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Exploring the choroidal vascular labyrinth and its molecular and structural roles in health and disease

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ABSTRACT

The choroid is a key player in maintaining ocular homeostasis and plays a role in a variety of chorioretinal diseases, many of which are poorly understood. Recent advances in the field of single-cell RNA sequencing have yielded valuable insights into the properties of choroidal endothelial cells (CECs). Here, we review the role of the choroid in various physiological and pathophysiological mechanisms, focusing on the role of CECs. We also discuss new insights regarding the phenotypic properties of CECs, CEC subpopulations, and the value of measuring transcriptomics in primary CEC cultures derived from post-mortem eyes. In addition, we discuss key phenotypic, structural, and functional differences that distinguish CECs from other endothelial cells such as retinal vascular endothelial cells. Understanding the specific clinical and molecular properties of the choroid will shed new light on the pathogenesis of the broad clinical range of chorioretinal diseases such as age-related macular degeneration, central serous chorioretinopathy and other diseases within the pachychoroid spectrum, uveitis, and diabetic choroidopathy. Although our knowledge is still relatively limited with respect to the clinical features and molecular pathways that underlie these chorioretinal diseases, we summarise new approaches and discuss future directions for gaining new insights into these sight-threatening diseases and highlight new therapeutic strategies such as pluripotent stem cell–based technologies and gene therapy.

1. Introduction

The choroid is one of the most complex vascular networks in the human body and is located between the sclera and the retinal pigment epithelium (RPE), which forms the outer blood-retinal barrier. Choroidal circulation is essential for maintaining retinal homeostasis, and a key function of the choroid is to support the high metabolically active photoreceptor cells located in the outer retina by delivering oxygen and nutrients and removing waste products. Importantly, the choriocapillaris is shaped in such a way that the contact area between the luminal surface of the choriocapillaris and Bruch's membrane (BM) is maximised, thus optimising the exchange of nutrients and metabolic waste (Nickla and Wallman, 2010; Yu et al., 2014a). Several other anatomical layers can be distinguished in the choroid, with Sattler's layer and Haller's layer together comprising the largest portion of the choroid's total thickness. These layers contain an abundance of arteries and veins in order to provide a high rate of perfusion. The choroid is comprised of a variety of cell types, including choroidal endothelial cells (CECs), melanocytes, fibroblasts, pericytes, vascular smooth muscle cells, non-vascular smooth muscle cells, intrinsic choroidal neurons, and immune cells. Together, these cell types support a range of functions, including regulation of the immune response, heat dissipation, and perhaps even the modulation of scleral growth during emmetropisation

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Abbreviations

AMD	age-related macular degeneration
BM	Bruch's membrane
CEC	choroidal endothelial cell
CFH	complement factor H
CNV	choroidal neovascularisation
CSC	central serous chorioretinopathy
DR	diabetic retinopathy
EC	endothelial cell
EDI-OCT	enhanced depth imaging optical coherence tomography
eNOS	endothelial nitric oxide synthase
FA	fluorescein angiography
GA	geographic atrophy
ICGA	indocyanine green angiography
iNOS	inducible nitric oxide synthase
MMD	myopic macular degeneration
mRNA	messenger RNA
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
OCT	optical coherence tomography
OCTA	optical coherence tomography angiography
PCA	posterior ciliary artery
PCV	polypoidal choroidal vasculopathy
PDT	photodynamic therapy
PLVAP	plasmalemmal vesicle-associated protein
REC	retinal endothelial cell
RPE	retinal pigment epithelium
scRNA-seq	single-cell RNA sequencing
SDD	subretinal drusenoid deposit
SD-OCT	spectral domain OCT
SNPs	single nucleotide polymorphisms
SRF	subretinal fluid
SS-OCT	swept-source OCT
TLR	Toll-like receptor
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor

(Nickla and Wallman, 2010).

During embryogenesis, differentiation of the choroid (mesodermal in origin) is highly dependent upon the RPE (ectodermal in origin), which secretes vascular endothelial growth factor (VEGF) and other growth factors basally towards the choriocapillaris (Blaauwgeers et al., 1999; Marneros et al., 2005). Interestingly, VEGF secreted by the RPE maintains the fenestrations in the choriocapillaris (Marneros et al., 2005), microscopic pores in specific cellular membranes of the endothelium that facilitate rapid transendothelial transport of small molecules such as glucose (Bosma et al., 2018). Signalling by the RPE also prevents inappropriate apoptosis or proliferation of CECs that may otherwise lead to choroidal atrophy or choroidal neovascularisation (CNV) (Schlingemann, 2004). CNV is further prevented by either the physical boundary formed by BM or – in the case of pathology – by local fibrotic scar tissue. Components of the extracellular matrix in the choroidal stroma also play an important role in maintaining the delicate balance between CEC atrophy and proliferation by regulating "survival" or anti-angiogenesis. Thus, age-related changes in BM and a disruption of the trophic interaction with the RPE are fundamental features of degenerative chorioretinal diseases such as age-related macular degeneration (AMD), CNV, and pathological myopia.

In addition to its role in degenerative diseases, the choroid is also involved in the pathogenesis of uveitis, the pachychoroid disease spectrum, and systemic diseases such as diabetes mellitus and hypertension. Here, we first review the choroid from a physiological perspective at both the anatomical and functional levels, focusing on the choroidal endothelium. Subsequently, we review transcriptomics studies based on CEC subpopulations and the phenotype of human primary cultured CECs, and we discuss the role of these studies in improving our understanding of the pathophysiology of chorioretinal diseases at the molecular level. Finally, these aspects of the choroid are discussed in the clinical context of a variety of chorioretinal diseases.

2. Anatomy and physiology of the choroid

2.1. Development of the choroid

During embryogenesis, the human eye develops three distinct vascular systems: the vitreous vasculature, the choroid, and the retinal vasculature, which begins to develop in gestational week 5, 6, and 12, respectively (Lutty and McLeod, 2018). Development of the mesoderm-derived choroid begins with haemovasculogenesis, which is the formation of blood vessels from haemangioblasts independent of the existing vasculature (Sequeira Lopez et al., 2003). This process is induced by differentiated RPE cells in the optic cup via signalling molecules, such as VEGF and basic fibroblast growth factor (bFGF) (Saint-Geniez and D'Amore, 2004). Islands of multipotent cells arise from these haemangioblasts and develop into vascular cords, which later interconnect (Lutty et al., 2010). By gestational week 9–11, a structurally immature capillary network has formed, and the first premature lumina are visible, forming the basis of the choriocapillaris (Lutty et al., 2010).

Development of larger choroidal vessels starts in gestational week 11–12 (Lutty et al., 2010). During this process, medium-sized blood vessels bud from the scleral side of the premature choriocapillaris. Formation of these vessels is no longer driven by vasculogenesis, but rather by angiogenesis, a fundamentally different process. Indeed, unlike vasculogenesis angiogenesis depends on endothelial cell (EC) precursors that are already present in existing vessels within the developing choroid (Chan-Ling et al., 2011; Lutty et al., 2010). The newly formed intermediate vessels are then believed to eventually anastomose with larger vessels protruding from the posterior wall and organise into a complex network (Lutty and McLeod, 2018; Saint-Geniez and D'Amore, 2004). Further expansion and remodelling of the choroidal vasculature occurs through gestational week 22 and includes the formation of fenestrations and maturation of the basal lamina formed by the ECs (Lutty and McLeod, 2018). In the developing choroid, genes that encode regulators of angiogenesis, including proteins involved in arterial specification and capillary sprouting, are expressed at high levels (Voigt et al., 2020). Furthermore, compared to the mature choroid, the developing choroid has high expression of CD34 (a marker for haematopoietic stem and progenitor cells) and actively suppresses the expression of the pro-inflammatory cell surface markers VCAM1 (vascular cell adhesion molecule 1) and ICAM-1 (intercellular adhesion molecule 1) (Voigt et al., 2020).

All other cell types in the choroid, including stromal cells, melanocytes, and pericytes, are derived from the cranial neural crest and migrate around the optic vesicle as undifferentiated mesenchymal cells during closure of the neural tube, followed by their final differentiation (Saint-Geniez and D'Amore, 2004).

2.2. Structural organisation of the choroid

Running posterior to anterior, the choroid extends from the optic nerve to the ora serrata, covering approximately 5/6th of the posterior surface of the globe. At the ora serrata, it forms a continuum with the

ciliary body. Together, the choroid, ciliary body, and iris comprise the uvea. In healthy individuals, the thickest part of the choroid is localised in the macular region, also the thickest part of the neuroretina (excluding the fovea). The maximum thickness of the macular choroid is approximately 350 μm, although this varies widely among individuals (Hoseini-Yazdi et al., 2019; Shin et al., 2012). At the periphery, choroidal thickness is approximately 264 μm (Hoseini-Yazdi et al., 2019). The innermost part of the choroid is attached to BM, which separates the choroid from the RPE monolayer. On the outmost side, the choroid is attached to the sclera via adhesive fibres in the region of the optic nerve, while the rest of the choroid is loosely attached to the scleral surface. Based on early histological studies by Haller and Sattler, the choroid is stratified into three layers from interior to exterior. The choriocapillaris which contributes to the structure of BM. The middle layer (known as Sattler's layer) contains medium-sized choroidal vessels, and the outer layer (known as Haller's layer) contains large choroidal vessels (Hayreh, 1975; Nickla and Wallman, 2010; Sattler, 1876).

The choroidal vasculature is organised as a continuum of diverging and converging blood vessels that have a predominantly segmental distribution and are characterised by watershed areas (i.e. overlap between the regions covered by blood vessels) and a lack of anastomoses at most levels (Fig. 1) (Hayreh, 1975; Spaide, 2020). Several orders of choroidal arteries and veins (i.e. first-order, second-order, etc.) have been described based on the number of branches and vessel diameters, and based on the characteristic filling patterns observed on fluorescein angiography (FA) and indocyanine green angiography (ICGA) (Hayreh, 1975; Lee et al., 2017; Spaide, 2020; Zouache et al., 2016). Inherent to this segmented anatomy, distinct clinical phenomena may be restricted to apparent anatomical borders, including ischaemic, metastatic, inflammatory, and degenerative diseases of the choroid, as discussed below (Hayreh, 1975, 1990, 2004). In the next several sections, we describe the organisation of the choroidal blood flow and the molecular features and functions of the choroidal layers, from the outermost layer to the innermost layer.

2.2.1. *Organisation of the choroidal blood supply*

Choroidal blood flow is determined primarily by the structure of the choroid and the anatomical organisation of its arteries and veins. Arterial blood to the choroid is supplied by the ophthalmic artery, which is a branch of the internal carotid artery. The ophthalmic artery itself has multiple branches categorised into orbital and optical branches (Bird and Stawicki, 2019). The orbital arteries of the ophthalmic artery include the posterior and anterior ciliary arteries, the central retinal artery, and the muscular arteries, with the posterior ciliary arteries (PCAs) supplying blood to the choroid. Typically, two medial PCAs supply the choroid at the nasal side of the optic nerve, and one lateral PCA supplies the choroid at the temporal side of the optic nerve (Fig. 1A) (Hayreh, 2004). These arteries appear to provide strictly segmental choroidal blood flow that typically includes the part of the choroid covering the anterior optic nerve head; however, anatomical variations are common (Hayreh, 2004). Due to this segmental nature of the PCAs, compromised blood flow in one PCA – for example, due to ischaemia or arteritis – can affect up to half of the choroidal circulation, including the respective region of the anterior optic nerve head (Hayreh, 1990, 2009). PCA occlusion – commonly referred to as anterior ischaemic optic neuropathy in clinical practice – is often complicated by optic disc oedema due to acute ischaemia of the optic nerve head, causing severe visual impairment (Hayreh, 2009). The portion of the optic nerve that extends from the optic nerve head is dependent on the blood supply provided by the pial vascular plexus, and ischaemia in this vasculature causes posterior ischaemic optic neuropathy (Hayreh, 2009).

At several points around the optic nerve, the lateral and medial PCAs subdivide into branches classified as either short PCAs (with 6–12 per eye) or long PCAs (with 2 per eye) (Fig. 1A) (Hayreh, 1975). The short PCAs cross the sclera taking the shortest possible route, while the long PCAs extend rather horizontally through the sclera to the anterior part of the eye, where they supply blood to the peripheral choroid and part of the ciliary body and iris. The short PCAs supply blood to triangular-shaped areas of the choroid. These triangular-shaped areas have the highest overlap in the posterior globe in the macular region, from which each of these triangular-shaped areas diverges radially in the anterior direction to the ora serrata (Fig. 1B). Infarction of both short and long PCAs can be caused by inflammation (a condition known as giant cell arteritis) and orbital trauma. Typically, these rare infarctions are visible as wedge-shaped defects on FA, a feature known as the "triangular sign of Amalric" (Hayreh, 1990; Nemiroff et al., 2017). Given the anatomical location of the short PCAs just outside the macula, where all watershed areas diverge, the centre of the macula is believed to be particularly vulnerable to generalised vascular choroidal disorders (Fig. 1B) (Hayreh, 1975, 2004).

Subdivisions of short PCAs form smaller segmental blood vessels that can appear as irregularly shaped geographical filling defects on FA following an ischaemic event (Hayreh, 1975, 1990, 2004). The arterial branches arising from each short PCA are organised to form a continuum gradually extending towards the choriocapillaris (Hayreh, 1975). The smallest subdivisions of short PCAs are non-communicating terminal arterioles, each of which has been proposed to supply a single demarcated choriocapillaris lobule, thus creating a characteristic mosaic pattern on FA and ICGA when applying artificially increased intraocular pressure (IOP) (Hanyuda et al., 2017; Hayreh, 1975, 1990). High-resolution histological analysis of the choroid has identified that the terminal arterioles in the choriocapillaris are located at the lobular periphery, and has localised the collecting venules of the choriocapillaris predominantly in the central part of the lobuli (McLeod and Lutty, 1994). Therefore, it has recently been proposed that the terminal arterioles localised at the edges may supply multiple lobules, with each lobule containing a centrally located collecting venule (Hanyuda et al., 2017). The arterioles that branch out from the short PCAs located in Sattler's layer and Haller's layer – which contain medium-sized and large-sized vessels, respectively – have not been mapped specifically to either layer.

Venous blood from the choroid and other uveal tissues is returned via the vortex vein system, which originates in numerous small venules that converge to form choroidal veins. These veins traverse the posterior pole, running parallel towards the equator, and typically merge into four vortex veins per eye, each of which drains its own quadrant of the globe (Fig. 2) (Jung et al., 2020; Paula et al., 2013). At the watershed zones, anastomoses are formed between the superior and inferior vortex veins and appear to be more pronounced in diseases in the pachychoroid spectrum, where they have been suggested to alleviate venous congestion (Jung et al., 2020; Matsumoto et al., 2020b; Spaide et al., 2020b, 2021). Notably, most choroidal veins merge just before exiting the sclera in a dilated venous structure called the ampulla (Paula et al., 2013; Spaide, 2020). Using ultra-widefield ICGA imaging, the anatomical configuration of the choroidal veins and vortex veins can be visualised out to the extreme periphery (Jung et al., 2020; Klufas et al., 2015). Interestingly, a recent study using ultra-widefield ICGA found that venous outflow is higher in the inferior quadrants than in the superior quadrants, possibly due to either gravitational forces or anatomical differences between the superior and inferior ophthalmic veins (Fig. 2) (Jung et al., 2020).

Vortex veins leave the sclera in the equator area and drain into the ophthalmic veins that terminate in the cavernous sinus. Outflow from the vortex veins can be blocked during scleral buckling procedures, which can lead to choroidal effusion, haemorrhage, and/or remodelling of choroidal blood flow (Spaide, 2020). Furthermore, backflow and thrombosis in the superior ophthalmic veins has been described in the context of sinus cavernosus arteriovenous malformations and sinus cavernosus thrombosis, which can lead to choroidal effusion or even serous retinal detachment reminiscent of central serous chorioretinopathy (CSC) (Spaide, 2020; Stiebel-Kalish et al., 2002). Another example

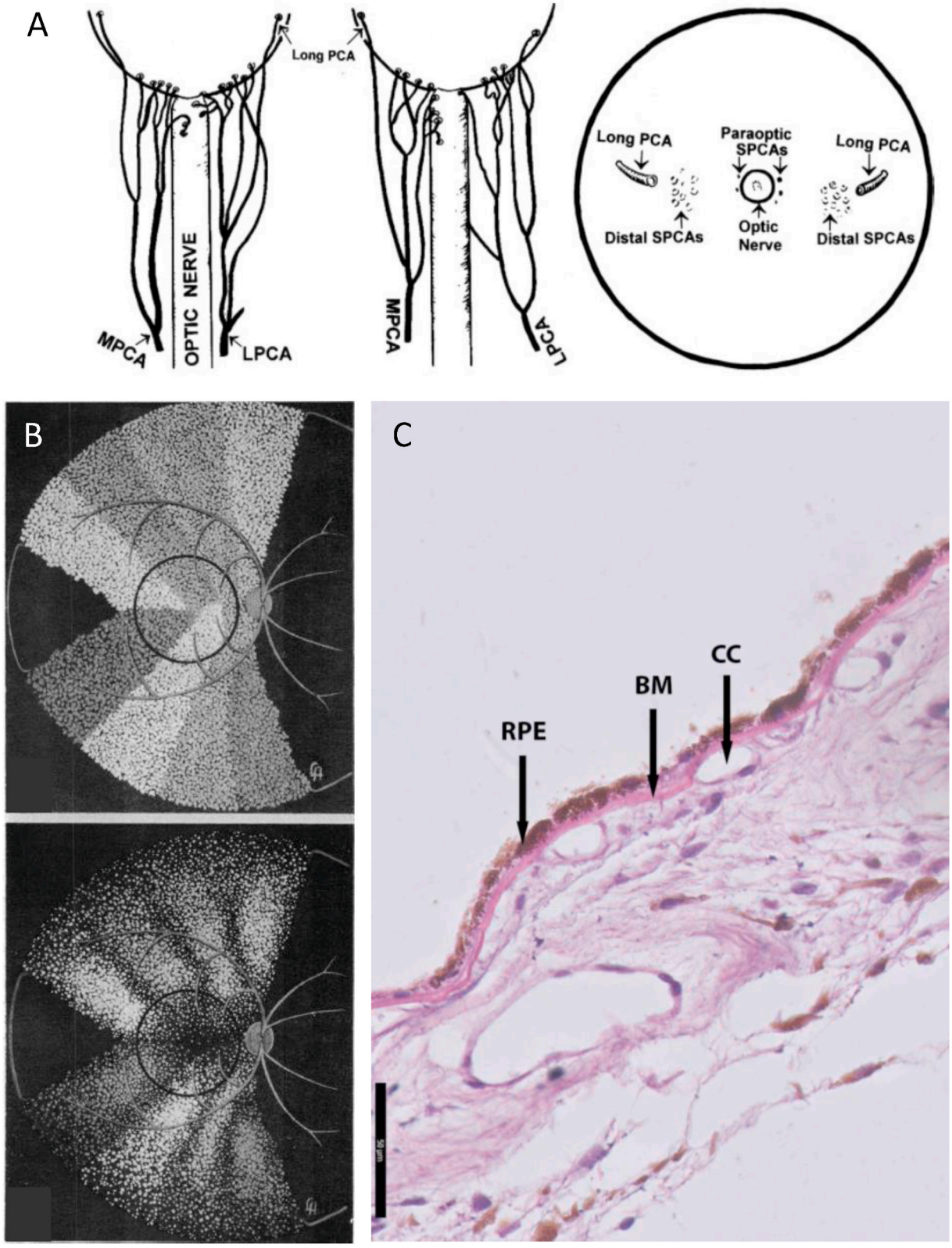


Fig. 1. An overview of the anatomy of the choroid. (A) Reproduced with permission from Hayreh (2004). Schematic representation of the branching pattern of medial and lateral posterior ciliary arteries in 2 eyes (illustrations at the left and the middle) and of the site of entry of the various branches of the posterior ciliary arteries, as seen at the back of the eye (illustration at the right). (B) Reproduced with permission from Hayreh (1975). Schematic representation of distribution of choriocapillaris supplied by various temporal short posterior ciliary arteries. (Top) Normal pattern. (Bottom) Postulated pattern caused by generalised chronic ischaemic disorder of the choroid, with reduction of choriocapillaris most marked in macular region and equatorial choroid. (C) A haematoxylin & eosin staining of a cross section of the human choroid showing the retinal pigment epithelium (RPE), Bruch's membrane (BM), and the choriocapillaris (CC). In the deeper layers, the lumen of a large vessel and the presence of other cell types resident in the choroid, such as melanocytes, can be observed. Scale bar = 50 μm.

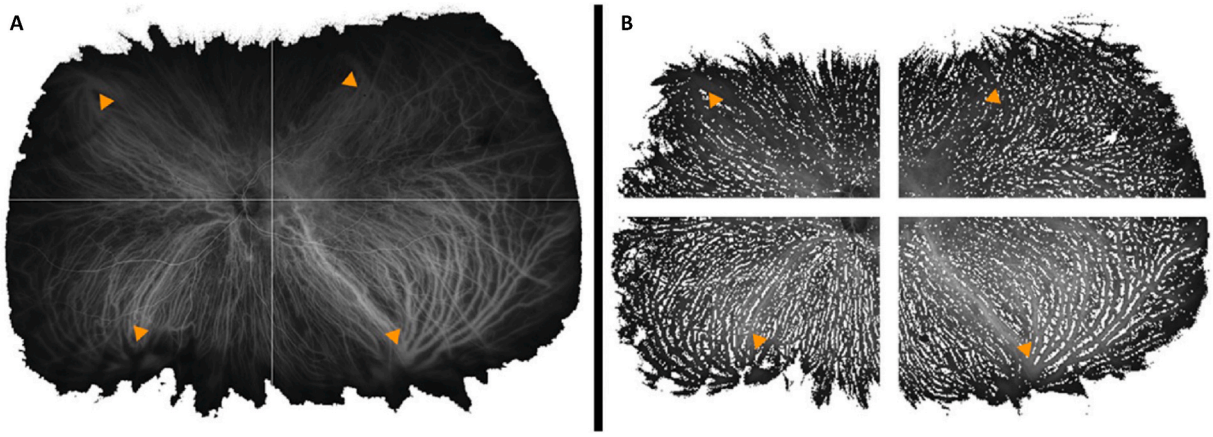


Fig. 2. Reproduced with permission from Jung (2020). Representative mid-phase ultra-widefield indocyanine green angiography images (Optos California; Optos, Inc., Dunfermline, UK) after removal of imaging artefacts from a left eye diagnosed with chronic central serous chorioretinopathy. (A) Multilevel image thresholding with Otsu's method and local adaptive threshold calculations based on pixel values and (B) after segmentation to quadrants based on the most central extension of the choroidal vessel draining into respective vortex veins (yellow arrowheads). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of venous outflow obstruction is uveal effusion syndrome, a condition presumed to be primarily caused by scleral abnormalities (Elagouz et al., 2010).

2.2.2. *Bruch's membrane*

BM is an acellular layered sheet of extracellular matrix approximately 2–4 μm thick located between the RPE and the choriocapillaris (Fig. 1C). BM plays an important role in both structure and transport function, which are relevant both in the normal functioning of the RPE/choroid complex and in the course of chorioretinal diseases. With respect to disease, BM is affected in AMD and in genetic disorders that affect connective tissue, including pseudoxanthoma elasticum, Alport syndrome, Marfan syndrome, and Sorsby's fundus dystrophy (Booij et al., 2010a). An important functional role of BM is to provide physical support for RPE cells and CECs, as well as elasticity to the eye. BM also permits the bi-directional diffusion of molecules between the choroid and the RPE, thus supporting photoreceptors and draining metabolic waste. Under physiological conditions, BM plays an essential role in maintaining the anatomical structure by inhibiting the migration and division of the RPE and CECs (Booij et al., 2010a).

Both the RPE and the choroid contribute to the molecular composition of BM, which has been widely studied in the context of AMD. Choroidal cells, including CECs, fibroblasts, and smooth muscle cells, have been suggested to produce approximately 75% of the proteins in BM, including fibronectin, collagens, heparan sulfate proteoglycans, and other proteoglycans. Furthermore, half of the remaining proteins in BM are believed to be produced predominantly by the RPE, with the remaining 12.5% being produced by both RPE cells and choroidal cells (Booij et al., 2010a; Sivaprasad et al., 2005; Whitmore et al., 2015). Histologically, BM consists of three layers enclosed by the basement membrane of the choriocapillaris on the outermost side and the basement membrane of the RPE on the innermost (Guyer et al., 2006). These three layers include the inner collagenous layer, the elastin layer, and the outer collagenous layers (Booij et al., 2010a; Hogan, 1961). The elastin layer of BM consists primarily of elastin fibres, collagen type VI, and fibronectin, and is 3–6 times thinner at the macula compared to the periphery. This thinness is believed to contribute to the macula's vulnerability to CNV, as the elastin fibres have important biomechanical and anti-angiogenic properties (Chong et al., 2005). The elastin layer is covered on both sides by collagenous layers, which are nearly identical to each other and consist primarily of type I, type III, and type V collagen fibres (Booij et al., 2010a). The basement membranes adjacent to the collagenous layers are also highly similar in content and have a composition similar to other basement membranes in the body, consisting of collagen type IV, laminin, fibronectin, heparan sulfate, and chondroitin/dermatan sulfate (Booij et al., 2010a). On the outermost side of BM, collagen type VI is present in the choriocapillaris basement membrane, where it is believed to play a molecular role in anchoring the choriocapillaris (Marshall et al., 1994). In contrast to the flat basement membrane of the RPE, which supports this highly organised monolayer of cells, the basement membrane of the choriocapillaris follows the irregular contours of the CECs in order to maximise surface contact and promote efficient transendothelial transport.

Interestingly, processes extending from the CECs can penetrate the basal lamina layer of the choriocapillaris and reach BM, similar to pseudopodia or microvilli (Guymer et al., 2004; Manche and Korte, 1990). Although the precise function of these 'foot-like' CEC processes is unclear, they have been suggested to have several functional roles, including mechanosensing tractional forces on BM, preventing collapse of the vascular lumina by anchoring BM, stabilising the microvascular wall, transporting metabolites and hormones, and phagocytosis (Guymer et al., 2004). Although these structures are believed to be present under physiological conditions, they may also play a pathological role, for example in the pathogenesis of CNV, as these CEC processes can pierce BM and may therefore serve as a starting place for neovascular outgrowth (Guymer et al., 2004). However, direct evidence of a relationship between these processes and CNV is lacking.

2.2.3. *Choriocapillaris*

The choriocapillaris is a vascular network located directly beneath BM, facilitating the transport of nutrients and respiratory gases in order to support the high metabolic demands of the outer retina (Fig. 1C) (Nickla and Wallman, 2010; Wangsa-Wirawan and Linsenmeier, 2003). Conversely, metabolic waste produced primarily by the photoreceptors is transported to the systemic bloodstream via the choriocapillaris. The choriocapillaris is a single layer comprised of a densely interconnected network of capillaries (Nickla and Wallman, 2010; Otsuji et al., 2002; Yu et al., 2014a). The highest density of capillaries is located in the macular area, where the choriocapillaris is thickest (reaching approximately 10 μm), which then gradually decreases in both density and thickness towards the periphery (Nickla and Wallman, 2010; Otsuji et al., 2002; Yu et al., 2014a). The choriocapillaris is best studied using histopathological sections, as the resolution achieved with optical coherence tomography (OCT) scans is currently insufficient for identifying details in this thin vascular layer (Ferrara et al., 2016; Spaide, 2021). Another emerging modality for imaging of the choriocapillaris is optical

coherence tomography angiography (OCTA), which is further discussed in section 4.1.1.3.

In the horizontal plane, the choriocapillaris is subdivided into non-communicating terminal lobules that resemble a 'honeycomb' pattern on FA, and the blood vessels entering these terminal lobules are abundant and highly anastomosed (Hayreh, 1975; Lee et al., 2017; Nickla and Wallman, 2010; Yu et al., 2014a). This specialised vascular architecture ensures the continuous and sufficient perfusion and oxygenation of the entire choriocapillaris, which is particularly important in the foveal avascular zone where the function of the outer photoreceptor segments is heavily dependent on the choroidal circulation. Structurally, the shape of the choriocapillaris allows for the optimal exchange of molecules to and from the outer retina. Specifically, the lumina of the choriocapillaris have a relatively large diameter in the horizontal plane (Yu et al., 2014a). Furthermore, the intercapillary spaces contain extracellular matrix and fibrous structures called 'intercapillary columns', which are believed to play a role in stabilising the vascular lumen (Booij et al., 2010a; Lengyel, 2004; Nickla and Wallman, 2010). Interestingly, erythrocytes travel approximately 5 times more slowly through the choriocapillaris compared to the retinal vasculature (Wajer et al., 2000); this difference may be due to the relatively large lumina of the choriocapillaris and the large blood volume in the choroid, as well as the lobular configuration of the choriocapillaris, through which erythrocytes traverse via numerous parallel routes (Hayreh, 1975; Wajer et al., 2000). Although choroidal haemodynamics are not fully understood, the slow movement of erythrocytes in the choroid may serve to optimise the transport of oxygen from the choriocapillaris to the photoreceptors, which consume the highest amount of oxygen per gram of tissue in the entire body (Alm and Bill, 1972; Wajer et al., 2000).

2.2.4. Sattler's layer and Haller's layer

The vasculature between the choriocapillaris and the suprachoroid has traditionally been subdivided into two distinct layers. Sattler's layer lies directly under the choriocapillaris and contains medium-sized blood vessels, whereas Haller's layer contains large vessels and lies under Sattler's layer. The stroma of Sattler's layer and Haller's layer contains collagen and elastic fibres, which support the structure and plasticity of these tissues. On histological sections, the anatomical borders are not clearly distinguishable (Fig. 1C), and the thickness of each layer varies among reports. This difference is in part due to the fact that different groups use different factors to define each layer, for example a cut-off value for vessel diameter versus the intensity of the intervascular tissue (Branchini et al., 2013b; Zhao et al., 2018). Moreover, histological measurements do not necessarily correlate with measurements obtained using an OCT scan due to shrinking of the tissue *ex vivo* and the presence of intravascular pressure *in vivo* (Li et al., 2016). Zhao and colleagues used EDI-OCT (enhanced depth imaging optical coherence tomography) to study nearly 2000 healthy participants and found that the subfoveal thickness of Sattler's layer and Haller's layer were 92 ± 39 μm and 155 ± 66 μm, respectively (Zhao et al., 2018). This relatively large study used OCT parameters to delineate both layers, defining Sattler's layer as hypo-intense lumens with hyper-intensive stroma, and defining Haller's layer as larger hypo-intense lumens with less dense intervascular tissue compared to Sattler's layer (Zhao et al., 2018). The authors reported that the thickness of these layers varied widely among the participants, even though they were healthy individuals without any known ocular disease. Furthermore, they found that both increasing age and longer axial length were correlated with reduced total choroidal thickness, generally reflected by thinning of Sattler's layer and Haller's layer rather than thinning of the choriocapillaris.

Although both layers can be distinguished on OCT, some anatomists consider Sattler's layer and Haller's layer to be a single layer consisting of a continuum of vessels with gradual changes in diameter (Borrelli et al., 2018). Indeed, these two layers do not appear to differ significantly with respect to their function, and this is reflected by similarities between these layers in terms of their extracellular matrix and cell types, as discussed further in section 2.2.6 (Nickla and Wallman, 2010). Despite their anatomical and physiological overlap, the distinction between Sattler's layer and Haller's layer has high clinical value for understanding chorioretinal diseases, which is discussed in Chapter 4.

2.2.5. Suprachoroid

The suprachoroid forms the transition zone between the choroid and the sclera, and consists of layers of loose connective tissue containing collagen fibres, fibroblasts, and melanocytes. The outmost layer of the suprachoroid is called the lamina fusca; a 30-μm thick highly pigmented layer that consists of flattened fusiform melanocytes and fibroblast-like cells that secrete extracellular matrix components (Hogan, 1971; Nickla and Wallman, 2010). In the human eye, the function of the suprachoroid is currently a topic of debate. In avian species, the suprachoroid contains fluid-filled sacs resembling lymphatic structures and is believed to play a role in regulating choroidal thickness in response to retinal defocus (Nickla and Wallman, 2010). However, whether these lymphatic-like structures are present in the suprachoroid in the human eye is controversial (Schroedl et al., 2008). In addition, the suprachoroid has been described as a space maintained by hydrostatic pressure and fluidic dynamics, as it is not always readily identifiable and/or translatable to histological sections obtained from enucleated eyes even when a suprachoroidal space was identified on OCT prior to enucleation (Li et al., 2016). Interestingly, clinical trials currently ongoing evaluate whether the suprachoroidal space can serve as a suitable site for drug delivery to the posterior segment of the eye (Chiang et al., 2018; Kansara et al., 2020).

2.2.6. Other cell types in the niche of the choroidal endothelial cell

Choroidal blood flow is regulated not only by CECs, but also by an interconnected network of various cell types that communicate via vasoactive factors (Benedicto et al., 2017; Rafii et al., 2016). Recently, single-cell RNA sequencing of both mouse and human choroid samples revealed the presence of stromal fibroblasts, smooth muscle cells, melanocytes, haematopoietic cells, and Schwann cells, along with CECs (Lehmann et al., 2020; Voigt et al., 2019).

Stromal fibroblasts secrete collagen and elastic fibres in order to provide structure and stability to Sattler's layer, Haller's layer, and the suprachoroid. Other cell types include large numbers of heavily pigmented melanocytes, which are present in Sattler's layer, Haller's layer, and the suprachoroid, where they play a role in maintaining ocular homeostasis by absorbing light, regulating oxidative stress, and mediating the immune response (Sitiwin et al., 2019). Non-vascular smooth muscle cells are also present in the human choroid and have been suggested to play a dynamic role in choroidal thickness, although this role is based primarily on studies involving birds and non-human primates (Nickla and Wallman, 2010; Reiner et al., 2018). Perivascular cells, including pericytes and vascular smooth muscle cells, contribute to the structural and functional stability of blood vessels and regulate blood flow (Yamazaki and Mukouyama, 2018). Vascular smooth muscle cells cover large-diameter vessels but do not cover capillaries (Yamazaki and Mukouyama, 2018). Pericytes support capillaries and venules and play an important role in the maturation and maintenance of vascular branching (Bergers and Song, 2005; Murfee et al., 2005; Voigt et al., 2019). Importantly, the precise distribution and significance of pericytes in the human choroid has not been examined. In mice, however, the distribution of pericytes in the choroid has recently been examined based on expression of the pericyte marker α-SMA (alpha-smooth muscle actin) (Kim et al., 2020b). However, it should be noted that this marker is also expressed in smooth muscle cells and fibroblasts (Corliss et al., 2019), cell types that are also present in the choroid; moreover, not all pericytes express α-SMA (Schlingemann et al., 1991). A variety of immune cells, including mast cells, macrophages, and T cells, are also found in both Sattler's layer and Haller's layer, where they play a central role in the immunological state of the eye. Finally, mesenchymal stem cell–like cells were recently reported to be present in the mouse choroid

(Lehmann et al., 2020).

