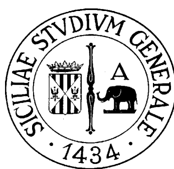
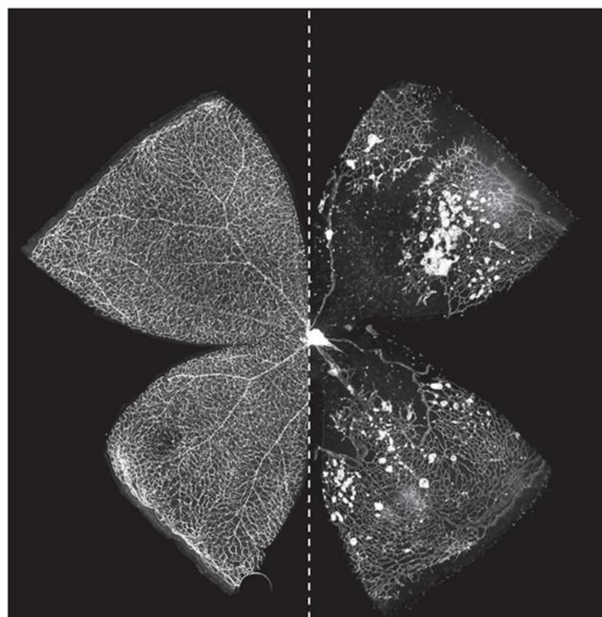


New pharmacological targets for the treatment of diabetic retinopathy

Ph.D. Thesis



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New pharmacological targets for the treatment of diabetic retinopathy

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PhD thesis

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS:	**3**
LIST OF ABBREVIATIONS**:**	**4**
ABSTRACT**:**	**6**
CHAPTER I**:**	**28**
CHAPTER II**:**	**58**
DISCUSSION AND CONCLUSIONS**:**	**85**

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List of abbreviations

DR: DIABETIC RETINOPATHY

NPDR: NON PROLIFERATIVE DIABETIC RETINOPATHY

PDR: PROLIFERATIVE DIABETIC RETINOPATHY

BRB: BLOOD RETINAL BARRIER

DME: DIABETIC MACULAR EDEMA

AMD: AGE-RELATED MACULAR DEGENERATION

ERK: EXTRACELLULAR SIGNAL-REGULATED KINASES

AGE: ADVANCED GLYCATION END PRODUCTS

HIF: HYPOXIA-INDUCIBLE FACTOR

MMP: MATRIX METALLOPROTEINASE

NF-KB: NUCLEAR FACTOR-KAPPA B

PLGF: PLACENTAL GROWTH FACTOR

PKC: PROTEIN KINASE C

RAGE: RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS

TNF-α: TUMOR NECROSIS FACTOR-ALPHA

VEGF: VASCULAR ENDOTHELIAL GROWTH FACTOR

$HREC_S$: HUMAN RETINAL ENDOTHELIAL CELLS

$COCL_2$: COBALT (II) CHLORIDE

MIRNAS: MICRORNAS

TGF-β: TRASFORMING GROWTH FACTOR-BETA

CSME: CLINICALLI SIGNIFICANT MACULAR EDEMA

CWS: COTTON WOOL SPOTS

BS: BASEMENTE MEMBRANE

IRMA: INTRARETINAL MICROVASCULAR ABNORMALITIES

RGCS: RETINAL GANGLION CELLS

PKC: PROTEIN KINASE C

HMGB: HIGH-MOBILITY GROUP BOX

ICAM-1: INTERCELLULAR ADHESION MOLECULE

VCAM: VASCULAR CELL ADHESION MOLECULE-1

IL: INTERLEUKIN

MCP: MONOCYTE CHEMOTACTIC PROTEIN

iNOS: INDUCIBLE NITRIC OXIDE SYNTHASE

COX: CYCLOOXYGENASE

HRE: HYPOXIA-RESPONSE ELEMENTS

VEGFR: VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR

HGF: HEPATOCYTE GROWTH FACTOR

PDGF: PLATELET-DERIVED GROWTH FACTOR

CTGF: PRO-FIBROTIC CONNECTIVE TISSUE GROWTH FACTOR

Abstract

Diabetic retinopathy (DR) is a secondary complication of diabetes mellitus and represent the most common cause of irreversible vision loss in working aged people in industrialized countries. DR is generally considered as microvascular complication of diabetes, although during the last decade several other etiopathogenic mechanisms have been proposed for this disease. The main cause of the vision loss in diabetic patients with proliferative diabetic retinopathy (PDR) is diabetic macular edema (DME), mainly caused by neovascularization, vessel leakage and blood retinal barrier (BRB) breakdown in the macula. Angiogenesis, a hallmark of the advanced stage of DR (proliferative diabetic retinopathy, PDR), occurs at the vitreoretinal interface and is often associated with tractional retinal detachment and vitreous hemorrhage. Neovascularization is basically driven by pro-angiogenic factors (e.g. VEGF-A, PlGF), inflammatory mediators (TNF-α, interleukins, chemokines) and oxidative stress-related elements. Chronic hyperglycemia is the primary causative factor of DR, however, etiopathogenesis of DR due to poor glycemic control is still unclear. Many other factors are involved during the early stages of DR such as the hypoxic microenvironment, which leads to the upregulation of pro-angiogenic and inflammatory mediators, indeed retinal hypoxia is a well-known trigger of VEGF release in diabetic retinopathy. Up to now therapies provided for DR, among laser photocoagulation or vitrectomy (only for PDR patients) are intravitreal anti-VEGF and corticosteroids. However, a key issue in the treatment of retinal disease in general is the heterogeneity of response to both these therapies, indeed various novel pharmacological targets are also being assessed in order to reduce these limitations. Therefore, retinal microvascular complications must be deeply investigated to better understand the pathophysiology of retinal degeneration during in DR, in order to search and

discover new pharmacological targets for treatment of diabetic retinopathy. To this purpose I focused my research project on PlGF and growth factors modulated during retinal hypoxia. Particularly, I have studied in-vitro and in-vivo the role of PlGF in modulation of angiogenic and inflammatory pathways in diabetic retinopathy. I have found that aflibercept (a decoy receptor for VEGFA and PlGF) and an anti-PlGF could be endowed of anti-inflammatory activity, blocking the ERK pathway and its consequential production of inflammatory mediators, such as TNF-α. Together with the demonstration of anti-inflammatory molecular action of aflibercept, I have found that PlGF can be considered a target for the treatment of DR, along with VEGFA. Moreover, considering that hypoxia is one of the main factors triggering neovascularization, I have also analyzed the modulation of growth factors and miRNAs, previously found to be linked to diabetic retinopathy, in an in-vitro model of retinal hypoxia (primary retinal endothelial cells (HRECs) treated with $CoCl_2$). In this context we hypothesized that specific dysregulated miRNA in diabetic retinopathy could be linked to hypoxia-induced damage in HRECs. Therefore, I have investigated the effects of chemical hypoxia on the expression of HIF-1α, VEGF, PlGF, and pattern expression of a focused set of miRNAs (miR-20a-5p, miR-20b-5p, miR-27a-3p, miR-27b-3p, miR-206-3p, miR-381-3p). Our results demonstrated that pattern expression of the above mentioned miRNAs correlated also with expression of VEGFA and TGFβ signaling pathway genes, but not with PlGF expression. We propose that retinal angiogenesis by HIF-1α nuclear translocation could be promoted also by up-regulation of PlGF and other factors such as miRNAs and their target genes of VEGF and TGFβ1 signaling pathways.

GENERAL INTRODUCTION

DIABETIC RETINOPATHY

Worldwide, diabetic retinopathy (DR) is among the leading causes of vision loss in working age, and due to due to burden of disease and consequent economic impact, DR represents a major public health concern [1]. DR is generally considered as a microvascular complication of diabetes mellitus, which is one chronic disease with high rate of incidence. In fact, according to the World Health Organization, it is estimated that the total number of people with diabetes will double from 171 million in 2000 to 366 million by 2030 [2]. The prevalence of DR among patients with diabetes is about 40%, and approximately 5-10% of these individuals have vision threatening conditions [3,4]. Chronic hyperglycemia is the primary causative factor of diabetic retinopathy, however, etiopathogenesis of DR is still unclear [5–7].

Ophthalmologists classify diabetic retinopathy mainly into two stages, the non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) [8]. The NPDR can be characterized by lesions due to chronic hyperglycemia, that can lead to damage of retinal capillaries. In NPDR the stability of capillary walls is decreased, and microaneurysms are typical clinical sign of NPDR (small outpouchings of the vessel lumens). Microaneurisms are called “dot-and-blot” hemorrhages, for their dot-like appearance. As soon as microaneurisms start leaking, NPDR can develop in macular edema and consequent impaired vision, due to deposition of fluid under the macula. The presence of this fluid, composed by lipids, leads to the formation of yellow deposits, called hard exudates. Clinically significant macular edema (CSME) is a term used to describe retinal thickening and/or adjacent hard exudates, that either threaten or involve the center of the macula. Clinically significant macular edema can be divided into center-

involved and non-center-involved macular edema. As diabetic retinopathy progresses, the affected vessels can be obstructed, then leading to impaired retinal perfusion. Retinal ischemia can cause the infarction of the nerve fiber layer, resulting in fluffy, white patches called cotton wool spots (CWS). Furthermore, NPDR is subclassified based on specific outcomes of retinal examination:

- early NPDR: at least one microaneurysm present on retinal exam;
- moderate NPDR: characterized by multiple microaneurysms, dot-and-blot hemorrhages, venous beading, and/or cotton wool spots;
- severe NPDR: the most severe stages of NPDR, are characterized by retinal cotton wool spots, venous beading, and severe intraretinal microvascular abnormalities (IRMA).

NPDR can progress to PDR, that has been accounted to extensive retinal ischemia [9], which can promote neovascularization in the vitreous and inner retina. In fact, the retina is a high oxygen demanding tissue, and under ischemic conditions retinal cells release angiogenic factors, like vascular endothelial growth factor (VEGF) and placental growth factor (PlGF). The overproduction of these factors results in the growth of abnormal new blood vessels (neovascularization). These vessels are typically fragile, fenestrated, brittle and leaky. Furthermore, abnormal vessels can growth in the optic disc (neovascularization of the disk, NVD) or elsewhere within the retinal periphery (neovascularization elsewhere, NVE). Leaking vessels can cause vitreous hemorrhages, and repeated vitreous hemorrhaging is associated with gliosis and fibrovascular scar formation. Moreover, contraction of fibrous tissue can result in tractional retinal detachment and sudden loss of vision [10,11], along with further activation of pro-fibrotic pathways. As soon as extensive vitreous hemorrhage occurs or NVD occupies more than one-third of disc area, the PDR patient is considered at high-risk of vision loss due to

retinal detachment [5,12]. Generally, the progression of diabetic retinopathy is related to abnormalities of the vasculature including permeability of the blood retina barrier (BRB), progressive microvascular damage involving vascular endothelial cell and pericyte, occlusion of capillaries, thickening of vascular basement membrane (BM) and excessive retinal neuronal and glial abnormalities [10].

Diabetic Macular Edema (DME)

In case diabetic retinopathy affects the macula, the disease is also termed ‘diabetic maculopathy’. Vascular leakage at the macula leads to macula swelling (diabetic macular edema, DME), which is the most common cause of blindness in diabetic patients [13,14]. Although DME can occur at any stage during the development of retinopathy, it is most prevalent during PDR, following progressive vascular and neural damage [15]. Diabetic maculopathy may be classified as 'central' or 'non-central', depending on edema localization with respect to the fovea. DME can also be 'focal' or 'diffused', based on edema extension Additionally, DME can be classified as 'ischemic' or 'non-ischemic', based on the involvement or preservation of the perifoveal capillary network, respectively [10]. Risk factors for DME include duration of diabetes, poor glycemic control, hypertension, proteinuria, and hypercholesterolemia [16]. Furthermore, several causative factors contribute to the pathogenesis of DME, including hypoxia and oxidative stress, upregulation of VEGF, alteration of the blood-retinal barrier (BRB), retinal vessel leukostasis, pericyte loss, and vascular hyperpermeability [17,18].

The retinal neurovascular system as a basis for understanding diabetic retinopathy

The retina is the innermost light-sensitive tissue of the eye, able to convert light to electrochemical signals, at first through photoreceptors, that transmitt electrochemical signals to retinal neuronal circuitry (bipolar, amacrine cells). Neuroretinal electrochemical stimuli are thereafter processed and collected by retinal ganglion cells (RGCs), that transmitt signals to the visual cortex by means of the optic nerve, that is constituted by RGCs axons. The retina is located in the posterior part of the eye and the neural tissue lines between the vitreous body and the choroid, which support the pigmented retinal epithelial layer (outer blood retinal barrier, BRB) with systemic circulation [19,20]. The retina is characterized by a complex vascular system, whose integrity is necessary for the correct retinal function, providing nutrients and oxygen to the inner and outer retina [1]. The retinal vascular system, similarly to central nervouse systems, is characterized by blood-retinal barrier (BRB), that similarly to blood-brain barrier ensures the regulation of microenvironment and is crucial for the integrity of vascular structure. In the retina, BRB consists of inner and outer components. Inner BRB (iBRB) is composed by junctions between endothelial cells (EC) and supporting perycites and astrocytes; outer BRB (oBRB) is formed by junctions between retinal pigmented epithelial cells (RPE) [21,22]. Diabetes can affect both iBRB and oBRB before and after neovascular events, involving endothelial cells, pericytes (at the capillary level), vascular smooth muscle cells (arteriolar/arterial level), glia, neuronal processes and associated immune cells, and if choroid is effected also RPEs [23].

Pericytes and endothelial cells

Pericytes have a crucial role in maintaining vascular stability and depletion of pericytes is a hallmark of DR. Pericytes envelope capillary walls and share basal lamina with endothelial cells, with which they directly interact through N-cadherin and connexin-43 hemi channels [20,24,25]. Pericytes wrap around retinal capillaries providing structural support as well as modulating endothelial cell function. The perycites to endothelial cells ratio is 1:1, indeed, perycites have a crucial role in maintainance of BRB structure and integrity. In the inner BRB, retinal endothelial cells form the physical barriers between vascular lumen and the retina. They are characterized by several types of junction (i.e. tight junctions, adherens junctions and gap junctions) that are involved in the regulation of signaling for cell migration, growth and apoptosis inhibition [20]. During neovascularization, endothelial sprouting is mediated essentially by ECs; they migrate to form new vessels and build the sprout; than leading to lumen formation. Onlyafter recruitment of perycites and smooth-muscle cells, the new vessel is formed [26]. Any ischemic event in the tissue, also caused also by toxic metabolites, can leadd to additional retinal neovascularization [21,27].

Retinal glia and neurons

Retinal glia, including Müller cells and astrocytes, provide metabolic support to neurons and they also play a critical role in maintaining the iBRB integrity [28]. Glial processes surround all the blood vessels in the retina and play a leading role for maintainence of normal retinal homeostasis [29]. For example, Müller cells regulate glucose flux between the circulation and retinal neurons and have a role in providing substrates for aerobic metabolism in neurons by gluconeogenesis [30].

Immune cells

The development of new blood vessel is also supported by microglia, monocyte-derived tissue macrophages of the central nervous system. It is well reported how retinal microglia is present in the retina before development of vascularization [31,32]._In the adult retina, ramified microglia cells were found in in the inner and outer plexiform layers, and can able to produce factors that support neuronal survival. During several types of trauma or insults, microglia became activated, shifting to an amoeboid morphology and producing a wide range of pro-inflammatory cytokines [33].

Pathophysiology of Diabetic Retinopathy

The pathogenesis of DR is complex and involves multiple interlinked mechanisms including metabolic modifications, mitochondrial dysfunction, vascular damage, apoptosis, inflammation, and oxidative stress [34,35]. Several biomechanical pathways have been proposed to better understand microvascular complications during DR along with sustained hyperglycemia: e.g. accumulation of advanced glycation end-products (AGEs), inflammation, polyol accumulation, activation of protein kinase C, neuronal dysfunction and oxidative stress [20,36]. All these pathological modifications lead to increased vascular permeability and capillary depletion, resulting in macular edema and neovascularization in the retina.

Hyperglycemia and Retinal Microvasculopathy

One of the earliest abnormalities observed in DR is related to retinal blood vessels, with the constriction of arteries and arterioles and blood flow anomalies [37–39]. Vessel abnormalities result in a series of metabolic and biochemical alterations, like: (i) induction of activation of several PKC isoforms (e.g. PKC-α, -β, -δ and –ε; in particular PKCβII isoform [40,41]; (ii)

alteration in function of ionic channels in smooth muscle cells (BK channels) present in the retinal arteriolar vasculature [42–44]. Moreover, retinal pericytes loss is another hallmark of the early events of DR. Several in in-vitro and in in-vivo studies report that hyperglycemia leads pericyte loss [45–47] or degenerated pericytes, also called "ghost cells". Therefore, decreased amount of functional pericytes, or their loss, lead to endothelial cell degeneration, microvascular destabilization and perfusion alterations [37,48,49]. Indeed, pericyte degeneration and loss lead to degenerated endothelial cells, leading to the impairment of the BRB [47,50] and to ischemic events caused by capillary occlusion.

