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Investigational Studies of Human Vitreous and Serum VEGF-A, VEGF-B and PlGF Remodeling the Paradigm in Patients with Ocular Pathology

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To my mother

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List of publications

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I. **Vascular endothelial growth factors and placenta growth factor in retinal vasculopathies: current research and future perspectives**
Joana Mesquita, João Paulo Castro de Sousa, Sara Vaz-Pereira, Arminda Neves, Luís A. Passarinha and Cândida T. Tomaz
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List of scientific communications

Oral scientific communications:

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II. **Quantification and comparison of VEGF-A, VEGF-B and Placental Growth Factor (PlGF) in the vitreous humor and plasma of patients with ocular pathology**, XII CICS Symposium, UBI, 6th July 2017, Covilhã, Portugal

Poster presentations:

I. **Quantification and comparison of VEGF-B in the vitreous of patients with diabetic ocular disease and a control group of patients with non diabetic ocular disease;** Joana Mesquita, João Paulo Castro de Sousa, Ana s. Rocha, Fátima Santos, João Monteiro, Luís A. Passarinha and Cândida T. Tomaz. Annual Meeting: Association for Research in Vision and Ophthalmology, ARVO Meeting Abstracts, *Invest Ophthalmol Vis Sci*, Orlando, EUA, 2014.

II. **Comparison of serum and vitreous PlGF in diabetic retinopathy patients and non-diabetic patients;**
Joana Mesquita, João Paulo Castro de Sousa, Paulo Tavares-Ratado, Sara Vaz-Pereira, Arminda Neves, Ana s. Rocha, Fátima Santos, Luís A. Passarinha and Cândida T. Tomaz. Annual Meeting: Association for Research in Vision and Ophthalmology, ARVO Meeting Abstracts, *Invest Ophthalmol Vis Sci*, Denver, EUA, 2015.

III. **Correlation between concentration of vitreous angiogenic cytokines (VEGF-B and PlGF) with central retinal thickness and macular volume in diabetic retinopathy patients;**
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IV. **Quantitative analysis and correlation of VEGF-A and VEGF-B in serum and vitreous humor of patients with proliferative vs. non-proliferative ocular disease;**
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V. **Quantitative research of VEGF-A, VEGF-B and PlGF in vitreous and serum in angiogenic ocular disorders;**
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I. **Intravitreal injection of ranibizumab for myopic choroidal neovascularization – case series of treatment in a real life setting**
Rita Flores, Ana Cabugueira, Joana Mesquita
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II. **Intravitreal fluocinolone acetonide 0.19 mg implant for chronic diabetic macular edema – early outcomes in a real world setting**
Pedro Simões, Miguel Cordeiro, Helena Urbano, Luísa Queiroz, Joana Mesquita, António Rodrigues
16th ESASO Retina Academy. June 23-25, 2016, Estoril, Portugal: Abstracts. (2016). Ophthalmic Research, 56(1), 1-52. http://dx.doi.org/10.1159/000446561

III. **Safety outcomes of intravitreal implant of fluocinolone acetonide 0.19 mg in clinical practice assessed by intraocular pressure measurement**
José António Dias, António Figueiredo, Joana Mesquita
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November 2 - 4, 2017 Bangkok, Thailand

Resumo alargado

Os fatores de crescimento endotelial vascular (VEGF) são importantes reguladores da proliferação, da migração e da permeabilidade das células endoteliais, não só durante a fase embrionária e vasculogénese, mas também na angiogénese fisiológica e patológica.
A família VEGF é constituída por sete membros, os quais incluem o VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F e o factor de crescimento placentário (PlGF), cada um com uma expressão única, especificidade e função. Estes membros com diferentes propriedades físicas e biológicas específicas, atuam através de três receptores tirosina quinase, VEGFR-1, VEGFR-2 e VEGFR-3, e dois receptores de sinalização neuropilina-1 (NRP-1) e NRP-2.
Quase 80% do globo ocular é constituído por uma substância clara, semelhante a um gel, designada por humor vítreo, que preenche o centro do olho e está em contacto contínuo com a retina. O humor vítreo é constituído por água (~ 99%), hialócitos, ácido hialurónico, ácido ascórbico, sais inorgânicos, glucose e uma rede de fibrilhas de colagénio.
No presente estudo, foram investigadas três citoquinas (VEGF-A, VEGF-B e PIGF) no humor vítreo e soro de doentes com patologia ocular através da quantificação por ELISA ("Enzyme-Linked Immunosorbent Assay").