2.3. The ageing choroid

Ageing is an established risk factor for a wide range of chorioretinal diseases. Thus, notable overlap exists between natural age-related choroidal changes and processes that can be interpreted as either the first signs of disease or susceptibility to chorioretinal pathology and disease progression. In this respect, age-related choroidal changes are particularly interesting, as they may provide important clues regarding the pathogenesis and treatment of a variety of chorioretinal diseases.

Using histopathological data based on 95 human eyes obtained from donors ranging from 0 to 100 years of age, Ramrattan et al. examined age-related changes in BM, the choriocapillaris, and choroidal thickness, and analysed these changes over a 10-decade lifespan (Ramrattan et al., 1994). On average, they found that the thickness of BM increased from 2.0 μm to 4.7 μm; during the same time frame, the density of the choriocapillaris decreased by 45%, and the diameter of the choriocapillaris lumen decreased by 34%. The authors also found that on average, total choroidal thickness decreased drastically from 193.5 μm to 84 μm (Ramrattan et al., 1994). More recently, age-related thinning of the choroid has been described in imaging studies using EDI-OCT (Ruiz-Medrano et al., 2017; Zhao et al., 2018); in these studies, the decrease in total choroidal thickness was found to be primarily due to atrophy of Haller's layer.

These findings with respect to age-related changes in choroidal structures were recently corroborated using OCTA. Interestingly, haemodynamics in the choriocapillaris can be assessed *in vivo* using long-wavelength swept-source OCTA, yielding several new findings (Sacconi et al., 2019; Zheng et al., 2019). For example, Zheng et al. found that loss of the choriocapillaris occurs primarily within the central 1 mm of the macula (Zheng et al., 2019). Although they had no explanation for this finding, the authors speculated that this preferentially localised loss of choriocapillaris vascularity may explain the localisation of CNV in AMD, which is often central and has been suggested to be triggered by hypoxia (Blaauwgeers, 1999; Zheng, 2019). In addition, Sacconi et al. used swept-source OCTA to study healthy individuals of various ages and found a marked decrease in the perfusion density of the choriocapillaris with increasing age; the authors attributed this finding primarily to a decrease in capillary diameter, not to a reduced number of capillaries (Sacconi et al., 2019).

Both thickening of BM and a decrease in submacular vascularity are normal changes that occur with ageing, and these changes may also be associated with a decreased capacity to remove the large amounts of metabolic waste products from the outer retina (Whitmore et al., 2015). The choroidal endothelium is a key player in this process by delivering these waste products to the systemic circulation. Several studies have found an age-related accumulation of various cellular and blood-derived products in the RPE/choroid complex, including minerals, lipids, and advanced glycation end-products (i.e. irreversibly damaged proteins). These materials can accumulate in various compartments and structures, including subretinal, sub-RPE, BM, and as intercapillary pillar deposits, where they affect local physiological processes (Bergen et al., 2019; Chirco et al., 2017a; Lengyel et al., 2004; Li et al., 2009; Whitmore et al., 2015). For example, thickening of BM with ageing reduces the normal transport function of the interface between the RPE, BM, and the choriocapillaris. This change can also precede the development of sub-RPE extracellular deposits localised between the basal lamina of the RPE and the inner collagenous layer of BM. These sub-RPE deposits – known in the clinic as drusen – are a hallmark feature of AMD and contain a wide variety of waste components derived from both local cellular (e.g. photoreceptor and RPE cells) and systemic (e.g. blood and choriocapillaris) sources (Bergen et al., 2019). Other deposits can include 'hard' drusen, which accumulate near collecting venules, and basal laminar and linear deposits, which are associated with capillary dropout (McLeod and Lutty, 1994) and are located primarily in the intercapillary pillars of the choriocapillaris (Lengyel et al., 2004).

In addition to the accumulation of toxic metabolic substances in the posterior pole of the eye, other key protective functions of the choroid are also known to decline with ageing, eventually causing a shift in the delicate balance between the clearance of waste products and cellular damage in the posterior eye (Bergen et al., 2019; Rozing et al., 2020). For example, a localised reduction in the activity and/or expression of several functional proteins such as CD34, complement factor H (CFH), and heparan sulfate proteoglycans, has been suggested to play a role in the development of several chorioretinal diseases (Bergen et al., 2019; Whitmore et al., 2015). Indeed, with ageing, expression of the leukocyte 'anti-adhesive' molecule CD34 (Strilić et al., 2009; Voigt et al., 2020), which is involved in inhibiting the migration of leukocytes into the choroidal stroma, thereby suppressing local inflammation (Sohn et al., 2014), is reduced in CECs. Another example is heparan sulfates, which are present on the cell surface and in the extracellular matrix in BM and in the choroid and are important for regulating local inflammatory stimuli (Booij et al., 2010a). Interestingly, during one's lifetime the levels of heparan sulfate in BM can decrease by approximately 50% (Keenan et al., 2014). Given that heparan sulfates anchor CFH, this decrease in heparan sulfates may perturb local regulation of the complement system, which is an important player in maintaining transport from the choriocapillaris through BM and the RPE (Chirco et al., 2017a).

Another component of the complement system, the membrane attack complex (MAC), is also known to accumulate in the choriocapillaris with ageing. The MAC is one of the major effectors of the complement system, and its accumulation is believed to be an important factor in the disease progression of AMD by causing the lysis of CECs in the choriocapillaris, particularly when either CFH expression is reduced or CFH activity is impaired due to a loss-of-function mutation (Toomey et al., 2018; Whitmore et al., 2015). Interestingly, this CEC lysis, which causes a feature known as 'ghost vessels' on histological sections of the choriocapillaris of AMD patients, is correlated spatially with extracellular accumulations and drusen, suggesting that normal ageing may trigger a vicious cycle as the basis for AMD (Chirco et al., 2017a; Toomey et al., 2018). During ageing, a variety of other choroidal cell types undergo specific changes. For example, CECs in the vortex vein system increase in size and show signs of cellular senescence (Yu et al., 2014b), and RPE cells decrease in number, have reduced numbers of melanosomes (pigment organelles), and accumulate lipofuscin (Chirco et al., 2017a). In addition, the expression of α-SMA decreases in perivascular cells (Kim et al., 2020b), and nerve fibres that help regulate choroidal blood flow degenerate (Jablonski et al., 2007). Moreover, with ageing choroidal melanocytes fuse, forming rosette-like structures consisting of 8–15 cells (Nag, 2015); these melanocytes also accumulate lipid droplets and lipofuscin granules, and have reduced numbers of melanosomes.

2.4. Regulation of the choroidal blood flow: a complex interplay of neural networks

Choroidal blood flow is tightly regulated in order to maintain a healthy, properly functioning neuroretina. Several mechanisms and processes have been found to modulate choroidal blood flow, including neuronal signalling in the retina, autoregulated changes in local pressure, and buffering by the vortex vein system (Reiner et al., 2018; Spaide, 2020). Retinal signalling can alter choroidal blood flow via neuronal networks based on metabolic demand (Reiner et al., 2018). This process circumvents the need to secrete vasoactive signalling molecules that would then have to diffuse through the tight outer blood-retinal barrier of the RPE and act in the context of high choroidal blood flow (Reiner et al., 2018). Neuronal regulation of choroidal blood flow also helps the choroidal circulation adapt to fluctuations in systemic blood pressure, as reviewed thoroughly by (Reiner et al., 2018). Indeed, transneuronal retrograde tracer studies and immunolabelling studies in rodents have shown that nearly the entire choroidal vasculature – with the exception of the choriocapillaris – is innervated by

neurons (Reiner et al., 2018). Similarly, orbital vessels, including the ophthalmic artery and PCAs, receive neuronal innervation, thus regulating blood flow through arterial branches at multiple levels. Deeper in the choroidal vasculature, neurons terminate at vascular smooth muscle cells and pericytes in the walls of arteries and veins, exerting contractile effects (Schrodl et al., 2014). Although a subject of controversy, intrinsic autoregulatory mechanisms within the choroid – possibly acting as a myogenic response to local changes in intraluminal pressure – may also play a role (Calzetti et al., 2018; Kiel, 1999; Nickla and Wallman, 2010; Reiner et al., 2018; Spaide, 2020). Finally, the vortex vein system has been suggested to function as a buffer, compensating for the pulsatile inflow of arterial blood into the choroid, in which the vascular systems are enclosed by the rigid sclera (Spaide, 2020). Spaide recently proposed such a mechanism and suggested that modulation of venous outflow may occur at the level of the ampulla; this so-called 'Starling resistor effect' would be analogous to the venous storage used by the brain to regulate intracranial blood flow (Spaide, 2020). How these various mechanisms interact – and the extent to which each of these mechanisms can affect choroidal blood flow – is largely unknown. However, the putative role of the central nervous system and the coupling between choroidal blood flow and metabolic demand, thermal adaptation, and systemic blood pressure, are discussed in detail in sections 2.4.1 and 2.4.2.

2.4.1. Neuroanatomy and coupling of the choroidal blood flow to systemic blood pressure regulation

The choroid is innervated by three different types of nerve fibres: efferent parasympathetic fibres from the pterygopalatine ganglion, sympathetic fibres from the superior cervical ganglion, and afferent sensory fibres innervating the trigeminal ganglion (Reiner et al., 2018; Spaide, 2020). Choroidal vasodilatation is mediated by parasympathetic fibres, which release vasoactive intestinal polypeptide, nitric oxide (NO) produced by neuronal nitric oxide synthase (nNOS), and acetylcholine. In contrast, choroidal vasoconstriction is mediated by sympathetic fibres, which release noradrenaline and neuropeptide Y. Finally, sensory fibres relay signals from the ophthalmic branch of the trigeminal nerve, exerting a vasodilatory effect by releasing neurotransmitter substance P and the neuropeptide calcitonin gene-related peptide (Reiner et al., 2018; Spaide, 2020). Transneuronal retrograde pathway tracing studies in rodents showed that preganglionic parasympathetic input into the pterygopalatine ganglion arises from the superior salivatory nucleus in the brainstem. In combination with immunolabelling, these studies also showed that neuronal populations in the dorsal part of the hypothalamic paraventricular nucleus signal to the superior salivatory nucleus. This finding is particularly interesting, as the paraventricular nucleus regulates systemic blood pressure and cerebral blood flow. In addition, the superior salivatory nucleus also receives input from aortic baroreceptors via the vagus nerve and the solitary tract nucleus. Moreover, several structures in the central nervous system that regulate systemic blood pressure via sympathetic innervation signal to the superior salivatory nucleus, ultimately connecting the regulation of systemic blood pressure by the central nervous system to both parasympathetic and sympathetic innervation to the choroid (Reiner et al., 2018).

Systemic blood pressure continuously fluctuates due to normal changes in physiological conditions, for example during physical activity, sleep, changes in body posture, and stress. The choroid adjusts ocular blood flow by either vasoconstriction or vasodilatation, thereby maintaining stable choroidal blood pressure during these conditions (Riva et al., 1997). Extreme fluctuations in systemic blood pressure – for example due to severe trauma – can pose a major threat to proper choroidal functioning and to the neuroretina due to increased vulnerability to hypoxia. In the event of a life-threatening drop in blood pressure, peripheral vasoconstriction serves as a protective mechanism by maintaining blood pressure in vital organs such as the central nervous system as long as possible. Interestingly, studies in rats have shown that this peripheral vasoconstriction mechanism does not include the vessels in the eyes; thus, like the central nervous system, the eye is also considered a privileged organ under 'emergency' conditions (Bill and Nilsson, 1985). Recently, Reiner et al. suggested that the cerebral nuclei that regulate cerebral blood flow during low blood pressure may also regulate choroidal blood flow under these conditions (Reiner et al., 2018).

2.4.2. Metabolic and thermal adaptation of choroidal blood flow

Presumably, choroidal blood flow is not regulated solely by autoregulatory mechanisms and sensory input from systemic blood pressure regulation via the autonomic nervous system. Indeed, the retina itself is believed to be capable of modulating choroidal blood flow based on metabolic demand (Reiner et al., 2018). This hypothesis is supported by a study by Huemer et al. involving 15 healthy human subjects, which found that choroidal blood flow – measured using laser Doppler flowmetry and laser interferometry – was higher in the light conditions than in the dark (Huemer et al., 2007). Interestingly, the authors also found that the decrease in choroidal blood flow when transitioning from light to dark was attenuated by administering the nonspecific NOS antagonist L-NMMA (NG-monomethyl-L-arginine), indicating that this mechanism for regulating choroidal blood flow is mediated – at least in part – by NO (Huemer et al., 2007). More recently, studies involving animal models indicated that the central nervous system is involved in modulating choroidal blood flow in response to either retinal illumination or exposure to flickering light, and this modulation is mediated by the suprachiasmatic nucleus and the paraventricular nucleus (Reiner et al., 2018). This signalling may be mediated via melanopsin-expressing, light-sensitive retinal ganglion cells (Sand et al., 2012); interestingly, melanopsin-expressing cells have also been found in the cornea, where they may also serve to control choroidal blood flow in response to light, possibly via projections to the superior salivatory nucleus and trigeminal axon projections, which reach the choroid (Matynia et al., 2016). The neuronal circuit connecting the central nervous system and the choroid differs from the mechanism in the retinal vascular network, which is mediated by direct neurovascular coupling. Unlike the autoregulation of choroidal blood flow, retinal autoregulation is mediated by local metabolic factors such as oxygen and carbon dioxide; thus, in mammals the retinal vessels are not innervated (Bill and Sperber, 1990; Reiner et al., 2018).

Heat dissipation is an important function of blood flow, working at the systemic level, as well as at the level of tissues such as the skin and the choroid (Parver, 1991; Song et al., 1989). Because of its high rate of blood flow, the choroid functions as an active heat sink by increasing blood flow in response to a local increase in temperature due to the absorption of light by choroidal melanocytes and the RPE (Geeraets et al., 1960; Parver, 1991). In addition, metabolic activity in the retina can also contribute to a rise in local temperature. Sensory trigeminal nerve fibres located within the choroid are believed to convey the local thermal state to the autonomous nervous system, thus representing another neuronal circuit by which the regulation of choroidal blood flow can protect the retinal environment (Parver, 1991; Reiner et al., 2018).

2.4.3. Intraocular pressure and the regulation of choroidal blood flow

Under physiological conditions, intraluminal pressure in the choriocapillaris and the choroidal veins is slightly higher than IOP (Spaide, 2020), ensuring that the choroidal vascular bed remains perfused even if IOP increases. The underlying mechanisms that maintain choroidal vascular filling are not completely understood, although both active mechanisms (e.g. myogenic, autoregulatory, and neuronal) and passive mechanisms have been suggested to play a role (Reiner et al., 2018; Spaide, 2020). Recently, Spaide proposed an interesting explanation based on a passive mechanism analogous to a Starling resistor. In this hypothesis, an increase in IOP causes a proportional decrease in venous blood outflow in order to maintain filling of the choroidal vasculature. In this analogy, the Starling resistors are the vortex veins and the ampulla, with the vortex vein system functioning to buffer venous blood

and the ampulla functioning to restrict venous outflow (Spaide, 2020).

From the opposite perspective, several studies have suggested that choroidal blood flow may play a role in maintaining IOP (Reiner et al., 2018). For example, Ruskell et al. found that surgical dissection of the parasympathetic nerves arising from the pterygopalatine ganglion – which is relevant for choroidal neuronal innervation – induced a sustained drop in IOP in macaques (Ruskell, 1970). In support of these early findings, other animal studies showed that stimulation of facial nerves causes a rise in IOP (Reiner et al., 2018). Although no direct evidence has been reported, a change in choroidal volume may underlie this effect, at least in part. In humans, however, whether choroidal blood flow affects IOP is currently unknown and warrants further study.

2.5. The role of the choroid in emmetropisation

Choroidal dynamics have been implicated in emmetropisation, a process during which the eye achieves a balanced refraction that occurs primarily during ocular development and continues after birth (Nickla and Wallman, 2010; Troilo et al., 2000). Evidence supporting a role for the choroid in emmetropisation comes from studies in chicks, in which choroidal thinning was found to be associated with increased ocular elongation and choroidal thickening with decreased ocular elongation (Nickla and Wallman, 2010). Interestingly, the authors found that positioning of negative lenses caused a substantial increase in axial length and proposed that this effect was mediated by increased production of scleral extracellular matrix proteins; in addition, the authors observed a second compensatory mechanism involving choroidal thinning, which further increased the depth of the vitreous chamber (Nickla and Wallman, 2010). Conversely, they found that positive lenses decreased ocular elongation and increased choroidal thickness, repositioning the retina back into focus. Evidence for the choroid as a secretory tissue that secretes growth factors which modulate scleral growth has been provided by Marzani and Wallman, in co-culture *in vitro* experiments using choroidal and scleral tissue (Marzani and Wallman, 1997). Similar findings were reported recently by Zhang et al., who found that choroidal thickness increased transiently in lens transplant recipients with high myopia (Zhang et al., 2020). Importantly, however, an open question is whether these changes in choroidal thickness are simply a consequence of retinal defocus or play a causal role in emmetropisation.

Regardless of whether choroidal dynamics are the cause or consequence, the precise role of the choroid in emmetropisation is likely to be more complex than merely adjusting choroidal thickness to keep the retina in focus. Although the exact mechanisms are poorly understood, the choroid is believed to also function as a secretory tissue that modulates scleral growth, and the choroid itself may also be affected by retinal signalling (Nickla and Wallman, 2010; Summers, 2013). For example, the retina has been suggested to target the choroid by direct signalling via growth factors traversing the RPE and/or via neural networks that signal via the central nervous system and terminate in the choroid (Nickla and Wallman, 2010; Wildsoet and Wallman, 1995). Given that the sclera itself is not innervated, the latter mechanism may be facilitated by choroidal neurons that secrete trophic signals, including dopamine, acetylcholine, and NO (Nickla and Wallman, 2010). Alternatively, growth factors released by the retina may diffuse through the choroid and directly act upon the sclera; thus, choroidal thickness may modulate emmetropisation by acting as a physical barrier (Nickla and Wallman, 2010).

The release of biological compounds by the choroid itself may also regulate scleral growth, either autonomously or guided by retinal signalling. In animal models, several factors produced predominantly by the choroid have been found to affect scleral growth and include retinoic acid, transforming growth factor beta, and tissue plasminogen activator (Nickla and Wallman, 2010; Summers, 2013). Although an open question is whether these choroid-derived factors play a significant role in emmetropisation in humans, evidence to support this notion has emerged from genome-wide association studies in humans with refractive error. However, these studies also revealed several refractive error–associated genes in other ocular tissues, including the neuroretina, RPE, and sclera (Tedja et al., 2018). Genetic variants specific to CECs have been identified in the *CD34*, *VEGFR1*, *BMP2*, *BMP4*, and *TNFSF12* genes, although the underlying pathophysiological mechanisms and the significance of CECs in the development of refractive error relative to other ocular cell types remains unclear (Tedja et al., 2018). Taking all available evidence into account, it is reasonable to speculate that the choroid plays a role in emmetropisation in the growing human eye; however, most of the proposed mechanisms cannot fully explain the complex molecular processes that regulate scleral growth. Given the correlation between high refractive error and the risk of ocular disease, this topic clearly warrants further study.

2.6. Transport mechanisms utilised by the choroidal endothelial cells

The choriocapillaris is often described as being relatively permeable to small molecules and solvents. Indeed, to a certain extent the choriocapillaris is relatively permeable under physiological conditions, whereas the RPE – which forms the outer blood-retinal barrier – prevents free diffusion between the extravascular choroidal space and the neuroretina (Törnquist et al., 1990). However, several elaborate transport mechanisms regulate the movement of different-sized molecules from the choriocapillaris to the extracellular space. Although little is known regarding these mechanisms in the human choriocapillaris, results obtained using animal models combined with electron microscopy and either fluorescent or radioactive molecules have yielded valuables insights (Nakanishi et al., 2016; Törnquist et al., 1990). These components, which include fenestrations, caveolae, vesiculo-vacuolar organelles, and coated pits, are discussed in detail below.

2.6.1. Fenestrations: small pores in the choroidal endothelium

Fenestrations are small 'pore-like' structures in cellular membranes that facilitate transcellular transport. These structures are both the most abundant and the smallest structures for nutrient transport in the choriocapillaris (Bosma et al., 2018; Grebe et al., 2019; Guymer et al., 2004; Nakanishi et al., 2016; Törnquist et al., 1990). Fenestrations are enriched in the RPE side of the choriocapillaris lumen, where they form a pattern reminiscent of a sieve plate when viewed using freeze-fracture electron microscopy (Guymer et al., 2004). Fenestrations are present at sites where the apical membrane of the CEC is close to the basal cellular membrane, thus forming a pore that directly connects the luminal space to the extravascular space (Bosma et al., 2018; Guymer et al., 2004). Although fenestrations are only 60–80 nm in diameter, most macromolecules are unable to pass due to the presence of diaphragms, which are raster-like structures formed by plasmalemmal vesicle-associated protein (PLVAP), which radially span the diameter of each fenestration (Bosma et al., 2018). These diaphragms serve to regulate the permeability of fenestrations and have been found in fenestrations and the stomata of endothelial caveolae. Fenestrations can also exist without diaphragms, for example in the liver (Bosma et al., 2018). Certain molecules such as glucose, fatty acids, and amino acids can diffuse freely through fenestrations, while macromolecules such as albumin are unable to pass (Grebe et al., 2019; Törnquist et al., 1990). Thus, glucose concentrations in the choroidal extravascular space are relatively high, estimated to reach 90–95% of the concentration in the blood (Törnquist et al., 1990). The large number of fenestrations appears to play an important role in maintaining a healthy functioning retina, particularly given that the choroid has been estimated to supply 80% of the glucose consumed by the retina (Törnquist et al., 1990).

2.6.2. Plasmalemmal vesicle-associated protein is essential for transendothelial transport

In 1985, the 'Pathologische Anatomie Leiden-Endothelium' (PAL-E) monoclonal antibody was used by Schlingemann and colleagues to detect a protein that was later identified as PLVAP (Schlingemann et al.,

1985). In their study, the authors found that this antibody strongly stained ECs in capillaries and veins, and – albeit to a much lesser extent – ECs in arteries (Schlingemann et al., 1985). Later, PLVAP was identified as a marker in fenestrations, caveolae, and transendothelial channels, and its expression has been reported in endothelial cells in a variety of tissues, including kidney and skin (Bosma et al., 2018). In the human choroid, the expression of PLVAP is highest in the choriocapillaris, with moderate expression levels in some larger vessels (Fig. 3) (Blaauwgeers et al., 1999; Schlingemann et al., 1997). Interestingly, PLVAP is the only known molecular component of fenestral diaphragms, and serves as a marker for identifying non-barrier endothelium (Schlingemann et al., 1985, 1988, 1997; Stan et al., 1999). Recently, Nakakura and colleagues found that localisation of PLVAP at fenestrations requires the microtubule cytoskeletal system, which is regulated by fibronectin-integrin α5β1 signalling (Nakakura et al., 2021). PLVAP also plays a role in the morphogenesis of fenestrations (Ioannidou et al., 2006), and its expression is both induced and maintained by basal secretion of VEGF from the RPE (Blaauwgeers et al., 1999; Kim et al., 2020a). The presence of numerous PLVAP homodimers form a 'wheel-like' structure that covers the gap in the fenestrations, decreasing this gap from 60 to 80 nm to as small as 6 nm (Bosma et al., 2018). In addition, PLVAP plays a major role in barrier function, as discussed in detail in section 3.4.2.

2.6.3. *Caveolae are cytoplasmic transport vesicles*

In the choroid, the transcellular transport (also known as transcytosis) of macromolecules is facilitated by caveolae, which are 'cave-like' microstructures expressed abundantly in ECs throughout the body (Grebe et al., 2019; Nakanishi et al., 2016). Caveolae are flask-shaped invaginations of the cellular membrane that facilitate the bi-directional movement of macromolecules from the luminal side of the cell to the basal side via cytoplasmic vesicles (Gu et al., 2017). The typical size of the invaginations is 50–100 nm, although they are observed with a narrowing of the structure's neck or a diaphragm-like filter that contains PLVAP (Gu et al., 2017; Nakanishi et al., 2016). The Caveolin-1 protein (encoded by the *CAV1* gene) serves as the main structural component in caveolae. Caveolae facilitate the receptor-mediated transport of a wide variety of ligands, nutrients, and other compounds, including albumin, transthyretin, cholesterol, low-density lipoproteins, transferrin, insulin, and numerous growth factors (Gu et al., 2017; Nakanishi et al., 2016). Importantly, caveolae play an essential role in transporting nutrients and other macromolecules that cannot readily pass through fenestrations (Gu et al., 2017). Thus, the transcytosis of bovine serum albumin (BSA) is impaired in the choriocapillaris of a knockout mouse lacking Cav1 (Nakanishi et al., 2016). In the eye, the transcellular transport of molecules by caveolae is more rapid and efficient than in other organs. Indeed, mouse studies using fluorescently labelled albumin have shown that transcytosis through CECs occurs within 30 min after injection in the blood stream, reaching the photoreceptors within 60 min (Nakanishi et al., 2016). Moreover, the rate of albumin extraction from the blood in the choriocapillaris is considerably higher than in other tissues, for example up to 6 times the rate of extraction measured in the kidney (Törnquist et al., 1990). These findings have clinical relevance, as the levels of both CAV1 and PLVAP – two key components of caveolae-mediated transcytosis – are reduced in the macular RPE/choroid complex in patients with AMD, a disease often linked to impaired transcellular transport (Bergen et al., 2019; Rozing et al., 2020; Yuan et al., 2010).

2.6.4. *Transendothelial channels, vesiculo-vacuolar organelles, and coated pits*

Electron microscopy studies using mouse tissues suggest that the fusion of caveolae forms transendothelial channels and vesiculo-vacuolar organelles (i.e. networks of interconnecting vesicles), which serve a function similar to caveolae to transport macromolecules (Gu et al., 2017; Klaassen et al., 2013; Nakanishi et al., 2016; Tse and Stan, 2010). However, their relationship to caveolae has been disputed, as these structures can still form in mice lacking Cav1, the principal component in caveolae (Nakanishi et al., 2016; Tse and Stan, 2010). Transendothelial channels are 60–80-nm openings that span the cytoplasm from the apical side of the cell to the basal side of the cell, and can be covered at both sides by PLVAP (Bosma et al., 2018; Nakanishi et al., 2016; Tse and Stan, 2010). Interestingly, these channels have been shown to facilitate the extravasation of leukocytes from the circulation into tissues during inflammation (Bosma et al., 2018; Keuschnigg et al., 2009). PLVAP, which is heavily expressed on cell membranes at the site of transmigration, appears to play an essential role in this process, as blocking PLVAP using a monoclonal antibody in a mouse model of acute inflammation reduced the transmigration of leukocytes by 85% (Keuschnigg et al., 2009). Transendothelial channels can also transport

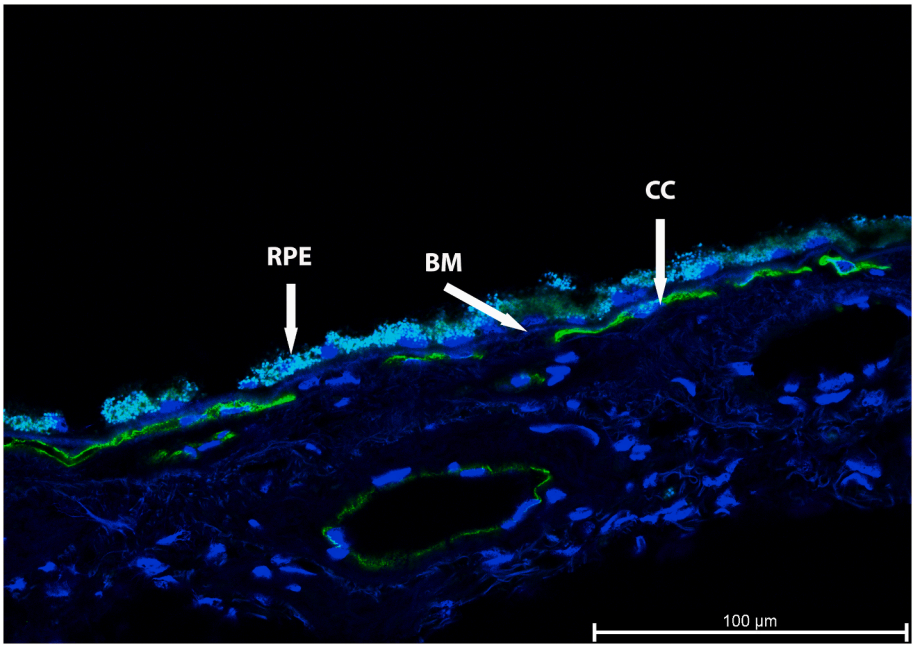


Fig. 3. Confocal image of human retinal pigment epithelium/choroid cryosection, showing immunofluorescent labelling of plasmalemmal vesicle-associated protein (PLVAP; green). Post-mortem retinal pigment epithelium/choroid complex was fixed with acetone and stained for PLVAP using a monoclonal antibody diluted 1:400 (ab81719; Abcam, Cambridge, UK). Note the intense staining of the choriocapillaris and to a lesser extent staining of some larger choroidal vessels. Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). The retinal pigment epithelium shows autofluorescence due to accumulation of lipofuscin. BM = Bruch's membrane; CC = choriocapillaris; RPE = retinal pigment epithelium. Scale bar = 100 µm. Original magnification 40x. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

relatively large plasma proteins such as albumin across CECs, suggesting that either they do not contain diaphragm filters formed by PLVAP or that the filter can open and close (Nakanishi et al., 2016; Tse and Stan, 2010). Like transendothelial channels, evidence suggests that the permeability of vesiculo-vacuolar organelles, which appear as multiple fused vesicles on electron microscopy, can also be modulated. Interestingly, experiments with mice suggested that VEGF and histamine – which is produced in the human choroid by mast cells – may serve to increase vascular permeability by acting on vesiculo-vacuolar organelles (Dvorak and Feng, 2001). Coated pits, which are present in the choriocapillaris, are specialised patches of receptor proteins at the cell surface that invaginate upon ligand binding and mediate transendothelial transport via endocytosis (Nakanishi et al., 2016). Although coated pits have been suggested to play a role in transporting low-density lipoproteins, their exact function is currently unknown, as both caveolae and transendothelial channels have been shown to transport these lipoproteins as well (Nakanishi et al., 2016).

In summary, transendothelial channels, vesiculo-vacuolar organelles, and coated pits are all present in CECs and serve to transport macromolecules and leukocytes (Bosma et al., 2018; Klaassen et al., 2013; Nakanishi et al., 2016). Future research regarding the specific functions of each of these specialised structures is needed in order to understand how they relate to and/or interact with caveolae and PLVAP, thus yielding new insights into how CECs transport nutrients and the role of these structures in specific chorioretinal diseases.

2.6.5. Oxygen dynamics in the choroid

Unlike most tissues with high metabolic demands, in which the capillaries are integrated and in close proximity to the tissue they supply, the photoreceptors are dependent upon oxygen that must diffuse from the relatively distant choroid. Using radioactively labelled microspheres of various sizes to estimate organ perfusion, Alm and Bill measured oxygen dynamics in the retina and choroid in cat eyes (Alm and Bill, 1972). They found that the choroid supplies 80% of the oxygen consumed by the neurosensory retina, with only 20% supplied by the retinal vasculature. Moreover, although the retina is one of the most metabolically active tissues in the body, the difference in oxygen tension between arterial and venous choroidal blood is extremely low (3% in the choroid, compared to 38% in the retinal vasculature) (Alm, 1992; Alm and Bill, 1973). Furthermore, the choroid has an extremely high rate of blood flow per gram of tissue compared to other tissues, for example 4 times higher than in the highly vascularised renal cortex and 37 times higher than in the brain and small intestine (Alm and Bill, 1972). Taken together, these results suggest that the choroid supplies a vast 'overcapacity' of oxygen to the retina; however, this may actually play an essential role in maintaining ocular homeostasis. Notably, HIF-1α, a subunit of the transcription factor hypoxia-inducible factor 1 encoded by the *HIF1A* gene is constitutively expressed by human photoreceptors (Hughes et al., 2010). Given that this protein is expressed under hypoxic conditions and helps cells adapt to hypoxia, its constitutive expression in photoreceptors suggests that oxygen reaching these cells is consumed almost immediately. Thus, the relatively high oxygen tension in the choroid may be required in order to ensure the sufficient diffusion of oxygen from the choriocapillaris to the relatively distant inner segments of the photoreceptors, where the mitochondria are located and oxygen is consumed (Yu and Cringle, 2001). This is particularly important during dark adaptation, during which rod cells consume the highest amounts of oxygen (Arden et al., 2005). Moreover, although currently untested, in the event of a sudden drop in systemic blood pressure, the choroid may serve as a temporary source of oxygen, thereby protecting the highly vulnerable neurosensory retina from oxygen deprivation.