Retinal Inflammation

Several studies were focused on the role of inflammatory processes in early stages of DR; although, specific inflammatory molecular mechanisms are still poorly understood. Chronic low-grade inflammation has been detected in different stages of DR, both in diabetic animal models and in patients [51,52], along with significantly increased systemic and local expression of proinflammatory cytokines; that affect endothelial cells, pericytes, Müller cells, and microglial cells, contributing to structural and functional abnormalities of the retina [53]. In particular, microvascular endothelium, activated by these cytokines and angiogenic growth factors, express pro-inflammatory molecules (e.g., IL-1β, IL-6, TNF-α, high-mobility group box-1 (HMGB1)) and chemokines (MCP-1) involved in leukocyte recruitment and activation [54–56]. Leukocyte-endothelium adhesion mediated by adhesion molecules has been implicated in leukostasis during diabetes. Sequential adhesive interactions between endothelial cells and leukocytes, are coordinated adhesion molecules (e.g. ICAM-1) present in the surface of endothelial cells, that interact with the leukocyte counter-receptor CD18 [57,58]. Furthermore, ICAM-1 is found to be highly expressed in the blood

vessels of the retina, choroid and fibrovascular membrane in patients with diabetes [59,60], and several studies have shown that ICAM-1 and CD18 knockout diabetic mice do not develop retinal vascular permeability, leukostasis, and retinal capillary degeneration [57]. All these inflammatory responses may contribute to neovascularization in the retina during DR, especially under hypoxic conditions. As regards the biochemical pathways involved in DR, expression of inflammatory cytokines might be mediated by activation of mitogen-activated protein kinases (MAPKs) [61], as well as ERKs. Activation of MAPKs and ERKs regulate several cellular processes, such as stress response, inflammation, proliferation, differentiation, apoptosis, and survival [62]. Moreover, ERK pathway can influence NF-κB activation, by the regulation of NF-κB-dependent genes expression, e.g. inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and tumor necrosis factor-alpha (TNF-α) [63].

Retinal Hypoxia

The retina is one of the most oxygen and glucose demanding tissue [64]. Retinal hypoxia represents an important causative factor for DR development, and plays a central role in progression of NPDR to, due to the release of some soluble factors such as cytokines, chemokine and growth factors which promote the growth of extraretinal neovascularization [65]. Ocular ischemic events are considered crucial for promotion of vascular abnormalities, due to endothelial cells adaptation to stress, and switching to anaerobic metabolism and upregulating several genes, like VEGF [66]. Furthermore, VEGF and other hypoxia-regulated growth factors are controlled by hypoxia-inducible factor (HIF) [67]. HIF is a heterodimer, HIF-1α (inducible subunit) and HIF-1β (constitutively expressed). Oxygen deprivation, induce HIF-1α, the active HIF heterodimer can translocate to the nucleus and bind the hypoxia-response elements (HREs) in DNA, leading to expression of inflammatory and pro-

angiogenic genes, therefore, promoting inflammation and angiogenesis, respectively [68,69].

Retinal Angiogenesis

Angiogenesis is a crucial mechanism in both physiological vascular development and during pathological conditions. Angiogenesis is related to proliferation of endothelial cells, that form new blood vessels under stimulation of some angiogenic factors [70]. The angiogenic process is characterized by several steps: first of all, a number of angiogenic growth factors activate the receptors present on resident endothelial cells; then, the endothelial cells begin to release specific enzymes (proteases, such as matrix metallopeptidases (MMPs)) that degrade the basement membrane, leading endothelial cells to leave the original vessel wall. After that endothelial cells start to proliferate into the surrounding matrix, mechanism promoted by adhesion molecules.

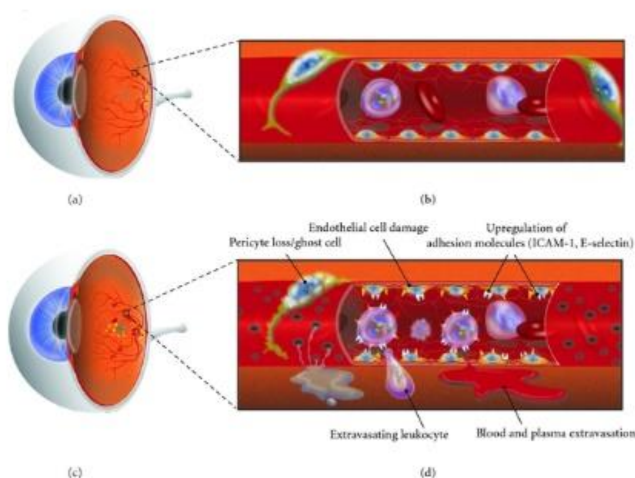

FIGURE 1: Normal eye (a) with intact vasculature (b). Accumulation of microvascular diabetic changes in the eye (c) manifest in adverse cellular changes with ultimate compromise to the blood-retinal-barrier (d) [71]

In case of dysfunctional physiological angiogenesis, altered homeostasis of metabolism could arise, due to irregular delivery of nutrients and oxygen, typical of several eye disorders, like DR [72]. The main regulators of angiogenesis are the vascular endothelial growth factors (VEGF-A, VEGF-B, VEGF-C, and VEGF-D) and the placenta growth factor (PlGF) [73–79]. VEGFs can bind to three tyrosine kinase receptors: VEGFR-1 (Flt-1), -2 (KDR), and -3 (Flt-4) [80]. VEGFR-1 [79,81] and VEGFR-2 [77,82–85] are the main receptors involved in angiogenesis. VEGFR-2 (also known as Flk1) is expressed on endothelial cells (ECs) and on circulating bone marrow–derived endothelial progenitor cells, and is also the principal mediator of VEGF-induced pathological angiogenic signaling. Binding to VEGFR-1 (also known as Flt1) leads to the activation of quiescent endothelial cells and promote vascular permeability [86–89]. Vascular endothelial growth factor (VEGF-A) is the pro-angiogenic factor; and is significantly increased in ocular tissues from patients with diabetes [90,91]. Moreover, VEGF-A is both an endothelial specific mitogen and vascular permeability factor; this means that it could be the causative factor for both proliferation and vasopermeability of new abnormal vessels in PDR [92]. All the mechanism linked to the progression of DR, are responsible of the overproduction of VEGF-A, including also hypoxic events. VEGFA expression consequently increases the expression of other proteins such as ICAM-1, which promotes leukostasis (and vascular leakage) and increases leukocyte counts in the retinas of diabetic animals and humans [51,59,93], exacerbating also inflammatory process. Besides stimulation of endothelial cell growth, VEGF-A can also promote the disassembly of junctions between endothelial cells, leading then to vascular permeability (BRB breakdown).

Recently, several papers have studied the role in retital diseases of another member of VEGF family, the placental growth factor (PlGF). Particularly,

PlGF has been implicated in pathological angiogenesis, especially in retinal disorders, although its function is less well understood [89], compared to VEGFA. Oppositely to VEGF-A, PlGF is not required during physiological angiogenesis but plays a role only during pathological conditions [86,89,94–100].

PlGF is secreted as a glycosylated homodimer and is able to induce angiogenesis through the activation of endothelial cells; for this reason, its role is widely studied also in other types of disease, like cancer and atherosclerosis. Recently, Seung-Ah Yoo et al. have demonstrated that PlGF is secreted by Th17 cells. Additionally, T cells released PlGF is functionally active in promoting angiogenesis [101]. As regards DR, Ao-Wang Qiu et al. demonstrated that Th17 cells infiltrate from systemic circulation into the eye, across retinal endothelium, leading to retinal inflammation [102], possibly due to PlGF production. Secreted PlGF specifically acts through VEGFR-1, that can bind also VEGF-A. On the contrary PlGF cannot activate VEGFR-2. Furthermore, recently it has been showed that VEGF-A and PlGF can form heterodimers, in case of cell co-expression of respective genes[103,104]. The VEGF-A/PlGF heterodimer can bind VEGFR-1, activate dimerization VEGFR-1/VEGFR-2, then stimulating endothelial cell migration, in vitro tube formation, and vasorelaxation via the nitric oxide pathway [105–108]. Moreover, it has been found that VEGF/PlGF heterodimer can lead to activation of a positive feedback, leading to further increase of VEGF-A release, that binds VEGFR-2. Therefore, PlGF may stimulate angiogenesis directly through VEGFR-1 but also indirectly through VEGFR-2 [86,87,99,109,110]. Moreover, PlGF acts through neuropilin receptor 1 (NRP1) [94,98,111–113], that is expressed in angiogenic vessels [114–117]. Moreover, Pan et al. demonstrated that blocking NRP1 inhibits vascular remodelling, leading to vessels more susceptible to treatment with anti-VEGF

agents [118], considering also that NRP1 acts as a co-receptor for several growth factors (VEGF-A, PlGF, TGF-β1, FGF2) [119]. As well as VEGF-A, PlGF is upregulated in endothelial cells exposed to in hypoxic conditions PlGF [120–122]. The hypoxic PlGF overexpression is driven by HIF-1α, which is able to recognize a hypoxia responsive element (HRE) located in the second intron of PlGF gene [120]. Tudisco L. et al demonstrated also, for the first time, that hypoxia activates PlGF expression also in vivo, in particular in lymphatic endothelial cells [123].

Fibrosis

Angiogenesis and subsequent fibrotic events occur with progression of PDR. Fibrosis can cause the formation of fibrovascular, tractional epiretinal membranes, which lead to retinal complications such as tractional retinal detachment and at least vision loss [124–127]. Fibrosis is a complex reparative process that is activated to restore damaged tissue, by means of remodeling extracellular matrix (ECM). Cell proliferation, ECM deposition and neovascularization are key mechanisms during PDR, usually stimulated by pathological conditions like hypoxia or inflammation, promoting formation of fibrotic tissue [128,129]. Muller cells regulating retinal homeostasis seem to play a role also in retinal fibrotic events [30,130–132]. In fact, along with microglia and astrocytes, Muller cells in response to retinal injury participate to fibrotic events, through production of inflammatory and angiogenetic mediators [132,133]. Fibrosis can also be promoted by retinal hypoxia, leading to a consequent overproduction of VEGF-A [134–136]. As mentioned above, inflammation promotes fibrovascular scarring primarily through IL-8 and TNF-α, angiogenic inflammatory cytokines, then promoting endothelial cell proliferation [128]. Several growth factor play a role in fibrosis, VEGF-A but also transforming growth factor-β (TGFβ), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), and the pro-

fibrotic connective tissue growth factor (CTGF) [137–139]. It was demonstrated also how CTGF f in concert with TGF- βprotein induces sustained fibrosis [140]. Furthermore, increased levels of CTGF were found in vitreous of patient with PDR [141,142] and CTGF could be a downstream mediator of TGF-β, main regulator of pro-fibrotic effects.

Pharmacological treatment of DR

Currently, only PDR can be pharmacologically or surgically treated, and no approved treatments already exist for NPDR. Clinical history of PDR has been revolutionized with anti-angiogenic treatments, that are invasive and costly.

The main PDR treatments are [47]:

Photocoagulation. This laser treatment (focal laser treatment) can stop or slow the leakage of blood and fluid in the eye. Focal laser treatment involves delivery of laser burns within the macula for individuals with DME. The laser is applied to leaking microaneurysms. This treatment does not have the same efficacy in visual improvement, if compared to anti-VEGF therapy.

Panretinal photocoagulation. This laser treatment (scatter laser treatment) can shrink the abnormal blood vessels. During the procedure, the areas of the retina away from the macula are treated with scattered laser burns. The burns cause the abnormal new blood vessels to shrink and scar.

Vitrectomy. This procedure uses a tiny incision in the eye to remove blood from vitreous. During vitrectomy scar tissue that is tugging the retina can be removed.

Anti-Inflammatory Therapy. Corticosteroids represents one of the oldest treatments available for DR, and they are still the standard acute therapy for inflammatory diseases, including vitreoretinal diseases, such as vein occlusion, DME, and uveitis [143]. Steroids have a wide spectrum of action,

as they are involved in different biological processes and are used for their anti-inflammatory and anti-edemigene properties. Steroids are powerful drugs for suppression of inflammation and reduction of edema, fibrin deposition, capillary hyperpermeability and phagocytic migration typical of the inflammatory response. Furthermore, they also antagonize the action of VEGF-A and stabilize and reconstitute the blood-retinal barrier [144]. At this time, three synthetic corticosteroids are used commonly for treatment of vitreoretinal disease: dexamethasone (DEX), fluocinolone acetonide (FA), and triamcinolone acetonide (TA) [143]. Corticosteroids have pleiotropic activity, including restoring the structural integrity of tight junctions and reducing paracellular permeability [145]. They also block intracellular signaling of inflammatory lipid mediators, such as prostaglandins and leukotrienes. In several human studies it has been demonstrated that action of corticosteroids results in broad-spectrum reductions of inflammatory molecules; Sohn HJ et al, demonstrated that patients with DME treated with TA showed a reduction of expression of multiple cytokines and chemokines in the aqueous humor, whereas bevacizumab reduced only levels of VEGF, without altering levels of inflammatory molecules [146]. Obviously, ophthalmologist have to consider all the side effects that can occur choosing therapy with corticosteroid. The most common side effects of steroids are cataract and increased IOP, during steroids clinical use for DME, along with conjunctival hemorrhage as side effect related to the administration [143]. Moreover, cell type–specific effects of glucocorticoids in ocular tissues have not well been studied, so it is important to better understand whether corticosteroids regulate different pathways in retinal diseases, in order to select the best therapeutic agent for patients.

Intravitreal anti-vascular endothelial growth factor therapy. The anti-VEGF therapies have revolutionized the treatment of DR. These medications (ranibizumab, aflibercept, pegaptanib, off-label bevacizumab), called vascular endothelial growth factor (VEGF) inhibitors (anti-VEGF), may help stop growth of new blood vessels by blocking the effects of growth signals the body sends to generate new blood vessels. The pegaptanib (Macugen, OSI/Eyetech, New York, NY, USA) was the first anti-VEGF approved by FDA, although its clinical usage was replaced by more effective anti-VEGF, such as the Fab fragment ranibizumab that is capable to bind VEGFA (Lucentis®, Genentech, Inc., South San Francisco, CA, USA), the recombinant decoy receptor aflibercept (EYLEA® or VEGF Trap-eye; Regeneron, Tarrytown, NY, USA) and the full-length monoclonal antibody (mAb) bevacizumab (Avastin®; Genentech, South San Francisco, CA, USA) (off-label), from which ranibizumab has been developed. Ranibizumab is a 48kDa antigen-binding fragment (Fab) fragment of a humanized monoclonal antibody (bevacizumab), produced in Escherichia Coli cells using recombinant DNA technology [147]. Ranibizumab has a higher affinity for VEGF-A than bevacizumab (Fab-12) [148]; it binds with high affinity all the VEGF-A isoforms (such as VEGF-A165, VEGF-A110 and VEGF-A121) reducing the activation of VEGFR1 and VEGFR2 receptors. The small size of this fragment enhances its diffusion from the vitreous to the retina and the choroid, improving the pharmacokinetic profile, compared to bevacizumab [149]. Aflibercept (EYLEA®) is an anti-VEGF approved by FDA in 2011 for the treatment of age related macular degeneration (AMD), for impaired vision due to secondary macular edema caused by retinal vein occlusion (Branch RVO or central RVO, Retinal vein occlusion) and for the treatment of visual impairment due to myopic choroidal neovascularization (CNV choroidal neovascularization). Recently, aflibercept was approved also for the treatment

of diabetic macular edema (DME, Diabetic macular edema). The recommended dose is 2 mg of aflibercept (50 microliters of injected solution). EYLEA® treatment generally start with one injection per month, for three consecutive months; the treatment interval is then extended to 8 weeks. Based on the physician's evaluation of visual and/or anatomic outcomes, the treatment interval may be maintained at 8 weeks or further extended using a treat-and-extend dosing regimen, where injection intervals are increased in 2- or 4-weekly doses to maintain stable visual and/ or anatomic outcomes. Aflibercept (VEGF-trap) is a fusion protein (115 kDa) bearing two binding domains of VEGF receptors, specifically the domain 2 (d2) of VEGFR1 and the domain 3 (d3) of VEGFR2 in the C-terminal region of aflibercept there is the fragment crystallizable region (Fc) of human immunoglobulin (Ig) [150]. The binding domain of aflibercept is named "VEGFR1d2_R2d3" [151]. Aflibercept is a soluble decoy receptor of VEGF-A, VEGF-B, and PIGF with high affinity (VEGF-A121, Kd =0.36 pM; VEGF-A165, Kd =0.50 pM; VEGF-B, Kd =1.92 pM; PIGF, Kd =38.9 pM) [100,152]. Moreover, aflibercept's binding affinity to VEGF-A165 is almost 100-fold greater than ranibizumab and bevacizumab [92,100,153,154].

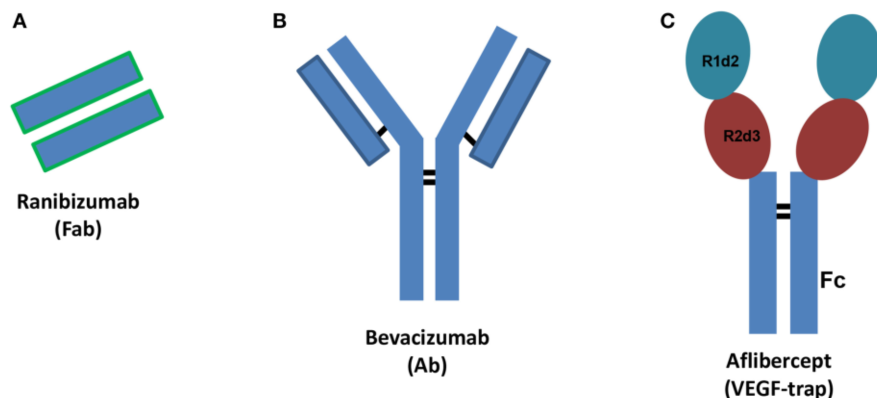

FIGURE 2: Structures schemes of ranibizumab (A), bevacizumab (B), and aflibercept (C). Ab stands for antibody, Fab stands for fragment antigen binding, Fc stands for fragment crystallizable region. R1d2 stands for domain 2 of vascular endothelial growth factor receptor VEGFR1 and R2d3 stands for domain 3 of VEGFR2. Black bars correspond to inter-chain disulfide bridges

Intravitreal ranibizumab has been proven to be more effective than laser monotherapy in patients with clinically-significant DME [155,156]. Furthermore, it has been demonstrated that intravitreal aflibercept was associated with better visual outcomes than laser therapy in patients with DME [157]. Additionally, aflibercept was found to be more effective of ranibizumab and bevacizumab, as regards as the improvement of visual acuity in patients with not advanced acuity loss [158]. Obviously some adverse effects are reported with all anti-VEGF therapy, considering first of all the short half-life of these agents (generally one or two injections every month are required), but also the physiological role of VEGF in the retina, so the use high-dose or for an unlimited period can be deleterious for the retina itself [159].

