THE ESSENTIAL ROLE OF THE CHORIOCAPILLARIS IN VISION: NOVEL INSIGHTS FROM IMAGING AND MOLECULAR BIOLOGY

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Abstract

The choriocapillaris, a dense capillary network located at the posterior pole of the eye, is essential for supporting normal vision, supplying nutrients, and removing waste products from photoreceptor cells and the RPE. The anatomical location, heterogeneity, and homeostatic interactions with surrounding cell types makes the choroid complex to study both in vivo and in vitro. Recent advances in single-cell RNA sequencing, in vivo imaging and in vitro cell modeling are vastly improving our knowledge of the choroid and its role in normal health and in macular degeneration (AMD). Histologically loss of endothelial cells (ECs) of the choriocapillaris occurs early in AMD concomitant with elevated formation of the membrane attack complex of complement. Advanced imaging has allowed us to visualize early choroidal blood flow changes in AMD in living patients, supporting histological findings of loss of choroidal ECs. Single-cell RNA sequencing is being used to characterize choroidal cell types transcriptionally and discover their altered patterns of gene expression in aging and disease. Advances in iPSC protocols and 3D cultures will allow us to closely mimic the in vivo microenvironment of the choroid in vitro to gain a better understanding of the mechanism leading to choriocapillaris loss in AMD.

Overview of the retina-choroid interface

Vertebrate photoreceptor cells are uniquely adapted to detect light and transmit visual information to retinal bipolar cells which ultimately reaches the visual cortex. As discussed elsewhere in this volume, the process of phototransduction involves a conformational change in 11-cis retinal to all-trans retinal, followed by the recycling and regeneration of this retinoid through a series of trafficking and enzymatic processes. In addition to recycling, a considerable fraction of retinoids are delivered to the retina daily from the circulation. Thus,

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alterations in the quality or quantity of the outer retina's vascular supply could profoundly affect dark adaptation (Owsley et al., 2016), discussed elsewhere in this volume.

Because this process is highly finessed, photoreceptor cells require an extracellular milieu that is tightly controlled, and even temporary perturbations in this microenvironment can lead to degeneration of photoreceptor cells, particularly rods. This microenvironment is maintained by the retinal pigment epithelium (RPE), where one RPE cell maintains 30 or more photoreceptor cells; the retinal Muller cells (which help provide retinoids to cones, as discussed elsewhere in this volume), and the interphotoreceptor matrix, which appears to be largely synthesized by the photoreceptor cells themselves and serves as the conduit for retinoids between the photoreceptor cells and RPE. Changes in the composition or volume of the fluid bathing photoreceptor cells can result in a sudden loss of vision.

At the same time, because of the retina's exceptional energy consumption (the highest in the body (Ames, 1992)) photoreceptor cells require a constant and large supply of oxygen and generate a high volume of CO_2, which must be converted to bicarbonate for removal through the action of carbonic anhydrases.

In summary, photoreceptor cells require a clear path of light through the retina with minimal distortion by vascular elements, a highly regulated microenvironment, and abundant metabolites. Collectively, these features all contribute to the need for a dense vascular supply located beneath (or external to) the photoreceptor cells. In humans, this need is met by the choroid (Figure 1).

Choroid: structure

The choroid is a component of the peripheral vasculature outside of the blood retinal barrier that occupies the anatomical compartment between the retinal pigment epithelium and the sclera. It may be divided between the vascular elements and the surrounding connective tissue, the choroidal stroma. From outer to inner submacular choroid, the vascular elements progressively narrow as they branch, with layers of large, intermediate, and small vessels in Haller's layer, Sattler's layer, and the choriocapillaris, respectively (Figure 2). Notably, the smallest choroidal capillaries are still very large compared to those of the retinal vasculature, with biophysical implications for both velocity and diffusion.

The choroid is supplied by the short posterior ciliary arteries (Figure 3) that enter the sclera at the posterior pole and drained by the vortex vein system at the eccentricity of the equator, where venules from the posterior pole converge and exit the eye through one of the ophthalmic vein branches. Each vortex vein typically drains around 20–25% of the choroid.

As in other vascular beds, the endothelial cells (ECs) of the choriocapillaris are supported by pericytes, which are normally distributed along the lateral and outer aspect of the endothelium. In contrast to retinal capillaries, the pericytes of the choroid do not circumscribe the endothelium and are not normally present between the endothelial lumen and the tissue it nourishes.

Choroid function:

The most obvious function of the choriocapillaris is supplying nutrients and oxygen and removing waste from the photoreceptor cells and RPE. The high level of photoreceptor metabolism results in CO_2, water, and heat as byproducts of oxidative phosphorylation or aerobic glycolysis (Hurley et al., 2015). Carbonic anhydrases in the RPE and in the choriocapillaris (including CA4, which is restricted to choriocapillaris ECs) interconvert CO_2 gas and bicarbonate ions. The removal of excess heat by the choriocapillaris (a consequence of both photoreceptor metabolism, highest in the dark, and absorption of photons by the RPE in the light) has been studied indirectly. Experimental elevation of intraocular pressure above the perfusion pressure of the choroid is associated with increased ocular temperature in both monkeys and humans, indicating that the normal choroidal circulation serves as a heat sink (Parver, 1991). The potential consequences of loss of choroidal flow in AMD (discussed below) and its impact on temperature at the photoreceptor/RPE/choroid interface has not been fully explored, however it is notable that RPE cells grown at elevated temperature show evidence of altered synthesis of extracellular matrix molecules (Sekiyama et al., 2012), which is a key feature of AMD (Chong et al., 2005; Grossniklaus et al., 1992; Sura et al., 2020).

The choroid also plays a critical role in accommodation in nonmammalian species by rapid shape changes (Nickla and Wallman, 2010) in contrast to the subtle and age-related thickness changes in humans, discussed below.

Cells of the choroid and their gene expression

While the vasculature of the choroid is very dense, especially at the level of the choriocapillaris where vessel lumens occupy more than 50% of cross-sectional area of the layer, ECs comprise a minority of choroidal nuclei (Voigt et al., 2019). Anatomically, in addition to the vascular cells lining blood vessel lumens, one can readily distinguish smooth muscle and pigmented melanocytes. Moreover, numerous nucleated cells, which include fibroblasts, mast cells, macrophages, lymphocytes, and nerve processes with supporting Schwann cells, are present in the choroid (Figure 4).

In addition to identifying choroidal cells morphologically, gene expression patterns have been characterized for individual populations of choroidal cells with single-cell RNA sequencing (scRNA-seq). Transcriptomic signatures have been identified for all major choroidal cell populations in post-mortem human donors and mice, including distinct populations of fibroblasts, melanocytes, pericytes, ECs, smooth muscle cells, myelinating and non-myelinating Schwann cells, and multiple classes of resident and infiltrating leukocytes (Gautam et al., 2021; Lehmann et al., 2020; Voigt et al., 2019). Additionally, subpopulations of arteries, veins, and choriocapillaris ECs have been described by enriching for PECAM1-expressing cells prior to scRNA-seq, providing a high-resolution view of gene expression along the choroidal vascular tree (Figure 5, Table 1) (Rohlenova et al., 2020; Voigt et al., 2019; Voigt et al., 2020c).

Moroever, these detailed expression atlases have allowed vision researchers to pinpoint which cell populations express genes previously implicated in AMD pathogenesis. For example, complement factor H (*CFH*) is a complement inhibitor with single-nucleotide variants strongly associated with AMD (Fritsche et al., 2016). However, CFH is a secreted protein, and the expression pattern of this important complement inhibitor has been a subject of debate. ScRNA-seq has clarified that *CFH* is most highly expressed by ECs (including the choriocapillaris), their surrounding pericytes, and fibroblasts in the choroid (Voigt et al., 2020a). Such gene expression information has been made rapidly available to the vision research community with web-based resources such as Spectacle (https://singlecell-eye.org) (Voigt et al., 2020b) and EndoDB (https://endotheliomics.shinyapps.io/murine_ectax/) (Khan et al., 2019).

