Retinal Microvasculature and Vortex Vein System Structure in Relation to Function and Pathogenesis

PhD thesis

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Summary

The precise architectural structure of the dual blood supply to the retina is intimately linked with its function, and arguably has the most important role in retinal cell survival and function through the fastidious delivery of oxygen and nutrients, and removal of metabolic waste. Retinal vascular diseases, including diabetic retinopathy, venous or arterial vascular occlusive disease, hypertensive retinopathy, inflammatory vascular disease, and retinopathy of prematurity, are a source of extensive morbidity and disability worldwide.1,2 They are associated with systemic cardiovascular diseases including ischaemic heart disease, hypertension and hypercholesterolaemia.3-10 Whilst it is generally anticipated that the highest metabolic demands of the inner retina correlate with the greatest supply of retinal microvasculature,11 this complex capillary structure relating to the heterogeneous metabolic demands of neuronal elements has not been clearly delineated in normal and diseased eyes. Additionally, it is the choroid that supports the outer retina’s high metabolic demands, with the entire choroidal circulation draining through the vortex vein system. There is an enormous paucity of information regarding this significant but undervalued vortex vein system, with little to no information on general morphology, endothelial phenotypic structure and endothelial structural barrier.

This thesis provides new, detailed information concerning the quantitative distribution of microvasculature within normal and diseased states of the human retina, as well as the regional heterogeneity of endothelial cells within the porcine vortex vein system.
Investigations performed on the normal and diseased human retina employing state-of-the-art-technology that permits continual monitoring of the intraocular pressure, perfusion pressure and perfusion flow rate, enabled visualisation of the precise in situ location of capillary networks within the different layers of the retina. From these experiments it was revealed that there were morphometrically different capillary networks located in the 1) nerve fibre layer, 2) retinal ganglion cell layer, 3) border of the inner plexiform layer and superficial boundary of the inner nuclear layer, and 4) boundary of the deep inner nuclear layer and outer plexiform layer. Additionally, quantification of the normal human capillary diameter and density between these four different networks showed significant differences between capillary diameters in all four networks, as well as the retinal ganglion cell layer accommodating the greatest capillary density.

In human donor eyes known to have a medical history of cardiovascular comorbidities but no known ocular disease, an association was found with significant changes in the capillary density of the inner retina of those with cardiovascular disease when compared with normal human eyes. Interestingly, even though the human donor eyes of those with cardiovascular disease had no known ocular pathology, vascular abnormalities were noted corresponding to the clinical appearances of microaneurysms, tortuous venules, and other retinal changes. This highlights that understanding retinal microvascular changes prior to clinical manifestation will enable insight into the prevention of retinal vascular diseases and resultant vision loss.

This thesis also demonstrates a new custom-built prototype speckle variance optical coherence tomography device as a potential clinical diagnostic tool in
accurate qualitative and quantitative retinal angiography in humans. This device was used to generate en face retinal capillary networks corresponding to the four retinal capillary networks identified in our previous studies, and exhibited the capacity to provide histology-like anatomical information about the human retinal capillary networks in vivo. This device may have great potential as a research and diagnostic tool in the management of retinal vascular diseases.

Application of our novel perfusion technique from the human retina to the porcine experimental model enabled the general structure and endothelial cell morphology of the vortex vein system to be described for the first time. Observations from this study revealed heterogeneity in endothelial cell morphology and a lack of smooth muscle cells in the ampulla region. This likely reflects the highly varied haemodynamic conditions and potential blood flow control mechanisms in the different regions of the vortex vein system.

Finally, experiments conducted on the endothelial cell f-actin, claudin-5, vascular endothelial cadherin and phosphorylated tyrosine labelling within the porcine vortex vein revealed regional phenotypic heterogeneity. Additionally, intramural cells were observed which were immune-positive for vascular endothelial cadherin and phosphorylated tyrosine. These findings provide novel insights into the potential regions of vulnerability and pathogenic mechanisms in the vortex vein system.

This thesis comprises of eight chapters. The first chapter introduces the entire thesis. Chapter 2 details the methodology for the acquisition of human and porcine eyes, microperfusion, immunohistochemical staining, tissue preparation and microscopy imaging techniques. Chapter 3 investigates the quantitative
distribution of blood vessels in different neural layers of the human retina. 

Chapter 4 reports the quantitative changes seen in the retinal microvasculature of patients with cardiovascular comorbidities but no known ocular disease, and compares this to the findings found in the third chapter. Chapter 5 correlates the human retinal capillary network information derived from a prototype speckle variance optical coherence tomography device with the histological findings found in the third chapter to determine the utility of this instrument for quantitative angiography. Chapter 6 details the findings of region-dependent endothelial heterogeneity in the porcine vortex vein system. Chapter 7 describes the regional differences present in endothelial cell f-actin, claudin-5, VE-Cadherin and phosphorylated tyrosine labelling within the porcine vortex vein system. Chapter 8 is the grand discussion integrating all the results.

Most of the contents of this thesis have already been published in international journals in the form of five papers for which I was the primary author.\textsuperscript{12-16} I was the major contributor to these papers, performing the research, data collection, statistical analysis, writing and intellectual input into each study. Furthermore, I am the co-author of four additional papers.\textsuperscript{17-20} I am completely responsible for the compilation of this thesis.
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CHAPTER 1

INTRODUCTION
1. Introduction

The brain represents only 2% of the body weight, however it receives 15% of cardiac output, 20% of total body oxygen consumption, and 25% of total body glucose utilisation.\(^1\) Within this lies perfusion to the retina, which has an energy demand on a per gram basis higher than that of the brain,\(^{21-23}\) and one of the highest rates of oxygen consumption of any tissue in the body per unit weight.\(^{21, 24, 25}\) It is crucial that sufficient vascular perfusion to the retina be maintained in order for normal function, with any decrease in supply leading to potentially blinding diseases.\(^{26}\) The significance of the circulation in the body is gargantuan: it is critical for cell survival and function via oxygen and nutrient delivery to the tissues; it is required for metabolic waste removal;\(^{27}\) it is the first organ system to develop in the embryo; almost all functional cells are within 20 – 30 µm from a capillary,\(^{28}\) of which there are approximately 10 billion capillaries in the human body; it encompasses a large surface area of approximately 500 – 700 m\(^2\) as shown by endothelial cell lining.\(^{29}\) The central role of the cardiovascular system is to maintain an adequate capillary flow.\(^{30}\) Thus, the health of vasculature supplying the retina is intensely crucial for maintaining its function and survival, with retinal vascular diseases contributing to extensive causes of morbidity and disability.\(^1, 2\)

The retina has a dual blood supply via the retinal and choroidal circulations, which are end-arterial systems without anastomoses.\(^{31, 32}\) Being a visual organ, the eye requires a limited supply of capillary networks within the human retina to support the immense energy demands of neuronal components without compromising the optical integrity of the light pathway to the outer retina. The retinal circulation, which supplies the inner retina, has been quoted to have a
mean flow velocity of 61 mm/s,\textsuperscript{33} high oxygen exchange,\textsuperscript{31, 34, 35} and is entirely 
regulated at a local level as it has no autonomic innervations.\textsuperscript{36, 37} Conversely, the 
choroidal circulation which supplies the outer retina, has a particularly high flow 
rate, low oxygen exchange,\textsuperscript{31, 34, 35} and intrinsic choroidal neurons acquiring 
sympathetic, parasympathetic and nitrergic innervations.\textsuperscript{38}

It has been demonstrated that oxygen tension and demands within the retina are 
markedly heterogeneous\textsuperscript{21} and the metabolic demands of distinct retinal layers are 
most likely satisfied, to varying extents, by capillary adaptations that function to 
increase the efficiency of regional nutrient delivery and waste removal. It has been 
demonstrated that the three highest regions of oxygen uptake are the inner 
plexiform layer, outer plexiform layer, and photoreceptor inner segments. 
Additionally, high metabolic demands were noted in the retinal ganglion cell 
layer.\textsuperscript{39} Nevertheless, the relationships between capillary network morphometry 
and the heterogeneous metabolic demands of neuronal elements remain 
unclarified in both normal and diseased human eyes.
1.1 Structure and function relationship of the retina

The relationship between microvasculature structure and function has been studied in many organs in our body. For example, the lung is morphologically characterised by a very large surface area with an extremely thin barrier between blood and air. The specific architecture of the capillaries surrounding the alveoli allows efficient gas exchange to occur. In the kidney, the glomerulus is a unique network of capillaries, and is specifically structured to serve in the first stage of the ultrafiltration process in producing urine. Likewise, it can be expected that the eye would also have a specific capillary structure within the retina that relates to its function. It has been demonstrated that as the choroid is only able to supply the outer retina of the oxygen requirements at normal atmospheric conditions, an intricate capillary network via the retinal circulation would be required.

1.1.1 Layered structure

The human retina consists of two primary layers: the neurosensory retina and the retinal pigment epithelium, averaging a thickness of only 300µm. The processing of visual information prior to cortical transmission is predominantly due to the layered configuration of neuronal components in the retina. The neurosensory retina is very densely packed with retinal neurons, and converts light stimuli into neural impulses prior to transmission to the brain via the optic nerve.

Retinal histology has been traditionally based on light microscopic findings comprising of ten layers (Figure 1-1).
1. The inner limiting membrane – border between vitreous and retina formed by the basement membrane of Muller cells and supporting astrocytes

2. The nerve fibre layer – comprises of retinal ganglion cell axons which converge to form the optic nerve

3. The retinal ganglion cells – nuclei and cell bodies of ganglion cells

4. The inner plexiform layer – synaptic connections between bipolar, amacrine and ganglion cells

5. The inner nuclear layer – nuclei of bipolar, horizontal, amacrine and Muller cells

6. The outer plexiform layer – comprises the synapses between the terminal processes of rods, cones, bipolar and horizontal cells

7. The outer nuclear layer – nuclei of the rod and cone cells

8. The external limiting membrane – zonula adherens between Muller cells, rods and cones

9. The rods and cones – photoreceptors

10. The pigment epithelium – monolayer of cuboidal and columnar epithelial cells which play a critical role in normal visual processing, including maintaining adhesion of the neurosensory retina and providing a selectively permeable barrier between the neurosensory retina and choroid.
Figure 1-1 Normal histology of the human retina

Toludine blue stained histological cross-section demonstrates the stratified structure of the human retina and choroid. ILM = inner limiting membrane; NFL = nerve fibre layer; RGC = retinal ganglion cell; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer; IS = inner segments; OS = outer segments; RPE = retinal pigment epithelium. Scale bar: 50μm.
1.1.2 Retinal oxygen demand and the capillary-neuronal relationship

Based on previous studies of the oxygen profile in various animal models of the layered retinal structure, there were three regions of high oxygen consumption identified: the inner segments of the photoreceptors, the outer plexiform layer, and the deeper region of the inner plexiform layer.\(^{21}\) The diversity of intraretinal oxygen consumption highlights a necessity for thorough research into the precise microvasculature structure, the correlation of distribution within the neural and glial components of the retina, and its relationship to oxygen supply and demand in normal and diseased human retina.

The neuron-glial-vascular unit maintains neuronal tissue health within the central nervous system (Figure 1-2).\(^{47}\) Concepts regarding the role of neurons in modulating regional blood flow, and thereby the local supply of glucose and oxygen, have undergone significant paradigm shifts over the past decade\(^ {48}\) with considerable research conducted on capillary-neuronal relationships within the human central nervous system.\(^ {49-52}\) Previous researchers favoured the hypothesis that local blood flow was controlled by negative feedback mechanisms, however there is now increasing evidence to suggest that neurons and glial cells play a vital role in controlling the capillary circulation,\(^ {48,53}\) with correlation of capillarisation and blood supply being modified to meet the metabolic demands of regional tissue.\(^ {50,54-56}\) In studies of rabbit brains, blood flow distributions were closely associated with capillary density and a cytochrome oxidase activity.\(^ {57,58}\) In adult rat brains, there were also correlations between capillary density, blood flow and glucose utilisation.
The high metabolic demands of retinal neurons\textsuperscript{21, 22, 59} are satisfied by a complex and morphologically unique system of capillary networks,\textsuperscript{12, 60-63} and the characteristics of each network with respect to capillary diameter and density, is varied relative to neuronal layer and retinal eccentricity.\textsuperscript{12, 64} Understanding the morphometric organisation of capillary systems in the human retina and the relationship they bear to distinct neuronal layers may increase our understanding of neurovascular coupling mechanisms that are important in retinal homeostasis.\textsuperscript{53} Detailed knowledge of neuron-glial-vascular interactions in the retina may also provide insights into pathogenic mechanisms relevant to retinal vascular diseases. It may also allow useful structure-function extrapolations between capillary morphometry and previously determined measurements on retinal metabolism\textsuperscript{42, 43, 65-72} to be performed.
Figure 1-2 Neuron-glial-vascular unit

Confocal flat mount image of the human retina demonstrating the intimate relationships between neuronal nuclei (blue), GFAP positive processes (green) and retinal arterioles (red). Scale bar: 20μm.
Oxygen is an essential substrate for retinal energy metabolism and plays a key role in moderating neurovascular-coupling mechanisms. Intraretinal oxygen distribution and uptake has been precisely quantified in the mammalian retina and the disparities in metabolic activity between somal, dendritic and synaptic retinal compartments have been previously demonstrated. Although it has been known for decades that the multi-layered retinal capillary network underlies the distribution of oxygen tension in the retina, the relationships between capillary network morphometry and the heterogeneous metabolic demands of neuronal elements have not been clearly elucidated. The retina is vulnerable to ischaemic damage: it requires a fine balance of capillary networks in an optical organ; it has a low average pO$_2$ of approximately 5mmHg – 20mmHg relative to the rest of the body; it has one of the highest rates of oxygen consumption in the body per unit weight, of which most is supplied for oxidative metabolism coupled to ATP synthesis. If there is an altered retinal homeostasis that requires any change in capillary network density, it is likely to modify the refractive properties of the light path to the outer retina. Similarly, changes in capillary morphometry as a means of improving retinal optics, may compromise nutritional supply to important neuronal elements.

Yu et al. and Cringle et al. provided a series of experimental models to calculate different intraretinal oxygen consumption rates of the different layers of the retina in a range of species (Figure 1-3). In vivo studies showed oxygen tension and demands within the retina are markedly heterogenous with three regions of dominant oxygen consumption: the inner plexiform layer, the outer plexiform layer and the inner segments of the photoreceptors.
Figure 1-3 The relationship between retinal cell layers, blood supply and intraretinal oxygen distribution

Retinal and choroidal micrograph of the normal monkey parafoveal region showing three regions of high oxygen uptake: inner plexiform layer (IPL), outer plexiform layer (OPL) and photoreceptor inner segments (IS). Scale bar: 100μm.
1.2 Retinal microvasculature

1.2.1 Previous research

The retinal microvasculature has been intensely studied due to its importance in retinal health and pathology, and previous work on the retinal microvasculature in animals has founded some of our basic knowledge of the retina. These include discovering there are different vascular densities in each of the four quadrants, the density of retinal capillaries decreases equally in the four quadrants, the vascularity of the retina is greatest in the peripapillary region near the optic disc, and the retinal vascular network being a contributor to the optical filtering properties of the eye.

However, information regarding the precise structure and location of the capillary networks within the human retina remains sparse due to limitations in immunohistochemical and microscopic imaging techniques. Previous investigators have employed a variety of different techniques to document the organisation of capillary networks in the primate retina. Trypsin digest, corrosion casting and colloidal iron techniques have provided valuable information concerning the three-dimensional morphology of capillary networks, however several limitations exist with these methods. First, there is usually inadvertent tissue damage as a result of these techniques, which thus limits localisation of capillary networks respective to retinal layers. Also, these techniques are subject to pressure artifact and are unable to be optically sectioned. Another technique used is histological sectioning, which allows positioning of the blood vessels in relation to the cell layer structure, however
three-dimensional information remains difficult to obtain. Yet another issue is that with many of the experiments based on animal models, there were notable differences compared to humans, with resultant extrapolation difficulties, in relation to structure and function. Post mortem human retinas also deteriorate rapidly, undergoing changes such as swelling, distortion and fragmentation.91

Unfortunately extensive research has largely been precluded owing to the difficulty of obtaining and preparing human tissue.92 More recently, fluorescein angiography93 and magnetic resonance imaging94 has been employed to study the retinal circulation, however the limited resolution offered by these techniques also hinders study at a cellular level and restricts co-localisation. Finally, confocal microscopy and immunofluorescent staining technology have been found to delineate three-dimensional retinal microcirculation and thus been utilised.57, 95

In addition to the technical limitations of previous investigators on the human retinal microvasculature, there has also been varying reports concerning the order and number of capillary networks in the human retina. Early trypsin digest studies in the twentieth century by Michaelson et al.61, 96 demonstrated a two-layered laminar pattern of capillaries in the retina using benzidine peroxidase techniques, whilst Toussaint et al.60 demonstrated a lack of capillary lamination. Subsequent to these earlier classical works, Snodderly et al.62, 85 and Gariano et al.63 published excellent studies in the 1990s and demonstrated the morphology of retinal capillaries as a complex consisting of a two inner and two outer capillary beds in human and non-human primate retinas.

It was due to the markedly heterogeneous oxygen tension and demands within the retina, technological difficulties and limitations of previous studies, and the
uncertainties in the literature, that the first study of this thesis (Chapter 3) was conducted. Using our recently developed novel technique of microperfusion and confocal imaging, whereby the central retinal artery of an intact eyeball was cannulated, perfused and confocal imaged, the precise structural architecture of the capillary networks within the different neural layers of the human retina was able to be quantitatively determined. Details of the methodology of this novel technique will be explained in Chapter 2. A major advantage of this methodology is that it allows complete labelling of the retinal microcirculation without inadvertently altering surrounding nonvascular structures. Triple-labelling of post-perfused tissue also allows accurate identification of the capillary network location within the retina and is able to confirm what was demonstrated in flat-mount confocal microscope images.

1.2.2 Current understanding

The central retinal artery arises from the ophthalmic artery, pierces the inferomedial aspect of the optic nerve, and runs through the centre of the nerve. After passing a constriction in the lamina cribrosa, it pierces the papilla centrally, and decreases in diameter to approximately 100µm upon emerging from the optic disc. The retinal vessels are functional end-arteries and continue to spread throughout the retina as capillary networks. Research up to this point of the retinal circulation has identified two distinct capillary beds serving the inner retina: a superficial and deep layer. The superficial capillary bed continues along the vitreal layers to perfuse the nerve
fibre layer and retinal ganglion cell layer. The deeper capillary bed dips below to the level of the inner nuclear and plexiform layers. Differences noted between these two networks include the vascular pattern, and higher density and presence of larger vessels in the superficial plexus. There are anastomoses between both networks and the capillaries are seen to course through from one network to the other. It has also been noted that the superficial network becomes increasingly three-dimensional around the macular region and optic disc, consisting of up to four capillary layers.

1.2.3 Retinal vascular diseases

Retinal vascular diseases involving both the microvasculature, such as diabetic retinopathy, and large vessels, such as retinal vein occlusion, are the most sight-threatening vascular disorders. Additionally, studies have examined the link between retinal microvasculature abnormalities and systemic vascular diseases. Cardiovascular comorbidities such as hypertension, hypercholesterolaemia and ischaemic heart disease, have also been shown to be associated with retinal vascular disease, with changes at the cellular level to basement membranes, mural cells and endothelial tight junctions in histopathologic and electron microscopy studies. Other observations include changes in capillary density and diameter leading to increased vascular resistance, and changes to capillary wall thickness and endothelial mitochondrial content in primates secondary to age.

Previous studies which demonstrated links and alterations in patients already had clinical manifestations of retinal disease, or aimed to induce diseased states, rather than demonstrating quantitative changes prior to the manifestation of known
retinal vascular disease.\textsuperscript{111-113} It is unknown as to whether the morphometrically organised capillary network in the retina is altered prior to observed clinical retinopathy in patients with retinal vascular disease or cardiovascular comorbidities. Understanding retinal microvascular changes prior to clinical manifestation will provide important insights into the prevention of retinal vascular diseases and thus prevent resultant vision loss. For this reason, the second study in this thesis (Chapter 4) was conducted, which concentrated on determining quantitative characteristics of the retinal microvasculature in diseased human eyes, and compared these quantitative measurements to normal human eyes in the first study (Chapter 3). Such knowledge is pertinent for the application to emerging state-of-the-art technology in imaging distinct retinal capillary networks to detect early retinal vascular disease.\textsuperscript{114}

1.2.4 \textit{In vivo} imaging

Our ability to study distinct retinal capillary networks in real-time and \textit{in vivo} has been limited.\textsuperscript{93, 94} Fluorescein angiography, although an excellent investigative tool, requires the administration of a contrast agent but is associated with many adverse effects.\textsuperscript{115-119} Non-invasive techniques that have the capacity to perform angiography without the administration of a contrast agent are thus highly desirable. Although retinal capillaries have low optical contrast in scanning laser ophthalmoscopy, recent developments of label-free flow contrast and forward scattering/offset pinhole techniques have been described in the literature using adaptive optics scanning laser ophthalmoscopy.\textsuperscript{120-123} Label-free, depth resolved optical angiography using Optical Coherence Tomography (OCT) for visualisation of capillary flow has also been reported in the literature. A few of the
various techniques for extracting flow contrast from the OCT data have been described including: phase variance,\textsuperscript{124} optical micro-angiography,\textsuperscript{125} speckle variance,\textsuperscript{126} phase-resolved,\textsuperscript{127} and split spectrum amplitude decorrelation angiography.\textsuperscript{128} Each of these techniques has their own advantages, but they operate on similar principles. Variations in the intensity and/or phase of the OCT signal caused by particle motion are detected by pixel to pixel comparison across repeat B-scan images acquired at the same location. The strength of these techniques is that they are sensitive to the slow flow of blood cells in capillaries.

A detailed review of flow contrast OCT comparing these techniques has recently been published.\textsuperscript{129} Whilst the image quality of flow contrast OCT are comparable for \textit{in vivo} imaging, the speckle variance (sv) OCT technique, described by Mariampillai and colleagues,\textsuperscript{126} is computationally simple, permitting the images to be generated in real time during acquisition.\textsuperscript{130, 131} The third study conducted in this thesis (Chapter 5) employs the use of a custom-built svOCT device\textsuperscript{132} to quantitatively study the different capillary networks in the human retina. These measurements were compared to our first study (Chapter 3) to determine whether there was a capability of the svOCT device for defining and providing detailed quantitative information of distinct capillary networks in the clinical setting.
1.2.5 Region of interest: 3mm superior to the optic disc

The region 3mm superior to the optic disc was used consistently in the first three studies of this thesis as an area of retina most representative of the majority of the retina. It was chosen specifically to avoid the macula; an area with vascular and cellular specialisations, markedly different to most other retinal eccentricities.46

Figure 1-4 Region of interest in retina

Fluorescent imaging of the flat-mounted retina showing region of interest (square) in relation to the optic disc edge and macula (circle).
1.3 Vortex vein system

Whilst the inner retina is supplied by the retinal microvasculature, it is the choroid that plays a vital role in sustaining the outer retina’s high metabolic demands.\textsuperscript{21, 24, 25} The outer retinal layers are completely avascular and depend on the metabolic support from retinal and choroidal vascular beds via diffusion.\textsuperscript{78} The choroidal blood supply is derived mainly from the long and short posterior ciliary arteries. Histologically, there are five layers of the human choroid: Bruch’s membrane, choriocapillaris, two vascular layers and the suprachoroid. The choroidal vascular layer consists of an outer component of major arteries and veins (Haller’s layer), and an inner component of medium-sized vessels of arterioles and venules (Sattler’s layer).\textsuperscript{34} The choroid also acts as a channel for vessels traversing to other areas of the eye and potentially has a thermoregulatory role. By affecting perfusion rates of the ciliary processes, choroidal blood flow regulation may also influence intraocular pressure.

