New Perspectives in Diabetic Retinopathy

Novas Perspetivas na Retinopatia Diabética

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Abstract

Diabetic retinopathy (DR) is a prevalent complication of diabetes and a leading cause of blindness and vision impairment on a global scale. Presently, there are no treatments that cure or permanently halt the progression of DR; existing interventions primarily target advanced stages of the disease, often after significant vision loss has occurred. There is an urgent need for innovative therapeutic approaches addressing early-stage DR before irreversible retinal damage occurs. The use of cellular and animal models is crucial for understanding the cellular and molecular mechanisms underlying DR, allowing for the development of more effective therapeutic strategies and advancement in the treatment of this devastating ocular complication. However, choosing the best preclinical model is challenging due to the high number of factors that need to be considered. Here, we provide an overview of the current state-of-the-art in the field of DR research and explore prospective avenues to expedite advancements, ultimately leading to the creation of efficacious and timely interventions.

Keywords: diabetic retinopathy; cellular and animal models; cellular and molecular mechanisms

Resumo

A retinopatia diabética (RD) é uma complicação prevalente da diabetes e uma das principais causas de cegueira e diminuição significativa da acuidade visual à escala global. Atualmente, não existem tratamentos que curem ou travem permanentemente a progressão da RD. As intervenções existentes visam principalmente as fases avançadas da doença, muitas vezes depois de ter ocorrido uma perda significativa da visão. Há uma necessidade urgente de abordagens terapêuticas inovadoras que tratem a RD nos seus estadios iniciais, antes de ocorrerem danos irreversíveis na retina. A utilização de modelos celulares e animais é crucial para a compreensão dos mecanismos celulares e moleculares subjacentes à RD, permitindo o desenvolvimento de estratégias terapêuticas mais eficazes e o avanço no tratamento desta complicação ocular devastadora. No entanto, a escolha do melhor modelo pré-clínico é um desafio devido ao grande número de fatores que têm de ser considerados. Aqui, apresentamos uma visão geral do estado da arte atual no campo da investigação da RD e exploramos vias prospetivas para acelerar os avanços, conduzindo, em última análise, à criação de intervenções eficazes e oportunas.

Palavras-chave: retinopatia diabética; modelos celulares e animais; mecanismos celulares e moleculares

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> INTRODUCTION

Diabetic retinopathy (DR) is a major complication of diabetes affecting 146 million people worldwide and a leading cause of visual impairment and blindness in working-age adults, according to the World Health Organization (WHO). DR exhibits a gradual progression over 10-15 years following the diagnosis of diabetes, being characterized in the early stages by mild retinal lesions and generally preserved visual acuity, and in the advanced stages (non-proliferative DR – NPDR, and proliferative DR – PDR) by structural lesions leading to compromised visual acuity. [1] Approximately one-third of diabetic patients exhibit signs of DR, with about one-

-tenth at risk of vision loss due to PDR or diabetic macular edema (DME), a complication associated with DR characterized by fluid accumulation in the central region of the retina known as the macula. [2]

The classification of DR into stages, as per the "Early Treatment of Diabetic Retinopathy Study (ETDRS)" classification, [3] relies on observable ophthalmologic changes and the occurrence of retinal neovascularization. The early stage, known as preclinical retinopathy, shows no alterations in the fundus. The disease can progress to mild NPDR, characterized by the presence of some microaneurysms. The third stage corresponds to moderate NPDR, characterized by microaneurysms, hard exudates (lipid deposits in the retina originating from lipoprotein leakage from the retinal microvasculature), cotton-wool spots, intraretinal microvascular abnormalities (IRMAs), and intraretinal hemorrhages or venous beading. Microaneurysm formation is associated with localized proliferation of endothelial cells, loss of pericytes, and alterations in the capillary basement membrane. [4] In severe NPDR, signs of retinal ischemia appear, consisting of cotton-wool spots, IRMAs, several regions with lack of capillary perfusion, and increased venous tortuosity. About 50% of patients with severe NPDR progress to severe PDR within one year. [5] PDR refers to a severe stage of DR characterized by the proliferation of new blood vessels on the surface of the retina and on the posterior surface of the vitreous (Figure 1). [6,7]

