Alpha-Smooth Muscle Actin Expression and Parafoveal Blood Flow Pathways Are Altered in Preclinical Diabetic Retinopathy

Dong An,1,2 Jonathan Chung-Wah-Cheong,1 Dao-Yi Yu,1,2 and Chandrakumar Balaratnasingam1–3

1Lions Eye Institute, Nedlands, Western Australia, Australia
2Centre for Ophthalmology and Visual Science, University of Western Australia, Perth, Western Australia, Australia
3Department of Ophthalmology, Sir Charles Gairdner Hospital, Nedlands, Western Australia, Australia

Correspondence: Chandrakumar Balaratnasingam, Lions Eye Institute, 2 Verdun Street, Nedlands, WA 6009, Australia; balaratnasingam@gmail.com.

DA and JC contributed equally to the work presented here and should therefore be regarded as equivalent first authors.

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PURPOSE. To investigate differences in alpha smooth muscle actin (αSMA) expression and parafoveal blood flow pathways in diabetic retinopathy (DR).

METHODS. Human donor eyes from healthy subjects (n = 8), patients with diabetes but no DR (DR−; n = 7), and patients with clinical DR (DR+; n = 13) were perfusion labeled with antibodies targeting αSMA, lectin, collagen IV, and filamentous actin. High-resolution confocal scanning laser microscopy was used to quantify αSMA staining and capillary density in the parafoveal circulation. Quantitative analyses of connections between retinal arteries and veins within the superficial vascular plexus (SVP), intermediate capillary plexus (ICP) and deep capillary plexus (DCP) were performed.

RESULTS. Mean age between the groups was not different (P = 0.979). αSMA staining was seen in the SVP and ICP of all groups. The DCP was predominantly devoid of αSMA staining in control eyes but increased in a disease stage–specific manner in the DR− and DR+ groups. The increase in αSMA staining was localized to pericytes and endothelia of terminal arterioles and adjacent capillary segments. Capillary density was less in the DCP in the DR+ group (P < 0.001). ICP of the DR− and DR+ groups received more direct arteriole supplies than the control group (P < 0.001). Venous outflow pathways were not altered (all P > 0.284).

CONCLUSIONS. Alterations in αSMA and vascular inflow pathways in preclinical DR suggest that perfusion abnormalities precede structural vascular changes such as capillary loss. Preclinical DR may be characterized by a “steal” phenomenon where blood flow is preferentially diverted from the SVP to the ICP and DCP.

Keywords: retina, macula microvasculature, capillary, smooth muscle actin/αSMA, diabetic retinopathy

Diabetic retinopathy (DR) is a progressive disorder of the retinal vasculature than can lead to severe vision loss.1–3 Early detection and intervention of retinal vascular dysregulation are a key strategy in mitigating irreversible neuronal injury in diabetes.4 The retinal microaneurysm is defined as the earliest clinical manifestation of DR5; however, histologic studies have shown that irreversible endothelia injury and pericyte loss have already occurred prior to the formation of microaneurysms.1,6 The pathophysiologic mechanisms underlying the earliest stages of DR before the occurrence of permanent cellular injury such as those seen in microaneurysms are less clear. Several proposed mechanisms of microaneurysm formation include vascular stasis,7 fat emboli,8 varicose formation,9 and pericyte loss leading to structural weakness.10 A greater understanding of these mechanisms is important, as it will serve to prevent vision loss in diabetes by facilitating earlier intervention.

Oxygen consumption in the retina, per gram tissue, is greater than that in most other organs in the human body.11 Blood flow to various vascular layers of the macula are constantly fluctuating in order to maintain the dynamic balance between energy supply and demand.12–14 Blood flow is regulated by key elements of the vascular apparatus, including pericytes, endothelia, and smooth muscle cells.15–18 Alpha smooth muscle actin (αSMA) is a filamentous contractile protein that is ubiquitously distributed throughout the vascular system and contributes to the control of vascular diameter, vascular tone, and retinal blood flow.15,19,20 In the brain, disease-induced alterations in the contractile elements of the vascular tree are inherently linked to the pathogenesis of major neurological diseases.21 Such changes can alter regional perfusion patterns and initiate or propagate neuronal injury. There is clinical and experimental evidence to implicate retinal perfusion abnormalities in patients with diabetes.22–24 These observations may
be explained by significant alterations to the contractile elements of the retinal vascular tree that control perfusion.

The organization of the macular circulation is highly specialized.\textsuperscript{,\textsuperscript{25,\textsuperscript{26}}} In a recent study, we showed that there is great variation in the distribution of \(\alpha\text{SMA}\) contractile proteins between parafoveal capillary beds, suggesting that the characteristics of physiologic perfusion and vulnerability to vascular injury may vary between retinal layers.\textsuperscript{25} The purpose of this report is to define disease stage-specific changes to contractile proteins, arterial inflow pathways, and venous outflow pathways within the parafovea in eyes of donors with diabetes. We performed a detailed histologic study that included eyes from donors without diabetes, donors with diabetes but without clinical manifestations of DR,\textsuperscript{3} and donors with DR. The results were used to hypothesize the pattern of microregional blood flow changes due to diabetes and their chronologic relationship to the onset of structural clinical changes such as capillary loss and microaneurysms.\textsuperscript{27} This report expands our understanding of the pathophysiology of DR and provides novel insights into how macula perfusion with respect to vascular topology might be disturbed in the earliest stages of DR.

