes in the OPL (Figure 26E). Some GS positive Müller
cell processes were seen around vessels at the deeper plexuses (Figure 26F-F’’), but they were not as closely associated with the vasculature as AQP4. This might be due to the main function of GS in converting glutamate to glutamine, and reduced excitotoxicity in the extracellular space (Linser et al., 1979; Bringmann et al., 2013), thus implying a less vasculature-related role.

In summary, I was able to detect local glial changes (regarding AQP4 but not CRALBP and GS expression) associated with capillary dropout in diabetic eyes with very early DR phenotypes.
Figure 26. Müller cells in normal and dropout capillaries in diabetic retina.

Immunohistochemistry using antibodies against AQP4 (A and B), CRALBP (C and D) and GS (E and F) shows Müller cell processes around normal and acellular capillaries from a donor in the diabetic with dropout subgroup.

A, C and E are consecutive sections of donors from diabetic with dropout subgroup, showing the same region with localised capillary dropout. B, D and F are magnified images of boxed areas in A, C and E respectively. Arrowheads point at acellular capillaries and arrows show normal capillaries. (A-B'') AQP4 expression is specially concentrated around glial cell endfeet, which remained present around acellular capillaries (B'', arrowheads), but staining intensity was notably reduced. (C-D'') CRALBP was highly expressed in RPE and Müller cell bodies, and only faintly in Müller processes. Its expression was weak around vessels (D'', arrows and arrowheads). (E-F'') GS was highly expressed in Müller cell endfeet at the ILM and OLM, and less concentrated at the vascular interface than AQP4.

Scale bar 50 μm in E and 25 μm in F.
4.3.3 Reactive glial markers

Astrocytes wrap around blood vessels in the SVC and sometimes DVC capillaries residing at IPL/INL boarder. Studies using model systems to study DR have widely reported upregulation of GFAP and glial activation in the retina (Rungger-Brändle et al., 2000b; Mugisho et al., 2017), and this was confirmed by investigators using post-mortem human retinae (Carrasco et al., 2008). However, in our diabetic cohort this was not the case, except within a laser lesion in our advanced DR case (ARVO poster). We found no GFAP upregulation in Müller cells (indicating a reactive state), and GFAP expression in retinal astrocytes was not affected either by ghost vessels in the SVC or at the inner boundary of the DVC (Figure 27B‴, arrowheads).

Another useful glial marker is crystallin Aα (CRYAA), which is a heat shock protein that is commonly upregulated under cellular stress conditions. We found it to be considerably upregulated in the case of an advanced DR, especially at the site of laser scar. However, in diabetic donors without DR, I did not notice any changes in labelling intensity around nonperfused DVC capillaries (Figure 27D‴, arrowheads) compared to normal capillaries (Figure 27D‴, arrows).

Collectively, I used a panel of widely accepted Müller cell markers, AQP4, CRALBP and GS and reactive glial markers, GFAP and CRYAA, to assess the glial component in the gliovascular unit around normal and pathological capillaries in the DVC. Results revealed a reduced staining intensity of AQP4 around pathological DVC capillaries, but no major anatomical changes at the glia vascular interface in nonperfused DVC capillaries, as can be revealed by immunohistochemistry.
Figure 27. Astrocytes and reactive Müller cell endfeet in capillary dropout.

Immunohistochemistry using anti-GFAP (A and B) and anti-CRYAA (C and D) antibodies shows processes of retinal astrocytes and Müller cells around normal and acellular capillaries from a donor in the diabetic with dropout subgroup.

A and C were consecutive sections showing the same region with localised capillary dropout. B and D were magnified images of boxed areas in A and C, respectively. Acellular capillaries lack endothelial lining (B'-B'' and D'-D'', arrowheads), but the glial interface formed by retinal astrocytes (B-B'', arrowheads) and Müller cell processes (D-D'', arrowheads) remained the same in normal and acellular capillaries (B-B'' and D-D'', arrows).

Scale bar: 50 μm in C and 25 μm in D.
4.3.4 NND is Consistent amongst Individuals

Having found that there are only minor alterations at the glial-vascular interface, I then wonder whether deeper capillary dropout has more effects on neural cell numbers. I therefore attempted to test this hypothesis by counting nuclei around capillaries in the DVC.

In order to determine the potential zone of influence of a single DVC capillary, I measured the nearest neighbour distance (NND) of deeper capillaries. Results revealed that NND is constant across individuals (**Figure 28A**) \( (p = 0.635, \text{ ANOVA}) \), giving an average NND of 4.65-fold of the mean reference. Furthermore, distribution analysis of NND revealed consistency between control and diabetic subgroups. Although the peak of DR NND did not shift (median value does not change), it was clearly lower than other groups (**Figure 28B**). This can be further described by the regularity index (RI), defined as the ratio of the mean NND over its standard deviation. Despite consistent NND across all groups, the RI of DR showed statistically significant RI reduction \( (p < 0.05) \); whereas there was no difference amongst control and diabetic subgroups \( (p > 0.05) \) (**Figure 28C**).