In-vitro model of DR

Eye colture models (in-vitro) can contribute to the characterization of the pathophysiology of diseases, including diabetic retinopathy. In vitro approaches can address several questions about cell response to high glucose (HG), lipids, glutamate, advanced glycation end products (AGEs) cytokines and oxidative stress. Furthermore, in-vitro models are generally aimed to preliminary screening of drugs (small molecules or biologics), in order to assess drug cell toxicity and efficacy, but also to understand key molecular pathways linked to diabetic retinopathy. These investigations can be carried out on several retinal cell types, pericytes [160], retinal capillary endothelium [161], retinal pigment epithelium [162,163], Müller glia [164] and microglia [165]. Considering that the retina is a multicellular tissue, and studies on one isolated cell-line could be a limited perspective on DR etiopathology, co-culture systems have been largely used to better investigate on some mechanisms, such as BRB integrity, by co-coltures of endothelial cells and perycites, with or without blood monocytes [46,166–170]. The most used in vitro model of DR is represented by mono-coltures o co-coltures of retinal cells challenge with high glucose levels (HG), resembling partially the main stimuli in diabetes mellitus, without events correlated to microvascular anomalies. Other stimuli linked to DR can be resembled in in-vitro model of the disease, such as inflammation (cytokine treatments) and hypoxia. In general, the experimental procedures to mimic hyperglycemia used glucose concentrations ranging from 25 mM to 50 mM. Retinal inflammation can be directly mimicked by inflammatory cytokines cell treatment [171–173], although high glucose levels can in turn lead to increase inflammatory cytokines cell expression [174–176]. Mono-coltures and co-coltures can also be challenged with advanced glycation endproducts (AGEs) [177]. Hypoxia can be induced in-vitro by chemical stimuli or physically, with a hypoxic

chamber (low percentage of O_2). Among chemical inducers, cobalt chloride ($CoCl_2$) has the advantage to be inexpensive and fast, and is commonly used to stimulate the hypoxic response in several cells types [178–180]. The mechanism of action of $CoCl_2$ is related to induced expression of the transcription factor HIF-1/3α [181], which activates the transcriptional induction of many oxygen dependent genes.

In-vivo model of DR

Animal models are used to investigate the pathogenesis of DR. Generally, the progression and complexity of this DR, require the use of various animal species, having different retinal anatomy, metabolism, diets. There are several types of animal models of DR, subclassified as:

- induced animal models of DR; generated through surgical injury, laser o chemical damage-genetic models[182].

Moreover, among induced animal models of diabetes and indeed diabetic retinopathy, the administration of streptozotocin represent the most widely used method [183], for the fast onset of the disease [184], due to impaired secretion of insulin. The most used animal species are rats and mouse [31]. Streptozotocin-diabetic rats have been widely used in drug research & development studies diabetic retinopathy. Streptozotocin-diabetic rats resemble features typical of the early stages of the retinopathy. In fact, afteronly one month of diabetes, retina of diabetic rats show capillary BM thickening, vasopermeability, loss of retinal pericytes and capillary closure [185,186]. Another model, largely used, is represented by Ins2Akita mouse. This strain contains a spontaneous and dominant point mutation in the insulin 2 gene, leading to type 1 diabetes, especially in males [186,187]. The symptoms in heterozygous mutant mice include hyperglycemia,

hypoinsulinemia, polydipsia, and polyuria, beginning around 3-4 weeks of age. Retinal complications (12 weeks after the onset of hyperglycemia) include increased vascular permeability, alterations in the morphology of astrocytes and microglia, increased apoptosis and thinning of the inner layers of the retina [188].

Chapter I

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Aflibercept regulates retinal inflammation elicited by high glucose *via* the PlGF/ERK pathway

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Abstract

Diabetic retinopathy (DR) is a secondary complication of diabetes. DR can cause irreversible blindness, and its pathogenesis is considered multifactorial. DR can progress from non-proliferative DR to proliferative DR, characterized by retinal neovascularization. The main cause of vision loss in diabetic patients is diabetic macular edema, caused by vessel leakage and blood retinal barrier breakdown. Currently, aflibercept is an anti-VEGF approved for diabetic macular edema. Aflibercept can bind several members of vascular permeability factors, namely VEGF-A, B, and PlGF. We analyzed the aflibercept-PlGF complex at molecular level, through an in silico approach. In order to explore the role of PlGF in DR, we treated primary human retinal endothelial cells (HRECs) and mouse retinal epithelial cells (RPEs) with aflibercept and an anti-PlGF antibody. We explored the hypothesis that aflibercept has anti-inflammatory action through blocking of PlGF signaling and the ERK axis in an in vitro and in vivo model of DR. Both aflibercept and the anti-PlGF antibody exerted protective effects on retinal cells, by inhibition of the ERK pathway. Moreover, aflibercept significantly decreased ($p < 0.05$) the expression of TNF-α in an in vitro and in vivo model of DR. Therefore, our data suggest that inhibition of PlGF signaling, or a selective blocking, may be useful in the management of early phases of DR when the inflammatory process is largely involved.

Introduction

Diabetic retinopathy (DR) is one of the main causes of vision loss in developed countries [1]. DR is a chronic progressive disease affecting retinal microvasculature, mainly due to uncontrolled hyperglycemia and other conditions such as hypertension [2,3]. The pathogenesis of diabetic retinopathy is still not completely clear, because hyperglycemia can activate multiple biochemical pathways [4,5]. DR can progress from non-proliferative diabetic retinopathy (NPDR), characterized by retinal microvascular damage, to proliferative diabetic retinopathy (PDR), characterized by retinal neovascularization. One complication of PDR is the diabetic macular edema, which is currently treated with steroids and anti-VEGF agents, injected into the vitreous cavity [6]. The hyperglycemia-induced pathogenesis of diabetic retinopathy has been related to several biochemical alterations, such as increased advanced glycation end-product (AGE) formation and activation of protein kinase C (PKC) isoforms [7–9]. These activated pathways were linked to increased oxidative stress, inflammation, and vascular dysfunction, which can either affect the inner blood retinal barrier (pericytes and endothelial cells) or the external blood retinal barrier (RPE, retinal pigment epithelium), thus leading to vascular occlusion, retinal ischemia and blood retinal barrier (BRB) breakdown. Furthermore, these events cause the upregulation of proangiogenic and inflammatory factors such as the vascular endothelial growth factor (VEGF) [10]. Vascular endothelial growth factor A (VEGF-A) is the main angiogenetic factor that contributes to the development of choroidal neovascularization [11,12]. VEGF-A belongs to the VEGF family, which includes other members: VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) [13,14]. VEGFs exert their biological effects by binding VEGF receptors (VEGFR-1, VEGFR-2 and VEGFR-3) [15]. VEGF receptors are expressed in various cell types, e.g. vascular endothelial cells, epithelial cells, pericytes, monocytes and macrophages [16]. The VEGF-A isoform is upregulated in response to ischemia, hypoxia, inflammation, and trauma. Therefore, the anti-angiogenic therapies have been shown to be effective for the treatment of several eye diseases (neovascular age-related macular degeneration, diabetic macular edema, neovascular glaucoma), via the pharmacological inhibition of VEGF-A signaling [17–21]. Aflibercept is a soluble VEGF decoy receptor (fusion protein) generated with Trap technology and it binds several members of the VEGF family: VEGF-A, VEGF-B and PlGF, which activate the VEGFR1 [12,22]. Therefore, aflibercept can further inhibit VEGFR1 signaling by trapping the PlGF ligand. The signaling of VEGFR1 has been reported to

modulate vascular development, angiogenesis, cell survival, inflammation and chemotaxis of inflammatory cells [23]. Moreover, PlGF is a multifunctional peptide associated with angiogenesis-dependent pathologies in several ocular diseases. In fact, vitreous levels of PlGF positively correlated with retinal ischemia in DR patients [24,25]. Furthermore, it has been demonstrated that a PlGF antibody reduces inflammation and angiogenesis in a mouse model of DR [26]. Regarding biochemical pathways, VEGF signaling is a complex pathway that includes various receptor/growth factor interactions and subsequent activation of kinases. Particularly, VEGF-A and PlGF binding to VEGFR1 leads to phosphorylation and activation of p38 mitogen-activated protein kinase (MAPK) and extracellular signalregulated kinases 1 and 2 (ERK1/2) [27], and also VEGFR2 signaling has been linked to ERK activation, e.g. for mesenchymal stem cell differentiation to endothelial cells [28]. Furthermore, expression of inflammatory cytokines might be mediated by activation of mitogen-activated protein kinases (MAPKs) [29]. The MAPKs, as well as ERKs, are kinases that regulate several cellular processes, such as stress response,

inflammation, proliferation, differentiation, apoptosis, and survival [30]. MAPKs in turn can influence VEGF levels, thus leading to increased vascular permeability and angiogenesis [31]. Moreover, it has been reported that the ERK pathway can influence NF-κB activation, by the regulation of NF-κB-dependent genes expression, e.g. inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and tumor necrosis factor-alpha (TNF-α) [32]. Firstly, we assessed at the atomistic level (molecular dynamics simulations and prediction of binding free energy of complexes) the interaction of aflibercept with PlGF and compared this complex with the all-atom model of aflibercept bound to VEGFA. After the in silico analysis, to study the mechanism of high glucose (HG)-induced inflammation in human retinal endothelial cells (HRECs) and retinal pigmented epithelial (RPE) cells, we hypothesized that the effects of HG on the expression of inflammatory cytokines might be mediated by the ERK pathway. In order to evaluate and discriminate the effects of aflibercept and anti-PlGF antibody on the ERK pathway, we evaluated the expression levels of VEGF-A, PlGF and TNF α in two in vitro models. Moreover, we analyzed TNF-α expression in retinas of diabetic rats.

Material and methods

In silico studies

Molecular modeling of the binding domain of aflibercept (VEGFR1d2_R2d3) was carried out as reported previously [33]. Structures of VEGF-A and PlGF were retrieved from the Protein Data Bank repository, PDB:1CZ8 and PDB:1RV6, respectively. In the 1CZ8 structure VEGF-A is in complex with Fab of bevacizumab, whereas in 1RV6 PlGF is in complex with domain 2 of VEGFR1. VEGFR1d2_R2d3, VEGFA and PlGF structures were optimized with an energy minimization protocol, before protein-protein docking. Protein-protein docking was carried out by accessing to the web server PyDock, as previously described [33]. VEGFR1d2_R2d3/VEGFA and VEGFR1d2_R2d3/PlGF complexes were subjected to all atom molecular dynamics simulations (MD) in water environment with GROMACS 4.6 [34]. Three equilibration steps were carried out before MD simulation (20 ns per each independent replica, 3 replicas per simulated complex). The simulation protocol was previously described [33]. We herein analyzed the root mean square deviation (RMSD), root mean square fluctuation (RMSF) and number of contacts (within 3.5 Å) at the protein-protein interface. Low and stable RMSD is commonly considered as an index of structural stability of protein during simulation. However, RMSF is more descriptive of conformational stability of proteins, than RMSD. Molecular Mechanics-Poisson Boltzmann Surface Area calculation (MM-PBSA) was carried out on three independent replicas of MD simulations of VEGFR1d2_R2d3/VEGFA and VEGFR1d2_R2d3/PLGF complexes. MMPBSA calculations are time-consuming and generally aimed at the evaluation of each energetic contribution (electrostatic, Van der Waals, polar and apolar free energy) to overall binding free energy [33].

Materials

Rabbit phospho-p44/42 MAPK (phospho-Erk1/2, catalog n. 9101), rabbit p44/42 MAPK (Erk1/2, catalog n. 9102), rabbit anti-TNF-α (catalog n. 11948) and rabbit anti-vinculin (catalog n. 13901) were purchased from Cell Signaling Technology (Danvers, MA); mouse anti- GAPDH (catalog n. 2118) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary goat anti-rabbit IRDye 800CW conjugated antibody (#926-32211) and secondary goat anti-mouse IRDye 680LT, (#926-68020) were purchased from LI-COR (Lincoln, NE). Aflibercept (Eylea ®) was purchased from Bayer Pharma (Berlin, Germany). The anti-PlGF was purchased from Thrombogenics (Leuven, Belgium).

In vitro studies

Human retinal endothelial cells (HRECs) were purchased from Innoprot® (Derio – Bizkaia, Spain). Cells were cultured at 37 °C, in humidified atmosphere (5% CO2), in Endothelial cell medium (ECM) supplemented with 5% fetal bovine serum (FBS), 1% ECGS (Endothelial Cell Growth Supplement) and 100 U/ml penicillin 100 µg/ml streptomycin. After reaching confluence (approximately 70%), cells were starved for 16 h; then the medium was shifted to an ECM supplemented with 2.5% FBS. Cells growth in medium containing 5mM glucose (physiological glucose concentration) served as control group. HRECs were also exposed to medium containing 40mM glucose (high glucose, HG) [35]. HRECs, exposed to HG, were treated with aflibercept (1, 10, 40 and 100 µg/ml) or anti-PlGF (1, 10, 25 and 50 µg/ml) for 48 h. Primary mouse RPE cells (pmRPE) were isolated from 6 to 8 weeks-old C57Bl6/J. Male mice C57Bl6/J, weighing 20–25 g, were purchased from Charles River (Calco, Italy). Retinas sampling from mice was carried out in accordance with European directives no. 2010/63/UE and Italian directives D.L. 26/2014. Experimental protocols were approved by the Italian Ministry of Health (authorization no. 695/2015- PR of July 17, 2015). Mouse sacrifice was carried out through cervical dislocation. Enucleated eyes from sacrificed mice were cleaned of fat and extra tissues and incubated in a 1:1 mixture of 0.8 mg/ml collagenase and 4% dispase. Then, the cornea and anterior segments were removed and the 'eye cup' was flattened with four incisions, rinsed in chelating agent (Versene; Invitrogen, Life Technologies, Carlsbad, CA), then incubated with 0.48mM EDTA and incubated for 10 min in a moisture box with 2% dispase. Eye cups were washed with PBS, therefore, retinas were gently removed and the RPE were digested with 2% dispase for 10 min at 37 °C. The digested eye cup was then washed with PBS in order to remove the dispase solution; 20 µL of DMEM 10% FBS was added and RPE were then gently scraped from the "eye cup" in a 12 well plate (1 eye/well), by rubbing the cup against the bottom of the well with a pipette tip. Finally, 20% FBS DMEM with antibiotics was added in the plate wells and cells were grown until confluence. RPE cells were pooled and growth in DMEM (Dulbecco's Minimal Essential Medium, Euroclone, Milan Italy) containing 20% FBS and standard antibiotics at 37 °C in a humidified environment containing 5% CO2. RPE cells (passage 5) were plated at 70–80% of confluence in DMEM+20% FBS. After 24 h, medium was replaced with DMEM+1% FBS containing normal glucose (5 mM) or high glucose (25 mM). The RPE cells were treated with equimolar amounts of aflibercept (1, 10 and 50 µg/mL) or anti-PlGF (1.3, 13 and 65 µg/mL) for 24 h.

Cell viability

HRECs viability was evaluated with quantification of LDH levels in cell media. Cells were seeded in 96-well plates at a density of $2x10^4$ cells/well, to obtain optimal cell density. LDH release was assessed with a commercial kit (Roche Diagnostics, Basel, Switzerland) and reported as LDH (% control): (absx÷absctrl+) × 100. In the equation absx is absorbance in the x well, and absctrl+ is the average absorbance of positive control cells (retinal endothelial cells lysed accordingly to manufacturer's protocol); absorbance values were corrected by subtracting blank. The absorbance values were measured at 450 nm using a microplate reader (VarioSkan, Thermo Fisher Scientific, Waltham, MA).

Western Blot

HRECs were cultured in 60mm Petri dishes ($2x10^5$). Proteins of whole cell lysates were extracted with RIPA Buffer, including protease and phosphatase inhibitors cocktail (Sigma-Aldrich, St. Louis, MO). Total protein content, in each cell lysate sample, was determined by means of the BCA Assay Kit (Pierce™ BCA Protein Assay Kit, Invitrogen, Life Technologies, Carlsbad, USA). Extracted proteins (30 µg) were loaded on 4–12% tris–glycine gel. After electrophoresis, proteins were transferred into a nitrocellulose membrane (Invitrogen, Life Technologies, Carlsbad, CA). Immunoblot was preceded by 30 minutes incubation with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) to membranes. Membranes were incubated overnight (4 °C) with appropriate primary phospho-p44/42 MAPK (1:500 dilution), p44/42

MAPK (1:500 dilution) and anti-GAPDH (1:500 dilution) antibodies. After overnight incubation, the membranes were then incubated with secondary fluorescent antibodies (1: 20,000 dilution) for 1 h at room temperature. Immunoblot was detected through Odyssey imaging system (LI-COR Biosciences, Lincoln, NE). Densitometry analyses of blots were performed at non-saturating exposures and analyzed using the ImageJ software (NIH, Bethesda, MD; available at http://rsb.info. nih.gov/ij/index.html). Values were normalized to GAPDH, which was also used as loading control. Total protein extracts from pmRPE cells were obtained by cell lysis in Tris-HCl 20mM pH 8, NaCl 150 mM, Triton X-100 1%, EDTA 10 mM, Glycerol 10% and ZnAc 1mM supplemented with protease and phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). The protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Milan, Italy). 100 µg of total protein extracts were separated in a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% nonfat dry milk in Tris-buffered

saline-Tween 0.1% (TBS-T) and then probed with antibodies against: p-ERK (1:1000), ERK (1:1000), TNF-α (1:1000) and Vinculin (1: 10,000), purchased from Santa Cruz Biotechnology, Heidelberg, Germany. The secondary antibodies were purchased from DAKO Agilent Santa Clara, CA (1:10,000). The signals were visualized by chemiluminescence using ECL substrate (Advansta, San Jose, CA). Densitometric analyses were performed using ImageJ software (NIH, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

Extraction of total RNA and cDNA synthesis

Extraction of total RNA, from HRECs, was performed with TRIzol Reagent (Invitrogen, Life Technologies, Carlsbad, CA). The A260/A280 ratio of the optical density of RNA samples in agarose gel electrophoresis blots (measured with Multimode Reader Flash, VarioSkan™) was 1.95–2.01, assessing the adequate RNA purity. cDNA was synthesized from 2 μg RNA with a reverse transcription kit (SuperScript™ II Reverse Transcriptase, Invitrogen, ThermoFisher Scientific, Carlsbad, CA).