Currently, DR is recognized as a multifaceted complication wherein neurodegeneration also plays a key role, being defined as a neurovascular (NVU) complication. In early-stage DR, pathogenic mechanisms involve impairment of the NVU and retinal capillary regression. [8] This is characterized by glial activation and neurodegeneration of retinal ganglion cells (RGC) and photoreceptors, as well as the loss of endothelial cells and pericytes, resulting in the breakdown of the blood-retinal barrier (BRB). Capillary occlusion due to the narrowing of capillaries and regression or loss of retinal capillaries due to cell loss contribute to the disruption of the BRB, leakage of fluid into the retinal tissue, and the development of ischemia. This ischemic environment stimulates the release of the important vascular endothelial growth factor (VEGF), which promotes the growth of abnormal blood vessels (neovascularization) as a compensatory mechanism to restore oxygen supply.

Treatment options for DR vary depending on the stage of the disease. In the early stages of DR, when there is no significant vision loss, with only small areas of microvascular lesions, treatment typically focuses on controlling risk factors such as blood glucose levels, blood pressure, and cholesterol through lifestyle changes and medications. As DR progresses to more advanced stages, such as PDR or DME, invasive treatment options may be necessary to prevent further vision loss, such as retinal photocoagulation, intravitreal administration of corticosteroids and anti-VEGF inhibitors, and vitrectomy. [9,10] However, these invasive treatments are merely palliative, ineffective in preventing DR onset or progression, and sadly, many patients do not adequately respond to the treatment. [9] Innovative therapeutic solutions for early disease stages are needed to avoid progression to severe forms leading to vision compromise. Also, novel, and more effective treatments for the disease's later stages remain an unmet need. Importantly, the identification of novel therapeutic approaches is inherently intertwined with and constrained by the extent of our understanding of the mechanistic complexities of pathophysiology, primarily attributed to limitations in existing disease models.

> OXIDATIVE STRESS AND INFLAMMATION IN DIABETIC RETINOPATHY

Chronic hyperglycemia-associated oxidative stress and low-grade inflammation are key factors in the onset and progression of DR. The precise mechanisms linking oxidative stress to inflammation and vice versa remain incompletely understood. Oxidative stress triggers cytokine production by activating transcription factors like NF-κB, STAT, and activator protein-1, which promote cytokine gene transcription. NF-κB activity is elevated in endothelial cells, pericytes, and glial cells in experimental DR models and diabetic patients' retinas. [11-13] Inhibiting NF-κB suppresses the production of pro-inflammatory cytokines, underscoring the pivotal role of NF-κB in diabetic retinal inflammation. [14]

Also, cytokines promote reactive oxygen species (ROS) production. Tumor necrosis factor (TNF), Interleukin (IL)-1β, and interferon-γ induce ROS generation in retinal pigment epithelial (RPE) cells through mitochondria and nicotinamide adenine dinucleotide phosphate oxidase (Nox) pathways. Chemokine (C-C motif) ligand 2 (CCL2) production during retinal vascular inflammation involves Nox pathway activation via NF-κB and Akt signaling. IL-1β intravitreal administration increases retinal oxidative stress akin to diabetes models. Pro-inflammatory cytokines induce ROS and nitric oxide (NO) formation, reducing small heat shock protein 27 (HSP27) levels and causing retinal endothelial cell apoptosis. [15]

