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# Evaluation of Nonperfused Retinal Vessels in Ischemic Retinopathy

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**PURPOSE.** Retinal ischemia has been traditionally assessed by fluorescein angiography, visualizing perfused vessels. However, this method does not provide any information about nonperfused vessels, and although it is often assumed that vessels in ischemic areas regress, we know little about how nonperfused retinal vessels change over time. Here, we aim to learn more about the long-term fate of nonperfused vessels in the retinal vasculature.

**METHODS.** Optical coherence tomography (OCT) was used to visualize perfusion as well as structural properties of the retinal vasculature in patients suffering from retinal vascular occlusions. In addition, postmortem tissue from a patient with long standing (6 years) central retinal vein occlusion (CRVO) was investigated, using immunohistochemistry on whole-mount retina and paraffin sections to visualize blood vessel components.

**RESULTS.** Comparing OCT angiography with enface OCT images revealed that in ischemic areas of the retina, nonperfused, larger vessels could be detected as hyperreflective structures in enface OCT images. Furthermore, analysis of a postmortem tissue sample from a CRVO patient with a large nonperfused region in the macula, revealed preservation of the basement membrane from all retinal vessels, including nonperfused, acellular vessels of all calibers.

**CONCLUSIONS.** Our data suggests long-term preservation of vascular basement membrane in ischemic retina. This has implications for therapeutic approaches aiming to alleviate retinal ischemia via the regeneration of damaged vessels.

**Keywords:** vascular occlusion, basement membrane, perfusion

Loss of perfusion to the retinal vasculature can lead to ischemia and consequent vision loss in several diseases, most notably in retinal vascular diseases such as retinal vein occlusion (RVO) and diabetic retinopathy. Retinal vein occlusion can cause significant ocular morbidity and is a common cause for significant visual loss among the elderly with a worldwide prevalence of 16 million.<sup>1</sup> The clinical classification of central, hemi, and branch RVO is based upon the location of vascular sequelae in the retina and the site of thrombus within the retinal vascular tree.<sup>2</sup> In central retinal vein occlusion (CRVO), this occurs in the main trunk of the central retinal vein either at, or proximal to the lamina cribrosa.<sup>2</sup> In branch retinal vein occlusion (BRVO), the blockage occurs distal to the first-order bifurcation of the retinal vein at sites of arteriovenous crossings, where the arteriole and vein share a common adventitial sheath.<sup>3,4</sup>

While the pathogenesis of RVO and its subtypes have been evidenced by several clinicopathological series, its pathophysiology is less clear.<sup>2,5</sup> Nonetheless, some insight into the impact of nonperfusion itself on downstream vascular cells has been gained by a primate study based on experimental BRVO using an argon laser to occlude vessels.<sup>6</sup> This led to rapid

degenerative changes (onset within hours) resulting in an almost complete loss of capillary endothelial cells and pericytes (within 1-3 weeks), with only empty basement membrane sleeves remaining. There was no evidence of recanalization. In contrast, a similar study (also in primates) showed recanalization of some basement membrane tubes.<sup>7</sup> Similarly, the largest histopathological study in humans to date (29 eyes), observed that recanalization can occur, but in this instance this was found only in the thrombi of the large central retinal veins.<sup>2</sup> Clinically, there is limited evidence for reperfusion of retinal vessels. Nevertheless, one clinical study has observed a reduction in areas of "retinal nonperfusion" on ultra widefield fluorescein angiograms (FA) on a small proportion (<10%) of 31 patients with BRVO receiving anti-VEGF intravitreal injections.<sup>8</sup>

Functional recovery of previous perfusion paths via the repopulation of empty basement membrane tubes with migrating endothelial cells and pericytes from neighboring, intact vessel regions, might be possible as long as the vascular basement membrane persists. However, how long and in what shape empty basement membrane tubes survive in the retina is not well understood and may depend on the specific



circumstance. For instance, Ashton described in a classic study that only very delicate, acellular strands remained after hyperoxia-induced capillary closure in kittens.<sup>9</sup> Similarly, we have shown transient, thin, collagen IV-positive strands in developing mouse retina after hyperoxia induced capillary obliteration.<sup>10</sup> In contrast, in mature retina the basement membrane is more substantial<sup>9</sup> and was found to persist up to 40 days in an ischemia/reperfusion model in cat.<sup>11</sup>