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Real-time RT-PCR was carried out with the LightCycler ® 2.0 (Real- Time PCR System Roche Life Science, Roche Diagnostics GmbH, Mannheim, Germany). The amplification reaction mix included iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Milan, Italy) and 1 μL (100 ng) of cDNA. Forty-five amplification cycles were carried out for each sample. Results were analyzed with the $2^{-\Delta\Delta Ct}$ method. VEGF and PlGF mRNAs expression (in vitro experiments) were normalized to β-actin mRNA levels. On the contrary, TNF-α mRNA (in vivo experiments) expression was normalized to 18S mRNA. Primers were purchased from Eurofin Genomics (Milan, Italy). Quantitative PCR experiments followed the MIQE guidelines. Forward and reverse primer sequences (for human and rat analyzed genes) are herein listed: i. human β-actin (Forward 5′-TCCACCTTCCAGCAGATGTG-3′, Reverse 5′-GCATTTGCGGTGGACGAT-3′); ii. human PlGF (Forward 5′-ATGTTCAGCCCATCCTGTGT-3′, Reverse 5′-CTTCATCTTCTCCCGCAGAG-3′); iii. human VEGF-A (Forward 5′-GAGGTTTGATCCGCATAATCTG-3′, Reverse 5′-ATCTTCAAGCCATCCTGTGTGC- 3′); iv. rat TNF-α (Forward 5′-ACCACGCTCTTCTGTCTACTG-3′, Reverse 5′CTTGGTGGTTTGCTACGAC-3′); v. rat 18S (Forward 5′-CATTCGAACGTCTGCCCTAT-3′, Reverse 5′GTTTCTCAGGCTCCCTCTCC-3′).

Enzyme-linked immunosorbent assay (ELISA)

HRECs were cultured in 60mm Petri dishes (2×10^5). VEGF-A and TNF-α levels were quantified in 200 μL cell media. Commercial ELISA kits (ENZ-KIT156-0001 and ADI-900-099, Enzo Life Science, Farmingdale, NY) were used for VEGFA and TNF-α detection, respectively. Total protein content in cell medium was analyzed prior to the ELISA assay, accordingly to the manufacturer's instructions.

In vivo studies

Sprague-Dawley rats (200–250 g) were purchased from Envigo (San Pietro a Nadisone, Udine, Italy). Animals were housed under standard conditions, with free access to standard chow and water, in a lightcontrolled (12-h light/12-h dark; lights on at 6 am) room with standard temperature and humidity conditions (21 ± 3 °C and 54 ± 4% humidity). Animals were randomly assigned to four experimental groups: 1) negative control group (Ctrl) non-diabetic rats (n=4) injected (i.p.) with citrate buffer, and after 15 days injected intravitreally with vehicle; 2) non-diabetic rats (n=4) treated with intravitreal injection of aflibercept (160 μg) (Ctrl+afl group); 3) diabetic rats (STZ group; glycemia > 250 mg/dl) injected (i.p.) with streptozotocin (STZ) and after 15 days injected intravitreally with vehicle (n=4); 4) diabetic rats (STZ+afl group; glycemia > 250 mg/dl) injected (i.p.) with streptozotocin (STZ) and after 15 days injected intravitreally with aflibercept (n=4). Animals were sacrificed 48 h after intravitreal injection (vehicle or 160 μg aflibercept). Rats were treated accordingly to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Protocols were approved by the Italian Ministry of Health (authorization no. 1172/2016-PR of November 13, 2016). Diabetes was induced with a single injection (i.p.) of streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO) as previously described [36,37]. After 12 h fasting, two groups of animals (STZ and STZ+afl groups) received a single injection of STZ (60 mg/kg body weight) in 10mM sodium citrate buffer, pH 4.5 (1 mL/kg dose/volume). The control group of animals received a (4 μL) injection of citrate buffer. Twenty-four hours post STZ injection blood glucose levels were measured. Rats with blood glucose levels>250 mg/dL were considered diabetic. Glycaemia was monitored with a blood glucose meter (Accu–CheckActive; Roche Diagnostic, Milan, Italy), along with rat body weight. Two weeks after STZ administration, diabetic rats received by intravitreal administration, 160 μg of aflibercept dissolved in PBS solution (final volume=4 μL). This dose, converted to human equivalent dose (HED) 1.3 mg/ml (160 μg/20 μL of ratvitreous=8 mg/ml×0.162 [38]), is comparable with the three monthly intravitreal

doses of aflibercept (2 mg/4.4 mL of human vitreous×3= 1.35 mg/ml aflibercept dose). Before intravitreal injection, animals were anesthetized by intravenous injection of 5 mg/kg Zoletil® (tiletamine HCl and zolazepam HCl, Virbac, Milan, Italy), and 1 drop in each treated eye of local anesthetic 0.4% oxybuprocaine (Novesina®, Novartis, Origgio, Italy). Vehicle treated animals were subjected to intravitreal injection of 4 µL of PBS solution (pH 7.4). Aflibercept treated animals received 4 µL intravitreal injection of aflibercept (40 µg/µL) solution. Forty-eight hours after treatment, rats were killed by CO2 inhalation, the eye enucleated, the retina collected and treated with TRIzol reagent in order to extract total RNA (methods 2.3.3 and 2.3.4) or RIPA buffer (ThermoFisher Scientific, Carlsbad, CA), including protease and phosphatase inhibitors cocktail (Sigma-Aldrich, St. Louis, MO) for ELISA quantification of retinal TNFα (methods 2.3.5). TNFα quantification in the rat retina was carried out following manufacturer's instructions of ELISA kit, and RIPA buffer was used as blank within plate preparation procedures. We loaded retinal lysate samples containing 40 µg proteins, determined as described in methods Section 2.3.2. TNFα levels were normalized to total retinal protein content.

Statistical analysis

All results are reported as mean ± SD, experiments were carried out at least 4 times, each run in triplicate. The results were analyzed using one-way ANOVA followed by Tukey-Kramer post-hoc multiple comparisons test. Differences between groups were considered significant for p-value < 0.05. Graph design and statistical analysis were carried out using GraphPad Prism 5 software (GraphPad Inc., San Diego, CA).

Results

PlGF interacts preferentially with the VEGFR1 domain 2 of aflibercept The output of PyDock is a score used for rough prediction of binding energy of protein complexes. Furthermore, the PyDock score provided some hints about the weight of each component to the binding free energy (ΔG binding) of VEGFA- and PlGF-aflibercept complexes. VEGFA binding to aflibercept, as predicted by PyDock, was characterized by a better desolvation energy (-12 Kcal/mol), if compared with PlGF-aflibercept complex (-2 Kcal/mol). Moreover, PyDock predicted for the PlGF/aflibercept complex a slightly better electrostatic energy (-18 Kcal/mol), compared to the VEGFA/aflibercept complex (-11 Kcal/ mol). Van der Waals (VdW) component of PyDock score was similar for PlGF and VEGF bound to the aflibercept binding domain, −38 and −34 Kcal/mol, respectively. In order to carry out binding energy prediction, with less approximations as applied by PyDock, 20 ns molecular dynamics simulations of the aflibercept binding domain (VEGFR1d2_R2d3) in complex with VEGFA and PlGF were carried (3 independent replicas for each system). Aflibercept binding domain stabilized more VEGFA (average RMSD 0.32 ± 0.01 nm, whole complex, over 2000 steps of three replicas) than PlGF (average RMSD 0.36 ± 0.01 nm, whole complex over 2000 steps of three replicas) (Fig. 1). RMSD was also calculated for each interactors of complexes: VEGFR1d2_R2d3, VEGFA or PLGF. VEGFA bound to the aflibercept binding domain was more stabilized (RMSD 0.20 ± 0.01 nm, over 2000 steps of three replicas), in comparison with PLGF bound to aflibercept (RMSD 0.30 ± 0.02 nm, over 2000 steps of three replicas). Furthermore, the analysis of root mean square fluctuation (RMSF) of α-carbons revealed that the VEGFR1d2_R2d3/VEGFA complex was more stable than VEGFR1d2_R2d3/PLGF (Fig. 2). We have calculated the number of contacts at protein–protein interface, applying a 0.35 nm cut-off, which takes into account coulombic and Van der Waals (VdW) interactions. The complex VEGFR1d2_R2d3/VEGFA formed during simulation 290 ± 2 contacts (over 2000 steps of 3 replicas); while the VEGFR1d2_R2d3/PlGF complex formed 260 ± 20 (over 2000 steps of 3 replicas) at protein–protein interface. Additionally, we have identified the main residue-residue interactions at the interface of each VEGFR1d2_R2d3/growth factor complex (Fig. 3, Table 1). Table 1 shows the interactions also shown in Fig. 3; these interactions mainly involved the domain VEGFR1_d2 of aflibercept and the loop 1 of growth factors. Moreover, VEGFA formed through Asp34 a stable salt bridge with the N atom in the amide bond of Glu159, which is in VEGFR2 domain 3 of aflibercept (not shown in Fig. 3). Furthermore, MM-PBSA calculations were carried on three replicas of MD of complexes. MMPBSA results compared with experimental binding affinity data [39] are shown in Table

2. The predicted binding energy of VEGFR1d2_R2d3/ PlGF complex was less negative (less favorable) than predicted binding energy of VEGFR1d2_R2d3/VEGFA, accordingly to experimental binding affinity results [39].

Aflibercept and anti-PlGF treatment protect human retinal endothelial cells from high glucose insult

We assessed the protective effect of aflibercept and anti-PlGF treatment on HRECs damaged by high-glucose levels, by LDH assay. After 48 h, high glucose levels induced a significant increase of LDH release (Fig. 4), compared to control cells grown in medium with physiological glucose levels (*$p < 0.05$ vs. Ctrl). LDH release was significantly reduced, in a concentration-dependent manner, after aflibercept (Fig. 4A) and anti-PlGF treatment (Fig. 4B), compared to cells grown in high glucose medium (HG group) (†$p < 0.05$ vs. HG). Modulation of VEGF-A cell release by aflibercept or anti-PlGF treatments was evaluated in HRECs, challenged with high glucose levels. After 48 h of high glucose concentration exposure, VEGF-A protein levels were significantly increased in HRECs, compared to control cells. The aflibercept (Fig. 5A) and anti-PlGF (Fig. 5B) treatments were able to reduce significantly ($p < 0.05$) VEGF-A protein levels. Moreover, we evaluated the expression of VEGF-A (Fig. 6A) and PlGF (Fig. 6B) by quantitative PCR measurements. The VEGF-A and PlGF mRNA levels were significantly increased in HRECs challenged with high glucose levels, compared to control cells. The results from the ELISA assay were confirmed by quantitative PCR and both aflibercept and anti-PlGF treatments reduced significantly ($p < 0.05$) the VEGF-A mRNA levels in HRECs exposed to hyperglycemia insult (Fig. 6A). Furthermore, similarly to the effects on VEGFA expression, the treatment with aflibercept and the anti-PlGF antibody decreased PlGF mRNA levels, in HRECs challenged with high glucose levels (Fig. 6B).

ERK/TNF-α inflammatory pathway is modulated by aflibercept and anti-PlGF treatment on retinal pigmented epithelial cells and endothelial cells

After 24 h of high-glucose exposure, the ERK phosphorylation levels were significantly increased in retinal pmRPE cells, compared with control cells (Fig. 7). While, after 48 h, the ERK pathway was significantly activated in HRECs exposed to high glucose levels, compared with control cells. Treatment with aflibercept and anti-PlGF significantly reduced ERK levels in a concentration-dependent manner both in retinal pigmented primary mouse cells (Fig. 7) and in human retinal endothelial cells (Fig. 8 A, B). We also tested the effect of aflibercept and anti-PlGF treatment on the expression of the inflammatory cytokine TNF-α,

which is downstream to the ERK pathway. The aflibercept and anti-PlGF antibody treatment significantly ($p < 0.05$) reduced TNF-α levels in pmRPE cells, compared with non-treated cells, 24 h after exposure to HG levels (Fig. 9). The anti-inflammatory action of aflibercept and anti-PlGF antibody in terms of TNF-α levels was also demonstrated in HRECs exposed to high glucose levels for 48 h (Fig. 10).

Retinal TNF-α levels in diabetic rats are attenuated by aflibercept

We tested the anti-inflammatory effect of aflibercept in an in vivo model of diabetic retinopathy. Diabetes was induced in rats by STZ (60 mg/kg), and after 15 days (glycemia > 250 mg/dL) we intravitreally injected aflibercept (160 μg). It should be noted that the dose of aflibercept used in our study (160 μg) was superimposable to the dose used in clinical practice [40]. Rats were sacrificed 48 h after pharmacological treatment. Diabetes induction significantly increased TNF-α expression in the retina of diabetic rats (STZ group), compared to control rats (citrate buffer, Fig. 11A). The intravitreal injection of aflibercept in non-diabetic rats did not affect retinal TNF-α levels compared to control non-diabetic rats. On the contrary, aflibercept significantly ($p < 0.05$) reduced the retinal TNF-α expression in diabetic rats (STZ+AFL, Fig. 11A), compared with vehicle-treated diabetic rats (STZ). TNF-α protein levels in retina of diabetic rats were measured with ELISA kit, accordingly to qPCR analysis TNF-α retinal levels were increased in diabetic rat, compared to control animals (Fig. 11B). Aflibercept intravitreal injection effectively decreased TNF-α protein levels in the retina of treated diabetic rats, compared to untreated animals (Fig. 11B)

Discussion

Aflibercept (VEGF-trap agent) is currently used for treatment of retinal diseases such as age-related macular degeneration (AMD), retinal vein occlusion and diabetic macular edema. It has been reported that aflibercept, through its binding to VEGF-A and PlGF, can effectively inhibit the signaling activated by these ligands [39]. Aflibercept therapeutic action is accounted mainly to the neutralization of VEGF-A activity, therefore preventing the binding and activation of its receptors (VEGFR1 and VEGFR2). The binding of PlGF to aflibercept was experimentally found to be less favorable (38.9 nM dissociation constant – KD), compared to VEGFA/ aflibercept complex formation (0.49 nM dissociation constant – KD). In agreement with experimental data [39] we demonstrated through in silico studies (molecular dynamics combined with MM-PBSA calculations), that PlGF forms less stable

non-covalent interactions with aflibercept, compared with VEGFA. The binding of PlGF and other growth factors to VEGFR1-2-3 receptors was previously studied, by molecular dynamics simulations [41]. However, the authors did not carry out specific energetic analysis of growth factors/receptor complex formation, and analyzed RMSD profile of a single replica of each complex [41]. On the contrary, we applied a previous published approach [33,42,43], that combines molecular dynamics, MM-PBSA calculation and statistical analysis to obtain prediction of energetic components of binding free energy of complex formation. Indeed, we described for the first time the binding of aflibercept (fusion protein and decoy receptor) to PlGF and compared its properties to aflibercept/VEGFA complex. Particularly, our in silico studies confirmed that PlGF preferentially binds the VEGFR1 domain 2 of aflibercept, as previously reported [44,45], while VEGFA forms a stable salt bridge with a residue of VEGFR2_d3 domain of the aflibercept binding domain. Moreover, we found that the binding of aflibercept to growth factors (VEGA and PlGF) is mainly guided by electrostatic interactions, whereas ranibizumab and bevacizumab interact with VEGFA mainly through VdW interactions [33]. It is known that VEGF-A plays a central role in both physiological and pathological conditions, whereas the role of PlGF seems to be specific to pathological conditions [36]. It has been demonstrated that the loss of PlGF significantly protects mice against retinal neovascularization and hypoxic ischemic retinopathy [37,38]. Indeed, the aflibercept's capability to bind both VEGFA and PlGF could deliver additional beneficial effects in diabetic retinopathy, compared with biologics that are capable of binding only VEGFA. Therefore, on the basis of previous reports, reporting the detrimental role of PlGF in the development and progression of diabetic retinopathy [24,46,47], we tested in vitro and in vivo the hypothesis that aflibercept, by binding also to PlGF, could exert an anti-inflammatory action in the diabetic retina. We demonstrated that inhibition of PlGF signaling improved the diabetes associated inflammation in retinal cells exposed to high glucose levels, through the administration of aflibercept and of an anti-PlGF antibody. The latter binds PlGF selectively, directly influencing the activation of VEGFR1 and indirectly the activation of VEGFR2 (heterodimeric form with VEGF-A), while as stated before aflibercept can bind different isoforms of VEGF, including PlGF. In the current study we used human retinal endothelial cells (HRECs) and mouse primary retinal pigmented epithelial cells (RPE) exposed to high glucose levels, as in vitro models of the early insult occurring in diabetic retinopathy and in two different areas of the retina. We demonstrated that both aflibercept and anti-PlGF antibody protected human RECs and mouse RPE cells from high glucose damage, by blocking the activation of the ERK pathway with the subsequent suppression of