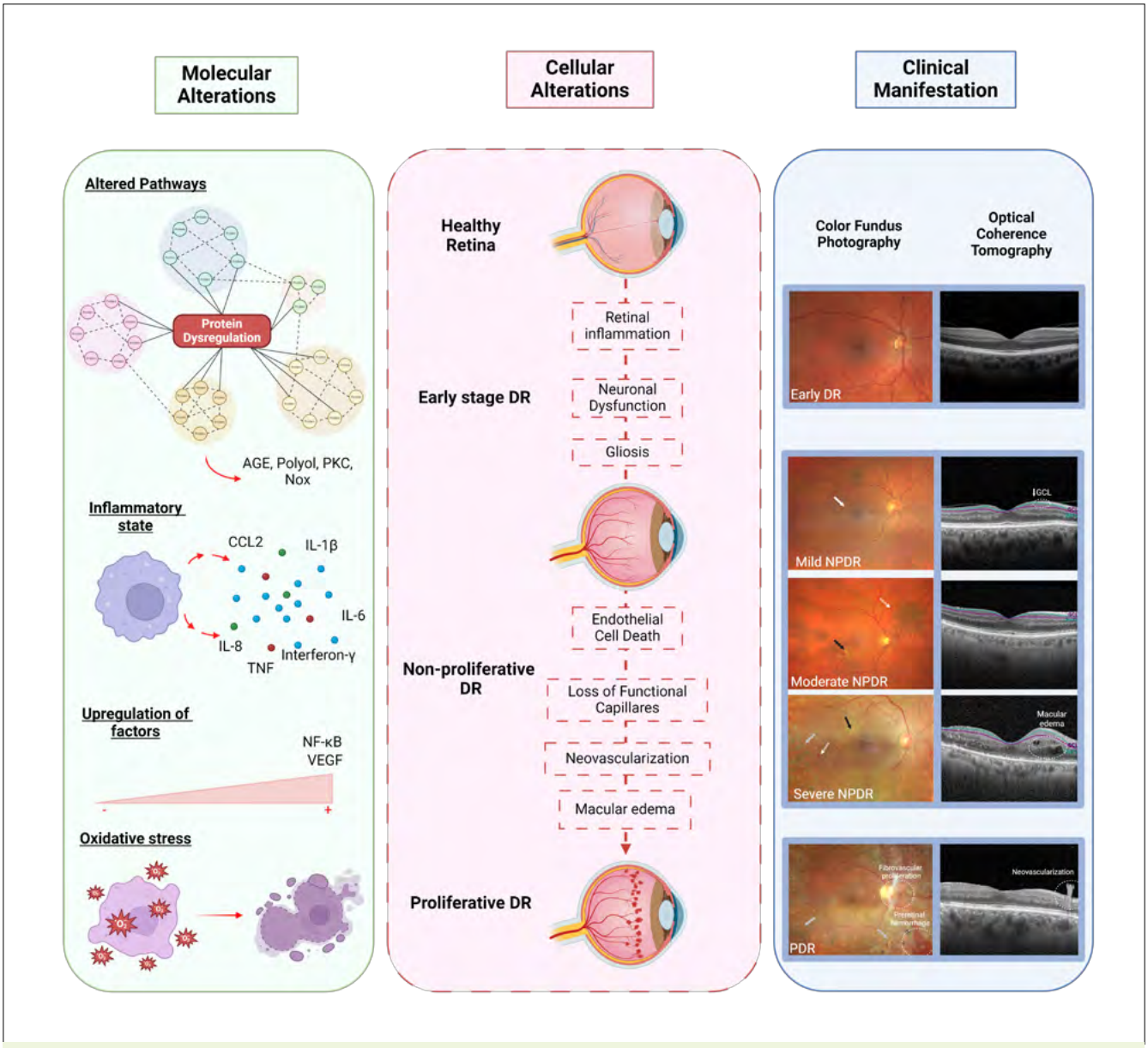

Figure 1 - Stages of diabetic retinopathy (DR) progression. Main molecular, cellular and clinical alterations occurring on the different stages of the disease: i) early DR where no clinical alterations are visible; ii) mild NPDR with few microaneurysms (white arrow); iii) moderate NPDR with hemorrhages (dash white arrow) and hard exudates (black arrow); iv) severe NPDR with several hemorrhages, LASER photocoagulation marks (grew arrow), hard exudates (black arrow), hemorrhages(dash white arrow); v) PDR with LASER photocoagulation marks (grew arrow), preretinal hemorrhage and neovascularization. Figure generated using BioRender (biorender.com/).

> ROLE OF LOCAL VASCULAR NETWORK

DR has traditionally been considered a microvascular disease, largely due to the fact that primary screening tests predominantly detect microvascular alterations. [16] However, beyond the observable changes such as microaneurysms and neovascularization, other retinal cell types are affected by diabetes, potentially contributing to visual impairment.