Para comparar e caracterizar os achados quantitativos destes fatores de crescimento, os doentes foram divididos em 2 grupos:
1. Doentes com doenças oculares neovasculares classificados posteriormente nos seguintes subgrupos de doentes: degenerescência macular da idade (DMI), oclusão da veia da retina (OVR) e retinopatia diabética (RD);
2. Doentes com doenças oculares não-neovasculares, ou seja, doentes com síndroma de tracção vitreomacular de etiologia idiopática ou doentes com descolamento retiniano regmatogénio.
Além disso, os fatores de crescimento foram comparados ou correlacionados entre:
1. Os grupos de doentes estudados (neovascular vs. não neovascular);
2. As diferentes patologias oculares;
3. As características clínicas de "baseline" do doente, como por exemplo, doentes naïve versus doentes não-naïve, tratamentos efetuados antes de serem submetidos a vitrectomia, presença de glaucoma e estadio de retinopatia diabética (retinopatia diabética não proliferativa vs. retinopatia diabética proliferativa).
Para uma investigação mais aprofundada, os valores médios das concentrações séricas e vítreas de VEGF-A, VEGF-B e PIGF foram correlacionadas entre si e entre as alterações estruturais ocorridas nestes doentes, avaliadas por tomografia de coerência óptica (OCT) e medições da acuidade visual (AV).
As principais conclusões deste estudo foram as seguintes:

1. Os níveis vítreos de VEGF-A, VEGF-B e PIGF encontram-se sobre-expressos em doentes com doença ocular neovascular em comparação com os doentes sem doença ocular neovascular;
2. Nos doentes diabéticos, os níveis de fatores de crescimento endotelial vascular no humor vítreo aumentaram com a progressão da doença, sendo as concentrações obtidas de VEGF-A, VEGF-B e PIGF, menores em doentes com retinopatia diabética não proliferativa e maiores em doentes com retinopatia diabética proliferativa. Contudo, os níveis séricos de VEGF-A, VEGF-B e PIGF apresentaram valores maiores em doentes com retinopatia diabética não proliferativa em comparação com os doentes com retinopatia diabética proliferativa;
3. Não se observaram diferenças estatisticamente significativas entre valores de concentração de VEGF-A, VEGF-B ou PIGF sérico entre doenças neovasculares e não neovasculares;
4. Observou-se uma correlação positiva e forte entre VEGF-A e VEGF-B, tanto no vítreo como no soro analisado, sugerindo um aumento patológico em simultâneo dessas citoquinas;
5. Não foram reportadas correlações entre os níveis séricos e os níveis vítreos para todos os factores de crescimento endotelial vascular: VEGF-A, VEGF-B e PIGF;
6. Foi encontrada uma correlação positiva entre VEGF-A ou VEGF-B e volume macular;
7. Através da análise descritiva de terapêuticas prévias administradas aos doentes com doença ocular neovascular, e das análises comparativas entre doentes naïve e não-naïve, confirmou-se a existência provável de doentes com resposta insuficiente às terapêuticas actualmente aprovadas, reconhecendo-se assim a necessidade de investigação de novos fármacos para o tratamento de doenças neovasculares.

Em resumo, todos os resultados sugeriram uma sobre-expressão dos níveis vítreos para as três citoquinas estudadas; as correlações entre fatores de crescimento de VEGF-A e VEGF-B, confirmaram o seu aumento em simultâneo em patologias neovasculares oculares; e as correlações com o estadio da retinopatia diabética e características funcionais e estruturais das doenças neovasculares, demonstraram a ação destas citoquinas na progressão da doença.