4.3.5 NND Remains Unchanged across Different Retinal Regions

To assess whether NND changes with respect to retinal eccentricity, multiple sections with varying distance to the optic disc-fovea axis were analysed for each group. As shown in **Figure 28D**, NND plotted against the distance to the optic disc-fovea axis showed no changes in the superior-inferior axis (DR and diabetic no dropout subgroup not shown).
**Figure 28. DVC capillary zone of influence.**

A, NND was calculated for each donor and then normalized to the height of the nuclei of RPE cells (over 10 RPE cells were measured per donor and the average value was used), so that results express fold change to the reference value. No difference was found between any two groups, meaning that the NND is consistent across groups. B, Frequency plot of the NND reveals an overlapping distribution pattern in control and two diabetic subgroups, while lower peak of the DR NND distribution was noticed. The dashed line shows the overall normalized mean NND of 4.65. C, Regularity index, calculated as mean/s.d. tends to decrease with increased severity of capillary loss. No difference was found amongst control and subgroups of diabetics, whereas RI of DR was significantly different from every one another. D, The NND was plotted against the distance from the optic disc-fovea axis along the superior-inferior axis. No linear relationship could be found in any group (diabetic no dropout subgroup and DR not shown). N = 3 (control), 4 (diabetic no dropout), 5 (diabetic with dropout), 1 (DR). Results are presented as mean ± s.d., n.s. denotes not significant and *P < 0.05. Statistical significance was tested by one-way ANOVA with Tukey’s post hoc comparison (A, C). Abbreviations: LOI, line of identity; NND, nearest neighbour distance.
4.3.6 INL Cell Loss in Early DR is Irrespective of DVC Dropout

Having determined the NND as a definition of deeper capillary zone of influence, I quantified the cells within a circular area (with the NND x 0.5 as its radius) from each capillary in the DVC. An example is shown in Figure 29A. Results showed a comparable number of cells around normal and nonperfused capillaries in diabetic with dropout subgroup (12.27 ± 1.80 vs 12.39 ± 1.65, p = 0.9994) and DR (8.42 ± 2.33 vs 8.35 ± 1.33, p > 0.999). However, compared to control, there was a general loss of 7% INL cells in the diabetic with dropout subgroup (13.25 ± 2.27 vs 12.33 ± 1.71, p = 0.457), which increased to 37% in DR (13.25 ± 2.27 vs 8.38 ± 1.89, p < 0.0001). Taken together, these results suggest that in diabetic patients with no DR, there is a subtle but statistically significant loss of INL cells in the diabetic group, which becomes much pronounced in DR. Importantly, this effect was pan-retinal and independent of local capillary dropout (Figure 29B).

4.3.7 Around 10% INL Interneurons are Lost at Early DR

Functional assessments revealed that cells contributing to the ERG b-wave may be affected in diabetic patients and diabetic rodent models. Thus, I used antibodies raised against PV and PKCα to label horizontal (Figure 29C) and bipolar cells (Figure 29E), respectively. Quantifying these interneurons within deeper capillary zones of influence showed result in line with our previous finding that cell loss is independent of local capillary dropout (Figure 29D and F). Of note, in the diabetic with dropout subgroup, a loss of around 10% PV+ horizontal cells (3.63 ± 1.07 vs 3.13 ± 0.74, p = 0.31) and PKCα+ bipolar cells (4.75 ± 1.39 vs 4.27 ± 0.65, p = 0.51) compared controls was identified. Although this difference was not statistically significant, it represented an interesting trend, because this cell loss was much more profound in the DR case, where around 60% of both interneuron types were lost compared to that in control (PV+ cells: 3.63 ± 1.07 vs 1.36 ± 0.84; PKCα+ cells: 4.75 ± 1.39 vs 1.97 ±
1.09; \( p < 0.0001 \) for both) (Figure 29D and F).

4.4 Discussion

In this chapter, I explored a potential correlation between localised neural cell loss and capillary dropout in the DVC.

4.4.1 Disruption of gliovascular units

Firstly, I checked whether localised DVC dropout directly impacted the glial interface surround capillary dropout. The main finding from this section was a downregulation and possible re-distribution of AQP4 at Müller endfeet, which, under healthy conditions, is concentrated at the glial-vascular interface where it contributes to water homeostasis in the retina (Nagelhus et al., 1999). This plays an important role in the development of DME (Daruich et al., 2018). Yet, how much downregulated AQP4 contributes to the oedema in diabetic patients remains unknown.

It is commonly reported that the BRB is also compromised in diabetic model systems (Robinson et al., 2012), which may also contribute to vascular leakage and oedematous changes. However, in my diabetic tissue cohort, I used antibodies raised against human IgG and did not notice a signs of serum leakage into the retina on cross sections (data not shown).