Indeed, retinal neurodegeneration is an early event in DR preceding vascular alterations. [9,16,17] The impact of hyperglycemia on the retina is profound, initiating a cascade of biochemical and molecular events that contribute to the development and progression of DR (Figure 1). There are several biochemical alterations that occur in the diabetic retina. For instance, the advanced glycation end-products (AGEs), that are formed through non-enzymatic glycation of proteins, can accumulate in retinal tissues, disrupting cellular function and promoting a pro-inflammatory state. [18,19] In addition, there is

dysregulation of polyol pathway enzymes, increased oxidative stress, abnormal activation of the protein kinase C (PKC) pathway and activation of pro-inflammatory cytokines. [18,19] The activation of these pathways leads to the degeneration of the neural retina in the inner retina. RGCs, located in the inner retina, are the more susceptible cells to hyperglycaemia, [20] and their loss has been detected in diabetic rats and diabetic patients either without or with only minimal DR. [17,21] In addition to RGCs, amacrine cells, and photoreceptors have shown an increased apoptotic rate in diabetic retinas. [22-24]

> MICROVASCULAR CHANGES IN DIABETIC RETINOPATHY

DR involves progressive changes in the retinal microvasculature alongside damage to the neural and glial cells that support retinal function. [25]

The retinal vascular endothelium is a monolayer of cells covering the vascular lumen within the retina, facilitating the supply and drainage of the inner retina. Functionally, it acts as a selective barrier, known as the inner BRB, regulating the passage of macromolecules while maintaining the local microenvironment. Simultaneously, it ensures the provision of oxygen and nutrients to the neural retina. However, under conditions like hyperglycemia, retinal endothelial cells become susceptible to damage. Hyperglycemia triggers a cascade of signaling events in endothelial cells, leading to metabolic and biochemical abnormalities. Superoxide anions, overproduced by the mitochondria under hyperglycemic conditions, initiate various biochemical pathways implicated in the pathogenesis of DR. [26] These pathways include increased flux through the polyol pathway, enhanced non-enzymatic glycation, elevated diacylglycerol production, and stimulation of PKC. Consequently, ROS production rises, exacerbating oxidative stress and causing biochemical and metabolic imbalances. [26]

Moreover, hyperglycemia-induced superoxide production can leave a lasting impact on vascular cells, contributing to the development of microvascular abnormalities. This phenomenon, termed metabolic memory, involves the accumulation of harmful agents like AGEs, perpetuating oxidative stress even after glycemic control is restored. The resultant epigenetic changes, including DNA methylation and histone modifications, further promote DR progression. [27] Histological examinations have revealed consistent features in diabetic retinas, including loss of retinal capillary pericytes and endothelial cells, pericyte dropout, and formation of acellular capillaries and microaneurysms. These changes lead to decreased retinal perfusion, capillary basement thickening, and induction of biochemical alterations. [28] Additionally, non-vascular cells like Müller cells and glial cells undergo apoptosis, contributing to retinal ischemia, and increased expression and release of several growth factors and pro-inflammatory cytokines. In fact, increased production of VEGF plays a pivotal role in pathological angiogenesis in PDR. [29] Increased levels of other factors such as angiopoietin-1 and -2, erythropoietin, and TNF were found to orchestrate protease production, endothelial cell proliferation, migration, and tube formation, ultimately determining the development and progression of PDR. [30,31]

> *IN VITRO* MODELS FOR DIABETIC RETINOPATHY

In vitro models of DR have had a crucial role in our understanding of this pathology. Specifically, two-dimensional (2D) models of DR contributed to the characterization of molecular and biochemical alterations that lead to retinal damage during diabetic conditions.

Retinal endothelial cell models have been extensively investigated to gain valuable insights into DR microvasculopathy. Most *in vitro* cell cultures use isolated endothelial cells, mainly from bovine, human and monkey. [32] These studies allowed to identify several causes of endothelial dysfunction in DR, such as the AGEs and their receptors (RAGE), disruption of peroxisome proliferator-activated receptor-γ (PPARγ), chronic inflammation, oxidative stress, and dysregulated growth factors, cytokines, and microRNA (miRNA) networks. [33]

Following, the loss of pericytes has great consequences on blood vessel remodeling in the diabetic eye. In the past years, studies found pericytes to be essential in the formation and maturation of BRB at the postnatal stage, while remaining indispensable in the adult stable retinal blood vessels. [34] Pericytes can either be isolated from human, murine, and bovine tissues, [35] or obtain commercially. For example, studies using this approach showed a novel inflammatory pathway mediated by macrophages and pro-apoptotic BIGH3 protein. [36]

Müller glia cells are the main macroglia that cross the whole retina, enclosing both the endothelial and the neuroretina cells. In later stages of DR, the inflammatory factors and cytokines secreted by activated Müller glia cells increases apoptosis and promotes further abnormal secretion of cytokines, leading to damage of the BRB. [37] These dysfunctions can be studied using either isolated cells from human tissue, or derived-human and rat cells lines, and more recently, through the isolation

of Müller glia cells from induced pluripotent stem cells (iPSC) derived-retinal organoids. [38]

Regarding inflammation, microglia cells are key players in the Central Nervous system (CNS) and the retina, constantly monitoring their surroundings for signs of damage. In DR, microglia activation occurs in the early stages, most likely promoting the pro-inflammatory environment and contributing to disease progression. [39] Research on human microglia in DR is still relatively limited, although some emerging studies on the matter, use commercially available immortalized human microglial lines, [37,40] microglia isolated from postmortem human retinal tissue [41] or differentiated from iPSCs, [42] but few studies address DR.

