QUANTITATIVE ASSESSMENT OF THE HUMAN RETINAL MICROVASCULATURE WITH OR WITHOUT VASCULAR COMORBIDITY

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Abstract

**Purpose:** To determine whether morbidity and mortality in patients with cardiovascular comorbidities, but no known ocular disease, is related to demonstrable quantitative changes in the retinal microvasculature.

**Methods:** 11 eyes from 8 donors with cardiovascular comorbidities as a diseased group were compared with 16 eyes from 14 donors free from vascular disease as a control group. All eyes had no known ocular disease. The retina was perfusion-fixed and labelled for endothelial f-actin using micro-cannulation techniques. The retinal microvasculature 3 mm superior to the optic disc was imaged with confocal scanning laser microscopy. Quantitative measurements of capillary diameter and density were obtained using two-dimensional image reconstructions. Pathological vascular changes in other regions of the retinal vasculature found in the diseased group were identified and reconstructed in two- or three-dimensions.

**Results:** Capillary densities were significantly different between each capillary network in the diseased group. There was a significant decrease in density between both the nerve fibre layer and retinal ganglion cell layer of the diseased group when compared to those layers in the control eyes. There were pathological vascular changes including microaneurysms and tortuous, dilated venules identified in the diseased group.

**Conclusions:** Cardiovascular comorbidities may be associated with changes to the capillary density within the human retinal microvasculature, prior to the manifestation of known ocular diseases. These differences in capillary density may have important correlations with neuronal function and facilitates the basis of understanding pathogenic mechanisms in retinal vascular disease.
Introduction

Previous studies have shown a positive correlation between cardiovascular disease and retinal microvascular abnormalities, but this has usually been in the presence of clinically known retinopathy. 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 provide important insights into the prevention of retinal vascular diseases and thus prevent resultant vision loss.

To date there has been only one study that has documented quantitative changes in perifoveal capillary networks in patients with vascular comorbidities. We have previously documented the quantitative characteristics of human retinal capillary networks using novel micro-cannulation techniques developed in our laboratory. Four capillary networks were identified in the normal human retina, with different quantitative and morphometric characteristics in each of the four retinal capillary layers. Despite these informative studies, there has been no study 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 this study is to describe whether patients with cardiovascular comorbidities reveal measurable alterations on 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.
Materials and Methods

This 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.

Definitions

In this study, “Control” has been defined as being free from known ocular disease or vascular disease. They have been used in this study as healthy, control eyes. “Diseased” has been defined as 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” has been defined as the perpendicular distance across the maximum transverse measurement of each vessel. “Capillary density” has been defined as the percentage quantity of area occupied by vessel lumens. “Laminar” is used to describe a single-planar orientation that is predominantly confined to a single retinal layer with minimal projections along the z-axis. “Retinal vascular pathological changes” are defined as an abnormal appearance of the retinal vasculature. “Three-dimensional” is used to describe the capillary networks that traversed prominent projections along the vertical z-axis and were not confined to a single plane.

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 also used in our previous work. 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 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 1 (Donors A to V).

**Perfusion Labelling of Retinal Vasculature**

Identical methodology from our previous reports for targeted retinal endothelial labelling has
been used in this study. Notably, this study follows directly from a previous paper, which
researched the normal microvasculature of the human retina. 

Briefly, after cannulation of the central retinal artery, blood was washed out from the retinal
vasculature with Ringer’s solution and 1% bovine serum albumin through cannulation of the
central retinal artery. It was then fixed with 4% paraformaldehyde in 0.1M phosphate buffer,
washed, perfused with 0.1% Triton-X-100 in buffer, washed and lastly labelled over the
course of two hours, and then had a final wash. 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 glycoglycans;
Claudin-5 (1:50; 1mg/mL; Sigma-Aldrich, St. Louis, MO) to label the transmembrane tight
junction proteins; Monoclonal Anti-Glia 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.
Tissue Preparation and Flat Mounting of the Retina

After labelling, the human eye was dissected at the equator to allow viewing of the posterior retina. The posterior retina was carefully dissected out and a few cuts made to the retina to enable flat mounting in glycerol before cover-slipping and imaging.

Microscopy

Initially, low magnification epifluorescence microscopy (E800; Nikon, Tokyo, Japan) images were taken of the whole-mount retina to ensure the precise location of the studied region, which was typically 3 mm superior to the optic disc. Confocal imaging was then performed using two Nikon C1 conofocal systems and a series of z-stacks were captured to visualize all vascular layers of the retina at this studied region. Other areas of the retinal vasculature were also studied in some eyes in which retinal vascular abnormalities were noticed.