2.6.6. Carbon dioxide transport in the choriocapillaris requires carbonic anhydrases

The enzyme carbonic anhydrase IV (CA4) was first identified as being expressed in the human choriocapillaris three decades ago by Hageman and colleagues (Hageman et al., 1991). CA4 is now considered to be one of the most specific markers of the choriocapillaris, with strong immunoreactivity at the level of the choriocapillaris ECs and – to a lesser extent – in larger choroidal vessels (Fig. 4). Carbonic anhydrases are a family of enzymes that help regulate the acid-base balance by converting carbon dioxide and water to bicarbonate, and vice versa. Although little is known regarding the regulation of ion transport at the RPE/choroid interface, carbonic anhydrases are believed to play an important role in this process, thereby facilitating the transport of carbon dioxide primarily from the photoreceptors to the bloodstream (Hageman et al., 1991). CA4 is localised on both the luminal and abluminal sides of the choriocapillaris, presumably on the extracellular surface of the cell membrane, thus ensuring close proximity to both the RPE and the bloodstream (Hageman et al., 1991). Interestingly, CA4 is the only membrane-bound carbonic anhydrase isozyme expressed in the human eye. Two other carbonic anhydrase isoforms – CA1 and CA2 – have been identified in the human eye, both of which are cytoplasmic and expressed at considerably lower levels than CA4 (Hageman et al., 1991; Voigt et al., 2019; Wistrand et al., 1986). Nevertheless, immunohistochemistry has shown that CA1 is expressed in endothelial cells in the choriocapillaris, and this isozyme is believed to function synergistically with CA4 in mediating the transendothelial movement of carbon dioxide (Wistrand et al., 1986). This expression of both membrane-bound and cytosolic forms of carbonic anhydrases has also been reported in the epithelium of renal proximal tubules, with CA2 serving as the cytoplasmic isoform (Hageman et al., 1991). Other cell types in the eye express only the cytoplasmic isoforms of carbonic anhydrases (Hageman et al., 1991; Wistrand et al., 1986). For example, CA1 is expressed in the capillary cells of the ciliary processes, and CA2 is

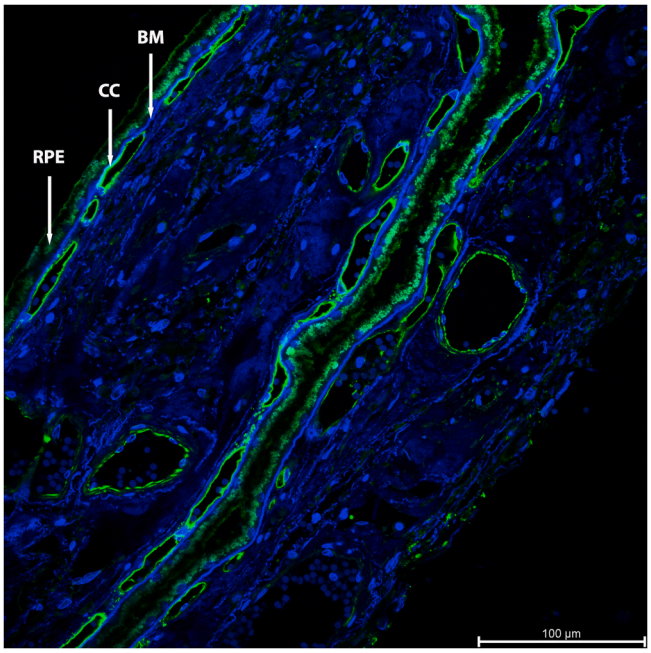


Fig. 4. Confocal image of human retinal pigment epithelium/choroid complex, showing immunofluorescent labelling of carbonic anhydrase 4 (CA4; green). Post-mortem retinal pigment epithelium/choroid complex was fixed with 4% paraformaldehyde and stained for CA4 using a polyclonal CA4 antibody diluted 1:100 (PA5-42003; Thermofisher Scientific, Waltham, MA, USA). There is intense staining of the choriocapillaris, as well as staining of some larger choroidal vessels. Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). The retinal pigment epithelium shows autofluorescence due to accumulation of lipofuscin. BM = Bruch's membrane; CC = choriocapillaris; RPE = retinal pigment epithelium. Scale bar = 100 µm. Original magnification 40x. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

expressed in the adjacent pigmented epithelial cells, where they are believed to coordinate the production of aqueous humour via the release of bicarbonate (Wistrand et al., 1986).

2.7. Immunological features of the choroid

2.7.1. Immune cell trafficking in the choroid

The choroid – and CECs in particular – plays a fundamental role in ocular immunity. Thus, dysregulation of this immunological state in the choroid contributes to a variety of chorioretinal diseases such as AMD and uveitis. In contrast to the inner eye, which is located behind the blood-retinal barriers, the choroid has a highly active immunological state in which various immune cells traffic continuously in both directions between the bloodstream and the choroidal stroma (McMenamin, 1997; Whitmore et al., 2015). This process is mediated by the relatively high expression of adhesion molecules, which enable immune cells to 'anchor' to CECs and invade the choroidal stroma for immune surveillance. An important adhesion molecule expressed in CECs, and a mediator of inflammation, is the protein ICAM-1 (intercellular adhesion molecule 1) (Skeie et al., 2010; Whitmore et al., 2015). This transmembrane glycoprotein is constitutively expressed on choriocapillaris ECs and is upregulated in response to inflammatory stimuli such as the complement system factor C5a or pro-inflammatory cytokines. Another important molecule expressed at the surface of CECs is CD34, which is believed to have anti-adhesion effects mediated by negatively charged residues (Sohn et al., 2014). Ageing has been associated with both an upregulation of ICAM-1 and a downregulation of CD34, and these changes are believed to contribute to creating a pro-inflammatory environment in which AMD can develop (Sohn et al., 2014; Voigt et al., 2020; Whitmore et al., 2015). In addition, the major histocompatibility complex (MHC) class I molecule is constitutively expressed within the choroid, particularly in the choriocapillaris (Goverdhan et al., 2005b). MHC-I and MHC-II are responsible for antigen presentation. Specifically, MHC-I presents peptide fragments of self-antigens to lymphocytes. Under physiological conditions, this does not trigger leukocyte extravasation and invasion; however, during infection or in the case of autoimmune uveitis, activation of MHC-I can trigger the invasion of $CD8^+$ cytotoxic T cells into the choroidal stroma (Mochizuki et al., 2013). Other immune cell types have been observed in the choroid and include phagocytes such as neutrophils and macrophages, antigen-presenting dendritic cells, and mast cells, all of which are involved in the local inflammatory response, with mast cells playing a specific role in the allergic response (Bhutto et al., 2016; Cherepanoff et al., 2010; Ezzat et al., 2008; McLeod et al., 2016; McMenamin, 1997; Voigt et al., 2019).

Ocular immunity is based on a complex and unique interplay between immune cells and ocular cells, giving rise to the eye's state of so-called immune privilege, in which physical barriers formed by tight junctions of the RPE (i.e. the outer blood-retinal barrier) and retinal vascular ECs (i.e. the inner blood-retinal barrier) play a key role (Klaassen et al., 2013). The inner and outer blood-retinal barriers protect the eye from unwanted fluctuations in solutes and proteins, as well as from the entry of invading pathogens. In addition, they play an important role in regulating inflammatory responses induced by immune cells, by forming a physical barrier that prevents immune cells from entering the inner eye and by establishing cell-cell interactions between the RPE and T cells. Thus, the RPE can interact with nearby lymphocytes, which can be either suppressed by the RPE or converted from $CD4^+$ effector cells to anti-inflammatory regulatory T cells (Mochizuki et al., 2013). This process protects the eye from lymphocytes that might recognise retinal autoantigens as pathogenic, thereby triggering autoimmunity. Furthermore, RPE cells can function as antigen-presenting cells, playing an important role in the transition zone between the immune-privileged inner eye and the outer blood-retinal barrier. During ageing, the eye's immune-privileged state can be weakened due to low but progressive levels of chronic oxidative damage to the retina (Chen and Xu, 2015). This process results in the activation of local microglia and low-grade activation of the complement system, creating a so-called 'para-inflammatory response' that can usually be controlled under healthy conditions. During disease, however, the para-inflammation response can be altered, damaging the blood-retinal barrier and causing chronic inflammation (Chen and Xu, 2015).

2.7.2. The complement system: maintaining the balance between immune system homeostasis and chorioretinal disease

The complement system – also known as the complement cascade – plays a key homeostatic role in the innate immune response in the choroid (Whitmore et al., 2015). This system consists of more than 30 proteins that circulate primarily in the bloodstream and can be activated via three distinct pathways, triggering a series of reactions mediated by the enzymatic cleavage of these proteins. These three pathways include: *i*) the classical pathway, which is initiated by the formation of antigen-antibody complexes; *ii*) the lectin pathway, which is triggered by mannose; and *iii*) the alternative pathway, which is activated by the direct binding of complement system components to non-self-biomolecules and pathogens. All three pathways lead to production of the enzyme C3 convertase, which cleaves complement factor C3 into C3b and C3a. The resulting release of peptides induces an inflammatory response, triggering phagocytes to either internalise targets or drive the formation of the membrane attack complex (MAC) (Whitmore et al., 2015). Although the complement system is important in initiating the immune response to pathogens, its overactivation can drive a variety of severe systemic conditions (Boon et al., 2009b; Ricklin et al., 2010). Interestingly, overactivation of the complement system – particularly the alternative pathway – plays a key role in chorioretinal diseases such as AMD (Anderson et al., 2010; Boon et al., 2009b, 2013). For example, using human CEC organ cultures Skeie and colleagues found that CECs express a receptor for factor C5a, which activates the complement system, causing increased expression of ICAM-1, which may ultimately increase the recruitment of leukocytes from the blood (Skeie et al., 2010). Components of the complement system in sub-RPE deposits can adhere to accumulating protein deposits and drusen, potentially triggering a vicious circle (Bergen et al., 2019), attracting phagocytes and stimulating MAC formation, ultimately resulting in the selective death of CECs (Bergen et al., 2019; Chirco et al., 2017a; Whitmore et al., 2015; Zeng et al., 2016).

Similarly, inhibition of the complement system in the choroid plays a major role in reducing the consequences of both ageing and chorioretinal disease. This process is mediated by the inhibitory complement factor CFH and other regulatory molecules such as completement factor I (CFI), which acts by inhibiting C3 activity, thus reducing activation of the terminal lytic step in the complement cascade (Boon et al., 2009b; Clark and Bishop, 2018; Clark et al., 2014). Importantly, CECs express heparan sulfate at the cell surface, which serves to anchor CFH, thus facilitating its function on the inner surface of the choriocapillaris (Keenan et al., 2014). CFH is also expressed on the RPE side of BM. Interestingly, CECs also express a truncated, alternatively spliced isoform of CFH called FHL-1 (Factor H Like-1) (Clark and Bishop, 2018; Clark et al., 2014). Unlike the full-length CFH protein, the smaller FHL-1 protein can diffuse freely through the extracellular matrix and is therefore believed to be more effective at protecting BM and the intercapillary septa from dysregulated complement activity (Clark et al., 2014). Other key molecular players can also contribute to complement dysregulation and include complement factor H–related (CFHR) proteins (Fritsche et al., 2010) such as CFHR4, which is localised to the choriocapillaris, BM, and RPE. CFHR4 is strongly associated with AMD and competes with CFH for C3 binding, thus preventing CFI-mediated C3 cleavage (Cipriani et al., 2020). Although the importance of the complement system in the innate immune response in the choroid is irrefutable, additional research is needed in order to fully understand how the complement system is regulated in the RPE/choroid complex, as well as how this system contributes to specific chorioretinal diseases,

as discussed in Chapter 4.

2.7.3. Toll-like receptors as a trigger of the inflammatory response

Toll-like receptors (TLRs) trigger the innate immune response by recognizing pathogen-associated molecular patterns, which are conserved molecular motifs expressed on the surface of pathogens. In humans, TLRs are expressed on the surface of antigen-presenting cells such as macrophages, where they also play a role in adaptive immunity by increasing the binding and interaction between these cells with naïve T cells. With respect to innate immunity, TLRs are also expressed by a wide range of non-immune cell types in the eye, including CECs, thus providing a mechanism for triggering an inflammatory response upon the detection of pathogens and/or vascular damage (Mochizuki et al., 2013). CECs express TLR1 through TLR6, as well as TLR9, thus overlapping to a large extent with the TLRs expressed in retinal endothelial cells (RECs) (Stewart et al., 2015). However, unlike CECs, RECs express TLR10 but do not express TLR1 (Stewart et al., 2015). Although hypothetical, these differences in TLR expression between CECs and RECs may explain why diseases such as punctate inner choroidopathy manifest primarily in the choroid, while other diseases such as systemic lupus erythematosus manifest in the retinal vasculature (Stewart et al., 2015).

With respect to the functional mechanism, a study using human cultured CECs found that activating TLR3 and TLR4 drives release of the pro-inflammatory cytokine interleukin 6 (IL-6) (Stewart et al., 2015). Interestingly, TLR4 is expressed at high levels in both CECs and RECs and has been associated with vascular diseases such as atherosclerosis and ischaemia/reperfusion injury, as well as angiogenesis (Stewart et al., 2015). Furthermore, TLR4 has been found to play a critical pathogenic role in a murine model of autoimmune uveitis (Mochizuki et al., 2013). However, whether this role can be translated to uveitis in humans remains unclear. For example, the presence of uveitis in patients with sarcoidosis is more prevalent in Japan (ranging from 50% to 93.5%) compared to other countries (25–60%), but does not appear to be correlated to variants in the *TLR4* gene (Asukata et al., 2009). Moreover, another study found no clear association between specific single nucleotide polymorphisms (SNPs) in the *TLR4* and various forms of AMD, including CNV (Despriet et al., 2008). Taken together, studies have confirmed that TLRs are expressed in human CECs and likely exert pro-inflammatory effects via the release of IL-6 release. Although a specific pathogenic role for TLRs in human chorioretinal disease has not been established, their clear expression in human CECs, their role in mediating other vascular diseases in humans, and their role in experimental animal models indicate that further research is needed in order to unravel their relevance in mediating chorioretinal diseases in patients.

2.7.4. Adrenomedullin: a multifunctional vasoactive peptide

Several lines of evidence suggest that the peptide hormone adrenomedullin (encoded by the *ADM* gene) plays a significant role in both ocular homeostasis and chorioretinal disease. Although adrenomedullin has primarily vasoactive and anti-inflammatory properties, protective effects against hypoxia and apoptosis effects have also been reported (Chu et al., 2001; Iesato et al., 2016). Adrenomedullin was initially discovered in human pheochromocytoma tissues (Kitamura et al., 1993), but has since been found to be expressed in a wide range of tissues and cell types, including vascular ECs, the RPE, and the neuroretina (Iesato et al., 2016; Owji et al., 1995; Udono-Fujimori et al., 2003). Because of its relatively short biological half-life, adrenomedullin is considered a local regulator of vascular function, which is believed to be by increasing blood flow and microvascular permeability (Udono et al., 2002). Elevated serum levels of adrenomedullin have been reported in various systemic vascular diseases, including hypertension, septic shock, heart failure, kidney failure, and atherosclerosis, and the levels are correlated to the severity of the disease (Udono et al., 2002). The biological and clinical significance of adrenomedullin was demonstrated in adrenomedullin knockout mice, in which vascular development is severely impaired, leading to death of the embryo at mid-gestation due to impaired angiogenesis (Iesato et al., 2016).

The main receptor for adrenomedullin is the calcitonin receptor-like receptor (CALCRL) (Iesato et al., 2016). In mice, this receptor is expressed at high levels in CECs, nearly 20-fold higher than in RECs, suggesting that adrenomedullin likely plays a specific role in the choroidal vasculature (Toriyama et al., 2015). Activation of CALCRL requires the simultaneous binding of adrenomedullin and either receptor activity-modifying protein-2 (RAMP2) or RAMP3 (Iesato et al., 2016). These co-ligands of CALCRL modulate the response to adrenomedullin, depending on their tissue-specific expression. Moreover, our group confirmed the presence of *CALCRL*, *ADM*, and *RAMP2* messenger RNA (mRNA) in human cultured CECs (Table 1). Elevated levels of adrenomedullin have also been found in the aqueous humour of patients with a variety of chorioretinal disorders (Udono et al., 2002). In contrast to patients with senile cataract, patients with active uveitis, proliferative vitreoretinopathy, or proliferative diabetic retinopathy (DR) have significantly higher levels of adrenomedullin (Udono et al., 2002). In addition, increased expression of adrenomedullin has been measured at the mRNA level in malignant uveal melanoma (Udono et al., 2000), and this peptide hormone has been found to increase the proliferation of tumour cells (Pio et al., 2001). In addition to its expression in CECs, adrenomedullin is expressed in three separate human RPE cell lines upon stimulation with inflammatory cytokines (Udono-Fujimori et al., 2003). Interestingly, adrenomedullin can bind to and functionally interact with CFH, suggesting that this peptide may play a role in modulating complement activity (Pio et al., 2001). Moreover, the functional consequences of this binding between adrenomedullin and CFH are reciprocal, increasing both the biological half-life of adrenomedullin and CFH activity by cleaving C3b (Pio et al., 2001). Despite these findings, whether this interaction between CFH and adrenomedullin plays a specific role within the context of chorioretinal diseases remains an open question.

In summary, adrenomedullin can interact with CFH and is expressed in both the RPE and CECs. Furthermore, the finding of elevated levels of adrenomedullin in chorioretinal diseases suggests that further study is needed in order to determine the precise role of this molecule in CECs (Udono-Fujimori et al., 2003).

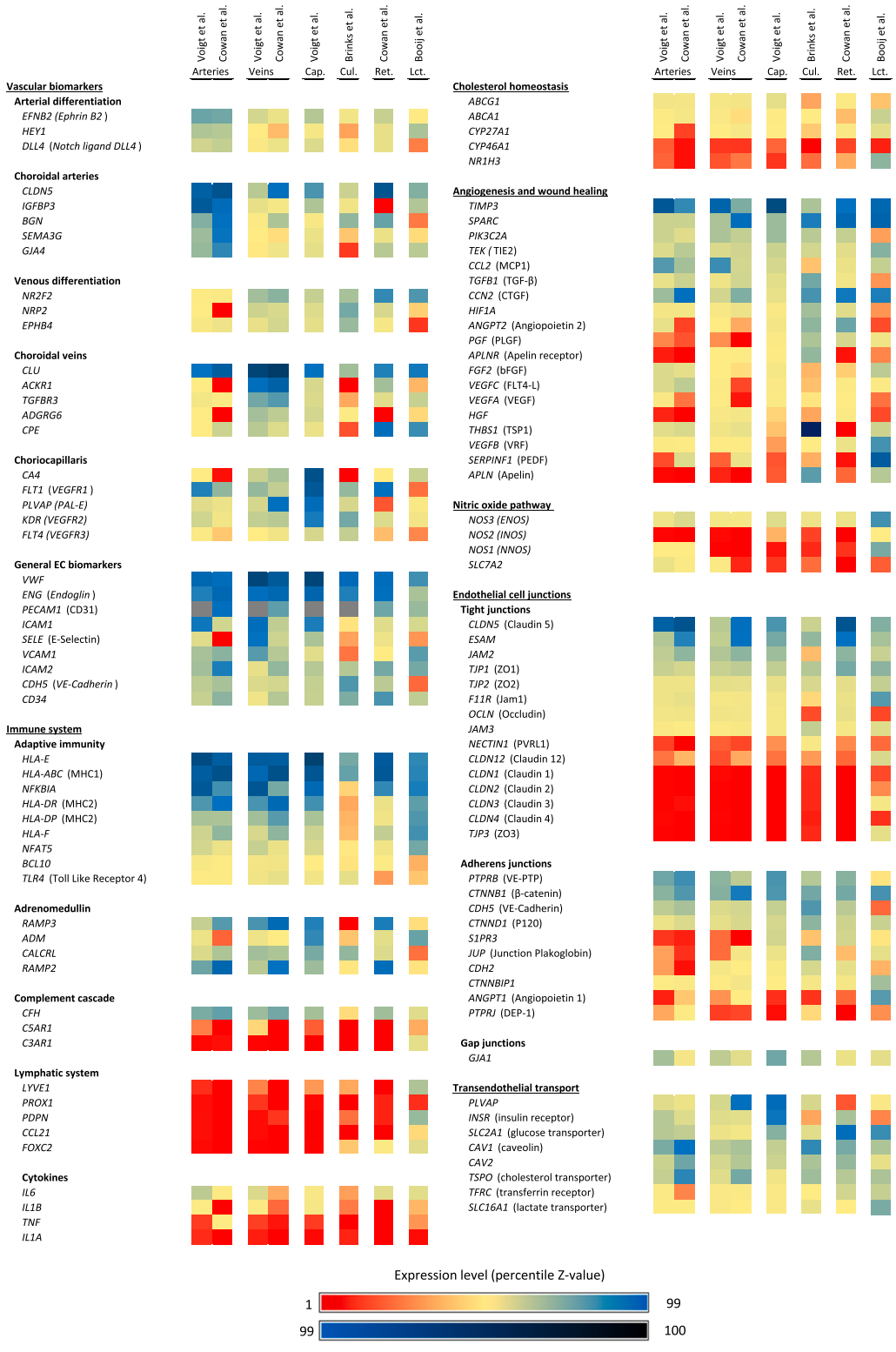
2.7.5. A choroidal lymphatic system?

The lymphatic system consists of a network of vessels that drain fluids and waste material from various organs; in addition, this system plays an important role in immunology and antigen presentation. In the human eye, whether the choroid contains a lymphatic system has been the subject of controversy for decades, for several reasons. First, lymphatic vessels (i.e. lymphatics) are difficult to identify using biomarkers due to their heterogeneity between various tissues. Second, putative biomarkers expressed by lymphatics can also be expressed by other cell types such as vascular ECs and macrophages. Moreover, it can be difficult to detect a lymphatic system in the absence of inflammatory stimuli (e.g. in the cornea). Nevertheless, in 2014 Schroedl et al. published a consensus statement regarding the possible detection of lymphatic vessels in the ocular system (Schroedl et al., 2014), in which they summarised the following four criteria and recommendations: 1) the presence or absence of erythrocytes/lymph-like fluid is insufficient to discriminate between lymphatic vessels and blood vessels; 2) the use of multiple markers for immunohistochemistry analysis is recommended; 3) the use of ultrastructural markers (e.g. based on electron microscopy) is recommended; and 4) the use of appropriate controls and documentation for both lymphatic vessels and blood vessels is recommended.

The following year, the same authors published a study in which they extensively examined the possible expression of lymphatic markers in the human choroid, using both immunohistochemistry and mRNA analysis to measure LYVE1, PDPN, PROX1, FOXC2, VEGFR3, and CCL21) (Table 1) (Schrodl et al., 2015). Based on apparent absence of

Table 1
Biomarker overview of the choroidal endothelial cell (CEC).

Table 1 Biomarker overview of the choroidal endothelial cell (CEC).



Expression levels of messenger RNA transcripts from different datasets are shown of CEC biomarkers and genes related to CEC function. Subpopulations of CECs from arterial, venous, and cappillary origin are identified. Moreover, datasets of primary cultured CECs (Cul.), intraretinal endothelial cells (Ret.), and microdissection laser capture of human choriocapillaris (Lct.) are shown. For each dataset, the Z-score of all genes was calculated based on the log2 counts per million (log2CPM) reads. To optimise scaling, the range of the color scale was adjusted to Z-scores within expression between

percentile 1–99, and Z-scores above the 99th percentile. Data of PECAM1 from the study by Voigt et al. is not included (gray boxes), since this marker was used for cell selection.

co-expression of these markers, the authors concluded that classic lymphatic vessels are not present in the adult human choroid (Schrodl et al., 2015). However, their results could not rule out the existence of a choroidal lymphatic-like system with features specific to the choroid. Previously, this group speculated that macrophages – which are abundant in the choroid and essential to lymphangiogenesis – might be involved in the formation of lymph channel–like structures under specific inflammatory conditions (Schroedl et al., 2008).

In 2015, Konia et al. reported results that were largely contradictory to the aforementioned findings (Koina et al., 2015). They examined both foetal and adult human choroidal tissue for the presence of lymphatic and/or lymphatic-like structures using an extensive array of lymphatic-specific and vascular-specific markers combined with immunohistochemistry and transmission electron microscopy. Their findings revealed that initial lymphatic channels are present just outside of the choriocapillaris. These dead-ended sacs, which appear to be in the optimal location for macromolecular absorption, fluid homeostasis, and immune surveillance, were found to converge into pre-collector channels and collector lymphatics in Haller's layer. However, similar to the findings reported by Schroedl et al., they did not identify continuous lymphatic channels. Interestingly, however, their electron microscopy data yielded evidence supporting the existence of lymphatic channels, as multiple ultrastructural features consistent with lymphatic channels were observed, including anchoring filaments, tall lymphatic ECs, and the absence of fenestrations and luminal erythrocytes (Koina et al., 2015). Nevertheless, they failed to identify lymphatics exiting the eye in order to connect to the sentinel lymph nodes (Koina et al., 2015).

In conclusion, evidence supporting the existence of a lymphatic system in the posterior human eye remains controversial and a topic of debate (Koina et al., 2015; Schroedl et al., 2014). Despite compelling evidence for the absence of typical lymphatics in the choroid (Schrodl et al., 2015), this evidence has been contradicted by other evidence (Koina et al., 2015) and by experimental data supporting the possible formation of atypical lymphatics under specific inflammatory conditions based on the presence of LYVE1-positive macrophages (Schroedl et al., 2008). Moreover, Nakao and colleagues found evidence for choroidal lymphatics in a mouse model of laser-induced CNV; however, these results could not be translated to surgically removed CNV membranes obtained from patients with uveitis and AMD (Nakao et al., 2013). Taken together, these findings indicate that further studies are clearly needed in order to definitively determine whether the human choroid contains a classic lymphatic or possibly an atypical lymphatic system.

2.8. The wound-healing response in the choroid

The choroidal wound-healing response is a highly complex process involving ECs, pericytes, fibroblasts, immune cells, epithelial cells, platelets, extracellular matrix, the coagulation cascade, and numerous growth factors (Ishikawa et al., 2016; Kent and Sheridan, 2003; Schlingemann, 2004). Much of our knowledge regarding the wound-healing response is deduced from the wound-healing response in the skin, which has been studied extensively and appears to have features that overlap between these two organs (Kent and Sheridan, 2003).

Several phases in the wound-healing response can be distinguished. Although some overlap exists between these phases, the wound-healing process generally proceeds as follows: *i*) clot formation, *ii*) creation of a fibrin matrix, *iii*) infiltration of phagocytes and phagocytosis, *iv*) angiogenesis, *v*) extracellular matrix formation, *vi*) scarring, and *vii*) re-epithelisation (Kent and Sheridan, 2003; Schlingemann, 2004). Histological studies using post-mortem human donor eyes, as well as imaging studies, have shown that this basic series of events is similar to the wound healing in response to choroidal rupture and during the formation of CNV (Kent and Sheridan, 2003). Typical CNV presents with a peripheral rim of fibrin with newly formed unstable vessels, a central region containing maturing vessels, and fibrosis (Schlingemann, 2004). Unlike the wound healing under physiological conditions, CNV is driven by hypoxia and is marked by unstable vessels that actively haemorrhage into the subretinal space, triggering an inflammatory response and the formation of fibrovascular tissue, which can readily cause irreversible damage to the retina (Kent and Sheridan, 2003; Schlingemann, 2004).

3. Structural and functional classification of the choroidal endothelium

Based on transcriptomics, structural features, and anatomical landmarks, CECs can be categorised into subpopulations of capillary, venous, or arterial origin (Table 1) (Booij et al., 2010a; Brinks et al., 2018; Cowan et al., 2020; Voigt et al., 2019). Below, we discuss the rationale for this classification, and we describe the structural properties of each subpopulation in the context of its specific function. A graphical abstract of the main CEC biomarkers related to specific CEC functions and their interaction with the RPE is shown in Fig. 5. Markers that are general to vascular endothelium, as well as markers that are specific to the choroid and can be used to classify CEC subpopulations, are discussed in section 3.1. A number of markers may be considered specific to certain choroidal functions and/or the unique environment in which the CECs reside, including the interaction between the choroid and the RPE, regulation of transendothelial transport, maintaining barrier function, and CNV. These functional aspects are discussed in detail in sections 3.2 through 3.5, and we discuss gaps in our current knowledge regarding CECs.

3.1. Classification of subpopulations of choroidal endothelial cells

Table 1 provides a meta-analysis based on four transcriptomics studies, summarising the expression of genes relevant to the various structural and functional subpopulations of CECs, shown as *Z*-scores on a colour scale. This analysis is based on several datasets, including: single-cell RNA sequencing (scRNA-seq) data from two studies (Cowan et al., 2020; Voigt et al., 2019), microarray data from laser-captured choriocapillaris tissues (Booij et al., 2010a), and bulk RNA sequencing of primary cultured CECs (our unpublished data). Specifically, Voigt et al. used anti-CD31 antibody-conjugated magnetic beads to select cells from the RPE/choroid complex from four non-related human donors (Voigt et al., 2019). The other scRNA-seq study by Cowan et al. also analysed the human RPE/choroid complex in both eyes obtained from four healthy donors (2 men and 2 women) (Cowan et al., 2020). Booij et al. performed a microarray analysis on laser-capture microdissected cells taken from the choriocapillaris of three healthy donors (Booij et al., 2010a). Finally, our unpublished bulk RNA-seq dataset was derived from primary CECs obtained from both eyes taken from ten subjects (5 men and 5 women) and cultured as previously described (Brinks et al., 2018).

3.1.1. Generic markers for vascular endothelial cells in choroidal endothelial cells

The RNA-seq analyses revealed that several generic markers for vascular ECs, including von Willebrand factor (VWF), CD31, and endoglin (ENG), are widely expressed in all subpopulations of CECs (Table 1). In recent decades, additional markers expressed in CECs have also been identified and were found to be specific for certain CEC subpopulations. For example, ICAM-1, MHC-I (HLA-ABC), VEGFR1, and PLVAP are expressed at high levels in CECs in the choriocapillaris but are also expressed in CECs of the deeper vascular layers of the choroid, albeit at significantly lower levels (Fig. 3) (Table 1) (Blaauwgeers et al., 1999; Cowan et al., 2020; Voigt et al., 2019). This difference may be due

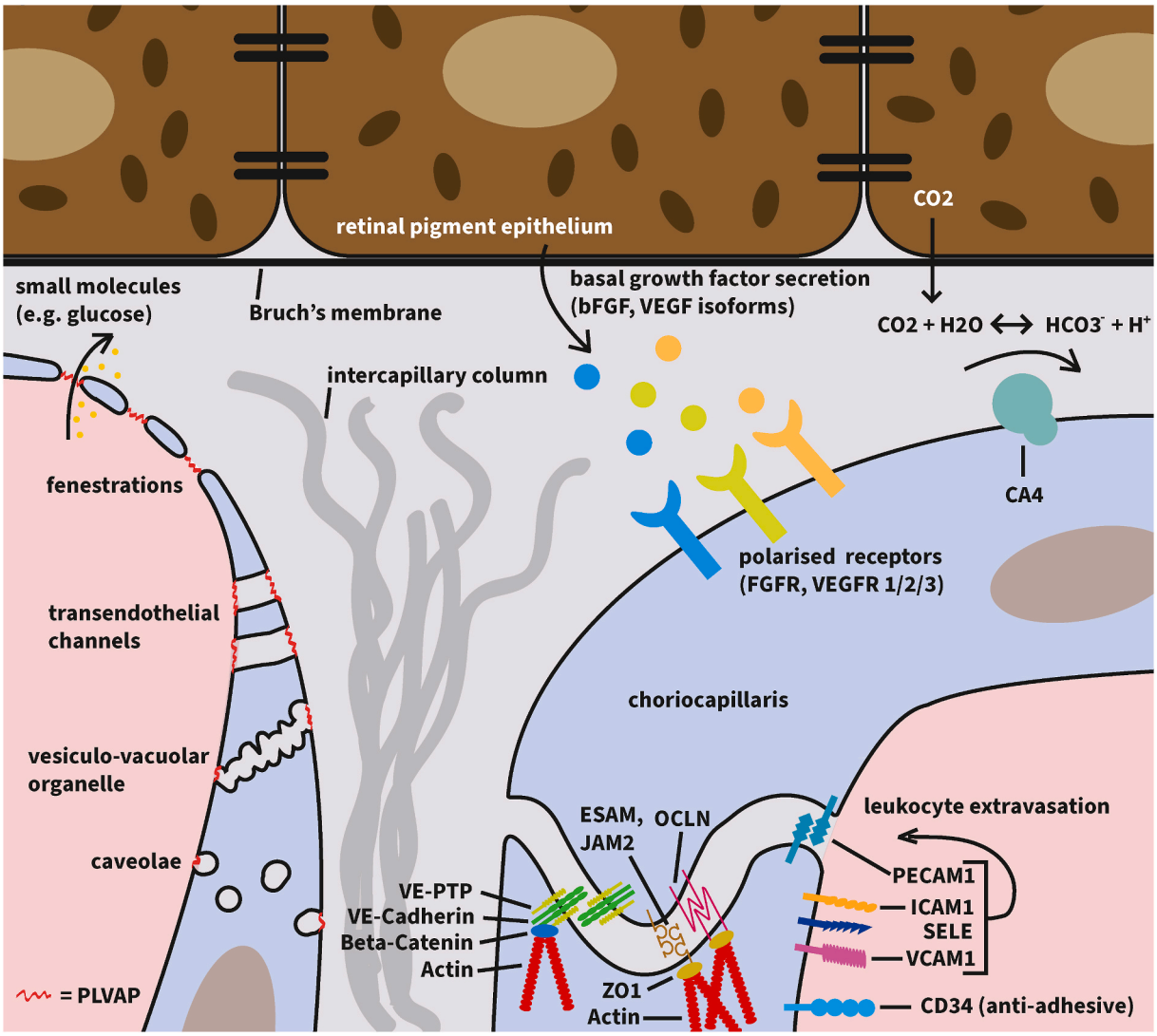


Fig. 5. Schematic representation of the choriocapillaris at the retinal pigment epithelium interface. Choroidal endothelial cells (CECs) have transportation structures (fenestrations, transendothelial channels, vesiculo-vacuolar organelles, and caveolae) which facilitate transcytosis. Diaphragms cover the openings of these transportation structures, of which the only known molecular component in CECs is plasmalemmal vesicle-associated protein (PLVAP). Intercapillary columns consist of extracellular matrix, which provides structural support to the choriocapillaris. The cellular junctions at the choriocapillaris consist of a variety of proteins, which have a barrier function but may also be dynamic (i.e. open and close) to facilitate paracellular transport and leukocyte extravasation. The choriocapillaris is highly dependent on growth factors secreted by the retinal pigment epithelium, such as vascular endothelial growth factor (VEGF). CECs express carbonic anhydrase 4 (CA4), an enzyme presumed to be involved in the export of carbon dioxide produced by the highly metabolically active outer segments of the photoreceptors.

to the highly active role that the choriocapillaris plays in the ocular immune response, particularly given the close proximity of the choriocapillaris to the outer blood-retinal barrier relative to the deeper vascular layers of the choroid. Thus, the relatively high expression levels of MHC-I and ICAM-1 – which contribute to antigen presentation and leukocyte extravasation, respectively – can be linked to the active role that the choriocapillaris plays in the choroidal immune response. Interestingly, however, although immunohistochemical studies have confirmed that MHC-I and ICAM-1 expression levels are higher in the choriocapillaris compared to the deeper layers in the choroid (Goverdhan et al., 2005a; McLeod et al., 1995; Mullins et al., 2006), this choriocapillaris-specific expression is not reflected in the mRNA levels of these markers as shown in Table 1 (Voigt et al., 2019). This discrepancy between protein levels and mRNA levels may be due to the fact that measuring an mRNA transcript does not always accurately reflect the corresponding protein levels (Edfors et al., 2016). Nevertheless, although mRNA levels can predict cell-specific expression, this finding underscores the importance of measuring protein levels in order to validate and/or complement transcriptomics studies, with immunohistochemistry providing the standard for measuring spatial specificity.