Choroidal circulation drainage occurs through the vortex vein system of the eye. The vortex veins exit the sclera and drain into the superior and inferior ophthalmic veins,\textsuperscript{34, 35} and are located in each quadrant of the eyeball near the equator.\textsuperscript{133} In addition to nearly all the venous drainage of the choroid, the vortex veins also drain the venous blood from the anterior portion of the eye. Numerous choroidal veins converge into a sacculated dilatation called the ampulla before entering the sclera to exit through a vortex vein;\textsuperscript{46, 134, 135} this entire drainage pathway forming a unique anatomical structure known as the vortex vein system.
The venous system is more complex than the arterial system in many respects, with venous diseases being ten times more frequent than arterial diseases. The vortex vein system is a drainage pathway for choroidal circulation, with a blood flow rate many times greater than that of the retinal circulation. It is thus essential to study the very significant but undervalued vortex vein system of the eye. The literature has been largely silent on this important vascular system. A PubMed search on “vortex vein” in the eye cites only 34 studies, of which 23 pertain to the human eye and none to the porcine eye. More than half of these studies were written before the year 2000. Most of the studies on vortex veins currently available are clinical case reports or series mainly on varix, occlusion, or post-surgical complications. These studies primarily investigated the macroscopic structural abnormalities of the vortex vein, usually via fundoscopy, optical coherence tomography or indocyanine green angiography. Of these studies, four in particular have drawn associations between macroscopic vortex vein abnormalities and disease. The first supported the hypothesis that uveal effusion was the result of choroidal congestion from impaired vortex vein drainage through the thickened sclera of nanophthalmic eyes. The second demonstrated occlusion of the vortex vein system causes degenerative changes in the retinal pigment epithelium and outer segment in rabbit eyes. Another study found vortex vein invasion to be associated with an increased risk of liver metastasis in those with uveal melamomas. Finally, a more recent study identified a correlation between vortex vein engorgement in the setting of polypoidal choroidal vasculopathy. Otherwise little is known about macroscopic vortex vein abnormalities and retinal disease.
With regards to microscopic abnormalities, the literature is absent of such studies for the vortex vein system, and pathological changes at a microscopic level have not yet been elucidated. To our knowledge, the vortex vein studies performed in our laboratory are the first to examine the micro architecture of the vortex vein system, its general morphology, endothelial phenotypic architecture, and vascular endothelium structural barrier. Changes at the microscopic level would occur prior to the clinical visualisation at a macroscopic level. We hope that by defining what is “normal”, it will allow future research and comparisons into the identification of abnormal vortex vein system states, which is a very important but untapped area of research.

In addition to the eye being an optical organ with high-energy demands yet possessing a limited blood supply, it is also a positive pressure organ with inevitable pressure gradients lending itself to several specific sites within the ocular vasculature that may potentially be at an increased risk of vulnerability to pathological changes. The vortex vein system is one of these areas, in particular the region whereby a very large volume of blood at a high velocity is required to exit the eye though a small and rigid canal in the sclera. A valuable approach to researching the vortex vein system at a microscopic level is to examine its vascular endothelium.

Research has shown that numerous vascular diseases are due to stressful changes in endothelial intracellular cytoskeleton, with the resultant excessive and prolonged endothelial activation leading to dysfunction and pathogenesis. As abnormal changes in haemodynamics affect blood flow patterns, and endothelial cells have a critical role in vascular biology and pathophysiology of
disease processes, it is important to identify any patterns which may point to preclinical vascular disease. The vortex vein system is geometrically complicated, and the culmination of high flow rates and large volumes of blood through this system has the potential to induce unusual flow patterns and haemodynamic forces. This may expose vascular endothelial cells within the vortex vein system to different haemodynamic pressure gradients and stressors such as shear and turbulent forces, predisposing the endothelium to pathogenesis. However, there is an enormous paucity of information regarding the vortex vein system, its architecture, endothelial cell intracellular structures, or role in pathogenesis. Therefore, we conducted a series of studies discussed in Chapters 6 and 7. These studies detail the quantitative findings of endothelial cells in the porcine vortex vein system, of which current knowledge on endothelial cells will now be discussed.

1.4 Endothelial cells and cell junctions

Endothelial cells, once considered simple inert cells, line the entire circulatory system. These extraordinary cells display a vast array of functional and adaptive characteristics, serving as a life-support system by adapting, extending and remodeling blood vessels to almost all parts of the body. They enable tissue growth and repair, and are the major determinants of health and disease in vasculature.

The endothelium in the adult human is composed of approximately $1 - 6 \times 10^{13}$ endothelial cells, covering a surface area of $1 - 7 \, \text{m}^2$, and weighs about 1 kg. The endothelial cell provides a permeability barrier for vasculature in addition to
modulating blood flow, blood vessel tone, immune response, growth regulation, coagulation, and production of extracellular matrix components. By facilitating the exchange of nutrients and toxic wastes between neurons and supporting glia, they form a crucial role in modulating retinal homeostasis.

The precise flow pattern and haemodynamic forces in the vortex vein system is not currently known. It has been well documented that haemodynamic forces induce endothelial phenotype differences in the cytoskeleton, endothelial cell and nuclei shape. Vascular endothelial cells are constantly exposed to haemodynamic forces and are highly sensitive to hemodynamic shear stresses, bearing the majority of the shear stress as it is in direct contact with blood flow. When abnormal shear stress of blood flow is sustained, variations of shear stress can functionally regulate the vascular tone, and also induce structural endothelial cell changes by slow adaptive remodeling. Evidence shows that shear stress contributes to regional and focal heterogeneity of endothelial gene expression, which is a factor in vascular pathology. Regions of flow disturbances near arterial branches, bifurcations and high curvatures can result in complex spatiotemporal shear stresses and these characteristics can predict regional susceptibility to atherosclerosis. Modifications in local vascular geometry can further alter shear stress characteristics at the endothelium. Mechanotransduction, which are flow-induced endothelial cell responses, involve a repertoire of cell-signaling events ranging from instantaneous ion fluxes, biochemical pathways, and gene and protein expression. Cell junctional proteins, stretch-activated ion molecules, glycocalyx, and cytoskeleton involved in shear stress transduction and signaling are localised to various endothelial compartments not directly exposed to flow. The endothelial cytoskeleton is
suitable to bring together these shear stress receptors.\textsuperscript{180} The cytoskeleton also has an important role in maintaining the normal structure and function of cells. Vascular endothelial cytoskeleton is associated with modification in cell shape and adherence in response to changes in hemodynamic both in vitro,\textsuperscript{181-183} ex vivo,\textsuperscript{184} and in vivo.\textsuperscript{185, 186} Haemodynamic factors are known to influence the organisation of endothelial cell cytoskeleton in situ,\textsuperscript{187} and aging has been shown to be associated with reorganisation of the actin cytoskeleton in endothelial cell culture as well as in animals.\textsuperscript{188, 189}

Site-specific changes of endothelial cell morphology in regions known to be vulnerable to venous occlusive diseases have been found to be present in the central and branch retinal veins of human donor eyes.\textsuperscript{18, 190} Endothelial cell injury, activation or dysfunction is a trademark of many vascular pathological states such as atherosclerosis, thrombosis, hypertension, aneurysms, or the loss of semi-permeable membrane function,\textsuperscript{174} and has lead to an influx of interest in endothelial cell biology. There has been a rapid expansion of knowledge on the vascular endothelium with over 100,000 publications. However, there exists a large bench-to-bedside gap in endothelial biomedicine with clinicians being largely unaware of the role of the endothelium in health and disease.\textsuperscript{191}

Knowledge of the process of endothelial cell injury would aid in our understanding of vascular disease pathogenesis and bridge the gap in clinical awareness.

Knowledge of the endothelial cell border junctional proteins is vital to understanding areas of vulnerability to pathophysiology. The cooperative efforts from tight junctions, gap junctions and adherens junctions, allow molecular
transport and permeability access at the endothelial cell border. These types of junctional proteins are dynamically regulated and allow the transportation of various sized molecules (Figure 1-6).

Tight junction proteins and the associated protein complex consist of the intracellular zonula occludens and transmembrane protein groups of claudins, junctional adhesion molecules (JAMs) and MARVEL domain containing occludin and tricellulin. Claudin-5 is important for controlling leakage of molecules smaller than 800Da. Also, phosphorylation of occludin and the increase in the presence of JAM-C at the endothelial junction is associated with increased permeability. Thus an observation of heterogeneity of immunohistochemistry in these labels may indicate a region of increased permeability and vulnerability.

Adherens junction of the endothelial cell maintains vascular integrity. Vascular endothelial cadherin (VE-Cadherin) is an endothelial specific adhesion protein situated at the adherens junctions. VE-Cadherin phosphorylation is modulated by haemodynamic conditions. In general, phosphorylation of VE-Cadherin is associated with increased permeability whilst dephosphorylation is associated with increased barrier function. It has been shown with in vivo experiments in mice that VE-Cadherin was predominantly tyrosine (Y658 and Y685) phosphorylated in the venous region of the diaphragm microvasculature with stress-induced junctional Src activation. It would be interesting to determine whether phosphorylated tyrosine is present in the vortex vein system, which may indicate regions of vulnerability for increased permeability. Delineation of the architecture and endothelial cytoskeleton of the vasculature which supports the retina is vital for understanding morphological changes prior to clinical
manifestation, and may provide important insights into the prevention of retinal vascular diseases and resultant vision loss.

Figure 1-5 Diagram of the endothelial cell to cell junction

This diagram shows the molecular architecture of the endothelial cell to cell junction, composing tight junctions and adherens junctions. The major membrane protein components which form tight junctions are claudin, occludin and junctional adhesion molecule. Vascular-endothelial cadherin is located at adherens junctions.
1.5 Key questions addressed and major hypothesis in this thesis

In summary, the basis of this thesis will exemplify the characteristics of various vasculature within the eye which support the retina. This will be conducted through a series of experiments involving detailed quantitative assessments of retinal capillary networks in normal and diseased human eyes, advances in emerging state-of-the-art technology in capturing capillary networks in patients in a clinical setting, and regional heterogeneity of endothelial cells and its junctional proteins in the human and porcine vortex vein system of the eye.

Four important issues are addressed in this thesis.
1.5.1 How does the structure and distribution of retinal microvasculature relate to its function in the human eye?

Studies are performed in normal human eyes to determine quantitative measurements of capillary morphometry, diameter and density. The major question is:

1. Where are the retinal microvasculature network layers located in relation to the retina?

The hypothesis is that the retinal microvasculature network layers are closely coupled to the demands of the retinal cellular layers, but also need to meet the functional requirements of the retina as an optical organ.
1.5.2 How does the retinal vasculature change with pathogenesis?

Comparisons are made between the microvasculature of normal human eyes and those with a history of vascular comorbidities but no clinically known ocular disease. The major questions are:

1. Do vascular comorbidities such as cardiovascular disease influence the retinal microvasculature networks?

2. What quantitative and morphological changes occur in the vasculature of those with cardiovascular disease?

The hypothesis is that the retinal microvasculature network is vulnerable to systemic vascular comorbidities which can cause structural changes in the retinal vasculature.
1.5.3 What recent advances in technology may aid in the early diagnosis and intervention of retinal diseases?

Quantitative comparisons were made between speckle variance optical coherence tomography (svOCT) and the normal retinal capillary network. The major questions are:

1. How accurate are svOCT images from patients compared to their corresponding histological counterparts?

2. Can this emerging state-of-the-art technology be used in a clinical setting?

The hypothesis is that the results with the microvascular distribution in human donor eyes in which the retinal microvasculature has been perfused, labelled, flat mounted, and imaged by confocal microscopy, can be used as a gold standard to determine whether svOCT can reliably image the entire vascular tree, with resolution to the capillary level.
1.5.4 Is there region-dependent endothelial heterogeneity within the vortex vein system?

Studies are performed to delineate the vortex vein system, both in general morphology and also at the level of the endothelial cytoskeleton. Additionally, endothelial cell f-actin, claudin-5, VE-Cadherin and phosphorylated tyrosine labeling within the vortex vein system was conducted. The major questions are:

1. What is the morphology of the vortex vein system?

2. What quantitative changes occur in the endothelial cytoskeleton of the vortex vein system?

3. Are there regional differences with f-actin, claudin-5, VE-Cadherin and phosphorylated tyrosine labelling?

The hypothesis is that vascular endothelial phenotype heterogeneity can be detected in the different regions of the vortex vein system.
The hypothesis tested in this thesis concerns the structure and distribution of both the vasculature supplying the inner retina and the vortex vein system that drains the choroid supporting the outer retina. As shown in other body organs, structure relates to function, and it is expected that the distribution of retinal microvasculature networks correlate with the neuronal layers requiring the greatest energy demands. It is equally important to investigate the potential clinical impact of these findings by comparing normal and diseased eyes. As previous studies have shown a positive relationship between cardiovascular disease and retinal microvascular abnormalities in those with clinically detectable retinopathy, of higher clinical relevance would be to determine whether there are altered changes in the retina observed prior to clinical retinopathy in those with vascular comorbidities. With changes occurring at a cellular level during pathogenesis, it is anticipated that there would be quantitative differences to the microvasculature with diseased states. Furthermore, there remains the need for a useful, safe, non-invasive, real-time clinical tool for reliable capillary morphology and structure imaging. Speckle variance optical coherence tomography is potentially able to fulfill these requirements and retinal capillary imaging from this technique is envisioned to provide comparable microvascular images to histology. Finally, it is predicted that the vortex vein system is a significant structure of the eye with unique morphology. Due to the maintenance of large volumes of blood through the vortex vein system, it is anticipated that there would be significant regional differences in the endothelial cytoskeleton, along with an understanding of potential regions of vulnerability.
CHAPTER 2

METHODOLOGICAL OVERVIEW
2. Methodological Overview

2.1 Aim

Materials and methods that could be generalised are provided in this chapter, with detailed specifications related to each study discussed in the methodological section of their respective chapters. In this chapter, the ethics approval, method of obtaining human and porcine eyes, our novel perfusion technique for labelling the retinal and choroidal circulation, tissue preparation of the retina and choroid, image acquisition, statistical techniques, and image analysis are disclosed.
2.2 Ethics approval

2.2.1 Human eyes

The study was approved by the human research ethics committee at The University of Western Australia. All human tissue was handled according to the tenets of the Declaration of Helsinki.

2.2.2 Porcine eyes

The study was approved by the University of Western Australia Animal Ethics Committee. All experiments were conducted in accordance with The Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.
2.3 Obtaining eyes

2.3.1 Human eyes

Over 70 eyes (41 human eyes and 30 porcine eyes) were used for this thesis. Human eyes used in this study were predominantly obtained from donors via the Lions Eye Bank of Western Australia (Lions Eye Institute, Western Australia). Eyes were received after removal of corneal buttons for transplantation.

Occasionally, patient eyes generously donated to Lions Eye Bank of Western Australia were unable to be used for corneal buttons. In this instance, myself or another medical doctor from our laboratory enucleated the donor eyes at the hospital bedside or morgue, after obtaining written consent from the family and the on-call hospital medical directive (usually an intensive care consultant).

2.3.1.1 Enucleation of human eyes

Briefly, eyelids were retracted and conjunctival peritomy was conducted to access the extraocular muscles. The eyeball was oriented and marked prior to dissecting all muscles, orbital fat and the optic nerve to release the eyeball. The eye was then placed in a cooler with carbogen bubbled Ringer’s solution, and then transported to the laboratory for perfusion. The demographic data, time of death, cause of death, time of enucleation and postmortem time to eye perfusion were recorded.

2.3.2 Porcine eyes

Post-mortem white landrace pig eyes were used and sourced from a local abattoir: the Linley Valley Abattoir. Freshly enucleated eyes were placed in carbogen
bubbled Ringer’s solution and delivered to our laboratory on ice, and used within the hour.

2.4 Perfusion technique

Our laboratory developed a new technique for the detailed study of the retinal and choroidal microvasculature distribution and their relation to neurons and glial cells at the cellular level of human cadaveric eyes.47

2.4.1 Retinal circulation

2.4.1.1 Technique

This novel technique involves cannulation of the central retinal artery with micropipettes to perfuse the retinal microvasculature (Figure 2-1 and Figure 2-2). Two 8-0 vicryl suture ties secure the central retinal artery to the cannula prior to perfusion. Assiduous precautions were taken to ensure all air bubbles were carefully eliminated prior to cannulation and securing of the vessel, and throughout the perfusion process. Syringe pumps (model 22; Harvard Apparatus, South Natick, MA) delivered an adjustable perfusate flow (typically 50 μL/min) with the perfusion pressure continuously monitored through conventional transducers (Cobe, Arvada, CO). These transducers were each connected to a bridge amplifier (model 5B38-02; Analog Devices, Norwood, MA) and recorded on a chart recorder (LR8100; Yokogawa, Tokyo, Japan). To ensure intravascular pressures did not exceed physiological range, the perfusate flow rate was calculated. In a study conducted on canine eyes, direct measurements of intravascular pressure indicated the pressure in the retinal artery is approximately
70% of the aorta. If this relationship holds true in humans, a mean systemic blood pressure of 100 mm Hg would translate to an average retinal artery intravascular pressure of approximately 70 mm Hg. With a normal intraocular pressure of 15 mm Hg, the average transmural pressure in the retinal artery would thus be approximately 55 mm Hg. Our donor eye intraocular pressures would be zero due to corneal removal. Thus, we chose a baseline flow rate of 50 \( \mu \text{L/min} \) as that produced an average transmural pressure in the central retinal artery close to 50 mm Hg.

### 2.4.1.2 Perfusate solutions

After cannulation, the following order of perfusate solutions were conducted:

1. Washout of residual blood with oxygenated Ringer’s solution and 1% bovine serum albumin for 20 minutes
2. Fixation with 4% paraformaldehyde in 0.1 M phosphate buffer solution for 30 minutes
3. Detergent with 0.1% Triton-X 100 in 0.1 M phosphate buffer solution for 7 minutes, to aid in the permeation of endothelial cell membranes
4. Washout with 0.1 M phosphate buffer solution for 30 minutes
5. Microfilaments and cell nuclei were then labelled over the course of two hours by using a mixture of various dyes depending on the study involved, for example, a mixture of phalloidin conjugated to Alexa Fluor 546 (30U; Invitrogen, Carlsbad, CA) and iodide dye (YO-PRO-1, 6.6 \( \mu \text{M} \); Invitrogen)
6. Residual dye was washed out from the vasculature with a further perfusion of 0.1 M phosphate buffer perfusion for 30 minutes
The eyes were then immersed in 4% paraformaldehyde in 0.1 M phosphate buffer overnight prior to flat mounting of the retina in glycerol for imaging.

![Perfusion setup diagram](image)

**Figure 2-1 Perfusion set up for the human donor eye**

The human eye is placed in an eye holder, cannulated and secured (A). This diagram shows dual perfusion of both the central retinal artery for staining the retinal microvasculature (red), and also cannulation of the posterior ciliary artery for perfusion staining of the choroidal microvasculature (green). (B) shows typical chart recordings reflecting the pressure change throughout perfusion of various solutions. CRA = central retinal artery; LPCA = long posterior ciliary artery; ON = optic nerve; EB = eyeball.
Figure 2-2 Magnified view of cannulation in the vessels in the human eye

This image shows a magnified view of the cannulation of the central retinal artery (CRA) and short posterior ciliary artery (PCA) in the human eye with micropipettes.
2.4.2 Choroidal circulation to view the vortex vein system

2.4.2.1 Technique

Similar techniques for retinal and choroidal perfusion in human eyes were applied to the choroidal circulation of the porcine eye to visualise the intracellular structures within and surrounding the vortex vein system.

The pig eye was placed temporal side facing up in the pig eye holder, and the superior temporal vortex vein (STVV), inferior temporal vortex vein (ITVV) and the long posterior ciliary artery (LPCA) located. In the first vortex vein study conducted (Chapter 6), the STVV was cannulated and secured by two 8-0 vicryl sutures and the ITVV placed in clear view to watch for the outflow of blood during perfusion. In the subsequent vortex vein study (Chapter 7), the temporal LPCA was cannulated with glass micropipettes tip sizes of 270 μm to 300 μm and the cannula secured with 8-0 vicryl sutures and attached to a syringe pump (model 22; Harvard Apparatus, South Natick, MA). Assiduous precautions were taken to ensure all air bubbles were carefully eliminated prior to cannulation. The pump supplied a modifiable flow of solution (typically 50 μL/min) and the perfusion pressure monitored through a pressure transducer (Cobe, Arvada, CO), connected to a bridge amplifier (model 5B38-02; Analog Devices, Norwood, MA) and recorded on a chart recorder (LR8100; Yokogawa, Tokyo, Japan). The perfusion pressure recorded ranged from 25 mmHg to 30 mmHg, and based on pilot studies, was the highest possible pressure at which the choroidal vasculature network could be entirely perfused without exceeding the physiological intraocular pressure or inducing tissue swelling. This equated to a flow rate of approximately 150 μL/min and an intraocular pressure range between 7 mmHg to 14 mmHg.
Figure 2-3 Perfusion set up for the adult porcine eye

(A) An adult porcine eye is placed on an eye holder and the superior temporal vortex vein has been cannulated with a micropipette. (B) A close-up view shows the two 8-0 vicryl suture ties to secure the superior temporal vortex vein (STVV) in place, as well the tying of outflow regions, namely long posterior ciliary artery (LPCA) and inferior temporal vortex vein (ITVV).
2.4.1.2 Perfusate solutions

Following cannulation, the following sequence of filtered solutions were perfused through the temporal vortex vein system:

1. 30-minute washout of blood with 1% bovine serum albumin in oxygenated Ringer’s solution at 150 μL/min, with observation of the outflow drainage of the perfusate through the ITVV

2. 30-minute fixation at 150μL/min with filtered 4% paraformaldehyde in 0.1M phosphate buffer

3. 8-minute wash with 0.1% Triton X-100 in buffer at 150 μL/min to aid in the permeation of endothelial cell membranes

4. 30-minute wash with 0.1M phosphate buffer solution at 100 μL/min

The flow rate was reduced after perfusion of Triton X-100 to avoid fluid leaking out into the extravascular stroma, as this non-ionic surfactant detergent permeabilises the membranes of living cells.

The perfusion labelling that follows is discussed in detail within the methodological section of the relevant chapters as they differed with each study.
2.5 Tissue preparation

2.5.1 Dissection and flat-mounting of the retina

The posterior globe was dissected at the equator to allow viewing of the posterior retina. The retina was dissected carefully around the optic disc edge. A few cuts were made in the peripheral retina to enable the retina to lie flat. A fluorescent image of the flat-mounted retina has been shown in Chapter 1 (Figure 1-5).

2.5.2 Dissection and flat-mounting of the choroid

The anterior portion of the eye was cut as close to the ora serrata to expose as much of the posterior portion of the globe. The retina and vitreous were dissected and removed gently to leave behind an intact choroid on the posterior globe. The superior temporal quadrant of the globe was located with the corresponding STVV and approximately a 1.5 cm x 1 cm portion of the choroid on the globe with the exiting STVV was cut from the rest of the posterior globe. From this smaller portion, the choroid had a layer of retinal pigment epithelial cells overlying it, which was removed with a cotton-tipped bud, and washed from the choroid with a buffer solution. The choroid was then gently peeled back from the sclera, and adherent regions cut from the sclera with vannas scissors. The section of the STVV that entered the sclera was cut at the sclera to release the choroidal segment of the vortex vein system. The dissected choroid was then flat mounted in glycerol, scleral side face up, before cover-slipping and imaging (Figure 2-4).
Figure 2-4 Dissection of porcine eye with a light iris

(A) An adult porcine eye with a light iris, prior to dissection. (B) Removal of retinal pigment epithelial cells with buffer-soaked cotton tip bud to reveal a pale-coloured choroid beneath. (C) Pale-coloured choroid on sclera with extra-ocular vortex vein. (D) Flat-mounting of the choroid.
Figure 2-5 Dissection of porcine eye with a pigmented iris

(A) An adult porcine eye with a pigmented iris is dissected to reveal the vortex vein system. (B) The extra-ocular vortex vein is shown. (C) is a magnified image of (A). (D) is the flat-mounted choroid prior to cover-slipping and imaging.
2.5.3 Dissection and flat-mounting of the intra-scleral and extra-ocular vortex veins

The sclera was carefully dissected into very small and thin regions to expose the vortex vein within the intra-scleral canal (Figure 2-6). These specimens were then flat mounted with glycerol onto glass slides with a concave centre before cover-slipping to allow imaging. For the extra-ocular vortex vein, the vein was carefully cut open with vannas scissors and flat mounted in glycerol, lumen side face up, before cover-slipping and imaging.

*Figure 2-6 Dissection of porcine eye intra-scleral canal and extra-ocular vein*

The intra-scleral canal has been dissected down its centre. Also, the extra-ocular vein can be clearly seen.
2.6 Microscopy image acquisition

2.6.1 Retinal studies

Whole-mount retinas were imaged using low magnification epifluorescence microscopy (E800 or i90; Nikon, Tokyo, Japan) before confocal imaging. Epifluorescence microscopy images were used for precise location of the retinal region, centred 3mm superior to the optic disk (Fig. 1), prior to confocal microscopy studies. Such measures ensured that retinal eccentricity used for capillary morphometric comparisons was consistent in each eye donor.

Two Nikon C1 systems equipped with either three lasers (wavelengths 405 nm, 488 nm and 532 nm) or four lasers (wavelengths 405 nm, 488 nm, 561 nm and 638 nm) were used for confocal imaging. Both microscopes were equipped with EZ-C1 software (v.3.20). Objective lenses that were used for confocal imaging included x4 (NA 0.2), x10 (NA 1.45) and x20 (NA 0.75) Plan-Apo lenses.

Using a motorised stage, a series of z-stacks were captured for each specimen beginning from the vitreal surface, at the level of the inner limiting membrane, to the outer retina. Each z-stack consisted of a depth of optical sections collected at 0.35µm increments along the z-plane. Images of different wavelengths were acquired sequentially.
2.6.2 Vortex vein system studies

Image acquisition for the vortex vein studies was similar to the retinal studies. All specimens were imaged using low magnification epifluorescence microscopy (E800 or i90; Nikon, Tokyo, Japan) in conjunction with the NIS Elements software V4.0 (Nikon, Japan) to scan and compile multiple different images into a single large fluorescent image. These images were used to identify the precise location of the eight regions studied prior to confocal microscopy studies.