4.4.2 Neurodegeneration

Secondly, the survival of cells around capillary dropout may be reduced due to a lack of oxygen and nutrients. For instance, Bek (1994) found that at sites of capillary dropout, histological cross sections showed pronounced tissue loss in the INL. However, it remains uncertain as to what extent the DVC contributes to the oxygen supply in the INL.
Figure 29. Identifying overall and subpopulation cell loss in the INL.

A, C and E, confocal microscopy image showing the measurements of INL cell nuclei within the zone of influence of DVC capillaries (circle, A). Examples of normal capillaries (magenta counting marks) and nonperfused capillaries (green counting marks) are shown. Horizontal (C) and bipolar cells (E) were visualized using antibodies against PV and PKCa, respectively. B, D and F, quantification of total cells (B) and interneurons (D and F) within deeper capillary zones of influence. Normal capillaries are shown by empty boxes, nonperfused capillaries are shown by filled boxes.

N = 3 (control), 4 (diabetic no dropout), 5 (diabetic with dropout), 1 (DR). Box represents 25th, median and 75th quartile, “+” shows the mean value. Whiskers represent max and min. n.s. denotes not significant and * P < 0.05, *** P < 0.0001. Statistical significance was tested by unpaired two-tailed Student's test (intragroup differences) or one-way ANOVA with Dunnett’s post hoc comparison with control (intergroup differences). Scale bar 25 μm.
Some clinical studies suggested a spatial association between disruption of the outer retina on OCT to the macular nonperfusion on FA in cases of NPDR (Scarinci et al., 2015, 2016), other studies showed that the outer retina receives most of its oxygen supply (around 90%) from the choroidal vasculature (Ahmed et al., 1993; Birol et al., 2007). Some suggested that the choriocapillaris blood flow is significantly slower in diabetic rats (Braun et al., 2009) and that choroidal blood pressure is reduced with increasing DR severity in human (Langham et al., 1991; Savage et al., 2004). Clinical imaging study also revealed nonperfused choroidal patches in human diabetic patients (personal communications with Edward Adams from UCL Institute of Ophthalmology). Yet diabetic rodent models presented that oxygenation of the outer retina is not affected diabetes (Lau et al., 2014).

In contrast, the oxygen tension in the inner retina is only around 1/3 of that in the outer retina (Linsenmeier et al., 2017) and the DVC may be an evolutionary adaptation to alleviate the lack of oxygen in the INL. Diabetic rat model have provided some insights that the oxygen consumption of inner retina is decreased in diabetic rabbits (Sutherland et al., 1990) and cats (Linsenmeier et al., 1998). It is further suggested that such reduced oxygen consumption lowers retinal flow (Small et al., 1987; Rimmer et al., 1993), as has been reported in diabetic patients (Clermont et al., 1997).

Results presented in the current chapter suggest that in diabetic retinæ, there is a general loss of INL cells, notably PKC+ bipolar cells and PV+ horizontal cells irrespective of the sites of DVC dropout. Although such cell loss was subtle (10%) in diabetic eyes without DR, the changes were much more pronounced in our advanced DR case, in which half of all INL cells were lost, with around 30% loss in bipolar and horizontal cells. This finding concurs with thinning of retinal nuclear layers in rodent diabetes models (Martin et al., 2004; Zhang et al., 2008) and clinical screening studies
Although it has been shown that there is increased neuron apoptosis in rodent diabetic models (Martin et al., 2004; Losiewicz et al., 2011) and in human post-mortem tissues (Carrasco et al., 2008), universal cell loss has not been confirmed in the STZ-induced mouse model (Zhang et al., 2008; Énzsöly et al., 2015; Moore-Dotson et al., 2016) or ZDR rat model of diabetes (Johnson et al., 2013; Szabó et al., 2017). However, these animal studies quantified the number of cells within a quite large area (~1mm). According to functional studies that the early functional abnormalities appear in small patches early in DR (Han et al., 2004), the significance of local cells loss will be easily diluted in a wider region. Therefore, the approach I took in the current study was to narrow down the effect of single dropout DVC capillaries to a circular region with a radius of no greater than 20 μm in order to detect and preserve the significance of subtle changes.

Akin to neurodegeneration in the brain, it is also possible that synapses start to be affected first, as they are also proposed to be strong candidates of generating the OP signal in ERG (Heynen et al., 1985). Observations from Sohn et al. (2016) also seem to support this idea, as they reported a significantly thinner NFL in diabetic post-mortem eyes whilst RGC density did not differ. In addition, Lavia et al. (2019) reported that SVC vessel density positively correlates to ILM/IPL thickness, yet a similar correlation was not revealed between DVC and INL/OPL thickness. However, given the heterogeneity of the types of synapses in human retina, this hypothesis is difficult to validate directly with post-mortem human retinal tissues.