**Materials and Methods**

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.

**Donor Eyes and Assignment of Groups**

The donor cohort was stratified into three groups: control group, diabetes without retinopathy group (DR–), and diabetes with retinopathy group (DR+). The control group was comprised of eyes with no history of retinal disease and no history of diabetes mellitus. The DR– and DR+ groups were comprised of eyes from donors that upon medical record review were confirmed to have a diagnosis of diabetes mellitus and were treated with an oral antiglycemic agent and/or insulin. On microscopic examination, eyes that demonstrated any histologic vascular alterations that characterize DR, including microaneurysms, capillary non-perfusion,\textsuperscript{27} retinal hemorrhage, and intraretinal microvascular abnormalities, were assigned to the DR+ group (Supplementary Figs. S1, S2).\textsuperscript{7,8} Eyes from donors with diabetes mellitus that did not demonstrate any features of DR were assigned to the DR– group. Additionally, 15 donor eyes were not studied due to postmortem tissue changes. These were excluded from the study. Postmortem tissue changes include significant loss of retinal integrity resulting in tears and breakdown and any significant clots detected within the vasculature resulting in absent or reduced perfusion downstream that is not part of DR pathology (Supplementary Fig. S3).

**Donor Retina Preparation**

Human donor eyes used in this report were obtained from DonateLife WA, the organ and tissue retrieval authority in Western Australia, Australia, and the Lions Eye Bank. Eyes were enucleated within 24 hours of death and prepared using our previously described techniques.\textsuperscript{25,\textsuperscript{28,\textsuperscript{29}}} No primary antibody control was utilized to test non-specific binding of secondary antibodies. No secondary antibody control was utilized to detect autofluorescence among the protein targets of interest. In brief, enucleated eyes were transported in oxygenated Ringer’s lactate solution and were then placed in a custom-built eye holder. The central retinal artery was cannulated for perfusion labeling using a glass micropipette (100-μm tip diameter) and perfused with the following solutions in sequence at a rate of 60 μL/min unless otherwise stated: 1% bovine serum albumin in Ringer’s solution for 20 minutes to wash out blood clots; 4% paraformaldehyde in 0.1-M phosphate buffer (PB) for 20 minutes; and 0.1-M PB for 20 minutes. Next, eyes were perfused using one or more of the following protocols (Table 1):

- **Lectin—**Lectin–fluorescein isothiocyanate (FITC; 40 μg, L4895; Sigma-Aldrich, St. Louis, MO, USA) and Hoechst stain (2.5 μL; H6024; Sigma-Aldrich) in 400 μL PB were perfused over a period of 30 seconds, stained for 12 minutes, and washed out using PB over 20 minutes. Lectin binds to the glycoproteins of endothelial cell membrane.\textsuperscript{30}
- **Filamentous actin (F-actin)/phalloidin—**Triton X-100 (0.1%; Sigma-Aldrich) in 0.1-M PB was perfused for 5 minutes, followed by 1 μg phalloidin tetramethylrhodamine B isothiocyanate (phalloidin–TRITC, P1951; Sigma-Aldrich) and Hoechst stain (2.5 μL in 1 mL PB) delivered over three 30-second perfusions 20 minutes apart, and then washed out using PB over 20 minutes. F-actin is found in cellular cytoskeleton and is most abundant in vascular smooth muscle cells.\textsuperscript{20}
- **Collagen IV—**Triton X-100 (0.1%) in 0.1-M PB was perfused for 5 minutes, followed by 10 μg rabbit anti-collagen IV antibodies (SAB4300738; Sigma-Aldrich), 10 μL 100% goat serum, and Hoechst stain (2.5 μL in 1 mL PB) delivered over three 30-second perfusions, each 20 minutes apart, and then washed out with PB perfusion over 20 minutes after a total of 1 hour of labeling time. Next, 20 μL of goat anti-rabbit (Alexa Fluor 488, ab150077; Abcam, Cambridge, UK) in 1 mL PB was delivered via three 30-second perfusions, each 20 minutes apart, followed by washout using PB after 1 hour. Collagen IV is a structural protein of basement membrane that is present throughout the vasculature.\textsuperscript{31} This procedure allows for clear visualization of vascular structures in diabetic retina without obscuration from label leakage that is sometimes seen in lectin and phalloidin labeling. Due to collagen IV having autofluorescence properties within the ultraviolet and blue spectrum, negative control was not performed, and secondary antibodies targeting this spectrum were avoided.
- **αSMA—**Triton X-100 (0.1%) in 0.1-M PB was perfused for 5 minutes followed by 20 μL mouse anti-αSMA antibodies (A2547; Sigma-Aldrich) and 10 μL 100% donkey serum in 1 mL PB delivered via three 30-second perfusions, each 20 minutes apart, then washed out using PB after a total of 1 hour of labeling time. Next, 20 μL of donkey anti-mouse (Alexa Fluor 647, ab150111; Abcam) in 1 mL PB was delivered via three 30-second perfusions, each 20 minutes apart, and washed out using PB after 1 hour of labeling time. αSMA is most abundant in smooth muscles cells and endothelium of small arteries and arterioles within the retinal microvasculature.\textsuperscript{20} It has also been
Table 1. Donor Demographic Information and Staining Protocols