Likewise with retinal studies, confocal images were obtained using two Nikon C1 systems equipped with either three lasers (wavelengths 405 nm, 488 nm and 532 nm) or four lasers (wavelengths 405 nm, 488 nm, 561 nm and 638 nm). We used the EZ-C1 software along with various objective Plan Apo lenses including x4 (NA 0.2), x10 (NA 0.45), x20 (NA 0.75), and x40 (NA = 1.0, oil) for confocal imaging.

A series of z-stacks was captured in eight specific regions of each specimen, beginning from the scleral surface of the choroid, down to the choriocapillaris. Confocal images were predominantly taken with the x20 and x40 Plan Apo lenses, with each z-stack consisting of a depth of optical sections collected at 0.35 μm and 0.3 μm increments respectively along the z-plane. Images of different wavelengths were obtained in succession.
2.7 Image preparation

The confocal images were processed and analysed using both ImagePro Plus (Media Cybernetics, Version 7.1) and Image J (version 1.43, available free online, National Institute of Health, USA, http://rsb.info.nih.gov/ij). Also used were Adobe Photoshop (version 12.1, Adobe Systems Inc.) and Adobe Illustrator CS5 (version 12.1.0, Adobe Systems Inc.) for preparation of images for this manuscript. Confocal images were pseudo-coloured with ImagePro Plus.
2.8 Statistical analysis for retinal studies

All data is expressed in terms of mean and standard error and were calculated using Sigmastat (Sigmastat, ver. 3.1; SPSS, Chicago, IL). Multiple measurements from eyes with data taken from right and left eyes of the same individual were analyzed using R (R Foundation for Statistical Computing, Vienna, Austria). One-way analysis of variance (ANOVA) testing was performed to compare measurements between layers or regions. The model used included “Right” or “Left” nested within “eye donor” as random effects using linear mixed modeling to test measurement differences between retinal layers or vortex vein system regions. The assignment of donor as a random effect was used to account for the effects of intra-“eye” correlation and similarly “Right” and “Left” to account for right and left eye correlation.

2.9 Statistical analysis for vortex vein studies

All data were analysed using R and expressed in terms of mean and standard error. A linear mixed model incorporating a random factor to account for multiple measurements within the eye was included. One-way analysis of variance (ANOVA) testing was performed to compare measurements between the eight different regions (choroidal vein, pre-ampulla, anterior portion of ampulla, mid-ampulla, posterior portion of ampulla, post-ampulla, intrascleral canal, and extra-ocular vortex vein) against endothelial cell and nuclei parameters of area, perimeter, length and width. We also performed a one-way ANOVA testing the correlation between the nuclei position in relation to the upstream pole of the endothelial cell border, as well as comparisons of the vessel diameter in the different regions. The comparisons were significant when $P < 0.05$. 
CHAPTER 3

HUMAN RETINAL
MICROVASCULATURE IN
NORMAL EYES
3. Human Retinal Microvasculature in Normal Eyes

3.1 Aim

Whilst it has been long known that the stratified retinal capillary network underlies the distribution of oxygen tension in the retina, the relationships between capillary network morphometry and the heterogeneous metabolic demands of neuronal elements remains unclarified. Furthermore there have not been any studies that have quantified these morphometric characteristics of capillary networks within the normal human retina.

The retina has the highest rate of oxygen consumption of any tissue in the body per unit weight, and the precisely organised retinal capillary networks support the immense energy demands of their neuronal components without compromising optical clarity. Previous studies have documented the location of vascular plexuses within the primate retina and have performed detailed quantitative measurements of the temporal and spatial sequence of vascular plexus formation. These have been critical for understanding physiological mechanisms that govern retinal angiogenesis and vascular development. Delineating the morphometric characteristics of capillary networks may identify important vascular adaptations that allow the unique metabolic demands of each retinal layer to be satisfied. It may also allow important correlations between capillary network morphometry and previously determined retinal oxygen measurements to be performed, thus allowing speculation concerning structure-function relationships.
in the primate retina. Finally, it may elucidate structural mechanisms that facilitate the fine balance between optical clarity and cellular nutrition in the human eye.

In this report a novel, micro-pipette and antibody-based perfusion methodology developed in this laboratory was used, together with confocal microscopic techniques, to examine the retinal circulation. The region of retina 3 mm superior to the optic disc was examined and the morphometric features of capillary networks, respective to distinct retinal layers, were quantified. A region superior to the optic disc was chosen for examination in an effort to avoid the macula: an area with vascular and cellular specialisations that is markedly different to most other retinal eccentricities. This chapter details the quantitative assessment of capillary networks in different neural layers of the human retina and is an important extension to previous studies that have studied retinal vascular characteristics.
3.2 Materials and Methods

3.2.1 Human Donor Eyes

A total of 16 eyes from 14 donors were studied. All eyes were obtained from the Lions Eye Bank (Lions Eye Institute, Western Australia). Eyes were received after removal of corneal buttons for transplantation. None of the donors had a known history of eye disease. The demographic data, cause of death and post-mortem time to eye perfusion for each donor are presented in Table 3-1. Twelve eyes were used for quantitative analysis, three eyes were used for co-localisation studies and one used for epoxy sectioning.

3.2.2 Perfusion labeling of retinal circulation

A detailed review of the novel perfusion-based techniques used for targeted retinal endothelial labeling has been discussed in Chapter 2.
<table>
<thead>
<tr>
<th>Donor ID</th>
<th>Age</th>
<th>Sex</th>
<th>Eye</th>
<th>Cause of death</th>
<th>Time to cannulation (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>22</td>
<td>M</td>
<td>L</td>
<td>MVA</td>
<td>15.0</td>
</tr>
<tr>
<td>B</td>
<td>32</td>
<td>M</td>
<td>L</td>
<td>MVA</td>
<td>20.0</td>
</tr>
<tr>
<td>C</td>
<td>49</td>
<td>F</td>
<td>L + R</td>
<td>Cancer</td>
<td>5.5</td>
</tr>
<tr>
<td>D</td>
<td>67</td>
<td>M</td>
<td>L</td>
<td>Cancer</td>
<td>9.5</td>
</tr>
<tr>
<td>E</td>
<td>23</td>
<td>M</td>
<td>L</td>
<td>Suicide</td>
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</tr>
<tr>
<td>F</td>
<td>66</td>
<td>M</td>
<td>L + R</td>
<td>Cancer</td>
<td>15.0</td>
</tr>
<tr>
<td>G</td>
<td>22</td>
<td>M</td>
<td>L</td>
<td>Suicide</td>
<td>15.0</td>
</tr>
<tr>
<td>H</td>
<td>53</td>
<td>M</td>
<td>L</td>
<td>MVA</td>
<td>14.0</td>
</tr>
<tr>
<td>I *</td>
<td>60</td>
<td>M</td>
<td>L</td>
<td>Collapse</td>
<td>18.0</td>
</tr>
<tr>
<td>J *</td>
<td>72</td>
<td>M</td>
<td>R</td>
<td>Drowning</td>
<td>15.0</td>
</tr>
<tr>
<td>K †</td>
<td>72</td>
<td>F</td>
<td>L</td>
<td>Sepsis</td>
<td>3.0</td>
</tr>
<tr>
<td>L ‡</td>
<td>60</td>
<td>M</td>
<td>R</td>
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<td>M</td>
<td>R</td>
<td>Melanoma</td>
<td>12.0</td>
</tr>
<tr>
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<td>39</td>
<td>M</td>
<td>L</td>
<td>Sepsis</td>
<td>20.0</td>
</tr>
</tbody>
</table>

*Table 3-1 Human Donor Details*

Age (years), sex (M = male or F = female), side (L = left or R = right) cause of death and time to cannulation for each eye donor is provided. Donor eyes that were flat-mounted for co-localisation studies are designated (*), the donor eye that was used for epoxy sectioning is designated (†) and the donor eye that was sectioned transversely for co-localisation studies is designated (‡). MVA = motor vehicle accident.
3.2.3 Tissue preparation and immunolabelling

After perfusion labelling, the retina was carefully dissected from the globe and flat-mounted.

Retinas from two separate donors (Table 3-1) underwent whole-mount immunolabelling using the protocol described by Xiao and Hendrickson. This permitted co-localisation of capillary networks relative to distinct retinal layers. In brief, the whole retina was washed in 0.1M phosphate buffer on a shaker for one to two days, floated in 1% sodium borohydride in phosphate buffer overnight, cryoprotected in 30% sucrose and stored at –80°C. The retina was subsequently thawed and maintained at 4°C on a slow speed shaker prior to immunolabelling. The retina was permeabilised overnight in solution comprising 0.1% sodium azide and 0.1% Triton-X 100 in 0.1M phosphate buffer. The solution was then changed to a blocking serum comprising 10% normal goat serum in phosphate buffer for overnight incubation. Primary antibodies were then added to the solution for incubation over three to four days. Excess antibodies were washed off by overnight incubation in 0.1M phosphate buffer. The retina was then incubated with secondary antibodies and nucleic acid label overnight and washed thoroughly in solution for at least two hours prior to mounting.

Retinal ganglion cells and their axons were labelled using rabbit anti-γ-synuclein (1:200, Abcam ab55424) and goat anti-rabbit secondary antibody conjugated to Alexa Fluor 488 (1:200, Invitrogen A11034). For identification of ON-bipolar cells and horizontal cells the primary antibodies that were used included mouse anti-Go-α (1:100, Millipore MAB3073) and rabbit anti-
parvalbumin (1:200, Swant PV 25) respectively. Secondary antibodies that were conjugated to these primary antibodies included goat anti-mouse antibody (Alexa Fluor 488; 1:200, Invitrogen A10680) and goat anti-rabbit antibody (Alexa Fluor 635; 1:200, Invitrogen A21070) respectively. Separate retinas were used for labelling retinal ganglion cells and horizontal cells as the secondary antibodies for both primary antibodies were derived from rabbit species.

One eye which did not undergo perfusion-labelling was used for immunolabelling co-localisation after frozen sectioning (Table 3-1). The same retinal eccentricity was examined. Transverse retinal frozen sections, 12μm in thickness, were dried onto Gold slides (Erie Superfrost PLUS, ERIIF-4981GLPLUS-006E; Biolab, Decatur, GA) and used for immunolabelling. Blood vessels were labelled using lectins from Triticum Vulgaris conjugated to TRITC (1:50; Sigma L5266). Nuclei were counter-labelled using bisbenzamide (H33258; 1.2µg/ml; Sigma-Aldrich, St. Louis, MO). Remaining primary and secondary antibodies that were used for co-localisation studies in these transverse sections were the same as that described above for whole-mount specimens.

3.2.4 Microscopy

Chapter 2 discusses the methodology for microscopy image acquisition for this paper. The precise location of the retinal region examined was centered 3mm superior to the optic disc (Figure 3-1).
Figure 3-1 Region of study

Colour fundus (A) and transverse retinal section (B) from healthy subjects demonstrate the clinical and histological features, respectively, of the retinal eccentricity chosen for capillary morphometry analysis. A region 3mm superior to the optic disc (fenestrated box) was examined. NFL = nerve fibre layer, RGC = retinal ganglion cell, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, ONL = outer nuclear layer, IS = inner-segments of photoreceptors, OS = outer-segment of photoreceptors, A = artery and V = vein. Scale bar = 1000µm (colour fundus) and 50µm (histology).
3.2.5 Study of capillary topography

Morphometric criteria previously used to define vascular plexuses in central nervous system and ocular studies were employed to divide the retinal circulation into different capillary networks.\(^ {46, 62, 85, 203}\) A change in capillary branching pattern\(^ {204, 205}\) and projected orientation\(^ {46, 205, 206}\) was used to distinguish the different capillary networks. Additionally, using the movie-sequence function available on Image J software to sequentially view the z-stack, it was possible to determine if capillary networks displayed a laminar, single-planar orientation that was predominantly confined to a single retinal layer or if they displayed a complex three-dimensional configuration that traversed the z-axis. In this report, the term laminar refers to capillary configurations that are predominantly confined to a single plane with minimal projections along the z-axis. Likewise, the term three-dimensional refers to capillary networks that are not confined to a single plane but instead demonstrate prominent projections along the vertical z-axis.

The movie-sequence function on Image J was also used to view capillary morphology simultaneously with nuclei and thereby locate the position of capillary networks within the retina. Transverse and flat-mount immunolabelling, with multiple antibodies, was also used to confirm the position of capillary networks relative to the nerve fibre layer (NFL), retinal ganglion cell (RGC) layer, bipolar cells and horizontal cells.
3.2.6 Quantitative study of capillary networks

A z-projected image of each capillary network together with previously defined histological parameters,\textsuperscript{18, 71, 85} was used to perform quantitative measurements. The following measurements were attained from each network as illustrated in Figure 3-2:

1. Capillary diameter – Defined as the perpendicular distance across the maximum chord axis of each vessel.
   
   Each image was partitioned into 9 equal areas and measurements were acquired from each area to ensure representative sampling.

2. Capillary density – Defined as percentage of the sample area occupied by vessel lumens.
Confocal images of capillary networks (A) were manually traced (B) prior to quantitative analysis. Each traced image was divided into 9 equal areas (red fenestrated lines) to allow representative sampling of capillary diameter. The perpendicular distance across the maximum chord axis of each vessel was used to measure capillary diameter (green). The area occupied by capillary structures, as a proportion of the total image area, was used as a measure of capillary density and expressed as a percentage. Scale bar = 50µm.
3.2.7 Statistical analysis

Chapter 2 describes the statistical analysis techniques employed for this study. Specifically for this study, statistical analysis also determined if age accounted for differences in capillary diameter and vascular density measurements between retinal layers. We tested the influence of age upon density using a linear mixed model incorporating age as the predictive factor and right and left eye as random factors to account for variation between right and left eye nested within donor eyes as another random factor. Adjustments were made for multiple comparisons. We also performed a one-way ANOVA testing the effect of cause of death upon vascular density using the same random effects model as described above. Cause of death was defined as either accidental or cancer.
3.3 Results

3.3.1 General

The mean donor age was 49.71 ± 5.19 years. We examined 11 left and 5 right eyes from 12 male and 2 female donors. The mean post mortem time to initial perfusion ranged from 3 to 22 hours with an average of 13.39 ± 1.68 hours.

All orders of the retinal microvasculature were well perfused after cannulation of the central retinal artery. Endothelial cells, smooth muscle cells and nuclei of the retinal vasculature were clearly labelled.

3.3.2 Capillary topography in the human retina

Morphometrically different capillary networks were consistently identified in the following locations:

1. Nerve fibre layer
2. Retinal ganglion cell layer
3. Border of inner plexiform layer (IPL) and superficial boundary of the inner nuclear layer (INL)
4. Boundary of deep INL and outer plexiform layer (OPL)

3.3.2.1 Nerve fibre layer

Capillaries in the NFL network were orientated parallel to the direction of RGC axon bundles and arose from branch vessels in the RGC layer. Capillaries in the NFL had linear trajectories with fewer inter-capillary anastomotic connections compared to other networks (Fig. 3). Co-localisation using γ-synuclein antibody
confirmed that the location of this network was within the NFL. Within the retinal eccentricity examined in this study there was an observed reduction in capillary density in the NFL as the distance away from the optic disk increased (Figure 3-3A).

**Figure 3-3 Nerve fibre layer capillary network (overleaf)**

Whole-mount confocal microscope image captured from a single laser channel (A) demonstrate the linear trajectory of capillaries and the relative absence of anastomotic connections between vessels. There is a reduction in the density of capillaries as the distance away from optic disk increases (left-to-right direction in image). Merged image with nuclei information (B) demonstrate a paucity of cells in this region. Triple-stained transverse retinal section (C) demonstrates the location of this network within the nerve fibre layer (NFL). Lectins stain endothelium, Hoechst stain nuclei and γ-synuclein stain retinal ganglion cell (RGC) axons. IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer and ONL = outer nuclear layer. Scale bar = 50µm.
3.3.2.2 Retinal ganglion cell layer

In the RGC layer (Figure 3-4), retinal arterioles were clearly observed to give rise to capillary networks. Retinal venules were also observed in this layer of the retina. Analysis of capillary trajectory in the RGC layer, using the movie-sequence function on Image J, demonstrated a complex vascular configuration. Many capillaries in the RGC layer projected along a single plane while some capillaries were observed to run obliquely at varying angles along the z-plane and form anastomoses with capillary networks in the NFL and outer retina. The configuration of capillaries in the RGC network was significantly more three-dimensional than the NFL network. Capillary density was also observed to be greatest in the RGC capillary network.

Figure 3-4 Retinal ganglion cell layer capillary network (overleaf) 12

Whole-mount confocal microscope image captured from a single laser channel (A) demonstrate the high density of capillaries in this network. Arterioles and venules are also observed in addition to capillaries. Merged image with nuclei information (B) demonstrate a high density of cells in this region. Triple-stained transverse retinal section (C) demonstrates the location of this network within the retinal ganglion cell (RGC) layer. Lectins stain endothelium, Hoechst stain nuclei and γ-synuclein stain RGCs. NFL = nerve fibre layer, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer and ONL = outer nuclear layer. Scale bar = 50µm.
3.3.2.3 Border of inner plexiform layer (IPL) and superficial boundary of the inner nuclear layer (INL)

The capillary network between the IPL and superficial portion of the INL also displayed a three-dimensional vascular configuration (Figure 3-5). However, capillary density in this layer appeared significantly less than the RGC layer with a pronounced reduction in the number of vessels that projected along a single plane. Co-localisation studies using ON-bipolar cell marker demonstrated that capillaries in this layer were situated amongst bipolar cell processes.

**Figure 3-5 Inner plexiform layer and superficial inner nuclear layer capillary network (overleaf)**

Whole-mount confocal microscope image captured from a single laser channel (A) demonstrate the low density of capillaries in this network. Merged image with nuclei information (B) demonstrate a large number of cells in this region. Triple-stained transverse retinal section (C) demonstrates that this network is located at the boundary between the inner plexiform layer (IPL) and inner nuclear layer (INL). Lectins stain endothelium, Hoechst stain nuclei and Go-α stain ON-bipolar cells. NFL = nerve fibre layer, RGC = retinal ganglion cell layer, OPL = outer plexiform layer and ONL = outer nuclear layer. Scale bar = 50µm.
3.3.2.3 Boundary of deep INL and outer plexiform layer (OPL)

The deepest capillary network was found at the level of horizontal cells in the INL (Figure 3-6). Co-localisation studies using Go-α antibody revealed that synapses of ON-bipolar cells were distributed among the capillary network. Similar to the NFL network, capillaries in this layer predominantly demonstrated a laminar configuration with most capillaries projecting along a single plane. Multiple closed loops were also observed in this capillary network.

*Figure 3-6 Deep inner nuclear layer and outer plexiform layer capillary network (overleaf)*

Whole-mount confocal microscope image captured from a single laser channel (A) demonstrate the planar configuration of this network with multiple closed capillary loops. Merged image with nuclei information (B) demonstrate a high density of cells in this region. Triple-stained transverse retinal section (C) demonstrates that this network is located at the boundary between the inner nuclear layer (INL) and outer plexiform layer (OPL). Lectins stain endothelium, Hoechst stain nuclei and Parvalbumin stain horizontal cells. NFL = nerve fibre layer, RGC = retinal ganglion cell layer, IPL = inner plexiform layer and ONL = outer nuclear layer. Scale bar = 50µm.
3.3.3 Quantitative analysis of capillary diameter

Mean capillary diameter for all networks was $8.26 \pm 0.03 \, \mu m$. Table 3-2 provides mean capillary diameter for each capillary network. Capillary diameter in NFL network was significantly greater than RGC ($P < 0.001$) and IPL networks ($P < 0.001$). Capillary diameter in deep INL network was also significantly greater than RGC ($P < 0.001$) and IPL ($P < 0.001$) networks. There was no difference in capillary diameter between NFL and deep INL networks ($P = 0.227$) and RGC and IPL networks ($P = 0.740$). Age did not influence capillary diameter in any of the networks (all $P > 0.050$).

Table 3-3 provides mean capillary diameter for patients that deceased from cancer or accidental means. There was no difference in capillary diameter between the two groups in RGC network ($P = 0.303$), NFL network ($P = 0.874$), IPL network ($P = 0.964$) and deep INL network ($P = 0.183$).

3.3.4 Quantitative analysis of capillary density

Table 3-2 provides mean density measurements for each capillary network for each individual eye specimen. Capillary densities were significantly different between networks. Capillary density was greatest in the RGC network and was significantly greater than the NFL network ($P < 0.001$), IPL network ($P < 0.001$) and deep INL network ($P < 0.001$). Capillary density was lowest in the IPL network and was significantly lower than the deep INL network ($P < 0.001$). NFL capillary density was also greater than IPL network ($P = 0.036$). Age did not influence capillary density in any of the networks (all $P > 0.050$).
Table 3-3 provides mean capillary density for patients that deceased from cancer and accidental means. There was no difference in capillary density between the two groups in RGC network \((P = 0.660)\), NFL network \((P = 0.886)\) and IPL network \((P = 0.455)\). Capillary density was significantly greater in the cancer group in the deep INL network \((P = 0.046)\).

Table 3-2 Quantitative capillary diameter and density data for each network

(overleaf)

The mean capillary diameter and density of 9 data points are displayed for each specimen for each capillary layer. Additionally, the overall mean and standard error for nerve fibre layer (NFL) network, retinal ganglion cell (RGC) layer network, inner plexiform layer and superficial inner nuclear layer (IPL/superficial INL) network and deep inner nuclear layer and outer plexiform layer (deep INL/OPL) network is provided. Numbers in brackets indicate sample number for each measurement.
<table>
<thead>
<tr>
<th>Specimen number</th>
<th>Capillary diameter (µm)</th>
<th>Capillary density (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NFL</td>
<td>RGC</td>
</tr>
<tr>
<td>1</td>
<td>8.02</td>
<td>7.81</td>
</tr>
<tr>
<td>2</td>
<td>9.29</td>
<td>8.00</td>
</tr>
<tr>
<td>3</td>
<td>8.21</td>
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<td>7.24</td>
</tr>
<tr>
<td>12</td>
<td>7.89</td>
<td>7.60</td>
</tr>
</tbody>
</table>

| Mean ± SE (n)   | 8.47 ± 0.05 (540) | 8.01 ± 0.05 (540) | 7.99 ± 0.06 (540) | 8.56 ± 0.06 (540) | 13.69 ± 0.01 (12) | 26.74 ± 0.01 (12) | 11.28 ± 0.01 (12) | 16.12 ± 0.01 (12) |
Table 3-3 Morphometric comparisons between cancer-death and accidental-death groups\textsuperscript{12}

Mean and standard error for nerve fibre layer (NFL) network, retinal ganglion cell (RGC) layer network, inner plexiform layer and superficial inner nuclear layer (IPL/superficial INL) network and deep inner nuclear layer and outer plexiform layer (deep INL/OPL) network is provided. Capillary density in the Deep INL/OPL network was significantly different between the two groups.

<table>
<thead>
<tr>
<th>Capillary Network</th>
<th>NFL</th>
<th>RGC</th>
<th>IPL / Superficial INL</th>
<th>Deep INL / OPL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cause of death</td>
<td>Accident</td>
<td>Cancer</td>
<td>Accident</td>
<td>Cancer</td>
</tr>
<tr>
<td>Capillary Diameter (µm)</td>
<td>8.30 ± 0.08</td>
<td>8.64 ± 0.08</td>
<td>8.02 ± 0.07</td>
<td>8.01 ± 0.07</td>
</tr>
<tr>
<td>Total Capillary Density (%)</td>
<td>13.17 ± 0.01</td>
<td>14.22 ± 0.01</td>
<td>27.25 ± 0.02</td>
<td>26.23 ± 0.02</td>
</tr>
</tbody>
</table>
3.4 Discussion

3.4.1 Key findings

The major findings from these experiments were:

1. The demonstration of four morphometrically different capillary networks in the human retina being present in the following regions:
   - NFL
   - RGC layer
   - Border of IPL and superficial boundary of INL
   - Boundary of deep INL and OPL

2. The identification of significant differences in capillary diameter between all four networks were shown with the smallest capillary diameter being present in the RGC and IPL networks

3. Vascular density was greatest in the RGC layer
3.4.2 Advantages to perfusion labelling

Studying morphometric variations between capillary networks may provide vital information concerning the energy requirements of regional neuronal structures via effective neurovascular coupling mechanisms. It may also allow useful structure-function extrapolations between capillary morphometry and previously determined measurements on retinal metabolism\(^{42, 43, 65-72}\) to be performed.