3.1.2. *Specific features of endothelial cells in the choriocapillaris*

Although the gene expression profile of ECs in the choriocapillaris can be distinguished from the gene expression profile in other choroidal ECs, there is also a large amount of overlap in gene expression with CECs in larger choroidal vessels (see section 3.1.3), primary cultured CECs (see section 3.1.5), and even RECs (see section 3.2) (Table 1). A single marker that is expressed exclusively in choriocapillaris ECs has not yet been identified; however, the simultaneous expression of CA4, PLVAP, and VEGFR3 may represent a gene expression profile unique to the choriocapillaris (Figs. 3 and 4) (Blaauwgeers et al., 1999; Hageman et al., 1991). Based on functional studies and immunohistochemical data, these three specific markers are correlated with specific features of the choriocapillaris (Fig. 5). The role of CA4 in carbon dioxide transport was discussed in section 2.6.6, while the role of PLVAP in physiological transcytosis and barrier function is discussed in sections 2.6.1 and 3.4.2,

respectively. Finally, the role of VEGFR3 is discussed in section 3.3.1.

Voigt et al. identified a choriocapillaris EC population based on their scRNA-seq study of the human choroid (Table 1) (Voigt et al., 2019). Interestingly, the five most differentially expressed genes in the choriocapillaris reported by Voigt and colleagues were *CA4* and *PLVAP*, as well as *IGFBP6*, *VWA1*, and *ADM*, which encode insulin-like growth factor-binding protein 6, von Willebrand factor A domain containing 1, and adrenomedullin, respectively. Expression of the *ADM* gene was discussed in detail in section 2.7.4; in contrast, the relevance of the high expression of *IGFBP6* and *VWA1* is currently unknown. Finally, other genes were found to be expressed at higher levels in the choriocapillaris endothelium compared to the endothelium of larger choroidal vessels include *SIPR3*, *SPARC*, *PCDH12*, *RGCC*, and the three genes that encode the VEGF receptors, *VEGFR1*, *VEGFR2*, and *VEGFR3* (Voigt et al., 2019).

3.1.3. Endothelial cells in Sattler's layer and Haller's layer: arteries and veins

Anatomical and clinical studies often characterise the medium-sized and large-sized vessels of the choroid as belonging to Sattler's layer and Haller's layer, respectively. Although these two layers can be distinguished based on anatomical landmarks (Zhao et al., 2018), which has proven to be of particular clinical value, these layers have not been distinguished based on markers or gene expression in the CECs present in the respective layers. Rather, two recent scRNA-seq studies suggest that the CECs in both Sattler's layer and Haller's layer can be classified as being either arterial or venous in origin (Table 1) (Cowan et al., 2020; Voigt et al., 2019).

To support their function, arteries have a relatively thick wall and are subject to pulsatile blood flow. In contrast, veins have a relatively thin wall, a large lumen, and lower blood pressure compared to arteries. These functional differences are reflected in the gene expression patterns of the respective vascular ECs, which are established during embryonic development (Wolf et al., 2019). These expression patterns are regulated primarily by Notch signalling, a highly conserved pathway that determines the arterial versus venous fate of ECs during development, resulting in arteries expressing EFNB2 (Ephrin B2) and veins expressing EPHB4 (Ephrin B4) (Wolf et al., 2019). These markers, as well as other generic markers of EC differentiation towards either an arterial fate (HEY1, DLL4, etc.) or venous fate (NR2F2, NRP2, etc.) are expressed at moderate to high levels in CECs, consistent with their respective arterial or venous differentiation (Table 1) (Aird, 2007; Cochrane et al., 2019; Wolf et al., 2019). Moreover, Voigt et al. found that additional arterial EC markers, including *CLDN5*, *GJA4*, and *SEMA3G*, were among the highest differentially expressed genes in arterial CECs when compared to their expression in CECs in capillaries and veins (Table 1) (Voigt et al., 2019). Other genes found to be expressed at high levels in arterial CECs included *BGN* and *IGFBP3* (Table 1) (Voigt et al., 2019). The *ACKR1* gene (atypical chemokine receptor 1, also known as *DARC*) has the highest differential expression in choroidal veins compared to the CECs in the choriocapillaris and arteries; this gene has been described as a marker for postcapillary and small collecting venules (Voigt et al., 2019). Interestingly, the *ACKR1* gene was recently linked to the interaction between adhesive leukocytes and the endothelium (Thiriot et al., 2017), a function that has also been attributed to ICAM-1, E-selectin (encoded by the *SELE* gene), and VCAM-1, all of which are also expressed at high levels in choroidal veins (Table 1). Additionally, the genes *CLU*, *TGFBR1*, *ADGRG6,* and *CPE* are expressed at higher levels in venous CECs compared to CECs from the choriocapillaris and arterial CECs, although the functional implications are currently unknown (Table 1) (Voigt et al., 2019). Recently, Cowan and colleagues provided independent support of these findings by reporting a similar gene expression pattern in human choroidal arteries and veins (Table 1) (Cowan et al., 2020).

Importantly, the gene signatures measured in ECs in various arteries and veins throughout the body can be quite heterogeneous (Aird, 2007). Thus, blood vessels in various tissues can express markers with different specificity, based on the nature of the vasculature and the organ that the vessels perfuse. This heterogeneity can be influenced by a variety of factors, including vessel size, shear stress, and epigenetics (Aird, 2007; Cochrane et al., 2019). Although arteries and veins can be classified based on the presence of classic markers for determining arterial and venous fate, respectively, the relevance of additional markers that may contribute to the tissue-specific heterogeneity of blood vessels has not been investigated with respect to the choroidal vasculature. Thus, future studies will likely provide a clearer understanding of the heterogeneity between vessel types, perhaps providing new insights into vascular diseases that preferentially affect a specific organ and/or vasculature. With respect to the choroid, these diseases may include PCA occlusion, arteritis, uveitis, and diseases within the pachychoroid spectrum.

3.1.4. Subtle differences between the central choroid and the peripheral choroid

The regional differences between the central and peripheral retina, RPE, and choroid have been studied rather extensively in recent decades, with microscopy, immunohistochemistry, and transcriptomics primarily using tissue punches taken from human donor eyes. In this section, we discuss the most relevant studies and their conclusions with respect to the choroid.

A large number of studies have assessed regional transcriptomics differences in the choroid by comparing macular versus peripheral areas using a variety of techniques. These studies usually involve paired tissue punches taken from the peripheral and central tissues of human donor eyes. The majority of these studies analysed gene expression in the RPE/choroid complex using either microarrays (Radeke et al., 2007; van Soest et al., 2007) or bulk RNA sequencing techniques (Li et al., 2014; Whitmore et al., 2014), thus analysing all cell types contained in each sample. This approach is not necessarily suited to identifying cell-specific gene expression profiles in cells such as CECs, particularly when it comes to drawing comparisons between these studies. First, many genes are expressed by several cell types, which makes it difficult to trace the expression of a specific gene to a specific cell type (i.e. the so-called dilution effect). Second, although most studies separate the retina from the RPE/choroid complex and analyse the tissues separately, contamination of neuroretinal cellular content can occur during dissection (Whitmore et al., 2014). Third, biopsies taken from different regions can contain different proportions of each cell type, potentially resulting in a relative overrepresentation or underrepresentation of gene expression by a specific cell type. This is presumably often the case with the choroid, which varies widely with respect to layer thickness (and thus also in the relative proportions of melanocytes and/or blood-derived cells in a given sample). Lastly, many other factors can influence the study outcome, including the technique used to isolate the samples, the location of the biopsies, post-mortem delays, patient age, genetic diversity between patients, and the techniques and/or methods used to analyse the dataset. A number of these limitations have been overcome by using a study design that considers several retinal layers at once and by optimising the bioinformatics analysis (Booij et al., 2010b). Nevertheless, defining regional differences in gene expression profiles in CECs remains difficult with this type of study, giving rise to the highly variable – and often low – degree of overlap when comparing findings drawn from these studies (Tian et al., 2015).

Studies using scRNA-seq can circumvent many of the aforementioned challenges associated with bulk RNA sequencing. To date, two such studies have analysed macular and peripheral samples of RPE/choroid complexes, yielding similar results (Cowan et al., 2020; Voigt et al., 2019). In their study, Voigt et al. found statistically significant – albeit, subtle – differences in gene expression between the macular and peripheral areas (Voigt et al., 2019). The most enriched genes in the macular CECs compared to peripheral CECs were the *SELE* gene in the choriocapillaris (log fold change: 0.97), the *PTGDS* gene in the choroidal veins (log fold change: 0.70), and the *EDN1* gene in the choroidal arteries (log fold change: 0.47) (Voigt et al., 2019). E-selectin, which

serves as a general marker for ECs, plays a role in leukocyte adhesion and extravasation (Vestweber, 2015; Voigt et al., 2019). Interestingly, the expression of PTGDS (prostaglandin synthase) is increased in response to shear stress (Helliwell et al., 2004). This is consistent with observations in animal studies in which choroidal blood flow – and thus shear stress – is markedly higher in the macula compared to the periphery (Nork et al., 2006). PTGDS also functions as an anti-coagulant, vasodilator, and inflammatory mediator (Helliwell et al., 2004). Finally, EDN1 (Endothelin 1), which is expressed predominantly in arteries, is a potent vasoconstrictor (Houde et al., 2016).

Genes found to be expressed at higher levels in the peripheral choroid include the *CP* gene, which encodes the iron-binding protein ceruloplasmin (Hellman and Gitlin, 2002), and *IGFBP5*, which encodes insulin-like growth factor-binding protein 5, a protein believed to suppress the inflammatory response in atherosclerosis (Xu et al., 2018). Similar observations with respect to region-specific differences in *PTGDS*, *EDN1*, *IGFBP5*, and *CP* expression were made by Cowan and colleagues (Cowan et al., 2020). Notably, Cowan et al. did not distinguish a separate population of choriocapillaris ECs in their study (Cowan et al., 2020).

Importantly, scRNA-seq can be subject to bias when quantifying transcripts (Chen and Zheng, 2018). Moreover, scRNA-seq studies are usually performed using a limited number of donors, due to the high costs associated with the technique and the scarcity of suitable human ocular tissues available for these studies. In addition, differences between individuals can further complicate the interpretation of studies that involve a limited number of subjects. For example, Mullins et al. performed a morphometric analysis of ICAM-1 expression in nine individuals using immunohistochemistry and observed significantly stronger macular ICAM-1 staining in six individuals, stronger peripheral staining in one individual, and no difference in the remaining two individuals (Mullins et al., 2006). Given these limitations with respect to scRNA-seq analysis, findings based on regional differences in gene expression should be validated at the protein level and using a sufficient number of subjects, particularly in the case of rather subtle differences.

Using human primary CEC cultures, our group compared the transcriptomes between the central choroid and the peripheral choroid. Bulk RNA sequencing was performed on cells derived from four donors, with a paired differential gene expression analysis between cultures derived from the central and peripheral choroid (Table 2). In total, we found 90 genes that were statistically significant enriched in central CECs, and 74 genes that were statistically significant enriched in peripheral CECs. Although agreement with the two scRNA-seq datasets is relatively low (Cowan et al., 2020; Voigt et al., 2019), we also found that *IGFBP5* expression was enriched in peripheral CECs (Table 2), consistent with increased expression of this gene in peripheral CECs in the choroidal veins and choriocapillaris as reported by Voigt et al. (2019). Similarly, expression of the androgen receptor (encoded by the *AR* gene), found to be slightly higher in macular venous and capillary CECs in the study by Voigt et al. (2019), was also found to be increased in our study using cultured primary CECs (Table 2). Moreover, we found enriched expression in central CECs of the *CALCRL* gene, which encodes for the receptor of adrenomedullin (Table 2). In peripheral CECs, we observed enriched expression for VEGF (encoded by the *VEGFA* gene) and the vitamin D receptor (encoded by the *VDR* gene) (Table 2). Importantly, further studies are needed in order to confirm these apparent regional differences. This is mainly because our data are in relatively low agreement with the scRNA-seq datasets (Cowan et al., 2020; Voigt et al., 2019), given the relatively small sample size of our analysis (n = 4), and because these observations were based on measurements made at the mRNA level. Ideally, validation at the protein level is performed using immunohistochemistry that includes both the central and the peripheral choroid within the same tissue section, which enables a direct comparison of protein expression between central and peripheral CECs.

Table 2
Differential gene expression between central and peripheral choroidal endothelial cells.

Top 50 significantly enriched in central CECs			Top 50 significantly enriched in peripheral CECs		
Gene	Expression	Fold change	Gene	Expression	Fold change
MEOX2	Low	2.9	*MSC*	Moderate	3.4
FOXP2	Low	2.8	*IL24*	Low	3.2
PDK4	Low	2.7	*KRT81*	Low	3.1
DCLK1	Moderate	2.6	*HEY2*	Low	2.6
MYRIP	Moderate	2.4	*B4GALNT1*	Low	2.6
SLC16A9	Moderate	2.2	*BMP8B*	Moderate	2.6
EPHA7	Moderate	2.1	*COL16A1*	Moderate	2.4
RUNDC3B	Moderate	2.1	*IL11*	Moderate	2.3
GIPC2	Moderate	2.1	*MATN2*	Moderate	2.3
CGNL1	Moderate	2.0	*COL9A3*	Moderate	2.3
LIFR	High	2.0	*IGFBP5*	Moderate	2.3
ADAMTS9	High	1.9	*SEMA3B*	Moderate	2.1
DPP4	Moderate	1.9	*OLFM1*	Moderate	2.1
AR	Moderate	1.9	*IRX3*	Moderate	2.1
SLC40A1	Moderate	1.9	*FOXF1*	Moderate	2.1
ADAMTS18	Moderate	1.8	*SERPINF1*	Moderate	2.1
KLHL3	Moderate	1.8	*APCDD1L*	Moderate	2.1
RORA	Moderate	1.8	*GPAT3*	Moderate	2.1
SESN3	Moderate	1.8	*ALDH3B1*	Moderate	2.1
CREBRF	Moderate	1.8	*BAIAP2L2*	Moderate	2.1
FAT4	High	1.8	*PMEPA1*	High	2.0
NHSL2	High	1.8	*PLPP4*	Moderate	2.0
NRCAM	Moderate	1.8	*LYPD1*	Moderate	2.0
PIK3C2A	High	1.8	*VDR*	Moderate	1.9
PRTG	Moderate	1.8	*PACSIN3*	Moderate	1.9
FGD4	Moderate	1.7	*VASN*	Moderate	1.9
ZDHHC21	Moderate	1.7	*MMP17*	Moderate	1.9
LRRC8B	High	1.7	*EMILIN1*	High	1.8
CALCRL	High	1.7	*TGFBI*	High	1.8
FOXO1	High	1.7	*PALLD*	Moderate	1.8
RUNX1T1	Moderate	1.7	*CD74*	High	1.8
RICTOR	Moderate	1.7	*GPR153*	Moderate	1.8
TGFBR3	Moderate	1.7	*FERMT3*	Moderate	1.8
LPAR6	High	1.7	*VEGFA*	Moderate	1.8
FAM171B	Moderate	1.7	*P3H3*	High	1.8
NFIB	High	1.7	*PHLDA2*	High	1.7
SH3BGRL2	Moderate	1.6	*ZNF703*	High	1.7
DGKE	Moderate	1.6	*CYB5R2*	Moderate	1.7
PREX2	Moderate	1.6	*FSTL3*	Moderate	1.7
N4BP2	Moderate	1.6	*AMIGO2*	Moderate	1.7
HECW2	High	1.6	*AEBP1*	High	1.7
MEF2C	High	1.6	*CD44*	High	1.7
SLC12A2	High	1.6	*PAK1*	Moderate	1.7
DNAJB4	High	1.6	*CLEC11A*	High	1.7
FAM214A	Moderate	1.6	*FAM20C*	High	1.7
PRRG1	Moderate	1.6	*BAMBI*	Moderate	1.7
MTUS1	High	1.6	*ARHGEF40*	High	1.7
VPS13C	High	1.6	*PFKFB4*	Moderate	1.7
PLCB1	Moderate	1.6	*TRPV2*	High	1.6
ABCB1	High	1.6	*TNFRSF12A*	High	1.6
TNFSF4	Moderate	1.6	*ST3GAL4*	High	1.6

Paired RNA sequencing analysis on the central and peripheral primary choroidal endothelial cells (CECs) derived from 4 human donors. Expression levels were calculated as average log2 counts per million (log2cpm) reads. Expression is shown as percentile of log2cpm reads; low expression (1st-10th percentile), moderate expression (10th-50th percentile), or high expression (>50th percentile). Statistically significant differences in gene expression between central and peripheral CECs are shown as fold change relative to the peripheral CECs (left column) or the central CECs (right column).

3.1.5. *The phenotypic properties of cultured human choroidal endothelial cells*

Cultured cells are often amenable to a wide range of experimental manipulations and can therefore be used to better understand the role of CECs in chorioretinal disease. Many studies use immortalised cell lines,

which are relatively easy to maintain and have the clear advantage of being available as a nearly infinite supply. Despite their advantages, however, commercially available immortalised cell lines derived from CECs often lose many of their functional properties that are present in the original *in vivo* cells. In this respect, freshly isolated primary CECs can better recapitulate the *in vivo* phenotypic properties, although these cells can also undergo de-differentiation, particularly when passaged many times (Brinks et al., 2018; Loeven et al., 2018; Makin et al., 2018; Peavey and Malek, 2020). For example, the RF/6A cell line, an immortalised endothelial cell line derived from macaque CECs, has been widely used for studying chorioretinal diseases, but is well-known to be subject to de-differentiation (Makin et al., 2018). Thus, many of the functional properties typically present in primary ECs, including endocytosis of low-density lipoprotein and the response to shear stress, are often impaired in RF/6A cells. Furthermore, the expression of generic EC markers such as VWF, CD31, and CDH5 is significantly reduced in RF/6A cells (Makin et al., 2018). For these reasons, RF/6A cells are not endothelial-like and may not be suitable as a model for studying chorioretinal diseases in a specific context.

Alternatively, several protocols have been developed for isolating and culturing human primary CECs from post-mortem ocular tissues (Fig. 6) (Brinks et al., 2018; Browning et al., 2005; Penfold et al., 2002; Sakamoto et al., 1995a). The majority of these protocols involve enzymatic digestion of the choroidal tissue, which is then filtered to obtain a single-cell suspension. Magnetic beads conjugated to an antibody directed against a generic EC marker such as CD31 are then used to isolate and culture the CECs. Nevertheless, issues related to primary CEC cultures can include contamination by microorganisms derived from the post-mortem tissue and the growth of unwanted cell types such as fibroblasts, which thrive in EC cultures and tend to proliferate at a faster rate than ECs (Van Beijnum et al., 2008). In our study, CECs were isolated and cultured using consistent conditions, and sequencing was

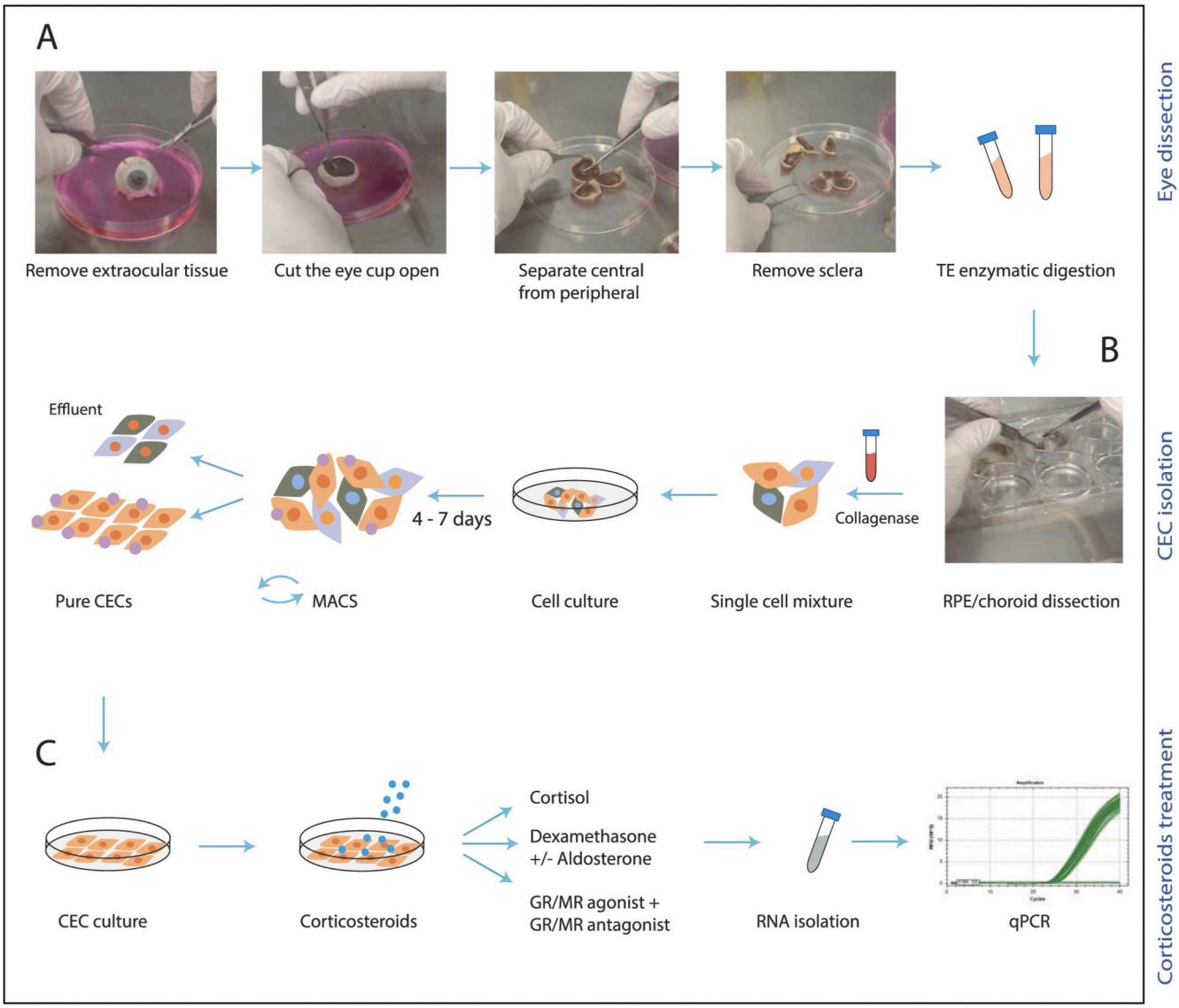


Fig. 6. Reproduced with permission from Brinks et al. (2018). Flowchart of the isolation of human choroidal endothelial cells (CECs). First (A), eyes were dissected to separate the choroid from the other layers and to divide the choroid into central and peripheral parts to be processed separately. The choroidal tissue was then enzymatically digested using trypsin/EDTA (TE). (B) The remnants of retinal pigment epithelium (RPE) were brushed away (RPE/choroid dissection). Single-cell suspension of the choroid was obtained by collagenase digestion. Cells were cultured for 4–7 days and the magnetic-activated cell sorting (MACS) was performed after reaching 70%–80% confluence, after which the same procedure was performed 4–7 days later. (C) Primary cultures of purified CECs were used during the different corticosteroids treatment experiments. Finally, quantitative polymerase chain reaction (qPCR) was performed to investigate the differential gene expression of the cultured CECs.

performed using confluent cells cultured after a second selection round using magnetic-activated cell sorting (Table 1) (Brinks et al., 2018). Although culture conditions have been optimised for ECs, and although most CEC markers are still expressed in primary cultures, a certain degree of de-differentiation is unavoidable with *ex vivo* cultures (Table 1) (Alizadeh et al., 2018; Peavey and Malek, 2020). Specifically, primary CEC cultures can lose their expression of the choriocapillaris marker *CA4*, and expression of genes related to leukocyte adhesion such as *ICAM1*, *SELE*, and *VCAM1* is substantially lower compared to *in vivo* CECs (Table 1). These changes in expression may be due to a lack of stimuli in culture compared to *in vivo*. Indeed, ICAM-1 expression can be strongly induced *in vitro* using pro-inflammatory stimuli (Caprio et al., 2008; Whitmore et al., 2015). Another interesting observation is the relatively high expression of genes related to angiogenesis and wound healing measured in primary CECs in confluent cultures (Table 1), including genes encoding the tip cell markers APLN and APLNR, both of which have been used as markers of a highly migratory cell type that causes angiogenic sprouting, thus giving rise to angiogenic tip cells present in endothelial cell cultures grown under standard conditions (Palm et al., 2016; Siemerink et al., 2013).

3.2. Choroidal endothelial cells versus retinal endothelial cells: similarities and differences

The human eye contains a dual vascular system in which the inner retina is perfused by the retinal vasculature, while the outer retina receives nourishment from the choroid. Although the ECs in the retinal vasculature and choroidal vasculature have certain similarities with respect to both structure and function, they also have substantial differences that should be considered with designing therapeutic strategies for treating retinal and chorioretinal diseases (Stewart et al., 2011).

3.2.1. Choroidal and retinal endothelial cells have unique niches

The choroidal and retinal vasculatures have pronounced differences at the level of the capillaries, more so than at the level of the arteries or veins. CECs in the choriocapillaris are highly dependent upon their close spatial relationship with the RPE, and their phenotype and structure is optimised for the transendothelial transport of both small and large molecules, which reach the photoreceptors by passing the BM and the RPE. In contrast, capillary RECs are closely arranged with the foot processes arising from pericytes and Müller glial cells, forming highly specialised tight junctions with neighbouring cells, similar to the blood-brain barrier (Klaassen et al., 2013). Based on the environmental, structural, and phenotypic differences between RECs and CECs, transendothelial transport is regulated differently between these two cell types. The most illustrative marker of these differences is perhaps the protein PLVAP, which is a transendothelial transport marker that is absent in barrier endothelium (Table 1) (Bosma et al., 2018; Schlingemann et al., 1985, 1988, 1997). Indeed, tracer studies using radiolabelled molecules have shown that nutrients can diffuse almost freely through the choriocapillaris, but are subsequently filtered as they cross the outer blood-retinal barrier formed by the RPE (Törnquist et al., 1990). In contrast, the inner blood-retinal barrier formed by the RECs is virtually impermeable, even to small molecules and sodium ions; instead, this vasculature relies on carrier-mediated transport mechanisms similar to those used by the blood-brain barrier, thus providing far more selective transendothelial transport compared to CECs (Klaassen et al., 2013; Törnquist et al., 1990). Notably, PLVAP can be upregulated in RECs if the inner blood-retinal barrier is compromised, for example during prolonged hyperglycaemia in diabetic macular oedema (Klaassen et al., 2013; Schlingemann et al., 1999).

3.2.2. Phenotypic and functional characteristics of choroidal endothelial cells and retinal endothelial cells

Transcriptomics and proteomics studies of CECs and RECs have shed new light on the phenotypes of these two cell types, as well as the molecular basis underlying their functional differences. Table 3 summarises the markers that are differentially expressed between CECs and RECs, and the most relevant markers are discussed below. Importantly, most studies comparing the phenotypic and functional differences between CECs and RECs use *ex vivo* samples and therefore may not adequately reflect the *in vivo* situation. Two separate transcriptomics studies analysed freshly isolated and primary cultured CECs and RECs and found that under basal conditions 9% of the transcripts are differentially expressed between these two cell types (Browning et al., 2012b; Smith et al., 2007). More recently, Smith et al. used proteomics and found that 14% of the proteins analysed differed in expression between freshly isolated CECs and RECs (Smith et al., 2018).

Under unstimulated conditions, both CECs and RECs express high levels of the canonical EC markers *VWF* and *CD31* (Browning et al., 2012b); moreover, both *VEGFR1* and *VEGFR2* are expressed at similar levels between these two cell types (Stewart et al., 2011). With respect to cellular junctions, *OCLN* and *CLDN5*, which encode tight junction proteins that are considered important gatekeepers of paracellular permeability, are expressed at approximately 5-fold higher levels in RECs than in CECs (Table 3) (Saker et al., 2014). On the other hand, expression of the *F11R* and *JAM3* genes, which encode tight junction components, and the *CDH5* gene, which encodes the adherens junction marker VE-Cadherin, is similar between CECs and RECs (Saker et al., 2014).

Interestingly, CECs and RECs have distinct expression profiles in terms of angiogenesis markers; these differences are present both under unstimulated conditions and during hypoxia, a highly potent inducer of angiogenesis (Table 3) (Browning et al., 2012b; Mammadzada et al., 2016). Unstimulated CECs can be distinguished from unstimulated RECs by their higher expression of angiogenesis-related genes, including *IL7*, *VCAM1*, and *PEDF*. Conversely, unstimulated RECs express higher levels of *ANGPTL4*, *PLGF*, and *VEGFA* compared to unstimulated CECs (Browning et al., 2012b; Mammadzada et al., 2016). Interestingly, during hypoxia *ANGPTL4* and *VEGFA* are strongly upregulated in CECs, but not in RECs (Mammadzada et al., 2016). The *F3* gene, which encodes coagulation factor III, is upregulated in both CECs and RECs during hypoxia; however, this effect is more pronounced in CECs, which is particularly interesting given that the *F3* gene was reported to be upregulated in CNV lesions in patients with AMD (Cho et al., 2011). Moreover, during hypoxia the *PLGF* and *EDN1* genes – both of which encode pro-angiogenic factors known to induce EC proliferation, migration, and tube formation – are upregulated only in RECs (Table 3) (Mammadzada et al., 2016; Van Bergen et al., 2019).

Angiogenesis has also been found to manifest differently between CECs and RECs at the functional level. Specifically, VEGF has a more pronounced effect on the proliferation rate of RECs compared to CECs (Browning et al., 2012b; Stewart et al., 2011). Interestingly, the addition of IGF-1 (insulin-like growth factor) to the culture media increased the proliferation rate of RECs but had no effect on CECs, consistent with the finding that components in the IGF-1 signalling pathway are expressed 3-6-fold higher in RECs compared to CECs (Browning et al., 2012b). In addition, the hypoxia-induced gene *IGFBP1*, which encodes a binding protein for IGF-1, is specifically upregulated in CECs, however a functional aspect of *IGFBP1* in CECs remains to be established (Mammadzada et al., 2016). Lastly, CECs and RECs differ in their response to high levels of glucose, a condition that damages ECs and leads to reduced barrier function (Klaassen et al., 2013; Lutty, 2017; Saker et al., 2014). Specifically, RECs are more susceptible than CECs to glucose-induced barrier dysfunction, with a relative increase of permeability of 20.6% versus 3.9%, respectively. Moreover, high glucose causes the downregulation of OCLN, CLDN5, and F11R in RECs but not in CECs (Saker et al., 2014), possibly due to the relatively low basal expression of these junction proteins in CECs. Notwithstanding, CECs are undoubtedly also sensitive to the damaging effects of hyperglycaemia, as discussed in section 4.4.

Taken together, these results suggest that CECs and RECs utilise distinct biomolecular mechanisms in response to both hypoxia and hyperglycaemia. These findings may have implications with respect to

Table 3
Overview of differential gene expression between choroidal endothelial cells and retinal endothelial cells.

Condition	Gene	Category	Differential expression	Validation	Reference
Basal conditions			*Fold change in CECs relative to RECs*		
	PLVAP	Transendothelial transport	–	protein	Blaauwgeers et al. (1999)
	VCAM1	Adhesion molecules	↑3.3	mRNA	Mammadzada et al. (2016)
	OCLN	Cellular junctions	↓5.3	mRNA	Saker et al. (2014)
	CLDN5	Cellular junctions	↓4.8	mRNA	Saker et al. (2014)
	IL7	Angiogenesis	↑32.0	mRNA	Mammadzada et al. (2016)
	PIK3C2A	Angiogenesis	↑7.9	mRNA	Browning et al. (2012b)
	PTGS1	Angiogenesis	↑4.4	mRNA	Mammadzada et al. (2016)
	PEDF	Angiogenesis	↑4.4	mRNA	Mammadzada et al. (2016)
	CXCL6	Angiogenesis	↑3.2	mRNA	Mammadzada et al. (2016)
	CCL2	Angiogenesis	↑3.1	mRNA	Mammadzada et al. (2016)
	MMP9	Angiogenesis	↑2.0	mRNA	Mammadzada et al. (2016)
	PGF	Angiogenesis	↓26.3	mRNA	Mammadzada et al. (2016)
	EDN1	Angiogenesis	↓12.0	mRNA	mRNA
	ANGPTL4	Angiogenesis	↓10.7	mRNA	Mammadzada et al. (2016)
	VEGFA	Angiogenesis	↓8.7	mRNA	Browning et al. (2012b)
	CD34	Angiogenesis	↓5.9	protein	Stewart et al. (2011)
	F3	Angiogenesis	↓5.9	mRNA	Mammadzada et al. (2016)
	HIF1A	Angiogenesis	↓2.2	mRNA	Browning et al. (2012b)
	CTGF	Angiogenesis	↓2.1	mRNA	Mammadzada et al. (2016)
	VEGFC	Angiogenesis	↓2.1	mRNA	Mammadzada et al. (2016)
	IGFBP7	IGF-1 signalling	↓4.1	mRNA	Browning et al. (2012b)
	IRS1	IGF-1 signalling	↓6.4	mRNA	Browning et al. (2012b)
	IGF1R	IGF-1 signalling	↓3.0	mRNA	Browning et al. (2012b)
Hypoxia			*Direction of regulation*		
	F3	Angiogenesis	↑ CECs, ↑ RECs	protein	Mammadzada et al. (2016)
	ANGPTL4	Angiogenesis	↑ CECs, - RECs	mRNA	Mammadzada et al. (2016)
	TSP1	Angiogenesis	↑ CECs, - RECs	protein	Mammadzada et al. (2016)
	IGFBP1	Angiogenesis	↑ CECs, - RECs	protein	Mammadzada et al. (2016)
	VEGFA	Angiogenesis	↑ CECs, - RECs	protein	Mammadzada et al. (2016)
	EFNA1	Angiogenesis	↑ CECs, - RECs	mRNA	Mammadzada et al. (2016)
	MDK	Angiogenesis	↑ CECs, - RECs	mRNA	Mammadzada et al. (2016)
	IL8	Angiogenesis	↓CECs, - RECs	protein	Mammadzada et al. (2016)
	PLAU	Angiogenesis	↓CECs, - RECs	protein	Mammadzada et al. (2016)
	PGF	Angiogenesis	- CECs, ↑ RECs	protein	Mammadzada et al. (2016)
	EDN1	Angiogenesis	- CECs, ↑ RECs	protein	Mammadzada et al. (2016)
Hyperglycaemia			*Direction of regulation*		
	CLDN5	Cellular junctions	- CECs, ↓ RECs	protein	Saker et al. (2014)
	OCLN	Cellular junctions	- CECs, ↓ RECs	protein	Saker et al. (2014)
	F11R	Cellular junctions	- CECs, ↓ RECs	protein	Saker et al. (2014)

CECs, choroidal endothelial cells, RECs, retinal endothelial cells.

developing new strategies for treating neovascularisation and diabetes-associated ocular diseases that are either retinal or choroidal in origin.