TNF-α release. This effect could be related to regulation of NF-kB signaling [32,48]. According to previous findings, we also found that high glucose induces the overexpression of PlGF and VEGF-A [49,50]. We found that HRECs, challenged with high glucose levels, were protected from cell death after treatment with aflibercept and anti-PlGF. Therefore, the inhibition of PlGF signaling may protect the inner blood retinal barrier. Moreover, aflibercept and anti-PlGF treatment decreased also VEGFA and PlGF release by HRECs challenged with high glucose levels. It has recently been demonstrated [26] that anti-PlGF antibody and aflibercept were able to attenuate the inflammatory response in a choroidal neovascularization mouse model, on the contrary, the selective anti-VEGF antibody had no effect on inflammation. These findings highlight a possible favorable effect of blocking PlGF, compared to canonic VEGF-inhibitors, for which possible concerns have been reported as regards potential side effects [51]. High glucose can activate several biochemical pathways, including AKT and ERK [52]. Besides AKT and ERK pathways, toll-like receptors [53] and receptors for advanced glycation end-products (RAGE) were found to be activated in several models of diabetic retinopathy; furthermore, their activation was directly linked to NFκB activation and transcription of pro-inflammatory cytokines, such as IL-1β [9,54,55]. Previous data, borrowed from cancer research, showed that PlGF activates ERK1/2 phosphorylation [56]; which is involved in triggering of inflammation [29,30,32]. Similar effects on ERK activation were also observed in conditions where VEGFA was overexpressed. We herein found that block of PlGF signaling, by aflibercept and anti-PlGF treatments, inhibited ERK phosphorylation in HRECs and primary mouse RPE challenged with high glucose levels (Fig. 12). Both aflibercept and PlGF treatments were able to inhibit EKR activation along with TNF-α release, the downstream protein of this pathway. The findings obtained from the in vivo model of diabetic retinopathy supported this hypothesis, because rat retinal TNF-α levels were significantly decreased by aflibercept treatment. The present findings show that aflibercept has an anti-inflammatory action through the PlGF/ERK pathway. Furthermore, our results support the development of a new strategy to counteract diabetic retinopathy by selective inhibition of the PlGF signaling pathway. Particularly, the present data suggest that PlGF signaling can modulate the retinal inflammatory response elicited by high glucose through ERK and TNF-α regulation. This approach could avoid the over-activation of VEGFR1 maintaining physiological actions of VEGF, without inducing VEGF inhibitors treatment-related complications. In conclusion, our data suggest that PlGF may be considered a selective target to manage diabetic retinopathy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Fig. 1. RMSD of VEGFR1d2_R2d3/VEGFA and VEGFR1d2_R2d3/PlGF complexes (3 independent replicas of 20 ns simulations). RMSD profiles of VEGFR1d2_R2d3/VEGFA and VEGFR1d2_R2d3/PlGF complexes. Black lines represent replica 1, red lines represent replica 2 and green lines represent replica 3 of each simulated system. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

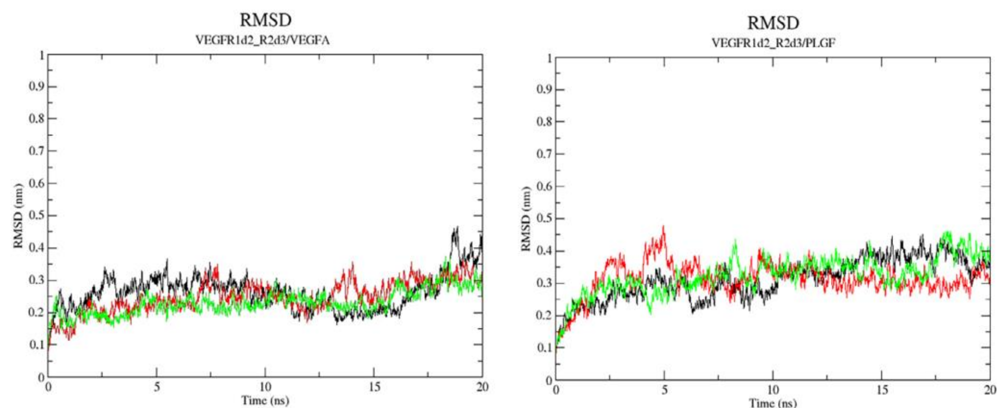


Fig. 2. Mean RMSF of complexes during 20 ns of simulations. Red line represents RMSF of VEGFR1d2_R2d3/PlGF and black line represents RMSF of VEGFR1d2_R2d3/VEGFA. The RMSF profile is the mean of 3 replicas of each simulated complex during 20 ns of MD simulations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

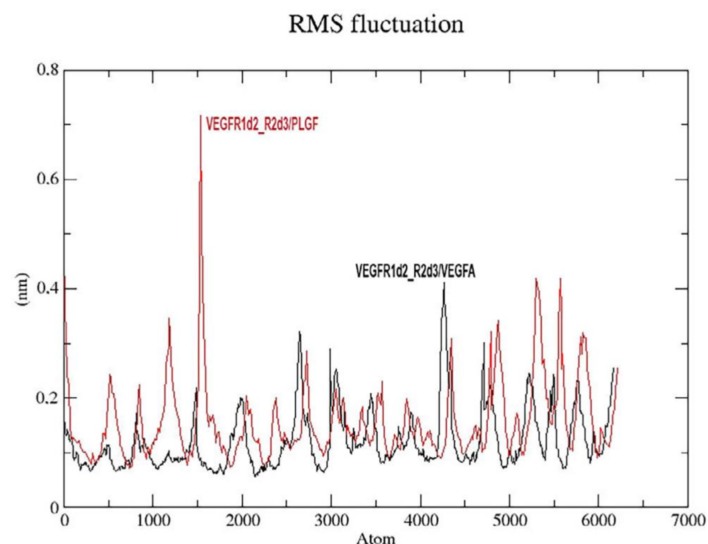


Fig. 3. Main contacts at VEGFR1d2_R2d3 and growth factor interface. Panel A. VEGFR1d2_R2d3/VEGFA; panel B VEGFR1d2_R2d3/PlGF. Magenta cartoon represents the VEGFR1d2_R2d3 bound to dimer of growth factors (green and cyan cartoons). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

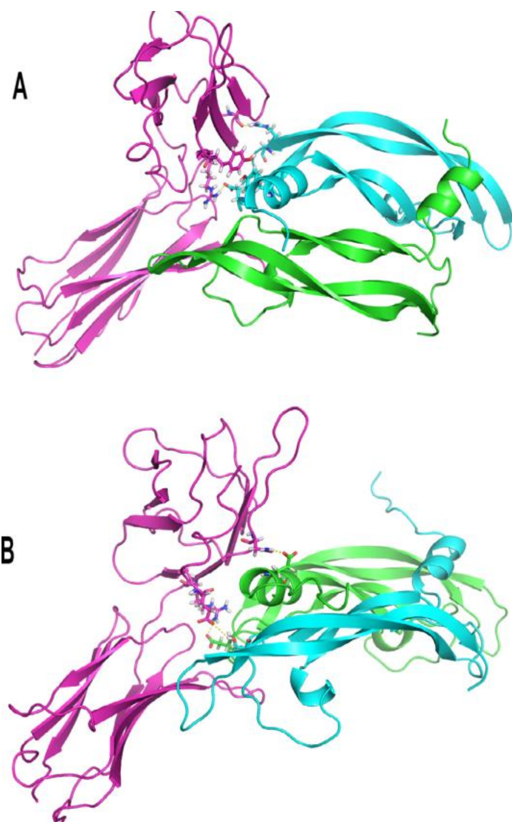


Table 1. Interactions between aflibercept binding domain and growth factors. Residue X of aflibercept directly interacts with residue Y of growth factors.

Complex	X-Y interacting residues
VEGFR1d2_R2d3/VEGFA	Asn91-Glu11 Hbond
	Glu16-Ser40 Hbond
	Arg96-Asp65 Hbond
VEGFR1d2_R2d3/PlGF	Glu73-Arg103 Hbond
	Arg96-Asp63 Hbond
	Tyr 92-Tyr 21 π-π stacking

Table 2. Energetic contribution to binding free energy.

Complex	Binding Parameters			MM-PBSA Energy terms (KJ/mol)				
	$K_{on}/10^5$ ($M^{-1}\ s^{-1}$)	$K_{off}/10^{-5}$ (s^{-1})	K_D (pM)	$\Delta E_{binding}$	ΔE_{VdW}	$\Delta E_{electrostatic}$	ΔG_{Polar}	Δ
VEGFR1d2_R2d3/VEGFA	410	2.01	0.49	-1440±90	-307±50	-1433±100	1050±100	-
VEGFR1d2_R2d3/PLGF	17.5	6.81	38.9	-1270±10	-280±40	-1170±40	840±70	-

Fig. 4. LDH assay. Aflibercept (1–100 μg/mL) (A) and anti-PlGF (1–50 μg/mL) (B) protect human retinal endothelial cells from high glucose-induced LDH release, 48 h after incubation. Data are shown as % of control LDH release. Ctrl stands for cells grown in medium containing 5mM glucose concentration. HG stands for cells grown in medium containing 40mM glucose concentration Values are reported as mean ± SD (n=8; each run in triplicate). *p < 0.05 vs. control; † p < 0.05 vs. HG, ‡ p < 0.05 vs 10 μg/mL aflibercept or anti-PlGF, • p < 0.05 compared to 25 μg/mL anti-PlGF treatment.

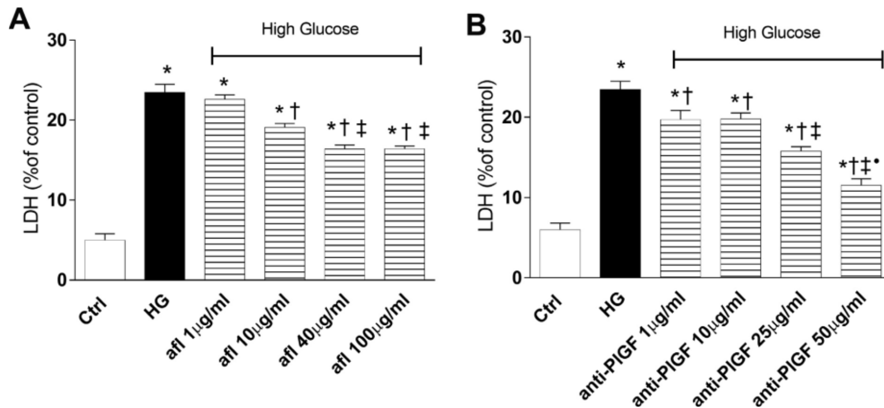


Fig. 5. VEGF-A ELISA. Aflibercept (1–100 μg/mL) (A) and anti-PlGF (1–50 μg/mL) (B) decreased VEGF-A release induced by high glucose levels, 48 h after incubation. Values are reported as mean ± SD (n=8; each run in triplicate). * p < 0.05 vs. control; † p < 0.05 vs. HG. ‡ p < 0.05 vs 10 μg/mL aflibercept or 25 μg/ mL anti-PlGF.

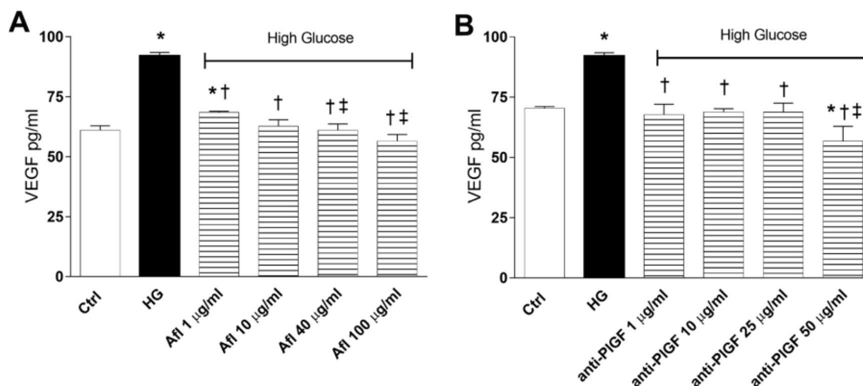


Fig. 6. Real-time PCR. Aflibercept and anti-PlGF reduced VEGF-A (A) and PlGF (B) mRNA expression. HRECs were treated for 48 h with high glucose concentrations (40 mM), aflibercept (40 µg/ml), and anti-PlGF (25 µg/ml). The mRNA levels were evaluated by qPCR. Each bar represents the mean value ± SD (n=4; each run in triplicate). *p < 0.05 vs. control; †p < 0.05 vs. HG.

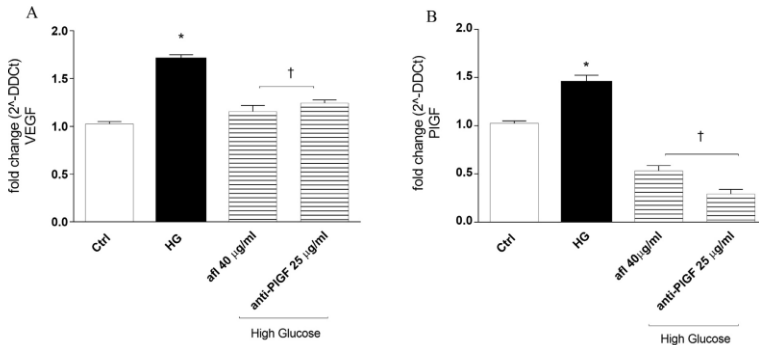


Fig. 7. Western Blot pERK with aflibercept and anti-PlGF in pmRPE cells. Aflibercept and anti-PlGF effect on the inflammatory pathway activated by high glucose (HG) in pmRPE cells. Immunoblot analysis of ERK1/2 phosphorylation in lysates from pmRPE treated for 24 h with HG, aflibercept (50 µg/mL) and anti-PlGF (65 µg/mL). Bar graphs show the densitometry analysis of each band, carried out with the Image J program. Each bar represents the means ± SD (n=4; each run in triplicate). * p < 0.05 vs. control; † p < 0.05 vs. HG.

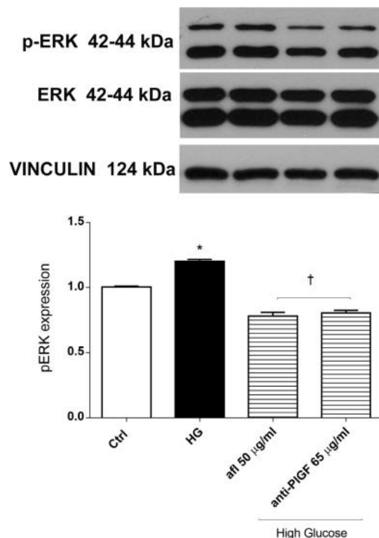


Fig. 8. Western Blot pERK with aflibercept and anti-PlGF. Aflibercept (A) and anti-PlGF (B) effect on the inflammatory pathway activated by high glucose (HG) in HRECs. Immunoblot analysis of ERK1/2 phosphorylation in lysates from HRECs treated for 48 h with HG, aflibercept (1–100 μg/mL) and anti-PlGF (1–50 μg/mL). Bar graphs show the densitometry analysis of each band, carried out with the Image J program. Each bar represents the means ± SD (n=4; each run in triplicate). *p < 0.05 vs. control; † p < 0.05 vs. HG.

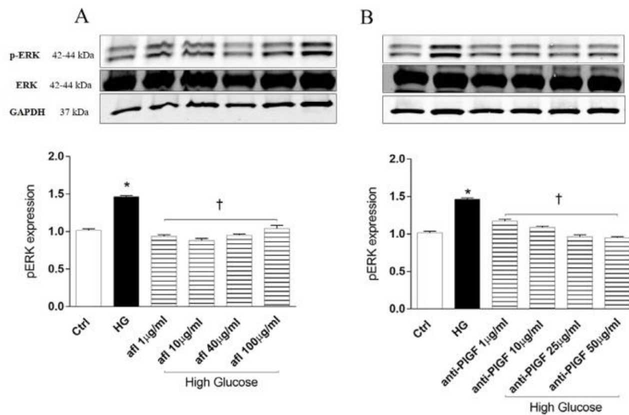


Fig. 9. TNF-α levels in RPE. Immunoblot analysis of TNF-α in lysates from pmRPE treated for 24 h with high glucose (HG), aflibercept (50 μg/mL) and anti-PlGF (65 μg/mL). Bar graphs show the densitometry analysis of each band, carried out with the Image J program. Each bar represents the means ± SD (n=4; each run in triplicate). *p < 0.05 vs control; †p < vs HG, ‡p < 0.05 aflibercept vs anti-PlGF treatment.

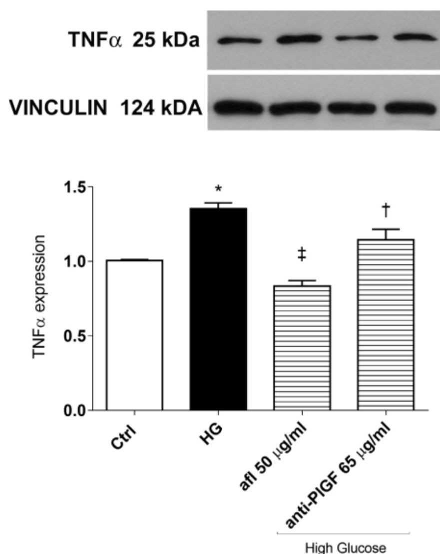


Fig. 10. TNF-α levels in HREC (ELISA). Aflibercept (1–100 µg/mL) (A) and anti-PlGF (1–50 µg/mL) (B) treatment decreased TNF-α release induced by 48 h exposure to high glucose (HG) levels. Ctrl stands for control cells, growth in medium with physiological glucose concentrations (5 mM). HG stands for cells grown in medium with high glucose (40 mM) levels. Bars represent mean values ± SD (n=8; each run in triplicate). *p < 0.05 vs. control; †p < 0.05 vs. HG, ‡ p < 0.05 vs 40 µg/ mL aflibercept or 25 µg/mL anti-PlGF.