| Eye | Group | Age (y) | Sex | Cause of Death | αSMA | Collagen IV | Lectin | F-Actin |
|-----|-------|---------|-----|----------------|------|-------------|--------|--------|
| 1   | Control | 18      | M   | Drug overdose  | ✓    | ✓           |        | ✓      |
| 2   | Control | 86      | F   | CVA           | ✓    | ✓           | ✓      | ✓      |
| 3   | Control | 86      | F   | Septic arthritis | ✓    | ✓           | ✓      | ✓      |
| 4   | Control | 46      | M   | Angiosarcoma  | ✓    | ✓           | ✓      | ✓      |
| 5   | Control | 46      | M   | Angiosarcoma  | ✓    | ✓           | ✓      | ✓      |
| 6   | Control | 72      | F   | Endometrial Ca | ✓    | ✓           | ✓      | ✓      |
| 7   | Control | 72      | F   | Endometrial Ca | ✓    | ✓           | ✓      | ✓      |
| 8   | DR–    | 66      | M   | CVA           | ✓    | ✓           |        | ✓      |
| 9   | DR–    | 55      | F   | Renal cell Ca | ✓    | ✓           | ✓      | ✓      |
| 10  | DR–    | 74      | M   | Trauma        | ✓    | ✓           | ✓      | ✓      |
| 11  | DR–    | 73      | M   | AMI           | ✓    | ✓           | ✓      | ✓      |
| 12  | DR–    | 58      | F   | AMI           | ✓    | ✓           | ✓      | ✓      |
| 13  | DR–    | 60      | F   | Endometrial Ca | ✓    | ✓           |        | ✓      |
| 14  | DR–    | 54      | M   | Pituitary adenoma | ✓    | ✓           |        | ✓      |
| 15  | DR+    | 50      | M   | Renal cell Ca | ✓    | ✓           | ✓      | ✓      |
| 16  | DR+    | 50      | M   | Renal cell Ca | ✓    | ✓           | ✓      | ✓      |
| 17  | DR+    | 70      | M   | Cardiomyopathy| ✓    | ✓           | ✓      | ✓      |
| 18  | DR+    | 70      | M   | Cardiomyopathy| ✓    | ✓           | ✓      | ✓      |
| 19  | DR+    | 59      | M   | AMI           | ✓    | ✓           | ✓      | ✓      |
| 20  | DR+    | 58      | M   | Acute pancreatitis | ✓    | ✓           | ✓      | ✓      |
| 21  | DR+    | 82      | M   | AMI           | ✓    | ✓           | ✓      | ✓      |
| 22  | DR+    | 82      | M   | AMI           | ✓    | ✓           | ✓      | ✓      |
| 23  | DR+    | 66      | M   | AMI           | ✓    | ✓           | ✓      | ✓      |
| 24  | DR+    | 70      | M   | DM complications | ✓    | ✓           | ✓      | ✓      |
| 25  | DR+    | 70      | M   | DM complications | ✓    | ✓           | ✓      | ✓      |
| 26  | DR+    | 45      | F   | DM complications | ✓    | ✓           | ✓      | ✓      |

CVA, cerebral vascular accident; Ca, carcinoma; AMI, acute myocardial infarction; DM, Diabetes mellitus. Eyes 2/3, 5/6, 7/8, 16/17, 18/19, 21/22, 23/24, and 26/27 were pairs of donor eyes belonging to the same donor.

Confocal Scanning Laser Microscopy

A confocal scanning laser microscope (Eclipse 90i and C1; Nikon Corporation, Tokyo, Japan) equipped with four solid-state lasers at wavelengths of 405, 488, 561, and 635 nm was used to scan flatmounted retina samples. For each retina sample, a whole-retina montage (NIS Elements; Nikon Corporation) was created using low-magnification images acquired with a Nikon 4× oil NA 1.3× objective lens with a field of view of 0.31×0.31 mm. Image stacks with optical sections 0.5 μm apart were acquired to visualize endothelial cells, pericytes, and precise locations of αSMA staining. Immunofluorescence labeling using Hoechst (405 nm), lectin–FITC (488 nm), phalloidin–TRITC (561 nm), and Alexa Fluor donkey anti-mouse (635 nm) were visualized via argon laser excitation with emissions detected through 450-, 561-, 605-, and 668-nm bandpass filters, respectively.

Image Analysis

Confocal image files were processed with IMARIS v7.4.2 (Bitplane, Zurich, Switzerland) and/or ImageJ (National Institutes of Health, Bethesda, MD, USA). Figures were compiled with Illustrator CC 25.4.1 (Adobe, San Jose, CA).