3.3. Regulation of choriocapillaris function by the retinal pigment epithelium

Proper signalling from a healthy RPE is essential for the normal development, structure, and function of the choroid, and vice versa. Signalling between the RPE and the choriocapillaris is based on the basal secretion of VEGF by the RPE and expression of the three VEGF receptors on the choriocapillaris endothelium, a process first described by Blaauwgeers and colleagues more than two decades ago (Fig. 7) (Blaauwgeers et al., 1999). This interaction begins during embryogenesis, when the RPE serves as a driving force for the developing choriocapillaris (Lutty and McLeod, 2018). Conditional knockout mice in which VEGF expression is deleted specifically in the RPE showed that the choroid depends on the RPE, particularly during embryogenesis (Marneros et al., 2005). The authors found that the loss of RPE-derived VEGF during embryogenesis led to severe microphthalmia, near-complete absence of the choroid, and disorganised and shortened photoreceptor outer segments. The release of VEGF by the RPE continues throughout life, playing an essential role in healthy choroid function and maintaining fenestrations (Blaauwgeers et al., 1999; Kim et al., 2020a).

Assays using RPE cells with transwell filters revealed the release of growth factors from the RPE is highly polarised, with up to 10 times more VEGF released on the basal side compared to the apical side (Blaauwgeers et al., 1999; Klettner et al., 2015). Notably, studies have shown that the trophic interaction between the RPE and the choriocapillaris is likely more complex than simply the release of VEGF, although the role of additional factors has not been examined in detail. One such factor is bFGF, which has been shown to play a role in the RPE-choriocapillaris interaction during embryogenesis (Saint-Geniez and D'Amore, 2004; Sakamoto et al., 1995b). Another growth factor that may be involved in this interaction is PlGF (placental growth factor), which is released on the basal side of the RPE in porcine cultured RPE/choroid complex explants (Klettner et al., 2015). Interestingly, RECs expressed 26-fold higher levels of PlGF (measured at the mRNA level) compared to CECs (Mammadzada et al., 2016), which may indicate that CECs require an external source of PlGF such as the RPE. Nevertheless, the functional role of PlGF signalling in CECs remains unknown (Klettner et al., 2015; Van Bergen et al., 2019). In summary, which growth factors other than VEGF are released by the RPE in order to regulate choroidal function – and vice versa – remains an open question. Given that the RPE is involved in a wide variety of chorioretinal diseases, understanding its interaction with the choroid may provide valuable insights into these diseases, thus warranting further study.

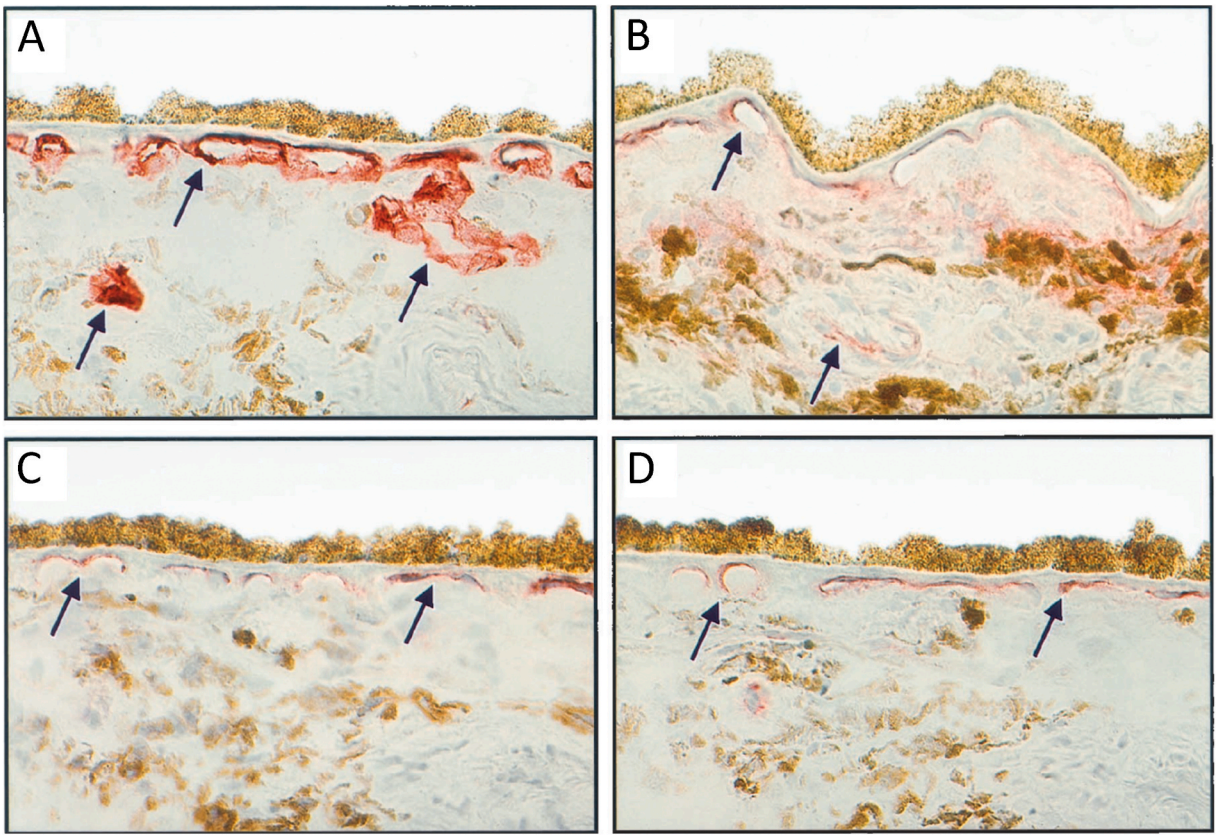


Fig. 7. Reproduced with permission from Blaauwgeers (1999). Immunoperoxidase staining of frozen tissue sections of human choroid with monoclonal antibody Pathologische Anatomie Leiden-Endothelium (PAL-E), recognizing fenestrated endothelium (A), and antibodies recognizing the vascular endothelial growth factor receptors (VEGFR): VEGFR1 (B), VEGFR2 (C), and VEGFR3 (D). Note the intense staining of the entire choriocapillaris and other choroidal vessels for PAL-E (A), in contrast to the localised staining of the choriocapillaris at the side of the retinal pigment epithelium cell layer for VEGFR2 (C) and VEGFR3 (D). VEGFR1 staining can be observed in the inner choriocapillaris and in a large vessel in the choroid (B). Staining of vascular structures is indicated by arrows.

3.3.1. VEGF receptors: a unique role for VEGFR3

The expression of VEGF receptors (VEGFRs) is essential for maintaining a healthy, properly functioning choroid. Indeed, both transcriptomics and immunohistochemistry studies found that VEGFR1, VEGFR2, and VEGFR3 are expressed in high levels in the choroid (Table 1 and Fig. 7) (Blaauwgeers et al., 1999). VEGFR2 and VEGFR3 have similar patterns of expression and localisation in the choroid; in contrast, VEGFR1 is more widely expressed than VEGFR2 and VEGFR3, and is also expressed in larger vessels (Blaauwgeers et al., 1999). Interestingly, the localisation of VEGFR3 is highly polarised at the RPE interface, underscoring the trophic relationship between the RPE and the choriocapillaris (Fig. 7) (Blaauwgeers et al., 1999). Even within the choriocapillaris ECs, VEGFR3 is localised adjacent to the RPE side of the cell membrane. Outside of the eye, VEGFR3 is expressed only in lymphatic ECs, in some vascular ECs in the kidney, and in other anatomical sites in which fenestrated capillaries are adjacent to the epithelium (Witmer et al., 2002), although it is expressed more widely during embryogenesis and vascular sprouting (Ward and Cunningham, 2015; Witmer et al., 2001; Wolf et al., 2019).

The precise function of VEGFRs in the choroid is poorly understood. However, it is interesting to note that VEGFR1 and VEGFR2 primarily bind VEGF-A, while VEGFR3 binds VEGF-C and VEGF-D (Blaauwgeers et al., 1999; Witmer et al., 2003). With respect to VEGFR1, given its more widespread localisation compared to VEGFR2, coupled with the fact that both VEGFR1 and VEGFR2 bind VEGF-A, VEGFR1 may play a different functional role than VEGFR2 in response to VEGF-A binding. Moreover, given the similar localisation and polarised expression of VEGFR2 and VEGFR3 in the choriocapillaris, it is reasonable to speculate that these two receptors may respond differently to specific RPE-derived VEGF ligands (Ikeda et al., 2006; Kannan et al., 2006; Witmer et al., 2003). If so, then a functional synergy may exist between VEGF-A and either VEGF-C or VEGF-D, for example in maintaining the highly fenestrated structure of the choriocapillaris, which is mediated by the release of VEGF from the basal side of the RPE (Blaauwgeers et al., 1999; Marneros et al., 2005). Thus, given its expression pattern in the choriocapillaris, its presence in highly specialised cell types, and its unique ligand-binding properties compared to VEGFR1 and VEGFR2, VEGFR3 may play a unique physiological role in the choriocapillaris.

3.4. Barrier function of the choroidal endothelial cell

3.4.1. Cellular junctions

The inner blood-retinal barrier is maintained by elaborate tight junctions in RECs, while the outer blood-retinal barrier is maintained by tight junctions in the RPE (Klaassen et al., 2013). Thus, CECs are not a component of the blood-retinal barrier; in contrast, CECs form a fenestrated, non-barrier endothelium (Witmer et al., 2003). Although cellular junctions in CECs have not been widely studied at the structural or functional level, transcriptomics and studies involving molecular tracers indicate that permeability of the choroidal endothelium is a tightly regulated process (Table 1) (Grebe et al., 2019; Nakanishi et al., 2016; Törnquist et al., 1990). In addition to mediating transcellular transport, the junctions formed between ECs also provide an essential bi-directional route for paracellular transport. Cellular junctions are functionally dynamic, meaning they are able to open and close in order to regulate paracellular permeability in response to various stimuli (Dejana, 2004; Vestweber et al., 2009). Moreover, cellular junctions are important for cell signalling, thus mediating cell survival, proliferation,

migration, and polarisation.

Three distinct types of endothelial junctions have been identified, namely adherens junctions, tight junctions, and gap junctions, each with its own function and components (Klaassen et al., 2013). The expression of the principal components of several cellular junctions in CECs is summarised in Table 1. The vascular beds of various tissues have unique properties in order to meet the tissue's specific function (Dejana, 2004). In the eye, this distinction is reflected in the stark contrast between the inner blood-retinal barrier (formed by the retinal vasculature) and the non-barrier endothelium of the choroidal vasculature. Although the blood-retinal barrier has been characterised extensively, its molecular basis is highly complex and is still not fully understood (Klaassen et al., 2013; Rudraraju et al., 2020). Moreover, studies that specifically characterised the choroidal paracellular route are sparse, although the basic properties of this barrier can be deduced from studies of other types of endothelium and from basal gene expression in human CECs (Table 1).

Adherens junctions comprise the principal cellular junctions in CECs, with VE-Cadherin (also known as CDH5) serving as the predominant membrane-spanning component (Fig. 5) (Vestweber et al., 2009). On the intracellular side of the plasma membrane, VE-Cadherin binds to adaptor proteins such as beta-catenin, which links the adherens junctions to the cytoskeleton, particularly actin filaments. Tight junctions are expressed predominantly in continuous barrier endothelium (for example, in the retinal vasculature), where they prevent the passive movement of most molecules and ions (Klaassen et al., 2013). Occludins and claudins, the two major transmembrane components in tight junctions, are not expressed in CECs (Table 1). Finally, gap junctions, which have not been well-characterised in CECs, are specialised intercellular connections believed to be involved in cellular communication by directly connecting the cytoplasmic contents of cells joined by gap junctions. Structurally, gap junctions are composed of proteins known as connexins and allow the passage of relatively small molecules, thus electrically and metabolically coupling cells connected via gap junctions (Klaassen et al., 2013). Gap junctions can also connect cells of different types, for example ECs and pericytes. CECs express gap junction components, at least at the mRNA level (Table 1), although the precise expression patterns and the functional role of gap junctions in CECs are currently unknown.

3.4.2. *Plasmalemmal vesicle-associated protein: a gatekeeper of transcellular permeability*

As discussed in section 2.6.1, PLVAP is an endothelium-specific protein that plays an essential role in the transport of plasma proteins due to its presence in various transcellular transport mechanisms, including fenestrations, caveolae, and transendothelial channels (Fig. 5). PLVAP contains an intracellular domain that binds to the cytoskeleton, a transmembrane domain, and an extracellular domain (Bosma et al., 2018). Extracellularly, PLVAP spans the gaps in fenestrations and caveolae, forming fenestral diaphragms and stomatal diaphragms, respectively; it also spans transendothelial channels, forming homodimers that act as a sieve (Fig. 3) (Bosma et al., 2018; Gu et al., 2017). Using electron microscopy, PLVAP homodimers show a radial organisation within these transport structures, reminiscent of radial spokes in a wheel (Bosma et al., 2018). Interestingly, PLVAP is the only molecular component identified in fenestral and stomatal diaphragms (Bosma et al., 2018); thus, it is not surprising that a *Plvap* knockout mouse lacks these diaphragms (Herrnberger et al., 2012; Stan et al., 2012). Strikingly, fenestrations, caveolae, and transendothelial channels still form in *Plvap* knockout mice; however, the lack of diaphragms within these structures causes impaired regulation of barrier function in a variety of endothelia and epithelia, resulting in a lethal phenotype (Stan et al., 2012). More specifically, these mice develop severe protein-losing enteropathy and leakage of plasma proteins, resulting in early death. Interestingly, mice in which *Plvap* is knocked out specifically in endothelial cells also develop this severe phenotype, while endothelial-specific reconstitution of *Plvap* rescues the phenotype, confirming that the Plvap protein plays an essential role in the barrier function mediated by ECs (Stan et al., 2012). In humans, mutations in the *PLVAP* gene have also been attributed to the development of protein-losing enteropathy, leading to infant mortality (Broekaert et al., 2018; Kurolap et al., 2018).

The importance of PLVAP in regulating barrier function has been demonstrated clearly (Wisniewska-Kruk et al., 2016); however, precisely how this protein is functionally regulated, and which intracellular and extracellular proteins interact PLVAP, remain poorly understood (Bosma et al., 2018). Interestingly, VEGF has been shown to potently induce PLVAP expression and increase the number of fenestrations (Bosma et al., 2018; Hofman et al., 2001; Marneros et al., 2005). This finding may explain why staining shows that PLVAP is more strongly localised at the choriocapillaris compared to the underlying larger choroidal vessels, possibly due to the dilution of RPE-derived VEGF as it diffuses into the choroidal tissue (Fig. 3). Another factor that may regulate PLVAP expression is PEDF (pigment epithelium-derived factor), as a *Pedf* knockout mouse has a complete lack of Plvap in CECs (Farnoodian et al., 2018). Importantly, these mice were global *Pedf* knockout mice, rather than a conditional knockout mouse lacking *Pedf* expression selectively in CECs or ECs; therefore, whether *Pedf* acts via autocrine regulatory mechanisms in CECs or via paracrine sources such as the RPE remains an open question. Nevertheless, the RPE is known to release PEDF primarily on the apical side, rather than towards the CECs on the basal side (Farnoodian et al., 2018). Interestingly, the function of PLVAP may also be regulated by changing its biochemical properties, for example by modifying anionic sites and/or components in the pericellular protein matrix (i.e., the glycocalyx) (Bosma et al., 2018).

In summary, it is reasonable to speculate that different vascular beds have specific mechanisms for regulating their selectivity with respect to vascular permeability. Thus, further studies are clearly needed in order to better understand the mechanisms that regulate PLVAP expression and/or function, particularly in the choroid.

3.4.3. *Vascular endothelial growth factor as a vascular permeability factor*

Although VEGF is best known for its role in angiogenesis, it can also be considered a pleiotropic factor, participating in a wide range of processes such as embryogenesis, the immune response, regulation of permeability, the response to hypoxia, wound healing, and CNV (Witmer et al., 2003). In the human posterior eye, VEGF is released primarily from the basal side of the RPE towards the choroid, but is also produced by CECs themselves (Table 1 and Fig. 7) (Blaauwgeers et al., 1999; Browning et al., 2012a). Importantly, RPE-derived VEGF has a "maintenance of differentiation" function on the choroid by maintaining fenestrations, caveolae, vesiculo-vacuolar organelles, and transendothelial channels, thus making VEGF essential for maintaining choroidal function (Blaauwgeers et al., 1999; Bosma et al., 2018; Marneros et al., 2005). In the absence of trauma or damage, with sufficient levels of anti-angiogenic factors in the choroid and BM such as TSP-1 (thrombospondin-1), pigment epithelium-derived factor (PEDF), and endostatin, the release of high levels of VEGF does not cause CNV, provided that the outer blood-retinal barrier formed by the RPE and BM is intact (Bhutto et al., 2008; Schlingemann, 2004; Schwesinger et al., 2001; Witmer et al., 2003). Schwesinger and colleagues generated a transgenic mouse in which VEGF is overexpressed specifically in the RPE and found that this mouse model developed increased choroidal thickness, higher capillary density, CNV, and increased vascular permeability; moreover, histological analyses of choroidal flat mounts revealed abnormal vessel formation, including elongated and tortuous vessels with dilated lumens (Schwesinger et al., 2001). This elegant study illustrates the fundamental role that VEGF plays in maintaining the structure and functioning of the choroid; in addition, both VEGF and localised damage or inflammatory stimuli are needed in order to trigger CNV, as discussed further in section 3.5.

In addition to its role in maintaining transendothelial transport in CECs, VEGF is also involved in regulating paracellular permeability,

although the underlying mechanism has not been examined in detail, particularly with respect to CECs. Here, we review the dynamics of adherens junctions based on landmark studies performed using *in vitro* and *in vivo* endothelial models (Dejana, 2004; Vestweber et al., 2009). Dejana and colleagues provided evidence supporting the role of VEGF in regulating paracellular permeability, either stabilising or disrupting the integrity of cellular junctions depending on the structural context and the presence of inflammatory stimuli. In confluent endothelial cell cultures in which the cells are connected by adherens junctions, VE-Cadherin is the primary protein spanning the intercellular space, with the intracellular domain forming a complex with the intracellular domain of VEGFR2 and other proteins, thus maintaining a stable monolayer of ECs (Dejana, 2004). Disruption of the adherens junctions by inflammatory factors such as histamine, thrombin, or TNF-α (tumour necrosis factor alpha) causes VEGFR2 to dissociate from the VE-Cadherin protein complex (Vestweber et al., 2009). In this pro-inflammatory context, the intracellular domain of VEGFR2 can be phosphorylated via the VEGF signalling cascade, ultimately driving the endocytosis of VE-Cadherin and increasing paracellular permeability (Dejana, 2004; Vestweber et al., 2009). In summary, pro-inflammatory signalling can act as a "sensitiser" of barrier breakdown by decoupling VEGFR2 from its complex with VE-Cadherin, thus drastically changing VEGF's biological mechanism of action. Although these studies were not performed specifically using CECs, it is reasonable to speculate that this dimorphic activity of VEGF may also occur in CECs, given that the proteins in the aforementioned studies are highly conserved in various endothelial cells. It should be noted that CECs constitutively express high levels of ICAM-1 (Table 1) (Whitmore et al., 2015). Interestingly, activation of ICAM-1 upon leukocyte binding phosphorylates VE-Cadherin at tyrosine residues, thereby disrupting complexes containing VE-Cadherin and promoting leukocyte extravasation (Vestweber et al., 2009). If these findings hold true for the choroid, destabilisation of VE-Cadherin may provide a physiological means of disrupting cellular junctions. Conversely, certain growth factors can prevent the dissociation of VEGF receptors from adherens junctions including PEDF and PlGF in RECs; however, these factors do not appear to be expressed at high levels in human CECs (Table 1) (Klaassen et al., 2013).

3.4.4. Nitric oxide as a regulator of vasodilatation and vascular permeability

NO, initially discovered as an "endothelium-derived relaxing factor", is a signalling molecule and neurotransmitter with a wide range of biological functions (Schmetterer and Polak, 2001). NO is a small free radical that can diffuse freely through cellular membranes. Due to its short biological half-life (on the order of several seconds), its effects are relatively local, and its bioavailability depends directly on its production by nitric oxide synthases (Schmetterer and Polak, 2001), a family of enzymes that includes neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) isoforms. The role of NO has been described in the context of vascular tone, ocular growth, angiogenesis, inflammation, barrier function, and protection against oxidative stress (Nickla et al., 2009; Polak et al., 2007; Schmetterer and Polak, 2001). Based on transcriptomics, all subtypes of CECs express eNOS. Moreover, arterial CECs also express nNOS, and CECs in the choriocapillaris express moderate levels of iNOS (Table 1). Immunohistochemistry has shown that in the choroid, iNOS and eNOS are expressed primarily by CECs. In addition, choroid-resident macrophages express high levels of iNOS, particularly in the pro-inflammatory environment of a CNV lesion (Bhutto et al., 2010; Cherepanoff et al., 2010).

The main NOS isoform expressed in CECs is eNOS, where it's primary function is to regulate local vascular tone, as shown by numerous studies involving animal models and human subjects (Bhutto et al., 2010; Polak et al., 2007). All three isoforms of NOS are inhibited by L-NMMA. Interestingly, intravenous injections of L-NMMA increases systemic blood pressure in humans, but decreases choroidal blood flow and fundus pulsation amplitude measured using laser Doppler flowmetry and interferometry (Polak et al., 2007). These observations underscore the role that NO plays in regulating choroidal blood flow, although further study is needed in order to determine which cell types and signalling pathways are exactly involved. Interestingly, VEGF is involved in regulating NO signalling. For example, intravenous injections of VEGF in rats causes a dose-dependent decrease in blood pressure and an increase in the choroid's permeability to albumin, and these effects were blocked by L-NMMA (Tilton et al., 1999). In addition, the coupling between VEGF signalling and NO production suggests that NO may play a pro-angiogenic role in CNV, given that VEGF is the primary driving force in CNV. Evidence supporting this functional role of NO in CNV was provided by Ando and colleagues, who generated NOS knockout mouse models and examined the effect of knocking out each NOS isoform on CNV formation and retinal neovascularisation (Ando et al., 2002). Surprisingly, they found that knocking out either iNOS or nNOS significantly decreased laser-induced CNV formation, whereas knocking out eNOS had no effect (Ando et al., 2002). In contrast, knocking out eNOS – but not iNOS or nNOS – reduced retinal neovascularisation in an oxygen-induced ischaemic retinopathy model (Ando et al., 2002). Taken together, these studies suggest that VEGF may play an important role in regulating NO signalling in the choroid, and may also play a pathological role in neovascularisation, in which specific NOS isoforms may be involved in CNV and/or retinal neovascularisation. Thus, further study is needed in order to determine the precise role of NO in CNV, as well as its potential as a therapeutical target via the selective inhibition of specific NOS isoforms.

In humans, the perivascular nerve fibres surrounding the choroidal arteries and veins express nNOS, and NO acts as a neurotransmitter to decrease vascular tone and induce vasodilatation (Bhutto et al., 2010). Moreover, based on results obtained using a rat model of diabetes mellitus, Sakurai and colleagues postulated that nNOS plays a role in diabetic choroidopathy (Sakurai et al., 2009). The authors found that 6 weeks after the onset of diabetes, choroidal nNOS expression was significantly reduced; they speculated that this drop in nNOS expression causes diabetes-induced dysfunction of parasympathetic perivascular nerve fibres, thereby contributing to the development of diabetic choroidal microangiopathy (Sakurai et al., 2009). In this respect, it is interesting to note that immunohistochemistry has shown that nNOS is also present in the RPE and in the outer nuclear layer of the neuroretina, possibly contributing to nNOS-mediated damage via an independent mechanism (Bhutto et al., 2010; Sakurai et al., 2009).

3.5. Choroidal neovascularisation

The biomolecular mechanisms that underlie CNV – which is essentially a wound-healing response – have been widely studied using both cell culture models and animal models of laser-induced CNV (Grossniklaus et al., 2010; Schlingemann, 2004). Other animal models for studying ocular angiogenesis have been reviewed elsewhere (Liu et al., 2017) and are not discussed further here. The processes that underlie CNV include the release of growth factors, inflammation, debris deposits, degeneration of the extracellular matrix, and atrophy of the RPE and choroid. Given the complex interplay between these processes, the full pathophysiology of CNV remains poorly understood. Nevertheless, the development of CNV is believed to require a concomitant increase in pro-angiogenic signalling together with a loss of anti-angiogenic signalling, followed by breakdown of the extracellular matrix and subsequent neovascularisation (Schlingemann, 2004). CNV can develop in a variety of chorioretinal diseases, the clinical aspects of which are discussed in Chapter 4. In this section, anti-angiogenic factors, pro-angiogenic factors, and the breakdown of BM and the extracellular matrix are discussed.

The growth factors that are presumably involved in CNV are summarised in Table 1. Anti-angiogenic factors are widely expressed in the choroidal tissue as extracellular matrix proteins and include thrombospondin-1 (TSP-1). Immunohistochemical analysis revealed

that eyes with AMD have decreased levels of TSP-1 in BM and in the choroidal vessels, which was suggested to have important implications for the manifestation of CNV (Uno, 2006). Furthermore, mouse CECs lacking TSP-1 expression have a pro-inflammatory phenotype and increased oxidative stress (Fei et al., 2014). Other factors that have been suggested to play a role in the development of CNV include adrenomedullin by inhibiting the attraction of macrophages (Yuda et al., 2012), and TIMP3 (tissue inhibitor of metalloproteinase 3) by inhibiting degradation of the extracellular matrix (Janssen et al., 2008). Moreover, the RPE plays an important role in maintaining angiogenic balance, for example by releasing PEDF; this potent anti-angiogenic growth factor is released primarily on the apical side of the RPE and is less present in the vitreous of patients with neovascular AMD compared to healthy controls (Palanisamy et al., 2019; Schlingemann, 2004).

Many hypotheses to explain the driving force behind the development of CNV suggest a central role for VEGF, the most potent pro-angiogenic factor identified to date (Grossniklaus et al., 2010; Schlingemann, 2004; Whitmore et al., 2015; Witmer et al., 2003). VEGF is also an essential survival signal for CECs and is important for maintaining fenestrations (Bosma et al., 2018; Marneros et al., 2005). Previously, Schlingemann et al. proposed that CNV may occur due to a sequence of events arising from an initial decrease in the permeability of BM for VEGF (Schlingemann, 2004). Reduced trophic action from VEGF secreted by the RPE would lead to atrophy of the choriocapillaris and a reduction in the number of fenestrations, a process closely associated with both ageing and AMD. Conversely, this can result in decreased oxygen transport from the choriocapillaris to the outer retina, which in turn causes the release of hypoxia-induced stress signals back to the choriocapillaris, eventually driving CNV formation towards either the sub-RPE space or into the subretinal space through defects in BM and the RPE (Schlingemann, 2004).

CNV can be considered multifactorial in origin, involving the formation of defects in BM and/or the RPE as crucial events (Grossniklaus et al., 2010). Activation of the immune system by an accumulation of debris and a loss of anti-angiogenic proteins in the extracellular matrix is often suggested to play a major role in breakdown of BM (Cherepanoff et al., 2010; Whitmore et al., 2015). In humans, histopathological studies suggest that early signs of AMD (e.g. the accumulation of extracellular debris, pigmentary changes in the RPE) or the presence of injured/apoptotic cells attract pro-inflammatory macrophages (Cherepanoff et al., 2010; McLeod et al., 2016; Yang et al., 2016). In contrast to the resident macrophages present in the choroid under physiological conditions (Cherepanoff et al., 2010), AMD-associated macrophages express iNOS and exert cytotoxic and pro-angiogenic effects (e.g. IL-17 release), thus damaging BM (Chan and Ardeljan, 2014; Cherepanoff et al., 2010; Whitmore et al., 2015). Ultimately, the breakdown of BM results in physical contact between the RPE and CECs, inducing the transmigration of CECs towards the RPE (Peterson et al., 2007). Animal models of CNV involving laser-induced damage to BM have shown that expression of the pro-angiogenic factors bFGF and HGF (hepatocyte growth factor) increases within hours of BM breakdown, with increased VEGF levels and the onset of early CNV occurring within 3 days (Hu et al., 2009).

4. Choroidal dysfunction in chorioretinal disease

In this chapter, we discuss a range of chorioretinal diseases in which the choroid – and more specifically, the CECs – have been found to play a prominent role. In each section, we discuss our current knowledge regarding the choroid and CECs based on imaging studies, histopathology, animal models, and human cell culture models. Moreover, given that in some virtually nothing is known regarding the specific role of CECs, for these topics we discuss these shortcomings in knowledge and suggest options for further study. First, we discuss imaging techniques in section 4.1. Current treatment modalities for chorioretinal diseases are discussed in section 4.12. Finally, we discuss advances in the field of regenerative medicine in sections 5.2. and 5.3.

4.1. Choroidal imaging techniques

In recent decades, newly developed choroidal imaging techniques have become indispensable in the diagnosis, treatment, and follow-up of chorioretinal diseases. Furthermore, the correlation between imaging techniques and histopathology has provided valuable insights into the pathophysiological mechanisms that underlie various chorioretinal diseases such as aneurysmal type 1 CNV (also known as polypoidal choroidal vasculopathy, or PCV) (Dansingani et al., 2016; Li et al., 2016, 2019; Mandadi et al., 2017; Mrejen and Spaide, 2013). In this section, we discuss the principles behind the techniques commonly used to image the choroid. In subsequent sections, we discuss the experimental and clinical applications of each of these techniques in the context of specific chorioretinal diseases.

4.1.1. Optical coherence tomography

The advent and widespread use of OCT has enabled ophthalmologists and scientists in the field of ophthalmology to study the various layers of the eye using a non-invasive tool that provides a cross-sectional view (Fig. 8). Because OCT is accurate, rapid, relatively cheap, and widely available, it has become one of the most important imaging modalities in ophthalmology. The imaging depth of traditional spectral domain OCT (SD-OCT) is relatively limited, and the high opacity of the RPE and choroidal melanocytes can make it difficult to obtain a clear image of the choroid. Over the last few years, however, several technical improvements have been introduced, providing for better visualisation of the choroid. These improvements have led to enhanced depth imaging OCT (EDI-OCT), swept-source OCT (SS-OCT), and optical coherence tomography angiography (OCTA), which are discussed in detail below.

4.1.1.1. Enhanced depth imaging OCT for improved choroidal visualisation. EDI-OCT provides imaging of the choroid at higher resolution and with a higher field depth, which is achieved by manually positioning the camera lens closer to the eye (Fig. 8) (Spaide et al., 2008). This technique has several advantages compared to conventional OCT, including a more reliable interpretation of the choroid-sclera interface, thus providing a more reliable measure of choroidal thickness (Spaide et al., 2008). Furthermore, EDI-OCT can provide anatomical features such as vessel diameter, vessel wall thickness, and hyperreflective spots in the choroidal stroma in more detail than with conventional OCT. However, the resolution achieved with EDI-OCT does not allow for detailed imaging of the choriocapillaris (Ferrara et al., 2016; Laviers and Zambarakji, 2014). Notably, compared to traditional OCT, EDI-OCT is more suitable for quantitatively measuring choroidal OCT biomarkers, which can be indicative of vascular pathology and therefore helpful in clinical diagnostics (Singh et al., 2019b). A widely used parameter is the choroidal vascularity index, which is defined as the ratio between the luminal surface area and the total surface area of the choroid (including the stroma), and is calculated upon binarisation of the luminal and stromal areas (Agrawal et al., 2016). However, automation of this calculation would improve its consistency, as the border between the desired regions can be difficult to define and is therefore subject to interobserver variability (Singh et al., 2019b).

4.1.1.2. Swept-source OCT for faster imaging and frontal plane reconstruction. SS-OCT has improved choroidal imaging even further. This technique uses a different light source and different detection methods than conventional SD-OCT, allowing for more rapid acquisition and the use of longer wavelengths (Huber et al., 2007; Unterhuber et al., 2005). The ability to use longer wavelengths is particularly beneficial for imaging pigmented tissues such as the melanocyte-containing choroid, which is obscured by the highly pigmented RPE (Ferrara et al., 2016). Furthermore, post-acquisition processing of SS-OCT images allows the

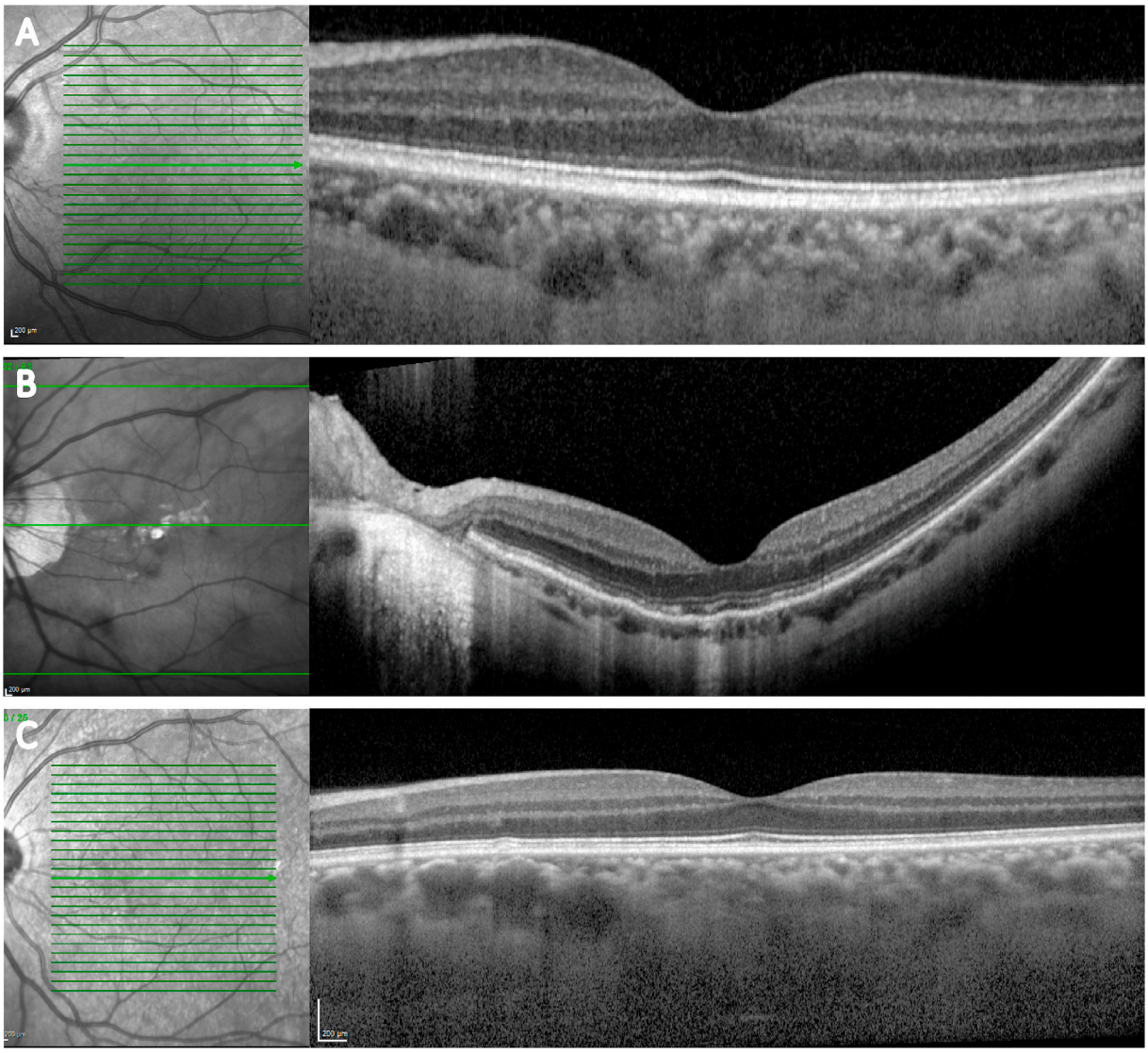


Fig. 8. Enhanced-depth imaging optical coherence tomography (EDI-OCT) from (A) the left eye of a healthy 51-year-old female (subfoveal choroidal thickness: 281 μm); (B) the left eye of a 72-year-old male with myopia-associated choroidal thinning (subfoveal choroidal thickness: 90 μm), who had previously received intravitreal injections with anti-vascular endothelial growth factor receptor medication for a myopic macular neovascularisation; (C) the left eye of a 37-year-old man with pachychoroid (subfoveal choroidal thickness: 481 μm) who was diagnosed with central serous chorioretinopathy in the fellow eye.

user to create a flattened reconstruction of the choroid in the frontal plane, resulting in what is known as en face OCT. These frontal plane reconstructions provide a direct overview of the organisation of the choroidal vasculature in the posterior pole, which can be particularly valuable for evaluating pachychoroid disorders, which are characterised by enlarged vessels in Haller's layer (Dansingani et al., 2016; Matsumoto et al., 2020a).