In addition to generating detailed expression databases, scRNA-seq has been used to investigate how important biological variables central to AMD pathogenesis influence gene expression in the choroid. For example, macular versus peripheral transcriptomes have been compared in postmortem human donor choroid, and cell types such as pericytes have been shown to demonstrate modest regional expression differences (Voigt et al., 2019). Likewise, scRNA-seq was utilized to compare choroidal EC gene expression between young (< 2 years old) and aging (> 60 years old) human donors (Voigt et al., 2020c). Choriocapillaris ECs from aging donors demonstrated increased expression of pro-inflammatory genes such as ICAM-1, while the young choriocapillaris strongly expressed anti-inflammatory molecules such as CD34. Collectively, these gene expression patterns suggest that the choroidal vasculature develops a pro-inflammatory phenotype with advancing age.

Choroidal changes in aging

Both anatomical and molecular changes occur in the aging choroid. Anatomically, choroidal thickness (i.e., the distance between Bruch's membrane and the inner surface of the sclera where the lamina fusca resides) decreases as a function of age, and this morphological feature of living humans was first noted by the application of enhanced depth imaging OCT (Margolis and Spaide, 2009; Spaide et al., 2008). Choroidal thicknesses have been measured predominantly subfoveally and show a normal distribution within a given population (Wei et al., 2013). The aging choroid exhibits a loss of approximately 3–4μm per year (Wakatsuki et al., 2015; Wei et al., 2013). Temporary increases in choroidal thickness can be experimentally achieved by increasing the blood volume (Arora et al., 2012), but these changes are relatively small compared to the age-related loss. In rodents, changes in choroidal thickness can be observed following experimental lesioning of central or peripheral nervous control of choroidal blood flow (Li et al., 2021; Steinle and Smith, 2002) with corresponding structural and functional deficits in the retina. In addition, choroidal thinning was also recently found to be related to levels of circulating C-reactive protein (CRP) (Chen et al., 2021b), an acute phase protein also elevated in patients with AMD (Seddon et al., 2004; Seddon et al., 2005). The mechanistic relationship between CRP and choroidal thinning is not well understood, however it is notable that CRP can activate the complement system, discussed below.

Loss of choroidal thickness is accompanied structurally by increased abundance and density of fibrillar collagen with corresponding decrease in ground substance in the choroidal stroma (Sohn et al., 2014). Both histological and swept source OCT studies suggest that the thickness of the choroid is unrelated to the density of the choriocapillaris and intermediate choroidal vessels, respectively (Sohn et al., 2014; Zhou et al., 2020).

Choriocapillaris degeneration:

In addition to choroidal thinning, there is a loss of choriocapillaris ECs in aging (Ramrattan et al., 1994). It is challenging to observe morphologic changes in AMD choriocapillaris cells with hematoxylin and eosin stained paraffin sections, and different investigators reached different conclusions about whether the choriocapillaris is lost in early AMD (Ramrattan et al., 1994; Spraul et al., 1996). We believe that this is due to the acellular choroidal capillary ghosts that present as clear lumens on H&E sections but can be readily discriminated using a label for intact ECs (Figure 6) (Lutty et al., 2020; Mullins et al., 2011).

Using the fucose-binding lectin *Ulex europaeus* agglutinin-I to label human choroidal whole mounts, Lutty and colleagues observed a loss of choriocapillaris area in early AMD from nearly 80% in controls to around 60% in early AMD eyes, a loss that was confined to the submacular choroid (Lutty et al., 2020). The degeneration of choriocapillaris and replacement of healthy capillaries with ghost vessels is further related to drusen density, in addition to drusen being spatially related with ghost choriocapillaris vessels (Mullins et al., 2011). The observed histological loss of choriocapillaris ECs is much more striking in eyes with geographic atrophy and this deterioration is observed under the still intact RPE (Sohn et al., 2019), consistent with recent OCT-A findings that widespread choriocapillaris flow voids (Kim et al., 2013; Zhang et al., 2018) precede atrophy in GA (Moreira-Neto et al., 2018; Moult et al., 2016). Based on available evidence, we propose that the most likely source of EC injury in aging eyes is the membrane attack complex (MAC) of complement, discussed below.

This loss, contributing to the impaired replenishment of bleached 11-cis retinal, likely contributes to the delayed dark adaptation observed in early AMD.

Biochemical changes in the aging choroid

A major feature of AMD is the accumulation of lipids and glycoproteins between the choriocapillaris and the cells it nourishes (reviewed in (Curcio et al., 2011)), and this accumulation can be in the form of discrete mounds (drusen) or in a linear confluent layer, which can further detach and form the appearance of soft drusen (Bressler et al., 1994; Chen et al., 2021a; Chen et al., 2020b). Other described changes include loss of the anti-angiogenic matricellular glycoprotein thrombospondin (Uno et al., 2006), increased advanced glycation end-products (Handa et al., 1999), decreased abundance of complement factor H (Bhutto et al., 2011), and accumulation of monomeric CRP (Bhutto et al., 2011; Chirco et al., 2018), which can both suppress and initiate complement activation in different contexts (Mihlan et al., 2011).

The activation of the complement system at the level of the choriocapillaris is of interest in the pathogenesis of AMD, especially considering the extensive evidence for genetic dysregulation of the complement system in numerous reports (recently reviewed (Armento et al., 2021)). The C5b-9 MAC of complement accumulates in aging choroid, with traces appearing in children and a steady increase through adulthood (Mullins et al., 2014). Biochemical quantification in human choroid using ELISA shows that the abundance of the MAC approximately doubles during normal aging, comparing young control (21–48 years) to aged control eyes (aged 71–96 years), and doubles again comparing aged control eyes to those with early AMD (77–99 years of age) (Mullins et al., 2014). We propose the joint findings that (a) the MAC accumulates at the level of the choriocapillaris, (b) choriocapillaris degeneration is a key early event in AMD pathogenesis and progression, (c) MAC levels are further related to the high risk Y402H variant in CFH (Chirco et al., 2018; Mullins et al., 2011), and (d) MAC can lyse primary choroidal ECs in vitro (Zeng et al., 2018), implicate the MAC as a major factor in AMD. Recent drug trials aimed at suppressing complement activation in atrophy have had variable results, but several of these are encouraging ((Wykoff et al., 2021), see below).

In Vivo Choroidal Imaging

Until a few years ago, in vivo imaging of the choroid was restricted to angiography that requires intravenous cannulation and injection of fluorescein and/or indocyanine green dye. In fluorescein angiography (FA), serial photographs are taken after dye injection using an excitation filter that allows blue light wavelengths to stimulate fluorescence and a barrier filter that allows yellow-green wavelengths from the fluorescent molecule to be imaged (Gass et al., 1967). In indocyanine green angiography (ICGA), different excitation and barrier filters are used (Alander et al., 2012). FA and ICGA can be performed simultaneously by coincident injection of both dyes and serial photography that alternates between two sets of filters.