Stratification of Macula Microvasculature Plexuses. The macula circulation was stratified into the superficial vascular plexus (SVP), which is between the inner limiting membrane and the outer nuclear layer; the intermediate capillary plexus (ICP), which is located in the inner half of the inner nuclear layer; and the deep capillary plexus (DCP), which is located between the outer half of the inner nuclear layer and the...
FIGURE 1. Comparison of αSMA staining distribution between an artery (a) and a vein (v) across the control group (A), DR– group (B), and DR+ group (C). Strong αSMA staining of the artery, arterioles, and capillaries adjacent to the artery was observed for all three groups. In the control group, αSMA staining was largely devoid from venules but demonstrated strong staining at venular junction sites and outer plexiform layer (OPL).55,26 Co-localization to nuclear label was used to stratify each plexus. Two-dimensional (2D) images were generated by projecting all confocal slices that comprised a single capillary plexus (using the 10× lens) and were used to attain quantitative measurements.

**Grading of αSMA-Labeled Images.** SVP, ICP, and DCP images from all donors were randomized (JC) and presented to two masked graders (DA, CB). The graders were instructed to grade each image as αSMA-label “positive” or “negative.” The result was used as a reliability index for qualitative analysis of αSMA staining. Qualitative analyses were performed for patterns of αSMA staining distribution along sections of retinal microvasculature. Quantitative image intensity measurements were not performed due to the following two factors: (1) retinas of different natural thickness result in variable depth of each plexus, thus requiring variable laser power for capture; and (2) the degree of diabetic retinal edema alters the vascular plexus depth and laser power required for capture. This variation occurs within retinal eccentricities and between individuals. The most representative images of αSMA distribution along retinal microvasculature were selected to study qualitatively.

**Capillary Density Measurements.** Images (1.27 × 1.27 mm) acquired from the superior, inferior, nasal, and temporal regions of the perifovea were used to quantify capillary densities of SVP, ICP, and DCP of each group. Using our previously published methods,26,36–38 a 3 × 3 grid was drawn on each of the 2D images, and the number of capillaries intersected by the grids were manually counted to represent the relative capillary density in this field (Supplementary Fig. S5B).

**Vessel Order Definitions.** The current study utilized the Horton–Strahler nomenclature (Supplementary Fig. S6) to assign vessel orders, which was utilized in our previous report.25 In brief, the system starts at the capillary level and proceeds centripetally. The order is increased if two segments of equal order join at a junction. Capillary is designated as c, pre-capillary arteriole as a1, and venule formed by adjoining capillaries as v1. In our previous report, we concluded, based on vessel tracing, that the retinal artery that branches from the artery of the arcade and courses toward the fovea is order a4.25 Arterioles that branch from a4 to supply the retinal plexuses are order a3. Large venules that drain a plexus into the SVP are v3, and the retinal veins located within the SVP are v4.25

**Arterial Inflow Analysis.** Arterial inflow pathways were analyzed based on our previously published methods.25 Criteria for differentiating retinal artery, arterioles, capillaries, venules, and veins were reported in detail within our previously published report.25 In brief, the IMARIS Surpass function was used to visualize the retinal vasculature at different angles of rotation to define inflow and outflow (green arrows). In the DR– group, additional αSMA staining was observed at venular junctions (green arrows) and in some short capillary segments (orange arrows). In the DR+ group, significantly more αSMA staining was found on the venous aspect of the vasculature. Some capillaries had more αSMA staining across an entire segment (orange arrows). Significantly more αSMA staining was seen along the distal portion of the vein and all of its tributaries (green arrow). A microaneurysm was seen adjacent to an area of capillary non-perfusion (white asterisk). αSMA, F-actin, and nucleus were false-colored yellow, red, and blue, respectively. All images are to the same scale. Scale bars: 150 μm.
**Figure 2.** Comparisons of the superficial vascular plexus among the control group (A, B), DR– group (C, D), and DR+ group (E, F). All images were acquired from within the macula region, superior to the fovea. The *left panel* contains images with collagen IV staining, and the *right panel* contains the same images with αSMA staining. Consistent staining patterns were observed for collagen IV across the three groups, where all orders of vessels demonstrated similar staining intensities. The $a_4$ and $v_4$ vessels are indicated on the collagen IV panel. For αSMA staining, retinal veins from both the DR– and DR+ groups demonstrated stronger staining compared to the control group. Arteries are indicated by red arrows and veins by green arrows in both panels. All images are to the same scale. Scale bar: 300 μm.
In the DR− group, more junctions where two or more venules converged (Fig. 1A). An exception was that veins and venules showed strong αSMA staining at junctions where two or more venules converged (Fig. 1A). In the DR− group, more αSMA staining was found on the venous aspect of the vasculature. Compared to the control group, in the DR− group there was also more αSMA staining outside of venular junctional zones (Fig. 1B). In the DR+ group, significant numbers of venules and capillaries were found to have more αSMA staining adjacent to retinal veins (Fig. 1C).

