Current understanding of the molecular and cellular pathology of diabetic retinopathy

David A. Antonetti[1]✉, *Paolo S. Silva*[2,3] *and Alan W. Stitt*[4]

Abstract | Diabetes mellitus has profound effects on multiple organ systems; however, the loss of vision caused by diabetic retinopathy might be one of the most impactful in a patient's life. The retina is a highly metabolically active tissue that requires a complex interaction of cells, spanning light sensing photoreceptors to neurons that transfer the electrochemical signal to the brain with support by glia and vascular tissue. Neuronal function depends on a complex inter-dependency of retinal cells that includes the formation of a blood–retinal barrier. This dynamic system is negatively affected by diabetes mellitus, which alters normal cell–cell interactions and leads to profound vascular abnormalities, loss of the blood–retinal barrier and impaired neuronal function. Understanding the normal cell signalling interactions and how they are altered by diabetes mellitus has already led to novel therapies that have improved visual outcomes in many patients. Research highlighted in this Review has led to a new understanding of retinal pathophysiology during diabetes mellitus and has uncovered potential new therapeutic avenues to treat this debilitating disease.

Diabetic retinopathy is one of the most common complications of diabetes mellitus (both type 1 and type 2) and remains a leading cause of loss of vision and blindness globally[1]. Diabetes mellitus affects many components of the eye, but the primary vision-threatening pathology occurs in the retina. Research described in detail in this Review reveals alterations to both neuronal and vascular cells of the retina in diabetic retinopathy. While a complete understanding of disease aetiology is needed, breakthroughs over the past few years in the treatment of diabetic retinopathy that focus on targeting vascular endothelial growth factor A (VEGFA) now provide effective treatment options in the clinic. However, anti-VEGF therapy is only effective in the late stages of diabetic retinopathy and requires regular intravitreous injections, and not all patients respond optimally[2,3]. The development of new therapeutic approaches for this disease is required for a range of reasons, including the increasing rate of diabetes mellitus globally and the need to prevent progression from the early stages of diabetic retinopathy, and because some patients do not respond to anti-VEGF therapy and anti-VEGF therapy is inappropriate in patients with ischaemic retinopathy.

This Review focuses on the current understanding of the molecular and cellular pathology of diabetic retinopathy with a primary focus on the cellular signalling between the neuronal and vascular retina that promotes formation of the inner blood–retinal barrier (BRB) of the retinal vasculature as an important point of intervention. Novel retinal biomarkers of changes in visual function identified by clinical imaging modalities, such as optical coherence tomography–angiography (OCT–A) and ultrawide field retinal imaging, are discussed. New therapies under investigation that might complement current laser treatment and anti-VEGF therapy are presented along with their mechanisms of action. Finally, the translational potential of novel approaches, such as the development of patient-derived cells and retinal organoids, for experimental investigation and the potential of tissue restoration are considered.

Classification of disease severity

Studies on the pathogenesis and treatment of diabetic retinal disease rely on the use of accurate methods to classify diabetic retinopathy that are reflective of its natural history. Diabetic retinopathy has been well described using the modified Airlie House classification scale as applied in the Early Treatment Diabetic Retinopathy Study (ETDRS)[4] and detailed in a 2017 position statement from the American Diabetes Association[5]. Altered retinal blood flow and vascular permeability[6], basement membrane thickening[7], loss of pericytes and the formation of acellular capillaries[8] contribute to clinically visible non-proliferative diabetic retinopathy lesions, such

[1]*Department of Ophthalmology and Visual Sciences, Department of Molecular and Integrative Physiology, Kellogg Eye Center, University of Michigan, Ann Arbor, MI, USA.*

[2]*Department of Ophthalmology, Harvard Medical School, Boston, MA, USA.*

[3]*Beetham Eye Institute, Joslin Diabetes Center, Boston, MA, USA.*

[4]*Centre for Experimental Medicine, Queen's University, Belfast, UK.*

✉*e-mail: dantonet@med.umich.edu*

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Key points

- Diabetic retinopathy is a leading cause of blindness that disrupts the normal interaction of the retinal neural and vascular components leading to vascular permeability, neovascularization and loss of proper neural function.
- Current effective therapeutic approaches target vascular endothelial growth factor, while a host of new therapies targeting vascular endothelial and pericyte signalling and inflammatory cytokines are being tested for diabetic retinopathy.
- Stem cell therapy for vascular regeneration holds potential for restorative therapeutic approaches in diabetic retinopathy.
- Understanding the neuronal and glial changes that drive loss of vision is rapidly emerging, and targeted approaches to directly test the relationship between the neurovascular unit and alteration in diabetic retinopathy are needed.

as microaneurysms, venous beading and intraretinal microvascular abnormalities. As ischaemia increases, patients might develop proliferative diabetic retinopathy (PDR), which is associated with a substantial risk of visual loss due to neovascular complications, such as vitreous haemorrhaging or retinal detachment as blood vessels grow into the vitreous[9] (FIG. 1).

Also linked to ischaemia, diabetic macular oedema (DME) develops as a result of increased permeability of retinal capillaries and from microaneurysms, leading to the accumulation of extracellular fluid and thickening of the normally compact macular tissue. As the severity of diabetic retinopathy increases, the risk of developing DME similarly increases[10]. Loss of vision from DME is correlated with the location and extent of retinal thickening on OCT scans, and also correlates with retinal blood vessel permeability and perfusion as assessed by fluorescein angiography[11,12]. Data from the ETDRS evaluating eyes with DME have shown that thickening involving the centre of the macula, termed centre-involved DME, is associated with a nearly tenfold greater risk of developing moderate loss of vision than thickening without centre involvement[13].

The retinal pigment epithelium (RPE) and the underlying choroid are also compromised during diabetes mellitus. The RPE provides a barrier that controls the exchange of metabolites from the rods and cones with the underlying choroidal vessels. Imaging focused on the RPE has revealed evidence of permeability of the RPE in patients with DME[14], which might be related to breakdown of the outer BRB[15] and activation of inflammation-linked pathways that drive pathology in the photoreceptors[16]. The RPE also shows impaired regulation of fluid outflow during diabetes mellitus that might be linked to dysfunction of the normal activity of Na–K ATPase pumps and aquaporin channels[17]. These outer retina changes occur concomitantly with what has been termed diabetic choroidopathy[18], which manifests as progressive non-perfusion of the choriocapillaris.