Twenty percent of fluorescein in the circulation remains unbound to albumin and readily diffuses across the fenestrations of the choriocapillaris (Wolfe, 1986). This causes early diffuse hyperfluorescence (the "choroidal flush") that obscures details of the choroidal circulation (Figure 7). Occasionally FA is useful in imaging the choroid, such as when there is delayed/absent choroidal perfusion in ocular ischemic syndrome, or in a hyperfluorescent "classic" choroidal neovascular membrane (1991). Unlike fluorescein, the ICG molecule remains protein-bound and normally remains in the circulation (Yoneya et al., 1998). The absence of ICG diffusion across the choriocapillaris enables visualization of the choroidal circulation (Figure 8) and of choroidal lesions such as choroidal hemangiomas. Two major strengths of dye-based angiography are the ability to visualize the temporal dynamics of dye transit and leakage, and to capture images despite poor fixation and significant media opacity. Weaknesses include the need for intravenous access, allergic contraindications and potential systemic adverse effects (Yannuzzi et al., 1986), slow image acquisition, and the lack of layer-specific angiography data.

Optical coherence tomography (OCT) is a newer technology that measures the reflection of light waves to reconstruct ocular anatomy (Huang et al., 1991). Early OCT systems

could not image the choroid well, but enhanced-depth spectral domain OCT enabled high-resolution imaging of the entire choroidal depth (Spaide et al., 2008), and swept source (SS) OCT can image the entire choroid even under retinal/sub-RPE lesions (Choi et al., 2013). Current versions of SS-OCT can image the entire posterior pole in a single, rapid scan (Russell et al., 2019). On OCT scans, the choroid consists of hyperreflective stroma and round to oval hyporeflective choroidal vessel lumens (Figure 9C, D). Many studies have catalogued choroidal thickness and choroidal vascularity (based on the ratio of lumen to stroma) in various conditions (Singh et al., 2019)(Figure 10). However, these studies are mostly limited to single OCT line scans of the subfoveal choroid, so there are few OCT studies of the choroid throughout the rest of the fundus. We recently compared normative values to human diabetic patients to show in vivo that the diabetic choroid is abnormal throughout the posterior pole (Russell et al., in revision), which had previously been demonstrated only with histopathology (Cao et al., 1998). A similar imaging strategy using SS-OCT can be applied to investigate choroidal dysfunction in many other retinal diseases such as AMD.

OCT angiography (OCTA) utilizes repeated OCT scans to identify areas where reflectivity varies from scan to scan (Spaide et al., 2018). Variable reflectivity is inferred to result from vascular flow, so algorithms impute the presence of blood vessels in these areas and reconstruct two-dimensional angiograms of detectable flow within individual tissue layers (Spaide et al., 2018). With SS-OCTA, the individual vessels of the choriocapillaris can be visualized (Choi et al., 2013). However, flow through larger choroidal vessels cannot be measured with modern OCTA systems because when light passes through the choroid, most of the photons scatter through the blood and the residual reflected light is scattered by the RPE, so there is no detectable signal (Zhou et al., 2020). Hence, large choroidal vessels appear dark on OCT images. With the rapid scanning speed of SS-OCT/OCTA, it is possible to capture the choroidal vasculature throughout the posterior pole in a single scan (Figure 9A, B). In comparison to dye-based angiography of the choroid, SS-OCT/OCTA are less effective with media opacity and poor fixation, but they are faster, safer, non-invasive, repeatable, and provide high-resolution, layer-specific angiography data.

New insights into choroidal biology

Improvements in imaging living patients, new knowledge of choroidal pathology in AMD, applications of cutting-edge molecular biology, and cellular studies for both disease modeling and transplantation have brought the choroid into renewed focus.

New Insights: Imaging:

The advent of SS-OCT/OCTA for imaging the choroid has begun to revolutionize clinical practice and our understanding of choroidal dysfunction. The most immediate clinical application of SS-OCTA is for diagnosis and longitudinal evaluation of choroidal neovascularization (CNV). CNVs can be difficult or impossible to definitively identify with dye-based angiography (1991), but they are usually easily visualized with a quick SS-OCTA scan (Figure 11) (Miller et al., 2017). SS-OCTA has shown that some eyes with age-related macular degeneration (AMD) have no subretinal or intraretinal fluid but do have CNVs (De

Oliveira et al., 2018; Querques et al., 2013). These lesions, also detected by histology (Chen et al., 2020a; Sarks, 1973), have been termed non-exudative CNVs (De Oliveira et al., 2018). They can persist for years without exudation (Yang et al., 2019), and it is not clear what triggers them to begin exudating. It has long been known that exudative AMD often evolves into geographic atrophy (GA) (Gemenetzi and Patel, 2017), but halting exudation with anti-VEGF injections may hasten GA (Gemenetzi and Patel, 2017). Conversely, slowing progression of GA via complement inhibition seems to increase the onset of exudative CNV (Wykoff et al., 2021). SS-OCTA in normal subjects has shown that choriocapillaris perfusion decreases with age in the macula, and this decrease is most pronounced in the fovea (Zheng et al., 2019). Longitudinal, in vivo SS-OCTA studies have demonstrated strong correlations between choriocapillaris ischemia and GA progression (Shi et al., 2021; Thulliez et al., 2019), and choriocapillaris flow correlates with retinal sensitivity in eyes with GA (Rinella et al., 2021).

We hypothesize that age, smoking, genetics, and metabolic factors cause choriocapillaris ischemia, which leads to dysfunction and atrophy of the RPE and outer retina in AMD (Chirco et al., 2017; Sohn et al., 2019; Whitmore et al., 2015). CNVs may then develop as an adaptive response to perfuse an ischemic RPE/outer retina to replace lost choriocapillaris vessels. If the flow through a non-exudative CNV exceeds the metabolic needs of the overlying tissue, exudation results. In contrast, if a non-exudative CNV fails to develop or cannot compensate for the metabolic needs of the overlying tissue, GA ensues. Therefore, one strategy for treating AMD is to promote non-exudative CNVs that safely and continuously nourish the RPE/outer retina. This will require a careful balance of molecular factors impacting angiogenesis, complement activation, and other pathways. SS-OCTA will be essential to identify eyes with choriocapillaris ischemia and non-exudative CNVs and to follow them longitudinally while titrating molecular therapy.

New Insights: The molecular biology of the choroid

ScRNA-seq has recently been applied to understanding AMD pathogenesis and this area is expected to greatly expand. In a recent report, gene expression was compared at the single-cell level between healthy mice and mice subjected to laser-induced choroidal neovascularization (CNV) (Rohlenova et al., 2020). In this study, choroids were isolated seven-days after laser-injury and enriched for PECAM1-expressing ECs. After clustering the cells, a distinct CNV endothelial population was identified that did not have any contributions from control choroids. This CNV-specific population demonstrated a gene expression pattern consistent with endothelial damage and was enriched in several metabolic pathways implicated in pathological angiogenesis (Rohlenova et al., 2020). In addition to this biological insight, this study provided further evidence that scRNA-seq is a valuable tool for interrogating small and rare populations of choroidal cells central to AMD pathogenesis.

Future studies will leverage scRNA-seq to further advance our understanding of AMD pathophysiology. While a total of two AMD-choroids have been examined with scRNA-seq to date (Voigt et al., 2019), larger cohorts of AMD and control donors may identify expression changes common to early or neovascular AMD. Likewise, scRNA-seq may help untangle important genotype-phenotype associations in AMD. For example, single-

nucleotide variants near the genes *HTRA1* and *ARMS2* confer substantial AMD risk (Fritsche et al., 2016), and scRNA-seq may highlight specific cell populations that harbor large gene expression changes in response such variants. Lastly, advances in spatial transcriptomics have the potential to map gene expression changes to the spatial location of disease. This technology may be incredibly useful in studying AMD by characterizing how gene expression is perturbed around drusen or within neovascular membranes.