Unlike previous investigators on this subject\(^{60, 61, 87, 88, 93, 94}\) a major advantage of the methodology employed in the present study is that it allows complete labelling of the retinal microcirculation without inadvertently altering surrounding non-vascular structures. Triple-labelling of post-perfused tissue also allowed accurate identification of capillary network location within the retina and confirmed what was demonstrated on flat-mount confocal microscope images.

3.4.3 Retinal capillary structure serving functional demands

Our findings reaffirm the studies conducted by Snodderly \textit{et al.}\(^{62, 85}\) and Gariano \textit{et al.}\(^{63}\) who demonstrated two inner and two outer capillary beds in human and non-human primate retinae. Similar to these previous authors\(^{62, 63, 85}\) we found that the innermost and outermost capillary networks displayed a single, planar configuration while that of the RGC and IPL demonstrated a complex three-dimensional configuration. Three-dimensional vascular configurations are believed to increase the efficiency of oxygen transfer and waste removal in metabolically active tissues\(^{203, 205}\). The variation in retinal capillary network morphology identified in the present study demonstrates important parallels to the human cerebral cortex where the microcirculation is adapted in accordance with
Regional neuronal demands.\textsuperscript{206-208} Previous researchers have demonstrated that the inner 1 mm of cerebral cortex demonstrates large meshes, the next 2 mm is filled with fine polygonal meshes and the outer 0.1 mm contains large quadrangular meshes that run parallel to the surface.\textsuperscript{206-208} Taken together our findings suggest that, similar to the brain, capillary networks in the retina are morphometrically adapted to serve the unique functional demands of each retinal layer.

### 3.4.4 Morphometric characteristics: capillary diameter

Utilising confocal microscope techniques and image analysis software we were able to quantify the morphometric characteristics of each capillary network and thus extend on the observations made by previous authors. We identified significant differences in capillary diameter between all four networks with the smallest capillary diameter being present in RGC and IPL networks. Mean capillary diameter is one measure of the rate at which a capillary network is able to exchange oxygen per unit volume of blood.\textsuperscript{209} A reduction in capillary diameter increases surface area/blood volume ratio resulting in greater oxygen exchange area for a given amount of blood. In the brain, differences in intra-cortical capillary network diameters correlate with regional variations in neuronal function.\textsuperscript{210} Lower mean diameters of capillary networks in RGC and IPL layers suggest high rates of oxygen exchange in this region of the retina. These morphometric findings correlate with our previous \textit{in vivo} functional studies where we demonstrated high oxygen uptake in the IPL.\textsuperscript{21} Capillary diameter measurements in the present study were also greater than previous reports\textsuperscript{62,85} and may reflect inter-species and tissue preparation differences.
3.4.5 Morphometric characteristics: capillary density

Within the grey matter of the CNS an increase in capillary density correlates strongly with an increase in blood flow and mitochondrial activity.\textsuperscript{211} Histological studies have shown significant variation in vascular density across neuronal layers in the brain.\textsuperscript{212} Total capillary density in the non-human primate fovea, peri-fovea and peri-papillary region has been measured as 40\%, 45\% and 60-70\% respectively.\textsuperscript{62,85} Vascular density is also known to vary depending on the eccentricity from central retina.\textsuperscript{63} To our knowledge, there have not been previous capillary density measurements of individual networks in the human retina. In this study, capillary density was greatest in the RGC layer implicating it as a metabolically intense region. The dependency of RGCs on a high density capillary circulation may be one reason why this subset of neurons could be particularly vulnerable to acute, transient and mild hypoxic stress.\textsuperscript{213}

3.4.6 Limitations

Although this study provides important new knowledge regarding retinal capillary topography in human eyes we acknowledge several limitations of the report. Firstly, the sample size of this study is relatively small and consists of only 16 human eyes. It is difficult to acquire human eyes from healthy individuals and we did not wish to waste human tissue by performing an extensive analysis when appropriate statistical tests permitted us to reliably identify differences between capillary networks, despite the relative small sample size. The other limitation of this work is that only one retinal eccentricity was examined. The aim of this study was to quantify the morphometric characteristics of capillary networks and
speculate upon structure-function relationships between vascular units and regional metabolic activity. It is expected that the morphometric characteristics of capillary networks will vary according to retinal eccentricity especially in specialised regions of the retina such as the fovea, macula and immediate peri-papillary tissue. Similar to the report by Snodderly et al.\textsuperscript{62,85} we observed a change in the NFL capillary network in a proximo-distal direction. Gariano et al.\textsuperscript{63} have demonstrated that deeper vascular layers disappear in the peripheral retina. Examination of the far peripheral retina may therefore possibly demonstrate significant alterations to capillary networks.

### 3.4.7 Summary

This study provides important insights into vascular mechanisms relevant to retinal homeostasis. It has been demonstrated that the presence of disease was a significant factor in determining capillary density and it is expected that regional capillary networks will be altered by ocular and systemic disease. It will therefore be important to perform similar studies using diseased human eyes as it may enhance our understanding of capillary-mediated mechanisms in retinal vascular disease.
CHAPTER 4

HUMAN RETINAL MICROVASCULATURE WITH CARDIOVASCULAR DISEASE
4. Human Retinal Microvasculature with Cardiovascular Disease

4.1 Aim

Having established in the normal human retina there are four morphometrically different capillary networks with significant quantitative characteristics in capillary diameter and density between each of the four retinal capillary layers, it was important to examine the same region in a diseased state.

Studies in the past have shown a positive correlation between cardiovascular disease and retinal microvascular abnormalities, but this has usually been in the presence of clinically known retinopathy.\textsuperscript{111-113} It is unknown as to whether the morphometrically organised capillary network in the retina is altered prior to observed clinical retinopathy in patients with cardiovascular comorbidities. Understanding retinal microvascular changes prior to clinical manifestation will offer important insights into the prevention of retinal vascular diseases and resultant vision loss.

At the time this chapter was conducted, only one study had documented quantitative changes in perifoveal capillary networks in patients with vascular comorbidities.\textsuperscript{214} To date there have been no studies to determine whether the representative area of retina would also reveal quantitative changes in capillary networks in patients with cardiovascular comorbidities, which may in turn lead to the development of clinical retinopathy.
The purpose of the following chapter is to describe whether patients with cardiovascular comorbidities reveal demonstrable quantitative changes in the retinal vasculature with no known ocular disease, leading to greater insight into ophthalmic disease processes. In addition to this, our findings may provide beneficial insights into previous reports that have determined a link between cardiovascular comorbidities and retinal vascular disease.
4.2 Definitions

In this study the following definitions have been used:

- **Control** – being free from known ocular disease or vascular disease. They have been used in this study as healthy, control eyes.

- **Diseased** – having a known history of cardiovascular disease or risk factors, such as ischaemic heart disease, stroke and hypertension, and are free from known ocular disease.

- **Capillary diameter** – the perpendicular distance across the maximum transverse measurement of each vessel.

- **Capillary density** – the percentage quantity of area occupied by vessel lumens.

- **Laminar** – a single-planar orientation that is predominantly confined to a single retinal layer with minimal projections along the z-axis.

- **Retinal vascular pathological changes** – an abnormal appearance of the retinal vasculature.

- **Three-dimensional** – the capillary networks that traversed prominent projections along the vertical z-axis and were not confined to a single plane.
4.3 Materials and Methods

4.3.1 Human donor eyes

All human donor eyes used in this paper had no known history of ocular disease. A total of 27 human eyes from 22 donors were studied. Of these, 11 eyes from 8 donors were carefully chosen based on having a known history of cardiovascular disease, and 16 eyes from 14 donors were used as the control group. The control eyes used in this study were from our previous study in Chapter 3. In the diseased group, patients had suffered from one or more of the following cardiovascular comorbidities: ischaemic heart disease, hypertension, atherosclerosis, cerebral vascular accident, cardiomyopathy, atrial fibrillation, hypercholesterolaemia, and vascular disease (Table 4-1). Eyes were acquired post removal of corneal buttons for transplantation from the Lions Eye Bank (Lions Eye Institute, Western Australia). Each donor’s demographic data, cause of death, and post-mortem time to cannulation are presented in Table 4-1 (Donors A to V).

Table 4-1 Donor Demographic Details: Age, Sex, Eye, Vascular Comorbidities, Cause of Death, and Time to Cannulation for Donor Eyes (overleaf)

Normal control eye data were taken from the study directly prior to this paper, which researched the normal microvasculature of the human retina. Age (in years); Sex (M = male, F = female); R = right; L = left; AF = atrial fibrillation; MVA = motor vehicle accident; – (donor had no cardiovascular comorbidities). Line separates disease (above) and control (below) eyes. Donor eyes with vascular abnormalities are noted with an asterisk (*).
<table>
<thead>
<tr>
<th>Donor ID</th>
<th>Age</th>
<th>Sex</th>
<th>Eye</th>
<th>Cardiovascular comorbidities</th>
<th>Cause of Death</th>
<th>Time to Cannulation (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>68</td>
<td>M</td>
<td>L</td>
<td>Ischaemic heart disease, hypertension</td>
<td>Cerebral vascular accident</td>
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</tr>
<tr>
<td>B</td>
<td>41</td>
<td>M</td>
<td>R</td>
<td>Atherosclerosis</td>
<td>Intracranial haemorrhage</td>
<td>17.0</td>
</tr>
<tr>
<td>C</td>
<td>51</td>
<td>M</td>
<td>L + R</td>
<td>Ischaemic heart disease</td>
<td>Myocardial infarction</td>
<td>19.0</td>
</tr>
<tr>
<td>D</td>
<td>65</td>
<td>M</td>
<td>L + R</td>
<td>Cerebral vascular accident</td>
<td>Intracranial haemorrhage</td>
<td>14.0</td>
</tr>
<tr>
<td>E</td>
<td>64</td>
<td>M</td>
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<td>F</td>
<td>65</td>
<td>M</td>
<td>R</td>
<td>Cardiomyopathy</td>
<td>Heart Failure</td>
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<td>G</td>
<td>75</td>
<td>F</td>
<td>L*</td>
<td>Hypertension, AF, vascular disease</td>
<td>Cerebral vascular accident</td>
<td>18.0</td>
</tr>
<tr>
<td>H</td>
<td>72</td>
<td>M</td>
<td>L* + R</td>
<td>Hypertension, hypercholesterolaemia</td>
<td>Myocardial infarction</td>
<td>12.0</td>
</tr>
<tr>
<td>I</td>
<td>22</td>
<td>M</td>
<td>L</td>
<td>-</td>
<td>MVA</td>
<td>15.0</td>
</tr>
<tr>
<td>J</td>
<td>32</td>
<td>M</td>
<td>L</td>
<td>-</td>
<td>MVA</td>
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</tr>
<tr>
<td>K</td>
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<td>L + R</td>
<td>-</td>
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</tr>
<tr>
<td>L</td>
<td>67</td>
<td>M</td>
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<td>-</td>
<td>Cancer</td>
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<tr>
<td>M</td>
<td>23</td>
<td>M</td>
<td>L</td>
<td>-</td>
<td>Suicide</td>
<td>22.0</td>
</tr>
<tr>
<td>N</td>
<td>66</td>
<td>M</td>
<td>L + R</td>
<td>-</td>
<td>Cancer</td>
<td>15.0</td>
</tr>
<tr>
<td>O</td>
<td>22</td>
<td>M</td>
<td>L</td>
<td>-</td>
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</tr>
<tr>
<td>P</td>
<td>53</td>
<td>M</td>
<td>L</td>
<td>-</td>
<td>MVA</td>
<td>14.0</td>
</tr>
<tr>
<td>Q</td>
<td>60</td>
<td>M</td>
<td>L</td>
<td>-</td>
<td>Collapse</td>
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<tr>
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<td>M</td>
<td>R</td>
<td>-</td>
<td>Drowning</td>
<td>15.0</td>
</tr>
<tr>
<td>S</td>
<td>72</td>
<td>F</td>
<td>L</td>
<td>-</td>
<td>Sepsis</td>
<td>3.0</td>
</tr>
<tr>
<td>T</td>
<td>60</td>
<td>M</td>
<td>R</td>
<td>-</td>
<td>Liver disease</td>
<td>3.5</td>
</tr>
<tr>
<td>U</td>
<td>59</td>
<td>M</td>
<td>R</td>
<td>-</td>
<td>Melanoma</td>
<td>12.0</td>
</tr>
<tr>
<td>V</td>
<td>39</td>
<td>M</td>
<td>L</td>
<td>-</td>
<td>Sepsis</td>
<td>20.0</td>
</tr>
</tbody>
</table>
4.3.2 Tissue preparation and immunolabelling

A detailed review of the novel perfusion-based techniques used for targeted retinal endothelial labeling has been discussed in Chapter 2.

Additionally, we used Phalloidin conjugated to Alexa Fluor 546 (30U; Invitrogen, Carlsbad, CA) to label the f-actin microfilaments; VE-cadherin (1:50; sc-6458; Santa Cruz Biotechnology) to label adhesion molecules at endothelial cell junctions; Concanavalin A (20µg/ml; Invitrogen, Carlsbad, CA) to label glucoglycans; Claudin-5 (1:50; 1mg/mL; Sigma-Aldrich, St. Louis, MO) to label the transmembrane tight junction proteins; Monoclonal Anti-Glial Fibrillary Acidic Protein (1:80; Sigma-Aldrich, St. Louis, MO) to stain astrocytes and glial cells; Bisbenzamide (H33258; 1.2µg/ml; Sigma-Aldrich, St. Louis, MO) or iodide dye (YO-PRO-1; 6.6µM; Invitrogen), to label the cell nuclei; and Goat anti-cellular retinaldehyde-binding protein (1:50; sc18757; Santa Cruz Biotechnology) to label the Muller cells.

After perfusion labelling, the retina was carefully dissected from the globe and flat-mounted.

4.3.4 Microscopy and image preparation

The methodology for both microscopy image acquisition and image preparation for this study is discussed in Chapter 2.

Furthermore, reconstruction of three-dimensional capillary networks were performed using Imaris software (version 7.4.2; Bitplane, Zurich, Switzerland).
4.3.5 Capillary network topography and quantitative analysis

For comparison consistency, the methodology used to study the capillary topography and quantitate the capillary networks was identical to the first paper on normal vasculature in the human retina. Using the movie-sequence function in Image J, we were able to view the z-stack sequentially and determine if the capillary networks were laminar or three-dimensional. This function also enabled viewing of the capillary morphology simultaneously with the surrounding nuclei, and thus confirmed the location of the capillary networks within the retina.

Once the confocal images had been prepared into a single z-projected image for each of the capillary networks, we were able to obtain measurements of the diameters and density of the capillary networks.

4.3.6 Statistical analysis

Statistical analysis techniques used in this study are described in Chapter 2. Additionally, we also performed a one-way ANOVA to analyse the differences between control and diseased eyes for both diameter and density, using the same random effects model as described.
4.4 Results

4.4.1 General

The mean donor age of the control group was 49.7 ± 4.9 years, taken from eleven left and five right eyes from two female and twelve male donors. The average post-mortem time before eyes were perfused was 13.39 ± 1.68 hours.

The mean donor age of the diseased group was 62.6 ± 3.39 years, taken from six left and five right eyes from one female and seven male donors. There was no statistical difference in the mean age of the two groups (P=0.09). The average post-mortem time before perfusion of eyes was 14.3 ± 1.20 hours.

After cannulation and perfusion of the central retinal artery, all orders of the retinal microvasculature were examined to ensure excellent perfusion and washout of blood from the retinal vasculature, as well as clear labelling of the endothelial cells, nuclei and smooth muscle cells.

4.4.2 General Comparisons in Capillary Topography in the Control and Diseased Human Retina Capillary Networks

The previous chapter highlights the consistent locations where morphometrically different capillary networks were found in the normal human retina.\textsuperscript{12} Vasculature networks of the diseased group were found in identical locations as the control group, and were located at:
1. Nerve fibre layer (NFL)
2. Retinal ganglion cell layer (RGC)
3. Border of the inner plexiform layer (IPL) and superficial boundary of the inner nuclear layer (sINL)
4. Boundary of deep inner nuclear layer (dINL) and outer plexiform layer (OPL).

Figure 4-1 compares confocal images of capillary networks in the retina from both the control and diseased group. In comparison to the control vasculature networks, the same vascular distribution pattern could be observed in the diseased group.

4.4.2.1 NFL capillary network

The NFL capillary network in the diseased group was similar to the control group, in that it was laminar in structure, and also orientated parallel to the RGC axon bundles linearly, and appeared to have fewer anastomoses compared to the other capillary networks. The main difference that could be observed was the marked decrease in capillary density of the network in the diseased group.

4.4.2.2 RGC layer capillary network

The RGC layer presented with the largest density of vasculature, and both retinal arterioles and venules were seen. Similar to the control group, capillaries were observed to not only project along a single plane but also seen to run at varying angles obliquely along the z-plane, and also form anastomoses with capillary networks in all capillary layers, forming a three-dimensional structure. It was also observed in this layer that there was a significant decrease in capillary density (P<0.05) in the diseased group compared to the control group.
4.4.2.3 IPL / sINL

In the diseased group, the IPL and superficial portion of the INL capillary network displayed a three-dimensional vascular configuration and was consistently the least dense capillary network compared to all other layers in a projected single plane, similar to the control group. Occasional capillary closed loops were seen in this region.

4.4.2.4 dINL / OPL

At the plane of the horizontal cells in the INL, the deepest capillary network could be found, for both control and diseased groups. This layer was laminar in morphology, and most capillaries were seen to project along a single plane. Closed loops were observed at this layer to be more numerous than any other layer.
Figure 4-1 Comparison of control and diseased capillary networks (overleaf)

(A) to (D) = Representative images of retinal vasculature from control group (A = Donor J; B = Donor M; C and D = Donor K). (E) to (H) = Representative images of retinal vasculature from diseased group (E to H = Donor D). Whole-mount confocal microscopy images capture both vascular and nuclei information at 3 mm superior to the optic disc. Phalloidin label the endothelial f-actin, and Hoechst or YO-PRO-1 labelled the nuclei. (A) The vasculature of the nerve fibre layer from the control group demonstrates a higher density of vasculature compared to the vasculature from the diseased group at the same region (E). This is also shown in the comparison of the vasculature in the retinal ganglion cell layer from the control group (B) and the diseased group (F) in the same region. In the retinal ganglion cell layer, venules and arterioles are also observed in addition to capillaries. (C) illustrates the control capillary network which lies between the inner plexiform layer and the superficial inner nuclear layer, while (G) shows the capillary network in this region from diseased group. (D) and (H) display the deepest capillary network of the control and diseased groups respectively, and lies at the border of the deep inner nuclear layer and outer plexiform layer. Scale bar = 100 µm.
4.4.3 Quantitative Analysis of Capillary Diameter

Mean capillary diameter for all networks was $8.92 \pm 0.05 \, \mu m$ ($n = 1980$). Table 4-2 provides the mean capillary diameter for each capillary network in patients with cardiovascular comorbidities. Diameters were not significantly different in each of the capillary networks ($P > 0.05$).

Figure 4-2 and Table 4-3 shows the comparison between the control and diseased networks. While capillary diameters in the diseased group were all larger than their control equivalents for all capillary networks, there was no statistically significant difference when comparing each of the four capillary networks of the diseased eyes against each of the four equivalent capillary networks of the control group ($P > 0.05$).

Figure 4-3 and Table 4-3 show the comparisons for diameter between ages below and above 60 years old. There was also no statistically significant difference in diameter when comparing each of the four capillary networks of the eyes below 60 years old with those above 60 years old ($P > 0.05$).

4.4.4 Quantitative Analysis of Capillary Density

Table 4-2 also provides the mean density measurements for each capillary network in patients with cardiovascular comorbidities. The capillary density was greatest in the RGC layer, followed by the deep INL/OPL, then IPL/superficial INL, and lastly the NFL. There was a significant difference between the capillary density of the NFL and RGC ($P < 0.001$), the NFL and deep INL/OPL ($P < 0.002$), the RGC and IPL/superficial INL ($P < 0.001$), the RGC and deep
INL/OPL (P < 0.003), and the IPL/superficial INL and deep INL/OPL (P < 0.006).

Figure 4-3 and Table 4-3 provide a comparison of capillary networks between the control and diseased groups. The average density measurements of all capillary networks in the diseased group were less than their control counterparts. There was a significant decrease in density between the NFL of the diseased group when compared to the NFL of the control eyes (P < 0.004), as well as a significant decrease when comparing the RGC layers of the diseased to the control groups (P < 0.02).

Figure 4-3 and Table 4-3 reveal the comparisons of densities in each capillary layer for those who were below and above 60 years old. There was no statistically significant difference between the densities of the four capillary networks between the different sets of ages (P > 0.05).

Table 4-2 Quantitative capillary diameter and density data for each network of the diseased human eyes

The mean capillary diameter and density of 9 data points are displayed for each specimen for each capillary layer. Additionally, the overall mean and standard error presented for diameter and density in patients with cardiovascular comorbidities in four capillary networks: nerve fibre layer (NFL), retinal ganglion cell (RGC) layer, inner plexiform layer and superficial inner nuclear layer (IPL/superficial INL), and deep inner nuclear layer and outer plexiform layer (deep INL/OPL). Bracketed numbers indicate the sample number for each measurement.
<table>
<thead>
<tr>
<th>Specimen number</th>
<th>Capillary diameter (µm)</th>
<th>Capillary density (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NFL</td>
<td>RGC</td>
</tr>
<tr>
<td>2</td>
<td>8.54</td>
<td>8.89</td>
</tr>
<tr>
<td>3</td>
<td>8.14</td>
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<td>10</td>
<td>10.58</td>
<td>11.74</td>
</tr>
<tr>
<td>11</td>
<td>11.83</td>
<td>11.46</td>
</tr>
<tr>
<td>Mean ± SE (n)</td>
<td>8.86 ± 0.09 (495)</td>
<td>8.93 ± 0.09 (495)</td>
</tr>
</tbody>
</table>
Table 4-3 Morphometric comparisons between control and diseased groups

Mean and standard error presented for capillary diameter and density in these four capillary networks: nerve fibre layer (NFL), retinal ganglion cell (RGC) layer, inner plexiform layer and superficial inner nuclear layer (IPL/sINL), and deep inner nuclear layer and outer plexiform layer (dINL/OPL). <60yo = less than 60 years old, >60yo = greater than 60 years old. Capillary density in the diseased NFL and RGC layer were significantly less than the normal control group. There was no significant difference between the capillary layers for age. Bracketed numbers indicate the sample number for each measurement.
Figure 4-2 Graphs showing the averaged data of capillary diameter and density in the control and diseased groups in each of the capillary networks of the retina at 3 mm superior to the optic disc (overleaf)\textsuperscript{13}

Layer 1 = Nerve Fibre Layer, Layer 2 = Retinal Ganglion Cell layer, Layer 3 = border of Inner Plexiform Layer and superficial Inner Nuclear Layer, Layer 4 = border of deep Inner Nuclear Layer and Outer Plexiform Layer. The diameter bar graph shows there were no significant differences in each of the capillary networks (P > 0.05). Density box plots show the mean value and the standard errors. Significant differences (P < 0.05) were noted between the densities of the control versus diseased eyes in Layer 1 and Layer 2, as indicated by the asterisk (*).
Figure 4-3 Graphs showing the average capillary diameter and density in the young (<60 years old) and elderly (>60 years old) groups in each of the different capillary networks (overleaf) \(^{13}\)

Layer 1 = Nerve Fibre Layer, Layer 2 = Retinal Ganglion Cell Layer, Layer 3 = border of Inner Plexiform Layer and superficial Inner Nuclear Layer, Layer 4 = border of deep Inner Nuclear Layer and Outer Plexiform Layer. The diameter bar graph shows there were no significant differences in each of the capillary networks (\(P > 0.05\)) above or below 60 years old. The density box plot shows the mean value and the standard errors. There were no significant differences (\(P > 0.05\)) between all capillary layers.
4.4.5 Pathological vascular changes

There were three eyes which were identified to have pathological vascular changes, corresponding to clinical appearances of microaneurysms, tortuous dilated venules, and retinal vascular abnormalities in the diseased group (Figure 4-4, Figure 4-5, and Figure 4-6). There were no vascular pathologies found in the control group studied.

4.4.5.1 Microaneurysms

Numerous bulges, which we refer to in this study as microaneurysms, were observed in the retinal capillary network from two of these donor eyes. The bulges were present in all four retinal vascular layers, and extended right out to the periphery of the retina in all four quadrants. Over 100 microaneurysms were seen per quadrant. On randomly sampling 30 microaneurysms, we found the largest external diameter of microaneurysms to range from 29.12 µm to 116.25 µm. Most of these microaneurysms were noted to be lined by endothelial cells with nuclei of different size and density. Some had large nuclei with speckled areas of increased density, whilst others were small and contiguously dense throughout. In general, a greater proportion of the endothelial cell nuclei were larger, less dense, more immature-appearing cells. No pericytes were seen. Claudin-5 which stained the transmembrane tight junctions, were noted to particularly highlight the microaneurysms (Figure 4-7). F-actin was contained within the vasculature and the nuclei were distinctly separated from each other, however most of the cell borders were indiscernible.
4.4.5.2 Tortuous vessels

Higher proportions of venules observed in the retinal vasculature in the diseased group were more tortuous in appearance and had dilated lumens. One particularly tortuous vessel from Donor G was found 3 mm nasal to the optic disc and was 39.47 µm in diameter (Figure 4-5). It was observed to be a derivation from the vasculature at the Retinal Ganglion Cell Layer (Layer 2), and appeared to be a draining tortuous vein. High magnification showed circumferential arrangement of the f-actin around the dilated vessel.