The RPE cells play a role in retinal homeostasis, in photoreceptor function, and contribute to the integrity of the outer BRB. In DR, RPE cells are involved in the DME, and loss production of the trophic factor pigment epithelial-derived factor (PEDF), leading to pericyte loss and endothelial damage. Primary cultures RPE can easily be isolated and grown from bovine, porcine, murine, and human retinal tissues. Plus, extensive studies have relied on a spontaneously immortalized cell line ARPE-19, [43] that replicates several characteristics of human RPE.

Moving into the inner retina, the RGCs collect optical information and transmit it to the brain via the optic nerve. In DR, they are more susceptible to hyperglycemia and become damaged in the early stages, [20] even before the onset of vascular damage, [44] having their function impaired, leading to their subsequent loss. [45] Early studies used the immortalized RGC-derived cell line, RGC5, to study the effects of hyperglycemia in RGCs. [46] Nevertheless, later data came to show concerns regarding the nature of the cell line. [46] Primary cultures of RGCs can also be established by isolating them from rodent retinas. [47] Human RGCs have recently been generated from iPSCs opening new possibilities for future studies in DR pathophysiology mechanisms. [48,49]

Understanding the role of photoreceptors damage in DR pathogenesis is a complex task, as they are reported to be notoriously difficult to isolate and grow in culture. Most studies on macular degeneration and retinal ciliopathies rely on a mouse SV40 T antigen transformed photoreceptor cell line 661W. [50,51] Exposure of the 661W cells to high glucose to mimic DR, led to significant findings on oxidative stress, gene expression, viability, apoptosis, and mitochondrial function. [52]

Nevertheless, all previously referred 2D models fail to reconstitute the complex *in vivo* physiological environment since they lack diverse cell types, tissue architecture, and mechanical and biochemical signaling dynamics.

The development of iPSCs, derived from somatic cells of an individual that can differentiate into other adult cell types, allowed the development of three-dimensional (3D) models, called organoids, for research and clinical applications. Organoids can model a variety of tissues and organs, and their complexities. Specifically for diabetes, studies were already performed using organoids of the pancreas, liver, gut, muscle, and adipose tissue, plus organs affected by its complications, such as blood vessels, [53] retina, [54] kidney, and nerves. [55,56]

Particularly, retinal organoids (RO) can recapitulate the human neuroretina with their cellular organization, cell-cell interactions, and were even found to be light-responsive. [57,58] In recent years, ROs have been used to model inherited retinal diseases [59], in applications in regenerative medicine, [60] and for screening of potential therapeutic drugs of toxicological studies. [61] We recently generated a model using ROs, mimicking neurodegeneration and glial reactivity occurring in early DR, before vascular alterations are visible. [54] In particular, this model reproduced loss of ganglion and amacrine cells, glial reactivity, inflammation, and elevated oxidative stress. [54]

> ANIMAL MODELS OF DIABETIC RETINOPATHY

Rodents serve as valuable models for investigating the etiology and pathogenesis of DR and for testing potential therapies. However, it is important to note that they may not perfectly replicate all aspects of human DR, particularly its proliferative stage characterized by neovascularization and retinal detachment.

Although no single animal model fully mimics all aspects of human DR, they provide valuable insights into its molecular and cellular mechanisms. Over the years, various genetic rodent models have been developed to simulate certain features of clinical DR and to evaluate new treatments. However, models involving transgenic animals overexpressing VEGF in retinal cells, while exhibiting neovascularization, do not fully replicate the metabolic changes associated with prolonged hyperglycemia or the progressive nature of DR seen in humans.