O enfoque na inibição de VEGF-A ou VEGF-B ou PIGF pode ter implicações benéficas e um grande impacto no tratamento das doenças neovasculares, não apenas em termos de melhor eficácia, mas também em termos de regressão da doença (no caso da diabetes).

A identificação de biomarcadores ou outras características da doença quantificáveis e a sua correlação com os níveis de VEGF-A, VEGF-B e PIGF no vítreo poderá conduzir a uma intervenção precoce na prevenção destas doenças, através da detenção da neovascularização ocular antes do aparecimento de sintomas clínicos, conduzindo assim à prevenção da cegueira.

Palavras-chave

Angiogénese; degenerescência macular da idade; doenças neovasculares; ELISA; factor de crescimento endotelial vascular A; factor de crescimento endotelial vascular B; factor de crescimento placentário; humor vítreo; inibição de VEGF; oclusão da veia da retina; retinopatia diabética; retina.

Abstract

Vascular Endothelium Growth Factors (VEGFs) are important regulators of endothelium cell proliferation, migration, and permeability not only during embryonic vasculogenesis but also in physiological and pathological angiogenesis. The VEGF family comprises seven members, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and Placental Growth Factor (PlGF); each one has a unique expression, specificity, and function. These members with different physical and biological properties act through three specific receptor tyrosine kinases, VEGFR-1, VEGFR-2, VEGFR-3, and two semaphorins receptors neuropilin-1 (NRP-1) and NRP-2.

Almost 80% of the volume of the eye is made up of a clear gel-like substance called vitreous humor that fills the center of the eye and is in continuous contact with the retina. It consists of water (~99%), hyalocytes, hyaluronic acid, ascorbic acid, inorganic salts, glucose, and a network of collagen fibrils.

In the present study, three cytokines (VEGF-A, VEGF-B and PlGF) were investigated in vitreous humor and serum of patients with ocular pathology, measured by Enzyme-linked immunosorbent assay (ELISA).

To compare and characterize the quantitative results obtained by ELISA, the patients were categorized into two groups:

1. Patients with neovascular ocular diseases were categorized into the following subgroups: age-related macular degeneration (AMD), retinal vein occlusion (RVO), and diabetic retinopathy (DR) patients and

2. Patients with non-neovascular ocular diseases: vitreomacular traction syndrome patients of idiopathic etiology or rhegmatogenous retinal detachment patients.

Additionally, the growth factors were compared or correlated between:

1. The studied groups of patients;

2. The different ocular pathologies;

3. With patient baseline clinical characteristics such as naïve vs. non-naive patients, previous treatments, the presence of glaucoma, and stage of diabetic retinopathy (non-proliferative diabetic retinopathy vs. proliferative diabetic retinopathy).

For further investigation, serum and vitreous concentration levels of VEGF-A, VEGF-B, and PlGF were correlated between each other and between changes assessed by optical coherence tomography (OCT) and visual acuity (VA) measurements.

The major conclusions of this study were as follows:

1. VEGF-A, VEGF-B, and PlGF vitreous levels are overexpressed in patients with neovascular ocular diseases in comparison with patients with non-neovascular ocular diseases;

2. In diabetic patients the vitreous vascular endothelial growth factors levels increase with the progression of the disease, being lower in non-proliferative diabetic retinopathy patients

and higher in proliferative diabetic retinopathy patients. However, serum levels of VEGF-A, VEGF-B and PIGF were higher in non-proliferative diabetic retinopathy patients in comparison with proliferative diabetic retinopathy patients;
3. There were no statistical differences between the concentration values of serum VEGF-A, VEGF-B or PIGF between neovascular and non-neovascular diseases;
4. There was a positive and strong correlation between vitreous and serum VEGF-A and VEGF-B, suggesting a simultaneous pathological increase in those cytokines;
5. There was no correlation between serum levels and vitreous levels of all growth factors: VEGF-A, VEGF-B and PIGF;
6. There was a correlation between VEGF A or VEGF-B and macular volume;
7. Through the descriptive analysis of previous treatments as well as the comparative analysis between naïve and non-naïve patients, the existence of patients with insufficient response to the current therapies was confirmed, and therefore, there is an unmet need for the research of new drugs to treat neovascular ocular diseases.