However, the natural history of nonperfused vessels and the long-term fate of empty basement membrane tubes in human pathology is poorly understood. This is largely due to the limitation of retinal imaging modalities for the visualization of nonperfused vessels. Although larger, nonperfused vessels can be seen on fundus photographs as ghost vessels (especially superficial ones), smaller or deeper nonperfused vessels have so far been invisible. The mainstay of imaging retinal vessels has been fluorescein angiography, which, by definition, only allows visualization of perfused vessels. But this has changed with the advent of optical coherence tomography (OCT)-based technology. Using enface imaging, and OCT angiography, we can now for the first time, delineate nonperfused retinal vessels *in vivo*. In this study, we demonstrate the feasibility of imaging nonperfused vessels using enface OCT and OCT angiography in patients with BRVO. We further provide clinicohistologic evidence, from a case of CRVO, for long-term basement membrane persistence in the entire retinal vasculature plexus.

## MATERIALS AND METHODS

### OCT Angiography and Enface OCT Imaging of BRVO Patients

All OCT angiography and enface OCT images were acquired and analyzed using the RTVue XR Avanti Spectral Domain OCT (Optovue, Inc., Fremont, CA, USA), using methodology described elsewhere.<sup>12</sup> The equipment was used to obtain and select good quality OCT angiograms (showing blood flow) and the corresponding enface OCT images (representing frontal sections of retinal layers) for multimodal analysis. Institutional review board (IRB) approval for the review of these patient records has been obtained at our institutions.

### CRVO Eye Tissue Donor

The medical history includes asthma, emphysema, carcinoid tumor, atrial septal defect, and two right-sided cardiac stents. Medication included warfarin, prednisone, spironolactone, digoxin, fluticasone/salmeterol, tiotropium, salbutamol, indapamide, mirtazapine, simvastatin, and lactulose. Institutional review board/ethic committee approvals for the review of this patient's records and the analysis of the postmortem eye tissue have been obtained at our institutions.

### Tissue Processing

The time elapsed between death and fixation of the eyes in this study was 3.5 hours. The eyes were fixed in 4% paraformaldehyde (PFA) for 64 hours and then kept in 1% PFA for 3 weeks. Subsequently, the anterior parts of the eyes were removed and the eyes were flattened (using four radial incisions) and photographed. A region that included the optic disc and fovea was dissected and processed for wax embedding. Because in one of our previously studied samples,<sup>13</sup> the retina split horizontally during embedding we used here a soft sponge to stabilize the tissue and apply gentle

pressure during wax processing. Once embedded, nasotemporal sections were cut at 6  $\mu$ m and mounted onto Superfrost plus slides (VWR International Ltd., Lutterworth, UK). Sections were deparaffinized with xylene and rehydrated through graded alcohols and processed for immunohistochemistry.

## Immunohistochemistry

Antigen retrieval was carried out by heating the slides to 125°C in 90% glycerol (molecular grade) and 10% 0.01M citrate buffer pH 6.0 for 20 minutes in a pressure cooker. Sections were then briefly washed in water, incubated for 1 hour at room temperature in blocking buffer (1% BSA, 0.5% triton X-100 in PBS) and then in primary antibody (diluted 1:200 in blocking buffer) either at room temperature for 1 hour or overnight at 4°C. Primary antibodies used were: rabbit anti-collagen IV (2150-0140; AbD Serotec, Kidlington, UK), goat anti-endoglin (AF1097; R&D systems, Minneapolis, MN, USA), mouse anti vimentin-Cy3 (C9080; Sigma-Aldrich, St. Louis, MO, USA), goat anti-GFAP (C9205; Sigma-Aldrich), mouse anti-alpha smooth muscle actin-Cy3 (C6198; Sigma-Aldrich). Sections were washed in washing buffer (0.1% tween20 in PBS) and incubated for 1 hour at room temperature in secondary antibodies (diluted 1:200 in blocking buffer; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Subsequently sections were washed in washing buffer, treated with Hoechst (10  $\mu$ g/ml in washing buffer) for 30 seconds, washed again and mounted in Moviol mounting medium (Sigma-Aldrich). Images were taken using a Leica DM IRB fluorescent microscope (Leica, Hicksville, NY, USA) and/or a Zeiss LSM700 confocal microscope (Zeiss, Jena, Germany).