New Insights: Disease modeling in the choroid

The blood vessels of the choroid, like other vascular beds, are lined with ECs. Immortalized EC lines (e.g., primate chorioretinal endothelial cells RF/6A), human umbilical vein endothelial cells (HUVECS) and iPSC-derived endothelial cells can all be used to study EC function in vitro. Although HUVECs are widely used it is important to note they have a different gene expression profile compared to native choroidal ECs (Browning et al., 2012), bringing to light the importance of tissue specific ECs. ECs have specialized functions and expression patterns depending on their anatomical location (Nolan et al., 2013). For example, ECs in the choroid uniquely express CA4 (Baba et al., 2009; Hageman et al., 1991). Given the importance of tissue specific ECs, our lab generated an immortalized choroidal EC line from human donor choroidal ECs (Voigt et al., 2019) and we have developed a stepwise protocol to differentiate CA4-positive choroidal ECs from induced pluripotent stem cells (iPSCs) (Songstad et al., 2015; Songstad et al., 2017; (Mulfaul et al., 2020).

The first step in modeling the choroidal vasculature in vitro is to generate tissue-specific ECs in monolayer. Single-cell suspensions of iPSC derived ECs form tube-like structures in Matrigel (Mulfaul et al., 2020) however these vascular structures are viable for only about 24 hours. *In vivo*, choroidal ECs require trophic signals from the RPE for both development and homeostasis (Saint-Geniez et al., 2009). To model the choroid *in vitro*, other cell types need to be co-cultured with choroidal ECs to mimic the *in vivo* tissue. Co-culture models of RPE and ECs on transwell inserts (Fan et al., 2002; Geisen et al., 2006; Spencer et al., 2017), collagen gel (Sakamoto et al., 1995) or amniotic membranes (Hamilton et al., 2007) have been used to study the interaction between RPE and choroidal ECs. Most co-culture systems use synthetic material that do not mimic Bruch's membrane.

Djigo and colleagues generated a tissue-engineered choroidal stroma by culturing choroidal stromal fibroblasts with ascorbic acid for six weeks to obtain ECM sheets which closely resembled the native human choroid. The tissue-engineered choroidal stroma supported growth of an RPE monolayer, a 3D vascular network of HUVECs and pigmented choroidal melanocytes (Djigo et al., 2019). Thus, this elegant system provides a non-synthetic alternative for RPE/choroid co-culture which supports vascular tube formation.

In addition to co-culture with RPE it is important to begin to culture choroid ECs in the presence of other supporting choroidal cell types to closely model the unique microenvironment of the choroid. A 3D RPE-choriocapillaris model using polyethylene glycol (PEG) hydrogels crosslinked with matrix metalloproteinase (MMP) degradable peptides highlights the importance of both RPE and mesenchyme in the development of

fenestrated choroidal EC-like vasculature. Manian et al. encapsulated iPSC derived ECs in a PEG-MMP hydogel in the presence of mesenchymal stem cells followed by plating RPE cells on the apical surface of the hydrogel. ECs formed fenestrated vascular networks in the hydrogel consistent with *in vivo* development of the choroidal endothelium and co-cultured RPE-EC were stable for up to 60 days in culture (Manian et al., 2021).

As more is learned about the cellular and extracellular milieu of the human choroid, 3D models have the potential to incorporate all cell types present in the choroidal vasculature and stroma *in vivo*. Bioprinting allows for a combination of cell types to be incorporated into a hydrogel support. Bharti and colleagues have used this approach to bioprint iPSC derived fibroblasts, ECs and pericytes on the basal side of a biodegradable scaffold and seeded RPE on the apical side. As the 3D cultures matured the scaffold was replaced with extracellular matrix components mimicking Bruch's membrane (Bharti et al., preprint). RPE developed apical processes, tight junctions, and basal infoldings and ECs formed fenestrated vascular capillary tubes. In the presence of human serum, the 3D cultures accumulated drusen-like deposits between the RPE and choroid and simulated CNV development using a Hif-1alpha activator, demonstrating the application of this model in the study of both dry and wet AMD.

In summary, the combination of innovations in *in vivo* imaging, gene expression analysis of single choroidal cells, quantitative biochemistry, and *in vitro* modeling of choroidal cells and their interactions are improving our understanding of this critical tissue. Future studies directed toward better understanding the environmental challenges faced by choroidal ECs, especially complement injury, and toward the rebuilding of the damaged choroid in AMD will offer improved opportunities for understanding and treating the choroidal EC loss observed in AMD.

Acknowledgments

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Summary points

- The choroid is the vascular supply for the photoreceptor cells of the mammalian eye and is thus essential for maintaining vision.

- Injury of the choriocapillaris layer of the choroid is an early initiating event in the pathogenesis of age-related macular degeneration and can precede the loss of the RPE in geographic atrophy yet this tissue has been understudied.

- The application of novel approaches—including quantitative histology, in vivo functional imaging, molecular analysis at the single cell level of resolution, and in vitro induced pluripotent stem cell (iPSC) modeling techniques--is providing new insights into this fascinating tissue.

Future issues

- In light of the role of complement in damage to the choriocapillaris, inhibiting complement is being explored as a method to protect against the growth of geographic atrophy.

- Imaging of the choriocapillaris in living patients will continue to improve and resolve some of the standing questions about flow voids and the relationships between the RPE, drusen and choriocapillaris health.

- Studying complex intercellular interactions using stem cell derived RPE, choriocapillaris endothelial cells and structural cells such as pericytes to recreate a choroid like microenvironment.

- Determining how genetic risk factors for AMD affect the physiology of the choroid prior to the onset of disease will provide insight into the mechanisms by which different variants contribute to macular disease.