ETDRS severity levels have been used to guide clinical practice recommendations for patient follow-up and treatment. In the ETDRS, different levels of severity were described — 13 at the eye level and 26 at the patient level — and have been used extensively in research and clinical trials. The American Academy of Ophthalmology formed a consensus panel and created a simplified classification called the International Clinical Diabetic Retinopathy and DME Disease Severity Scale[19]. This scale simplified descriptions of the categories of diabetic retinopathy; however, it is not a replacement for ETDRS levels of diabetic retinopathy in large-scale clinical trials or studies in which precise diabetic retinopathy classification is necessary. Despite advances in retinal imaging, the current diabetic retinopathy classification scales have not incorporated new approaches, such as ultrawide field imaging for the retinal periphery or OCT for macular oedema or neuroretinal changes. The current grading scales are still largely based on clinically visible retinal microvascular lesions and do not include neurodegenerative changes that might occur early and distinct from vascular changes[20]. The evolution of diabetic retinopathy classifications is inevitable and should include measures that will enable more accurate prognoses to be given and improve prediction of patient outcomes. But until then, the ETDRS severity levels should remain the standard for determining disease severity in both clinical and research settings.

While the pathological changes that occur during diabetic retinopathy are often considered progression, it remains possible that environmental or genetic factors promote a specific pathology. Epidemiological studies have quantified the risks of developing diabetic retinopathy or DME and have shown significant differences between patients with type 1 diabetes mellitus (T1DM) or type 2 diabetes mellitus (T2DM)[21]. Both degree of glycaemic control and diabetes mellitus duration are notable risk factors in the development to diabetic retinopathy and DME[21]. However, the 25-year rate of developing some degree of diabetic retinopathy is over 95% in patients with T1DM and only 60% in those with T2DM[10]. Furthermore, the 10-year rate of developing DME is 20% in patients with T1DM, 25% in patients with T2DM taking insulin and 14% in those with T2DM not taking insulin[22]. In general, patients with T1DM tend to develop diabetic retinopathy and PDR while patients with T2DM taking insulin are more at risk of developing DME. Future research to improve our understanding of what causes patients to present with DME, PDR or aspects of inflammation and whether these represent progression or separate pathologies is greatly needed.

Multiple large-scale clinical studies have shown that good glycaemic control is essential to prevent progression of diabetic complications and diabetic retinopathy[23]. A meta-analysis of multiple population-based studies of diabetic retinopathy revealed that HbA_{1c} levels, blood pressure and serum levels of total cholesterol are associated with the incidence and progression of retinopathy but explain diabetic retinopathy progression and PDR development in only 9% and 10% of affected patients, respectively[24]. Therefore, additional factors probably contribute to disease pathology. For example, a study published in 2018 has suggested that very long-chain (VLC) fatty acids that incorporate into VLC ceramides affect endothelial barrier properties[25]. Diabetes mellitus leads to loss of elongases, including ELOV4, and alters the retinal lipid profile[26]. Depletion of ELOV4 can reduce endothelial barrier properties while overexpression promotes barrier properties and reduces the effects of diabetes mellitus on permeability of the endothelial barrier in vivo[25].

Vitreous
The clear gel filling the space between the retina and the lens.

Choroid
The vascular bed adjacent to the retinal pigmented epithelium (RPE) supporting the rods and cones of the outer retina that are on the opposing side of the RPE.