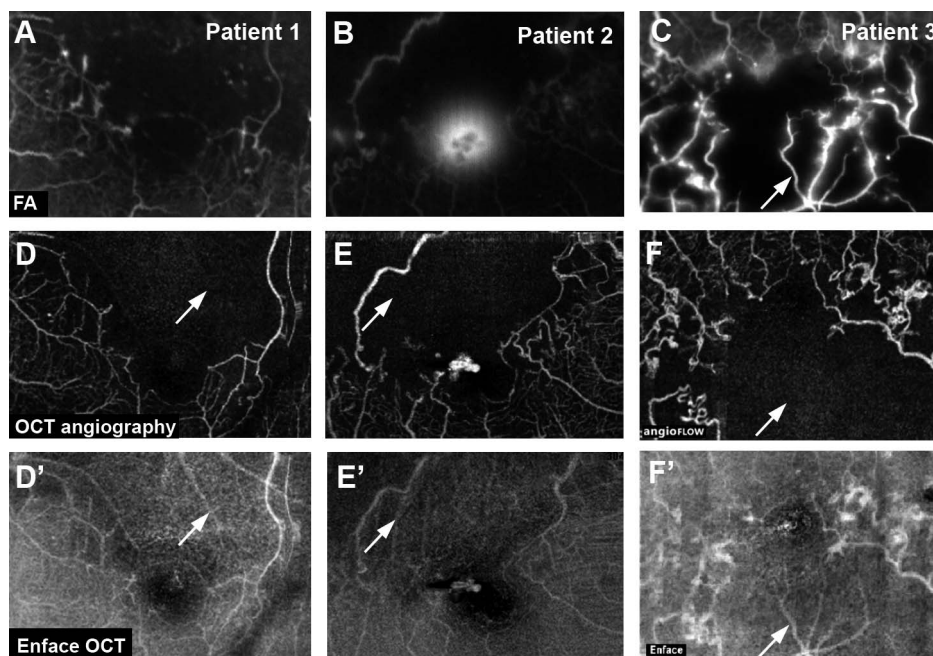
## RESULTS

### Vascular Structures in Ischemic Retina

In order to learn more about the relationship between vascular structures and vascular perfusion, we compared FA, OCT angiography, and enface OCT images from BRVO patients. Three selected chronic BRVO patients (15, 44, and 60 months since occlusion) are shown in Figure 1. In patient 1 (Figs. 1A, 1D, 1D') and in patient 2 (Figs. 1B, 1E, 1E') FAs and OCT angiography were recorded on the same day and nonperfused areas correspond well in the two imaging modalities. In patient 3 (Figs. 1C, 1F, 1F') the FA was taken 5 years earlier and shows a cluster of vessels (arrow in Fig. 1C) that is no longer perfused in the angiography OCT (Fig. 1F). In all three patients, the Enface OCT (Figs. 1D', 1E', 1F') revealed vascular structures (ghost vessels) in nonperfused areas that appeared similar to vessels in perfused areas, with regards to signaling intensity as well as network patterning (arrows in Fig. 1), suggesting vessels may maintain light scattering properties in OCT in the absence of perfusion. Notably, the ghost vessels seen in patient 3 (arrow Fig. 1F') show the same pattern as the still perfused vessels in the FA 5 years earlier (arrow Fig. 1C). We hypothesize that the OCT signal of ghost vessels originates from remaining basement membrane. To establish in more detail, the anatomical basis of this OCT signal, we carried out a histologic study on a postmortem sample from a CRVO patient.