4.1.1.3. Optical coherence tomography angiography: visualising erythrocytes in motion. The latest advance in OCT technology is OCTA, which allows for the detailed assessment of (neo)vascular structures in the en face plane. This imaging technique is based on the movement of erythrocytes, allowing vascular structures to be detected by the distinct reflection of light from the dynamic erythrocytes compared to the static surrounding structures. Software can then be used to reconstruct the vasculature in high resolution with high contrast at the border of the vascular lumen.

OCTA is particularly suitable for imaging the retinal microvasculature and – to a lesser extent – the choriocapillaris (Borrelli et al., 2018; Ferrara et al., 2016; Spaide et al., 2018). OCTA can provide images of these superficial vascular structures at a contrast and resolution not possible with conventional dye-based angiography techniques such as FA and ICGA. On the other hand, only FA and ICGA allow for the detection of vascular leakage and hyperpermeability, and therefore OCTA cannot be considered a replacement for FA or ICGA (Spaide et al., 2018). Current limitations of OCTA include an inability to reliably measure blood flow rate, and to detect flow signals below a certain threshold. Moreover, OCTA has a relatively limited imaging depth, as the signal is obscured by the highly pigmented RPE and choroid (Spaide et al., 2018). Nevertheless, by using a longer wavelength (i.e. 1050 nm) it has become possible to obtain better images of the choriocapillaris and detect abnormalities such as atrophy and flow voids (Borrelli et al., 2018; Ferrara et al., 2016; Spaide et al., 2018). Unfortunately, Sattler's layer and Haller's layer cannot be assessed using current OCTA techniques, mainly because of the limited imaging depth due to the high pigmentation in the posterior pole of the eye (Spaide et al., 2018). Thus, either SS-OCT or dye-based angiography is more suitable for imaging deep vascular layers, with the choice of modality depending on the clinical context.

Interestingly, because the choriocapillaris can be examined using

non-invasive techniques such as OCTA, studies can now investigate whether OCTA imaging of the choriocapillaris can have clinical value by serving as a surrogate tool for measuring systemic microvascular damage (Spaide et al., 2018). This may be particularly valuable for systemic conditions such as diabetes mellitus and hypertension. Although OCTA is an actively developing technique, it has already shown promising clinical value in a variety of chorioretinal diseases such as uveitis and AMD, and for the detection and follow-up of (subclinical) CNV (Borrelli et al., 2018; Ferrara et al., 2016; Schneider and Fowler, 2018; Spaide et al., 2018). The principal challenges in the improvement of OCTA imaging with respect to choroidal vascular diseases are optimisation of the imaging depth and reduction of artefacts and noise due to pigmentation (Borrelli et al., 2018; Ferrara et al., 2016; Spaide et al., 2018). The current applications of OCTA in these specific diseases are discussed in the respective sections in this chapter.

4.1.2. Fluorescein angiography

FA was first developed six decades ago by Novotny and Alvis and has since become highly valuable for imaging the retinal vasculature and for detecting possible weaknesses in the inner and/or outer blood-retinal barrier (Fig. 9) (Novotny and Alvis, 1961). FA relies on systemically administered (either by intravenous injection or orally) fluorescent molecules that are too large to pass the healthy, intact blood-retinal barrier (Törnquist et al., 1990). Importantly, FA can be used to measure blood flow dynamics over time, allowing for the user to roughly distinguish the arterial, capillary, and venous phases. However, it should be noted that FA is not the optimal technique for choroidal visualisation, primarily because the choroid is obscured by the pigmented RPE and melanocytes present in the choroid itself, and because the fluorescein molecules diffuse rapidly out of the fenestrated choriocapillaris, further preventing a clear visualisation of the choroidal vasculature (Spaide et al., 2018; Yannuzzi, 2011). Nevertheless, FA has several advantages in the context of chorioretinal disease. For example, the extravasation of fluorescein appears as hyperfluorescence, and flow deficits appear as hypofluorescence on optical images. Moreover, loss of to the RPE barrier's integrity appears as hyperfluorescence on FA, which may be observed secondary to choroidal dysfunction, as seen in diseases that are part of the pachychoroid spectrum. This is typical for central serous chorioretinopathy (CSC), in which a leakage "hot spot" can be observed due to focal disruption of the RPE barrier (Van Rijssen et al., 2019b), as discussed in section 4.3.3.2. Another phenomenon is pooling of fluorescein, which emerges as areas of hyperfluorescence in the late phase of the FA due to the extravasation of fluorescein into fluid-filled cavities; typically, this finding is observed in the context of pigment epithelial detachments or subretinal fluid (SRF) accumulation, for example as seen in CSC (Khalil et al., 2015). Finally, FA is suitable for visualising aberrations in larger choroidal vessels, for example occlusion of a PCA or subsegmental branches, which in clinical practice are commonly referred to as wedge-shaped infarctions or the "triangular sign of Amalric" (Hayreh, 1990; Nemiroff et al., 2017).

4.1.3. Indocyanine green angiography

ICGA is a dye-based angiography technique using indocyanine green and near-infrared (790–805 nm wavelength) light (Fig. 9). Because near-infrared light is not readily absorbed by the pigments present in the RPE and choroid, ICGA provides a clear visualisation of the choroidal vasculature (Yannuzzi, 2011). The molecular weight of indocyanine green is 775 Da, and the dye has high protein-binding capacity (98%), which prevents free diffusion of the dye through the choriocapillaris fenestrations, thus providing enhanced imaging of the larger choroidal vessels in Sattler's layer and Haller's layer (Yannuzzi, 2011). This is in stark contrast with FA, in which the dye rapidly extravasates in the choriocapillaris and prevents visualisation of large choroidal vessels, in addition to the disadvantage of light absorption by pigment (Spaide et al., 2018; Yannuzzi, 2011). Different phases in ICGA can be distinguished as the dye passes through the arterial, capillary, and venous structures in the choroidal circulation. For example, a delay in arterial fill can be observed in the early phase which typically appears as an area of hypofluorescence with indistinct borders (Van Rijssen et al., 2019b). This delayed arterial filling has been suggested to be the result of congested blood flow in the choriocapillaris and choroidal veins, which can be detected during early/mid-phase ICGA (Prünte and Flammer, 1996). In the mid phase, focal or diffuse areas of hyperfluorescence with indistinct borders – indicating hyperpermeability of the choriocapillaris – are typical findings in diseases within the pachychoroid spectrum (Fig. 9) (Dansingani et al., 2016; Gass, 1967; Spaide et al., 2021; Van Rijssen et al., 2019b). Lastly, the late phase of ICGA is ideally suitable for detecting choroidal haemangioma, which appears as a delineated structure in the late phase due to extravasation of the indocyanine green dye through the leaky tumour vasculature, a phenomenon referred to as pooling or a "wash-out" effect (Yannuzzi, 2011). In general, the presence of these abnormalities which can resemble more subtle changes such as CNV or capillary nonperfusion, are best detected using a confocal scanning laser ophthalmoscope, which obtains images with higher resolution than a conventional fundus camera and a scanning laser ophthalmoscope (Staurenghi et al., 2005).

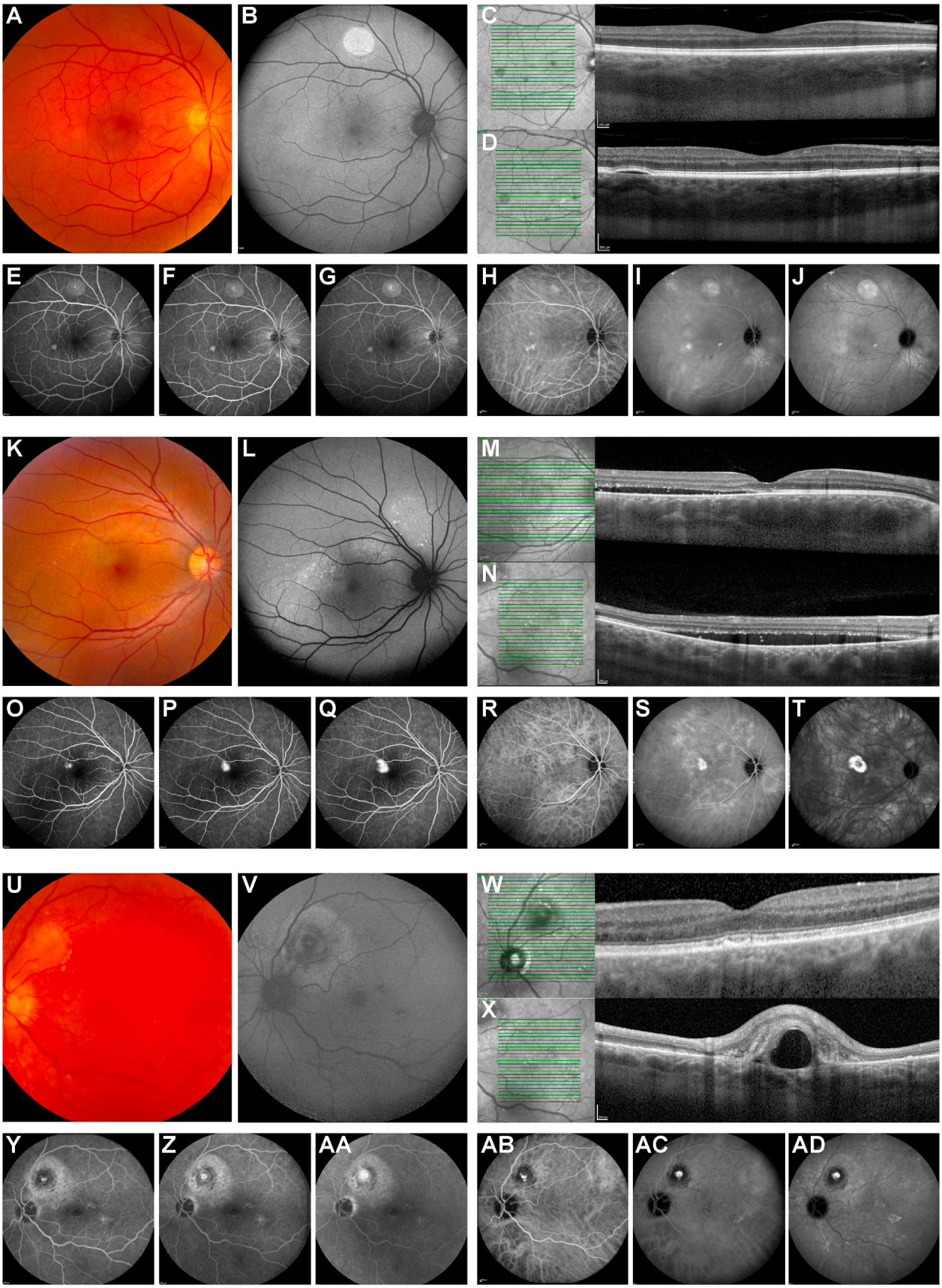
ICGA has clearly emerged as the gold standard for imaging the choroidal vasculature. However, performing ICGA in combination with FA still has high value in specific clinical contexts. For example, a finding of large hyperfluorescent areas with indistinct borders on ICGA is an important differential diagnostic sign when attempting to distinguish CSC from other chorioretinal diseases; however, this finding does not necessarily confirm the diagnosis (Van Rijssen et al., 2019b); in these patients, overlapping, more focalised areas of leakage visible on FA – known as leakage "hot spots" – may be useful for confirming the diagnosis, as these lesions suggest the presence of RPE damage that occurred secondary to a larger region of choroidal dysfunction (Van Rijssen et al., 2019b).

A unique property of ICGA is the ability to perform ultra-widefield imaging, which provides a view of the peripheral retina up to an angle of 200° (Klufas et al., 2015). This allows for the entire vortex vein system to be visualised, which can be used to assess the anatomy of choroidal venous outflow (Spaide et al., 2021; Verma et al., 2020). Moreover, quantification on the brightness of the ICGA dye has been used as a surrogate for detecting choroidal venous outflow congestion in pachychoroid disorders (Fig. 2) (Jung et al., 2020). The clinical and experimental applications of ultra-widefield ICGA are discussed in detail in section 4.3.

4.2. Choroidal aspects of age-related macular degeneration

4.2.1. Risk factors and disease hallmarks of age-related macular degeneration

AMD is a multifactorial, progressive chorioretinal disease marked by depositions in the RPE/choroid complex. For instance, these depositions occur between the basal lamina of the RPE and the inner collagenous layer of BM; the focal form of these deposits is commonly referred to as drusen (Bergen et al., 2019; Curcio, 2018; DeAngelis et al., 2017; Mitchell et al., 2018; Rozing et al., 2020). Similar deposits can occur between the outer retina and the RPE and are called reticular pseudodrusen – or subretinal drusenoid deposits (SDDs) – and are closely associated with an additional clinical phenotype of AMD (Sivaprasad et al., 2016; Spaide, 2018b). As its name suggests, the primary risk factor for AMD is ageing (Smith et al., 2001). Although AMD is not considered a direct physiological consequence of ageing, the ageing retina has certain overlapping features with AMD and can also provide the background on which AMD can develop (Mitchell et al., 2018; Spaide, 2018b; Zheng et al., 2019). In addition to ageing, other major risk factors for developing AMD include smoking and genetic factors (Chakravarthy et al., 2010; DeAngelis et al., 2017), as well as exposure to sunlight, cardiovascular disease, low intake of antioxidants, and obesity (Chakravarthy et al., 2010; Mitchell et al., 2018; Seddon et al., 2003).



(caption on next page)

Fig. 9. An overview of common clinical presentations within the pachychoroid spectrum. (A–J) Multimodal imaging of the right eye of a 63-year-old man with pachychoroid pigment epitheliopathy. The fundus photograph of this patient (A) showed retinal pigment epithelium (RPE) changes and detachments, both in and outside the macula. Fundus autofluorescence (FAF; B) showed both hypo- and hyperautofluorescent changes. Enhanced-depth imaging optical coherence tomography (EDI-OCT; C, D) scanning revealed a subfoveal choroidal thickness of 529 μm, RPE alterations, and pigment epithelial detachments. Fluorescein angiography (FA; E-G) showed hyperfluorescent changes, but no clear leakage. On indocyanine green angiography (ICGA; H-J) multifocal hyperfluorescent choroidal zones with indistinct borders, typical for the changes seen in choroidal vascular hyperpermeability associated with the pachychoroid spectrum, were visible. (K–T) Multimodal imaging of the right eye of a 29-year-old man with central serous chorioretinopathy. Fundus photography (K) showed RPE changes and a serous detachment of the macula. FAF (L) revealed diffuse hyperautofluorescent changes. On EDI-OCT (M, N) subretinal fluid accumulation was observed in the macular and extramacular area, together with a subfoveal choroidal thickness of 521 μm. FA (O–Q) revealed an area of focal leakage in the macula. ICGA (R–T) showed multifocal hyperfluorescent areas with indistinct borders, typical for pathological pachychoroid, and a focal area of active leakage of the choroidal vasculature corresponding with the area of focal leakage on FA. (U-AD) Multimodal imaging of the left eye of a 64-year-old man with polypoidal choroidal vasculopathy. Fundus photography (U) showed a large lesion superior to the optic nerve head and macular pigmentary changes. FAF (V) revealed a large irregular hypoautofluorescent lesion with a hyperautofluorescent border, together with hypoautofluorescent changes near the macular region. The EDI-OCT scan (W) showed a lesion that suspicious of polypoidal choroidal vasculopathy. The subfoveal choroidal thickness was 386 μm (X). FA (Y-AA) revealed some leakage from the polypoidal lesion with pooling of fluorescein. ICGA (AB-AD) showed a hyperfluorescent lesion that was already present in the early-phase, surrounded by hypofluorescence, in combination with multifocal hyperfluorescent choroidal zones with indistinct borders, that point to a context of a central serous chorioretinopathy/pachychoroid background. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

All drusen subtypes contain a variety of minerals, proteins and/or lipids, which can arise from cellular debris of CECs, the RPE, photoreceptor outer segments, and blood proteins (Bergen et al., 2019; Curcio, 2018; Tan et al., 2018; Thompson et al., 2015). The extensive accumulation of debris during the pathogenesis of AMD leads to an increase in both the size and number of these deposits, causing physiological and structural changes in the affected tissue area (Balaratnasingam et al., 2017; Bergen et al., 2019; Rozing et al., 2020). The end stage of AMD has two forms. The first form, geographic atrophy (GA), is characterised by atrophy of the RPE; the second form, CNV, can be more specifically defined as three subtypes of CNV (type 1 CNV through type 3 CNV) (Spaide, 2018b; Spaide et al., 2020a). Recently, MNV (macular neovascularisation) was suggested as a more suitable term than CNV, given that clinicopathological and anatomical studies indicate that the neovascularisation does not necessarily originate from the choroid (Spaide, 2018b; Spaide et al., 2020a). Type 1 CNV (previously referred to as "occult" neovascularisation based on FA findings) arises from the choroidal vasculature and is characterised by the formation of new blood vessels under the RPE. Type 2 CNV (previously called "classic" neovascularisation based on FA findings) is characterised by the growth of these vessels through the RPE into the subretinal space (Spaide et al., 2020a). If both type 1 CNV and type 2 CNV are present in substantial proportions, this presentation is known as mixed type 1 and type 2 CNV (Chen et al., 2020). Finally, type 3 CNV (also known as "retinal angiomatous proliferation") arises from the deep retinal vasculature and does not necessarily involve the choroidal circulation, although it can result in the formation of anastomoses between the retinal circulation and the choroidal circulation (Spaide, 2018b). Interestingly, type 3 CNV has been suggested to develop due to detached RPE cells, which release VEGF into the subretinal space rather than towards the choroid (Spaide, 2018b). In general, advanced AMD with CNV is associated with SRF and/or intraretinal fluid, haemorrhage(s), and – eventually – scar formation, resulting in a decrease in visual acuity (Elshout et al., 2017; Rozing et al., 2020).

4.2.2. *Imaging of choroidal pathology in age-related macular degeneration*

Imaging studies have provided ample evidence suggesting that the choroid plays a critical role in the pathogenesis of AMD. Although studied extensively, no clear association has been found between AMD and choroidal thickness, which can be increased, decreased, or unchanged during disease progression (Gattoussi et al., 2019; Koh et al., 2017; Mrejen and Spaide, 2013; Zhao et al., 2018). Koh and colleagues used EDI-OCT to measure the choroidal vascularity index – which is based on binarisation of EDI-OCT images and quantifies the vascular lumen area relative to the total choroid area, including the stroma – in AMD patients, revealing a statistically significant loss of vasculature in patients with atrophic AMD and patients with neovascular AMD (including treatment-naïve neovascular AMD) compared to healthy controls (Koh et al., 2017). Interestingly, they found that the choroidal vascularity index was also increased in the patients' unaffected fellow eyes, despite having no clear clinical signs of AMD, suggesting that a loss of choroidal vasculature may precede the clinical manifestation of AMD (Koh et al., 2017).

Interestingly, the presence of subretinal drusenoid deposits (SDDs) has been associated with choroidal thinning and reduced choroidal volume, which is not the case with soft drusen (Alten et al., 2013; Spaide, 2018b). Moreover, SDDs are frequently located in watershed zones, indicating that they might be related to chronic vascular insufficiency (Alten et al., 2013). Consistent with these observations, OCTA revealed reduced choriocapillaris density in eyes with SDDs compared to healthy controls (Alten et al., 2016). Importantly, the presence of either SDDs or soft drusen also has clinical value for predicting the course of AMD, as both SDDs and soft drusen are known risk factors for the development of GA and CNV (Chen et al., 2021; Curcio, 2018; Jaffe et al., 2020; Spaide, 2018b). A study using OCTA in patients with AMD showed that the reduced choriocapillaris flow in these patients extends beyond the borders of GA, suggesting that choroidal dysfunction plays a major role in the development of GA (Schneider and Fowler, 2018). Additional evidence of choroidal abnormalities in AMD was provided by a study using ultrasound in healthy controls, patients with atrophic AMD, and patients with neovascular AMD (Coleman et al., 2013). The authors found that compared to the controls, the patients with atrophic AMD had a decreased blood-to-tissue ratio, possibly indicating choroidal ischaemia; in contrast, the patients with neovascular AMD had an increased blood-to-tissue ratio, suggesting increased blood supply, inflammation, or microvascular abnormalities (Coleman et al., 2013).

4.2.3. *Genetic risk factors underlying age-related macular degeneration*

Genetics studies have been instrumental at unravelling the increased susceptibility for AMD, genotype-phenotype relationships, and – most important – the underlying molecular mechanism. The most relevant genetic risk factors are discussed below, with a focus on the potential role of CECs. Polymorphisms in the *CFH* gene (which encodes complement factor H) and other genes linked to the complement system such as *CFB*, *C3*, and *C9* have been associated with AMD (Anderson et al., 2010; Boon et al., 2009b; Fritsche et al., 2016; Geerlings et al., 2017; Toomey et al., 2018). Moreover, rare variants in some of these genes, including *CFH*, contribute to the familial occurrence of the early-onset cuticular drusen phenotype (Boon et al., 2008b; Geerlings et al., 2017), which is a subtype of AMD, as well as severe renal diseases such as membranoproliferative glomerulonephritis type 2 (also known as dense deposit disease) (Boon et al., 2009b, 2013). Interestingly, individuals carrying several common genetic variants have a substantially increased or decreased risk of AMD, which can be estimated using a genetic risk score and can be useful for predicting the risk of progression to advanced stages of AMD (Despriet et al., 2006; Heesterbeek et al., 2019).

However, it should be noted that these risk scores were developed only recently; therefore, their reliability and clinical value are still being studied. Variants in the *CFH* gene and other complement genes have also been linked to CSC, but not to systemic activation of the complement system (de Jong et al., 2015; Hosoda et al., 2018; Kaye et al., 2020; Schellevis et al., 2018b; van Dijk et al., 2017b). Interestingly, specific AMD-associated risk variants in the *CFH* and *ARMS2* (Age-Related Maculopathy Susceptibility 2) genes have been found to reduce the risk of CSC – and vice versa (de Jong et al., 2015; Miki et al., 2014), suggesting overlap between AMD and CSC with respect to their pathogenesis.

In support of these genetics studies, data obtained from biochemical and histopathological studies show that AMD is associated with altered activation of the complement system (Hageman et al., 2001; Hogan, 1965; Sarks, 1976). Based on genetic risk factors in the complement system, serum analyses revealed that AMD is associated with systemic dysregulation of the complement system (Heesterbeek et al., 2020; Scholl et al., 2008; Smailhodzic et al., 2012). Heesterbeek et al. recently examined a large cohort of 695 patients with AMD and found that serum complement activation (determined using the C3d/C3 ratio) was correlated with the early, intermediate, and advanced stages of AMD, including GA and CNV (Heesterbeek et al., 2020). Moreover, they found that patients carrying AMD risk alleles in either the *CFH* or *CFB* gene had higher complement activation and more progressive AMD (Heesterbeek et al., 2020). Previously, Scholl et al. studied systemic complement activation in patients with AMD and found that all of the activation products in the alternative complement pathway were elevated in these patients; moreover, the authors found that elevated plasma levels of these components were associated with variants in the *CFH* gene (Scholl et al., 2008). These findings were subsequently confirmed by Smailhodzic et al., who found an association between systemic complement activation and the risk variant in the *ARMS2* gene, suggesting a common pathway involving CFH and ARMS2 in the pathogenesis of AMD (Smailhodzic et al., 2012).

Interestingly, CFH is expressed at high levels in CECs and the RPE (Table 1), where it exerts the same biological function action as systemic CFH, namely to inhibit complement activation and prevent subsequent cellular damage (Bhutto et al., 2011; Whitmore et al., 2015). Thus, although currently only speculation, the pathogenic and/or protective effect of *CFH* gene variants may also be exerted locally at the level of the CECs, as discussed in section 4.2.4. This notion is supported by the fact that CECs interact closely with CFH and the putative role that CECs play in the pathogenesis of both AMD and CSC (de Jong et al., 2015; Langford-Smith et al., 2014; Whitmore et al., 2015). On the other hand, the ARMS2 protein is present in the choroidal extracellular matrix and may play a role in the pathogenesis of AMD via oxidative stress (Kortvely et al., 2016). Interestingly, De Jong et al. speculated that variants of the *ARMS2* gene may be associated with perturbations in the choroidal extracellular matrix, possibly increasing the susceptibility for cellular detachment (de Jong et al., 2015). Again, this suggests a link between specific gene variants and the pathogenesis of AMD and CSC at the level of the CECs, albeit in a converse relationship.

4.2.4. The pathophysiological background of age-related macular degeneration

Although both genetic and environmental risk factors in AMD have been studied extensively, the precise pathophysiological mechanisms have not been identified (Bergen et al., 2019; Rozing et al., 2020). Although experimental data point towards important roles for the RPE, BM, and the choriocapillaris, to date none of these tissues has been implicated as the principal origin of AMD (Rozing et al., 2020). Indeed, AMD is a multifactorial disease in which degeneration can occur in any combination of the aforementioned tissues and can ultimately progress to GA or CNV (Bergen et al., 2019; Spaide, 2018b). Interestingly, both clinical characteristics and the disease course can vary widely between patients, further obscuring our understanding of the underlying pathophysiology (Rozing et al., 2020; Spaide, 2018b).

In their recent review, Rozing and colleagues proposed a "2-hit" hypothesis to explain the pathophysiology of AMD (Rozing et al., 2020). The first hit is the accumulation of molecular and cellular damage to the RPE/choroid complex, which is largely in overlap with the normal healthy ageing eye. The second hit is the inflammatory response to this accumulation; this response can be modified by genetic and/or environmental factors, which may eventually result in advanced AMD with the development of either GA or CNV. This 2-hit hypothesis integrates most of the known risk factors and molecular mechanisms that have been attributed to AMD as the result of efforts by many researchers over the last few decades (Rozing et al., 2020). Here, we discuss the pathophysiology of AMD from the perspective of this 2-hit hypothesis, and we take other hypotheses and considerations into account as well. Importantly, AMD has been described as a progressive disease in which specific events can occur that accelerate the disease progression above the effects associated with normal ageing, including excessive complement activation and neovascularisation (Booij et al., 2010a).

As discussed above, the first hit in the 2-hit hypothesis is the accumulation of molecular and cellular damage to the RPE/choroid complex (Rozing et al., 2020). The posterior eye is particularly vulnerable to this type of damage due to the high metabolic activity in this region. Moreover, terminally differentiated cell types such as RPE cells are highly sensitive to damage, as they have an extremely limited regenerative capacity and are therefore highly dependent on adequate blood supply and the proper functioning of the various routes for removing waste products (Rozing et al., 2020). The accumulation of molecular and cellular damage can be either positively or negatively influenced by various environmental factors such as smoking and diet, as well as genetic factors such as variants in the *CFH* and *ARMS2* genes (Bergen et al., 2019; Despriet et al., 2006). The formation of drusen is a consequence of this first hit, driven by a complex series of processes that include oxidative stress, lipid accumulation, mineral and protein deposits, and decreased clearance and autophagy of cellular waste and debris (Bergen et al., 2019; Rozing et al., 2020). Biomolecules trapped in the sub-RPE deposits can be modified by oxidation, thus attracting complement system components and eventually triggering an inflammatory response (Bergen et al., 2019).

CECs may play a major role in the development of sub-RPE deposits, independent of the immune response triggered by the sub-RPE deposits. Campos and Abu-Asab found that patients with AMD present with signs of a decline in the transport capacity of the choriocapillaris (Campos and Abu-Asab, 2017). Moreover, using electron microscopy the authors examined the ultrastructural properties of the RPE/choroid complex and found reduced polarisation of the CECs in the choriocapillaris (Campos and Abu-Asab, 2017). Interestingly, CEC polarisation is believed to play an important role in the transport function of the choriocapillaris, as polarised CECs form a thin, highly fenestrated sheet between the vascular lumen and BM (Campos and Abu-Asab, 2017; Guymer et al., 2004). Indeed, CECs that are no longer polarised have an abnormally localised nucleus and a loss of fenestrations, which may -in part-explain the loss of CEC-mediated transendothelial transport in AMD (Campos and Abu-Asab, 2017). Although the underlying mechanism by which CECs lose their polarisation is currently unknown, one possibility may be impaired signalling via VEGF, a major trophic factor for CECs and CEC polarisation (Blaauwgeers et al., 1999). In this respect, it is interesting to note that in their study Campos and Abu-Asab included 7 elderly (>80 years of age) patients with atrophic AMD, with no control group (Campos and Abu-Asab, 2017). Therefore, additional ultrastructural studies that include both healthy controls and younger patients are needed in order to determine whether a causal relationship exists between the loss of polarisation and the loss of fenestrations in AMD.

The first hit (i.e. the accumulation of deposits) is then followed by an inflammatory response and – in progressive cases – the manifestation of GA and/or CNV (Rozing et al., 2020). The immune system plays a pivotal role in this process, and components in drusen are known to

attract immune effectors (Bergen et al., 2019; Hageman et al., 2001; Xu et al., 2009). A shift in the complement system towards activation can be caused by drusen deposits, but can also be caused by a loss of the immunosuppressive factors normally present in the healthy choroid, thus accelerating the development of a local pro-inflammatory response (Chirco et al., 2017a; Schlingemann, 2004). For example, the complement system is generally regulated less efficiently in the elderly due to an ageing-related loss of binding sites for the inhibitory complement factor CFH (Keenan et al., 2014). Activation of the complement system ultimately leads to formation of the membrane attack complex (MAC), a harmful product of the complement cascade that can cause the lysis of CECs, thereby affecting the transport capacity of the choriocapillaris (Whitmore et al., 2015).

Macrophages may also contribute to the development of AMD (Cherepanoff et al., 2010; Guillonneau et al., 2017), as they can be attracted to drusen in both early-stage AMD and advanced AMD (Cherepanoff et al., 2010; Guillonneau et al., 2017). Interestingly, macrophages reside in the choroid under physiological conditions, but immunosuppressive signals from the RPE and extracellular proteins in the choroidal stroma can prevent their differentiation into a pro-inflammatory phenotype (Guillonneau et al., 2017). When macrophages reach an inflammatory state, they can release harmful pro-inflammatory signalling molecules such as IL-6 and TNF-α. Interestingly, circulating monocytes (the precursor of macrophages) expressing IL-6 and TNF-α are particularly prevalent among patients with neovascular AMD, suggesting that pro-inflammatory macrophages may migrate to the site of CNV (Guillonneau et al., 2017). Although the extent to which macrophages contribute to AMD is currently unknown, their abundance at the sites of GA and CNV, as well as their ability to augment the pro-inflammatory state, suggests that their role is significant (Guillonneau et al., 2017). Despite the lack of direct evidence, the role of CECs in this process is likely, particularly given the high expression of adhesion molecules such as ICAM-1 and E-selectin (Table 1). Interestingly, ICAM-1 expression is particularly high in the macular area, and *in vitro* stimulation of human RPE/choroid complex tissues with complement factor C5a upregulates *ICAM1* expression in the CECs (Mullins et al., 2006; Skeie et al., 2010), providing further evidence that CECs likely play a role in recruiting macrophages in a pro-inflammatory context.

Taken together, the available data clearly show that AMD is a complex chorioretinal disease with a multifactorial origin. Future studies should investigate the mechanisms that underlie the formation of early drusen, as well as the high variability between patients with respect to the progression from drusen formation to the development of GA and/or CNV. Both genetic factors and environmental factors undoubtedly play a major role in this process and may explain – at least in part – some of this variability (Anderson et al., 2010; Rozing et al., 2020). Nevertheless, CECs likely play a prominent role in AMD, although the extent to which mechanisms that cause AMD originate in the CECs remains an open question.