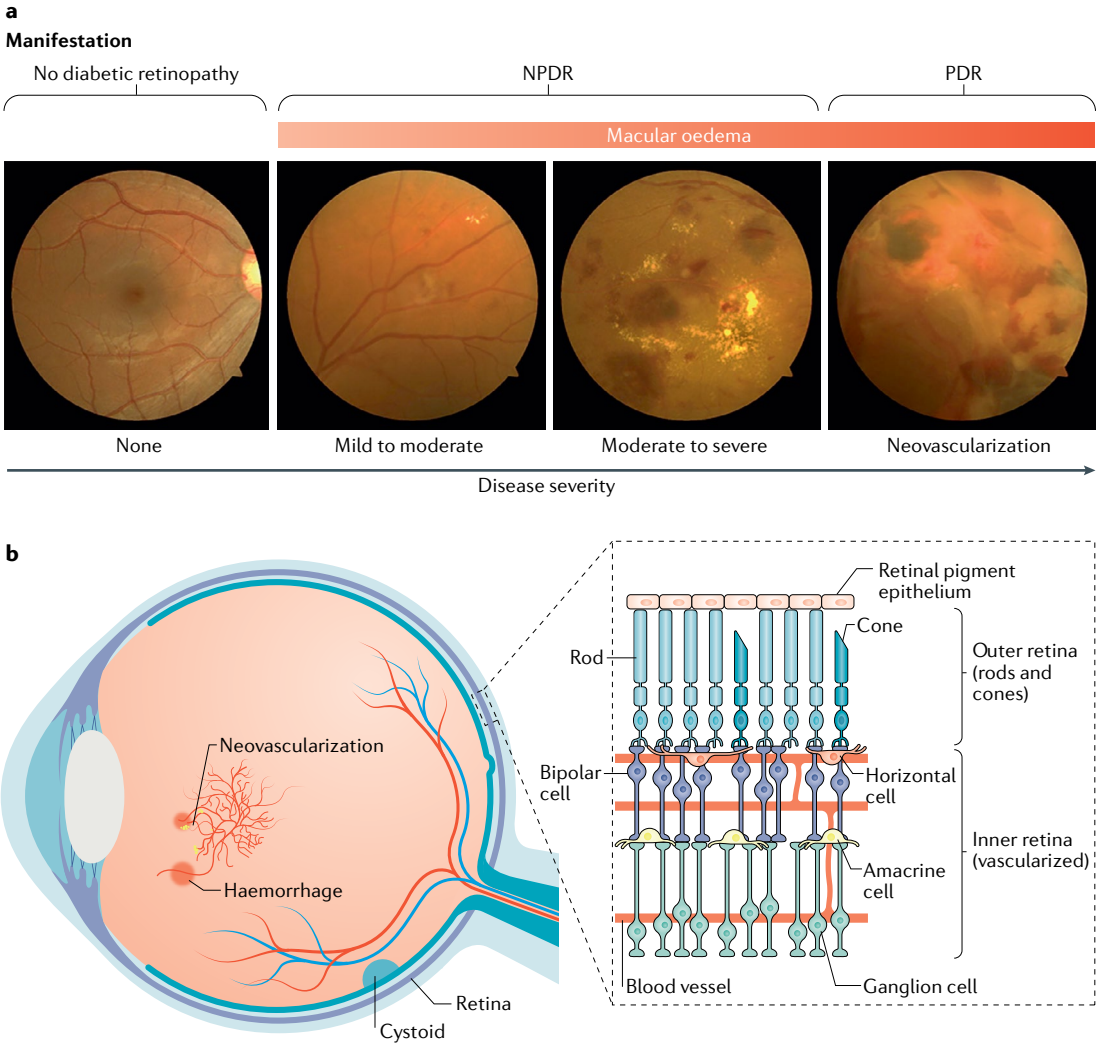
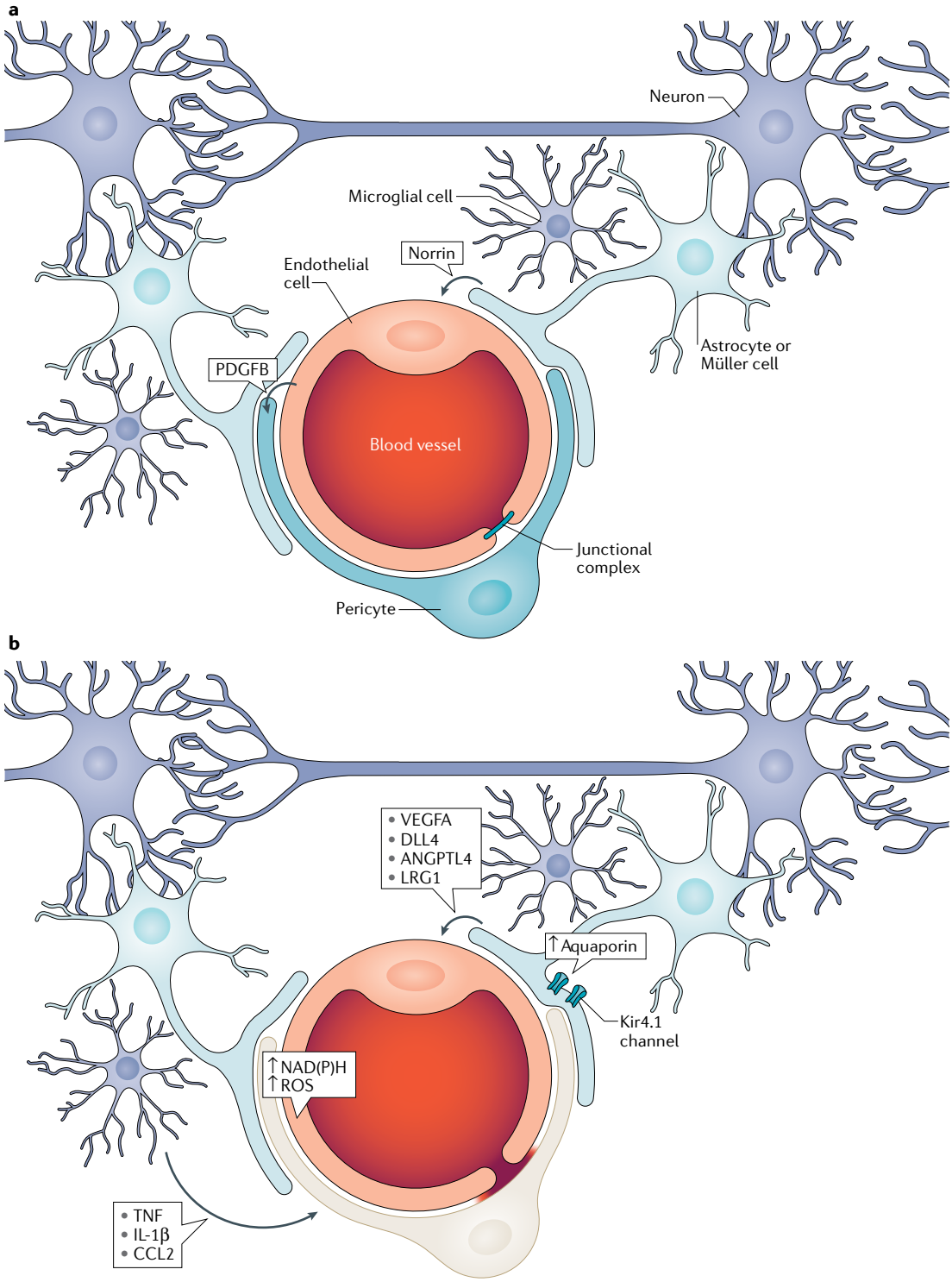


Fig. 1 | **Diabetic retinopathy manifests with multiple pathologies. a** | Patients with diabetes mellitus might have no readily observable alterations to the retina on fundus photography. Alternatively, microvascular abnormalities, haemorrhages, microaneurysms and venous beading reveal evidence of a disease process that might range from mild to severe and can occur in patients with non-proliferative diabetic retinopathy (NPDR). Patients with proliferative diabetic retinopathy (PDR) have neovascularization in the retina that might lead to retinal detachment. Diabetic macular oedema (DME) can occur in both NPDR and PDR. **b** | Schematic diagram of a cross section of the eye. Vessel leakage, neovascularization and cystoid formation due to DME are indicated. The cross section of the retina shows the organization of ganglion cells and bipolar cells in the inner retina versus rods and cones in the outer retina. Blood vessels in the inner retina make the inner blood–retinal barrier and the retinal pigment epithelium forms the outer blood–retinal barrier.