### Statistical Analysis

Data were analyzed using SigmaPlot 12.0 (SYSTAT Software, Chicago, IL) and R (R Foundation for Statistical Computing, Vienna, Austria). One-way ANOVA was used to compare age among the groups. Two-way ANOVA was used to compare capillary densities within and between each group and each vascular plexus. For inflow and outflow branching pathway analysis, the proportions of each branching pathway were compared among groups using one-way ANOVA. $P \leq 0.050$ was considered significant. Results are presented as mean ± standard deviation.

### Results

#### General

Twenty-eight eyes from 20 donors were used in this study. After microscopic examination, eight eyes from five donors were assigned to the control group, seven eyes from seven donors were assigned to the DR− group, and 13 eyes from eight donors were assigned to the DR+ group. Detailed donor demographic information and immunohistochemical labeling protocols can be found in Table 1. Mean ages of the control group (57.0 ± 26.2 years), DR− group (62.9 ± 8.3 years), and DR+ group (62.5 ± 12.0 years) were similar ($P = 0.979$).

#### Heterogeneous αSMA Staining Between Retinal Artery and Vein

Representative full-projection images of parafoveal vasculature in each group are provided in Figure 1. In the control group, vessels on the arterial aspect demonstrated strong αSMA staining, and vessels on the venous aspect demonstrated weaker or no αSMA staining (Fig. 1A). An exception was that veins and venules showed strong αSMA staining at junctions where two or more venules converged (Fig. 1A).

### Table 2. Analysis of Capillary Density Count Using ANOVA

| Capillary Density | Control | DR− | DR+ |
|-------------------|---------|-----|-----|
| SVP               | 40.4 ± 10.8 | 46.6 ± 13.1 | 47.1 ± 12.0 |
| ICP               | 46.2 ± 11.4 | 46.8 ± 13.5 | 48.1 ± 13.2 |
| DCP               | 36.9 ± 8.2  | 41.1 ± 17.6 | 20.4 ± 18.6 |

|          | Control vs. DR− | Control vs. DR+ | DR− vs. DR+ |
|----------|-----------------|-----------------|-------------|
| $P$      | 0.350           | 0.293           | 0.898       |
|          | 0.968           | 0.927           | 0.882       |
|          | 0.309           | $<0.001$        | $<0.001$    |

#### Staining Patterns of Retinal Vascular Plexuses

##### Superficial Vascular Plexus

All orders of retinal vasculature demonstrated complete labeling of collagen IV across the control, DR−, and DR+ groups (Figs. 2A, 2C, 2E). Arteries, arterioles, capillaries, venules, and veins demonstrated similar levels of collagen IV stain intensity. Arteries and arterioles demonstrated strong αSMA staining across the groups (Figs. 2B, 2D, 2F). There was more αSMA staining in both the DR− and DR+ groups in veins and capillaries compared to the control group (Figs. 2B, 2D, 2F). Capillary density for the SVP was similar across all three groups (all $P > 0.050$) (Table 2).

##### Intermediate Capillary Plexus

Collagen IV staining demonstrated a similar intensity of staining for all groups (Figs. 3A, 3C, 3E). Microaneurysms in the DR+ group appeared brightly hyperfluorescent with collagen IV stain (Fig. 3E). Compared to the control group, αSMA staining was stronger in the DR− group (Figs. 3B, 3D). In DR+ group, there was evidence of significantly more αSMA staining in some capillary segments (Fig. 3F). Capillary density for the ICP was similar across all three groups (all $P > 0.050$) (Table 2).

##### Deep Capillary Plexus

Collagen IV stain demonstrated complete DCP labeling for both the control and DR−groups (Figs. 4A, 4C). Microaneurysms and sites of capillary nonperfusion were clearly identified in the DR+ group (Fig. 4E). There was minimal αSMA staining within the DCP of the control group (Fig. 4B). The DR− group demonstrated segmental hyperfluorescence of αSMA staining along the vascular course (Fig. 4D). The DR+ group demonstrated significantly more αSMA staining compared to both groups, with many long segments of capillaries displaying hyperfluorescence (Fig. 4F). DCP capillary density of the DR+ group was significantly less compared to both the control and DR−groups (both $P < 0.001$).