### Clinical Features of CRVO Tissue Donor

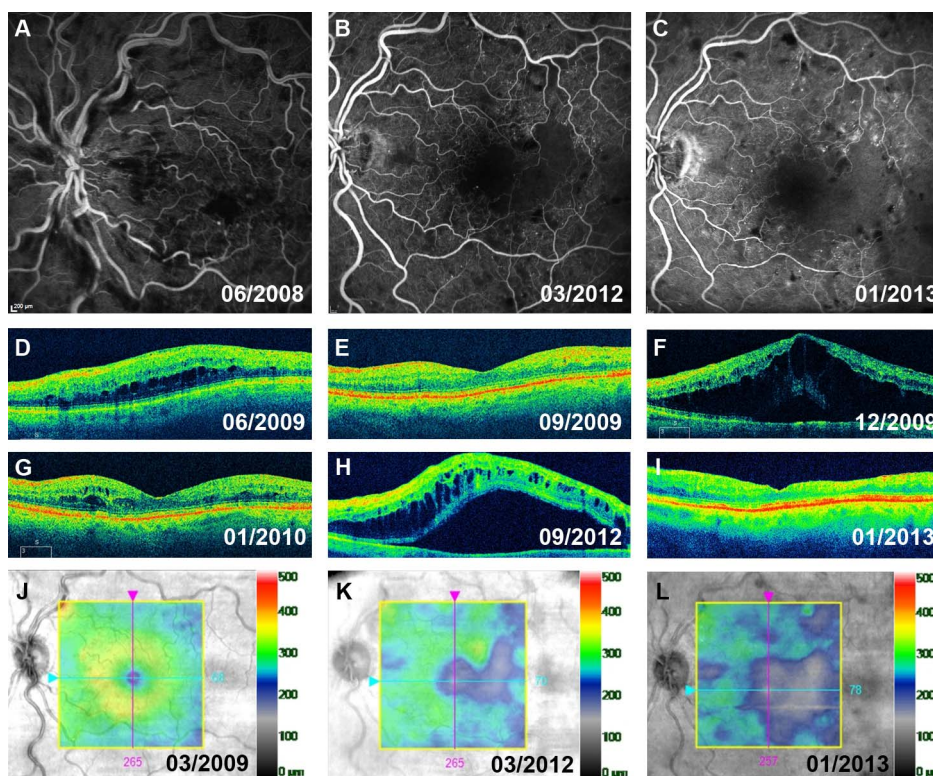
The female donor died from respiratory failure in January 2013, at age 81. She presented in June 2008 with deteriorating left central vision (VA 20/50 due to nonischemic CRVO). She was treated with an intraocular Avastin injection, to which she responded well (VA 20/26 a month later) and monthly



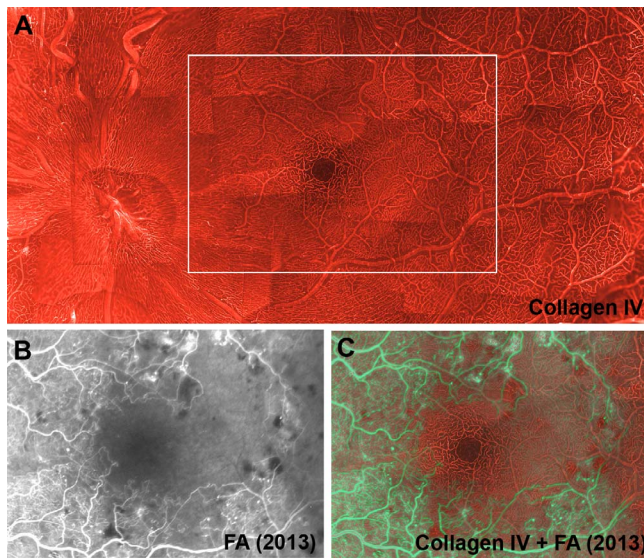
**FIGURE 1.** Fluorescein angiography (A-C), OCT angiography (D-F) and enface OCT (D'-F') imaging in three BRVO patients reveals vascular structures (*white arrows*) in nonperfused regions that have similar signal intensity and patterning to vessels in perfused areas. Fluorescein angiography and OCT imaging was done on the same day in patient 1 and 2. In patient 3 the FA has been taken 5 years earlier.

injections were continued (for a total of 41 injections). Fluorescein angiography at the initial visit showed a small ischemic region around the fovea (Fig. 2A). This ischemic region progressively expanded over the next 4.5 years up to

the point of death (Fig. 2B, 2C). During this period recurrent cystoid edema ensued (Figs. 2D-I), accompanied by progressive thinning of the retina that roughly correlated with the expansion of the ischemic area (Figs. 2J-L).



**FIGURE 2.** Clinical data from the CRVO eye donor. Fluorescein angiograms illustrate the appearance and enlargement of nonperfused areas over 5 years (A-C). During this time the patient received regular anti-VEGF injections with reoccurring edema (D-I), which was accompanied by a progressive thinning of the retina in nonperfused areas (J-L).