Markers of disease activity

The ETDRS standardized grading scale is based on 30° retinal images from seven standard defined retinal fields and characterizes the extent of retinal lesions located in the posterior pole. However, ultrawide field imaging has demonstrated that retinal lesions can appear or develop outside the ETDRS fields[27–32]. Predominantly peripheral lesions (PPL) are severe diabetic retinopathy lesions with a greater extent outside versus inside standard ETDRS fields. Eyes with PPL have been shown to have increased retinal non-perfusion compared with eyes without PPL[33]. The cause of PPL is currently unknown but might involve loss of autoregulation in retinal arterioles or microvascular degeneration causing capillary non-perfusion and retinal ischaemia[33]. PPL are present in ~50% of eyes with diabetic retinopathy and identify a more severe level of diabetic retinopathy in ~10% of eyes compared with the use of standard ETDRS field imaging[27,29]. Moreover, if PPL is present in an eye at baseline, the risks of the worsening diabetic retinopathy and the development of advanced, sight-threatening retinopathy over the subsequent 4 years are increased 3.2-fold and 4.7-fold, respectively[29]. These findings suggest that PPL might become a robust marker of diabetic retinopathy progression. Another marker might be the presence of vitreous hyper-reflective foci on OCT scans. In a study of 97 patients, the numbers of foci, presumed to represent inflammatory cells, were increased in patients with DME compared with control individuals or patients with diabetes mellitus but without DME[34]. Future longitudinal analyses could reveal whether these scans provide true biomarkers for disease progression.

Posterior pole
The posterior segment of the human retina visible during ophthalmoscopy made up of the optic disc (optic nerve head) and macula, or avascular central area including the fovea, or thinned retina with high cone density responsible for high visual acuity.



OCT–A allows the unprecedented ability to assess retinal vascular detail and might reveal important changes not previously observed by traditional methods available to ophthalmologists. OCT–A allows the noninvasive mapping of retinal vessels and blood flow, which enables visualization of the retina and choroidal vasculature[35,36]. Both the superficial vessels and deep retinal vascular layers can be readily differentiated with OCT–A, which enables the identification of the specific retinal capillary layers responsible for the underlying disease[37]. OCT–A gives a deeper understanding of how capillaries change over the course of diabetes mellitus and in response to treatments for diabetic eye disease, which might provide novel insights into approaches for treating diabetic retinopathy. In addition, interest in the use of metabolomics to identify biomarkers has increased in the past few years. Metabolomic analysis of vitreous and serum samples have identified dysregulation in pathways such as the pentose phosphate pathway, arginine to proline pathway, polyol pathway and ascorbic

◀ Fig. 2 | **The neurovascular unit and cytokine signalling in diabetic retinopathy.** **a** | Proper retinal functions require an intimate relationship between the retinal blood vessels in the inner retina and neurons, glia (astrocytes and Müller cells) and pericytes. Glia provide the Norrin signalling that is required for blood–retinal barrier formation. Endothelial cells recruit pericytes by platelet-derived growth factor subunit B (PDGFB) signalling and pericytes promote barrier properties of the endothelium by an unknown mechanism. **b** | In diabetic retinopathy, glia have increased levels of aquaporin and Kir4.1 channels, which contributes to swelling, and now produce vasoactive substances such as vascular endothelial growth factor A (VEGFA) and the associated Delta-like protein 4 (DLL4), angiopoietin-related protein 4 (ANGPTL4) and leucine-rich α2-glycoprotein 1 (LRG1) that promote permeability, angiogenesis or both. Loss of pericytes leads to hyper-responsiveness of endothelial cells to VEGF signalling. Furthermore, inflammatory cytokines, such as tumour necrosis factor (TNF), IL-1β and CC-chemokine ligand 2 (CCL2) among many others, are produced by microglia and other retinal cells as well as adherent inflammatory cells. In addition, hyperglycaemia induces direct endothelial dysfunction through a change in redox state (NAD(P)H and reactive oxygen species (ROS)). The retinal pigment epithelium also undergoes dysfunction with increased cytokine production (not shown). Collectively, these changes disrupt the neurovascular unit and alter normal retinal function.

acidic pathway[38,39]. However, further research is necessary to establish causative and longitudinal associations with diabetic retinopathy.

Retinal neural–vascular interaction

The retinal neurovascular unit refers to the interdependency between the vascular endothelial cells and pericytes, glia, neurons and retina-resident immune cells. While the vasculature provides the required nutritional support for the neural tissue, the neural and glial cells, along with pericytes, signal to the vascular endothelial cells of the BRB, which provides tight control of the neural environment (FIG. 2a). Loss of this BRB in diabetic retinopathy contributes to increased retinal vascular permeability and loss of vision[40,41]. Similarly, neurovascular coupling, or the control of blood flow by the retinal neural tissue, is also altered in the retina in animal models[42] and patients[43]. Diabetes mellitus also affects Müller glia, leading to mis-localized active transport mechanisms of inwardly rectifying channels at the capillary–Müller glial interface that contributes to swelling of Müller glia in the retina of patients with diabetes mellitus[44]. Changes to Kir4.1 and aquaporins on Müller glia are consistent findings in diabetic animal models[45] and these changes can be rectified by blocking the accumulation of lipoxidation end products[46]. Furthermore, the Müller glial response in diabetes mellitus might amplify inflammation by activating microglia through $P2X_7$ purinergic receptors, which leads to neuroinflammation and vascular damage, including leakage[47]. Indeed, microglial activation has a considerable effect on the retinal neurovascular unit and neuroinflammation-driven breakdown in the inner BRB in diabetic retinopathy[48].

Vascular endothelial changes. Vascular endothelial changes have so far been the only successful therapeutic target for diabetic retinopathy. Laser photocoagulation has long provided an effective means of controlling proliferation and oedema in many patients[49]. However, success in treating diabetic retinopathy has evolved in the clinic by targeting factors that drive microvascular abnormalities. Vascular changes in diabetic retinopathy have been attributed, in part, to elevated levels of VEGFA, which signals to retinal endothelial cells and alters blood vessel permeability and promotes neovascularization[50,51]. Multiple, multicentre clinical trials have demonstrated that targeting VEGFA with antibodies or trap (a modified soluble receptor blocking VEGFA action) can effectively reduce DME, prevent further loss of vision and, in some patients, improve vision[52–54]. Among patients with PDR, anti-VEGFA therapy prevents or reverses neovascularization, with 43% of treated patients demonstrating resolution of neovascularization after 2 years and only 27% worsening since the previous visit[55]. However, for PDR and DME[56], clinical studies have revealed that approximately half of patients receive benefit while the others remain unresponsive to anti-VEGFA therapy, which suggests that other factors might drive disease pathology in diabetic retinopathy.