##### Endothelial Cells and Pericytes of the Deep Capillary Plexus

All orders of DCP vasculature, including $a1T$, capillaries, and venules, stained positively for F-actin in the DCP across the three groups (Figs. 4A, 4C). Microaneurysms and sites of capillary nonperfusion were clearly identified in the DR+ group (Fig. 4F). There was minimal αSMA staining within the DCP of the control group (Fig. 4B). The DR− group demonstrated segmental hyperfluorescence of αSMA staining along the vascular course (Fig. 4D). The DR+ group demonstrated significantly more αSMA staining compared to both groups, with many long segments of capillaries displaying hyperfluorescence (Fig. 4F). DCP capillary density of the DR+ group was significantly less compared to both the control and DR−groups (both $P < 0.001$).
FIGURE 3. Comparisons of the intermediate capillary plexus among the control group (A, B), DR– group (C, D), and DR+ group (E, F). The left panel contains images with collagen IV staining, and the right panel contains the same images with αSMA staining. Collagen IV staining patterns were similar between the control and DR– groups. In the DR+ group, a microaneurysm can be seen (red arrow). There was more αSMA staining in the DR– and DR+ groups compared to the control group. Many capillaries of the DR+ group appeared hyperfluorescent (green arrows). All images are to the same scale. Scale bar: 300 μm.
FIGURE 4. Comparisons of the deep capillary plexus among the control group (A, B), DR– group (C, D), and DR+ group (E, F). The left panel contains images with collagen IV staining, and the right panel contains the same images with αSMA staining. Collagen IV staining patterns were similar between the control and DR– groups. The DR+ group demonstrated several capillary non-perfusion sites (yellow asterisks) and microaneurysms (red arrows). Levels of αSMA staining across the three groups differed, as minimal αSMA staining was seen in the control group (B), but more αSMA staining was found in the DR– group. There was evidence of focal hyperfluorescence of capillary segments (D; green arrows). The DR+ group showed significantly more αSMA staining, with many capillary segments showing hyperfluorescence (F; green arrows). The microaneurysm seen in the DR+ panel is highlighted in an inset. All images are to the same scale. Scale bar: 300 μm.
FIGURE 5. Illustration of αSMA staining patterns of the DCP in the control group using three-dimensional (3D) high-resolution microscopy. (A) The 3D stack shows the connections between the ICP and the DCP via pre-capillary arterioles designated as α1. (B) The projected view of all slices illustrates the sites of αSMA termination as α1 enters the DCP; pericytes that expressed αSMA can be seen along the α1 (green arrow). (C) The spatial relationship between an endothelial cell and the pericyte along α1. (D) αSMA expression by the pericyte. (E, F) Endothelial cells of the more distal segment of DCP capillary did not express αSMA (magenta arrows). The pericyte also did not express αSMA (green arrows). αSMA, F-actin, and nucleus were false-colored yellow, red, and blue, respectively. Scale bars: 30 μm.
**Figure 6.** αSMA staining patterns of the DCP in the DR– group. (A, B) αSMA staining can be seen within a1 and the proximal segment of capillary (c) downstream of a1. (C) The transition sites of αSMA expression/non-expression within DCP capillaries are indicated by blue arrows; panel C is an inset of A and B. (D) A capillary segment of interest is marked by a green asterisk. A pericyte that expressed αSMA is indicated by a green arrow. (E) Magnified view of this pericyte, as well as an αSMA-expressing endothelial cell (magenta arrow indicates an elongated cell nucleus). Beyond this endothelial cell, the capillary no longer expressed αSMA. αSMA, F-actin, and nucleus were false-colored yellow, red, and blue, respectively. Scale bars: 100 μm.

In this region expressed αSMA (Figs. 6D, 6E). In the DR+ group, additional αSMA staining was found in the DCP both downstream of a1 and at venular junctions adjacent to v3 (Fig. 7). Some distal capillary segments of the DCP away

**Figure 7.** αSMA staining patterns of the DCP in the DR+ group. (A) The points of inflow (a1) and outflow (v3) are marked on a DCP section labeled with F-actin. (B) There was significantly increased αSMA staining in the DCP. (C) Sites of increased αSMA staining included capillary segments adjacent to a1 (magenta arrows) and v3 (green arrows). αSMA, F-actin, and nucleus were false-colored yellow, red, and blue, respectively. Scale bar: 150 μm.
FIGURE 8. Illustration of αSMA staining pattern of the DCP in the DR+ group. (A) A 3D stack was rotated and visualized through the x,z-axis to highlight the connection between the ICP and DCP via the a1 arteriole. (B) Segments of both the ICP and DCP capillaries (Cap); the 3D stack was also viewed through the x,y-axis. The three insets highlight (C) a a1 bifurcation site, (D) an ICP capillary, and (E) a distal segment of DCP capillary. All three capillary segments were found to have αSMA-expressing pericytes (green arrows) and endothelial cells (magenta arrows). In addition, the capillary at the a1 bifurcation was found to have an enlarged diameter (11.5 μm; red asterisk). αSMA, F-actin, and nucleus were false-colored yellow, red, and blue, respectively. Scale bars: 30 μm.

Vascular Branching Analysis

Inflow Pathway Analysis. In the control group, 150 a3 arterioles were identified; 167 were identified from the DR− group and 172 from the DR+ group. In the control group, 79.3% of arterioles first supplied the SVP. Numerous connecting vessels were found between the SVP and ICP; 20.7% of arterioles directly supplied the ICP, bypassing the SVP. There was no evidence of direct arteriolar supply to the DCP from an a4 artery. Arteriolar supply to the DCP was via a1 vessels originating from the ICP. In the DR− group, 52.7% of arterioles supplied the SVP and 47.3% supplied directly to the ICP. In the DR+ group, 51.7% of arterioles supplied the SVP and 48.3% supplied directly to the ICP. Similar to the controls, both DR− and DR+ groups had no
Table 3. Summary of Inflow and Outflow Pathway Analyses

|                      | Inflow Pathway Analysis, α3 Arteriole | Outflow Pathway Analysis, v3 Venule |
|----------------------|--------------------------------------|-----------------------------------|
|                      | N                     | SVP–ICP–DCP, % (n) | ICP–DCP, % (n) | N                     | SVP, % (n) | ICP, % (n) | DCP, % (n) |
| Control              | 150                   | 79.3 (119)          | 20.7 (31)     | 518                   | 42.2 (219) | 34.9 (181) | 22.7 (118) |
| DR–                  | 167                   | 52.7 (88)           | 47.3 (79)     | 865                   | 42.4 (367) | 35.1 (304) | 22.4 (194) |
| DR+                  | 172                   | 51.7 (89)           | 48.3 (83)     | 1004                  | 41.3 (415) | 36.3 (365) | 22.3 (224) |