- In eyes that have lost vision due to AMD, restoring the choriocapillaris with patient derived endothelial cells will be a necessary component of restoring vision.
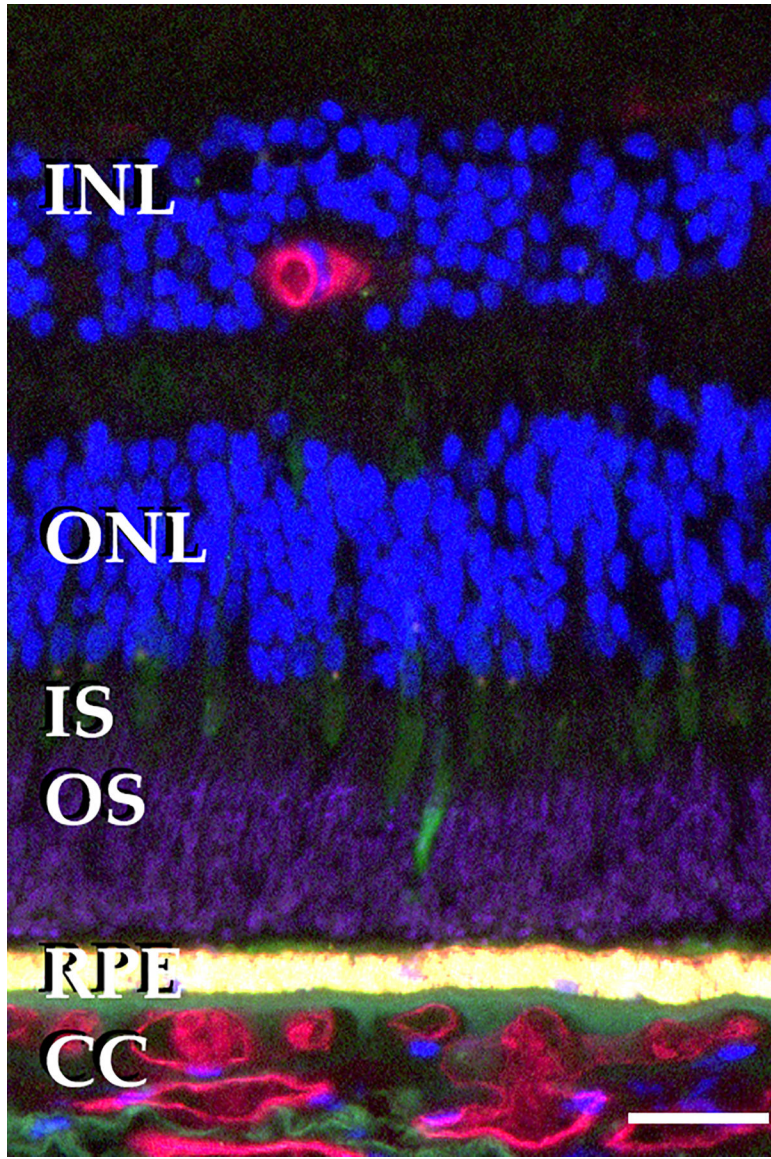
Figure 1.
Relationship of photoreceptor cells, RPE and choriocapillaris. Section of human retina labeled with antibodies directed against rhodopsin (magenta), pooled anti-cone opsins (green), UEA-I, which binds vascular endothelial cells of the retina and choroid (red) and DAPI, which labels DNA (blue). Note the relatively sparse vasculature in the inner retina, absence of vascular elements in the outer retina, and dense, large diameter vessels in the choriocapillaris. Scalebar = 25μm. INL, inner nuclear layer; ONL, outer nuclear layer; IS, inner segments; OS, outer segments; RPE, retinal pigment epithelium; CC, choriocapillaris.
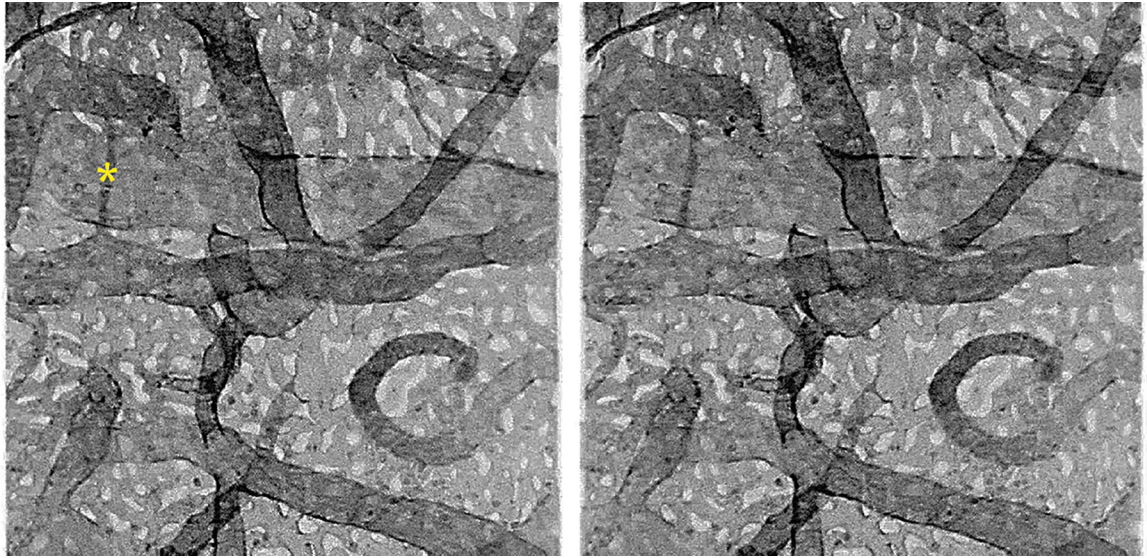
Figure 2.
Confocal projection stereo pair of the human choroidal vasculature labeled with UEA-I lectin. Note the very dense honeycomb appearance of the choriocapillaris (superficial) and the complexity of the intermediate and large vessels. A potential A-V shunt is indicated (asterisk).
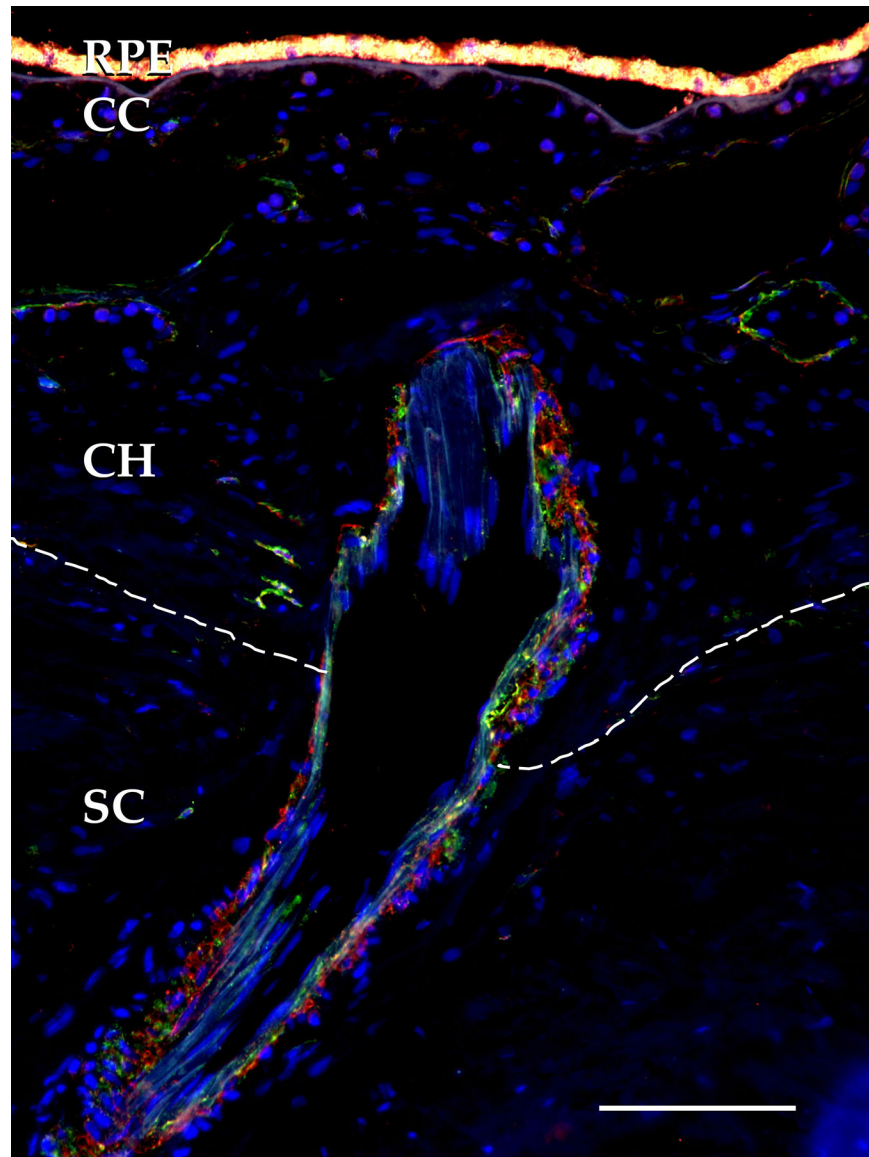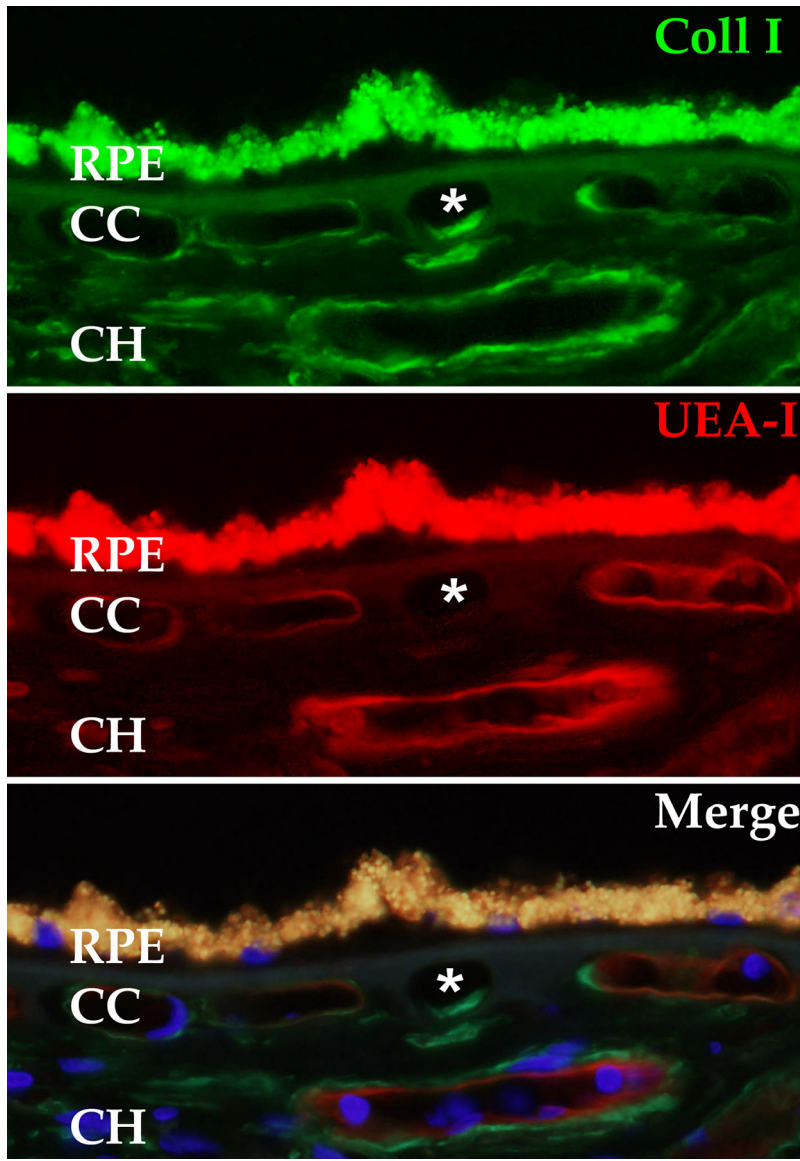
Figure 3.
Short posterior ciliary artery penetrating the sclera (SC, margin indicated by dotted line) to supply the choroidal arterioles and ultimately choriocapillaris. Red, CC, choriocapillaris, CH, Saller's and Hatler's layers of the choroid, RPE, retinal pigment epithelium. Red, anti-smooth muscle actin antibody; green, anti-vascular adhesion protein antibody, scalebar = 100μm.
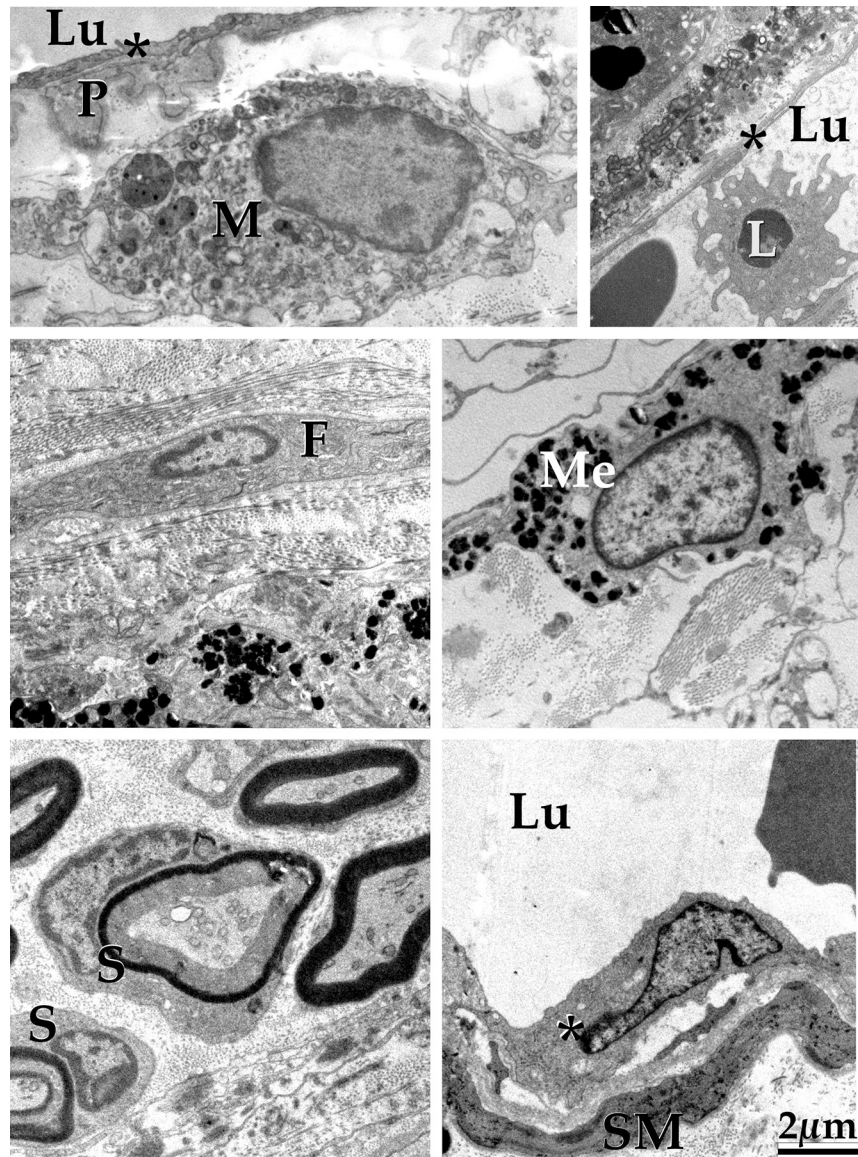
Figure 4.
In addition to endothelial cells (asterisks), cells of the human choroid include immune cells, both within the stroma (M, macrophage) and circulating (L); fibroblasts (F); melanocytes (Me); Schwann cells (S), smooth muscle cells (SM) and pericytes (P). Lu, vascular lumen. Scalebar = 2μm.
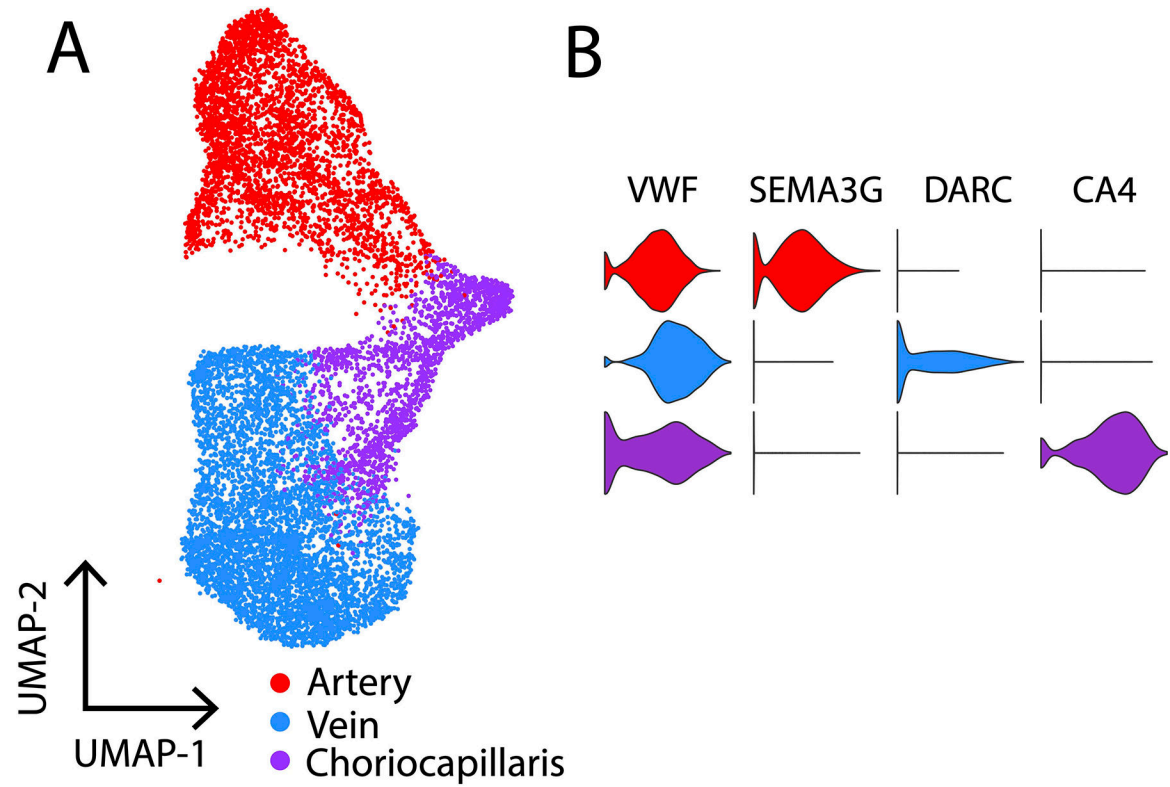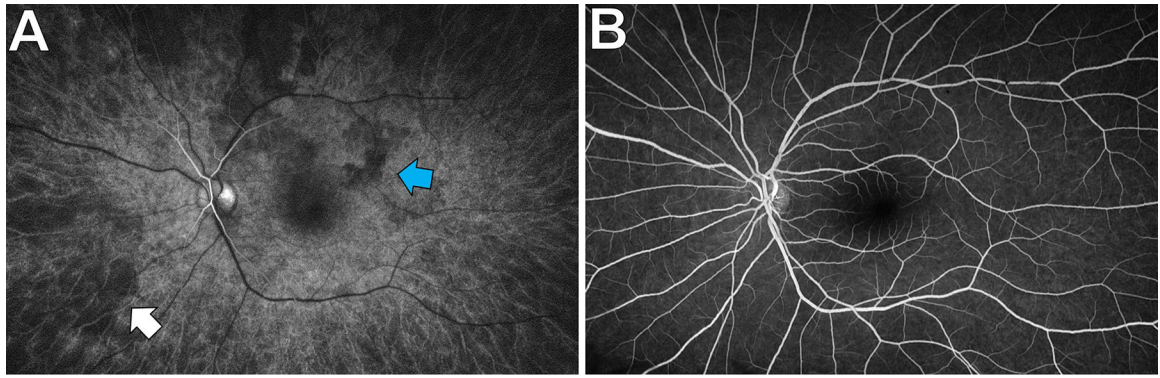
Figure 5:

Choroidal arteries, veins, and choriocapillaris endothelial cells identified with single-cell RNA sequencing. **A.** UMAP plot of 10,576 choroidal endothelial cells isolated from 6 human donors (Voigt et al., 2020c). Each point represents the multidimensional transcriptome of one cell. Clusters of cells (red, blue, purple) are classified into different endothelial cell types (arteries, veins, choriocapillaris). **B.** Violin plots demonstrate that all choroidal endothelial cells highly express the pan-endothelial cell gene *VWF*. Choroidal arteries specifically express *SEMA3G*, choroidal veins specifically express *DARC*, and the choriocapillaris specifically expressed *CA4.*
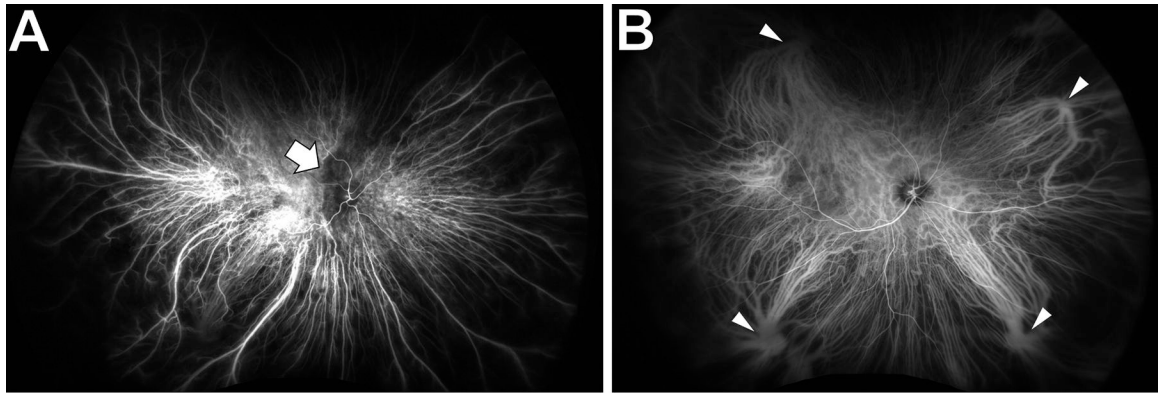
Figure 6:
Ghost choriocapillaris vessel (asterisk) beneath intact RPE monolayer. This degenerated capillary segment appears normal when the choroid is labeled with antibodies directed against type I collagen (green, top panel), but is unreactive with the endothelial cell binding lectin UEA-I (red, middle panel). Lower panel depicts merged channels with DAPI nuclear counterstain. RPE, retinal pigment epithelium; CC. choriocapillaris, CH outer choroid.