### 4.4.3 Limitations

Abnormal cells, such as senescent cells, do not directly contribute to a loss of INL cells, but they may be functionally impaired, thus can potentially influence ERG results. Studies have revealed a greater number of senescent cells, especially
neurons and vascular cells, in older human post-mortem retina tissues, these cells were also found to be spatially associated with retinal microaneurysm (López-Luppo et al., 2017). In addition, detecting the activity of senescence associated β-galactosidase enzyme (SA-β-gal) has been used to identify senescent cells. It has been reported that diabetes can induce cell senescence especially in the inner retina (Rojas et al., 2017) and isolated retinal vasculature (Shosha et al., 2018).

Attempts have been made in this study to detect senescent cells in our post-mortem tissues using canonical cell cycle markers such as p16 and p21, as reported in López-Luppo et al., (2017). However, staining results had high background and were not satisfactory, possibly due to prolonged fixation delay of our tissue. Optimisation of the protocol is needed to achieve better results. Moreover, efforts have also been made to detect SA-β-gal activity in our tissues. Yet this was not feasible in our study, as the technique requires fresh tissue with short fixation delay and fixation time (Levitsky et al., 2013) or snap frozen tissue (Kuilman et al., 2010) to preserve the enzyme’s activity. In order to identify apoptotic cells, I have also tried to stain our tissue with anti-activated caspase 3 antibodies, which did not yield any useful data, possibly because this molecule is short-lived. TUNEL staining, which detects DNA breaks formed during apoptosis, would be a better choice; however, my attempts to do this were hindered by the COVID19 pandemic.
4.5 Summary

In this chapter, I determined the zone of influence of DVC capillaries by taking the nearest neighbour approach. This zone was shown to be consistent across individuals. INL cells were then quantified within the zone of influence of normal and acellular DVC capillaries. Results revealed a 10% panretinal cell loss, including bipolar cells and horizontal cells, in the INL of diabetic retinas. However, this cell loss was independent of presumed local DVC dropout. This study provides histological evidence from human tissue supporting the hypothesis that neurodegeneration may precede capillary dropout in diabetic retinas without DR.
Chapter 5.

Conclusion Remarks and Outlook
5.1 Final remarks

In this thesis, I investigated the vascular and cellular changes in post-mortem tissues in the early phase of two common retinal ischemic diseases, ROP and DR.

In Chapter 2, I have advanced our understanding about the histopathology of eyes from human premature infants receiving oxygen therapy. I performed morphometric analysis on human premature retinal vasculature and revealed changes in the branching profile and capillary free zones. I provided quantitative evidence on how the retinal vascular network may be altered by increased or decreased levels of oxygen. In addition, I have elucidated the spatial correlation of retinal astrocytes, proliferating cells, the growing vasculature and activated microglial cell. My analysis also draws attention to a potentially important (but so far neglected) pathogenic contribution from retinal astrocyte gliosis in ROP. Finally, this study provides the first direct line of evidence showing strongly elevated expression of VEGF at the ridge and vanguard in the ROP retina which correlated closely with PAX2.

In Chapter 3 and Chapter 4, I have developed a method to assess the level of vascular dropout in human post-mortem retinal tissue, which provided direct histology evidence showing a considerable level of vascular damage especially in the deeper plexus of the retinal vasculature in the diabetic retina. Moreover, I have found a subtle but significant level of INL cell loss in diabetic eyes that is independent of localised influence from deeper capillary dropout. Taken together, my results contribute to another line of evidence supporting the hypothesis that neurodegeneration precedes vascular lesion in DR. My research on DR have made a significant contribution to our current knowledge on human DR. As such, my work investigating the incidence of capillary dropout has been presented on international conferences twice (ARVO 2018 and EASDec 2020) and so does my work on the consequence of dropout (ARVO 2019).
5.2 Future Work

5.2.1 Characterising Disturbed Cell-Cell Interaction

Due to technical reasons, in situ hybridization results presented in this thesis were very limited. An obvious future avenue to explore will be to investigate expression of other signalling molecules that could potentially influence the astrocyte-endothelial cell interaction at early and late postnatal tissues. Selvam et al. (2017) have reviewed such interaction networks, according to which we have purchased probes for future in situ hybridization. This includes, but is not limited to, genes that are involved in the cross talk between endothelial cells, pericytes and retinal astrocytes (PDGFA, PDGFB, PDGFRA and PDGFRB) (Fruttiger et al., 1996), or are likely to take part in the genesis of developmental retinal vascular pathologies (such as NDP and NPR1), or those contribute to the astrocyte-endothelial cross talk during development (APLN, APLNR and LIF) (Sakimoto et al., 2012).