Sample sizes of α3 arterioles and v3 venules are indicated in parentheses. Inflow pathway indicates the course that the α3 arteriole took to reach the deep plexus. Outflow pathway indicates the plexus of origin of the v3 venule.

direct arteriolar supply to the DCP from the α4 artery. Both groups had significantly lower SVP arteriole proportions and higher ICP arteriole proportions compared to the control group (both P < 0.001) (Table 3).

Outflow Pathway Analysis. In the control group, 518 v3 venules were identified; 42.2% of these originated from the SVP, 34.9% from the ICP, and 22.7% from the DCP. In the DR– group, 865 v3 venules were identified; 42.4% of these originated from the SVP, 35.1% from the ICP, and 22.4% from the DCP. In the DR+ group, 1004 v3 venules were identified; 41.3% of these originated from the SVP, 36.3% from the ICP, and 22.3% from the DCP. There was no difference in venular outflow pathway proportions for the SVP (P = 0.638), ICP (P = 0.284), or DCP (P = 0.818) across the three groups (Table 3).

DISCUSSION

The purpose of this study was to define stage-specific changes to vascular αSMA expression and macula blood flow pathways in DR. As it is difficult to perform in vivo imaging of human retinal capillary blood flow, we used three-dimensional quantitative structural data of the vascular network to infer knowledge regarding macula perfusion alterations in the different stages of DR. Our findings suggest that micro-regional blood flow changes precede clinically detectable retinal structural alterations in DR57,40; therefore, detection of such changes could serve as a surrogate marker of early vascular dysregulation in diabetes.

The energy required to support neuronal and glial function is immense but also highly variable between retinal layers.14,41,42 Accordingly, the morphology and spatial organization of the macula circulation demonstrates regional specializations that reflect the unique energy requirements of each retinal layer.55,43 As the extent of neural activity is rapidly and constantly fluctuating, vascular specializations are also required to facilitate precise temporal modulation of retinal blood flow.20,43 αSMA is a filamentous protein that confers contractile properties to cells and thereby regulates regional blood flow by controlling vascular diameter and resistance.19,34,41 In our previous reports we defined the topographic distribution of αSMA in the healthy human macula and demonstrated non-uniform distribution among the superficial, intermediate, and deep capillary plexuses.20,25 One of the most interesting findings was the relative paucity of αSMA in the capillaries of the deep plexus and the abrupt termination of αSMA staining in α1 arterioles that connect the ICP to the DCP.55 This particular pattern of αSMA distribution has also been described in cortical networks of the brain where αSMA termini are commonly seen in second-order arterioles and represent the boundary between ensheathing and mesh pericytes.45 Because mesh pericytes of the brain have a limited capacity to regulate blood flow, it is proposed that they have a significantly lower expression of αSMA.

In this report, we have demonstrated that the pattern of αSMA expression is altered in the earliest stages of DR before the onset of clinically visible structural changes such as microaneurysms. Specifically, there was more αSMA expression in the capillaries of the ICP and DCP in the DR– group compared to controls. A stage-specific increase in αSMA expression continued to occur in the ICP and DCP following the onset of clinical DR, such as the loss of capillaries and appearance of microaneurysms. There is some overlap in the physiologic properties of retinal and cortical vascular networks. In the brain, one of the most reliable means of determining if vessels less than 10 μm in diameter have contractile properties is to assess the magnitude of αSMA expression.46,47 Another important property of human vascular networks is that they can acquire contractile properties de novo in pathologic states by increasing αSMA expression.48 As an example, following experimental cardiac ischemia, pericytes near blockage sites can adapt contractile characteristics by increasing αSMA expression.48 Similarly, in the brain following ischemic stroke, persistent pericyte-mediated contraction marked by elevated αSMA expression has been found to inhibit capillary re-perfusion.21 In this study, we found that higher expression of αSMA was localized to both pericytes and endothelia, providing evidence that perfusion abnormalities in DR may be regulated by numerous elements of the vascular system and not just pericytes. Alterations in αSMA expression may influence vascular resistance49 and hemorheologic properties such as red blood cell deformability and adherence.55 Such changes may in turn alter regional oxygen handling and exchange by RBCs.