Interestingly, a study published in 2020 showed significant correlations between inflammatory cytokines and VEGF and, in particular, that the inner BRB is regulated by localization of the tight junction protein claudin 5 via activation of rho-associated coiled-coil-containing protein kinase (ROCK). Administration of ripasudil, a selective ROCK inhibitor, attenuated retinal inflammation and claudin 5 redistribution. When combined with an anti-VEGF agent, this ROCK inhibitor was synergistic in suppressing cytokine upregulation, monocyte and macrophage infiltration, macrophage and microglia activation and claudin 5 redistribution, an effect that was demonstrated preclinically but also in patients resistant to anti-VEGF therapies alone[57]. These data indicate that inflammation might be a key mechanism in the responsiveness to anti-VEGF therapy in DME.

Associated with VEGFA, Notch signalling might be altered in diabetic retinopathy. In vascular angiogenesis during retinal development, the VEGFA signal stimulates an endothelial cell with the highest VEGFR2 response to become a tip cell that migrates towards the VEGF source and signals to neighbouring cells to become the proliferating stalk cells of the angiogenic sprout. This cell to cell communication utilizes the Notch signalling pathway with Delta-like protein 4 (DLL4) and Notch receptor[58]. The levels of both DLL4 and the typical Notch antagonist Jagged 1 have been found to be increased in diabetic mouse models and in human endothelial cells in a glucose-dependent manner[59]. Intra-ocular injection of either ligand induced a modest increase in retinal permeability that was dependent on Notch, as conditional gene deletion of Notch prevented the permeability response. Furthermore, administration of a Notch–trap reduced retinal permeability in a diabetic animal model[59].

Pericyte–endothelial interactions. Diabetes mellitus alters the normal pericyte–endothelial interaction in the retina. Studies using targeted genetic deletion of pericytes have revealed that pericyte coverage of retinal vessels is required for proper formation of the BRB[60]. Platelet-derived growth factor subunit B (PDGFB) signalling to pericytes controls vessel stabilization as deletion of the PDGFB retention signal, which localizes the growth factor to the pericellular space, also causes deterioration of retinal vessels[61]. In addition, administration

of a PDGF receptor-β blocking antibody induces retinal haemorrhage and permeability in a FOXO1-dependent manner[62]. Interestingly, this study also revealed that loss of retinal pericytes in adult mice using inducible, targeted diphtheria toxin expression does not confer leaky retinal vessels as observed in other organs such as lung and skin. Instead, loss of pericytes makes the retinal vasculature highly susceptible to VEGFA signalling, with a dramatic increase in haemorrhage and vascular permeability to dextran[62]. Pericytes control endothelial expression of angiopoietin 2 and VEGFR2 through transcription factor FOXO1, with loss of pericytes dramatically promoting VEGF signalling[62]. This heightened response of retinal vascular endothelial cells to VEGFA after pericyte loss has stark implications for the well-established loss of retinal pericytes in diabetes mellitus. In addition, chronic hyperglycaemia reduces PDGF receptor tyrosine kinase signalling, which promotes pericyte apoptosis and diabetic vasculopathy through activation of protein kinase Cδ (PKCδ) and increased expression of the tyrosine phosphatase Src homology 2 domain-containing phosphatase 1 (SHP1)[63,64]. Together, these studies provided a mechanistic link explaining the effect of diabetes mellitus on pericyte loss through reduced PDGFR signalling, with loss of pericytes promoting increased VEGFA signalling, permeability and ultimately loss of vessels.

Norrin signalling. Glial cells provide WNT signalling to retinal vascular endothelial cells that are required for formation of the BRB and replicating this signalling might provide alternative therapies for diabetic retinopathy. The cytokine Norrin is not a WNT molecule but like WNT, Norrin signals through the Frizzled 4 (FZD4) receptor complex[65]. Gene deletion studies of Norrin, the receptor FZD4 or the co-receptors low-density lipoprotein receptor-related protein 5/6 (LRP5/6) or tetraspanin 12 (TSPAN12) have revealed that this signalling complex is required for both retinal angiogenesis[66] and BRB formation[67,68]. Importantly, both *Ndp* and *Fzd4*-knockout mice show high retinal vascular permeability that correlates with reduced endothelial cell border immunostaining of the tight junction protein claudin 5, and increased expression of the transcytosis marker and plasmalemma vesicle associated protein[67,68]. Furthermore, this phenotype can be reversed by the expression of a stabilized form of β-catenin, which reveals the role of the canonical WNT pathway[67].

Studies have begun to explore whether Norrin signalling could be used to restore vascular function in animal models of diabetic retinopathy. Norrin treatment might reduce the avascular area and inhibit neovascularization in oxygen-induced retinopathy models[69] and transgenic expression of Norrin might reduce vaso-obliteration and promote vascular growth[70,71]. Norrin can reverse VEGF-induced permeability in retinal endothelial cell culture and in rat retina when co-injected intravitreally with VEGF; in addition, intravitreal injection of Norrin reverses retinal permeability in rats with diabetes mellitus[72]. Interestingly, these studies have revealed that VEGF actually promotes Norrin signalling by increasing the membrane content of the FZD4 co-receptor TSPAN12. The addition of Norrin after VEGF promotes barrier induction, which suggests a potential novel approach to vascular restoration. It is important to ascertain whether Norrin expression, as well as that of other WNT signalling mediators, changes during diabetes mellitus to determine whether neuronal changes in WNT signalling alter BRB in patients with diabetes mellitus.

Inflammation. A variety of studies have suggested that cell signalling through inflammatory factors might contribute to diabetic retinopathy pathogenesis. Vitreous proteomic analyses have identified a host of altered inflammatory factors in the vitreous or aqueous humour at various stages of diabetic retinopathy[73,74], many of which are highlighted here. Gene deletion and cytokine capture studies in animal models have provided strong evidence for the role of tumour necrosis factor (TNF)[75,76] in diabetic retinopathy and evidence of leukostasis involving intercellular adhesion molecule 1 or its binding partner CD18 (REF.[77]). Studies of human vitreous fluid have found that levels of IL-1β and TNF are elevated in patients with PDR[78–80]. Levels of IL-6, IL-8 and CC-chemokine ligand 2 (CCL2) are also elevated in patients with DME and PDR[81]. Conversely, levels of anti-angiogenic mediators, such as pigment epithelium-derived factor, have been reported to be low in patients with diabetes mellitus and in patients with active PDR[82].