5.2.2 Recreating Human ROP in Rodents

We have found extensive retinal astrocytes hyperplasia in the eyes from premature infants received oxygen therapy. However, a similar gliotic response is not present in classic OIR model systems (Bucher et al., 2013; Lajko et al., 2016; Duan et al., 2019). However, McMenamin et al. (2016) and unpublished work from our group showed that exposing mouse pups to 65% hyperoxia from P0 to P7 can lead to some gliosis. Recently, an intriguing study from Perelli et al. (2019) reported that exposing mouse pups from P0 to P4 in 75% hyperoxia followed by normoxia generates vascular and astrocytic features resembling those observed in human ROP retina. Previous work from our lab, exposing mouse pups to hyperoxia at younger ages followed by returning to normoxia, has also shown a range of phenotypes and some presented arrested vasculature at P14. It is unclear whether this is the case for
neonatal OIR model from Perelli et al. (2019), therefore, results from their study will need to be carefully validated. In the meanwhile, attempts have been made in our group to expose mouse pups from P0 to different postnatal time points in order to find the optimal protocol that could recreate over-proliferative retinal astrocytes in the mouse model. Once this have been achieved, studying the role of these astrocytes and glial-vascular interaction in ROP-like mice might be possible.

5.2.3 Changes in Müller Cells and Apoptotic INL Cells

After having identified universal reduction of INL neurons in the diabetic retina, it would be interesting to also quantify Müller cell numbers, as they are also known to be affected by diabetes. Moreover, Carrasco et al. (2008) have identified an increase in TUNEL+ cells in the diabetic post-mortem retina, and notably in the INL. However, the level of dropout, especially at the DCV, is unknown. In addition, aging is one of the main driving factors for cell apoptosis (Horan et al., 1995). Therefore, using our well characterised tissues included in the current thesis, it will be possible to evaluate whether diabetes induces cell apoptosis in the INL. Using double-staining, would allow to pin down the subtype of INL cells susceptible to apoptosis. I hope that completion of the additional experiments outlined here will provide a comprehensive view of INL cell loss during early DR in human.

5.2.4 Senescent Cells in Ischemic Retinopathies

A vital question to ask in the cases of ischemic retinopathy is what triggers the vascular pathology at a specific location? A recent study proposed a pathogenic role of senescent cells and neutrophils in the retinal vasculature. By using the OIR mouse model, researchers show that neutrophils invade pathological vasculature and exude extracellular traps. Senescent endothelial cells were shown to stimulate the release of these extracellular traps from neutrophils via a senescent secretory pattern with
upregulated cytokine-related genes (Binet et al., 2020). Elevated levels of cytokines can recruit immune cells such as leukocytes. In experimental diabetic monkeys, accumulated leukocytes in the capillary were found upstream to capillary occlusion sites (Kim et al., 2005), and more intriguingly in the DVC (Schröder et al., 1991).

Although the cytokines will be tricky to be directly revealed by immunohistochemistry, it would be really intriguing to identify whether there are more endothelial cells in the diabetic with dropout subgroup underwent or are undergoing cellular senescence, which will exaggerate the release of cytokines from neighbouring neutrophils.

5.2.5 Testing Hypotheses on the Genesis of DR

Based on the results presented in the current thesis, we have received a PhD studentship funded by Moorfields Eye Charity to continue investigating the mechanism of DR by using human post-mortem samples.

To start with, I have reviewed a number of hypotheses on the genesis of DR which had or are undergoing clinical trials discussed in Section 1.3.3 and Appendix A as part of the current thesis, there are more to be updated. One lesson we learnt from the current thesis was to make the most out of human samples and learn from human diseases. The emerging new therapeutic strategy targeting the KKS system, described in Section 1.3.4, is backed up by extensive histological and clinical studies (Ma et al., 1996; Kim et al., 2007; Gao et al., 2008) and has received promising results in treating angioedema (Bork et al., 2007; Schneider et al., 2007). In fact, this KKS story started from the discovery of upregulation of carbonic anhydrase with DR severity; subsequent animal studies also validated the role of carbonic anhydrase in inducing vascular permeability (Gao et al., 2007). This investigation strategy of valuing and initiating ideas by studying human diseases is in accordance with what we have been complying with in this thesis.
The emerging omics technique have made learning from tissue more worthy. In the recent few years, our knowledge on the retina has been expanded greatly by omics technique. For instance, proteomics on human post-mortem retina tissue revealed differential metabolic preferences in the centre versus periphery, which provides mechanistic insights into disease susceptibility of different regions (Velez et al., 2018). Furthermore, the single cell transcriptomics have broadened views on progenitors during retinal development (Hoshino et al., 2017; Lu et al., 2020) and molecular profile of retinal cells in the adult human retina (Lukowski et al., 2019).

Using these omics technology, there are few things worth investigating in the context of ischemic retinopathies:

1. In NPDR cases, perform single-cell transcriptome analysis on samples from defined ischemic region and nearby perfused, normal region. Which genes expression are altered in the ischemic region? Can also perform KEGG or ontology analysis on the results to see what pathways are specifically involved.