4.4.5.3 Retinal vascular abnormalities

Pathological retinal vascular changes were not limited in the location that was selected to perform quantitative assessments of capillary density and diameter. These pathological vascular changes were seen to occur in the different capillary networks and were associated with other cellular changes, particularly with glial cells in the retina. In one particular eye (Donor H), there were over 1000 retinal vascular abnormalities seen per quadrant. Claudin-5, glial fibrillary acidic protein, and cellular retinaldehyde-binding protein label were used for illustrating the pathological alterations of vascular endothelial cells, astrocytes and Muller cells in the microaneurysms. Figure 4-6 is a three-dimensional reconstructed confocal projected image from Donor H showing that microaneurysms were located in various capillary networks and associated with alterations of glial cells and possible leakage from wall of microaneurysm, forming a relatively large retinal vascular abnormality. Whole-mount confocal images captured (Figure 4-7) demonstrate Claudin-5 labelling the transmembrane tight junction proteins, which particularly in highlight the microaneurysms. Close relationship between
microaneurysms and glial cells was evidenced by co-labelled Claudin-5, glial fibrillar acidic protein and cellular retinaldehyde-binding protein. Under microscopy, they fluoresced brightly and were different from the bulging outpouchings from retinal vasculature. They had a “burst” appearance with some minor leakage. They may potentially be representative of burst microaneurysms which were leaky.
Figure 4-4 Microaneurysms (overleaf) 13

(A) is a confocal projected image of 24 optical sections which show capillary microaneurysms within Donor H’s retinal vasculature from the diseased group using the x20 objective lens. Saccular microaneurysms (arrowheads) were predominantly noted. (B) is a confocal projected image of 17 optical sections with the x60 objective lens and shows a microaneurysm which is seen as saccular capillary outpouching containing proliferated endothelial cells. (C) is a z-series of 110 optical sections with the x60 objective lens and captures a round-shaped saccular microaneurysm amongst a background of numerous nuclei of neural cells in the inner nuclear layer. (D) shows a magnified image of (C) with less stacked images of 72 optical sections, to allow identification of the proliferated endothelial cells on the wall. F-actin staining on the wall of this microaneurysm shows remarkably uneven intensity. The cell boundary of most endothelial cells cannot be clearly defined except an endothelial cell (outlined in yellow). The difference in nuclei size and density staining of the endothelial cells is also found in this microaneurysm. (E) and (F) are schematic diagrams of images (C) and (D) respectively. Both (E) and (F) show the outline of microaneurysms and highlight the difference in nuclei size and density. Some nuclei are relatively larger with a speckled chromatin-filled appearance shown in purple (F) while some nuclei show relatively dark in staining (pyknotic-like appearance) shown in blue (F). These changes of endothelial cells may indicate varying stages of endothelial proliferation. Scale bar: A = 100 µm, B = 17.5 µm, C = 33.3 µm, D = 15.9 µm.
Figure 4-5 Tortuous retinal venule (overleaf)

(A) shows a large sausage-shaped, dilated and tortuous retinal venule with irregularities in f-actin staining and shape in the vessel wall amongst sparse vasculature from Donor G in the diseased group. It comprises of 19 optical sections to complete a confocal projected image using the x20 objective lens. Phalloidin label the endothelial f-actin, and Hoechst label the nuclei. (B) is the lower portion of this vascular abnormality magnified and captured using the x60 objective lens. (C) and (D) are schematic diagrams of the vasculature in images (A) and (B) respectively. These schematics highlight with clarity the tortuosity of the vasculature. Scale bar: A = 100 µm, B = 33.3 µm.
Figure 4-6 Retinal vascular abnormality (overleaf) 13

(A) is a confocal projected image of the beginnings of a small retinal vascular abnormality comprising of 61 optical sections using the x20 objective lens from Donor H. Claudin-5 label the transmembrane tight junction protein, Hoechst label the nuclei, glial fibrillary acidic protein label the astrocytes, and cellular retinaldehyde-binding protein label the Muller cells. (B) is a three-dimensional reconstruction of (A) showing the diseased vasculature from the sclerad surface. (C) is a confocal image of (A). Vasculature is labelled in red, the Muller cell processes in blue, and the astrocytes and nuclei are labelled in green. The white arrow points towards microaneurysms in (A) and correlate to the three-dimensional reconstructions in (B), and the yellow arrows point towards a larger retinal vascular abnormality. The retinal vascular changes are seen to be in closer association with the Muller cells, but a limited association with the astrocytes. Scale bar: A = 100 µm, B = 200 µm.
Whole-mount confocal images capture the four different vascular layers in a diseased eye (Donor H) at 6 mm nasal to the optic disc. (A) to (D) show Claudin-5 labelling the transmembrane tight junction proteins and particularly highlight the microaneurysms. (E) to (H) demonstrate labelling of Claudin-5 (green), glial fibrillary acidic protein (blue) and cellular retinaldehyde-binding protein (red). After merging the images, the microaneurysms appear yellow, demonstrating the co-localisation of the Claudin-5 and cellular retinaldehyde-binding protein. Microaneurysms are seen to be present in all layers of the retinal vasculature. As they are relatively large compared to the capillaries, some of the microaneurysms are seen to traverse several retinal vasculature layers. (A) and (E) Nerve Fibre Layer (Layer 1); (B) and (F) Retinal Ganglion Cell Layer (Layer 2); (C) and (G) Border of Inner Plexiform Layer and superficial Inner Nuclear Layer (Layer 3); (D) and (H) Border of deep Inner Nuclear Layer and Outer Plexiform Layer (Layer 4). Scale bar = 100 µm.
4.5 Discussion

4.5.1 Key findings

The major findings from this study are as follows:

1. Cardiovascular comorbidities were found to have an association with changes in capillary density within the retina. Specifically, there was a significant decrease in capillary density of inner retina in donors with cardiovascular comorbidities when compared to the control group.

2. Pathological vascular changes were identified in the vasculature of three out of eleven (27.3%) donors with cardiovascular comorbidities despite no known ocular disease. Specifically, vascular abnormalities found corresponded to clinical appearances of microaneurysms, tortuous venules, and other retinal changes. Close association of microaneurysms and glial cells in different capillary networks are also demonstrated.

It is important to note that only in these three eyes were vascular anomalies noted, and there were no other findings of these vascular abnormalities seen in any of the other human donor eyes studied, regardless of age or disease.

These findings may suggest that retinopathy likely begins much earlier than the onset of clinically detectable disease and we potentially should counsel at-risk patients with multiple cardiovascular comorbidities much earlier to prevent retinopathy.
4.5.2 Endothelial cells and cardiovascular comorbidities

Endothelial cells within the morphometrically organised capillary network play an indispensable role in modulating retinal homeostasis.\textsuperscript{42, 47, 48, 215} The intricate morphometric design of the capillary networks is believed to be strongly associated with the metabolic demands of neuronal subtypes within the region,\textsuperscript{21} enabling the heterogenic metabolic demand of the neurons and supporting glia to be met.\textsuperscript{12, 218} Theoretically, patients with cardiovascular comorbidities have the potential to alter the blood-retinal barrier and thus play an important role in influencing retinal nourishment.\textsuperscript{219, 220} By demonstrating quantitative changes in parameters such as capillary density and diameter, important information regarding how retinal disease processes are formed in patients with cardiovascular disease is more clearly understood.\textsuperscript{205, 221}

Cardiovascular comorbidities including ischaemic heart disease, hypertension and hypercholesterolaemia, is associated with retinal vascular disease.\textsuperscript{3-10} Alterations at the cellular level to basement membranes, mural cells and endothelial tight junctions have been shown in patients with cardiovascular disease and risk factors in histopathologic and electron microscopy studies.\textsuperscript{107, 108} However, previous studies which demonstrated changes in patients already had clinical manifestations of retinal disease, or aimed to induce diseased states. Our study not only supports existing data but also augments previous studies in that we have shown demonstrable quantitative changes prior to the manifestation of known retinal vascular disease. An example of clinical relevance would be the detection of retinal disease in people with diabetes before the development of clinical manifestation of diabetic retinopathy.
4.5.3 Capillary density

All capillary layer densities were lower in the retina of patients with cardiovascular comorbidities compared to normal retina, and were significantly so in the nerve fibre layer and the retinal ganglion cell layer. A decrease in capillary density may be the normal progression of retinal vascular disease processes, as confirmed by similar results in previous studies.\textsuperscript{103} Whilst capillary network layer three (border of IPL and superficial boundary of INL) and four (boundary of deep INL and OPL) were not significantly different between the patients with cardiovascular comorbidities and normal control patients, this may be due to the diseased patients being in the earliest stages of retinal vascular disease. Perhaps, given more time to allow for further retinal disease processes to occur, layer three and four of the retinal capillary network may also be significantly decreased.

A clinical example of relevance once capillary density has decreased is cotton-wool spots. Cotton-wool spots are usually caused by ischaemia in the nerve fibre layer, with the pathogenesis being described as an interruption of axoplasmic transport.\textsuperscript{222} Ischaemia may then lead to retinal vascular pathological changes, such as induced vessel dilatation.

4.5.4 Capillary diameter

It was shown in our study that all capillary diameters were larger (statistically not significant) in the eyes of diseased patients compared to the control group. These finding were shown in a similar study,\textsuperscript{214} and represented similar reports to cerebrocortical models of hypoxia\textsuperscript{223} and hypercapnic hyperemia.\textsuperscript{224-226} An increase in the capillary diameter allows a higher blood flow volume and therefore
increased tissue oxygenation. A possible hypothesis for an increased capillary diameter in patients with cardiovascular comorbidities but no known ophthalmic disease, may be due to a relatively hypoxic retina in these eyes compared to normal eyes, leading to a compensatory increase in capillary diameter to provide nourishment to hypoxia-sensitive neuronal tissue. These findings aid us to further understand the relationship between cardiovascular comorbidities and retinal vascular disease processes.

4.5.5 Early pathological vascular changes

Even though all Donors had no known presence of ocular disease, pathological vascular changes were also found in this study with clinically similar appearances to microaneurysms and tortuous vasculature. Of these, microaneurysms serve as an important finding in the clinical setting, particularly with regards to diabetic retinopathy. Microaneurysms are often graded as being the first and earliest important clinical sign characteristic of diabetic retinopathy, and serves as a catalyst for the diagnosis of diabetic retinopathy.

4.5.6 Microaneurysms

Stitt et al. has documented in detail four distinct groups of microaneurysms, and described the several stages in the formation of diabetic retinopathy microaneurysms. The microaneurysms we observed in our study were most likely Type I or Type II, however given our novel technique of perfusion, we were unable to observe the polymorphonuclear or red blood cells in the lumen, and made our observations by the endothelial cell structures instead.
Another important study written by Moore et al.\textsuperscript{230} classified microaneurysms into three categories corresponding to morphology: saccular, fusiform and focal bulges. In our study, we predominantly saw saccular microaneurysms.

Hypertension is another important risk factor in the development of microaneurysms. The exudative phase of retinal microvasculature anomalies usually shows classical features such as microaneurysms and haemorrhages in hypertension.\textsuperscript{3} A recent classification on hypertensive retinopathy revealed that retinal signs such as microaneurysms, cotton-wool spots, haemorrhages and hard exudates are associated with moderate hypertensive retinopathy.\textsuperscript{231} The three human donor eyes in which we observed vascular abnormalities had hypertension. This could potentially be a significant reason why these observations were noted in these eyes. Other risk factors in which microaneurysms would be important include smoking and pregnancy.

Macula oedema stemming from leaky diabetic microaneurysms is one of the leading causes of vision loss in working-aged people in the developed world,\textsuperscript{232} thus further research into this area is crucial. Knowledge in the past regarding microaneurysm structure and location have predominantly been histopathological studies with light microscopy or electron microscopy on trypsin-digested retinal flat mounts.\textsuperscript{229,233} More recently confocal-images,\textsuperscript{230} OCT,\textsuperscript{232,234} and fluorescein angiograms\textsuperscript{235} have also be utilised.

Our findings support previous data on the size and origin of the microaneurysms. Our random sampling of 30 microaneurysms diameters ranged from 29 µm to 116 µm, which was similar to the 14 µm to 136 µm range reported in another confocal study,\textsuperscript{230} and the 43 µm to 266 µm range reported in an OCT study.\textsuperscript{232} As shown
in Figure 4-7, there were also microaneurysms seen originating in all four retinal layers, as well as occupying between one to four retinal layers.

To our knowledge, there have been no studies which have observed the individual endothelial cells and nuclei which encapsulate the microaneurysms. Our data reveals there are multiple different-appearing nuclei framing the microaneurysm. Some nuclei were small and pyknotic-like, and other nuclei were larger, irregular in shape, and appeared to have less chromatin. Thus the difference in the nuclei shape, size and density could be due to the varying stages of cellular proliferation as the microaneurysm develops. Whilst f-actin staining was visible in the vasculature, most endothelial cell borders were indiscernible in the microaneurysm. However this is to be expected with proliferating cells which have not completely differentiated.

4.5.7 Tortuous vessels

Another finding in one of the diseased eyes (Donor G) showed a particularly tortuous vessel. Tortuosity of vasculature in the human body is common and has been linked with diabetes mellitus, atherosclerosis, hypertension, aging, and genetic defects.\(^{236-239}\) It has been found to be generally asymptomatic when mild, however when severe could lead to end organ ischaemia.\(^{240}\)

Specifically, retinal vessel abnormalities such as retinal vasculature tortuosity, has been found to be in association with diabetes mellitus, hypertension, retinopathy, genetic disorders, lower high-density lipoprotein cholesterol level, ischaemic heart disease, stroke and cerebral vessel disease.\(^{89, 241-250}\)
Our study found the tortuous vessel to be draining into a main retinal vein. Many more studies research arterial tortuosity, however there are few which examine venous tortuosity. In one population-based cross-sectional study, it was found that retinal venules were significantly more tortuous than retinal arterioles. As it is known that the venous system is highly complex with diseases being ten times more frequent in the venous than arterial system, it is thus important to study the venous system of the eye in more depth.

4.5.8 Retinal vascular abnormalities

Lastly, there were multiple retinal vascular abnormalities seen in two diseased eyes, up to 30 per quadrant. As they had a “burst” appearance with minor leakage, they may potentially be representative of burst leaky microaneurysms. It is unclear whether this may have occurred as part of the perfusion and staining process from our novel technique, or whether these were already present. As we closely monitor the input perfusate pressure at the entry point to the eye and keep it in the physiological range it is most likely that these leakage sites were already present in the diseased eye.

The relationship between microaneurysms and leakage is not yet understood, however understanding its occurrences and the processes of leaky microaneurysms resulting in vision loss would be important. We have been able to demonstrate a three-dimensional reconstruction of retinal microaneurysms in Figure 6. Further research into areas such as microaneurysms, tortuous vasculature and retinal lesions using three-dimensional techniques could be utilised to obtain more detailed information which we have otherwise been unable to obtain using two-dimensional studies.
4.5.9 Limitations

We acknowledge several limitations of this study. Firstly, the sample size is too small to conduct detailed studies on specific cardiovascular comorbidities or risk factors. Secondly, due to the limitation with all post-mortem histopathologic studies, we were unable to determine all the details of the patient’s systemic past medical history. We specifically attempted to provide information on the latest eye examinations for each donor eye, however due to confidentiality issues we were unable to obtain this information, and thus is a potential limitation, particularly with regards to the nature of progression in retinal diseases.

Confounding variables may have also been present, but all efforts were made to choose donor eyes carefully by evaluating all known medical history available, and including data from donor eyes with as few known variables as possible. In addition, the majority of the quantitative findings of this study were generally limited to the region 3 mm superior to the optic disc, and may be only relevant to this area.

4.5.10 Summary

In conclusion, we found cardiovascular comorbidities were associated with a significant decrease in the capillary density of the human inner retina. Also, pathological vascular changes were described. These findings may suggest that retinal vascular diseases have the potential to commence earlier than clinically detectable retinal diseases.
CHAPTER 5

SPECKLE VARIANCE OPTICAL COHERENCE TOMOGRAPHY

COMPARISONS WITH THE NORMAL HUMAN RETINAL MICROVASCULATURE
5. Speckle Variance Optical Coherence Tomography Comparisons with the Normal Human Retinal Microvasculature

5.1 Aim

Our previous work discussed in Chapter 3 provided an in-depth quantitative histological analysis of the different capillary networks in the normal human retina.\textsuperscript{12, 64} Subsequent to this we were also able to demonstrate in Chapter 4 that the presence of cardiovascular morbidity had a selective influence on capillary networks prior to the onset of clinically detectable retinal disease.\textsuperscript{214} Specifically, there was significant non-uniformity in the magnitude of disease-induced change between capillary networks that supplied predominantly somal, axonal and synaptic compartments within the retina. Therefore, it is likely that selective capillary network disease may also be critically linked to the pathogenesis of diabetic retinopathy and other microvascular diseases.

A need remains for the development of a non-invasive technique for meaningful and reliable capillary morphology and structure imaging.\textsuperscript{252} Speckle variance optical coherence tomography (svOCT) is a modern non-invasive technique which may offer retinal capillary information in real time.\textsuperscript{129, 131, 253} It remains unknown as to whether detailed capillary network information can be derived from svOCT imaging.
In this Chapter, we utilise a custom-built svOCT device\textsuperscript{132} to quantitatively study the different capillary networks in the human retina. This custom-built svOCT device was used instead of other widely available commercial OCT angiography devices, as it was found to be more comparable to our previous histological data. We leveraged the real time svOCT imaging capability for visual feedback to optimise the image quality during acquisition on research subjects. The custom-built svOCT device was used to demonstrate the capacity to image and isolate individual capillary networks at 3 mm superior to the optic disc. Then comparisons are made to our previous quantitative histological report in Chapter 3.\textsuperscript{12} This chapter also demonstrates the capability of this device for defining and providing detailed quantitative information of these distinct capillary networks for use in the clinical setting.
5.2 Materials and Methods

5.2.1 Ethics approval

This study was approved by the human research ethics committees at The University of Western Australia and The University of British Columbia. All live-patient imaging was performed with informed consent at the Eye Care Centre in Vancouver. All human tissue was handled according to the tenets of the Declaration of Helsinki.

5.2.2 Human donor eyes

A total of 12 eyes from 10 human donors were used for histological study with confocal scanning laser microscopy, from our previous study in Chapter 3.12 Donor eyes were acquired from the Lions Eye Bank (Lions Eye Institute, Western Australia) following removal of corneal buttons for transplantation. All human donor eyes used in this study had no known history of ocular disease.

5.2.3 Perfusion labelling, tissue preparation and microscopy

The details of perfusion labelling of retinal capillary networks, tissue preparation and confocal scanning laser microscopy for these eyes have already been described in depth in Chapter 2 and 3.

5.2.4 Speckle Variance OCT Imaging of Human Subjects

Speckle variance OCT images of human subjects were acquired from a Graphics-Processing-Unit (GPU)-accelerated svOCT clinical prototype (Figure 5-1).131, 254 The details of the acquisition system have previously been published.132 The OCT
system was based on a 1060nm swept source with 100 kHz A-scan rate (Axsun Inc) and 500 MSPS digitizer (AlazarTech Inc). The light source spectrum had a full-width half-maximum (FWHM) bandwidth of 61.5nm, which corresponded to a coherence length of ~6 μm in tissue. The sample arm optics were configured to deliver a beam of ~1.5 mm diameter at subject’s pupil, with the fast axis of galvanometer mounted mirrors (6210H, Cambridge) oriented for a vertical scan. The size of the focal waist on the retina was estimated using the Gullstrand-LeGrand model of the human eye\(^{255,256}\) to be \(\omega_o = \sim 7.3 \, \mu m\) (calculated using Gaussian optics), corresponding to a lateral FWHM of ~8.6 \(\mu m\) (calculated as \(\text{FWHM} = \sqrt{(2ln2)\omega_o}\)). The scan area was sampled by a 300x300(x3) grid on a ~1x1 mm field (corresponding to ~3.3 \(\mu m\) between A-scans) of view in ~3.15 seconds (Figure 5-2). Patient alignment was performed using a wider field, high speed, low resolution en face imaging mode with the OCT system, providing visual targets to guide the subject’s fixation so that the centre of the scan area was ~3 mm superior to the ONH. These intensity-only OCT intensity images were not saved. The larger vessels provided landmarks for the acquisition of the ~1x1 mm\(^2\) svOCT data at this location. In order to have data suitable for accurate capillary analysis, the svOCT data was acquired from a region free of large blood vessels.

For the speckle variance calculation, three repeat acquisitions at each B-scan location were acquired. En face visualization of the retinal microvasculature was processed and displayed in real-time using an open source svOCT code program developed for the GPU.\(^{131}\) The GPU software permitted dynamic selection of the retinal layers used for generating the en face svOCT image. Real-time processing to improve the svOCT image quality included: brightness and contrast adjustment to threshold out low values of speckle variance, and filtering to remove streak
The in vivo scan dimensions on the retina were calculated using a reduced eye model (single refractive surface), adjusted for the eye length of each participant measured using the Zeiss IOL Master 500. The scan dimensions on the retina were calculated as the length of an arc traced out by the OCT beam as it was scanned in angle (corrected for Snell’s law refraction) assuming a circle of radius equal to the subject’s eye length. The index of refraction for the eye media (vitreous) was approximated as \( n \approx 1.33 \). The svOCT en face images were cropped to match the dimensions of the images used for the ex vivo confocal microscopy analysis. Images were acquired from both eyes of seven subjects.
Figure 5-1 Acquisition and processing engine

Image of human imaging with custom-built svOCT device. Other specifications include an A-scan rate of 100 kHz, volume size of 1024 x 300 x 900, and acquisition time of ~3.15 seconds.
Figure 5-2 OCT volumetric imaging

Diagram illustrating how svOCT images were generated. The scan area was sampled by a 300x300(x3) grid on a ~1x1 mm field (corresponding to ~3.3 μm between A-scans) of view in ~3.15 seconds to generate volumetric data. En face view of the retinal capillary networks was processed and shown in real-time using an open source GPU algorithm. A-scan, a single depth profile; B-scan, a sequence of A-scans; Volumetric scan, multiple B-scans; En face view, front view of the retina.
As already mentioned in the previous two chapters, Chapter 3 demonstrated the presence of four different capillary networks in the retina in the region 3 mm superior to the human optic disk. These networks are located in:

1. Nerve fibre layer (NFL)
2. Retinal ganglion cell (RGC) layer
3. Border of inner plexiform layer (IPL) and superficial boundary of the inner nuclear layer (sINL)
4. Boundary of deep inner nuclear layer (dINL) and outer plexiform layer (OPL; Figure 5-3).

Chapter 3 details how co-localisation of the position of nuclei with respect of endothelial microfilaments to define different capillary networks was conducted.

The analysis of the OCT volumes was performed on data, already processed into intensity and speckle variance images using the GPU-accelerated program. The Inner Limiting Membrane (ILM) and Bruch’s Membrane / Retinal Pigment Epithelium (BM/RPE) complex were automatically segmented based on the intensity data using a 3D Graph Cut based algorithm tool implemented in Matlab. The quality of the segmentation and absence of artifacts was confirmed visually by a member of the image acquisition team. Given the small scan area and distance from the ONH, the retinal layers were reasonably uniform in thickness for each individual volume. Any curvature or tilt in the retinal surface was captured by the segmentation algorithm. The retinal vascular layers were semi-automatically delineated for each volume by applying a manually
determined offset to the Graph Cut segmentation of the BM/RPE surface. The appropriate offset for each vascular layer boundary was determined by visualizing the placement of the segmenting surface as an overlay on an intersecting OCT intensity B-scan image selected near the middle of the volume. The semi-automatic delineations of the vascular layers on a representative OCT B-scan are shown in Figure 5-3, with the enumerated list above corresponding to the regions bounded by the following colours: (1) NFL, orange lines; (2) GCL, red lines; (3) IPL/sINL, yellow lines; and (4) dINL/OPL, green lines. The en face images of the four capillary networks were generated by projecting the svOCT processed data within the depth region selective of a particular vascular layer. The value of the offsets used to define the boundaries was fine-tuned based on the qualitative appearance of the en face svOCT images. The en face image data was used for direct comparison of the vascular networks between svOCT and histology.
Figure 5-3 Region of study (overleaf)  

Transverse histological retinal section stained with toluidine blue (A) and a B-scan image acquired using svOCT (B) illustrate the various retinal layers at the eccentricity located 3 mm superior to the optic disk. Coloured dashed lines demarcate the retinal layers where different capillary networks have previously been co-localised and also illustrate how individual capillary networks were stratified from OCT and histological images. Although OCT and histology images were acquired from the same eccentricity the influence of normal anatomical variation and the effects of post-mortem changes in the histological specimen has resulted in some discrepancies in the dimensions of retinal layer thicknesses between the two images. Nerve Fibre Layer (NFL) capillary network (orange dashed lines); Retinal Ganglion Cell layer (RGC) capillary network (red dashed lines); the capillary network at the border of the Inner Plexiform Layer and superficial Inner Nuclear Layer (IPL/sINL; yellow dashed lines); the capillary network at the border of the deep Inner Nuclear Layer and Outer Plexiform Layer (dINL/OPL; green dashed lines); ONL = outer nuclear layer; IS = inner-segment of photoreceptors; OS = outer-segment of photoreceptors; RPE = retinal pigment epithelium. Scale bar = 50µm.
5.2.6 Image Analysis

Details of the image preparation used prior to statistical analysis have been described in Chapter 2. Additionally, z-projection of images that comprised each of the four capillary networks was performed prior to quantitative analysis. Images were also inverted prior to measurement to allow better identification of capillary margins.