Studies have demonstrated that targeting inflammation by inhibiting atypical protein kinase C (aPKC) might control vascular permeability in the retina. The aPKC isoforms contribute to endothelial permeability from a variety of inflammatory factors and growth factors, including VEGF, and also contribute to NF-κB activation[83,84]. Reducing aPKC activation with a small-molecule inhibitor or conditional expression of a dominant-negative form of the kinase reduced endothelial permeability and recruitment of monocytes and granulocytes in animal models of retinal inflammation[85]. Beyond broad-spectrum, the use of anti-inflammatory approaches such as corticosteroids that are already in clinical use, targeting specific cytokines based on measurement of patient vitreous or aqueous cytokine profiles to improve therapeutic options remains an exciting possibility[86].

Given sufficient time, the development of diabetic retinopathy is nearly universal in patients with diabetes mellitus[10]; however, the development of PDR plateaus at 60%, even in patients who have had diabetes mellitus for more than 50 years[10,87]. Therefore, there might be protective mechanisms that delay or prevent the progression to PDR[88]. Proteomic analysis identified elevated concentrations of photoreceptor-secreted retinol binding protein 3 (RBP3) in the vitreous of patients resistant to advanced diabetic retinopathy, despite diabetes mellitus durations of over 50 years[89], which is consistent with previous findings that RBP3 is reduced in the general patient population with diabetic retinopathy[90]. Retinal cell-based assays and rodent models have demonstrated that RBP3 can prevent diabetes mellitus-induced vascular permeability and altered retinal function measured by electroretinography[89]. RBP3 might have a role in

protection against the progression of diabetic retinopathy by decreasing the expression and signalling of inflammatory cytokines and VEGF. Furthermore, RBP3 can reduce glucose uptake into Müller glia cells by binding and inhibiting glucose transporter 1, thereby mitigating the effects of chronic hyperglycaemia[89]. Further exploration of the normal physiological role of RBP3 in mediating glucose uptake is required; however, these studies have provided novel insights into retinal metabolism and potential therapeutic approaches to the treatment of diabetic retinopathy.

Novel pathogenic pathways

The kinin–kallikrein system. Studies have identified a range of alternative neurovascular signalling pathways that lead to leakage and/or neovascularization in addition to VEGF. Amongst the most promising targets is the kinin–kallikrein system. Carbonic anhydrase 1 (CA1) and activation of plasma kallikrein have been identified in the vitreous of patients with advanced diabetic retinopathy[91]. Subsequent studies established that plasma kallikrein cleavage of kininogen generates bradykinin, which acts through bradykinin receptors on the blood vessels to induce permeability. Inhibitors of plasma kallikrein can block or reduce retinal permeability in animal models of diabetes mellitus and in response to direct CA1 and plasma kallikrein injection[92], but VEGFA administration cannot, suggesting a distinct pathway of vessel permeability. Currently, plasma kallikrein inhibitors are being tested in clinical trials in patients with DME (NCT03466099).

Angiopoietin-like 4. Experimental studies have implicated angiopoietin-related protein 4 (ANGPTL4) in diabetic retinopathy. Elevated levels of ANGPTL4 were initially found in aqueous fluid from the anterior chamber of the eye of patients with DME and the level of ANGPTL4 correlated with the ability of the aqueous fluid to induce permeability in an endothelial cell culture assay[93]. ANGPTL4 is downstream of gene transcription regulated by hypoxia-inducible factor and can induce endothelial permeability[94]. Interestingly, ANGPTL4 binds to neuropilin and activates the small G protein RhoA[93]. Neuropilin is a co-receptor for VEGFR2; however, the ability of ANGPTL4 to induce permeability is independent of VEGFR2, as demonstrated in knockdown studies in cell culture[93]. A soluble form of neuropilin was able to block ANGPTL4 and VEGF-induced permeability in cell culture and mice[93]. It should be noted that there are a number of conflicting reports of the role of ANGPTL4 in permeability. For example, studies have revealed that ANGPTL4 can reduce permeability in a mouse stroke model and can specifically attenuate VEGF-induced vascular permeability in endothelial cells by inhibiting Src phosphorylation and activation[95]. Furthermore, ANGPTL4 can inhibit the expression of genes that encode pro-inflammatory proteins and promote the expression of genes that encode anti-inflammatory proteins in macrophages in cell culture and in a myocardial infarct animal model[96]. Clearly additional studies on the complex role of ANGPTL4 are needed. However, another neuropilin binding protein, semaphorin 3A (SEMA3A), also induces retinal permeability and conditional knockout of neuropilin prevents SEMA3A-induced permeability but not VEGF-induced permeability[97]. Targeting neuropilin might provide a novel option to treat diabetic retinopathy and might prevent induction of vascular permeability from multiple sources if no toxicity is associated with this therapy.

Leucine-rich α2-glycoprotein 1. Gene expression studies of pathological angiogenesis identified elevated expression of leucine-rich α2-glycoprotein 1 (LRG1), which promotes neovascularization. LRG1 modifies transforming growth factor-β (TGFβ) signalling by binding to the accessory receptor endoglin and promoting a pro-angiogenic signalling pathway[98]. *Lrg1*-knockout mice have a modest delay in retinal vascular development but pathological angiogenesis can be dramatically reduced in *Lrg1*-knockout mice and in animal models treated with an anti-LRG1 antibody[98]. Currently, a phase I/IIa clinical trial of a humanized monoclonal antibody to LRG1 called magacizumab (information on this antibody is available in a preprint) is underway and could potentially lead to an additional therapeutic option for PDR (UKRI)[99].

Direct effects of hyperglycaemia. Finally, the direct effect of hyperglycaemia on endothelial cells has been extensively studied. However, a study from 2018 provides an intriguing model of hyperglycaemia-regulated epigenetic control of oxidative stress in endothelial cells. Using small interfering RNA and pharmacological inhibition of DNA methylation and hydroxymethylation, the investigators provide evidence for hyperglycaemia increasing levels of 5-hydroxy methyl cytosine and levels of NF-κB-induced activation of RAS-related C3 botulinum toxin substrate 1 (RAC1)[100]. RAC1 is an essential component of NADPH oxidase 2 and promotes reactive oxygen species (ROS) production. NADPH oxidase 2 is activated early in hyperglycaemia-induced endothelial cell dysfunction and contributes to mitochondrial production of ROS. These findings provide a novel model to link hyperglycaemia-induced epigenetic gene regulation to ROS production and mitochondrial dysfunction[101].