4.3. The pachychoroid spectrum: dilated Haller's layer vessels and hyperpermeability of the choriocapillaris

4.3.1. Defining the pachychoroid spectrum

The pachychoroid spectrum is a subset of chorioretinal diseases with specific choroidal abnormalities, as well as various degrees of focal abnormalities at the level of the RPE and outer retina (Cheung et al., 2019; Dansingani et al., 2016). The clinical spectrum of pachychoroid disorders includes: uncomplicated pachychoroid, pachychoroid pigment epitheliopathy, CSC, peripapillary pachychoroid syndrome, pachychoroid neovasculopathy (either with or without serous detachment of the neurosensory retina), and pachychoroid aneurysmal type 1 CNV (formerly known as polypoidal choroidal vasculopathy, or PCV) (Cheung et al., 2019; Siedlecki et al., 2019). Pachychoroid, which literally means "thickened choroid", is a hallmark feature of diseases in the pachychoroid spectrum, although this is an oversimplification, as simply having a thicker-than-average choroid does not cover the entire definition (Fig. 8C) (Dansingani et al., 2016; Spaide, 2021). In fact, the term "pachychoroid" itself is difficult to define, as choroidal thickness can fluctuate considerably due to normal physiological processes such as ageing, the circadian rhythm, and changes in systolic blood pressure (Brown et al., 2009; Usui et al., 2012). Moreover, not all patients who are clinically diagnosed with a disease considered part of the pachychoroid spectrum actually present with pachychoroid, and the pattern of increased choroidal thickness can also vary across the posterior globe (Cheung et al., 2019; Ferrara et al., 2014; Sakurada et al., 2020). Nevertheless, many clinicians define pachychoroid as subfoveal choroidal thickness >300 μm (Dansingani et al., 2016; Mrejen and Spaide, 2013), even though the use of a specific cut-off value for choroidal thickness in defining a pachychoroid spectrum disease remains a topic of debate, for the reasons mentioned above (Spaide, 2021). Perhaps at least as important as increased choroidal thickness is the presence of choriocapillaris hyperpermeability on ICGA and the presence of pachyvessels (dilated choroidal veins) on ICGA and OCT (Figs. 8C and 9). Choriocapillaris hyperpermeability on ICGA is characterised by focal or diffuse areas of hyperfluorescence without distinct borders (Fig. 9S) (Gass, 1967). Pachyvessels are grossly dilated veins in Haller's layer, associated with thinning of the overlying smaller vessels and the choriocapillaris, and are often found in a spatial relationship with retinal or RPE abnormalities (Figs. 8C and 9N) (Baek et al., 2019; Cheung et al., 2019; Dansingani et al., 2016; Phasukkijwatana et al., 2018). Although both of these factors – i.e. choriocapillaris hyperpermeability and pachyvessels – are specific to the pachychoroid spectrum, as with pachychoroid itself their absence does not necessarily exclude the diagnosis of a pachychoroid spectrum disease (Spaide, 2021). In summary, the precise definition of the pachychoroid spectrum remains controversial, and future studies are needed in order to establish a clear classification system. Such a system would not only define certain parameters such as the pachychoroid, but will also be valuable in establishing clear definitions of conditions that are considered to be part of the pachychoroid spectrum.

4.3.2. Pachyvessels and intervortex vein anastomoses

Pachyvessels are choroidal veins in Haller's layer with an enlarged lumen due to congested blood flow. Typically, several pachyvessels originating from the macular area can cross the posterior pole in parallel, running diagonally towards the periphery. Pachyvessels are best visualised using either ICGA or en face SS-OCT (Cheung et al., 2019; Dansingani et al., 2016; Spaide et al., 2021). Interestingly, at the distal end of the pachyvessels, the enlarged lumen can end abruptly, reminiscent of a partially inflated long party balloon (Cheung et al., 2019; Dansingani et al., 2016). Although it is tempting to speculate based on these observations that a downstream obstruction and/or congestion of blood flow may play a role in the development of pachyvessels, the precise pathophysiological mechanism is currently unknown. The dilated vessels in Haller's layer can affect the overlying choriocapillaris, which can become thin and disrupted, showing as delayed choriocapillaris filling on ICGA (Cheung et al., 2019; Dansingani et al., 2016; Kishi et al., 2018). These deficits in choriocapillaris flow have been suggested to cause chronic hypoxia in the RPE and outer retina, structures that are highly dependent on choriocapillaris flow for nourishment (Teussink et al., 2015).

ICGA has proven to be invaluable for studying and diagnosing pachychoroid disorders, for several reasons. First, choriocapillaris hyperpermeability – which can be readily detected using ICGA – is a well-established finding in pachychoroid disease, particularly in both pachychoroid pigment epitheliopathy and CSC. This hyperpermeability has been suggested to occur due to increased hydrostatic pressure, although solid evidence supporting this hypothesis is currently lacking (Dansingani et al., 2016; Prünte and Flammer, 1996). Second, ultra-widefield ICGA can be used to visualise the trajectory of

pachyvessels reaching far into the periphery of the posterior eye (Klufas et al., 2015). Moreover, quantification of ultra-widefield ICGA images in healthy individuals revealed that venous outflow is higher in the inferior quadrants than in the superior quadrants (Jung et al., 2020); the authors reported that this difference in brightness may be due to gravity and/or anatomical differences between the superior ophthalmic vein and the inferior ophthalmic vein. Interestingly, this non-symmetrical venous drainage was more pronounced in patients with CSC and patients with pachychoroid pigment epitheliopathy compared to the healthy controls, suggesting that congested outflow is a pathophysiological phenomenon associated with pachychoroid disorders (Jung et al., 2020).

Another interesting finding obtained using ultra-widefield ICGA is that intervortex vein anastomoses are more prevalent in patients with a pachychoroid disorder compared to healthy controls (Spaide et al., 2020b). Similar results were reported by Matsumoto et al. using en face OCT (Matsumoto et al., 2020a). In their study, Spaide et al. also reported more anastomoses in the macular area in patients with CSC and patients with pachychoroid-associated neovascularisation; in contrast, the anastomoses were more prevalent around the optic nerve in patients with peripapillary pachychoroid syndrome (Spaide et al., 2020b). Although further studies are needed in order to determine the pathophysiological relevance of these anastomoses in pachychoroid disorders, their presence may provide valuable insight into these diseases and may aid in developing a more suitable classification system (Spaide et al., 2021).

4.3.3. Central serous chorioretinopathy

4.3.3.1. Risk factors for developing central serous chorioretinopathy. Several intriguing risk factors have been associated with CSC, including the use of corticosteroids, pregnancy, and having a type A personality (Nicholson et al., 2018). Furthermore, males are more likely to develop CSC, with a 6-fold higher prevalence among men compared to women (Kitzmann et al., 2008). The use of corticosteroids is the strongest extrinsic risk factor. Interestingly, the increased risk associated with the use of corticosteroids is similar among the various routes of administration, including oral delivery, intra-articular injection, and nasal administration, indicating a pre-existing increased susceptibility to developing CSC (Ge et al., 2020). In this respect, it is interesting to note that endogenous hypercortisolism – i.e. Cushing's syndrome – is also a well-known risk factor for developing CSC (Bouzas et al., 1993; Brinks et al., 2021; van Dijk et al., 2016).

4.3.3.2. Choroidal imaging in central serous chorioretinopathy. The first condition attributed to the pachychoroid spectrum was CSC (Guyer et al., 1994; Prünte and Flammer, 1996). A diagnosis of CSC is established primarily based on the presence of serous SRF together with choroidal hyperpermeability on ICGA (van Dijk and Boon, 2021; Van Rijssen et al., 2019b). On OCT, the majority of patients with CSC present with pachychoroid, pachyvessels, and an accumulation of SRF between the RPE and the outer retina (Chhablani et al., 2020; Kaye et al., 2020; Van Rijssen et al., 2019b). FA together with ICGA is required to confirm the diagnosis of CSC; specifically, one or more hyperfluorescent "hot spots" of focal leakage through the RPE are often seen on FA, surrounded by a larger area of hyperfluorescent changes with an indistinct border on ICGA (Van Rijssen et al., 2019b). The SRF is believed to be the result of extravasated fluid leaking from the choroidal vasculature, which causes secondary damage to the RPE and the subsequent leakage of fluid into the subretinal space. Interestingly, the areas that indicate choroidal dysfunction on ICGA are often considerably larger than the areas of RPE alteration and SRF accumulation, suggesting that the choroid plays a major role – and perhaps the primary role – in the pathogenesis of CSC (Chhablani et al., 2020; Gass, 1967; Guyer et al., 1994; Kaye et al., 2020; Liew et al., 2013; Nicholson et al., 2013; Prünte and Flammer, 1996). Moreover, choroidal abnormalities typically appear prior to the manifestation of RPE abnormalities and SRF accumulation, and patients with unilateral CSC often present with pachychoroid and pachyvessels on OCT and/or choroidal hyperpermeability on ICGA in the fellow eye (Dansingani et al., 2016). Interestingly, the choroidal hyperpermeability on ICGA often persists even after the SRF has resolved (Mrejen and Spaide, 2013). In addition, impaired pumping function of the RPE has also been suggested to play a causal role in the pathophysiology of CSC (Marmor, 1988).

4.3.3.3. Genetics and pathogenesis of central serous chorioretinopathy. A variety of gene variants have been reported to increase the risk of CSC, including variants in *ARMS2*, *C4B*, *CFH*, *CDH5*, *GATA5*, *NR3C2*, *PTPRB*, *SLC7*A5, *TNFRSF10A*, and *VIPR2*, which have been reviewed in detail elsewhere (Kaye et al., 2020; Van Rijssen et al., 2019b). Here, we discuss the most notable genes that are potentially associated with CEC function. A familial form of CSC has been reported in association with the *PTPRB* gene, which encodes the protein tyrosine phosphatase receptor type B (Schellevis et al., 2019b; van Dijk et al., 2019; Weenink et al., 2001). Interestingly, this enzyme is believed to be involved in the regulation of vascular permeability, as it can associate with the extracellular domain of VE-cadherin and modulate phosphorylation of important adherens junctions proteins (Fig. 5) (Dejana, 2004). As discussed in section 3.4.3, VE-Cadherin and VEGFR2 interact to maintain EC barrier function, and both are expressed at high levels in CECs (Table 1). Another possible link between gene variants in CSC and CEC barrier function was reported by Schubert et al., who found that four common SNPs in the *CDH5* gene (encoding VE-Cadherin) were significantly associated with CSC (Schubert et al., 2014). Interestingly, experimental data support the notion that corticosteroids exert their pathogenic effect on CECs by breaking down VE-Cadherin, a major component of adherens junctions (Schubert et al., 2014). Another interesting genetic association was described by De Jong et al., who found that chronic CSC was associated with variants in the *ARMS2* and *CFH* genes (de Jong et al., 2015). Strikingly, variants in these genes that increase the risk of AMD were found to protect against CSC – and vice versa – as discussed in section 4.2.3. Furthermore, independent genome-wide genetic association studies confirmed the association between gene variants in the complement system – for example, in the *CFH* gene – and CSC (Hosoda et al., 2018; Schellevis et al., 2018a). Moreover, we reported that a variant in the *NR3C2* gene, which encodes the mineralocorticoid receptor, increases the risk of CSC, thus providing a possible genetic basis to explain the link between chronic stress and CSC (van Dijk et al., 2017a). Genetic factors associated with the risk of developing acute CSC have not been studied as thoroughly, although SNPs in the *CFH* gene and copy number variations in the *C4B* gene have been found to increase the risk of acute CSC, suggesting that acute CSC and chronic CSC may share certain pathogenic mechanisms (Mohabati et al., 2019).

Despite the discovery of these putative risk factors and recent advances in imaging techniques, the pathogenic mechanism in CSC remains poorly understood (Daruich et al., 2015; Kaye et al., 2020; Spaide et al., 2021; van Dijk and Boon, 2021). Interestingly, CECs are sensitive to corticosteroids and may therefore play a role in the pathogenesis of CSC (Brinks et al., 2018; Daruich et al., 2018; Kaye et al., 2020). The downstream activity of one of the main corticosteroid receptors – the glucocorticoid receptor – can be modulated by the androgen receptor, for which testosterone is the principal ligand (Kroon et al., 2020). Although currently only speculative and not yet examined in the context of CSC, this suggests that an interaction between corticosteroids and androgens may play an additional role in the development of CSC (Schellevis et al., 2019a).

Taken together, these results indicate that a variety of factors contribute to the pathogenesis of CSC, including choroidal dysfunction, the complement system, hormonal imbalance, and corticosteroid use. Thus, optimising the clinical classification of CSC, which is currently a

topic of debate among retinal specialists (Singh et al., 2019a), may help researchers attribute these specific aspects of CSC to subtypes of CSC, advancing our understanding of this complex disease.

4.3.3.4. Treatment of central serous chorioretinopathy. The ideal treatment for CSC is currently a subject of controversy (Mehta et al., 2017; Van Rijssen et al., 2019b); however, a growing body of evidence – including recent large prospective randomised trials – suggests that the most effective treatment strategy for chronic CSC is photodynamic therapy (PDT) with reduced settings (van Dijk et al., 2018; Van Rijssen et al., 2019a, 2019b). Indeed, half-dose and half-fluence PDT have been shown to provide long-term complete resolution of SRF in 67–97% of the patients (Van Rijssen et al., 2019b). On the other hand, a sizeable percentage of chronic CSC cases are therapy-resistant and have an increased risk of recurrent episodes and a permanent decline in both visual function and vision-related quality of life (Breukink et al., 2017). The beneficial effects of PDT are believed to be mediated primarily by reducing choroidal dysfunction, in contrast to treatment options such as focal laser coagulation and micropulse laser treatment, which primarily target the RPE (Maruko et al., 2010; van Dijk et al., 2018; Van Rijssen et al., 2019b). For example, PDT often causes a transient decrease in total choroidal area and total luminal area, as well as a sustained decrease in subfoveal choroidal thickness, supporting the notion that PDT primarily affects the choroid (Iovino, 2020). The mechanisms by which PDT affects the choroid are discussed in further detail in section 4.12.1. Taken together, the experimental and clinical data discussed in section 4.3.3 clearly indicate that the choroid plays a central role in the pathogenesis of CSC. Therefore, future studies should focus on these choroidal abnormalities, thus increasing our understanding of CSC, facilitating the development of new therapeutics, and guiding the optimisation of existing treatments.

4.3.4. Pachychoroid pigment epitheliopathy

Pachychoroid pigment epitheliopathy was first described in 2013 by Warrow et al. and is considered either a precursor or forme fruste (atypical presentation) of CSC (Ersoz et al., 2018; Shinojima et al., 2020; van Dijk and Boon, 2021; Warrow et al., 2013). Importantly, pachychoroid pigment epitheliopathy does not present with SRF accumulation. Though largely overlapping clinically with CSC, pachychoroid pigment epitheliopathy is characterised by pachychoroid together with serous pigment epithelial detachments, RPE changes, decreased fundus tessellation, changes in fundus autofluorescence, and indistinct hyperfluorescent changes on ICGA characteristic of choroidal hyperpermeability (Cheung et al., 2019; Dansingani et al., 2016; Warrow et al., 2013). The changes in the pigment epithelium often occur above focal areas of choroidal thickening and choroidal congestion (Warrow et al., 2013). The decreased fundus tessellation has been suggested to serve as a factor for differentiating pachychoroid pigment epitheliopathy from other diagnoses within the pachychoroid spectrum, although fundus tessellation also depends on choroidal thickness and axial length and thus should not be considered a sufficiently reliable parameter (Spaide, 2021). Thus, aside from the absence of SRF accumulation, whether a pathophysiological basis can distinguish between pachychoroid pigment epitheliopathy and CSC remains an open question that warrants further study (Warrow et al., 2013).

4.3.5. Type 1 choroidal neovascularisation and polypoidal choroidal vasculopathy

Neovascularisation occurring under the RPE – or type 1 CNV – can manifest in the context of AMD (as described in section 4.2.1), but it can also manifest in cases with normal choroidal thickness, an atrophic choroid, or pachychoroid (i.e. pachychoroid neovasculopathy) (Dansingani et al., 2016; van Dijk and Boon, 2021). The pathophysiology of type 1 CNV appears to vary depending on clinical factors such as the presence of drusen, pachydrusen, SDDs, angiographic features on multimodal imaging, genetic factors, and race (Cheung et al., 2019; Dansingani et al., 2016; Spaide, 2018a; van Dijk et al., 2020a). Notably, the characterisation and classification of type 1 CNV in various contexts is a topic of ongoing research designed to improve our understanding with respect to both the pathophysiology and the optimal treatment strategy for these specific lesions (Spaide et al., 2020a).

In this context, a topic of controversy regarding the classification of neovascular diseases is PCV, which has been suggested to be either an aneurysmal variant of type 1 CNV or a distinct neovascularisation entity, primarily due to its "polypoidal" appearance (Spaide et al., 2020a; van Dijk et al., 2020a). PCV was first described by Yannuzzi as "peculiar polypoidal, subretinal, vascular lesions associated with serous and haemorrhagic detachments of the RPE" (Yannuzzi et al., 1990). The polypoidal lesion is often described as an aneurysmatic vascular anomaly, which can be – but is not necessarily – connected to a branching vascular network believed to serve as the vessels that feed and drain the lesion (Spaide et al., 1995; Tan et al., 2015; Yannuzzi, 2011). This branching vascular network is located between the RPE and BM, and electron microscopy and histopathology indicate the ingrowth of blood vessels through BM into the sub-RPE space, forming a polypoidal vascular network (Kawamura et al., 2013; Li et al., 2019).

In conclusion, despite having distinct genetic factors and clinical properties (van Dijk et al., 2020a), how these polypoidal lesions develop, and the extent to which their pathophysiology overlaps with typical type 1 CNV, remains unclear. Moreover, a specific role for CECs remains an open question. Future advances in OCTA techniques – for example, improvements in the reliability of detecting the direction of flow or the presence of low flow phenomena – may provide new insights into the diagnosis, classification, and our understanding of the pathophysiology of PCV (van Dijk et al., 2020a).

4.4. Diabetic choroidopathy

Diabetes mellitus is a chronic condition characterised by a state of hyperglycaemia, affecting all of the small and large blood vessels, including the retinal and choroidal vasculature. Diabetic choroidopathy is an inflammatory disease associated with changes in the choroidal vasculature (Lutty, 2017). Hyperglycaemia induces a systemic inflammatory response, and patients with diabetes mellitus can show elevated serum levels of TNF-α (Chen et al., 2017). This systemic inflammation has suggested to lead to choroidal vascular damage mediated by increased expression the pro-inflammatory adhesion molecules ICAM-1 and P-Selectin on the cell surface of CECs (Lutty, 2017). The resulting pro-inflammatory state of the CECs is believed to promote the extravasation of leukocytes, eventually leading to capillary occlusion by leukocytes, resulting in vascular dropout (Lutty, 2017). On the other hand, the vascular damage in diabetes mellitus can lead to hyperpermeability, which is well-described in diabetic retinopathy (DR) (Daruich et al., 2018; Klaassen et al., 2013). At the molecular level, hyperglycaemia induces a pro-inflammatory state in ECs and affects barrier function, leading to structural changes in both the macrovasculature and microvasculature. Clinical and histopathological studies of diabetic choroidopathy have described the widespread effects of hyperglycaemia in all choroidal vascular layers (Lutty, 2017). These effects can include the appearance of microaneurysms, atrophy of Sattler's layer and Haller's layer, tortuosity of large choroidal vessels, CNV, and dropout of the choriocapillaris (Ferrara et al., 2016; Lutty, 2017; Yang et al., 2019). Below, we review diabetic choroidopathy from several perspectives, including histopathology (section 4.4.1) and imaging (section 4.4.2), and we discuss the effects of hyperglycaemia on the inflammatory response and the barrier function of CECs at the molecular level (section 4.4.3).

4.4.1. Histopathological and morphological findings in diabetic choroidopathy

Histopathology flat mounts of choroidal tissue can be used to

examine morphological properties and quantify choriocapillaris density. Using this approach, Lutty and McLeod described a technique for staining CECs based on phosphatase activity, which specifically stains viable CECs, thus revealing atrophic and/or nonviable CECs, which lack enzymatic activity. More recently, Lutty and colleagues used this technique to examine choroid tissues from diabetic subjects and found decreased numbers of viable ECs in the choriocapillaris, ranging from a focal pattern, to a diffuse pattern, to areas with a complete loss of ECs (Lutty, 2017). Moreover, the effects of hyperglycaemia appeared to be substantial, as the authors estimated that choriocapillaris dropout was four times higher in diabetics compared to non-diabetic controls (Lutty, 2017). The authors also described deposits of debris on BM reminiscent of the basal linear deposits observed in AMD, which correlated spatially with the loss of ECs in the choriocapillaris (Lutty, 2017). Furthermore, another finding using histopathology on the choroids from diabetic patients was "intrachoroidal microangiopathy", which are neovascular lesions associated with diabetic choroidopathy. These lesions contained microaneurysms and were frequently located in the outer choroid, near the lamina fusca. Taken together, these results indicate that diabetic choroidopathy significantly reduces the viability of CECs and leads to both vascular atrophy and vascular remodelling throughout the entire choroidal vasculature (Lutty, 2017).

4.4.2. Imaging modalities in diabetic choroidopathy

Here, we describe the various imaging modalities commonly used in diabetic choroidopathy, including conventional OCT, en face OCT, OCTA, FA, and ICGA. In general, OCT studies that have attempted to identify an association between choroidal thickness and diabetes mellitus are inconsistent with respect to finding either a decrease or increase in choroidal thickness (Lutty, 2017; Melancia et al., 2016). There are several possible explanations for these varying results. First, normal physiological fluctuations in choroidal thickness (irrespective of the presence of diabetes mellitus), the use of different OCT systems, and/or different measurement parameters may play a role (Brown et al., 2009; Melancia et al., 2016; Usui et al., 2012). Moreover, the effect of diabetes mellitus on the choroidal vasculature is often studied in the clinical context of various stages of DR; therefore, several studies of diabetic choroidopathy included patients who previously received treatment for DR such as anti-VEGF therapy and/or panretinal photocoagulation, which may have affected choroidal thickness independent of diabetes-induced changes (Ferrara et al., 2016; Lutty, 2017; Melancia et al., 2016). Thus, whether diabetes mellitus directly affects choroidal thickness remains unclear. In this respect, studies involving patients with diabetes mellitus who have not yet received any treatment for diabetes mellitus and have no known ocular comorbidity may provide important insights (Campos et al., 2017).

Studies using en face OCT (for a description of this technique, see section 4.1.1.2 above) have provided a clear view of the vascular remodelling associated with diabetic choroidopathy, consistent with histopathological findings (Ferrara et al., 2016). Specifically, Ferrara et al. found vascular remodelling in a cross-sectional study involving 76 patients with diabetes mellitus, with irregular, tortuous, and beaded choroidal vessels with focal dilatation and narrowing present in all 76 patients (Ferrara et al., 2016). Moreover Wang et al. performed a quantitative analysis of SS-OCT images of patients with various stages of DR by calculating the percentage of the area occupied by choroidal vessels within the macular region, finding reduced choroidal vascular density in all patients compared to aged-matched non-diabetic controls. Moreover, the authors found that the reduction in choroidal vascular density was correlated with the severity of DR, ranging from a 6% reduction in non-proliferative DR cases to a 12% reduction in proliferative DR cases (Wang et al., 2017).

Recently, Yang and colleagues used OCTA to examine choriocapillaris flow in patients with diabetes mellitus (Yang et al., 2019). Interestingly, the findings in both the en face OCT study and the OCTA studies are consistent with the histopathological findings in human choroid samples obtained from eyes of diabetic patients (Lutty, 2017; Yang et al., 2019). In their OCTA study, Yang et al. measured capillary flow density in 43 healthy controls and 103 diabetic patients who either had no evidence of DR or presented with either non-proliferative DR or proliferative DR; they found that capillary flow density was decreased in the eyes with signs of advanced DR, including proliferative DR and eyes with diabetic macular oedema (Yang et al., 2019). However, whether this reduction in choriocapillaris flow plays a pathogenic role in the development of proliferative DR and/or diabetic macular oedema remains an open question.

Previously, Weinberger and colleagues used FA and ICGA to examine 42 patients with non-proliferative DR (Weinberger et al., 1998). The authors found that half of these patients had a diffuse pattern of hyperfluorescence in the late phase of the ICGA, corresponding to areas of retinal nonperfusion on FA and retinal oedema. This overlap in hyperfluorescence between FA and ICGA suggests that focal areas of the diabetes-related choroidal dysfunction can affect the overlying neuroretina, for example by breaking down the outer blood-retinal barrier (Weinberger et al., 1998). The authors also found that in the very late phase of ICGA (at 40–60 min), 12% of patients had a lobular pattern of spotty hyperfluorescence and hypofluorescence known as a "salt and pepper" pattern, which they hypothesised was the result of selective choriocapillaris filling (Weinberger et al., 1998). Furthermore, 85% of the microaneurysms (saccular extensions of the vascular wall) were visible on both FA and ICGA, whereas 13.4% were observed solely on ICGA, suggesting the presence of saccular dilatation of the choroidal vessels. Using laser Doppler flowmetry to study the effects of diabetes on the choroid – which is limited to the foveal region, where the retinal vasculature does not interfere with the measurements – several groups found a significant reduction in blood flow attributed to increased vascular resistance (Lutty, 2017; Melancia et al., 2016; Nagaoka et al., 2004). Interestingly, Nagaoka et al. measured reduced choriocapillaris flow on laser Doppler flowmetry in patients with diabetes mellitus without clinical signs of DR compared to age-matched healthy controls, suggesting that choroidal damage may precede the manifestation of DR (Nagaoka et al., 2004). The authors hypothesised that this decrease in choriocapillaris flow due to hyperglycaemia may lead to a chronic state of hypoxia in the neuroretina. Ultimately, given that hypoxia drives VEGF expression, this may contribute to the progression of DR, which in turn may promote neovascularisation and diabetic macular oedema.

In summary, the results obtained from imaging studies clearly demonstrate that the choroidal vasculature is prone to hyperglycaemia-induced damage. Notably, several imaging studies found an association between HbA1c (haemoglobin A1c) levels (which provide an indication of blood sugar) and choroidal vascular damage, including hyperfluorescent spots on FA and ICGA, vascular remodelling on en face OCT, and choriocapillaris loss on OCTA, thus supporting the notion of a causal link between diabetes mellitus and widespread damage to the choroidal vasculature (Lutty, 2017; Yang et al., 2019). Importantly, the extent to which diabetic choroidopathy plays a role in diabetes-associated damage to the neuroretina remains poorly understood, and studying this potential relationship is frequently obscured by the concomitant presence of DR (Ferrara et al., 2016; Nagaoka et al., 2004). Furthermore, the effects of the commonly used treatments for proliferative DR, which include anti-VEGF injections and panretinal photocoagulation, have not been examined in detail with respect to their potential to damage the choroidal vasculature (Ferrara et al., 2016).

4.4.3. Differential effects of hyperglycaemia between choroidal and retinal endothelial cells

Here, we discuss the effects of hyperglycaemia on RECs and CECs, highlighting the specific properties of these two types of endothelial cells. Saker et al. compared the effects of exposing primary cultured RECs and CECs to high glucose levels (Saker et al., 2014). Under normal glucose conditions, they found that compared to CECs, RECs express significantly higher levels of the tight junction components CLDN5 (with

4.8-fold higher expression) and OCLN (with 5.3-fold higher expression). Upon exposure to high glucose, the permeability in RECs increased significantly (by 20.6%), together with decreased expression of CLDN5, OCLN, and F11R. In striking contrast, high glucose had virtually no effect on permeability in CECs (increasing by only 3.9%), and had no effect on the expression of tight junction proteins (Saker et al., 2014). Based on these data, it is reasonable to speculate that unlike CECs, RECs are relatively sensitive to diabetes-related vascular damage mediated by changes in the paracellular junctions. This preferential sensitivity of RECs may be attributed to the fact that the inner blood-retinal barrier is maintained by RECs with elaborate paracellular junctions and cell-cell contacts with pericytes and Müller glial cells (Klaassen et al., 2013). Nevertheless, the pathological effects of hyperglycaemia on the retinal vasculature are also conveyed via transcellular routes, characterised by the pathological expression of PLVAP, a protein that is not expressed in the retinal vasculature under healthy conditions (Bosma et al., 2018; Klaassen et al., 2013).

Taken together, the results discussed in section 4.4 indicate that hyperglycaemia significantly affects both the choroidal microvasculature and the choroidal macrovasculature, and compelling evidence suggests that these effects are mediated via a pro-inflammatory state in the CECs. As seen in clinical practice and at the molecular level, hyperglycaemia exerts strikingly different effects on the choroidal vasculature compared to the retinal vasculature. This difference may provide new opportunities for further research, including studies focusing on the causal link between diabetic choroidopathy and damage to the neuroretina, aside from the primary DR.

4.5. The effect of myopia on choroidal structure and function, and myopia-related complications

The prevalence of myopia has increased rapidly in recent decades, particularly in developed countries (Morgan et al., 2018). Currently, as many as 80–90% of young adults (age 17–18 years) in developed countries in East and Southeast Asia are myopic. Several causal factors have been identified as underlying this high prevalence, the most important of which are insufficient exposure to natural daylight and intensive education programmes (Morgan et al., 2018). In addition, genetics also play an important role in the development of refractive error (Tedja et al., 2018), which we discussed specifically for the choroid in section 2.5. Next to an increase in the overall prevalence of myopic refractive errors, the prevalence of high myopia – often defined as refractive error >5 dioptres – has also increased in recent decades by up to 10-fold in East and Southeast Asia (Morgan et al., 2018). These statistics reflect a major challenge in ophthalmology and eye care, as high myopia is strongly associated with an increased risk of several complications such as myopic macular degeneration (MMD) and rhegmatogenous retinal detachment (Fang et al., 2019; Ohno-Matsui et al., 2015, 2018). There is no effective treatment for myopia, although an interesting topic of current research is the administration of low-dose atropin in myopic children. A recent study in humans reported that atropin may play a role in the inhibition of myopia-induced choroidal thinning (Sander et al., 2019).

The effect of myopia on the choroid is both complex and poorly understood. It is well-established that severe elongation of the globe – as occurs in high myopia – is strongly correlated with thinning of the retina, choroid, and sclera. With respect to the choroid, studies using ICGA have revealed that atrophy of all choroidal vessel layers can be observed in patients with high myopia (Ohno-Matsui et al., 2016). In severe cases, complete loss of the choriocapillaris and atrophy of the RPE and retina can be observed, a condition known as patchy chorioretinal atrophy, in which these atrophic areas contain only large choroidal vessels (Mrejen and Spaide, 2013; Ohno-Matsui et al., 2016). A panel of experts in the field of myopia recently proposed a uniform classification system for MMD, a spectrum of myopia-associated fundus abnormalities with chorioretinal atrophy as the common denominator (Ohno-Matsui et al., 2015). This classification system consists of five categories of MMD and other myopia-associated lesions, including posterior staphyloma and three “plus signs” (lacquer cracks, myopic CNV, and Fuchs’ spot). In increasing severity, MMD is classified as follows: no myopic retinal lesions (category 0), tessellated fundus only (category 1), diffuse chorioretinal atrophy (category 2), patchy chorioretinal atrophy (category 3), and macular atrophy (category 4) (Ohno-Matsui et al., 2015). The additional myopia-associated lesions described within this classification system are strongly associated with MMD, but can occur in any category and are strongly associated with further loss of visual function (Ohno-Matsui et al., 2015).

The risk for developing complications of MMD not only depends on the severity of the myopia, but also on ageing. Indeed, MMD in teenagers with high myopia is rare, although peripapillary atrophy is observed in around 39% of this group, which may be attributed to mechanical stretching of the globe (Ohno-Matsui et al., 2016). A severe complication associated with MMD is myopic CNV, which is the most common cause of CNV in patients under the age of 50 (Mrejen and Spaide, 2013; Ohno-Matsui et al., 2016). This type of CNV tends to arise from lacquer cracks, which are tears in BM in the posterior globe characteristic of pathological myopia (Mrejen and Spaide, 2013; Ohno-Matsui et al., 2016). Another peculiar feature associated with pathological myopia is the manifestation of posterior staphyloma, a demarcated area in which the posterior globe bulges towards the exterior with pronounced thinning of the choroid and sclera (Ohno-Matsui and Jonas, 2019). Interestingly, the thinning effect of posterior staphylomas is more pronounced in the choroid compared to the retina and the sclera, and the choroid has even been described to be almost absent in these regions (Ohno-Matsui and Jonas, 2019). The pathogenesis of posterior staphylomas is currently unknown, although severe choroidal thinning and/or atrophy may affect nourishment of the sclera, thereby resulting in scleral thinning and outward bulging of the posterior eye due to intraocular pressure (Ohno-Matsui and Jonas, 2019). Though plausible, only limited evidence supports this hypothesis, and posterior staphyloma can also develop in patients with retinitis pigmentosa that have a normal choroidal thickness (Ohno-Matsui and Jonas, 2019). Lastly, myopic eyes are at risk for developing punctate inner choroidopathy, an inflammatory disease characterised by multifocal white-yellow chorioretinal lesions (Ohno-Matsui et al., 2016). Although the pathophysiology of punctate inner choroidopathy is unknown, a histopathological study on excised neovascular membranes of a punctate inner choroidopathy patient revealed aggregation of lymphocytes in the inflamed choroid of these patients (Pachydaki et al., 2012).

4.6. Posterior uveitis: the inflamed choroid

Uveitis is the collective term for a variety of intraocular inflammatory conditions (Pichi et al., 2020). Uveitis, which can be either infectious or non-infectious in origin, currently accounts for 5–20% of all cases of legal blindness in the Western world, and approximately 25% of cases in developing countries (de Smet et al., 2011). The choroid, which plays a central role in ocular immunity as discussed above, is involved in many forms of uveitis, although the pathophysiology is often complex and poorly understood, as multiple tissues and the immune system are simultaneously involved (de Smet et al., 2011; Pichi et al., 2020). Uveitis can be confined to the anterior, intermediate, and/or posterior segment of the eye, or it can manifest in all three areas causing a condition known as panuveitis. Common clinical presentations of posterior uveitis in which the choroid is involved are discussed in the following paragraphs.

4.6.1. Birdshot chorioretinopathy

Birdshot chorioretinopathy is characterised by the bilateral presence of multiple white lesions on fundoscopy, retinal vasculitis, and cystoid macular oedema. The underlying pathogenesis is currently unknown, although over 96% of the cases have the HLA-A29 (histocompatibility leukocyte antigen-A29 class I) serotype (Cao et al., 2016). A

histopathology case report described foci of lymphocytes that were aggregated in the deep choroid but spared the choriocapillaris, possibly representing the large hyporeflective white lesions seen on EDI-OCT (Mrejen and Spaide, 2013). Similarly, Pichi et al. described intact choriocapillaris flow above active lesions on OCTA, with reduced or absent choriocapillaris flow in advanced lesions marked by depigmentation and atrophy of the choroidal stroma (Pichi et al., 2017). The characteristic birdshot lesions appear on ICGA as hypofluorescent spots that overlie and outnumber the lesions seen on fundoscopy (Cao et al., 2016). Therefore, ICGA may be an important tool for early diagnosis, as ICGA can reveal the presence of lesions before lesions become visible on fundoscopy (Cao et al., 2016). Studies are also investigating whether ICGA may serve a valuable tool for monitoring treatment efficacy and disease recurrence by quantifying the birdshot lesions. With respect to treatment options, corticosteroids and immunomodulatory agents can be effective at driving the disease into remission (Cao et al., 2016).