2. On cross sections cutting through a defined area of ischemia, performing spatially resolved transcriptomics (Shah et al., 2016; Vickovic et al., 2019) and metabolomics (Sun et al., 2019) can provide information on altered gene expression and/or metabolites in the pathological areas compared to normal areas.

There are many other objectives worth exploring on human post-mortem tissues, and these emerging omics techniques have made learning from human more efficient than ever before.
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Appendices
Appendix A. Clinical Trials of Diabetic Retinopathy

The table below presents promising clinical trials acting on several pathways that are shown to be effective in treating experimentally induced diabetes related DR vascular and/or neuronal problems. This was researched by Qian Yang.

Table 8. List of clinical trials on diabetic retinopathy.

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Severity Description</th>
<th>Size</th>
<th>Duration (year)</th>
<th>Key findings</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-inflammation agents</td>
<td>Bilateral mild-to-severe NPDR or early PDR with/without DME</td>
<td>3711</td>
<td>7</td>
<td>• Aspirin did not reduce the risk of vision loss compared with placebo.</td>
<td>(Early Treatment Diabetic Retinopathy Study Research Group, 1991)</td>
</tr>
<tr>
<td>650 mg aspirin tablets vs placebo</td>
<td>Persistent DME involving central fovea persisting ≥ 3 months after adequate laser treatment</td>
<td>69</td>
<td>5</td>
<td>• More TA treated eyes achieved long term improvements in VA ≥ 5 letters than placebo group.</td>
<td>(Sutter et al., 2004; Gillies et al., 2009)</td>
</tr>
<tr>
<td>4 mg TA vs saline</td>
<td>DME involving central fovea</td>
<td>840</td>
<td>2</td>
<td>• Eyes treated with higher dosage of TA had more prominent improvement of VA.</td>
<td>(Diabetic Retinopathy Clinical Research Network, 2008)</td>
</tr>
<tr>
<td>1 mg or 4 mg TA injection vs focal/grid photocoagulation</td>
<td>DME involving central fovea</td>
<td>196</td>
<td>4</td>
<td>• More patients with FA implant had improvement in VA ≥ 3 lines at 6 months, but such benefit was lost after 3 years.</td>
<td>(Pearson et al., 2011)</td>
</tr>
<tr>
<td>Description</td>
<td>n</td>
<td>Duration</td>
<td>Notes</td>
<td></td>
<td></td>
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<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>0.2 or 0.5 µg/d FA insert (Iluvien) vs sham injection with/without rescue</td>
<td>953</td>
<td>3</td>
<td>Patients with baseline DME history of ≥ 3 years had doubled benefit from FA insert. Adverse effects: Eyes with FA inserts were more likely to require cataract surgery; around 40% FA implanted eyes had adverse effect caused by increased IoP. Iluvien was proved by FDA for treating DME patients without significant elevation of IoP with steroids (2014). (Campochiaro et al., 2012)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.35 or 0.7 mg of dexamethasone implant (Ozurdex) vs sham injection</td>
<td>1048</td>
<td>3</td>
<td>Mean reduction of CRT was more significant in Ozurdex treated group. Adverse effects: Incidence of cataract was three times higher in treated group; around 35% patients treated with Ozurdex developed IoP elevation, which was slightly lower than using FA implants. (Boyer et al., 2014)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.7 mg of Ozurdex implant vs 1.25 mg ranibizumab</td>
<td>88</td>
<td>1</td>
<td>The fraction of patients gaining ≥ 10 letters in BCVA was comparable in two groups. Ozurdex treated eyes showed more evident reduction in CRT than those treated with ranibizumab. Adverse effects: 11% implanted eyes lost ≥ 10 letters, mainly due to cataract. (Gillies et al., 2014)</td>
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**Neuroprotective agents**

<table>
<thead>
<tr>
<th>Description</th>
<th>n</th>
<th>Duration</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>400 µg/day octreotide 12-25 years of T1D history, mild DR</td>
<td>20</td>
<td>1</td>
<td>Difference in DR severity before and after treatment was unchanged. (Kirkegaard et al., 1990)</td>
</tr>
<tr>
<td>200-5,000 µg/d subcutaneous octreotide vs conventional diabetes treatment</td>
<td>46</td>
<td>1.25</td>
<td>Fewer Octreotide treated patients progressed to high risk PDR. (Grant et al., 2000)</td>
</tr>
</tbody>
</table>
The incidence of vitreous haemorrhage was significantly lower in octreotide treated eyes after 3 years.