Altered neuronal function

While most clinical focus has been on vascular pathology during diabetic retinopathy, there is now wide recognition that the impact of diabetes mellitus more broadly affects the cells of the retina. In addition to the clinically visible vascular defects, evidence suggests that changes occur in the neural retina as well. For example, apoptosis of non-vascular cells has been consistently identified in animal models of diabetic retinopathy[102]. Longitudinal studies of patients with diabetes mellitus have suggested that retinal degeneration occurs, as observed by thinning of the nerve fibre and ganglion cell layer (termed retinal neurodegeneration), without evidence of vascular pathology[103]. A number of changes in retinal function have been characterized that might occur before clinically observable vascular pathology, including a reduced electrical response as measured by electroretinography and diminished contrast sensitivity[104,105]. A study in mice found that apoptosis in the diabetic retina depended on

protein regulated in development and DNA damage response 1 (REDD1)[106]. REDD1 promotes dephosphorylation and inhibition of AKT kinase activity, allowing the transcription factor FOXO1 to promote cell death. Depletion of REDD1 in retinal neural cell culture prevented hyperglycaemia-induced apoptosis and deletion of REDD1 in mice reduced diabetes mellitus-induced retinal apoptosis and attenuated aspects of loss of vision, most prominently loss of b-wave intensity on scotopic electroretinograms and loss of contrast sensitivity. Some factors might directly affect both vascular and neural tissue such as endothelin, which affects vascular and neural tissue through different receptors subtypes. Topical administration of an endothelin antagonist in a diabetic mouse model prevented neurodegeneration[107].

Neuronal and vascular dysfunction. While both vascular and neuronal changes clearly occur during diabetic retinopathy, the question remains as to whether neural or vascular dysfunction initiates the disease process or whether the alterations are coincident but unrelated. In the European Consortium for the Early Treatment of Diabetic Retinopathy (EUROCONDOR) study in 449 patients with diabetes mellitus, patients with no vascular defects were compared with patients with mild vascular defects as assessed by ETDRS scoring, with alterations in retinal function measured using multifocal electroretinography and in retinal structure using OCT. The study found that 61% of patients without microvascular disease showed abnormalities related to neurodegeneration assessed by multifocal electroretinography or OCT[108]. Conversely, 32% of patients with visible microvascular disease did not show any signs of neurodegeneration. It is important to note that the lack of observable vascular defects does not confirm unaltered vessel function. However, the authors raise the possibility of a distinct disease aetiology in diabetic retinopathy with subtypes of diabetic retinopathy that might require distinct interventions. The use of conditional gene regulation targeting specific cell types is necessary to begin to elucidate the causal relationship between retinal vascular and neural changes observed in animal models of diabetes mellitus. Furthermore, longitudinal studies of patient populations assessing vascular and neuronal alterations and retinal function are needed to clarify potential differences in disease progression that will inform therapeutic approaches.

Growing clinical evidence indicates that neurovascular changes occur in the brain of patients with diabetes mellitus, especially in those with T2DM, which leads to an increased risk of dementia[109] or Alzheimer disease[110]. Evidence is also growing for an association between retinal vessel abnormalities and cognitive impairment and dementia[111], with the possibility of retinal imaging as an effective biomarker for neurodegenerative diseases. Proteomic analysis of the vitreous has identified changes in proteins associated with Alzheimer disease and Parkinson disease[112]. While these findings are intriguing, more research with mechanistic detail is needed to explore the potential role of diabetes mellitus in brain neurodegeneration and similarities with or differences from the retinal changes in diabetic retinopathy.

Oxidative stress. Extensive studies have revealed the role of oxidative stress in the pathology of diabetic retinopathy[113]. NF-E2-related factor 2 (NRF2) is a transcription factor that is a master regulator of a host of genes that act in a cytoprotective manner and provide cellular antioxidant gene products. NRF2 is normally bound and inhibited by KEAP1, which results in NRF2 being targeted for degradation. Stress-induced alterations in KEAP1 binding stabilizes NRF2, allowing expression of antioxidant gene products and cellular protection. Deletion of the gene that encodes NRF2 exacerbates the degree of retinal ischaemia and increases pre-retinal neovascularization in the oxygen-induced retinopathy model[114]. The oxygen-induced retinopathy model takes advantage of the plasticity of the neonatal retina and creates a central retinal ischaemia that drives pathological angiogenesis. By using broad neuronal conditional knockout, glial-specific knockout and endothelial knockout, the investigators demonstrated that this effect on vascular development, particularly the increased avascular area, was driven by neuronal cells. Loss of NRF2 increases expression of semaphorin 6a (SEMA6A), which acts extracellularly on endothelial cells through Notch signalling. In addition, the vascular pathologies in the *Nrf2*-knockout animals were reversed by lentiviral delivery of short hairpin RNA targeting SEMA6A[114].

The role of NRF2 signalling in diabetes mellitus was demonstrated by increased vessel permeability and loss of visual acuity in diabetic mice with deletion of the gene that encodes NRF2 (REF.[115]) and an ischaemia reperfusion model was used to demonstrate the role of NRF2 in retinal ganglion cell protection[116]. Ischaemia reperfusion has previously been shown to model aspects of VEGF-dependent vascular permeability, inflammation and retinal cell loss observed in diabetes mellitus, with more dramatic effects over a shorter, 2-day time-frame than diabetes mellitus, which might take weeks to months for pathology to manifest[117,118]. Visual acuity is protected with a NRF2-activating drug in the ischaemia reperfusion model[119], which suggests that this approach might provide therapeutic benefit for neurons in ischaemic retinal diseases, including diabetic retinopathy.

Potential for regenerative medicine

Investigators have begun to explore the potential for retinal vascular regeneration as a method of restoring proper vascular function in diabetes mellitus. In a clinical case study, spontaneous reperfusion of an ischaemic retina followed by recovery of visual acuity has been reported following radiation retinopathy[120], which suggests that return of adequate blood flow can restore retinal function. Spontaneous reperfusion of the ischaemic diabetic retina has been reported[121,122]. However, this spontaneous reperfusion is generally rare in diabetic retinopathy and the well-established failure of wound repair in diabetes mellitus might also lead to defects in normal vascular reparative processes in the diabetic retina that might contribute to the observed progressive vascular degeneration. The deficiencies in vascular repair processes related to diabetes mellitus are not well understood; however, it is widely appreciated that patients with diabetes mellitus have exacerbated cardiac and peripheral limb

ischaemia through reduced collateral vessel development and abnormal repair following infarct[123].