The following measurements were performed (Figure 5-4):

1. Capillary diameter
   - Defined as the perpendicular distance across the maximum chord axis of each vessel. Each image was partitioned into nine equal areas and five measurements were acquired from each area to ensure representative sampling. Capillary diameter measurements were acquired from regions of the image where capillary margins were clearly defined. Great measures were taken to ensure that, whenever possible, capillary diameter measurements were acquired from all regions of each square within the grid. This measure avoided over-sampling of one particular region. A total of 2160 diameter measurements (540 per layer) were taken from the 12 donor eyes and a total of 2520 diameter measurements (630 per layer) were taken from the 14 svOCT patient eyes.

2. Capillary density
   - Density was measured using two indices: number of vessels per 100µm and inter-capillary distance.\textsuperscript{259} Our measurements were performed on x20 confocal images, collected using the 1024 resolution. As a result, the
settings on our confocal system would scan a square measuring 636.5 µm x 636.5 µm when collecting the images. The number of vessels per 100 µm and the inter-capillary distance from the vessel intersections were calculated using the following formulae:

- Number of vessels per 100 µm = 
  \[
  \frac{\text{Number of vessel intersections}}{\text{Length}} \times 100
  \]

- Inter – capillary distance = 
  \[
  \frac{\text{Length}}{\text{Number of vessel intersections}}
  \]

where Length is 636.5µm

A total of 1316 density measurements were taken from 12 donor eyes and a total of 1829 density measurements were taken from 14 svOCT patient eyes.
Figure 5-4 Quantitative capillary network measurements (overleaf)\textsuperscript{14}

A representative histological image (A) and svOCT image (B) of the deep inner nuclear layer and outer plexiform layer capillary network illustrates how capillary diameter and number of vessels per 100 µm were determined. Each image was first inverted (C) and (D), respectively, to allow easier identification of vessel margins. A grid was then placed on each image to partition it into 9 equal segments (yellow lines). The perpendicular distance across the maximum chord axis of each vessel was used to measure capillary diameter in each segment (green line). The number of capillary intersections per 100 µm was determined by counting the total number of vessels intersecting the yellow line, dividing by 636.5 (the total length and width of each image in µm) and then multiplying by 100. Inter-capillary distance was derived from this calculation. Scale bar = 100 µm.
Manual tracing (Figure 5-5) was performed on five eyes from svOCT images and five eyes that were imaged with confocal scanning laser microscopy. Capillary density was determined by calculating the percentage of the sample area occupied by vessel lumens. Results were compared between svOCT and histological images. Manual tracing was only performed on the NFL capillary network and the dINL/OPL capillary network because these networks were previously shown to be planar with a relatively one-dimensional trajectory. The morphologies of the other networks are three-dimensional and are therefore more challenging to manually trace. Results of the comparisons between svOCT and histology, using manual tracing techniques, were compared with the results of capillary density using the indices described in point 2 above. This analysis allowed us to determine if the number of vessels per 100µm and inter-capillary distance were reliable indices for comparing capillary density between histology and svOCT.
A representative histological image (A) and svOCT image (B) of the deep inner nuclear layer and outer plexiform layer capillary network, and their respective manually traced images (C) and (D), illustrate how capillary density was calculated. Following manual tracing, the area occupied by capillaries was expressed as a percentage of the total area of tissue. In histological images the capillary lumens demonstrated a smooth contour while non-uniform varicosities (arrow heads) were seen in svOCT images. Scale bar = 100µm.
5.2.7 Statistical analysis

We performed analysis of variance (ANOVA) to determine differences between various capillary networks (NFL, RGC, IPL/sINL and dINL/OPL) and also between histology and svOCT eyes. We used linear mixed modelling to examine differences in retinal layer measurements. Statistical significance was defined as $P < 0.05$. 
5.3 Results

5.3.1 General

The mean donor age of the control histology group was 39.71 ± 3.68 years, taken from nine left and three right eyes of one female and nine male donors. The average post-mortem time before eyes were perfused was 14.80 ± 1.68 hours. The mean age of the svOCT research volunteer group was 45.58 ± 5.30 years. 14 eyes from one female and six male subjects with no history of eye disease were imaged. There was no statistical significant difference in age between the two groups ($P = 0.455$).

5.3.2 Capillary Network Topography in Histology and svOCT Images

Figure 5-6 illustrates the highly comparable morphological appearance of capillary networks between histological and svOCT images. As noted in Chapter 3,12 capillaries in the NFL network were largely linear in organisation and ran parallel to the direction of RGC axons in the NFL. Beneath the NFL capillary network was the RGC network, where not only capillaries but also arterioles and venules were seen. The IPL/sINL capillary network was three-dimensional in structure and was observably similar in density to the NFL capillary network. The deepest network was located at the level of the dINL/OPL and was the most easily distinguishable layer in histology and svOCT images. This layer was observed to have multiple closed loops and was also laminar in structure. Overall, we found that svOCT images had more background noise than what was apparent on histology.
Figure 5-6 Comparison of capillary network morphometry between histology and svOCT (overleaf)\textsuperscript{14}

Histology images (left panel) and svOCT images (right panel) demonstrate the comparable morphological features of the nerve fibre layer capillary network (A and B), the retinal ganglion cell layer capillary network (C and D), the capillary network at the border of the inner plexiform layer and superficial boundary of the inner nuclear layer (E and F) and the capillary network at the boundary of the deep inner nuclear layer and outer plexiform layer (G and H). Scale bar = 100\textmu m.
5.3.3 Quantitative Analysis of Capillary Diameter

The average capillary diameter for all networks in the histology and svOCT data was 8.26 ± 0.03 µm (n = 2160) and 8.80 ± 0.04 µm (n = 2520) respectively.

Mean capillary diameter values for each layer in svOCT and histology images are detailed in Table 5-1 and Figure 5-7. Within the histology images, capillary diameters were significantly different between networks ($P < 0.001$). Post hoc analysis revealed that the mean diameter was significantly different between all networks ($P = < 0.001$) except the NFL and dINL/OPL network ($P = 0.227$), and RGC and IPL/sINL network ($P = 0.740$). Within the svOCT images, capillary diameters were significantly different between all networks (all $P < 0.014$) except the NFL and IPL/sINL network ($P = 0.426$).

Comparisons between histology and svOCT data revealed no significant difference in capillary diameter in the NFL network ($P = 0.891$), IPL/sINL network ($P = 0.151$) and dINL/OPL network ($P = 0.168$). There was a significant difference in capillary diameter in the RGC network between histology and svOCT images ($P < 0.001$). Capillary diameter was greater in svOCT images.
**Figure 5-7** Graphs showing capillary diameter measurements

Graphs visually show the difference between histology and svOCT measurements of capillary diameter. Asterisk (*) denotes statistical significance.
5.3.4 Quantitative Analysis of Capillary Density

The mean values for the indices that were used to measure capillary density are provided in Table 5-1 and Figure 5-8 for both svOCT and histology images.

Within histology images, the inter-capillary distance and vessels per 100µm were significantly different between all networks \((P < 0.006)\) except between NFL and IPL/sINL network \((P > 0.137)\) and RGC and dINL/OPL network \((P > 0.368)\).

Within the svOCT images, these capillary density indices were also significantly different between all networks \((P < 0.045)\) except between NFL and IPL/sINL networks \((P > 0.394)\).

Density comparisons between histology and svOCT eyes demonstrated no differences in the RGC network for these indices \((P = 0.497)\). For the remaining capillary networks there were significant differences between svOCT and histology images for all of these indices \((P < 0.012)\). Density was greater in svOCT images.


Table 5-1 Quantitative capillary diameter and density measurements for histology and svOCT images\textsuperscript{14}

Mean and standard error presented for each parameter are presented. **NFL** = nerve fibre layer; **RGC** = retinal ganglion cell; **IPL** = inner plexiform layer and superficial inner nuclear layer (IPL/sINL); and deep inner nuclear layer and outer plexiform layer (dINL/OPL). Sample number for each measurement appear in brackets.
**Figure 5-8 Graph of indices used for measurement in density**

Graphs showing the mean values for the indices that were used to measure capillary density for both svOCT and histology images. Asterisk (*) denotes statistical significance.
5.3.5 Results of manual tracing

The NFL capillary network demonstrated a mean density of 10.20 ± 0.02% in the histology images and 16.97 ± 0.02% in the svOCT images. These density measurements were significantly different between the two imaging modalities ($P = 0.044$). The dINL/OPL network demonstrated a mean density of 16.04 ± 0.01% in the histology images and 25.61 ± 0.01% in the svOCT images. These density measurements were also significantly different between the two imaging modalities ($P = 0.006$).
5.4 Discussion

5.4.1 Key findings

The major findings in this study are as follows:

1. The morphological characteristics of human retinal capillary networks seen on svOCT imaging are comparable to what has previously been demonstrated histologically and is consistent with reports in the literature that have used other OCT-based angiography techniques.\(^{260-262}\)

2. With the exception of the RGC capillary network, there was no difference in mean capillary diameter measurements derived from svOCT and histology images for any network.

3. Capillary density measurements are significantly greater in svOCT images than histology for all networks, with the exception of the RGC network.

5.4.2 Revisiting previous work

The retina is particularly vulnerable to ischemic injury due to the absence of collateral circulations.\(^{263, 264}\) We have previously shown that in the presence of systemic cardiovascular disease, morphological changes to capillary networks precede functional visual changes.\(^{214}\) An in depth understanding of the anatomy of human retinal capillary networks and the ability to detect change, \textit{in vivo}, may therefore potentially aid in the clinical management of retinal vascular disorders. The utility of fluorescein angiography, magnetic resonance imaging and adaptive optics techniques to stratify the retinal circulation into various capillary networks
remains unclarified.\textsuperscript{93, 265, 266} svOCT is a non-invasive, non-contact technique that is able to provide angiographic information without the administration of contrast.

### 5.4.3 Customised svOCT

The customised svOCT device detailed in this report has the capability to provide both structural and functional information simultaneously in real-time and may therefore have many potential clinical applications in ophthalmology. The svOCT detects the presence of motion based on the changes of the speckle patterns in the OCT images. The acquisition of the three B-scans required to generate a single svOCT depth profile requires only \textasciitilde0.01s, which is fast enough to be largely free of artifact.

The same fundus location in Chapter 3 was used, that is 3 mm superior to the optic disc, in order to correlate the microscopic characteristics of different capillary networks of the svOCT images to histology.\textsuperscript{12} We found that svOCT was able to reliably stratify the retinal capillary circulation into the NFL network, RGC network, the capillary network bordering the IPL and superficial border of the INL, and the capillary network bordering the OPL and deep boundary of INL. Similar to our previous histological study, we observed that the innermost and outermost capillary networks on svOCT demonstrated a laminar configuration while IPL and deep INL networks demonstrated a complex three-dimensional configuration.

### 5.4.4 Comparison results for capillary diameter

When quantitative comparisons were made between svOCT and histology we found no difference in mean capillary diameter values for all networks with the
exception of the RGC capillary network. The mean diameter in svOCT images for the latter network was higher. Additionally, we observed the contour of capillary lumens to be different between svOCT and histology images. In histological images the capillary walls were smooth and undulating while the appearance of the capillary thickness in the svOCT images in all networks demonstrated non-uniform varicosities. The width of a capillary in the svOCT image is sensitive to the bloods cells at the particular vessel location being imaged within the time length of the three B-scans used to calculate the speckle variance (~0.01s). We speculate that the irregular morphological appearance of capillaries in svOCT images reflect pulsatile flow and the non-uniform distribution of erythrocytes and leukocytes along the capillary length. Previous modelling studies have shown that red blood cells (RBCs) within a capillary bed travel in groups and that the velocities of RBCs are not constant. It may also reflect regional pericyte constrictions within the length of a capillary segment. A closer study of the distribution of the capillary varicosities on svOCT images may aid our understanding of mechanisms that control oxygen and nutrient delivery in the human retina.

5.4.5 Comparison results for capillary density

Comparisons between svOCT and histology yielded similar results for capillary density if manual tracing techniques or the indices of capillary density were used. This suggests that the number of vessels per 100µm and inter-capillary distance were reliable indices of capillary density. There were significant differences in capillary density measurements between svOCT images and histology for all networks with the exception of the RGC network. Density measurements in
svOCT images were consistently greater than histology. The resolving power of the svOCT is not as high as the images of donor retinal specimens acquired with the confocal scanning laser microscope. Although the coherence length of the OCT system is ~6 µm, shadow artifacts due to blood vessels in the anterior planes may affect the resolution realized in the svOCT images. Some of the vessels visualised in the svOCT images in the deeper layers may therefore be due to these artifacts. This could potentially have resulted in some degree of double counting during quantitative analysis and may partly explain the increased density measurements in svOCT images. However, the influence of shadow artifact on the NFL is expected to be negligible as there are no capillary structures anterior to it. As svOCT density measurements were greater than histology in this network it suggests that the difference is likely to be a true finding rather than one due to artifact. Additionally, the difference in density measurements between svOCT and histology may also have been influenced by the limited sample size of the study. Previous work has shown that there is some degree of inter-individual variation in retinal layer thickness measurements between normal human eyes. Variations in retinal layer thickness measurements are likely to translate into variations in capillary density measurements between normal human eyes. With a larger sample size it is possible that the differences between histology and svOCT may become insignificant.

5.4.6 Limitations

We acknowledge several limitations of this study. The volume acquisition time with the current svOCT prototype is longer than the acquisition time of regular intensity-only volumes with current commercially available spectral domain OCT
devices. Consequently, motion artifact may degrade the quality of capillary images in those patients that are unable to reliably fixate. During the acquisition of the volume, slight eye motions can also contribute to spatial distortions along the slow axis. Additionally we observed greater background noise in svOCT images compared to histology and this may potentially induce image artifact or obscure fine capillary detail in some networks.

It will be important to resolve the reason for the appearance of varicosities along the capillary length in svOCT images as this may provide vital information about retinal vascular homeostasis. Investigating whether svOCT has the capacity to detect changes in retinal blood flow may also have great relevance to clinical ophthalmology.

5.4.7 Summary

This study demonstrates that svOCT has the potential to provide in vivo, histology-like information about the different capillary networks in the human retina. It could therefore serve as a powerful tool for studying retinal vascular diseases. The capacity of this device to produce images in real-time is also particularly advantageous for patient assessment in the clinical setting. A major advantage of this study is that we compared svOCT findings to those in Chapter 3, which utilised perfusion-labelling techniques of post-mortem samples to ensure complete histological identification of the retinal circulation. It has previously been shown that this technique ensures precise labelling of the capillary bed and therefore serves as a “gold standard” for comparing in vivo imaging technology.47
CHAPTER 6

ENDOTHELIAL CELLS IN THE
PORCINE VORTEX VEIN
SYSTEM
6. **Endothelial Cells in the Porcine Vortex Vein System**

6.1 **Aim**

The last three chapters have detailed the retinal microvasculature supply of the inner retina. However it is the choroid that plays a critical role in sustaining the outer retina’s high metabolic demands. The major drainage pathway for the choroidal circulation is the vortex vein system of the eye, and it supports a blood flow rate many times greater than that of the retinal circulation. Venous diseases are known to be complex and ten times more common than arterial diseases, yet there is very little known about the very significant but undervalued vortex vein system of the eye. Although previous studies have focused mostly on large vessels and cultured cells, and demonstrated that endothelial structural remodelling occurs in response to haemodynamic shear stress, recent studies have identified location specific phenotypic differences of endothelium at the level of venous microvasculature. Endothelial phenotypic heterogeneity is a core property of the endothelium and has been described at the level of cell morphology, function, gene expression, and antigen composition. Endothelial phenotypes are organ-specific and site-specific within the same organ, and can be indicative of their normal physiological or pathophysiological states. Studies of endothelial cell morphology in the central and branch retinal veins in human donor eyes have found site specific changes at these locations known to be vulnerable to venous occlusive diseases. It is therefore of interest to study other site-specific...
venous regions within the eye for other possible locations of endothelium vulnerability.

Additionally, it is known that vascular endothelial cells which line the inner wall of all vasculature are continually exposed to haemodynamic forces. They receive information through active transport, direct permeation, or indirect changes in smooth muscle and other vessel wall components. An immediate response to protection against haemodynamic disruptions is achieved by a sudden transformation of the endothelium to a vasoconstrictive, pro-coagulant, and pro-inflammatory state, resulting in various changes in its structure and behaviour, and ultimately dysfunction and pathogenesis. Thus, endothelial cells have a critical role in vascular biology and pathophysiology of disease processes, as they may point to preclinical vascular disease.

A culmination of high flow rates and large volumes of blood through a geometrically complicated vortex vein system has the potential to induce unusual flow patterns and hemodynamic forces, exposing vascular endothelial cells to various hemodynamic forces in different regions of the vortex vein system. It is possible that different haemodynamic pressure gradients and stressors such as shear and turbulent forces within the vortex vein system may affect the endothelium and present with special geographic patterns for specific regions. Exposure to such complicated haemodynamics may predispose the vortex vein endothelium to pathological phenotype, followed by vascular disease. It is therefore important that a detailed investigation of the endothelial cell intracellular structures within the vortex vein system of the eye be conducted in order to
understand its functional role and determine whether there could be pathological changes with age or disease.

The purpose of this chapter is to discover, determine, and describe if there are region-dependent differences in the endothelial cells morphologies within the vortex vein system. It is proposed that there would be haemodynamic changes due to special vascular distribution patterns, which would thus lead to a regional heterogeneity of endothelial cells.
6.2 Materials and Methods

6.2.1 General

Porcine eyes from the local abattoir were used for the present study. Porcine eyes were chosen as our baseline animal model due to the similarity of the porcine eye in anatomy and physiology to the young normal human eye, and will thus enable good comparisons to future studies of human eyes. Porcine eyes with relatively light iris pigmentation were selected as these eyes also had less fundus pigmentation which allowed the vortex vein structures to be more easily visualised.

6.2.2 Porcine eye preparation

The details of obtaining porcine eyes are discussed in Chapter 2. The eyes were then oriented via fundoscopy and the superior oblique muscle. All musculature and orbital fat were carefully dissected away to expose the vortex veins exiting the sclera from the equator at each quadrant. The superior temporal vortex vein (STVV) was isolated and noted for position and length. The feasibility of successfully cannulating the STVV was very dependent on its length. Only eyes with a sufficiently long STVV for cannulation were included in the analysis (n = 10).

6.2.3 Perfusion labeling of the vortex vein system

Chapter 2 describes the technique used for perfusion labelling of porcine eyes in this chapter. Additionally, the following steps were taken:
1. Wash out of blood within the vortex vein system by perfusing a filtered solution of 1% bovine serum albumin in oxygenated Ringer’s solution through the STVV for 20 minutes, observing the outflow drainage of the perfusate through the ITVV

2. Filtered 4% paraformaldehyde fixative through the STVV for 20 minutes

3. Washout of 0.1M filtered phosphate buffer over 10 minutes

4. Filtered 0.1% Triton X-100 in 0.1M phosphate buffer solution was perfused over 5 minutes to aid in the permeation of endothelial cell membranes

5. Washout of 0.1M filtered phosphate buffer over 10 minutes

Before perfusion labelling, the ITVV and all other potential outflow arteries and veins were tied off with one to two 8-0 vicryl sutures. Microfilaments (f-actin) and cell nuclei were then labelled by using a mixture of phalloidin conjugated to Alexa Fluor 546 (30U; Invitrogen, Carlsbad, CA) and an iodide dye (YO-PRO-1; 6.6 µM; Invitrogen), perfused slowly over a period of three hours. Once staining was completed, a final washout of 0.1M filtered phosphate buffer solution was perfused for 10 minutes to remove any excess dye. The rationale for selecting f-actin labelling is that the f-actin peripheral border staining can define the endothelial cell shape, and the cytoplasmic f-actin stress fibre bundles could indicate high shear stress associated with high blood flow. In addition, smooth muscle cells can also be labelled if present.

The eyes were then immersed in a solution of 4% paraformaldehyde overnight before dissection and flat mounting of the choroid and vortex vein for imaging.
The orientation, and location of the STVV of each pig eye were carefully noted and documented photographically.

### 6.2.4 Intravascular VE-Cadherin immunolabelling

The same protocol applies as to that described above of the phalloidin labelling, except that instead of the fluorescent probe, a filtered solution consisting of a 10% donkey serum in 0.1M phosphate buffer was perfused over one hour. Then, a goat anti-VE cadherin primary antibody in buffer (1:50; sc-6458; Santa Cruz Biotechnology) over two hours, a 30 minute 0.1M phosphate buffer wash, perfusion of Alexa Fluor 546 donkey anti-goat antibody in buffer (A11056, 1:200; Invitrogen) over 2 hours, and a final wash of 0.1M phosphate buffer over 30 minutes.

### 6.2.5 Flat mounting of the vortex vein system

The details of tissue preparation of flat mounting the choroid, intra-scleral canal and extra-ocular vortex veins have been described in Chapter 2. Once dissected, the choroid was flat mounted in glycerol, sclera side face up, before coverslipping and imaging (Figure 6-1A).

### 6.2.6 Microscopy and image preparation

Microscopy image acquisition and image preparation of the vortex vein system has also been described in Chapter 2.
6.2.7 Region identification and definition

Eight study regions were identified and defined as follows:

1. Choroidal vein – individual small choroidal veins which do not directly drain into the major vortex vein.
2. Pre-ampulla – the region where two or more choroidal veins converge into a single channel prior to the ampulla region.
3. Anterior portion of the ampulla – the region following the convergence of all pre-ampullae into a single channel, located in the anterior third of the ampulla.
4. Mid-ampulla – the largest diameter region in the single channelled vessel which forms after the convergence of all pre-ampullae.
5. Posterior portion of the ampulla – the outflow region, located in the posterior third of the ampulla.
6. Post-ampulla – the region of outflow in the single channelled vessel prior to entering the sclera.
7. Intra-scleral canal – the vessel portion which travels through the sclera.
8. Extra-ocular vortex vein – the vortex vein, which usually exits the sclera at the equator.

6.2.8 Detailed quantitative study of venous endothelial cell morphology

Detailed measurements of endothelial morphology were carried out in eight of the ten pig eyes. Only endothelial cells with a clear cell border identified were included for detailed measurements. At each of the eight regions examined, the
venous endothelial cells were studied in detail, with measurements taken for endothelial cell and nuclei area, perimeter, length, width, and the nuclei position relative to the upstream pole of the cell (Figure 6-2). The nuclei position is expressed as a percentage of the total cell length. Measurements of vessel diameter were carried out in all ten eyes.

6.2.9 Statistical analysis for vortex vein studies

Chapter 2 describes the statistical analysis techniques used to quantify and compare the different measurements of the eight different regions in the vortex vein system.
6.3 Results

6.3.1 General appearance

The temporal side of the porcine vortex vein system was well perfused after cannulation. The endothelial cells, smooth muscle cells and nuclei of the vortex vein system vasculature were clearly labelled. The only exception is the f-actin labelling of the extra-ocular vortex vein where cell border was much more difficult to delineate using f-actin, due to the masking effect of the strong f-actin labelling in smooth muscle cells. VE-cadherin was used to label the cell border of vortex vein endothelium in one specimen.