Figure 7.
Choroidal visualization on a normal fluorescein angiogram. **A**: Seven seconds after dye injection, the retinal arteries have begun to fill. The large choroidal vessels are visible (white arrow) and there is patchy choroidal flush (blue arrow) in the posterior pole. **B**: Thirty seconds later, the retinal circulation has filled, the choroidal flush has become homogenous, and the large choroidal vessels are no longer visible.
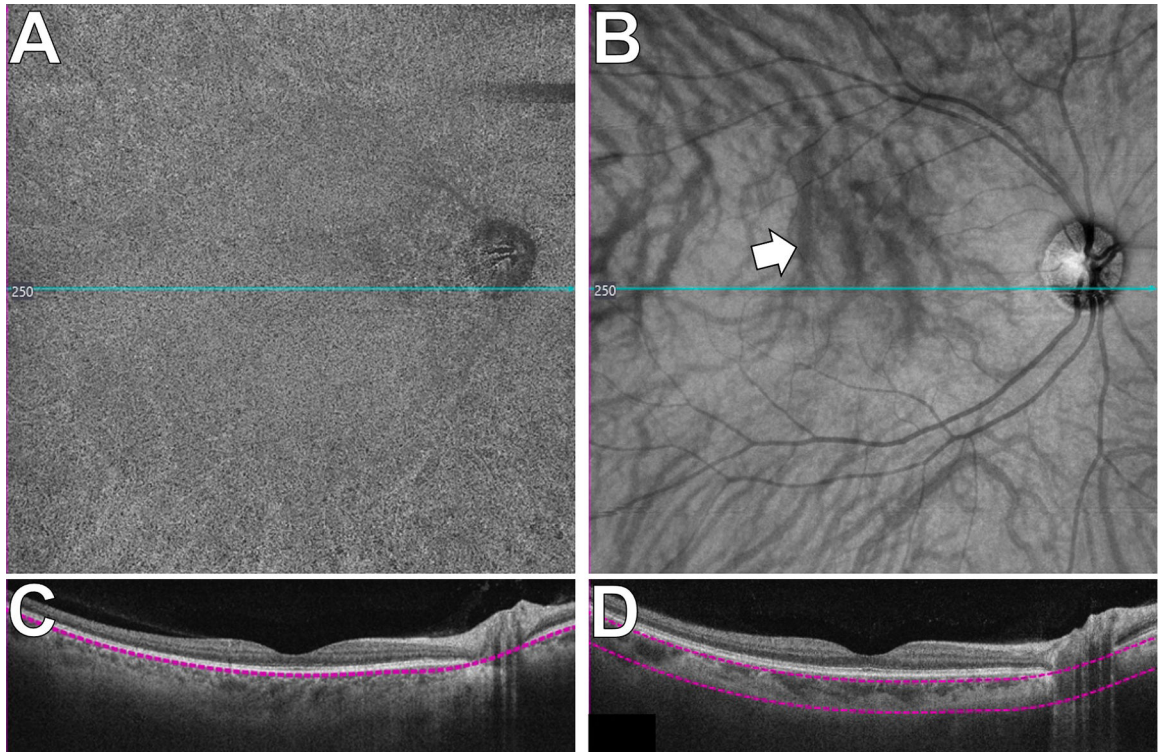
Figure 8.
Choroidal visualization on a normal indocyanine green angiogram.
A: Sixteen seconds after dye injection, the retinal and choroidal circulations have begun to fill. There is patchy choroidal filling by the nerve (arrow).
B: Thirty seconds later, the entire retinal and choroidal circulations have filled. There is no choroidal flush to obscure the choroidal vessels. Vortex veins are indicated by arrowheads.
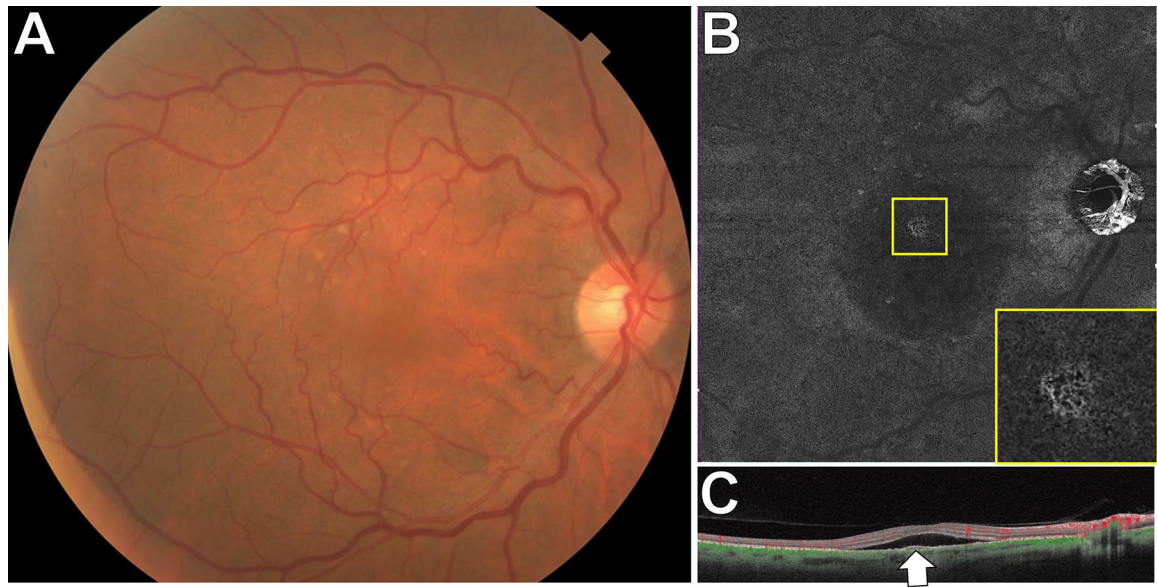
Figure 9.
Choroidal visualization via swept source optical coherence tomography (SS-OCT) in the central 12×12mm of a normal retina.