Interestingly, the Joslin Medalist T1DM cohort show normal levels of endothelial progenitor cells and circulating progenitor cells compared with other cohorts of patients with diabetes mellitus, which suggests that endogenous protective factors might provide a protective effect in the Medalist cohort[124]. Indeed, increasing evidence suggests that diabetes mellitus suppresses resident progenitor cells that would normally be activated by injury[125,126]. This finding is especially true for a side population of cells that have a progenitor phenotype in the endothelium[127,128]. Lineage tracing experiments in mice have shown that these self-renewing progenitors can become activated by vessel damage, after which they re-establish a viable endothelium and restore perfusion[128–132]. Although currently unknown, altered retinal progenitor cells could account for the observed deficits in repair in diabetic retinopathy.

In view of the damage to the retinal neurovascular unit related to diabetes mellitus, a strategy that could replace damaged endothelium is attractive and has clear translational potential. Cell therapy using vasoactive progenitors has received attention, as such cells are recruited to sites of capillary loss where they promote reperfusion[133]. Various cell types, including $CD34^+$ cells[134], Lin^- haematopoietic stem cells[135], $CD44^{hi}$ cells[136] and circulating angiogenic cells[137], have all been shown to enhance tissue repair of ischaemic tissues in preclinical models, including in the retina. Although described as endothelial progenitor cells, many such populations are, in fact, heterogeneous mixtures of myeloid cell types with no evidence of incorporation into the vasculature[138]. Unfortunately, because of the lack of definitive markers, the results of the majority of studies using the heterogeneous and poorly defined endothelial progenitor have been inconsistent[139]. However, an ongoing, phase I retina-focused trial using $CD34^+$ cells has demonstrated that intravitreous delivery is safe[140], and a clinical trial for various retinopathies, including diabetic retinopathy, is currently ongoing (NCT01736059). This advance is encouraging, although in the context of diabetic retinopathy the use of vasoactive progenitors needs to be approached with some caution, as evidence suggests that progenitors that carry myeloid markers might actively participate in pro-inflammatory responses[141].

Perhaps the most promising cell type from a therapeutic perspective is an endothelial progenitor cell type called endothelial colony-forming cells (ECFCs) that are isolated from peripheral adult blood or umbilical cord blood. These cells have been proven to be homogeneous and distinct from haematopoietic stem cells and cells sorted on $CD34^+$[138]. ECFCs possess many endothelial and progenitor cell characteristics and lack the haematopoietic markers CD45 and CD14. They also possess de novo endothelial tube-forming potential in vitro and in vivo and can form de novo vessels or be directly incorporated into pre-existing capillaries[142–145]. In vivo, ECFCs seem to share properties with side population cells that are present in the vasculature[127] and can become activated by vessel damage, at which point they are incorporated into the endothelium[129–131]. Emerging preclinical studies have validated the potential for ECFCs in the treatment of diseases where vascular insufficiency is a cardinal feature, such as stroke, peripheral artery disease, heart disease and diabetic retinopathy[146]. ECFCs that are delivered to the vitreous migrate to ischaemic retina and activate vascular repair[147–149]. In diabetic mice, administration of ECFCs combined with recombinant angiopoietin 1 gene therapy prevented barrier dysfunction and restored vision, as measured by opto-kinetic functional readouts[150].

While most attention has been focused on replacement of damaged endothelial cells, other damaged cells might also need to be restored in the retina of patients with diabetes mellitus. For example, replacing lost pericytes might be possible using mesenchymal stromal cells as stromal cells have been shown to migrate adjacent to retinal vessels and adopt a pericyte-like phenotype, which maintains vascular integrity[151,152]. Although not yet studied in diabetic retina, there is also potential for replacement of defective Müller glia, RPE and perhaps even neurons[153].

Patient-specific cells and organelles

The potential of induced pluripotent stem cell (iPSC) technology to produce retinal organoids has already led to considerable advances in ophthalmology and vision science. So-called retina-in-a-dish approaches have been developing apace in the past few years and they have provided great insight into both developmental biology and retinal neurodegenerative diseases[154,155]. Indeed, the use of iPSC-derived, patient-linked cells is already leading to advances in ophthalmic disease fields, such as in understanding the pathogenesis of drusen formation in iPSC–RPE from patients with macular degenerative disease[156]. Diabetic retinopathy research has been heavily reliant on animal models and while this strategy has led to many important advances, the clinical fidelity of the research has always been limited. Studies using retinal organoids for diabetic retinopathy research might help circumvent this limitation. There are already findings from the use of iPSC–RPE derived from donor patients with T2DM that have revealed decreased barrier function and attenuated autophagic capacity compared with iPSC–RPE from control individuals without diabetes mellitus[157]. Depending on the pluripotency approach used, these iPSC-derived cells might carry an epigenetic imprint and have DNA methylation signatures characteristic of their somatic tissue of origin[158]. Use of iPSC has the potential to combine laboratory studies with clinically relevant cells to more fully understand diabetic retinopathy phenotypes from a molecular perspective with the clear potential to develop more patient-specific therapeutic approaches.

Conclusions

Diabetes mellitus remains a leading cause of vision impairment worldwide. While the precise aetiology of metabolic dysregulation contributing to loss of retinal functions remains to be fully elucidated, targeting the VEGFA cytokine signalling that drives microvascular pathologies has proven effective in preventing disease progression and improving vision for many patients.

Studies exploring the cellular communication of the neurovascular unit in the retina and the alterations that occur in diabetes mellitus might provide additional targets to treat those patients who do not respond to current therapies. Disease management of diabetic retinopathy might by further improved with the development of novel biomarkers that take advantage of the unique availability of retinal imaging. However, a better understanding of the disease aetiology, the factors that might drive DME or PDR and the specific cytokines or factors that mediate specific disease processes, and additional information on the genetic basis of susceptibility to or protection from diabetic retinopathy, are needed. Accurate phenotypic description of populations of patients coupled with analysis of altered cytokine profiles in vitreous or aqueous fluid might lead to precision medicine with improved patient outcomes. In basic research, studies that utilize conditional gene targeting to explore cell communications in vivo are needed to elucidate the functional relationship of the cells in the neurovascular unit and the contribution to vascular and neuronal dysfunction in diabetic retinopathy. Finally, regenerative and restorative approaches provide hope for restoring retinal function lost as a result of diabetes mellitus.

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