6.3.2 Drainage pattern of the vortex vein system

Figure 6-1 shows a vortex vein system in the superior temporal quadrant from a porcine eye. Smaller choroidal veins from the anterior and equatorial portions were observed to converge into larger choroidal veins. Approximately three or more of these larger choroidal veins converged to form a few large vessels, known as the pre-ampulla. Typically, five to eight pre-ampullae were present anteriorly, before the outflow further merged to form a single vessel: the ampulla. Venous drainage from the posterior globe also appeared to run anteriorly and feed into the ampulla. The ampulla was approximately 2.0 mm in length and 1.0 mm in width, being widest in the middle portion. The post-ampulla was immediately downstream of the ampulla before entering the sclera as a single vessel with a slightly narrower width. This single vessel would then enter the sclera and exit the globe as the superior temporal vortex vein.
Figure 6-1 Regions of study (overleaf)$^{15}$

(A) and (D) are photographic images of the dissected choroidal and scleral portions of a porcine superior temporal vortex vein system. The images have been oriented with the top of the images pointing anteriorly. (A) is a photographic image of the intra-ocular choroidal regions taken from the scleral aspect with the ampulla facing up. The green arrow points to the large, anteriorly draining choroidal vessels and pre-ampulla which appear as clear tubular spaces in amongst the heavily pigmented extravascular choroidal tissue. The blue arrow points to the smaller but more numerous choroidal vessels that drain the posterior globe. The purple arrow corresponds to the mid-ampulla and the cut edge where the vortex vein meets the sclera can be seen in the middle of this image as a reflective bulb. (B) is the fluorescence image of (A) labelled for f-actin. Choroidal vessels are clearly visible as fluorescent, white curvy lines running towards the ampulla. The regions studied were outlined using different colours. (C) is a schematic drawing of the corresponding regions studied with a solid colour fill. Note that not all the choroidal venules have been outlined in B or C as they were too numerous. The numbering for the regions correspond as follows: 1 = Choroidal vein (blue); 2 = Pre-ampulla (pale green); 3 = Anterior portion of the ampulla (orange); 4 = Mid-ampulla (purple); 5 = Posterior portion of the ampulla (yellow); 6 = Post-ampulla (red). (D) is a photographic image of a bisected superior temporal vortex vein inside the sclera and as it exits the sclera. The upper half of the image shows the intra-scleral portion of the single exiting vessel within white scleral tissue outlined in green. The curvy, opaque lower half of the image contains the extra-ocular vortex vein outlined in orange. (E) is the
schematic drawing of these two portions with a solid colour fill. 7 = Intra-scleral canal (dark green); 8 = Extra-ocular vortex vein (orange).
Figure 6-2 Measurement of endothelial cells and their nuclei

(A) shows a confocal image of the post-ampulla region labelled for f-actin (red) and nuclei (green). Yellow dashed lines traced a few endothelia for visualisation of measurement methods. (B) is an enlarged schematic diagram of the endothelial cells outlined. Each endothelium was measured for its cell area, perimeter, length and width. The same parameters were measured for their nuclei. The position of the nuclei within the cell was measured from the upstream pole of the cell to the centre of the nucleus as illustrated by the light blue line. Arrow: direction of flow. Scale bar: 20 µm.
6.3.3 Vessel diameter measurements

The average diameter of each region are shown in Table 6-1. The diameter of one specimen from Region 8 was difficult to measure hence has not been included.

All diameters measured were sampled from flat-mount preparations, therefore the results could have been affected by collapse of the vessel wall. The post-ampulla region was often folded due to the anatomic nature in this location. The diameter of the intra-scleral canal was also difficult to measure; however it was similar in size to Region 8 as shown in Figure 6-1.

Table 6-1 Vessel diameter measurements in the different regions

<table>
<thead>
<tr>
<th>Region</th>
<th>Vessel Diameter (µm)</th>
<th>Number of Data Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 = choroidal veins</td>
<td>121.1 ± 3.33</td>
<td>100</td>
</tr>
<tr>
<td>2 = pre-ampulla</td>
<td>292.5 ± 14.71</td>
<td>40</td>
</tr>
<tr>
<td>3 = anterior portion of ampulla</td>
<td>744.1 ± 65.17</td>
<td>10</td>
</tr>
<tr>
<td>4 = mid-ampulla</td>
<td>957.9 ± 72.54</td>
<td>10</td>
</tr>
<tr>
<td>5 = posterior portion of ampulla</td>
<td>681.9 ± 65.63</td>
<td>10</td>
</tr>
<tr>
<td>6 = post-ampulla</td>
<td>438.7 ± 35.01</td>
<td>10</td>
</tr>
<tr>
<td>8 = extra-ocular vortex vein</td>
<td>744.7 ± 40.09</td>
<td>9</td>
</tr>
</tbody>
</table>
6.3.4 **Overall change of endothelial cell shape in the vortex vein system**

The porcine choroidal venous endothelial cells took on a spindle shape in most cells and a rounded end in some cells (Figure 6-3A). Further downstream, there was a gradual but significant shortening of cell length accompanied by a corresponding narrowing of cell width through the pre-ampulla, anterior portion of the ampulla, ampulla and posterior portion of the ampulla regions (Figure 6-3B, Figure 6-4, and Figure 6-6). This is reflected in the gradual drop in cell area observed through these regions (Figure 6-6A). The posterior portion of the ampulla is a transitional region where different cell shapes were consistently observed. Downstream from the posterior portion of the ampulla, dramatic differences in endothelial cell shapes were observed. At the post-ampulla region, the endothelial cells were very elongated (Figure 6-5A) and there was a sharp decrease in cell width (Figure 6-6). However, once the vessel entered the sclera and exited the globe, the endothelial cells became much more rounded in shape (Figure 6-5B and 6-5C) and were the smallest and shortest compared with all other regions of study (Figure 6-6).

6.3.5 **Quantitative analysis of endothelial cells in different regions**

Results of the mean area, perimeter, length and width of endothelial cells in each of the eight different regions are shown in Figure 6-6. Regions were statistically analysed in two ways: first by comparing all regions against a control, and secondly by comparing each individual region against its adjacent regions.
Using Region 1 as a control, there was statistically significant difference ($P < 0.001$) in all regions for endothelial cell area. Endothelial cell perimeter showed statistically significant differences in all regions compared to Region 1, except Region 2 ($P > 0.050$). There were no statistically significant differences between Region 1 and 2, and Region 1 and 3 for endothelial cell length ($P > 0.050$), however all other regions were statistically significant against the control ($P < 0.001$). Region 1 and 8 showed no statistical differences in endothelial cell width ($P > 0.050$) but were significant in all other regions against Region 1 ($P < 0.001$).

Comparing individual regions to their adjacent regions showed a significant reduction in endothelial cell area compared to the immediate upstream region from Regions 1 to 7, and a significant increase from Regions 7 to 8 (all $P < 0.005$). Perimeter was significantly different between all adjacent regions (all $P < 0.050$) except for between Regions 1 and 2, and Regions 4 and 5 ($P > 0.050$). Endothelial cell lengths were significantly different between Regions 3 and 4, Regions 5 and 6, Regions 6 and 7, and Regions 7 and 8 (all $P < 0.001$). Both perimeter and length of the endothelial cells showed an overall shortening with the exception of a surge in both length and perimeter measurement in Region 6. Endothelial cell widths showed consistent narrowing from Regions 1 to 6, and then a stark increase in width from Regions 6 to 8. Widths were significantly different between all adjacent regions (all $P < 0.001$) except for between Regions 2 and 3, and Regions 3 and 4 ($P > 0.050$).
6.3.6 Overall change in endothelial nuclei parameters through the regions

The venous endothelial nuclei assume an oval shape throughout and appear narrowest inside the intra-scleral canal. A gradual reduction of the endothelial cell nuclei area was observed from Regions 1 to 7. This corresponded with a gradual decrease in nuclei width through the corresponding regions. An abrupt and significant drop in nuclei length, width, area and perimeter was observed in the intra-scleral canal region (Figure 6-7). However, as the vessel exited the globe, the endothelial cell nuclei area, width, perimeter and length all increased.

6.3.7 Quantitative analysis of endothelial cell nuclei in different regions

Figure 6-7 illustrates the results of the mean area, perimeter, length and width of endothelial cell nuclei in each of the eight different regions.

Using Region 1 as a control, there was a significant difference between all regions for endothelial cell nuclei area (P < 0.001) except for Region 2, and Region 3 (P > 0.05). Endothelial cell nuclei perimeter was significantly different between all regions against the control except for Region 5 (p > 0.050). All regions showed a statistically significant difference against Region 1 for endothelial cell nuclei length (P < 0.001). There was a significant difference between all regions against the control for endothelial cell nuclei width (P < 0.001) except Region 8 (P > 0.050).
Comparing individual regions to adjacent regions revealed a significant difference between all regions for endothelial cell nuclei area (all P < 0.005) except between Regions 1 and 2, and Regions 2 and 3 (P > 0.050). Endothelial nuclei area showed a consistent decrease from Region 1 to 7, and a sudden increase from Region 7 to 8. Perimeter was significantly different between Regions 1 and 2, Regions 2 and 3, Regions 3 and 4, and Regions 6 and 7 (P < 0.050). Endothelial cell nuclei length was significantly different between all regions (all P < 0.005) except between Regions 4 and 5. Both perimeter and length of the endothelial cell nuclei showed an increase from Region 1 to 3, followed by a decrease in Region 3 to 5, an increase in Region 6, a steep drop in Region 7, and an increase in Region 8. Endothelial nuclei width was also significantly different in all regions (all P < 0.001) except between Regions 3 and 4, with a steady decrease from Region 1 to 7, and then a sharp increase in Region 8.

6.3.8 Quantitative analysis of nuclei position

The position of the nuclei was presented as a percentage in relation to the upstream pole of the endothelial cell (Figure 6-8). Endothelial nuclei were found to be positioned most down-streamed in the post-ampulla region, corresponding with the narrowest region within the ampulla, and which also contains the narrowest endothelial cells in the whole vortex vein system. A significant difference was found between Region 1 and 3 (P < 0.025) when using Region 1 as the control. Between adjacent regions, a significant difference in nuclei position occurred between Regions 6 and 7 (P < 0.050) corresponding to the abrupt change in nuclei parameters.
6.3.9 Overall change of smooth muscle cell distributions in the vortex vein system

The distributions of smooth muscle cells in the vortex vein system were interesting. There was clear evidence that smooth muscle cells were present in all regions studied except the mid-ampulla (Figure 6-4B), posterior portion of the ampulla (Figure 6-4C), and post-ampulla (Figure 6-5A) regions. Endothelia can be clearly visualised in these regions where there was a scant distribution of smooth muscle cells (Figures 6-4B, 6-4C and 6-5A).

Figure 6-3 Choroidal vein and pre-ampulla regions (overleaf)

Confocal images of venous endothelium (traced in yellow) in the choroidal vein (A) and pre-ampulla (B) regions. The endothelia and the vascular smooth muscle cells were labelled with phalloidin (red) for f-actin microfilaments and their nuclei counterstained with YO-PRO-1 (green). Endothelia in the choroidal vein and pre-ampulla regions have intact cell border labelling for f-actin, do not contain stress fibres, and are aligned in the direction of flow (white arrow). Blue arrows point to f-actin labelling in smooth muscle cells and showed prominent parallel stress fibres slinging at various angles to the direction of flow in the choroidal veins. In the pre-ampulla, however, smooth muscle cell f-actin tended to be plaque like although some thick stress fibres were still present. Scale bar: 20 µm.
Figure 6-4 Ampulla region (overleaf)\(^{15}\)

Confocal images of venous endothelium in the anterior portion of the ampulla (A), mid-ampulla (B) and posterior portion of the ampulla (C) regions. A few endothelial cells in each region have been outlined (yellow dashed lines) for ease of comparison. The endothelia and the vascular smooth muscle cells were labelled with phalloidin (red) for f-actin microfilaments and their nuclei counterstained with YO-PRO-1 (green). Flow direction is indicated by the white arrow and is applicable to all images in this panel. The endothelia appear to have an intact cell border f-actin labelling and no cytoplasmic stress fibres. A reduction in endothelial cell size may be seen going from the anterior portion of the ampulla to the mid-ampulla, and then to the posterior portion of the ampulla. The posterior portion of the ampulla (C) was a transitional region where great variation in endothelial cell size and length were found. A few smooth muscle cells (blue arrows) were found in the anterior portion of the ampulla but not in the mid-ampulla or posterior portion of the ampulla. Scale bar: 20 µm.
Confocal images of the venous endothelium in the post-ampulla (A), intra-scleral canal (B) and extra-ocular vortex vein (C & D) regions. The endothelia and the vascular smooth muscle cells were labelled with phalloidin (red) for f-actin microfilaments and YO-PRO-1 (green) for their nuclei in images A to C. Image D shows clear VE-cadherin labelling of endothelial cell border in the extra-ocular vortex vein region. A few endothelial cells in each region have been outlined (yellow dashed lines) for ease of comparison. The endothelia do not contain stress fibres and cell border f-actin appears intact. The narrowest endothelia were found in the post-ampulla region. Downstream from that, much shorter endothelia were found in the intra-scleral region and very rounded endothelia were found in the extra-ocular vortex vein region. Smooth muscle cells (blue arrows) were found in the intra-scleral and extra-ocular regions. Arrow: direction of flow. Scale bar: 20 µm.
Graphs showing the mean endothelial cell area, perimeter, length and width according to regions. $N$ values for regions 1 = 132, region 2 = 127, region 3 = 101, region 4 = 139, region 5 = 188, region 6 = 102, region 7 = 96 and region 8 = 76. Standard errors are shown. The asterisk (*) indicates a significant difference against Region 1 after ANOVA analysis and taking into account possible confounding variation introduced by left or right eye. (A) There is a gradual decrease in cell area through the regions with the smallest cells observed in the intra-scleral canal. This decrease in cell area corresponded with the gradual reduction in cell perimeter (B), length (C) and width (D) through the corresponding regions. An exception was observed at the post-ampulla region (Region 6) where there is a leap in cell length and perimeter accompanied by a sharp fall in cell width. There was a sharp rise in cell width in Regions 7 to 8.
Figure 6-7 Endothelial nuclei measurements

Graphs showing averaged data of endothelial nuclei area, perimeter, length and width according to regions. N values for regions 1 = 132, region 2 = 127, region 3 = 101, region 4 = 139, region 5 = 188, region 6 = 102, region 7 = 77 and region 8 = 56. Standard errors are shown. The asterisk (*) indicates a significant difference against Region 1 after ANOVA analysis and taking into account possible confounding variation introduced by left or right eye.
Figure 6-8 The intracellular location of endothelial cell nuclei in their different regions.

The position of the nuclei is expressed as a percentage of total cell length in relation to the upstream pole of the cell. N values for regions 1 = 132, region 2 = 127, region 3 = 101, region 4 = 139, region 5 = 188, region 6 = 102, region 7 = 77 and region 8 = 56. The plot shows the mean value and the standard errors. The asterisk (*) indicates a significant difference against Region 1 after ANOVA analysis and taking into account possible confounding variation introduced by left or right eye.
6.4 Discussion

6.4.1 Key findings

There are four main findings in this study:

1. There were significant differences in endothelial cell and nuclei parameters of area, perimeter, length and width in the specific regions identified within the porcine vortex vein system.

2. The greatest transitional change in endothelial cell pattern was between the posterior portion of the ampulla and the post-ampulla regions (with cells becoming significantly thinner), and the post-ampulla to the extra-ocular vortex vein regions (with cells becoming distinctly shorter and wider).

3. The greatest change in endothelial cell nuclei pattern was between the post-ampulla and the intra-scleral canal regions (with a steep decline in cell nuclei length), and the intra-scleral canal and the extra-ocular vortex vein regions (with a sharp rise in cell nuclei width). The most significant change of the nuclei position from the upstream pole of the cell was between the post-ampulla and the intra-scleral canal regions.

4. Interestingly, a lack of smooth muscle cells was found in the ampulla region.
6.4.2 Causes and mechanisms of regional differences

While studies observing the gross structure of vortex veins have been conducted to date there is no clarity of knowledge as to the exact intracellular structures that form the vortex vein system, nor their regulatory outflow mechanisms, in both normal and diseased states. To our knowledge, this study is the first of its kind in relation to describing and quantifying the vortex vein system of the porcine eye.

It is interesting to know the causes and mechanisms of regional differences of endothelial and smooth muscle cells in the vortex vein system. It is assumed that many factors could be involved such as genetic, evolutional and/or environmental, as well as aging and pathogenic factors. The results from this study are found from young, healthy pig eyes. It is therefore very unlikely that the regional differences in endothelial phenotype are caused by aging or pathological factors. However, the information of gene expression of endothelium in different regions of the vortex vein system is not currently available.

6.4.2.1 Haemodynamic forces

Vascular endothelial cells are continually exposed to haemodynamic forces and in turn modulate their structure and function. It is expected that the geometrically complex vortex vein system with a high flow rate has significantly uneven haemodynamic forces in the different regions. Unfortunately, haemodynamic information is also not available in the literature. A plausible explanation for the stark variability between the endothelial cells and nuclei is that our results indicate the existence of significant regional differences of
haemodynamic forces with the highest shear stress in the post-ampulla region. The “artery-like” appearance of endothelial cells in the post-ampulla region (elongated cell shape accompanied by the presence of stress fibres) within a vein is suggestive of a high shear stress as venous endothelia are generally free from cytoplasmic stress fibres. This region-dependent phenotypic difference of endothelium would be especially important if there was endothelial cell injury in the pathological eye with endothelial dysfunction, as regrowth of the endothelium shows morphologically different and functionally compromised cells. 281

6.4.2.2 Environmental factors

Environmental factors may also play a part and may be relevant to older, abnormal eyes, as blood flow velocity has been shown to change with factors such as herbal extracts and smoking 282. Many unknown questions of the vortex vein system will require extensive research experimentally and clinically. The current data may provide valuable experimental model and baseline information of the vortex vein system from normal healthy eyes. This information may be useful for further study on the physiological and pathological roles of the vortex vein system.

6.4.3 Limitations

While this study has provided important new knowledge regarding the vortex vein system in porcine eyes, several limitations of the present study exists. Further studies on other structures which may modulate the endothelium are needed. Such studies would include investigating smooth muscle cells and nerve tissue which may contribute to the regulation of blood flow.
6.4.4 Summary

Vascular endothelial cells are vital for normal physiological function, and pathological processes occur in the absence of their survival. Extensive research points to pathognomonic processes with endothelial dysfunction after prolonged and excessive endothelial activation, thus it is important that we understand abnormal changes in haemodynamics which affect blood flow pattern and subsequently the endothelium. Given that the incidence of venous pathology is about ten times higher than arterial disease, it is important to study the very significant but undervalued vortex vein system of the eye. This study has provided essential, novel, and interesting insights into the vortex vein system of the porcine eye. It will be interesting to continue research in this area to determine not only what the other intracellular structures of and within the vortex vein system of porcine eyes proves important in its function, but also to investigate this in normal and abnormal human eyes.
CHAPTER 7

REGIONAL HETEROGENEITY

OF ENDOTHELIAL CELL

JUNCTIONAL PROTEIN

LABELLING IN THE PORCINE

VORTEX VEIN SYSTEM
7. Regional Heterogeneity of Endothelial Cell Junctional Protein Labelling in the Porcine Vortex Vein System

7.1 Aim

The vortex vein system of the eye is the primary drainage conduit for the choroidal circulation, but there has been minimal research conducted on its role in both retinal and choroidal diseases despite venous diseases having a ten-fold greater incidence compared with arterial diseases. Whilst research has been conducted on the pathogenesis of venous diseases, research into the mechanism of pathophysiology of the vortex vein system of the eye has been scarce.

In our previous chapter, significant endothelial cell phenotype heterogeneity was found in the eight different regions of the human and porcine vortex vein system, and indicates remarkable haemodynamic variations within the vortex vein system. Previous demonstrations of morphological heterogeneity were based on endothelial cell border f-actin labeling which appeared intact. However, with such noticeable and measureable morphological heterogeneity, an important question to be addressed is whether junctional proteins at the endothelial cell border also have regional differences within the vortex vein system. Previous studies have supported endothelial adherens and tight junctions being modulated by haemodynamic forces and having a role in the regulation of vascular
permeability and inflammation. However, we still know very little overall about the structural barrier of the vascular endothelium in the choroidal and vortex vein system. Knowledge of junctional proteins and whether there are any regional differences are important for the further understanding of the normal vortex vein system and its venous pathologies.

This chapter aims to determine whether there are regional differences within endothelial cell junctional proteins, f-actin and phosphorylated tyrosine within the vortex vein system. We propose that there would be regional differences as there is significant endothelial cell phenotype heterogeneity within different regions of the vortex vein system.
7.2 Materials and methods

7.2.1 General

Twenty post-mortem white landrace pig eyes were used, sourced from a local abattoir. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the University of Western Australia Animal Ethics Committee.

7.2.2 Preparation

Preparation of porcine eyes prior to labelling has been discussed in Chapter 2 and Chapter 6. Specific methodology for perfusion labelling will now be discussed.

7.2.3 Phalloidin perfusion labelling

Eyes chosen for perfusion labeling for actin microfilament additionally had the inferior temporal vortex vein and other potential outflow arteries and veins tied off with 8-0 vicryl sutures. Microfilaments and cell nuclei were then labelled using a mixture of phalloidin conjugated to Alexa Fluor 546 (30U; A22283, Invitrogen, Carlsbad, CA) and an iodide dye (YO-PRO-1; 6.6µM; Invitrogen), which was slowly perfused over a period of three hours. Once staining was completed, a final 10-minute washout of 0.1M filtered phosphate buffer solution was perfused to remove excess dye.

The eyes were subsequently immersed in a fixative solution of 4% paraformaldehyde overnight prior to dissection and flat mounting of the choroid and vortex vein system for imaging.
7.2.4 Claudin-5 immunolabelling

After the phosphate buffer wash, a 1mL filtered solution of 10% donkey serum in 0.1M phosphate buffer was perfused over an hour. This was followed by another hour of perfusion of a rabbit anti-claudin-5 primary antibody (1:50; SAB4200538, Sigma-Aldrich, St. Louis, MO), and a further wash with 0.1M phosphate buffer for 45 minutes. Donkey anti-rabbit IgG secondary antibody (1:200; Alexa Fluor 488 A21206, Invitrogen, Carlsbad, CA) with Hoechst (H33258; 1:1000, Sigma-Aldrich, St. Louis, MO) in 0.1M phosphate buffer was perfused over an hour before a final 45 minute wash with phosphate buffer.

The three porcine eyes perfused with claudin-5 and Hoechst were immediately dissected and flat-mounted for imaging.

7.2.5 VE-Cadherin and phosphorylated tyrosine immunolabelling

For the nine eyes labelled with VE-Cadherin and phosphorylated tyrosine, a 1mL filtered solution of 10% donkey serum in 0.1M phosphate buffer was perfused over one hour. This was followed by another hour perfusion of goat anti-VE Cadherin primary antibody (1:50; sc-6458; Santa Cruz Biotechnology, Santa Cruz, CA) in buffer and phosphorylated tyrosine from mouse (1:250; Abcam, England), followed by a further wash with 0.1M phosphate buffer for 45 minutes. Donkey anti-goat IgG secondary antibody (1:200; Alexa Fluor 488 or 555 A11055, Invitrogen, Carlsbad, CA or ab150130, Abcam, Cambridge, UK), with Hoechst (H33258; 1:1000, Sigma-Aldrich, St. Louis, MO) in 0.1M phosphate buffer was perfused over an hour before a final 45 minute wash with 0.1M phosphate buffer at 100 µL/min.
Porcine eyes perfused with VE-Cadherin, phosphorylated tyrosine and Hoechst were immediately dissected and flat-mounted for imaging.

### 7.2.6 Control images

Images were taken from eyes perfuse labeled using the same protocol as above, but with omission of the primary antibodies.

### 7.2.7 Flat mounting, microscopy, image preparation and statistical analysis

Details of these techniques have been discussed in Chapter 2.

### 7.2.8 Semi-quantitative analysis of intramural cells

Measurements of intramural cells were carried out in nine porcine eyes stained with phosphorylated tyrosine. Confocal images of each of the eight regions of each porcine eye were taken at 636.5 µm × 636.5 µm. These images were then cropped to a size of 318.25 µm × 318.25 µm for semi-quantitative analysis. For each 318.25 x 318.25 µm image, the number of intramural cells was manually counted, and the region examined to determine if the endothelial cells had phosphorylated tyrosine labeling in the cytoplasm and/or cell border.
7.3 Results

7.3.1 General

Twenty adult porcine eyes were perfused for this study. Cannulation of either the temporal long posterior ciliary artery or the superior temporal extra-ocular vortex vein resulted in the temporal side of the porcine vortex vein system being well perfused. Phalloidin labelled the f-actin microfilaments in endothelial and smooth muscle cells, claudin-5 and VE-Cadherin labelled the endothelial cell borders, and phosphorylated tyrosine labelled the intramural cells. YO-PRO-1 and Hoechst were used to stain the nuclei. Our previous chapter showed heterogeneity in endothelial cell morphology throughout the different regions of the porcine vortex vein system, along with a lack of smooth muscle cells in the ampulla region. The focus of this chapter however, is on eliciting further information on f-actin, junctional proteins and phosphorylated tyrosine in the eight different regions of the vortex vein system.