High-fat diet (HFD) feeding protocols are commonly used to induce obesity and early T2D conditions in rodents, including insulin resistance (IR). However, retinal microvascular lesions like those observed in human DR typically develop only after prolonged HFD consumption. For instance, studies on C57BL/6 mice fed HFD for 16-20 weeks showed RGC dysfunction, while increased retinal vascular permeability, a key feature of early DR, occurred only after 48 weeks of HFD feeding. [62,63] Furthermore, in some cases, HFD-induced obesity may lead

to neural retinal dysfunction even before the onset of systemic hyperglycemia. Another study using Swiss mice fed HFD for 8 weeks showed increased body weight and fasting glucose levels, with subsequent inflammatory changes in retinal tissue and elevated levels of VEGF inducers after 16 weeks of HFD feeding. [64]

A widely accepted rodent model for studying retinal complications in T2D involves combining a HFD with streptozotocin (STZ) injection to induce chronic hyperglycemia. [65] C57BL/6J mice fed an HFD for 12 weeks and then injected intraperitoneally with a low dose of STZ (30 mg/kg) for 7 consecutive days, followed by another 12 weeks, exhibited features of early-stage DR, including loss of pericytes, formation of acellular capillaries, increased retinal vascular leakage, oxidative stress (evidenced by increased ROS production and NOX2 expression, along with decreased superoxide dismutase 2 (SOD2)), pro-inflammatory state (elevated TNF, IL-1β, and VEGF), and increased apoptosis. [66] Similarly, Sprague-Dawley rats, when fed an HFD combined with a single STZ injection at the same dosage, developed early-stage DR features after 16 weeks, including abnormal b-waves and outer nuclear layer (ONL) thickness reduction compared to controls. [67]

Another animal model of DR involves subjecting rodents to combined stressors commonly encountered in human diets, such as an HFD combined with high sugar intake, such as fructose. Wistar rats fed a high-fat and fructose diet (HFFD) for 56 weeks and injected intraperitoneally with low-dose STZ multiple times can recapitulate aspects of diet-induced T2DM observed in humans. As early as week 20, these rats show retinal morphological lesions, including retinal parenchyma thickening and pathological neovascularization. [68]

> CONCLUSIONS

DR is a public health problem with a major impact on the economy of developed countries. The costs of treatment correspond to only a part of the costs of the disease, since patients with worse visual acuity not only require increased eye care but also other care with indirect medical costs, with comorbid conditions such as depression (~ 30%), falls leading to hip fractures and other related injuries. It is, therefore, a disease that affects not only the quality of life of these patients, but it is also an economic burden for society.

Addressing DR effectively necessitates a dual approach. Firstly, improving early diagnosis, through advancements in retinal imaging tools and functional diagnostics, coupled with the identification of novel biomarkers - structural, functional, and molecular – can lead to the discovery of new surrogate endpoints, thus improving the robustness of clinical trials. Simultaneously, urgent attention must be directed towards elucidating the disease's triggering mechanisms, thereby identifying new therapeutic targets tailored for early-stage intervention to prevent progression towards severe forms that compromise vision. Such developments can potentially control the progression of retinopathies before vision is adversely affected. Novel effective treatments for the disease's later stages, where vision loss occurs, remain also an unmet need.

DR research strongly relies on experimental models, which bridge the gap between preclinical stages and clinical treatments. The generation of human-based models derived from hiPSCs represents a cutting-edge approach for both fundamental research and clinical use. Indeed, RO-based models offer greater versatility, enabling precise control over experimental conditions, such as drug concentrations and nutrient supply, which can prove challenging to regulate consistently *in vivo* models. Despite lacking vascular and microglia components, the integration of ROS with vascular organoids through assembloids presents a viable strategy, [69] generating more complex models that serve as a powerful platform for DR research. Also, the rapid development of microphysiological systems (MPS) is contributing to the improvement of *in vitro* disease models, potentially refining drug discovery [70] as well as compliance with reducing animal-based studies. Furthermore, the integration of artificial intelligence and machine learning algorithms may facilitate the identification of predictive biomarkers for early disease detection and personalized treatment approaches.

Ultimately, by pursuing a multidimensional approach that integrates basic research, translational studies, and innovations in healthcare development, it is possible to promote the development of effective and timely interventions for DR, thus mitigating its devastating impact on global vision health. <

Conflicts of interests/*Conflitos de interesses*:
The authors declare that they have no conflicts of interests./*Os autores declaram a inexistência de conflitos de interesses.*

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