VA was well maintained in octreotide treated group, while that in control arm significantly decreased (Boehm et al., 2001)

### PKC inhibitors

<table>
<thead>
<tr>
<th>32 mg/d Ruboxistaurin vs placebo</th>
<th>Moderate to severe NPDR</th>
<th>685</th>
<th>3</th>
<th>Reduced risk of moderate visual loss in treated group, but result was not statistically significant.</th>
<th>Did not delay DR progression</th>
<th>(The PKC-DRS Study Group, 2006)</th>
</tr>
</thead>
</table>

| 32 mg/d Ruboxistaurin vs placebo | DME | 686 | 3 | No notable difference in DME progression between two groups. | (The PKC-DMES Study Group, 2007) |

**Abbreviations:**

BCVA, best corrected visual acuity; CRT, central retinal thickness; DME, diabetic macular oedema; FA, fluocinolone acetonide; FTH, foveal thickness; IoP, intraocular pressure; PRP, panretinal photocoagulation; SOC, standard of care (additional laser or observation); TA, triamcinolone Acetonide; VA, visual acuity.
### Appendix B. Ethical Approval from Shenzhen Eye Hospital

<table>
<thead>
<tr>
<th>批号</th>
<th>深眼伦理20190717-02号</th>
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<tbody>
<tr>
<td>项目名称</td>
<td>单纯视网膜脱离玻璃体手术研究</td>
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<tr>
<td>研究单位</td>
<td>深圳市眼科医院</td>
</tr>
<tr>
<td>主要研究者</td>
<td>张国明</td>
</tr>
<tr>
<td>审查类别</td>
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<td>□紧急会议审查</td>
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<td>审查日期</td>
<td>2019年7月17日</td>
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</tr>
<tr>
<td></td>
<td>2. 试验方案（20190705）：</td>
</tr>
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<td></td>
<td>3. 知情同意书（20190705）。</td>
</tr>
<tr>
<td>出席人员</td>
<td>到会14人，回执1人，缺席0人</td>
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</tbody>
</table>

审查意见

根据国家《涉及人的生物医学研究伦理审查办法（试行2007）》、《药物临床试验质量管理规范（2003）》、《医疗器械临床试验规定（2004）》、WMA《赫尔辛基宣言》和CIOMS《医学伦理学研究国际道德指南》的伦理原则，经本伦理委员会审查同意按所批准的临床研究方案，知情同意书、招募材料及样本研究。

请遵循GCP原则，遵循伦理委员会批准的方案开展临床研究，保护受试者的健康与权利。研究开始前，请申请人完成临床试验注册。研究过程中，若变更主要研究者，对临床研究方案、知情同意书、招募材料等的任何修改，请申请人提交修正案审查申请。发生严重不良事件时，请申请人及时提交严重不良事件报告。

请按照伦理委员会规定的年度和定期跟踪审查频率，申请人在截止日期前1个月提交研究进展报告；亦可向研究安全报告委员会提交各中心研究进展的汇总报告；当出现任何可能显著影响试验进行或增加受试者危险的情况时，请申请人及时向伦理委员会提交书面报告。

研究纳入了不符合纳入标准或符合排除标准的受试者，符合中
Figure 30. Ethical approval for the ROP study.

This scanned document shows the ethical approval from Shenzhen Eye Hospital (深圳眼科医院) for the using of premature eyes collected at Shenzhen from Dr. Guoming Zhang (张国明) group for histology study. This approval was obtained on 5th August 2019. Approval code is 20190717-02.
Appendix C. UEA stained whole mount image of case 1.
Appendix D. UEA stained whole mount image of case 2.
Appendix E. UEA stained whole mount image of case 3.
Appendix F. UEA stained whole mount image of case 4 (ROP).
Appendix G. GFAP staining of the ROP retinal whole mount.

![Figure 31. GFAP immunolabeling on ROP whole mount.](image)

Co-labelling of the ROP retina whole mount tissue with GFAP and UEA to reveal mature astrocytes and vasculature, respectively (A). Dense GFAP+ gliotic process networks (A’) are posterior to the protruding vessels from the tufts (A’’). Due to extensive haemorrhage in this region, staining results have high background in both channels. Scale bar is 100 µm.
Appendix H. *VEGF and KDR* expression in a normal developing eye.

**Figure 32.** *VEGF and KDR* expression in a normal developing eye.

**A,** Immunohistochemistry with anti-PAX2 and -UEA antibody showing retinal astrocytes and vasculature, respectively.

**B and C,** *In situ* hybridization using probes designed against *VEGF* and *KDR* genes. Dashed line shows the approximate location of the most distal vessel according to A. Note the strong VEGF expression around and anterior to the developing distal vasculature (B). In contrast, KDR is steadily expressed at the INL throughout the retina (C).

Consecutive sections are used and aligned; red scale bar represents 1mm.
Appendix I. Whole mount images of UEA-stained human retinas
Figure 33. Collection of UEA-stained whole mount images of all donors.