Retinal blood flow is dynamic, and regional perfusion is coordinated by numerous checkpoints within the vascular tree.45 Kornfield and Newman17 used flicker stimulation to study blood flow regulation in the rodent retinal circulation with several important findings. First, they found that functional hyperemia in the retina is driven principally by the active dilation of arterioles. They also demonstrated differential regulation of blood flow between the capillary plexuses. Finally, they demonstrated a correlation between the amount of αSMA expression and the degree of vascular contractility. The marked alterations in αSMA staining demonstrated in this study suggest that the control points for blood flow may be modified in DR. Although αSMA expression was higher in the DCP, closer observation revealed that the pattern of additional αSMA staining was not diffuse but rather localized to capillaries in close proximity to α1 and v3 segments. This study was not designed to determine the etiology of the localized αSMA changes in the DCP, but we speculate that it may represent critical sites where endothelial wall shear stress patterns are altered due to vascular bifurcation or the presence of hemodynamic gradients.50 Functional
FIGURE 9. Schematic representation of αSMA distribution and changes in the development of diabetic retinopathy. Insets provide magnified views of regions of interest. (A) In the control group, αSMA (yellow dots) was predominantly localized within arteries, arterioles, and capillaries on the arterial aspect of the circulation (red). For the venous aspect (blue), αSMA was localized to major venular junctions only. Within the connecting arterioles (a1) between the ICP and DCP, αSMA expression was found to terminate abruptly prior to reaching the DCP (inset I; black arrow). The locations of each vascular plexus are indicated in the retinal layers panel. (B) The DR– group showed additional αSMA expression along veins, venules, and capillaries on the venous aspect of the circulation. Compared to the control group, there was
studies are required to determine if αSMA changes represent a compensatory mechanism that increases oxygenation to regions of increased metabolic demand in the DCP or if they serve to propagate neuronal injury. The "no-reflow" mechanism has been observed following cardiac, 48,52,53 and cerebral ischemia, 53,54 and it describes a phenomenon whereby abnormal and sustained capillary constriction due to pericytes overexpressing αSMA limits blood flow and propagates neuronal damage to an already ischemic region. A disease stage-specific increase in αSMA in the DCP suggests that a similar mechanism may be occurring in DR and may explain why the earliest ischemic changes in DR are seen within the DCP. 55–57 

The occurrence of a significantly greater number of inflow pathways that directly connect retinal arteries to the ICP in preclinical DR and clinical DR eyes implicates the establishment of a "steal" effect in diabetes where blood flow is preferentially diverted from the SVP to the ICP and DCP. Kornfield and Newman 17 demonstrated a similar physiologic "steal" effect in rat eyes using flicker stimulation that induced significantly greater dilation of intermediate layer capillaries than in capillaries of the superficial and deep vascular layers. The ICP supplies the neurons and synapses of the superficial portion of the inner nuclear layer and the deep portion of the inner plexiform layer, respectively. 25,26 The preferential diversion of blood flow to the ICP may reflect a disproportionate increase in energy demands in these retinal compartments in diabetes. 58 We did not find a significant difference in the frequency of venous draining pathways between the control and DR groups, although there was a stage-specific increase in the degree of αSMA staining within elements of the venous circulation in DR. In our previous work, we showed that endothelia of the retinal veins have contractile properties, and retinal vein tone can be modulated by vasoactive factors such as endothelin-1 and adenosine. 59 The relevance of increased expression of αSMA in the retinal venous circulation in diabetes is twofold: (1) it implies that the venous circulation may play a critical role in modulating retinal blood flow changes in diabetes as a compensatory or pathologic mechanism; and (2) systemic or ocular therapies that target the vasoactive properties of the retinal circulation may be useful for reversing retinal vascular damage in diabetes. 

In 1993, Cringle and colleagues 60 published a seminal report comparing retinal blood flow by hydrogen clearance polarography between streptozotocin-induced diabetic rats and controls. They demonstrated a 47% increase in mean retinal blood flow, as well as greater heterogeneity in retinal blood flow, characteristics after 5 to 6 weeks of experimentally induced diabetes. Retinal blood flow changes were observed prior to the occurrence of pathologic retinal vascular abnormalities. The current report provides a histologic basis for understanding these perfusion abnormalities and suggests that alterations in αSMA expression and arterial inflow pathways within the vascular tree may underlie retinal blood flow changes in diabetes. Recently we described a technique whereby analysis of successive algorithm-aligned optical coherence tomography angiography (OCTA) frames can be used to derive a measure of spatial and temporal variations in human retinal perfusion. 43 With the increasing availability of OCTA technology it may be possible to harness such techniques to facilitate rapid and non-invasive detection of the earliest stages of DR before the onset of structural changes such as pericyte loss and the formation of microaneurysms. 7,61,62 

Strengths of this study include the large sample size (total of 28 human donor eyes) and the precise quantitative evaluation of vascular parameters using high-resolution confocal scanning laser microscopy. Precise labeling of the retinal vasculature was also achieved using our well-established, perfusion-based antibody-labeling techniques that obviate many of the shortfalls associated with immersion tissue labeling and section labeling. 53 Collectively, these techniques permitted us to propose the sequences and interrelationships among αSMA expression, blood flow pathways, and capillary density alterations in the development of DR as summarized in Figure 9. We acknowledge that this study was limited by the investigation of only one vascular contractile protein—αSMA, the distribution of which was analyzed in a largely qualitative manner. The distribution and significance of other contractile proteins such as myosin and its isoforms remain to be elucidated.

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