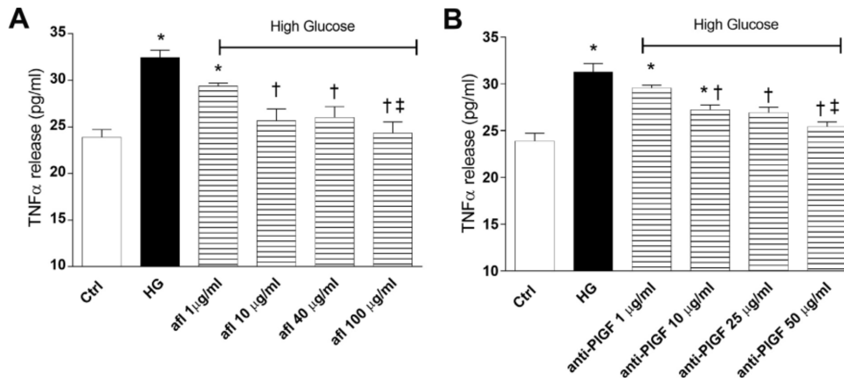


Fig. 11. Aflibercept reduces TNF-α mRNA expression (A) and protein levels (B) in the retina of streptozotocin-induced diabetic rats (STZ+afl group). Ctrl stands for non-diabetic rats. Ctrl+afl stands for non-diabetic rats treated with an intravitreal injection of aflibercept. STZ stands for diabetic rats treated with vehicle. STZ+afl are diabetic rats that were treated with aflibercept (160 µg; intravitreally). Each bar represents the mean value ± SD (n=4; each run in triplicate). *p < 0.05 vs. control; †p < 0.05 vs. STZ.

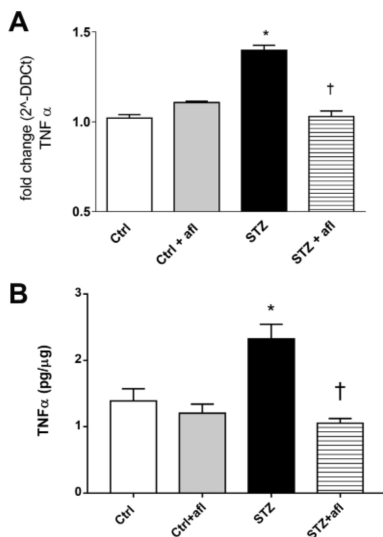
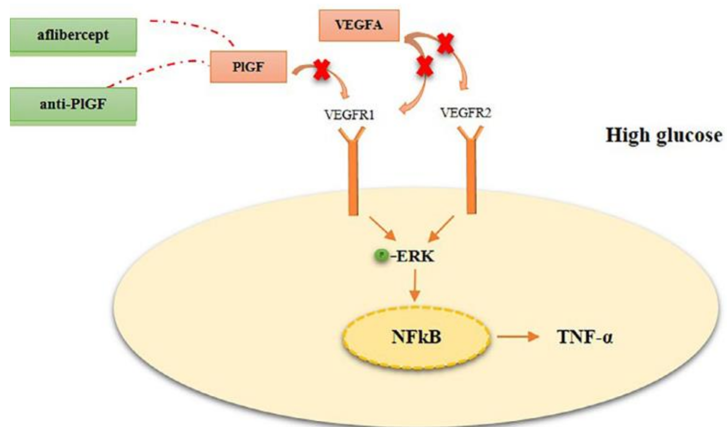


Fig. 12. High glucose-induced damage in retinal endothelial cells. Proposed anti-inflammatory activity of aflibercept and anti-PlGF.



Chapter II

Submitted to Frontiers in Pharmacology

Stabilization of HIF-1α in human retinal endothelial cells modulates expression of miRNAs and pro-angiogenic growth factors

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Abstract

Retinal hypoxia is one of the causative factors of diabetic retinopathy and is also one of the triggers of VEGF release. We hypothesized that specific dysregulated miRNAs in diabetic retinopathy could be linked to hypoxia-induced damage in human retinal endothelial cells (HRECs). We investigated in HRECs the effects of chemical ($CoCl_2$) hypoxia on the expression of HIF-1α, VEGF, PlGF, and of a focused set of miRNAs. We found that miR-20a-5p, miR-20b-5p, miR-27a-3p, miR-27b-3p, miR-206-3p, miR-381-3p correlated also with expression of TGFβ signaling pathway genes in HRECs, challenged with chemical hypoxic stimuli. In conclusion, our data suggest that retinal angiogenesis would be promoted, at least in early phases, by up-regulation of PlGF and other factors elicited by HIF-1α such as miRNAs, VEGFA and TGFβ1.

Keywords: Hypoxia-inducible-factor-1α; VEGF; TGFβ; retina; diabetic retinopathy; inflammation

Introduction

Diabetic retinopathy (DR), a complication of diabetes, is a microvascular disease with a strong inflammatory imprinting. Vascular endothelial growth factor (VEGF) is a key player in retinal neovascularization and intraocular injections of anti-VEGF agents are currently the established therapies for diabetic macular edema, along with steroids (Bandello et al., 2012). Although not fully elucidated, alterations in retinal hemodynamics and reduced blood flow may be detrimental for diabetic retinopathy, along with uncontrolled hyperglycemia (Schmetterer and Wolzt, 1999; Schmidl et al., 2015). Furthermore, during DR progression, local or global changes in retinal oxygenation may cause the development of hypoxic areas (B. Arden and Sivaprasad, 2012) and oxidative stress (Bucolo et al., 2006). Similar to the etiopathogenesis of retinopathy of prematurity (ROP), induction of hypoxia-inducible factor-1 α (HIF-1α) may be responsible for the production of vascular endothelial growth factor (VEGFA), which is the main cause of retinal neovascularization (Abu El-Asrar et al., 2012; Aiello et al., 1994; Arjamaa and Nikinmaa, 2006). Furthermore, HIF-1α/VEGFA crosstalk in ocular neovascularization has been widely confirmed and investigated (Ozaki et al., 1999; Rodrigues et al., 2016). In particular, the opportunity of HIF-1α inhibition has also been explored for treatment of retina neovascularization (D'Amico et al., 2015, 2017; Iwase et al., 2013; Zeng et al., 2017).

HIF-1α can also induce the placental growth factor (PlGF) (Charnock-Jones, 2016; Mitsui et al., 2018; Zimna and Kurpisz, 2015), an emerging target in retinal neovascular diseases (Kwon and Jee, 2018; Lazzara et al., 2019; Lee et al., 2018; Saddala et al., 2018; Van Bergen et al., 2019). HIF-1α is involved in expression of several microRNAs (miRNAs), that when include the hypoxia responsive elements (HREs) in their promoter region are named HypoxamiRs (Bertero et al., 2017; Nallamshetty et al., 2013). Indeed, HypoxamiRs, regulated by HIF-1α dependent or independent mechanisms, are tightly involved in molecular and cellular changes triggered by hypoxia (Bertero et al., 2017; Cottrill et al., 2014; Gee et al., 2014; Greco et al., 2014). HypoxamiRs target genes belonging to the VEGFR2 signaling pathway (Gupta et al., 2018); this pathway is relevant in endothelial cells for angiogenesis (Abhinand et al., 2016), and represents the target of current approved treatments for neovascular retinal degenerations (Bandello et al., 2012). We recently evidenced the dysregulation of expression pattern of 8 miRNAs (miR-20a-5p, miR-20a3p, miR-20b-5p, miR-106a-5p, miR-27a-5p, miR-27b-3p, miR-206-3p, and miR-381-3p) in retina and serum of diabetic mice, representing intriguing and potent mediators in the DR pathological

mechanisms (Platania et al., 2019). HREs were found in promoter region of miR-20a, miR-20b, miR-106, miR-27a, that indeed, have been enlisted as Hypoxamirs (Nallamshetty et al., 2013). Besides the presence of HREs in miR-20a, miR-20b, miR-106, miR-27a, in several hypoxic experimental settings also miR-206-3p, miR-381 and miR-27b were found to be modulated (Choudhry and Mole, 2016; Gupta et al., 2018; Lu et al., 2018; Yue et al., 2013). Therefore, we hereby hypothesized that these miRNAs could also be involved in activation of HIF-1α/angiogenic axis in retinal endothelial cells. With this aim, we stabilized, by cobalt chloride treatment, HIF-1α protein in human retinal endothelial cells (HRECs), in order to analyze the activation of HIF-1α/VEGFA-PlGF axis, along with expression of a focused set of miRNAs, previously found to be dysregulated in an *in vivo* model of diabetic retinopathy (Platania et al., 2019). A bioinformatic approach guided the identification and *in vitro* validation of alternative targets of miRNAs, dysregulated after inhibition of HIF-1α degradation. We analyzed the expression of genes of the TGFβ signaling pathway, which is an emerging target in diabetic retinopathy (Li et al., 2018; Stafiej et al., 2018) and was found to be one of top pathways in the enriched HypoxamiRs target genes (Gupta et al., 2018).

Material and Methods

Reagents

Mouse monoclonal anti-HIF-1α (catalog n. sc-13515), mouse anti-GAPDH (catalog n. 2118) antibodies were purchased from Santa Cruz Biotechnology, Inc. (CA, USA), and Cell-Signaling Technology (Leiden, The Netherlands), respectively. Secondary goat anti-mouse IRDye 680LT, (catalog n. 926-68020) were purchased from LI-COR (Lincoln, NE, USA). Cobalt chloride (0.1 M solution, catalog n. 15862) from Sigma-Aldrich (Saint Louis, MO, USA).

Cell Culture

Human retinal endothelial cells (HRECs) were purchased from Innoprot® (Derio – Bizkaia, Spain). Cells were cultured at 37 °C, in humidified atmosphere (5% CO_2), in Endothelial cell medium (ECM) supplemented with 5% fetal bovine serum (FBS), 1% ECGS (Endothelial Cell Growth Supplement) and 100 U/ml penicillin 100 µg/ml streptomycin. HRECs (cell passage number 4) for each experiment were seeded setting $4x10^5$ as final cell density.

Induction of chemical hypoxia in vitro

Cobalt chloride ($CoCl_2$) is commonly used to stabilize HIF-1α, because it inhibits the HIF-1α degradation, as shown in several *in vitro* settings, including primary human retinal endothelial cells cultures (HRECs) as previously described (Gao et al., 2008; He et al., 2019; Hu et al., 2012; Li et al., 2017). Preliminary studies were carried out and HRECs cultures were treated with various concentrations of $CoCl_2$ (100–200 μM), in order to assess cell tolerability for 24 hours with MTT test (supplementary data). The concentration used for all experiments was 200 μM, accordingly to previous $CoCl_2$ concentrations tested on retinal ganglion cells (Balaiya et al., 2012; Li et al., 2017). Cells were seeded in Petri dishes (passage number 4, cell density $4x10^5$); after reaching confluence (approximately 80%), cells were treated with $CoCl_2$ for 30 minutes, 2 and 8 hours to induce HIF-1α accumulation/nuclear translocation.

Western Blot

HRECs were cultured in 60 mm Petri dishes (cell density $4x10^5$). Proteins from cell lysates were extracted with RIPA Buffer, including protease and phosphatase inhibitors cocktail (Sigma-Aldrich, St. Louis, MO, USA). Total protein content, in each cell lysate sample, was determined by the BCA Assay Kit (Pierce™ BCA Protein Assay Kit, Invitrogen, Life Technologies, Carlsbad, CA, USA). Extracted proteins (40 μg) were loaded on 4-12% tris-glycine gel. After electrophoresis proteins were transferred into a nitrocellulose membrane (Invitrogen, Life Technologies, Carlsbad, CA, USA). Immunoblot was preceded by addition of Odyssey Blocking Buffer (LI-COR Lincoln, NE, USA) to membranes. Therefore, membranes were incubated overnight (4°C) with appropriate primary HIF-1α (1:200 dilution) and anti-GAPDH (1:500 dilution) antibodies. GAPDH was selected as control for protein expression, accordingly to previous reports (Ao et al., 2015; Botlagunta et al., 2011; Evrard et al., 2016; Gao et al., 2019). After overnight incubation, the membranes were then incubated with secondary fluorescent antibodies (1: 10,000 dilution) for 1 h at room temperature. Immunoblot was detected through Odyssey imaging system (LI-COR, Lincoln, NE, USA). Densitometry analyses of blots were performed at non-saturating exposures and analyzed using the ImageJ software (NIH, Bethesda, MD, USA; available at http://rsb.info.nih.gov/ij/index.html). Values were normalized to GAPDH, which was also used as loading control (see supplemental information for whole gel membranes immunoblots).

Extraction of total RNA and cDNA synthesis

Extraction of the total RNA was performed with TRIzol Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA). The A260/A280 ratio of the optical density of RNA samples (measured with Multimode Reader Flash di Varioskan™) was 1.95–2.01. This RNA purity was confirmed with the electrophoresis in non-denaturing 1% agarose gel (in TAE), that showed an adequate RNA purity, concentration, and integrity. cDNA was synthesized from 2 µg RNA with a reverse transcription kit (SuperScript™ II Reverse Transcriptase, Invitrogen, ThermoFisher Scientific, Carlsbad, CA, USA).

Real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) for PlGF and VEGFA

Real-time RT-PCR was carried out with LightCycler ® 2.0 (Real-Time PCR System Roche Life Science). The amplification reaction mix included iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) and 1 µl (100 ng) of cDNA. Forty-five amplification cycles were carried out for each sample. Results were analyzed with the $2^{-\Delta\Delta}$Ct method. VEGF and PlGF mRNAs expression were normalized to human 18S mRNA levels. Primers used in qPCR for 18S, VEGF-A, PlGF expression are: 18S (human) Forward (5'-AGTCCCTGCCCTTTGTACACA-3'), Reverse (5'-GATCCGAGGGCCTCACTAAAC-3'); PlGF (human) Forward (5′-ATGTTCAGCCCATCCTGTGT-3′) Reverse (5′-CTTCATCTTCTCCCGCAGAG-3′); VEGF-A (human) Forward (5'-GAGGTTTGATCCGCATAATCTG-3') Reverse (5'-ATCTTCAAGCCATCCTGTGTGC-3').

Analysis of miRNAs

HRECs total RNA, including small RNAs, was obtained following the miRNeasy Mini Kit (21700400, Qiagen), according to the manufacturer's protocol "Purification of Total RNA, Including Small RNAs, from Animal Cells". Particularly, for miRNAs isolation, Syn-cel-miR-39-3p miScript miRNA Mimic 5 nM (MSY0000010, Qiagen) was added to each sample before RNA purification in order to monitor miRNAs isolation efficacy. RNA quality and concentration were determined by using NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA). Gene Amp PCR System 9700 (Applied Biosystems Thermo Fisher Scientific, Carlsbad, CA, USA) was used for reverse-transcription phase. Mature miRNAs were converted in cDNA according the MiScript II Reverse Transcription Kit (218161, Qiagen, Germantown, MD, USA), starting from 615 ng of total RNA.CFX96 Real-

Time System C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA) was used to evaluate the expression levels of hsa-miR-20a-5p (Accession number MIMAT0000075), hsa-miR-20b-5p (Accession number MIMAT0001413); hsa-miR-27a-3p (Accession number MIMAT0000084), hsa-miR-27b-3p (Accession number MIMAT0000419), hsa-miR-206-3p (Accession number MIMAT0000462) and hsa-miR-381-3p (Accession number MIMAT0000736). Real time PCR was carried out with miScript SYBR Green PCR kit (218073, Qiagen, Germantown, MD, USA) and specific miScript primer Assays (MS00003199, MS00003206, MS00003241, MS00031668, MS00003787 and MS00004116, Qiagen, Germantown, MD, USA). The expression of the 6 miRNAs analyzed was normalized by using Ce_miR-39-3p (MIMAT0000010) as control (MS00019789, Qiagen, Germantown, MD, USA).

TGFR pathway qRT-PCR

Total RNA (615 ng) was subjected to reverse-transcription reaction with the Gene Amp PCR System 9700 (Applied Biosystems Life Technologies, Carlsbad, CA, USA) and Quantitect Reverse Transcription kit (205311, Qiagen, Germantown, MD, USA), following the manufacturer's protocol "Reverse Transcription with Elimination of Genomic DNA for Quantitative, Real-Time PCR". The expression levels of human TGFB1 (Gene ID 7040), TGFBR1 (Gene ID 7046), TGFBR2 (Gene ID 7048) and SMAD2 (Gene ID 4087) genes were evaluated by real time PCR measurement, by using a CFX96 Real-Time System C1000 Touch Thermal Cycler (BioRad Laboratories, Inc), Quantitect SYBR Green PCR Kit (204143, Qiagen, Germantown, MD, USA) and specific Quantitect Primer Assays (QT00000728, QT00083412, QT00014350 and QT00004207, Qiagen, Germantown, MD, USA) following the manufacturer's protocol "Two-Step RT-PCR (Standard Protocol)". Human GAPDH (Gene ID 2597) (QT00079247, Qiagen, Germantown, MD, USA) was used as control to normalize the expression of the 4 genes analyzed; accordingly to previous reports (Ao et al., 2015; Botlagunta et al., 2011; Evrard et al., 2016; Gao et al., 2019; Jiang and Xu, 2019; Lin et al., 2015; Rosen et al., 2015; Shao and Yao, 2016).