7.3.2 F-actin microfilaments

The characteristics of endothelial cell structure and smooth muscle cells were clearly labeled with phalloidin, and can be seen in column two of Figures 7-1 and 7-2. The peripheral endothelial cell borders appeared relatively even in thickness and intensity throughout Region 1 to 6 of the vortex vein system. They were seen most clearly in the ampulla region due to the lack of smooth muscle cells. The most noticeable feature of difference with f-actin staining was in Region 7 and Region 8, where visualization of the endothelial cell borders was difficult due to a thinner border and less intense stain. Additionally, endothelial cells were difficult
to visualize in the extra-ocular vortex vein due to an abundance of closely meshed smooth muscle cells surrounding the vein. Smooth muscle cells were seen in all regions of the vortex vein system except the ampulla. F-actin stress fibers were sparse within the venous endothelial cytoplasm, and if present, did not appear to show a distinct pattern.

7.3.3 Claudin-5 and VE-Cadherin junctional proteins

The endothelial cell junctions throughout the vortex venous system were clearly identified with claudin-5 and VE-Cadherin labeling, demonstrating the presence of tight and adherens junctions respectively. Representative images in each of the eight regions for both labels are shown in column three and four of Figures 7-1 and 7-2. Whilst both junctional proteins demonstrated continuous labeling, it was interesting to note several differences, one of which was the thickness of the endothelial border.

Claudin-5 had thinner width endothelial cell border staining compared to VE-Cadherin, and did not stain evenly throughout the entire endothelial cellular border. Within claudin-5 staining of the vortex vein system, the endothelial cell border width appeared to be relatively uniform from Regions 1 to 5, became thicker in Regions 6 and 7, and then was not easily identifiable in Region 8. There were also occasional areas of patchy uptake within the endothelial cytoplasm, which was more noticeable in the extra-ocular vortex vein. The unevenness and thinner width of claudin-5 staining in the endothelial cell border may indicate a weakening or absence of tight junctions in certain regions within in the vortex vein system, and may suggest that there is more potential for vascular leakage within the vortex vein system.
VE-Cadherin had a more even distribution and thicker width endothelial cell border staining than claudin-5. VE-Cadherin staining of the endothelial cell borders within the vortex vein system showed generally even widths and intensity in all regions except for Regions 7 and 8, where it appeared to be particularly thick around the downstream pole. Endothelial cells were also usually poorly stained and difficult to visualize in Region 6. Of note, VE-Cadherin and phosphorylated tyrosine were also picked up by intramural cells.

### 7.3.4 Phosphorylated tyrosine labelling

The final column in Figure 7-1 shows double labeling of VE-Cadherin and phosphorylated tyrosine. An increasing presence of phosphorylated tyrosine was observed from Regions 6 to 8 inside the endothelial cells. Qualitative assessment of phosphorylated tyrosine labeling at the endothelial cell border found an increasing presence in Regions 6 to 8 (Figure 3), with all specimens studied to have endothelial cell border phosphotyrosine labeling in Region 8 overlapping with VE-Cadherin labelling.

As mentioned, VE-Cadherin and phosphorylated tyrosine co-localized in intramural cells. These are shown in more detail in Figure 3. The intramural cells were of varying shapes and sizes; however the majority of them appeared to have a cellular border with two long processes extending from a central cellular body. Their processes often extended over four endothelial cell widths in length.
Figure 7-1 Distribution of Phalloidin, Claudin-5, VE-Cadherin and Phosphorylated Tyrosine in Regions 1 – 4 (overleaf)\textsuperscript{16}

Representative confocal images cropped to 132.145 x 132.145 μm to show the venous endothelium in the choroidal vein (A), pre-ampulla (B), anterior portion of the ampulla (C), and mid-ampulla (D) regions are shown against different labels. The first column shows negative control images obtained with omission of primary antibodies with Hoechst labels the nuclei. The second column showed f-actin (red) and nuclei (blue) of endothelia and vascular smooth muscle cells labelled with phalloidin and YO-PRO-1. The third column shows Claudin-5 (red) labeling at the endothelial tight junctions and nuclei have been labelled with Hoechst (blue). The fourth column shows VE-Cadherin (red) labeling at the endothelial adherens junctions and their nuclei counterstained with Hoechst (blue). In the last column, phosphorylated tyrosine (green) and VE-Cadherin (red) labelling in endothelia are shown. Non-endothelial intramural cells which are VE-cadherin and phosphotyrosine positive are observed in the first four regions of the porcine vortex vein system. They consist of two long spindle-like processes extending from a central cellular body. Arrow: direction of flow. Scale bar: 30 μm.
Representative confocal images of the venous endothelium in the posterior portion of the ampulla (E), post-ampulla (F), intra-scleral canal (G), and extra-ocular vortex vein (H) regions are shown against different labels. In order to show the endothelia and intramural cells clearly, images were cropped to 132.145 x 132.145 µm. The first column shows negative control images obtained with omission of primary antibodies with Hoechst labels the nuclei. Column two shows f-actin filament (red) and nuclei (blue) labelling. Both venous endothelial cells and vascular smooth muscle cells may be seen. Column three shows Claudin-5 (red) and nuclei (blue) labelling at the endothelial tight junctions. Column four shows VE-Cadherin (red) labelling at the endothelial adherens junctions and their nuclei counterstained with Hoechst (blue). Column five shows the location of phosphorylated tyrosine (green), VE-Cadherin (red), and Nuclei (blue). There is a presence of phosphorylated tyrosine within the cellular border and cytoplasm, particularly in Region 7 and 8. Arrow: direction of flow. Scale bar: 30 µm.
Figure 7-3 General distribution of intramural cells (overleaf)

Confocal images of the porcine vortex vein system endothelia have been taken from the choroidal vein (A), pre-ampulla (B), anterior portion of the ampulla (C), mid-ampulla (D), posterior portion of the ampulla (E), post-ampulla (F), intrascleral canal (G), and extra-ocular vortex vein (H). In order to show the endothelia and intramural cells clearly, images were cropped to 173.173 x 173.173 µm. The porcine vortex vein system has been labelled for VE-Cadherin (red), phosphorylated tyrosine (green) and nuclei (blue). Co-localisation of phosphorylated tyrosine and VE-Cadherin (yellow) may be seen in the endothelial cell cytoplasm, endothelial cell border and in the intramural cells. Arrow: direction of flow. Scale bar: 30 µm.
7.3.5 Semi-quantitative analysis of intramural cells in different regions

The average number of intramural cells per square millimeter in each region is presented in Table 7-1 and Figure 7-4. Statistical analysis was conducted in two ways: comparison of all regions against a control, and comparison of each individual region against its adjacent regions.

Using Region 1 as the control, there was a statistically significant difference (P < 0.001) between Region 1 and 5, Region 1 and 6, Region 1 and 7, and Region 1 and 8. Regions 2, 3 and 4 had comparable numbers of intramural cells to Region 1 (P > 0.05).

Comparison of individual regions to their adjacent regions showed a steady reduction in the number of intramural cells from Region 1 to 6 with a statistically significant decrease between Region 4 and 5 (P < 0.03), and Region 5 and 6 (P < 0.008). There was also a significant increase from Regions 7 to 8 (P < 0.0001).
**Figure 7-4 Intramural cells vs Region**

Graph showing the mean number of intramural cells against each region.

Standard errors of the mean are shown. The asterisks (*) indicate a statistically significant difference ($p < 0.05$) against Region 1.
Table 7-1 The intramural cell count in the eight different regions of the vortex vein system

<table>
<thead>
<tr>
<th>Region</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of intramural cells per square mm</td>
<td>210.6 ± 23.80 (9)</td>
<td>201.8 ± 25.41 (9)</td>
<td>182.0 ± 22.15 (9)</td>
<td>148.1 ± 32.87 (9)</td>
<td>87.7 ± 26.02 (9)</td>
<td>2.2 ± 1.54 (9)</td>
<td>4.4 ± 3.08 (9)</td>
<td>49.4 ± 6.98 (9)</td>
</tr>
</tbody>
</table>

Mean and standard error of mean presented for the number of intramural cells in each of the eight regions of the porcine vortex vein system.

Numbers in parentheses indicate the number of eyes included for each measurement.
7.4 Discussion

7.4.1 Key findings

There are five major findings in this study:

1. The endothelial cell structures of the vortex vein system revealed regional differences with f-actin, claudin-5, VE-Cadherin and phosphorylated tyrosine labeling.
2. Cytoskeleton labeling was relatively even in thickness and intensity throughout Region 1 to 6, but less intense in Regions 7 and 8.
3. Claudin-5 and VE-Cadherin labeling were clearly identified demonstrating the presence of tight and adherens junctions respectively. Except for Region 7 and 8, VE-Cadherin had a fairly uniform distribution and thicker width endothelial cell border staining than claudin-5.
4. There was an increase in the overlapping of phosphorylated tyrosine and VE-Cadherin labeling in the endothelial cell border and cytoplasm in Regions 6 to 8.
5. There was a presence of intramural cells in Regions 1 to 4, which were VE-Cadherin and phosphorylated tyrosine positive. There were significant differences in the mean number of intramural cells against different regions of the vortex vein system.
7.4.2 Contrasts and comparisons: the most changes occur in Region 6 to 8

The vortex vein system anatomy and endothelial cell morphology has been previously studied. However, to our knowledge, this is the first study that describes the regional differences in f-actin, junctional proteins and phosphorylated tyrosine labeling in endothelial cells of the porcine vortex vein system.

In general, the immunohistochemistry staining and intensity of endothelial cell borders for f-actin, claudin-5, VE-Cadherin and phosphorylated tyrosine were comparable in Regions 1 to 5. This correlated with the measurements of area, perimeter, length and width of endothelial cells of the porcine eye, in that there were also on the whole gradual changes rather than abrupt distinctions in Regions 1 to 5. In our previous chapter, the striking differences in endothelial cell regional heterogeneity were clearest in Regions 6 to 8, indicating significant haemodynamic differences. The post-ampulla region revealed “arterial-like” endothelial cells, which likely underwent significant forces of high shear stress. This contrasted the endothelial cell shape in the intra-scleral canal and extra-ocular vein, which revealed a rounded endothelial cell appearance, which likely underwent a decrease in haemodynamic forces.

Interestingly, the most changes in immunohistochemistry staining also appeared in Regions 6 to 8. We found a substantial increase in phosphorylated tyrosine staining in these regions, a decrease in f-actin staining for the intra-scleral canal and extra-ocular vein, thicker cell borders in the intra-scleral canal for VE-
cadherin adherens junction protein, and decreased staining in the extra-ocular vortex vein for claudin-5.

7.4.3 Tyrosine phosphorylated VE-Cadherin

Recent studies reported that VE-cadherin is phosphorylated on tyrosine in the veins, but not in the arteries. From mechanistic studies, tyrosine phosphorylation is involved in the priming of VE-cadherin for internalisation in association with a weakened barrier function. Although a preferential presence of phosphorylated tyrosine on VE-cadherin was not found throughout the microvascular porcine vortex vein system, an increased presence was observed in Regions 6 to 8. VE-cadherin in adherens junction are targets of signalling pathways for agents that increases vascular permeability. Since tyrosine phosphorylated VE-Cadherin is a necessary step in some of the signalling pathways to increased permeability, the observed co-localization of phosphorylated tyrosine and VE-Cadherin may be suggestive of the increased vulnerability of these positively labeled areas of the post ampulla, intra-scleral canal, and extra-ocular vortex vein to breach of blood barrier. In addition, the continuous appearance of VE-cadherin is comparable to in vitro endothelial cells that have been exposed to laminar flow after 24 hours.

7.4.4 Potential region of vulnerability

From these observations, it could be speculated that haemodynamic forces gradually increase, along with the volume of blood, from Regions 1 to 5, prior to a sudden increase in shear stress as blood exits through a restrictive channel in the sclera. From Regions 6 to 8, where the greatest changes occur with both
endothelial cell phenotype heterogeneity and junctional proteins, it could be argued that these regions may be the most vulnerable to venous pathogenesis. Vascular integrity is maintained by endothelial adherens junctions, with VE-Cadherin having a crucial role in the regulation of vascular permeability. A disruption of its function is associated with increased vascular fragility and tissue oedema, and thus the decrease in VE-Cadherin staining in the high shear stress post-ampulla region may be indicative of a region of vulnerability. Whilst it has been noted that VE-Cadherin phosphorylation is unlikely without permeability-increasing agents, we were able to observe phosphorylation particularly in the intra-scleral and extra-ocular vortex vein. This may indicate that whilst uncommon without inflammatory agents, the appearance of this observation in young adult porcine eyes may suggest an increase in permeability and vulnerability of the adherens junctions in these regions, and may potentially be linked to vortex vein pathologies.

7.4.5 Intramural cells

Intramural cells have also been observed in these studies that were VE-cadherin positive. To our knowledge, these cells that have been observed predominantly in Regions 1 to 5 of the vortex vein system have not yet been described. Our study observed the mean number of intramural cells was similar in Regions 1 to 4; with the highest mean number in Region 1 and a gradual decrease to Region 5. Intramural cells were rare in Regions 6 and 7 before reappearing occasionally in Region 8 (Figure 7-4). It is interesting to note the intramural cells were predominantly present in Regions 1 to 5, as this also correlated with the general stability of endothelial cell morphology and different immunohistochemistry
labeling. It would be interesting to identify these cells. We had additionally labelled the vortex vein system for LYVE and GFAP, however these did not reveal specificity for these intramural cells. Further studies are required to identify the specific cell type of these intramural cells. It is speculated that the intramural cells discovered in the vortex vein system may be pericytes or macrophages.

7.4.6 Limitations

While this chapter has contributed novel observations regarding the vortex vein system in porcine eyes, our focus was primarily on regional differences in different immunohistochemistry staining of the endothelial cells and thus have not further characterized these intramural cells. Further study to determine permeability differences using permeability-inducing agents such as histamine or VEGF may also provide more detailed information on the relative vulnerability of the different regions of the vortex vein system.

7.4.7 Summary

Significant morphological heterogeneity has been described in the eight different regions of the porcine and human vortex vein system, indicating remarkable haemodynamic variability. With such impressive differences, it is important to determine whether there are also regional changes in junctional proteins at the endothelial cell border within the vortex vein system. Whilst the vortex vein system of the eye is the primary drainage pathway for the choroidal circulation, and it is known that venous diseases outweigh arterial diseases ten-fold, minimal research regarding their role in retinal and choroidal diseases has been pursued. In this study, we have found significant regional differences with endothelial cell
junctional proteins, f-actin and phosphorylated tyrosine within the vortex vein system, which has also correlated and supported existing data on endothelial cell phenotype heterogeneity. These findings may be a potential link to understanding regions of vulnerability within the vortex vein system and aid in our knowledge of venous pathologies. Intramural cells have also been discovered in the vortex vein system, and it is believed to be of great value to further investigate and identify these cells.
CHAPTER 8

GENERAL DISCUSSION
8. **General Discussion**

Retinal vascular diseases continue to contribute to extensive causes of morbidity and disability worldwide,¹ ² yet there is uncertainty on the precise architectural structure of the dual blood supply in relation to retinal function, and only a rudimentary understanding of the pathophysiological causal sequence of retinal vascular diseases. A thorough understanding of the intimate relationship between structure and function within the vasculature supplying the retina is important for several reasons: (1) it may elucidate critical pathognomonic mechanisms and characterisations involved in retinal vascular disease, (2) it may allow segregation of specific architectural vascular models which represent early or manifest clinical disease and thus allow diagnosis and monitoring of disease in patients, and (3) it may potentially aid in identifying therapeutic targets to terminate or reverse disease progression.

This thesis provides new, detailed information regarding the quantitative distribution of microvasculature structure within physiological and diseased states of the human retina, as well as regional heterogeneity of endothelial cells within the porcine vortex vein system.

Employing our novel perfusion technique to enable visualisation of the precise location of capillary networks within the different layers of the retina revealed four capillary networks that were complex and morphometrically variable located at 1) nerve fibre layer, 2) retinal ganglion cell layer, 3) border of the inner plexiform layer and superficial boundary of the inner nuclear layer, and 4) boundary of the deep inner nuclear layer and outer plexiform layer.
Additionally, the capillary diameter in the retinal ganglion and inner plexiform layer networks were significantly less than the other networks, and capillary density was greatest in the retinal ganglion cell layer. The inner and outermost capillary layers were laminar in structure and contrasted the complex three-dimensional networks supporting the high synaptic activity of the inner plexiform layer. The specific three-dimensional vascular configuration of retinal capillary networks aids in increasing the efficiency of oxygen transfer and waste removal, and is critical considering the high metabolic demands of the inner retina, which needs to be supplied from a single central retinal artery to a collective retinal circulation spanning a total area of approximately 1000 mm².

The efficiency of the spatial distribution and regulatory capability of the retinal vasculature to match blood flow to local metabolic demands, and its impairment in retinal vascular disease, remains a topic of great interest. The current understanding of microvasculature formation suggests that the vascular supply is inherently fixed due to the stochastic process of angiogenesis and the physical limitations of tissue, with oxygen consumption being a variable. It has been shown that capillary networks and blood flow are by nature heterogeneous. Within vascular beds, there are constant dynamic adaptations that govern the diameter and density of the blood vessels based on shear stress to avoid shunting of blood away from target tissues. There are both upstream and downstream regulatory signaling mechanisms which control this. Whilst dynamic adaptation does occur, it does not fully account for the ability to supply distal tissues, and as such, still lends to the following question of how exactly are the high metabolic demands of avascular layers of the retina supplied, such as the photoreceptor inner segments? It is therefore likely that the precariously balanced avascular retinal
regions must have an extremely efficient means of oxygen extraction given its
distance of up to $75\mu m$ from the dual vascular circulations$^{83}$ in conjunction with
its high oxygen tension requirements. This is a question that continues to educe investigation.

Due to the nature of terminal retinal arteries and the absence of collateral
circulation, the retina is particularly vulnerable to ischemic injury. Capillaries
represent the major site of nutrient delivery and toxic substrate exchange, between
neurons and vasculature.$^{49-52}$ This thesis explored the retinal microvasculature in
both the normal and abnormal state. Comparison between the eyes of healthy,
normal donors and those with cardiovascular comorbidities revealed a significant
decrease in density in the nerve fiber layer and retinal ganglion cell layer of the
diseased group, potentially stemming from capillary drop-out and loss of
photoreceptors.$^{214}$ This may also imply disordered homeostatic signaling
mechanisms with resultant predisposition to retinal vascular disease and
ischaemia. The third and fourth capillary networks of those with cardiovascular
comorbidities were not significantly different to control patients, however this
may just be a reflection of being in the earliest stages of retinal vascular disease.
There were also pathological vascular changes including microaneurysms and
tortuous dilated venules despite no history of ocular disease, which suggests
retinopathy likely begins much earlier than the onset of clinically detectable
disease.

A solid understanding of the mechanism behind how or why the avascular region
(photoreceptor layer) in the human retina is able to adapt to the limited vascular
supply provided, would enable application of this knowledge to clinical
pathology, not solely for ophthalmological diseases but in many other systemic
diseases. Many vascular insufficiency problems such as ischaemic heart disease,
peripheral vascular disease and stroke, occur as a result of inadequate mismatch of
oxygen through blood flow to the target tissues.

Also demonstrated in this thesis is a new prototype svOCT device, which could be
useful as a clinical diagnostic tool in accurate qualitative and quantitative retinal
angiography in humans. It exhibits the capacity to provide dynamic, histology-like
anatomical information about the human retinal capillary networks in vivo,
providing great potential as a research and diagnostic tool in the management of
retinal vascular diseases. However, a few limitations exist: significant movement
artifact may hinder crisp images, there is currently a limited resolution and depth
of penetration precluding extensive microvascular visibility, and there is the
expense and availability of the device. Additionally, there were no statistical
differences in mean capillary diameter between svOCT images and histology for
all networks other than the retinal ganglion cell capillary network, and capillary
density measurements were significantly greater in svOCT images except in the
retinal ganglion cell capillary network. These findings demonstrate remarkably
comparable features of the svOCT to histological studies. A possible reason for
increased diameter and density in any of the networks may be due to the
differences between fixed versus dynamic live specimens, whereby an active
process of capillary dilation may occur in vivo. Eventually, it can be anticipated
that with further and thorough investigation on the retinal microvasculature in
diseased states in vivo with comparisons to physiological states, compilation of
specific structural vascular models which represent early or manifest clinical
disease may be viable. This may be the start of a revolutionary and exciting method of clinical diagnosis and monitoring of disease progress in patients.

This thesis is also the first to document the general structure and endothelial cell morphology of the vortex vein. Functionally, the vortex vein system has a role in controlling optimum choroidal blood flow. Understanding disturbed venous blood flow leading to drainage obstruction, stasis, inflammation or thrombus may be observed through endothelial flow patterns and phenotypic changes leading to endothelial dysfunction. This thesis documents significant regional differences in the endothelial cell and nuclei length, width, area and perimeter observed throughout the porcine vortex vein system. Most notably, very narrow and elongated endothelia were found in the post-ampulla region, along with a lack of smooth muscle cells in the ampulla region. It is likely that the highest shear stress occurs immediately downstream from the ampulla, with evidence to suggest this via observed nuclei position which tended to be located further downstream at this region. Increasing downstream positioning of the endothelial cell nuclei in the post-ampulla and intra-scleral canal was then abruptly changed to a rounding and shortening of both the endothelial cell and nuclei in the extra-ocular vortex vein. This corresponds with the sudden change between intra- and extra-ocular positive pressure compartments, and reflects the markedly variable haemodynamic conditions and potential blood flow control mechanisms.

Finally, results from experiments on the endothelial cell f-actin, claudin-5, VE-Cadherin and phosphorylated tyrosine labelling within the porcine vortex vein revealed regional phenotypic heterogeneity. This provides original insights into the potential regions of vulnerability, particularly at the post-ampulla, intra-scleral...
canal and extra-ocular vortex vein regions. Highlights of this study include:
adherens and tight junction proteins are present in vortex vein endothelia; VE-
cadherin distribution is generally even, with the exception of Regions 6, 7 and 8;
Claudin-5 labelling is generally absent from extraocular vortex vein endothelia;
and there was an increased presence of phosphorylated tyrosine is observed in
Regions 7 and 8. Additionally, newly described intramural cells, which were
immune-positive for VE-Cadherin and phosphorylated tyrosine, were noted.
There were significant differences in the mean number of intramural cells in the
different regions of the vortex vein system, with presence of these cells in the
choroidal veins, pre-ampulla, anterior portion of ampulla and mid-ampulla
regions. The culmination of these findings on the vortex vein system highlights
the importance of understanding this undervalued system.

There are probably two main reasons why the clinical importance of the vortex
vein system has not been discovered. Firstly, the vortex vein system is not easily
visualised clinically. Some information of the intra-ocular components can be
obtained using fundus imaging devices including spectral domain optical
coherence tomography and indocyanine green angiography, but not to the detail
of our histological studies. On the other hand, the intra-scleral and extra-ocular
components of the vortex vein cannot be visualised clinically with fundus
imaging, but require penetration depth and resolution devices. Secondly, there
is a paucity of vortex vein system studies at a detailed laboratory level. The
studies described in this thesis were the first in defining the regions of the porcine
vortex vein system, and areas of endothelial cell vulnerability.
An interesting observation found was the distribution and appearance of smooth muscle cells. These were noticeably irregular and regionally dependent, and were not like typical vascular smooth muscle cells. Potentially they may be non-vascular smooth muscle cells, but regardless, the smooth muscle cells were present in all regions except the ampulla. The wall of the ampulla was exceedingly thin with of one layer of endothelium, and smooth muscle cells buttressing before and after the ampulla, suggesting collapsibility. This assimilates well with the Starling resistor concept; an elastic fluid-filled collapsible tube mounted inside a pressurised chamber. Studies have suggested this concept as an effective variable resistor which controls flow, and has been described in cardiac, respiratory, nephritic and total peripheral vascular resistance. Whilst this thesis has not determined the Starling resistor effect in the vortex vein system, it is a fascinating concept and one that will require further investigation.

There is a large bench-to-bedside gap in knowledge and awareness of the role of the endothelium in health and disease. Yet there is a tremendous untapped potential for the endothelium to be used as a therapeutic target. Studies in the past have focused on cultured cells and large vessels, demonstrating haemodynamic shear stress causing endothelial structural remodeling. Vascular endothelial cells are critical for normal physiological function, with pathological processes occurring in the absence of their survival. Extensive research indicates pathognomonic processes with endothelial dysfunction after prolonged and excessive endothelial activation, and is therefore an important pathogenic inception for many systemic diseases and ocular pathology including diabetic retinopathy, retinal ischemic diseases, inflammation and
glaucoma.\textsuperscript{229, 316-325} Endothelial therapeutic targeting may be an invaluable resource in the future.

The findings in this thesis are two-fold: the precise structure of the distinct capillary networks of the retinal microvasculature is critical to the survival and function of the retina and has been reported \textit{in vivo}; and the vortex vein system is an architecturally unique, haemodynamically complex and functionally valuable system. The structure of both vasculature systems are intricately related to the function of the retina and it is foreseeable for both the \textit{in vivo} microvasculature in pathology and the vortex vein system to become an area for further ophthalmic research.
9. References


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