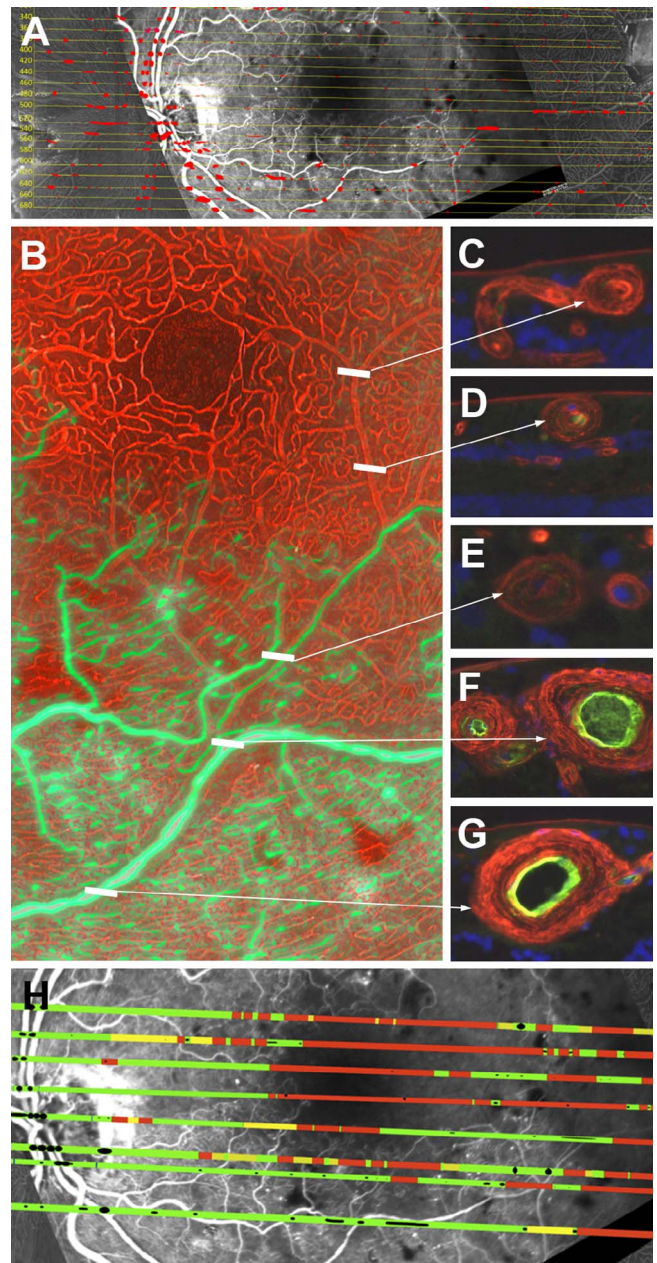
**FIGURE 3.** Immunohistochemistry visualizing collagen IV on postmortem tissue from the CRVO patient shows the pattern of an intact retinal vasculature (A). Mapping against the FA (taken 3 weeks before death) shows that basement membrane persists in nonperfused vessels (B–C).

### Postmortem Histology on Retinal Whole Mount

We dissected a sample including the optic nerve and the macula from the fixed eye and used an anti-collagen IV antibody to visualize basement membrane in whole-mount immunohistochemistry. Remarkably, this revealed the pattern of a complete retinal vasculature (Fig. 3A). We use the last available angiogram from the patient (Fig. 3B), which was obtained 3 weeks before death, to compare the basement membrane network with actual perfusion (Fig. 3C). This showed that in this patient there was no correlation between the external appearance of the retinal vessel basement membrane and the internal perfusion of the vessel.