MicroRNA or TGFβ signaling pathway genes expression determination analysis

CFX Manager™ Software (Bio-Rad, Hercules, CA, USA) was used to calculate Cycle threshold (Ct) values. Data analysis was carried out with the $2^{-\Delta\Delta}$Ct method. Particularly, ΔCt value for each miRNA or gene profiled was calculated as $\Delta Ct = Ct_{miRNA} - Ct_{Ce_miR\text{-}39\text{-}5p}$ or as $\Delta Ct = Ct_{gene} - Ct_{GAPDH}$. Then, ΔΔCt was calculated as $\Delta Ct_{time\ x} - \Delta Ct_{time\ 0}$, where time x

is the analyzed time point and time 0 is the expression of the target miRNA normalized to Ce-miR-39-5p or of the target gene normalized to GAPDH (Livak and Schmittgen, 2001). Where data are reported as fold-regulation, this was the inverse negative of fold change ($2^{-\Delta\Delta Ct}$) for fold change values lower than one (down-regulation). In case of up-regulation, the fold-regulation was equal to fold change ($2^{-\Delta\Delta Ct}$) for fold change values greater than 1.

Bioinformatics

In order to explore alternative factors and pathways regulated by miRNAs dysregulated with induction of chemical hypoxia in human retinal endothelial cells, we predicted the combinatorial effect of hsa-miR-20a-5p, hsa-miR-20b-5p, hsa-miR-27a-3p, has-miR-27b-3p, has-miR-260b-3p and has-miR-381-3p on biological pathways by means of the DIANA miRPath webserver (Vlachos et al., 2015). The miRNA:target interactions were analyzed with application of Tarbase algorithm (Riffo-Campos et al., 2016), which is based on experimental validated miRNA:target interaction.

Statistical Analysis

All results were reported as mean ± SD from four independent in-vitro experiments, were each group was triplicated in plates as technical replicate. The results were analyzed using one-way ANOVA followed by Tukey-Kramer post-hoc multiple comparisons test. Differences between groups were considered significant for $p\text{-value} < 0.05$. Graphs design and statistical analysis were carried out with GraphPad Prism 5 software (GraphPad Inc., San Diego, CA, USA).

Results

Chemical hypoxia in HRECs and angiogenic factors

$CoCl_2$ treatment, by inhibition of HIF-1α degradation, significantly increased stabilization of HIF-1α protein in HRECs (Fig 1). HIF-1α is a well-known inducer of VEGFA and PlGF (Charnock-Jones, 2016; Mitsui et al., 2018; Ozaki et al., 1999; Rodrigues et al., 2016), but the HIF-1α protein levels did not correlate with expression pattern of VEGFA, within the analyzed time-points (Figure 2A). Two hours after $CoCl_2$ treatment, VEGFA expression increased, compared to control HRECs. While, after 8 hours, VEGFA levels significantly ($p<0.05$) decreased, compared to levels detected 2 hours after, $CoCl_2$ treatment. On the other hand, the expression pattern of PlGF correlated with HIF-1α protein levels, within the analyzed time-points (Figure 2B).

Expression analysis of miRNAs induced by CoCl2 treatmet of HRECs

Six miRNAs (miR-20a-5p, miR-20b-5p, miR-27a-3p, miR-27b-3p, miR-260b-3p, miR-381-3p), out of eight analyzed, were found to be significantly ($p<0.05$) dysregulated in HRECs, treated with 200 µM $CoCl_2$, compared to control cells (Figure 3). All dysregulated miRNAs were found to be significantly ($p<0.05$) up-regulated, 2 h after $CoCl_2$ treatment, compared to control cells (Figure 4A). On the contrary, four miRNAs were significantly ($p<0.05$) dysregulated (up-regulated) 8 h after $CoCl_2$ treatment, compared to control cells (Figure 4B). Furthermore, after 8 h of exposure to $CoCl_2$, five miRNAs (miR-20a, miR-20b, miR-27a, miR-27b, miR-206-3p) were significantly down-regulated ($p<0.05$), compared to levels detected in cells treated for 2 h with 200 µM $CoCl_2$, except for miR-381-3p (Figure 4C).

TGFβ signaling pathway in HRECs challenged with $CoCl_2$

A bioinformatic approach was used to predict the combinatorial effect of miR-20a-5p, miR-20b-5p, miR-27a-3p, miR-27b-3p, miR-206-3p and miR-381-3p on biological pathways. The pathways dysregulated by these miRNAs were predicted by means of DIANA miRPath, applying the Tarbase algorithm, which generates, as output, pathways related to experimental validated miRNA:mRNA interactions (Vlachos et al., 2015). Based on this bioinformatic approach, we found that the TGFβ signaling pathway was the top-scored among the pathways significantly ($p<0.05$) dysregulated by hypoxia-induced miRNA in HRECs (Figure 5). The HIF-1α pathway was predicted to be regulated by miR-20a-5p, miR-20b-5p, miR-27a-3p, miR-27b-3p, miR-260-3p, miR-381-3p, according to the *in vitro* model of retinal chemical hypoxia. Moreover, PI3K-AKT, MAP kinases and Jak-STAT signaling pathways were

predicted to be modulated by the six miRNAs, that were dysregulated in HRECs treated with $CoCl_2$. Therefore, we focused our study on analysis of transcription of TGFβ signaling pathway genes (*TGFB1* encoding for TGFβ1, *TGFBR1* encoding for the TGFβR1 receptor, *TGFBR2* encoding for the TGFβR2 receptor and *SMAD2* encoding for SMAD2), in HRECs treated with 200μM $CoCl_2$ (table 2). These genes were significantly ($p<0.05$) dysregulated in HRECs, 2 and 8 hours after $CoCl_2$ treatment (Figure 6). Furthermore, we correlated gene expression with dysregulated miRNAs in the analyzed time-points (Figure 7, Table 1). After 2h of $CoCl_2$ treatment, *TGFBR2* and *TGFB1* gene expression increased significantly, TGFBR1 decreased ($p<0.05$) (Figure 7A), and all analyzed miRNA were significantly up-regulated, particularly miR-27a. The mRNA of *TGFBR1* is an experimental validated target of miR-20a, miR-20b, miR-27a, miR-27b and miR-381, therefore the up-regulation of this miRNAs significantly decreased the *TGFBR1* mRNA levels (Table 1). Moreover, *TGFB1* and *TGFBR2* are experimental validated targets of miR-27a and miR-20a, respectively, which even if overexpressed did not reduce the expression of these two genes 2 h after $CoCl_2$ treatment (Figure 7A). Eight hours after $CoCl_2$ treatment, miR-20a, miR-27a, miR-27b and miR-381-3p, were significantly ($p<0.05$) up-regulated in HRECs, compared to control cells (Figure 7B). This pattern of miRNA expression positively correlated with *TGFB1*, *TGFBR2* and *SMAD2* expression (Figure 7B). Although not significantly, 8h after $CoCl_2$ treatment, mRNA levels of TGFβ1, TGFβR2 and SMAD2 were higher, compared to HRECs treated for 2 h with $CoCl_2$ (Table 1). On the other hand, TGFβR1 mRNA expression levels were significantly upregulated 8 h after $CoCl_2$ treatment, compared to cells treated for 2 h with $CoCl_2$ (Figure 7C). This expression pattern negatively correlated with down-regulation of miR-20a, miR-20b, miR-27a, miR-27b and miR-206, according to the opposite trend observed 2 h after chemical hypoxia.

Discussion

Previous data report that eight miRNAs (miR-20a-5p, miR-20a3p, miR-20b-5p, miR-106a-5p, miR-27a-5p, miR-27b-3p, miR-206-3p, and miR-381-3p) were significantly dysregulated both in serum and retina of diabetic mice (Platania et al., 2019). Because retinal hypoxia is detrimental in diabetic retinopathy, exacerbating retinal damage and angiogenesis (Abu El-Asrar et al., 2012; Aiello et al., 1994; Arjamaa and Nikinmaa, 2006), we aimed at testing the hypothesis that these miRNAs would be modulated in human retinal endothelial cells, treated with $CoCl_2$ in order to stabilize HIF-1α.

In diabetic retinopathy, the role of angiogenesis linked to hypoxic events (i.e. increased VEGFA production stimulated by HIF-1α) has been largely proven (Arjamaa and Nikinmaa, 2006; B. Arden and Sivaprasad, 2012; Kurihara et al., 2014; Li et al., 2017). Furthermore, HIF-1α can induce expression of another pro-angiogenic factor, the placental growth factor (PlGF) (Lazzara et al., 2019; Tudisco et al., 2014). In this study we found a correlation, in terms of time-dependent expression, between HIF-1α and PlGF, after $CoCl_2$ treatment (Figure 2). Instead, VEGF mRNA levels did not correlate with HIF-1α protein (Figure 2). For this reason, we hypothesized that other factors could regulate VEGFA expression in an *in vitro* model of chemical hypoxia, such as miRNAs. Involvement of miRNAs in eye neovascular diseases has been widely studied (Martinez and Peplow, 2019; Natoli and Fernando, 2018; Platania et al., 2019; Romano et al., 2017). We found that six miRNAs (miR-20a-5p, miR-20b-5p, miR-27a-3p, miR-27b-3p, miR-206-3p, miR-381-3p), out of eight tested, were dysregulated in human retinal endothelial cells after $CoCl_2$ treatment (Figure 3). These miRNAs have been previously found to be either HypoxamiRs (bearing HREs in their promoting region) or linked to hypoxic microenvironment (Choudhry and Mole, 2016; Gupta et al., 2018; Lu et al., 2018; Nallamshetty et al., 2013; Yue et al., 2013). After 8 hours, similarly to VEGFA expression, we found a shift in expression pattern of miRNAs, compared levels detected 2 h after $CoCl_2$ treatment (Figure 4). Experimental validated miRNA:VEGFA mRNA interactions were found for miR-20a-5p and miR-20b-5p (Platania et al., 2019), and in hepatocellular carcinoma for miR-381-3p (Tsai et al., 2017; Wang et al., 2018). Therefore, VEGFA expression levels could be related to the expression pattern of miRNAs, 2 to 8 hours after stabilization of HIF-1α, because VEGFA is a target of miR-20a, miR-20b, miR-381, and indirectly of miR-27b (Veliceasa et al., 2015). On the contrary, PlGF is not a validated or predicted target of miRNAs dysregulated in HRECs treated with $CoCl_2$. Particularly, the role of PlGF in regulation retinal angiogenesis, under hypoxic stimuli, is still unknown. On the other hand, several reports support the detrimental role of PlGF in the pathogenesis and progression of diabetic retinopathy (Carmeliet et al., 2001; Huang et al., 2015), likely through HIF-1α, or indirectly by miRNAs and the PI3K/AKT signaling pathways (Figure 4) (Jin et al., 2018; Zhou et al., 2016).

Therefore, our hypothesis is based on retinal angiogenesis regulated by miRNAs under hypoxic stimuli, and miRNAs can be considered alternative and/or ancillary components to VEGFA and PlGF pathways. Indeed, we analyzed other putative miRNAs gene-pathway targets and identified, through a bioinformatic approach, the TGFβ signaling pathway as the top-scored pathway dysregulated by identified miRNAs (Figure 4). Then, we found that

miRNAs dysregulated after $CoCl_2$ treatment (miR-20a-5p, miR-20b-5p, miR-27a-3p, miR-27b-3p, miR-206-3p, miR-381-3p) influenced mRNA levels of TGFβ1, TGFβR1, TGFβR2 and SMAD2, according to experimental validated miRNA:mRNA interactions (Figure 6-7, Table 1). Particularly, the expression of TGFβR1 receptor, which is target of most of analyzed miRNAs (Table 1), correlated with expression pattern shift of miRNAs at 2 h and 8 h after $CoCl_2$ treatment. Several reports support a detrimental role of TGFβR1 in diabetic retinopathy, particularly, TGFβR1 immunoreactivity was found to be increased in retinal capillaries of diabetic rats (Gerhardinger et al., 2009; van Geest et al., 2010) The HIF-1α/ TGF-β1 axis, and related stimulation of angiogenesis, has been investigated in different experimental settings (Han et al., 2013; Mingyuan et al., 2018), including endothelial cells (Iruela-Arispe and Sage, 1993; Peshavariya et al., 2014). On the contrary, few reports demonstrated a putative link between HIF-1α/miRNAs/TGFβ signaling pathway and angiogenesis (Xing et al., 2014). Furthermore, only one study analyzed the role miRNAs in regulation of hypoxia-TGFβ-angiogenesis pathway in a model of corneal neovascularization (Zhang et al., 2019b). According to our findings, miR-27 was reported to be involved in regulation of HIF-α/TGFβ axis, at least in an *in vitro* model of cardiac ischemia (Zhang et al., 2019a). However, there are still no evidences about a putative link in retinal disease between hypoxia, miRNAs, VEGFA and TGFβ pathway. High throughput miRNA expression analysis on retinal endothelial cells, challenged with chemical hypoxic stimuli, could reveal the involvement of other miRNAs, along with the focused set analyzed in this study. However, those high throughput analyses are expensive and need quantitative qPCR validation (de Ronde et al., 2018). Despite the small set of analyzed miRNAs, our study suggested that ocular neovascularization, during hypoxia, would be promoted by the up-regulation of PlGF and other factors induced by HIF-1α/miRNAs, eg. VEGFA, and genes of the TGFβ1 signaling pathway (Figure 8). Indeed, the present findings highlighted that pro-angiogenic factors are worthy to be further explored as potential targets for pharmacological modulation of local retinal hypoxic events, which are generally transient but detrimental in retinal degenerations.

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Author Contributions Statement

CB, MD, SR made substantial contributions to conception, design, and interpretation of data. FL, MCT, CBMP carried out experiments. FL, MCT, CBMP, FP, CG, carried out formal analysis of data. FL, MCT, CBMP, CB wrote initial draft of the manuscript. CB, MD, SR, FD, MG reviewed the manuscript critically for important intellectual content and gave final approval of the version to be submitted.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability statement

The raw data supporting the conclusions of this manuscript will be made available by the authors, under reasonable request, to any qualified researcher.

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Figure 1. $CoCl_2$ treatment induces HIF-1α stabilization in human retinal endothelial cells.

Western blot of HIF-1α and GAPDH in HRECs exposed to $CoCl_2$ for 30 minutes, 2 h and 8 hours. Densitometric analysis was carried out and each bar represents the mean value ± SD. *$p < 0.05$ *vs.* control; †$p < 0.05$ *vs.* 30 min $CoCl_2$ treatments; (n=4).

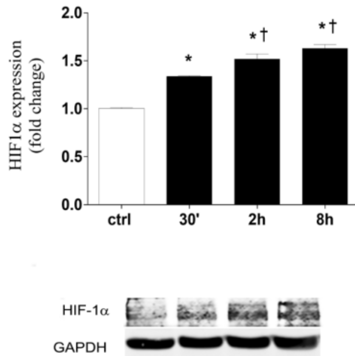


Figure 2. Chemical hypoxia induces VEGFA and PlGF expression in human retinal endothelial cells. $CoCl_2$ treatment increased VEGF-A (A) and PlGF (B) mRNA expression. HRECs were treated with $CoCl_2$, for 2 h and 8 h. The mRNA levels were evaluated by qRT-PCR. Each bar represents the mean value ± SD. *$p<0.05$ $CoCl_2$ *vs*. control (ctrl); †$p<0.05$ 8h *vs*. 2h $CoCl_2$ treatment; (n=4).

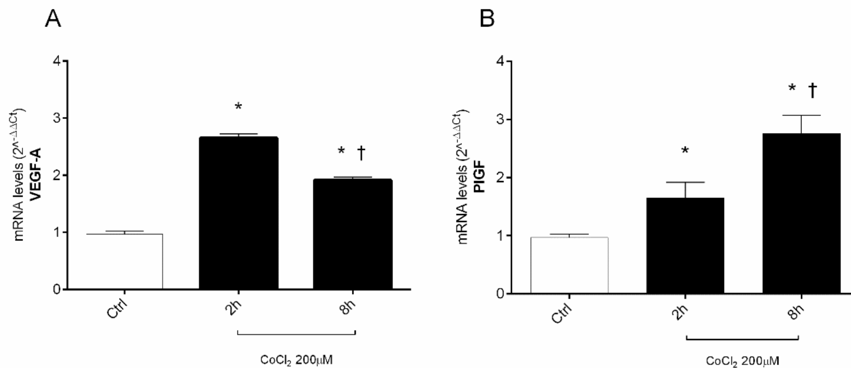


Figure 3. Pattern expression of miRNAs in HRECs treated with $CoCl_2$, for 2 and 8 h treatment. Expression of miRNAs was analyzed with qRT-PCR. Each bar represents the mean value ± SD. *$p<0.05$ 200 μM $CoCl_2$ *vs*. control (ctrl); †$p<0.05$ 8h *vs*. 2h $CoCl_2$ treatment; (n=4).

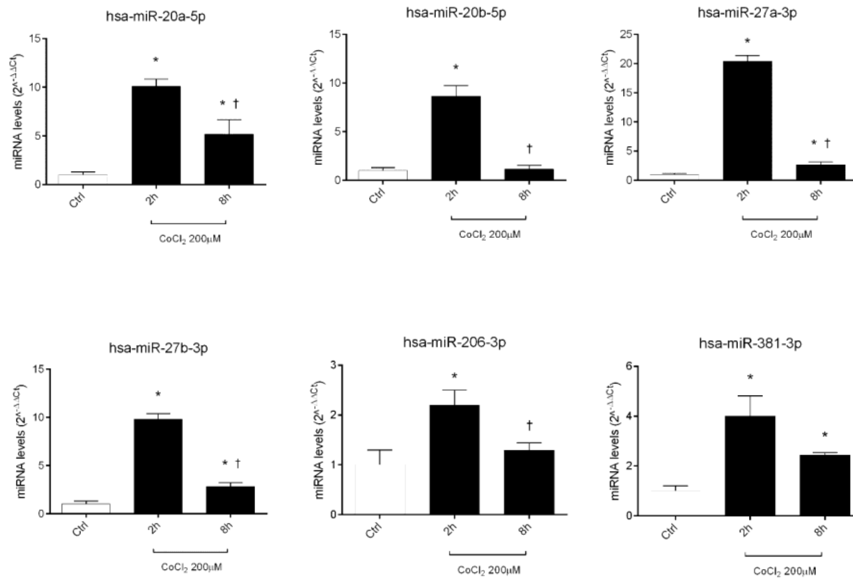
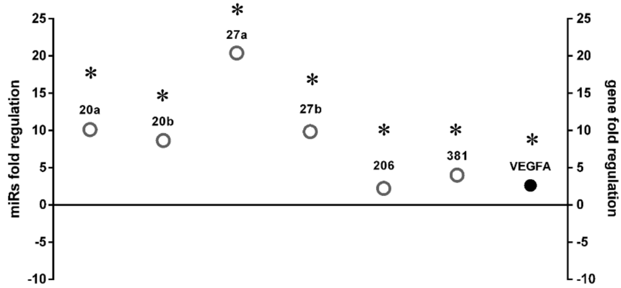


Figure 4. HIF-1α stabilization induced miRNAs and correlation with VEGFA expression. Correlation of miRNAs and VEGFA expression (Fold regulation). (A) *$p<0.05$ 2 h $CoCl_2$ treatment *vs.* control; (B) *$p<0.05$ 8 h $CoCl_2$ *vs.* control (ctrl); (C) * $p<0.05$ 8h *vs.* 2h $CoCl_2$ treatment; (n=4).