4.6.2. Vogt-Koyanagi-Harada disease

Vogt-Koyanagi-Harada disease is a putative autoimmune disease in which an inflammatory response is mounted against melanin-containing cells, affecting the uvea, skin, meninges, and inner ear (Pichi et al., 2017). With respect to the ocular manifestations, histopathological studies suggest that the inflammatory response initially manifests in the choroidal stroma, subsequently progressing to the RPE and retina (Pichi et al., 2017; van Dijk and Boon, 2021). A study using EDI-OCT in 8 patients with acute phase Vogt-Koyanagi-Harada disease found a marked increase in average choroidal thickness to 805 μm; moreover, the patients responded well to treatment with oral corticosteroids, reducing average choroidal thickness to 341 μm (i.e. within the normal range) within 2 weeks (Maruko et al., 2011). Thus, choroidal thickness measured using EDI-OCT has been suggested as a useful parameter for follow-up and for titrating the corticosteroid treatment (Maruko et al., 2011; Mrejen and Spaide, 2013). Interestingly, OCTA has also been suggested as a useful tool for monitoring disease activity, based on the flow deficits in the choriocapillaris that co-localise with the hypofluorescent spots on ICGA (Pichi et al., 2017). These areas of reduced flow on OCTA – believed to be due to choriocapillaris ischaemia – were found to improve with corticosteroids, and this was correlated with the reduction in choroidal thickness (Pichi et al., 2017).

4.6.3. Multifocal choroiditis

Despite what its name suggests, multifocal choroiditis is a form of uveitis that does not appear to primarily involve the choroid, although studies suggest at least some degree of choroidal involvement (Mrejen and Spaide, 2013; van Dijk and Boon, 2021). Although several subtypes of multifocal choroiditis have been described, including punctate inner choroidopathy and multifocal inner choroiditis, the clinical relevance of this distinction is unclear (Mrejen and Spaide, 2013; Slakter et al., 1997). A commonly occurring complication associated with multifocal choroiditis is CNV, occurring in up to 60% of cases (Dolz-Marco et al., 2017). Mrejen and Spaide described the characteristic lesions in multifocal choroiditis as “mound-like sub-RPE lesions that contain homogeneously moderately reflective material” on OCT (Mrejen and Spaide, 2013). These sub-RPE lesions can erupt and affect the outer retina, and in some cases can ultimately progress into “punched-out” atrophic scars (Dolz-Marco et al., 2017; Mrejen and Spaide, 2013; Pichi et al., 2020). Despite observations that direct involvement of the choroid appears limited, Slakter and colleagues performed ICGA in 14 patients diagnosed with multifocal choroiditis and found that half of the patients’ eyes had large (200–500 μm) hypofluorescent spots in the posterior pole; interestingly, however, none of the lesions were correlated with the lesions detected using either fundoscopy or FA (Slakter et al., 1997). In another study, Mrejen and Spaide reported that treatment with corticosteroids caused flattening of the sub-RPE lesions and decreased the thickness of the underlying choroid in some patients (Mrejen and Spaide, 2013). Although the pathophysiology of multifocal choroiditis is largely unknown, several factors indicate that the role of the choroid warrants further study, including the hypofluorescent spots on ICGA, the decrease in choroidal thickness upon treatment with corticosteroids, and the high prevalence of CNV in these patients.

4.6.4. Serpiginous choroidopathy

Serpiginous choroidopathy is a rare, chronic, bilateral inflammatory disease associated with atrophy and scarring of the RPE and the inner choroid (Pichi et al., 2017). The pathophysiology is currently unknown, although it is believed to be of immunological origin, given that immunosuppressive agents are generally effective. Findings on OCT can include multifocal spots of inflammation characterised by local choroidal thickening, decreased choriocapillaris reflectivity, and a loss of the normal dotted pattern of the choriocapillaris (Pichi et al., 2020). Interestingly, patients with a confirmed diagnosis of tuberculosis have an increased risk of developing serpiginous-like choroiditis (Gupta et al., 2003). However, whether this represents a distinct disease with features overlapping with serpiginous choroidopathy – or whether it should be considered a pathogenic mechanism of serpiginous choroidopathy – remains an open question. Mandadi et al. examined 16 patients with tubercular serpiginous-like choroiditis using EDI-OCT, OCTA, and ICGA, finding sharply defined areas of choriocapillaris flow void on OCTA that were correlated with hyporeflective spots on ICGA (Mandadi et al., 2017). Furthermore, they found that areas of choriocapillaris atrophy on EDI-OCT overlapped with the presence of medium-to-large vessels on OCTA, reflecting areas of inflammation (Mandadi et al., 2017). These findings are particularly interesting with respect to determining the value of using OCTA in the diagnosis and follow-up of superficial choroidopathies, and may have added clinical value for other types of uveitis as well (Pichi et al., 2017).

4.7. Preeclampsia and HELLP (haemolysis elevated liver enzymes and low platelets) syndrome

Pregnancy can be associated with conditions that cause choroidal dysfunction and/or changes in choroidal thickness. One such condition is preeclampsia, which occurs in approximately 2–8% of all pregnancies. Although the cause is largely unknown, preeclampsia presumably results from the mother’s response to the placenta. The most common symptoms of preeclampsia include hypertension and proteinuria (Evcimen et al., 2019); however, vision-related symptoms are estimated to occur in 25–50% of patients and can include serous retinal detachment, retinal oedema, and retinal haemorrhage (Evcimen et al., 2019; Kim et al., 2016). In most cases, the hypertension and the ocular symptoms resolve spontaneously within a couple of weeks after delivery (Gooding et al., 2012).

Several studies have shown that preeclampsia is also associated with increased choroidal thickness in the macula compared to healthy, non-pregnant women and women with a healthy pregnancy, with a reported average choroidal thickness ranging from 371 to 391 μm in the preeclampsia groups (Evcimen et al., 2019; Kim et al., 2016; Sharudin et al., 2020). In their study involving 7 women with preeclampsia, Kim et al. found that choroidal thickness returned to the normal range within 1 week after delivery (Kim et al., 2016). Interestingly, other studies of choroidal thickness measured during a healthy, uneventful pregnancy yielded conflicting results, reporting either an increase in choroidal thickness or no change in choroidal thickness (Roskal-Wałek, 2017). It is important to note that FA and ICGA are not recommended in pregnant women due to potential toxicity to the foetus; therefore, most clinical studies regarding preeclampsia used fundoscopy and/or OCT.

In approximately 10% of cases, preeclampsia can progress to HELLP (haemolysis elevated liver enzymes and low platelets) syndrome, a severe, potentially life-threatening complication of pregnancy (Sheth and Mieler, 2001). Previously, Erbagci et al. examined the occurrence of ophthalmological complications in a prospective cohort of 107 patients with HELLP syndrome and found hypertensive fundus changes in 18

patients (16%), bilateral asymmetric bullous retinal detachments in 4 patients (3.7%), and cortical blindness in 3 patients (2.7%); moreover, all of these complications resolved in virtually all cases (Erbagci et al., 2008). In addition, case reports of women who received ICGA shortly after pregnancy have provided valuable insights into the choroidal changes that occur in association with HELLP syndrome. For example, Van Rysselberge recently reported a case of a pregnant woman who underwent a late-stage emergency pregnancy termination due to HELLP syndrome; an ICGA performed 12 h post-partum revealed bilateral multifocal patchy regions of choroidal hypoperfusion, suggestive of choroidal ischaemia (Van Rysselberge, 2020). The authors also noted that these hypofluorescent lesions on early phase ICGA correlated spatially with the so-called "Elschnig spots" visible on fundus examination, which are demarcated RPE lesions due to nonperfused choriocapillaris areas caused by hypertension (Van Rysselberge, 2020). These observations are consistent with a previous case report by Song et al., who also reported numerous hypofluorescent spots on early phase ICGA image, which transformed into hyperfluorescent spots on middle-to-late phase ICGA; they hypothesised that these findings represented a leakage of the fluorescent dye into the nonperfused area due to choroidal vascular damage (Song et al., 2013).

The molecular and cellular pathophysiology of preeclampsia and HELLP syndrome is currently unknown; however, "systemic endothelial dysfunction" has been proposed to play a central role (Brennan et al., 2014; Tomimatsu et al., 2017, 2019). This endothelial dysfunction is believed to be caused by the placental release of soluble VEGFR1 into the maternal circulation due to insufficient placental perfusion (Tomimatsu et al., 2019). Soluble VEGFR1 can be distributed systemically, binding the pro-angiogenic factors VEGF and PlGF, ultimately decreasing both NO production and the release of vasoconstrictors such as EDN1 (Tomimatsu et al., 2019). Interestingly, the *VEGF*, *EDN1*, and *eNOS* genes are all expressed at high levels in CECs (Table 1). Furthermore, CECs are highly dependent on their trophic relationship with the RPE that secretes VEGF from its basal side (Blaauwgeers et al., 1999; Marneros et al., 2005). Thus, it is reasonable to speculate that high concentrations of circulating soluble VEGFR1 – as occurs in preeclampsia – may interfere with the critical cellular communication between the choroid and the RPE, leading to severe choroidal dysfunction.

4.8. Choroidal haemangioma

Choroidal haemangioma can present as either a circumscribed lesion or –in the case of Sturge-Weber syndrome – as a diffuse vascular tumour (Blasi et al., 2010; Francis et al., 2019; Shields et al., 2001). The most common complaint among patients with choroidal haemangioma is blurred vision, which is reported in 81% of the cases; in contrast, other symptoms such as metamorphopsia, visual field defects, and floaters are less common (Shields et al., 2001). Moreover, the presence of subretinal haemorrhage and metamorphopsia are prognostic factors associated with poorer visual function (Shields et al., 2001).

A circumscribed choroidal haemangioma typically appears as an orange mass on fundoscopy, with a hypofluorescent region on fundus autofluorescence and a multilobular vascular pattern with a surrounding hyperreflective halo on en face OCT (Flores-Moreno et al., 2016). Other OCT findings can include SRF and intraretinal fluid, as well as hyperplasia and fibrous metaplasia of the RPE (Blasi et al., 2010; Shields et al., 2001). Clinically, it can be difficult to distinguish a choroidal haemangioma from a choroidal melanoma or choroidal metastasis (Balasubramaniam et al., 2017). Vascular loops visible on infrared imaging and peritumoural vascular expansion on ICGA can indicate the presence of a choroidal haemangioma; thus, absence of these signs may be helpful in diagnosing either a choroidal melanoma or metastasis (Balasubramaniam et al., 2017).

Verbraak et al. first reported that single spot PDT can be used to safely and effectively treat choroidal haemangioma, with efficacy when applied at either 50 or 100 J/cm^2 radiance exposure (Verbraak et al., 2003, 2006). Subsequently, Blasi and colleagues performed a prospective study to examine visual outcome in 27 patients with choroidal haemangioma following treatment with PDT (100 J/cm^2) and verteporfin (6 mg/m^2 body surface area). The authors found that following treatment, all of the tumours were reduced in size, and macular exudation was fully resolved; moreover, no recurrences were detected. With respect to clinical outcome, the average improvement in visual acuity after 5 years was 18.5 ETDRS letters (Blasi et al., 2010). Interestingly, although the authors reported no adverse events or complications, Singh et al. previously reported delayed choroidal atrophy in 2 out of 10 patients with choroidal haemangioma treated with PDT, which they speculated could be a potential side effect of the treatment (Singh et al., 2004).

Recently, Francis and colleagues reported possible overlap in the underlying genetic factors between the circumscribed type of choroidal haemangioma and the diffuse type of choroidal haemangioma associated with Sturge-Weber syndrome (Francis et al., 2019). Specifically, although both tumour types commonly contain a mutation in the *GNAQ* gene (encoding the G protein α_q subunit), they contain two distinct mutations occurring in different codons (Francis et al., 2019). The protein encoded by the *GNAQ* gene acts downstream of the mitogen-activated protein kinase pathway, a major regulator of cell proliferation and survival. The mutation in *GNAQ* associated with the diffuse type of choroidal haemangioma causes excessive proliferation of CECs and vascular malformation (Bichsel et al., 2019; Francis et al., 2019). On the other hand, the mutation in *GNAQ* associated with the circumscribed type of choroidal haemangioma has also been found to occur in choroidal melanomas, leading to the hypothesis that this mutation may be specific to CECs in choroidal haemangioma and melanocytes in choroidal melanoma, possibly explaining why the same mutation can give rise to two distinct pathologies (Francis et al., 2019). Nevertheless, it is interesting to note that pharmacological inhibitors of the G protein α_q subunit are available, warranting future preclinical studies to determine whether this protein may serve as a viable therapeutic target (Francis et al., 2019).

4.9. Choroidal rupture

Choroidal rupture occurs as the result of a high-impact traumatic event that causes tearing of the RPE, BM, and the choroid (Aguilar and Green, 1984; Patel et al., 2013). Despite the fact that a choroidal rupture usually occurs after a trauma, it may also occur within the course of exudative AMD. Approximately 70% of traumatic eye injuries are closed globe injuries and are often caused by sports balls, other relatively large objects thrown at the eye, or accidents involving industrial tools or instruments (Ament et al., 2006). Choroidal ruptures can be subdivided into either direct ruptures or indirect ruptures (Aguilar and Green, 1984). A direct choroidal rupture involves injury at the site of impact in the anterior part of the eye, commonly leading to a rupture that is parallel to the ora serrata. Indirect choroidal ruptures – which are estimated to account for approximately 80% of cases (Ament et al., 2006) – are characterised by a choroidal tear on the site of the eye opposite to the impact, due to anteroposterior deformation of the globe (Patel et al., 2013). Indirect choroidal rupture (also known as a contrecoup injury) can often include tearing around the optic disc, at the site where the shear stresses are believed to converge (Patel et al., 2013). There is currently no treatment available for choroidal rupture in the acute phase (Patel et al., 2013). Moreover, a study involving 111 cases of choroidal rupture found that 68% of cases included the macula and were associated with poor visual outcome, with only 22% of cases recovering to driving vision (Ament et al., 2006).

An earlier histopathology study of 47 eyes with choroidal rupture examined at various time points after injury, ranging from several hours to 25 years, revealed that haemorrhage occurred within the first few hours after choroidal rupture (Aguilar and Green, 1984). In addition, the authors found that fibroblastic growth occurred within 2 weeks, and scar

formation occurred 3–4 weeks after rupture. Moreover, the scarring was either restricted to the choroid or extended into the retinal and/or vitreous space to various degrees. Finally, the authors observed hyperplasia of the RPE at the lesion's margin in 19% of cases (Aguilar and Green, 1984).

The recovery of choroidal rupture is associated with the formation of new vessels, which usually regress during the scarring process. On the other hand, a so-called secondary CNV can develop at a later stage; this complication associated with choroidal rupture can increase the risk of retinal damage, vitreous haemorrhage, and lead to a further decline in visual function (Ament et al., 2006). Secondary CNV is estimated to develop in 10–20% of choroidal rupture cases, and because it usually develops within the first year following the traumatic event, follow-up visits are warranted during this period (Patel et al., 2013). Interestingly, the central choroid is apparently more susceptible to developing CNV compared to the peripheral choroid. Although the reason for this is unclear, possible explanations include differences in blood flow and/or hydrostatic pressure between the central choroid and peripheral choroid (Ament et al., 2006). If diagnosed early, secondary CNV can often be treated effectively using anti-VEGF therapy (Barth et al., 2019).

4.10. Retinal dystrophies with secondary choroidal degeneration

The retinal dystrophies comprise a spectrum of degenerative disorders with autosomal recessive, autosomal dominant, X-linked, and mitochondrial inheritance patterns. Many retinal dystrophies originate from a genetic defect that primarily affects either the photoreceptors or the RPE, which then causes progressive choroidal atrophy in more advanced stages of retinal degeneration (Boon et al., 2008a). For some forms of retinal dystrophy such as central areolar choroidal dystrophy and choroideraemia, which tissue and/or cell type is primarily affected by the underlying genetic defect is currently unknown (Schachat et al., 2017).

A dystrophy that affects primarily the CECs has not been described. In the clinic, it can be difficult to determine which ocular tissue is primarily affected, as the onset of degeneration can appear to occur simultaneously in the retina, RPE, and choroid. Retinal dystrophies can be classified based on the pattern of degeneration, which often starts as patches of atrophy localised to either the macular or peripheral area (Boon et al., 2008a; Schachat et al., 2017). Macular dystrophies can be particularly difficult to distinguish from AMD-associated GA (Saksens et al., 2014; Smailhodzic et al., 2011). Central areolar choroidal dystrophy primarily affects the macula, with demarcated patchy atrophy of the photoreceptors, RPE, and choriocapillaris, with a decline in vision occurring around 30–60 years of age (Boon et al., 2009a). The disease progresses with loss of photoreceptors and severe atrophy of the RPE and choriocapillaris occurs, with large choroidal vessels becoming clearly visible on FA. Although its name suggests a primarily choroidal dystrophy, central areolar choroidal dystrophy initially affects the photoreceptors due to a mutation in the peripherin-2 protein (encoded by the *PRPH2* gene) that prevents normal formation of the photoreceptor outer segments, with subsequent atrophy of the RPE and – eventually – the choroid (Boon et al., 2008a). Other chorioretinal dystrophies such as gyrate atrophy and choroideraemia initially spare the macular area and present with patches of degeneration in the periphery, which progressively increase in number until they become confluent, eventually involving the macular area in the advanced stages (Quinn et al., 2019; van Schuppen et al., 2018). Not surprisingly, the rate of visual decline varies widely between the various forms of retinal dystrophy, and the disease course can even vary between affected family members carrying the same mutation (Ponjavic et al., 1995). This suggests that additional modifying factors can affect disease progression, given that most mutations cause a complete loss of expression of the affected gene (Boon et al., 2007, 2008a).

4.11. Age-related choroidal atrophy

Age-related choroidal atrophy (ARCA), first described by Spaide in 2009, has been suggested to represent a specific type of macular degeneration (Spaide, 2009). ARCA has been described as a distinct clinical entity characterised by severe choroidal atrophy, an apparent loss of medium-sized choroidal vessels, loss of choroidal pigmentation, and a tessellated fundus appearance (Spaide, 2009). In the retrospective case series that first described this type of macular degeneration, the average subfoveal choroidal thickness was 69.8 μm in 28 eligible eyes in 17 patients, after high myopic eyes (>6 dioptres) were excluded (Spaide, 2009). Interestingly, 10 of the 28 eyes (36%) also presented with late AMD (either CNV or GA), indicating possible pathogenic overlap between ARCA and AMD. Although soft drusen – a typical finding in AMD – were not present, SDDs (i.e. reticular pseudodrusen) were observed in 12 of the 28 eyes (43%) included in the study. Notably, 6 of the 17 patients also presented with glaucoma with concurrent peripapillary atrophy, indicating a possible correlation between glaucoma and ARCA (Spaide, 2009). Importantly, evidence of pathophysiological mechanisms that would support the classification of ARCA as a distinct disease entity is currently lacking. Thus, future studies designed to identify and classify this disease are clearly needed, as they may provide important new insights into degenerative chorioretinal diseases and may lead to new treatment options.

4.12. Perspectives on μcurrent treatment strategies for chorioretinal disease

4.12.1. Selective targeting of the choroid: the advantages of photodynamic therapy

PDT is considered both safe and effective for treating a range of choroidal vascular diseases, including CSC, choroidal haemangioma, and aneurysmal type 1 CNV (Chan et al., 2010; Schmidt-Erfurth and Hasan, 2000; van Dijk et al., 2020b; Van Rijssen et al., 2019b; Verbraak et al., 2003, 2006). PDT is based on the benzoporphyrin derivative verteporfin, a photosensitising compound that can be activated locally in the posterior eye using a 689-nm wavelength laser. Verteporfin is lipophilic, which means it can be taken up by cells via endocytosis, where it accumulates intracellularly (Gavini et al., 2019). Because CECs have high lipoprotein endocytosis activity, verteporfin accumulates selectively in these cells (Loeven et al., 2018). Moreover, studies in rabbits have shown that verteporfin is also taken up by photoreceptors and the RPE (Haimovici et al., 1997). Both cellular models and animal models have shown that light-activated verteporfin induces the cellular production of reactive oxygen species (ROS), leading to platelet aggregation and eventually causing thrombosis and occlusion of choroidal blood vessels (Eales et al., 2018b; Schmidt-Erfurth and Hasan, 2000). Interestingly, neovascular CECs have a particularly high rate of verteporfin endocytosis compared to normal CECs, presumably due to their abnormally high metabolic activity (Schmidt-Erfurth and Hasan, 2000).

As mentioned above, PDT has an excellent short-term and long-term safety profile for treating diseases such as CSC, choroidal haemangioma, and aneurysmal type 1 CNV (Van Rijssen et al., 2019b). Moreover, only rare cases of short-term or long-term vision loss were reported, primarily in early studies using full-dose PDT for treating neovascular AMD (Treatment of age-related macular degeneration with photodynamic therapy TAP study group, 2004; Van Rijssen et al., 2019b). Notably, the treatment paradigm likely plays an important role in determining safety and outcome, given studies in primate CNV models, which found that the dose of verteporfin and the timing of the laser application are crucial factors for determining the optimal treatment effect and for preventing irreversible damage to photoreceptors and large choroidal vessels (Schmidt-Erfurth and Hasan, 2000).

Why PDT has little or no effect on healthy tissues is currently unknown, although one plausible explanation is that CECs – and neovascular CECs in particular – take up verteporfin at the highest rate

among the various cell types. Another possible explanation is that all cell types are not equally susceptible to the damaging effects of verteporfin. For example, Eales and colleagues recently proposed that cells that are already under oxidative stress are more susceptible to the damaging effects of ROS, as is the case for neovascular CECs, which are driven by hypoxic stimuli (Eales et al., 2018a). Importantly, however, this hypothesis was based on cell culture experiments in which malignant glioblastoma cells were exposed to hypoxia; thus, whether these *in vitro* results hold true in the clinical setting warrants further study (Eales et al., 2018a).

In addition to driving the production of ROS, impaired autophagy – a process that mediates the degradation and recycling of cellular components – has also been suggested as a mechanism of action for verteporfin. For example, Konstantinou et al. found that verteporfin triggers the formation of cross-linked oligomers and high molecular weight protein complexes, which may interfere with autophagy and/or the cytoskeleton, eventually leading to cell death (Konstantinou et al., 2017). Conversely, in tumour cells, verteporfin can accumulate in lysosomes, structures that are essential to the process of autophagy (Gavini et al., 2019). This accumulation is believed to trigger the destabilisation of lysosomal membranes, subsequently leading to dysregulation of the autophagy pathway. Importantly, although CECs presumably have high lysosomal transport activity, whether these aforementioned effects play a role in verteporfin-containing CECs remains to be confirmed. Finally, another study used immunohistochemistry in neovascular membranes of AMD patients excised at different intervals after PDT (Tatar et al., 2007). A temporary decrease in the expression of MMP9 (matrix metalloproteinase 9) and endostatin was observed, but at longer intervals after PDT an increase in the expression of these proteins was observed, compared to neovascular membranes not treated with PDT. The increase in MMP9 was speculated to be due to the recurrence of CNV, as it is involved in the breakdown of extracellular matrix. On the other hand, endostatin, also increased in samples analysed at a long interval after PDT, is an endogenous inhibitor of angiogenesis (Tatar et al., 2007).

In conclusion, PDT is a safe and effective option for treating a variety of choroidal vascular diseases (Van Rijssen et al., 2019b). The fact that PDT appears to selectively target abnormally functioning CECs is a major advantage over thermal laser treatment, which can damage the full thickness of the retina, RPE, and choroid (Schmidt-Erfurth and Hasan, 2000). Further studies regarding the mechanisms by which photosensitising compounds are beneficial for treating choroidal vascular diseases may provide important new insights into this treatment modality, as well as the underlying pathophysiology. In addition, clinical studies designed to optimise the dose of verteporfin and the laser settings may further improve the efficacy of PDT for treating choroidal vascular diseases.

4.12.2. Anti-vascular endothelial growth factor treatment and choroidal side effects

The introduction of intravitreal anti-VEGF therapy has revolutionised the treatment of chorioretinal diseases associated with CNV. This treatment involves intravitreal injections of monoclonal antibodies that bind VEGF, thereby preventing the downstream activation of VEGF receptors, a key pathway in the development of CNV (Mettu et al., 2020). However, it should be noted that VEGF signalling is also essential for maintaining the choriocapillaris and its fenestrations, and compromised VEGF signalling between the RPE and the choriocapillaris causes the de-differentiation and atrophy of CECs (Blaauwgeers et al., 1999; Marneros et al., 2005). Thus, anti-VEGF therapy could – at least theoretically – have an adverse effect on the choroid, particularly in the context of diseases such as AMD in which the trophic relationship between the RPE and the choriocapillaris is already compromised (Rozing et al., 2020). This is especially relevant when the anti-VEGF injections are continued for a relatively long period of time, as this may affect the physiological function of the choriocapillaris, ultimately leading to atrophy of the choroid and/or RPE (Young et al., 2014). Although several studies have found a statistically significant correlation between the number of anti-VEGF injections and the occurrence of RPE and choroidal atrophy, whether a causal relationship exists is unclear, given that RPE and choroidal atrophy is a characteristic feature of the disease progression in AMD (Branchini et al., 2013a; Lois et al., 2013; Young et al., 2014). Moreover, even in patients with pre-existing GA, anti-VEGF therapy can improve visual outcome without affecting the natural course of the macular atrophy (Casalino et al., 2021; Sadda et al., 2018; Spooner et al., 2020). Importantly, some groups have suggested that intravitreal anti-VEGF injections may be associated with a higher incidence of myocardial infarction, stroke, and/or mortality, although other groups have found no such association (Yashkin et al., 2016). Nevertheless, while anti-VEGF therapy is clearly beneficial with respect to improving visual outcome in a number of chorioretinal neovascular diseases (Mettu et al., 2020), future studies may focus on selectively targeting anti-VEGF therapy to the neovascular tissue, thus reducing the risk of side effects and – ideally – increasing treatment efficacy.

4.12.3. Autologous retinal pigment epithelium/choroid patch surgery

Advanced atrophic AMD causes severe vision loss due to degeneration of the RPE and the underlying choroid. The idea of autologous transplantation of the RPE/choroid complex was first introduced three decades ago by Peyman and colleagues, although the benefits with respect to visual improvement are limited (Peyman et al., 1991). In brief, this technique involves the removal of an RPE/choroid patch from a peripheral region of the patient's eye; this patch is then transplanted to an area of GA or an area in which the CNV has been surgically removed (Parolini et al., 2020; Van Meurs et al., 2004). A recent review of 48 patients who underwent RPE/choroid patch surgery for neovascular AMD found that best-corrected visual acuity improved slightly – albeit significantly – from 20/252 prior to surgery to 20/187 at the final follow-up visit (ranging from 2 to 9 years following surgery). However, postoperative complications were relatively common and significant and included retinal detachment, macular atrophy, and subretinal haemorrhage in 11.4%, 7%, and 4.5% of the patients, respectively (Parolini et al., 2020). Moreover, a previous study by Van Meurs and colleagues found a high rate of proliferative vitreoretinopathy and no improvement in visual outcome (Van Meurs et al., 2004).

5. Future directions and therapeutic perspectives

5.1. Stem cell–based approaches for treating chorioretinal atrophy

Stem cell–based technologies represent a promising approach for regenerating the RPE and choroidal tissue in patients with degenerative chorioretinal disease. Several clinical trials have tested the use of stem cell suspension transplants in patients with advanced AMD by injecting multipotent ocular progenitor cells using a suprachoroidal surgical approach; however, this approach has not yet been successful, and its failure is commonly attributed to the fact that the transplanted cells may not fully differentiate and integrate into the diseased atrophic extracellular matrix (Singh et al., 2020). Moreover, injected cell suspensions may fail to form a monolayer. Therefore, a more promising strategy may be based on the use of patches of precultured stem cell–derived RPE embedded in a scaffold, which is then surgically implanted into the area of GA (García Delgado et al., 2019; Singh et al., 2020). Both phase 1 and phase 2 clinical trials suggest that this approach is safe, and studies are currently examining whether this treatment may provide long-term improvement of visual function in patients with advanced AMD (Singh et al., 2020). Notwithstanding, these promising results on autologous stem cell–based therapies may not be ideal, as the transplanted cells contain the same genetic makeup and risk profile. On the other hand, with respect to AMD, the long ageing process and debris accumulation is presumably required for the disease to manifest; thus, autologous stem cell–based therapies may still be a viable option, depending on the

underlying genetic risk factors. With respect to retinal dystrophies, the underlying genetic defects can be overcome by repairing the genetic defect *ex vivo* before implantation.

Another challenge associated with patch transplantation is that a multilayer transplant that includes the photoreceptors, RPE, and choroid – rather than only the RPE – may be required in order to achieve clinical improvement, given that several anatomical layers are affected. This poses a major challenge, as all tissue layers would need to integrate with the existing tissue and rapidly receive sufficient perfusion in order to avoid becoming ischaemic. Previously, Chirco et al. reported an innovative technique in which the human choroidal tissue was decellularised by removing the cellular material, thus leaving a scaffold for the stem cell–derived cells to attach (Chirco et al., 2017b). Interestingly, the authors found that primary CECs were able to integrate into the scaffolds; thus, in theory it might be possible to culture a patch of RPE/choroid tissue, which can then be implanted into an area of either GA or excised CNV.

In addition to stem cell–derived RPE, culturing CEC-like stem cell–derived ECs is a promising new approach (Kaye et al., 2020; Mulfaul et al., 2020). However, the extent to which these cells would be able to integrate into the existing tissue, resulting in meaningful functional improvement, remains an open question. To be successful, the stem cell-derived RPE would need to integrate into the viable RPE tissue and form a stable monolayer; while on the other side of the membrane, the stem cell-derived ECs would need to connect to the choroidal circulation. Interestingly, postoperative reperfusion of autologous transplanted RPE/choroid patches has been reported, indicating that integration of the new vasculature may be possible (Parolini et al., 2020). Lastly, perhaps the largest challenge associated with multilayer transplantation is that the photoreceptor axons will need to integrate into the existing neuroretina in order to become a fully restored functional unit (Lin et al., 2020).

5.2. Gene therapy–based strategies for treating chorioretinal disease

Gene therapy is rapidly emerging in the field of regenerative medicine. The eye is particularly amenable to this technique, given that it is an easily accessible, relatively small organ suitable for intravitreal and suprachoroidal drug delivery. Thus, the eye is currently at the forefront of retinal and subretinal gene therapy–based strategies for treating monogenic forms of retinal dystrophy; indeed, voretigene neparvovec (sold under the brand name Luxturna®) was the first *in vivo* gene therapy in humans approved by the US Food and Drug Administration. A range of other retinal gene therapy trials are currently in phase 1, phase 2, and phase 3 (Dias et al., 2018). Interestingly, some research groups are also focusing on using gene therapy to prevent CNV in patients who have not yet begun to show signs of neovascularisation (Askou et al., 2019; Grishanin et al., 2019; Reid et al., 2018). This approach uses adeno-associated virus constructs carrying genetic material that encodes a protein of interest, for example the VEGF-binding protein aflibercept (Grishanin et al., 2019). The goal of this therapy is to replace the need for repeated intravitreal injections of anti-VEGF compounds by modifying the ocular cells to constitutively express aflibercept. Moreover, these viral constructs are designed to integrate only in a specific cell type, thus reducing potential side effects (Askou et al., 2019). Experiments using these viral vectors in primate and rodent models of laser-induced CNV have shown that virus-based expression of anti-VEGF protein in ocular cells inhibits CNV formation (Grishanin et al., 2019; Reid et al., 2018). A phase 1 clinical trial involving 8 patients with advanced neovascular AMD evaluated the safety profile of a subretinal injection of a virus-based vector that drives the expression of anti-VEGF molecules, finding that the injection was well-tolerated and did not lead to either ocular or systemic side effects after a follow-up period of 36 months (Constable et al., 2017). An exploratory analysis indicated that there was no progression of AMD during follow-up (Constable et al., 2017). In summary, although more data are needed regarding the efficacy and safety profile (Guimaraes et al., 2021), gene therapy–based strategies are highly promising, with the potential to substantially improve the treatment of CNV and advance the use of gene therapy in humans for treating a variety of diseases (Bucher et al., 2020; Grishanin et al., 2019).

5.3. The rationale behind understanding a disease at the cell-specific level

The choroid plays an essential role in a wide range of ocular processes, ranging from providing vascular support to the outer retina to regulating ocular immunity. Advances in our understanding the molecular basis of chorioretinal diseases have been highly valuable with respect to developing treatments for chorioretinal diseases such as CNV, which has a high disease burden and drastically reduces both visual acuity and quality of life. However, numerous challenges remain in order to fully unravel the complex nature of many other choroidal diseases, including the pachychoroid diseases and uveitis, which are diseases for which an effective treatment is often not available.

In this review, we highlighted the variable nature of CEC subpopulations, emphasising the highly specialised CECs in the choriocapillaris. We show that CECs are similar in many respects to ECs in other organs, but also have unique functions, gene expression profiles, and a close relationship with the RPE. For example, the major differences between CECs and RECs with respect to their cellular niche, phenotype, and functional response to pathological stimuli, underscore the importance of studying chorioretinal diseases in a site-specific and cell type–specific manner. Ultimately, this approach will likely facilitate the development of targeted therapies for diseases in which either CECs or RECs play a specific role. Therefore, obtaining a thorough characterisation of the CEC phenotype and the interaction between these cells and the RPE is essential, particularly for developing protocols for differentiating stem cells into choroidal-like ECs. In this respect, spatially resolved transcriptomics is an interesting development in the field of transcriptomics that may help to thoroughly characterise CEC subpopulations (Marx, 2021). Using this technique, the location of specific mRNA transcripts is mapped to the tissue structure, yielding information that is not possible to obtain using bulk RNA sequencing or scRNA-seq. This technique may also reveal the precise location of gene expression in specific CEC subpopulations, as well as provide information regarding polarised gene expression specifically on the side of the choriocapillaris that faces the RPE.

In this manuscript, we also summarised the current state of knowledge regarding the structural and molecular basis of the CEC, CEC subpopulations, and the advantages and disadvantages of using primary CECs derived from human post-mortem donor eyes. Limitations with using primary CECs include the scarcity of donor eyes, lack of the donor's medical history due to privacy regulations, and the potential de-differentiation of primary CECs when maintained in culture. Interestingly, new innovative technologies may enable researchers to circumvent some of these limitations, for example by using induced pluripotent stem cells, enabling patient-specific research with readily accessible sources of donor material such as peripheral blood. However, further studies are needed in order to optimise the use of stem cell–based technologies, particularly with respect to differentiating the resulting cell types into specific phenotypes such as the choroidal endothelium. As discussed in this review, the use of primary CECs, together with *in vivo* data and transcriptomics studies, may serve as a benchmark for the development of such protocols. These future studies will ultimately improve our understanding of the broad spectrum of chorioretinal diseases and facilitate new approaches to targeting tissue-specific pathological processes, thereby minimising the risk of side effects while maximising therapeutic efficacy and patient outcome.

Author contributions

J. Brinks: writing, conceptualisation, methodology, investigation,

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