### Matching Perfusion With Vessel Histology

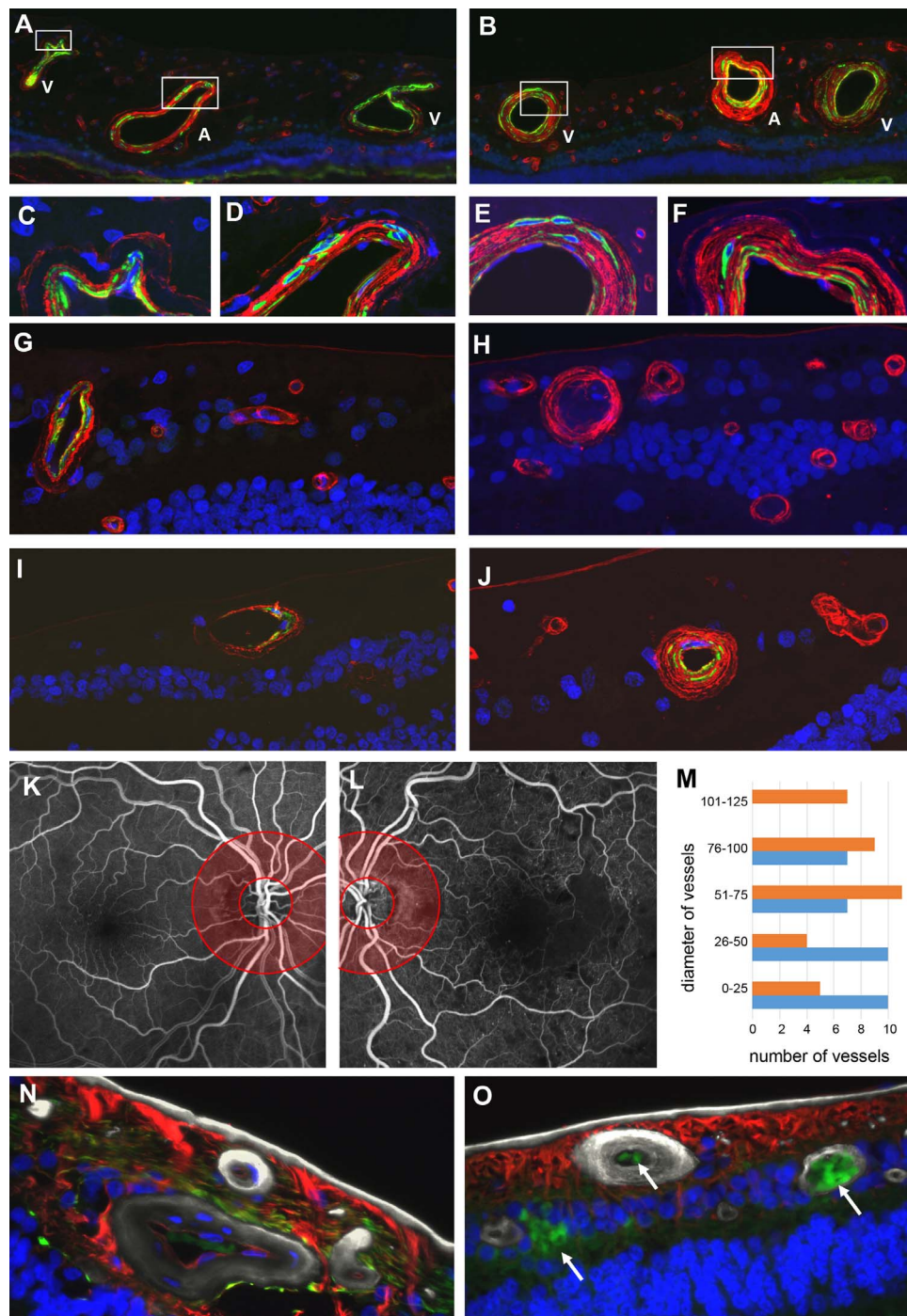
In order to learn more about the lumen of the retinal vessels, we cut serial wax sections from the entire tissue sample. To correlate the sections with the images from the whole-mount stain and the ante-mortem FA, we used the distribution of large blood vessels within individual sections to spatially map all sections as previously described.<sup>13,14</sup> This is illustrated in Figure 4A where the nasalt temporal location of vessels (red dots in Fig. 4A) is marked on every 20<sup>th</sup> section (yellow lines in Fig. 4A). This allowed us to follow an individual vascular path in cross sections. Immunohistochemistry with antibodies against collagen IV (red, labelling basement membrane) and endoglin (green, labelling endothelial cells) showed that endothelial cells were completely absent from the profile of a small, nonperfused arteriole near the fovea (Fig. 4B–D). No endoglin staining could be found either in a partially perfused section of the vessel further upstream (Figs. 4B, 4E). However, in well-perfused sectors of the artery, further upstream, strong endoglin staining indicated the presence of a complete endothelium (Figs. 4B, 4E, 4G). Mapping the presence or absence of endoglin staining on sections throughout the macula showed spatial correlation with perfusion in the angiogram (Fig. 4H).