A: SS-OCT angiogram of the choriocapillaris. The speckled pattern is normal.

B: En face SS-OCT of the large choroidal vessels. The choroidal vessels appear dark (arrow; see text). The overlying retinal vessels cause shadowing artefact.

The blue lines in **A** and **B** indicate the position of the OCT line scans in **C** and **D**, respectively. The purple segmentation lines in **C** and **D** define the depth of tissue depicted in the en face images (**A** and **B**).

Figure 10.
SS-OCTA of choroidal neovascularization in age-related macular degeneration. **A**: This 64 year-old patient was referred with a diagnosis of subretinal fluid from central serous chorioretinopathy. There were multiple large, soft drusen bilaterally.**B**: A 12×12mm SS-OCTA segmented to include detectable flow at the level of the RPE and a few microns below it showed a CNV (magnified view, inset). **C**: The corresponding SS-OCT line scan had an RPE elevation corresponding to the CNV.
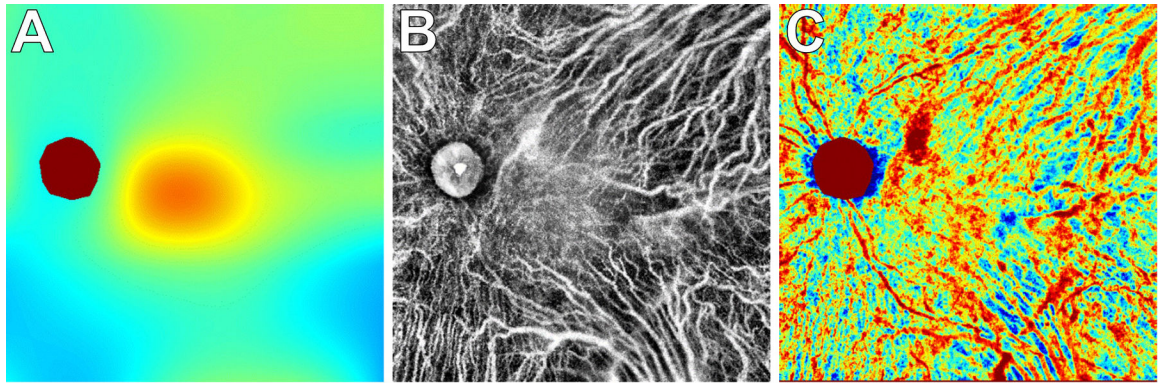
Figure 11.
En face SS-OCT imaging to create choroidal thickness and vascularity maps.
This patient with proliferative diabetic retinopathy was imaged with 12×12mm SS-OCT
to generate a choroidal thickness map (**A**), choroidal vascularity map (**B**), and choroidal
vascularity index map (**C**). Areas with a thicker choroid or larger CVI measurements are
depicted with warmer colors in the respective topographic heat maps (A, C). See Russell et
al. (manuscript in revision) for more details.

Table 1:

Genes enriched in different choroidal endothelial cell populations.

The top-ten genes enriched in choroidal arteries, veins, and choriocapillaris (CC) endothelial cells were identified by differential expression (Voigt et al., 2020c).

Rank	Artery genes	Vein genes	CC genes
1	IGFBP3	DARC	CA4
2	ADAMTS1	CLU	RGCC
3	CLDN5	TIMP1	PLVAP
4	HEY1	TGFBR3	VWA1
5	GJA4	GPR126	STC1
6	SEMA3G	NR2F2	ADM
7	GJA5	ACTN1	BTNL9
8	FAM107A	PKHD1L1	EDNRB
9	BGN	SELE	IGFBP6
10	CP	CPE	ITIH5