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. Reconstruction of three-dimensional capillary networks were performed using Imaris software (version 7.4.2; Bitplane, Zurich, Switzerland).

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.

**Statistical Analysis**

Sigmastat (Sigmastat, ver. 3.1; SPSS, Chicago, IL) was used to calculate all data in terms of mean and standard errors. R (R Foundation for Statistical Computing, Vienna, Austria) was used to analyse multiple measurements from diseased eyes with data from single eyes as well as those with measurements from the right and left eye of the same individual. To compare measurements between the layers, one-way analysis of variance (ANOVA) testing was performed. Our analysis model included “Right” or “Left” nested within “Eye donor” as random effects, and used linear mixed modelling to test measurement differences between the retinal layers. “Eye donor” was assigned as a random effect and used to account for the effects of intra-“eye” correlation as well as “Right” and “Left” to account for right and left eye correlation. 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.
Results

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.

General Comparisons in Capillary Topography in the Control and Diseased Human Retina Capillary Networks
Our previous report highlights the consistent locations where morphometrically different capillary networks were found in the normal human retina. 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 inner plexiform layer (IPL) and superficial boundary of the inner nuclear layer (INL). (4) Boundary of deep INL and outer plexiform layer (OPL).
Figure 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.

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.

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.

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.

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.

Quantitative Analysis of Capillary Diameter

Mean capillary diameter for all networks was 8.92 ± 0.05 µm (n = 1980). Table 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 2 and Table 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 3 and Table 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 ($P > 0.05$).

### Quantitative Analysis of Capillary Density

Table 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 3 and Table 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 3 and Table 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).

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 (Figures 4 – 6). There were no vascular pathologies found in the control group studied.

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 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.

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 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.

Pathological retinal vascular changes were not limited in the location which we selected to perform quantitative assessments of capillary density and diameter. It also these pathological vascular changes could be occurred in the different capillary network and associated with other cellular changes particularly glial cells in the retina. In one particular eye (Donor H), there were up to 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 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 7) demonstrates Claudin-5 labelling the transmembrane tight junction proteins particularly in highlight the microaneurysms. Close relationship between microaneurysms and glial cells was evidenced by co-labelled Claudin-5, glial fibrillary acidic protein and cellular retinaldehyde-binding protein. Under microscopy, they fluoresced brightly and were different to 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.

**Discussion**

The major findings from this study are as follows. Firstly, 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. Secondly, 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.

Endothelial cells within the morphometrically organised capillary network play an indispensable role in modulating retinal homeostasis.\textsuperscript{10, 12-14} 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{13-17} enabling the heterogenic metabolic demand of the neurons and supporting glia to be met.\textsuperscript{5, 18} 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{19, 20} 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{21, 22}

Cardiovascular comorbidities including ischaemic heart disease, hypertension and hypercholesterolaemia, have been shown in existing studies to be associated with retinal vascular disease.\textsuperscript{9, 23-29} Changes 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. However, previous studies which demonstrated links and alterations 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. Once clinical manifestations of retinal disease are present, particularly at the macula, accelerated and permanent vision loss can develop.

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, and similar results have been confirmed in previous studies. 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. Ischaemia may then lead to retinal vascular pathological changes, such as induced vessel dilatation.

It was shown in our study that all capillary diameters were larger (non-significant) in the eyes of diseased patients compared to the control group. These finding were shown in a similar study, and represented similar reports to cerebrocortical models of hypoxia and
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 allow us to further understand the relationship between cardiovascular comorbidities and retinal vascular disease processes.

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.

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. 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. 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.\(^4^2\) 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,\(^4^3\) 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.\(^4^0, 4^4\) More recently confocal-images,\(^4^1\) OCT,\(^4^3, 4^5\) and fluorescein angiograms\(^4^6\) 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 \(\mu\)m to 116 \(\mu\)m, which was similar to the 14 \(\mu\)m to 136 \(\mu\)m range reported in another confocal study,\(^4^1\) and the 43 \(\mu\)m to 266 \(\mu\)m range reported in an OCT study.\(^4^3\) As shown in Figure 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.

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.\textsuperscript{47-50} It has been found to be generally asymptomatic when mild, however when severe could lead to end organ ischaemia.\textsuperscript{51}

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.\textsuperscript{52-62}

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.\textsuperscript{60} As it is known that the venous system is highly complex with diseases being ten times more frequent in the venous than arterial system,\textsuperscript{63, 64} it is thus important to study the venous system of the eye in more depth.\textsuperscript{65}

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.