**FIGURE 4.** The position of larger vessels (red dots) in serial wax sections (yellow lines) was mapped onto the angiogram and collagen IV-stained whole mount (A). Immunohistochemistry to visualize endothelial cells (endoglin in green) and basement membrane (collagen IV in red) along the path of an arteriole (B) shows an intact endothelium in well-perfused sectors but a lack of endothelial cells in partial- or nonperfused sectors (C–G). Mapping the distribution of endoglin staining within vessels (in green for intact, yellow partial, and red absent staining) onto the angiogram shows a spatial correlation between the nonperfusion and the absence of endothelial cells (H).

### Thickening of Vessel Walls

In Figures 4E, 4G it is noticeable that the collagen IV-positive basement membrane appears abnormally thick. We therefore compared the vessel walls from the affected eye and the unaffected contralateral eye. The main vessels near the optic nerve head (Fig. 5A–F) displayed a markedly thickened tunica media in the affected eye (Figs. 5B, 5E, 5F). This appears to have increased the stiffness of the vessels as veins in the



**FIGURE 5.** Comparison of vessel walls from the unaffected (A, C, D, G, I) and the affected eye (B, E, F, H, J) show increased vessel wall thickness and decreased lumen sizes. Basement membrane is stained in red (collagen IV) and smooth muscle cells in green (smooth muscle actin). Larger vessels are shown in A-F (white boxes in A, B indicate magnified details in C-F). Arteries are indicated by A and veins by V. Smaller vessels and capillaries are shown in G-J in the central (G, H) and the peripheral (I, J) retina. There is an absence of smooth muscle cells in the nonperfused, central region in the affected eye (H). Measuring lumen size in the corresponding FA (K, L) in a ring-shaped area around the optic disc (indicated by red ring), confirmed the decreased lumens in vivo in the affected eye (M). The number of vessel lumens counted, of a certain diameter (measured in arbitrary units) are shown in orange for the unaffected and in blue bars for the affected eye. Visualization of glial cells in the affected eye (N, O) shows Müller cells in red (vimentin), retinal astrocytes in green (GFAP), and vessel basement membranes in white (collagen IV). Vessels in a still perfused area (between optic disc and fovea) show vimentin positive endothelial cells in their lumen (N), whereas vessels in the nonperfused area (temporal to fovea) do not contain vimentin positive structures (Müller cells or endothelial cells) within their lumen (O). Autofluorescent red blood cells (in green) can be seen inside and outside vessels (white arrows in [O]).

affected eye maintained a circular shape in cross sections (Fig. 5B), in contrast to the collapsed appearance in the contralateral eye (Fig. 5A). However, the hypertrophic tunica media contained only marginally more  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA)-positive cells. Increased collagen deposition was also found in smaller vessels (Figs. 5G–J). In nonperfused areas small vessels did not contain any smooth muscle cells (Fig. 5H), suggesting that not only endothelial cells but also mural cells have died in these regions.

The thickened vessel walls appear to result in decreased lumen diameters (Figs. 5A, 5B, 5I, 5J). To test whether lumens were also reduced in vivo, we measured the diameter of vessels around the optic disc in the FA that were obtained 3 weeks before the patient's death. This showed that calibers were larger in the contralateral eye (Figs. 5K, 5M) compared with the CRVO eye (Figs. 5L, 5M). Continuous narrowing of the lumen may lead to occlusion, but the vast majority of the vascular structures still had a lumen remaining. To assess whether glial cells invading the vessel lumen may have contributed to occlusions, as has been previously suggested,<sup>15,16</sup> we visualized Müller cells and retinal astrocytes (Figs. 5N, 5O) in the CRVO eye by staining for vimentin and glial fibrillary acidic protein (GFAP). In perfused retina, endothelial cells (which also express vimentin) can be seen lining the vessel lumen (Fig. 5N). In contrast, in nonperfused retina we could not find any vimentin- or GFAP-positive structures (Fig. 5O), demonstrating the absence of endothelial cells, Müller cells, and retinal astrocytes inside the vascular lumen (Fig. 5O). On the other hand, autofluorescent, red blood cells (arrows in Fig. 5O) could be seen occasionally inside vessel lumens, but it is not clear whether these represent obstructions in the living patient or whether they may have pooled postmortem.