This figure presents UEA-staining of all donor eyes used in this thesis, donor number corresponds to that shown in Table 6. All eyes were subject to whole mount UEA staining as described in Section 3.2.4.1, image as described in Section 3.2.5. Raw images were processed as described in Section 3.2.6. All whole mounts are equally scaled. Scale bar shown in the DR eye (donor 14) is 1mm.

Abbreviation: DR, diabetic retinopathy

People contributed to the production of these whole mount images: Qian Yang (43%), Marina Yasvoina (36%), Meaghan O'Neill (14%) and Almas Dawood (7%).
Appendix J. List and comments of other antibodies tested

The following antibodies have also been tested for immunostaining on sections during my PhD. Although results from them were not shown in the main context of the thesis, it may provide useful information to others to learn from my studies.

Table 9. List of other antibodies tested during PhD.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalogue number</th>
<th>Comments</th>
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<tbody>
<tr>
<td>B-galactosidase</td>
<td>Abcam</td>
<td>AB6645-1</td>
<td>Did not work ¹</td>
</tr>
<tr>
<td>B-galactosidase</td>
<td>Cortex</td>
<td>CR7001RP1</td>
<td>Did not work ¹</td>
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<tr>
<td>Caspase-3</td>
<td>Abcam</td>
<td>AB2302</td>
<td>Did not work ¹</td>
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<tr>
<td>ChX10</td>
<td>Exalpha</td>
<td>X1179P</td>
<td>Supposed to stain bipolar cells, but result had heavy background and varied between paraffin and frozen sections, not reliable, thus discarded ¹</td>
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<tr>
<td>Fibronectin</td>
<td>Chemicon</td>
<td>AB2033</td>
<td>Basement membrane ¹</td>
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<tr>
<td>FoxO1</td>
<td>Cell Signaling</td>
<td>2880T</td>
<td>Choroidal capillary ¹</td>
</tr>
<tr>
<td>Flt-1/VEGFR1</td>
<td>Santa Cruz</td>
<td>SC9029</td>
<td>Did not work ¹</td>
</tr>
<tr>
<td>GNAO1</td>
<td>Chemicon</td>
<td>MAB3073</td>
<td>Plexiform layer in differentiated regions ³</td>
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<td>HNF-4α (H-1)</td>
<td>Santa Cruz</td>
<td>sc-374229</td>
<td>Did not work ¹</td>
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<td>Insulin Receptor α</td>
<td>Santa Cruz</td>
<td>sc-57344</td>
<td>Did not work ¹</td>
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<tr>
<td>Islet1</td>
<td>Abcam</td>
<td>ab20670</td>
<td>Amacrine cells in differentiated regions ³</td>
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<td>KCNJ10</td>
<td>Sigma</td>
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<td>KCNJ10</td>
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<td>Did not work ¹</td>
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<td>Chemicon</td>
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<td>Kir4.1</td>
<td>Alomone</td>
<td>APC-035</td>
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<td>LHX2</td>
<td>Invitrogen</td>
<td>PA5-78287</td>
<td>Interneuron, possibly bipolar cells, at INL of differentiated regions 3</td>
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<td>mGluR6 / GRM6</td>
<td>novus</td>
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<td>A small subset of interneurons and strong at photoreceptors 3</td>
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<td>ONECUT2/OC-2</td>
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<td>Horizontal cells 3</td>
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<td>abcam</td>
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<td>Santa Cruz</td>
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<td>PAX2 (3C7)</td>
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<td>Abcam</td>
<td>ab79839</td>
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<td>PDGFR-β</td>
<td>R&amp;D systems</td>
<td>AF385</td>
<td>Did not work with any AR 2 on paraffin sections, but worked well on OCT frozen sections without AR.</td>
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<td>BioRad</td>
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<td>PPARα</td>
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<td>Stains some nuclei at GCL (intense) and most INL nuclei and photoreceptor nuclei 1</td>
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<td>Horizontal cells and some bipolar cells 3</td>
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<td>Thrombin</td>
<td>Novus</td>
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<td>invitrogen</td>
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<td>ab33157</td>
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<td>NBP2-34494</td>
<td>Endothelial cells, especially around large vessels, barely in capillaries</td>
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</table>

Abbreviation: AR, antigen retrieval; INL, inner nuclear layer; NFL, neural fibre layer; GCL, ganglion cell layer

1: these antibodies have only been tested with heat induced AR method using sodium citrate (pH 6.0) in paraffin embedded tissues

2: by saying any AR, I meant this antibody has been tested with all of the AR methods below, and none yielded reliable and/or conclusive staining results.

Protease induced AR: proteinase K or trypsin (both at 0.05% for 20min at 37 degree Celsius)

Heat induced AR: Tris-EDTA (pH 9.0); sodium citrate (pH 6.0 or 9.0); EDTA (pH 8.0)

Others: KMnO4 followed by oxalic acid.

3: these antibodies have only been tested on sections from 21-week GA and/or the ROP eye sections without AR.
This is the end of the thesis.