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.

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.
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**Legends**

**Figure 1** – Comparison of the capillary network in control and diseased groups. (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.*

**Figure 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. 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 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.

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.

**Figure 4** – (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: $A = 100 \, \mu m$, $B = 17.5 \, \mu m$, $C = 33.3 \, \mu m$, $D = 15.9 \, \mu m$.

Figure 5 – (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: $A = 100 \, \mu m$, $B = 33.3 \, \mu m$.

Figure 6 – (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. 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.*

**Figure 7** – 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.*
Tables

Table 1. Donor Demographic Details: Age, Sex, Eye, Vascular Comorbidities, Cause of Death, and Time to Cannulation for Donor Eyes.

(Insert Table 1 here, please refer to next page)

Age (in years); Sex (M = male, F = female); R = right; L = left; AF = atrial fibrillation; MVA = motor vehicle accident; Time to cannulation (in hours). Donor eyes with vascular abnormalities are noted with an asterisk (*). Line separates disease (above) and control (below) eyes. Normal control eye data were taken from the study directly prior to this paper, which researched the normal microvasculature of the human retina.⁶
<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</th>
</tr>
</thead>
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<td>A</td>
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<td>M</td>
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<td>Ischaemic heart disease, hypertension</td>
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<td>B</td>
<td>41</td>
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<td>R</td>
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<tr>
<td>C</td>
<td>51</td>
<td>M</td>
<td>L + R</td>
<td>Ischaemic heart disease</td>
<td>Myocardial infarction</td>
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<tr>
<td>D</td>
<td>65</td>
<td>M</td>
<td>L + R</td>
<td>Cerebral vascular accident</td>
<td>Intracranial haemorrhage</td>
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<td>M</td>
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<tr>
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<td>L</td>
<td>-</td>
<td>Sepsis</td>
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Table 2. Quantitative capillary diameter and density data for each network of the diseased human eyes.

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>Capillary network</th>
<th>NFL</th>
<th>RGC</th>
<th>IPL / Superficial INL</th>
<th>Deep INL/OPL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary Diameter (μm)</td>
<td>8.86 ± 0.09 (495)</td>
<td>8.93 ± 0.09 (495)</td>
<td>8.93 ± 0.10 (495)</td>
<td>8.96 ± 0.10 (495)</td>
</tr>
<tr>
<td>Total Capillary Density (%)</td>
<td>8.15 ± 0.01 (11)</td>
<td>21.42 ± 0.02 (11)</td>
<td>9.69 ± 0.01 (11)</td>
<td>14.19 ± 0.01 (11)</td>
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</tbody>
</table>
Table 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/superficial INL), and deep inner nuclear layer and outer plexiform layer (deep INL/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.

<table>
<thead>
<tr>
<th>Capillary Network</th>
<th>NFL</th>
<th>RGC</th>
<th>IPL / Superficial INL</th>
<th>Deep INL/OPL</th>
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</thead>
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<tr>
<td></td>
<td>Control</td>
<td>Diseased</td>
<td>Control</td>
<td>Diseased</td>
</tr>
<tr>
<td>Capillary Diameter (µm)</td>
<td>8.47 ± 0.05 (540)</td>
<td>8.86 ± 0.09 (495)</td>
<td>8.01 ± 0.05 (540)</td>
<td>8.93 ± 0.09 (495)</td>
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<tr>
<td>Total Capillary Density (%)</td>
<td>13.69 ± 0.01 (12)</td>
<td>8.15 ± 0.01 (11)</td>
<td>26.74 ± 0.01 (12)</td>
<td>21.42 ± 0.02 (11)</td>
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<tr>
<td>&lt;60yo</td>
<td>&gt;60yo</td>
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<tr>
<td>Capillary Diameter (µm)</td>
<td>8.48 ± 0.05 (540)</td>
<td>8.85 ± 0.09 (495)</td>
<td>8.27 ± 0.06 (540)</td>
<td>8.65 ± 0.09 (495)</td>
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<tr>
<td>Total Capillary Density (%)</td>
<td>12.02 ± 0.01 (12)</td>
<td>9.96 ± 0.02 (11)</td>
<td>26.43 ± 0.01 (12)</td>
<td>21.76 ± 0.02 (11)</td>
</tr>
</tbody>
</table>
References