## DISCUSSION

In the enface OCT images from the three BRVO patients presented in this study, we could identify vascular structures in nonperfused regions of the retinal vasculature. At this point, it is not possible to establish with certainty which anatomic structures within nonperfused vessels have sufficient light scattering properties to generate this OCT signal. However, a previous OCT study on large vessels around the optic disc has shown hyper scattering from the upper and lower edge of vessels, which could also be found in an occluded vein in a BRVO patient, strongly suggesting that the enface OCT signal originates from the vessel wall.<sup>17</sup>

Whether in our BRVO patients the nonperfused vascular structures still contained living endothelial cells or pericytes is not known, but in our clinicopathological CRVO case study there was a clear correlation between nonperfusion and lack of endothelial cells. Furthermore, there was also a lack of smooth muscle cells in the ischemic region and most vessels appeared to be acellular, suggesting also a loss of pericytes. This is in line with observations in model system where vascular occlusion has led to rapid loss of endothelial cells and pericytes.<sup>6,7,11,18,19</sup> Our CRVO patient converted from nonischemic to ischemic RVO, which occurs frequently in nonischemic cases.<sup>20–24</sup> The nonperfused area slowly expanded over the course of several years, suggesting a progressive loss of vascular cells over time. The remaining acellular basement membrane tubes, however, were preserved for many years. It therefore seems possible that the nonperfused vascular structures seen in the enface OCT are basement membrane remnants from occluded vessels.

Acellular basement membrane tubes have been referred to by numerous investigators as “string” vessels (extensively review by WR Brown<sup>25</sup>), but this term tends to describe

structures that are significantly thinner than the original vessels. In contrast, the outer diameter of the collagen IV-positive basement membrane tubes in our CRVO case study were similar to normal vessels, and the term “string” vessels may therefore not be appropriate here. Although it is known that the basement membrane in the retinal vasculature becomes progressively thicker with age,<sup>26–29</sup> the thickening of basement membranes in our CRVO case was much more substantial than normal, age-related changes. This was observed throughout the entire retinal vasculature in the affected eye, but not in the contralateral eye, suggesting the changes are disease specific. In fact, thickening of vascular walls has been previously noticed by Green et al.<sup>2</sup> in some of the cases in their CRVO histopathologic study.

The causes of the excessive basement membrane thickening in our case are not known. Nonetheless, endothelial cell proliferation has been shown in BRVO animal models<sup>19,30</sup> and in other vasodegenerative conditions, such as diabetic retinopathy and radiation retinopathy.<sup>31,32</sup> In some instances, basement membrane duplication has been described,<sup>32–34</sup> which could have been deposited in sequence by proliferating and dying endothelial cells<sup>35</sup>; a mechanism that may have contributed to the basement membrane broadening in our CRVO case. Endothelial cell turnover might also be linked to the conversion of nonischemic to ischemic CRVO—the expansion of the nonperfused region. However, what sort of insults might cause endothelial cell death in this situation is open to speculation. Our CRVO patient had received more than 40 bevacizumab injections and it could be argued that this might have damaged the remaining endothelium. However, clinical evidence indicates the contrary about blocking VEGF in RVO, where anti-VEGFs appear to be beneficial and reduce expansion of nonperfused regions.<sup>36,37</sup>

Persistent basement membrane in vasodegeneration conditions in the retinal vasculature may also have therapeutic implications, as the remaining structures can potentially provide a scaffold for vascular regrowth.<sup>38</sup> Re-endothelialization of acellular basement membranes has been observed numerous times in model systems,<sup>7,32,33,39,40</sup> as well as in patients.<sup>2,35</sup> Also, reperfusion in RVO patients seems to be promoted by anti-VEGF therapy,<sup>8,36</sup> which is probably based on re-endothelialization of persistent basement membranes. Furthermore, cell therapy approaches, aiming to regenerate ischemic retinal vasculature<sup>41,42</sup> will depend on a persistent extracellular template. Thus, our finding of persistent basement membrane after occlusion, demonstrates that therapies aiming for reperfusion in ischemic eye disease are possible in principle.

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