A



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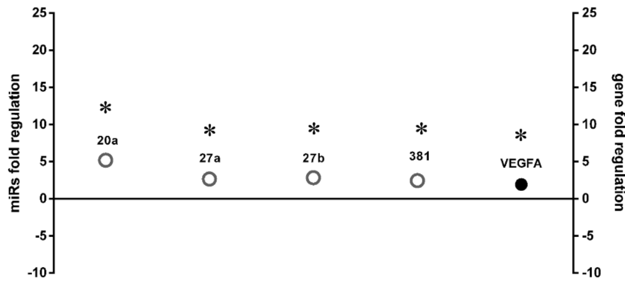

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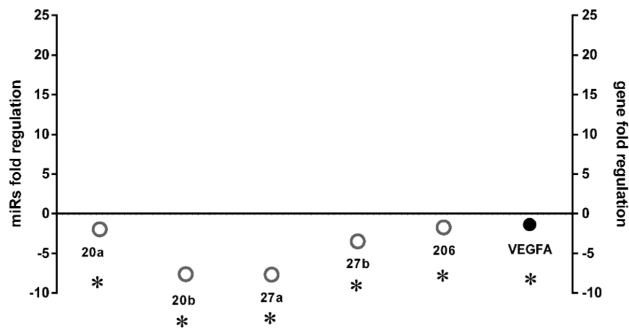


Figure 5. 3D scatter plot of pathways regulated by HypoxamiRs in HRECs, challenged with $CoCl_2$. Blue dots represent #gene projection of #miRNAs dimension. Green dots represent p-value projection of #genes dimension. Red dots represent #miRNA projection of p-value dimension.

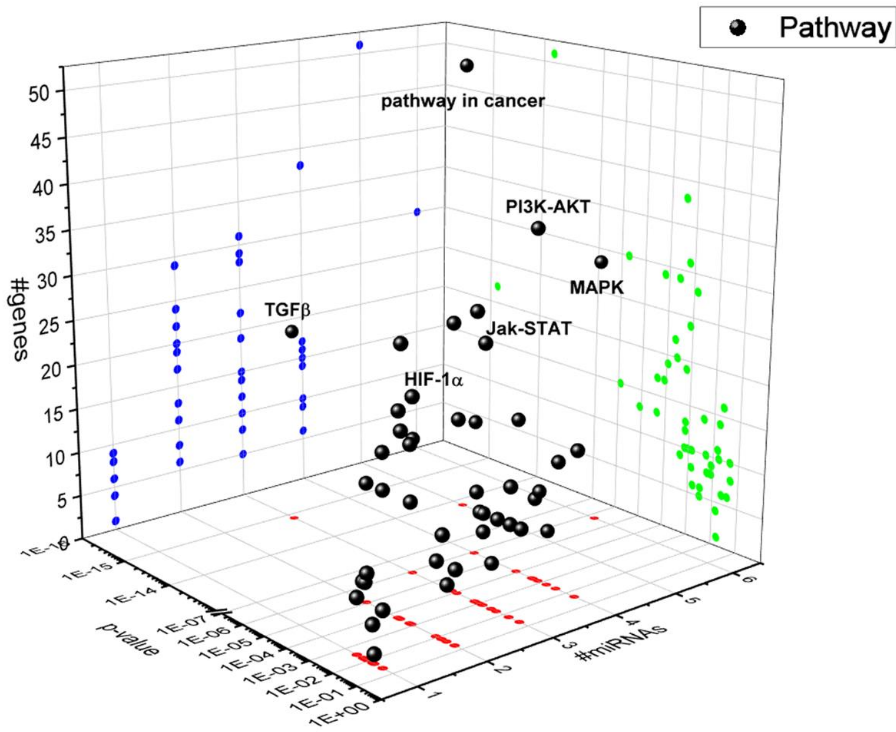


Figure 6. Expression of genes of TGFβ signaling pathway in HRECs treated with $CoCl_2$, for 2 and 8 hours.. The mRNA levels were evaluated by qRT-PCR. Each bar represents the mean value ± SD. *$p<0.05$ $CoCl_2$ *vs*. control (ctrl); †$p<0.05$ 8h *vs*. 2h $CoCl_2$ treatment. (n=4)

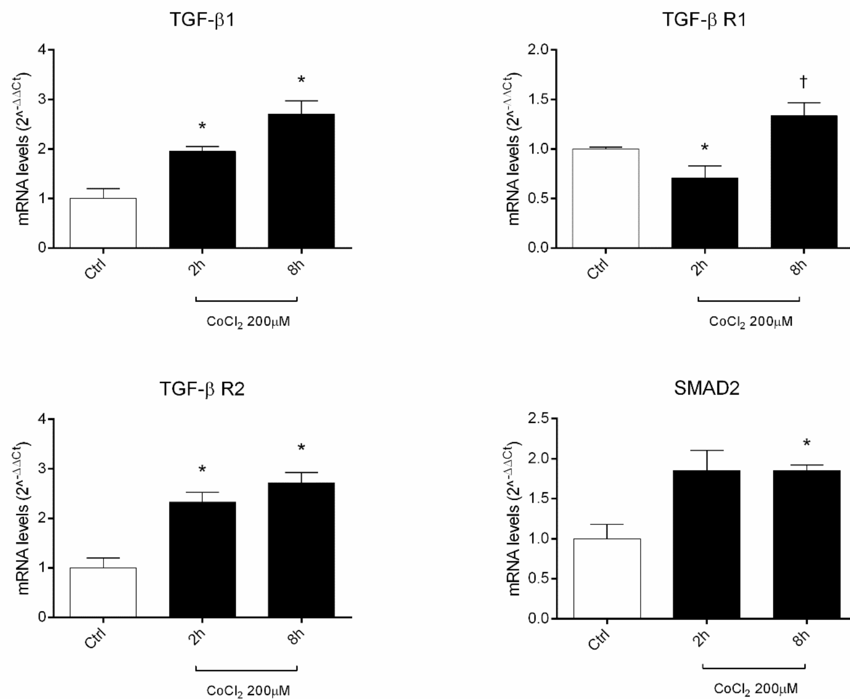


Figure 7. Correlation of miRNAs and TGFβ signaling pathway genes expression in HRECs, under chemical hypoxic stimuli. Fold regulation of miRNAs and TGFβ signaling genes: (A)

*p<0.05 2 h CoCl2 treatment *vs.* control (ctr); (B) * p<0.05 8 h CoCl2 treatment *vs.* control (ctr); C) *p<0.05 8 h *vs.* 2h $CoCl_2$ treatment; (n=4).

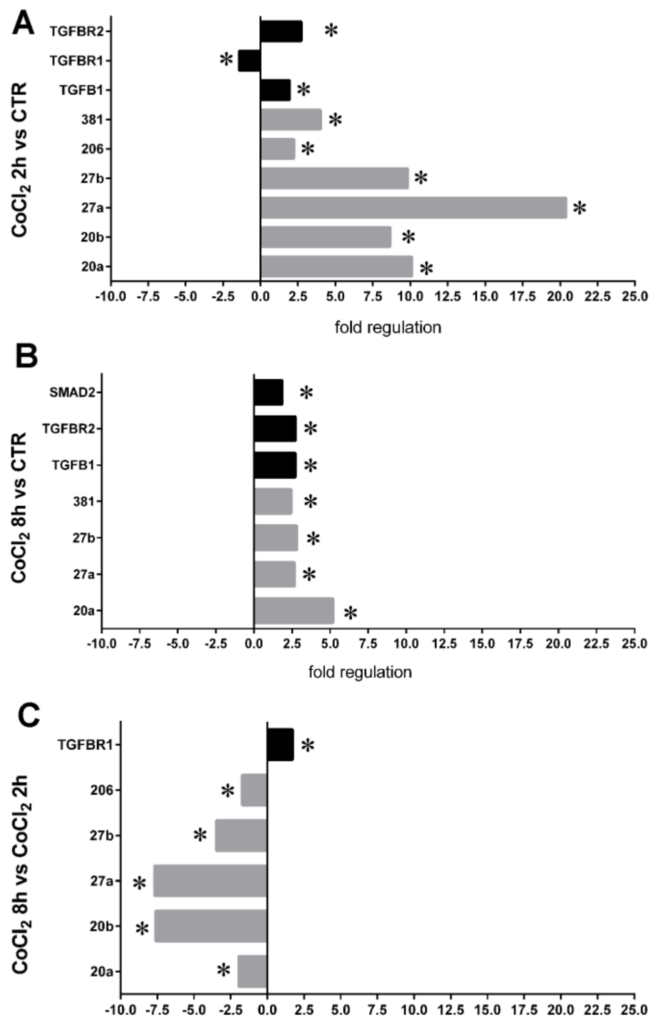
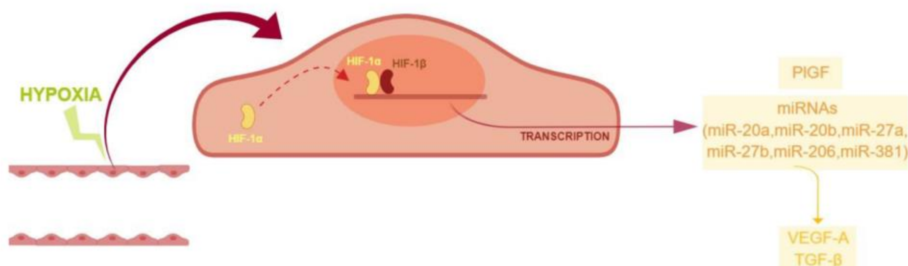


Figure 8. Proposed model of angiogenic shift in retinal endothelial cells exposed to chemical hypoxia. $CoCl_2$-induced hypoxia leads to the stabilization of HIF-1α, with the subsequent translocation into the nucleus and transcription of hypoxia-related genes.



Tables

Table 1. Differential expression of genes of the TGFβ signaling pathway. The miRNAs, targeting each gene, were predicted with application of Tarbase, or whenever written with microT-CDS algorithm.

Gene	Fold regulation $CoCl_2$ 2h vs CTR (p value)	Fold regulation $CoCl_2$ 8h vs CTR (p value)	Fold regulation $CoCl_2$8h vs 2h (p value)	Regulating miRNAs (tarbase)
TGFB1	1.9466 ($p<0.01$)	2.7128 ($p<0.05$)	1.3936	miR-27a
TGFBR1	**-1.419 ($p<0.05$)**	**1.3352**	**1.7166 ($p<0.05$)**	**miR-20a, miR-27a, miR-27b, miR-20b, (microT-CDS), miR-381 (microT-CDS)**
TGFBR2	2.327 ($p<0.05$)	2.7213 ($p<0.05$)	1.1694	miR-20a, miR-20b
SMAD2	1.5014	1.8547 ($p<0.05$)	1.2354	miR-20b, miR-206 (microT-CDS), miR-381 (microT-CDS)

Discussion and conclusions

Diabetic retinopathy has long been known as a microvascular disease, but increasing evidences suggest that retinal neurodegeneration and inflammation are implicated also in pathogenesis. In recent years, research is based on the discover of new targets and on the development of new agents targeting factors and receptors modulating neurodegenerative and inflammatory pathways.

In the last decade, intravitreal anti-VEGF agents have become the first-line therapy for DME and PDR, such as aflibercept. Aflibercept therapeutic action is accounted mainly to the neutralization of VEGFA activity, therefore preventing the binding and activation of its receptors (VEGFR1 and VEGFR2) Furthermore, aflibercept is the only anti-VEGF agents that binds also PlGF, although with lower affinity (38.9 nM dissociation constant – KD), compared to VEGF-A (0.49 nM dissociation constant – KD). As regards the role of PlGF in diabetic retinopathy, PlGF seems to be a pharmacological target alternative to VEGFA; because PlGF has been reported to activate angiogenesis in pathological conditions [36,189], such as diabetic retinopathy [24,46,189], while VEGFA has a neurotrophic role either in physiological and pathological conditions.

Aflibercept could counteract PlGF and in our study, reported in chapter I, we investigated both in vitro and in vivo the hypothesis that anti-VEGF/PlGF drug could also exert an anti-inflammatory action in the diabetic retina. We found that retinal endothelial cells (HRECs) and primary mouse retinal pigmented epithelial cells (mRPEs), challenged with high glucose levels (to mimic hyperglycemia in vitro) were protected from cell death after treatment with aflibercept and anti-PlGF, by blocking the activation of the ERK

pathway with the subsequent suppression of TNF-α release. This effect could be related to regulation of NF-kB signaling [32,48].

Moreover, data obtained from the in vivo model of diabetic retinopathy confirmed this hypothesis, because rat retinal TNF-α levels were significantly decreased by aflibercept intravitreal treatment. Therefore, as reported in chapter I, our results support the development of a new therapeutic strategy to counteract diabetic retinopathy, by inhibition of the PlGF signaling pathway, alternative or adjuvant to inhibition of VEGFA pathway.

Subsequently, we explored new pharmacological targets for diabetic retinopathy, by studying another crucial aspect of the pathogenesis of diabetic retinopathy, the hypoxic microenvironment. We highlighted in retinal endothelial cells, markers of pro-angiogenic process, induced by $CoCl_2$ treatment (chemical hypoxia) (miRNAs and TGFβ signaling genes), alternative to VEGFA. Considering that eight miRNAs were found to be dysregulated both in retina and serum of diabetic mice, then we analyzed those miRNAs also in hypoxic microenvironment, as described above [190]. Indeed, we demonstrated that six miRNAs (miR-20a-5p, miR-20b-5p, miR-27a-3p, miR-27b-3p, miR-206-3p, miR-381-3p), out of eight tested, were dysregulated in vitro after chemical hypoxia induction ($CoCl_2$ treatment).

As reported in chapter II, we identified through a bioinformatic approach the TGFβ signaling pathway, as one of the top-scored pathways dysregulated by identified miRNAs. We demonstrated that $CoCl_2$-hypoxia-induced miRNAs (miR-20a-5p, miR-20b-5p, miR-27a-3p, miR-27b-3p, miR-206-3p, miR-381-3p) can promote angiogenesis and fibrosis through modulation of VEGFA, TGFβ1, TGFβR1, TGFβR2, SMAD2. PlGF expression, on the basis of bioinformatic studies, was regulated only by HIF-1α and not by any of miRNAs dysregulated by chemical hypoxia, at least in this model. VEGFA and HIF-1α are strongly related to angiogenesis during DR [191–194].

Moreover, HIF-1α has been recently showed to modulate also transforming growth factor-β1 (TGF-β1)/Smad signaling in different experimental settings [43, 44], confirming our data, shown in chapter II. In this study (Chapter II), for the first time, we analyzed the link between retinal HIF-1α pathway activation from one side and miRNAs, VEGFA and TGF- β1 from the other side. As already reported, we found that $CoCl_2$ treatment induced a significantly reduction of HIF-1α degradation with the subsequent upregulation of VEGF-A and PlGF, in HRECs [197–201]. Particularly, the role of PlGF in regulation of retinal angiogenesis under hypoxic stimuli is still unknown, although in $PlGF^{-/-}$ mice retinal ischemia did not show typical signs of neovascularization or vessel tortuosity [47]. Considering the detrimental role of PlGF in development of diabetic retinopathy and, on the basis of our findings (HIF-1α/PlGF signaling activation), PlGF would likely promote angiogenesis through alternative pathways, such as p-ERK signaling [15, 53].

Hypoxia and local retinal hypoxic events are known detrimental factors triggering retinal degeneration, although are generally transient. Nevertheless, our findings presented in chapter II, highlighted novel pro-angiogenic factors, induced by chemical hypoxia (miRNAs and TGFβ signaling genes) that are worthy to be further studied as potential pharmacological targets to handle retinal neovascular diseases. Although in the first study we identified PlGF as a key factor in molecular mechanism activated during hyperglycemic condition, in the second study we were not able to demonstrate a strong connection between miRNAs/TGFb and PlGF (contrary to VEGFA). Further studies would be interesting to investigate the role of PlGF in hypoxic microenvironment, considering its emerging role in pathophysiology of DR. A high-throughput screening of miRNAs potentially related to PlGF would address this issue, although not extensive

bioinformatics data are currently available regarding miRNA-mRNA PlGF interactions.

In conclusion, we tried to add new hints and puzzle pieces about the etiopathogenesis of DR, addressing several hypotheses and trying to evaluate novel and promising pathways implicated in this pathology. Currently, the first-line pharmacological therapy for PDR is still represented by intravitreal injection of anti-VEGFs. However, the latter pharmacological approach by frequent, invasive and expensive treatments, that overall increase the economic impact and burden of disease, that generally decrease the compliance of patients. Indeed, search of effective drugs or adjuvant treatments is still an unmet medical need in the management of DR, and our studies are addressing this issue.

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