Taken altogether, these results suggest an overexpression of vitreous levels for the three studied cytokines. The correlations between VEGF-A and VEGF-B growth factors confirmed they may be simultaneously increased in neovascular eye pathologies. The correlations with diabetic retinopathy stage as well with structural and functional characteristics of studied neovascular disorders demonstrated the action of these cytokines in the progression of the disease.
Targeting VEGF-A or VEGF-B or PIGF inhibition may have beneficial and impactful implications in the treatment of neovascular diseases, not only in terms of better outcomes but also in the regression of disease (in the case of diabetes).
The identification of serum markers or other disease characteristics is easily measurable. Their correlation with vitreous levels will also lead to early intervention in disease prevention through the detention of ocular neovascularization before the appearance of clinical symptoms, subsequently leading to the prevention of blindness.

Keywords

Angiogenesis; age-related macular degeneration; diabetic retinopathy; enzyme-linked immunosorbent assay; neovascular diseases; placental growth factor; retina; retinal vein occlusion; vascular endothelial growth factor-A; vascular endothelial growth factor-B; VEGF inhibition; vitreous humor.

Thesis overview

The work presented in this PhD thesis was carried out at the Universidade da Beira Interior, Centro de Investigação em Ciências da Saúde (CICS-UBI) between September 2013 - 2017.
This thesis is structured in four chapters:

1. The first chapter is divided in seven main sections and a review paper. The chapter consists of a literature revision related to the studied subject. On the first section it is highlighted the structure of the eye with emphasis on anatomy and physiology. The second section comprises an overview of eye diseases with focus on the posterior eye diseases. The third and fourth sections briefly reviewed ocular drug delivery, angiogenesis and retinal diseases. The fifth and six sections are appraisals of the vascular endothelial growth factor and correspondent VEGF receptors and neuropilins. On the seventh section it is discussed the actual treatment of retinal diseases. The last section of the chapter comprises a review paper of the existing literature concerning quantification of vitreous levels VEGF-A, VEGF-B and PIGF and their respective roles in eye diseases.
2. The chapter II describes the global aims of the study.
3. The chapter III contains the research work developed during these four years and includes 3 original research papers:

(1) Paper II –VEGF-B Levels in the Vitreous of Diabetic and Non-Diabetic Patients with Ocular Diseases and Its Correlation with Structural Parameters;
(2) Paper III – Serum and vitreous placental growth factor in diabetic retinopathy patients: Relationship with disease severity and optical coherence tomographic parameters;
(3) Paper IV – Quantitative analysis and correlation of VEGF-A and VEGF-B in serum and vitreous of patients with neovascular vs. non-neovascular ocular diseases;

4. The chapter IV of this dissertation includes a general discussion.
5. The chapter V consists of the conclusion remarks &future directions obtained with this work.
6. The last chapter (chapter VI) incorporates the references used in the introduction and discussion of the thesis.

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Abbreviations

AGEs	Advanced glycation end-products
AMD	Age-related macular degeneration
CRT	Central retinal thickness
BAB	Blood-aqueous barrier
BCVA	Best corrected visual acuity
BOB	Blood-ocular barrier
BRB	Blood-retinal barrier
BRVO	Branch retinal vein occlusion
CME	Cystoid macular edema
CRVO	Central retinal vein occlusion
CSME	Clinical significant macular edema
DM1	Diabetes mellitus type 1
DM2	Diabetes mellitus type 2
DME	Diabetic macular edema
DR	Diabetic retinopathy
DRVS	Diabetic retinopathy vitrectomy study
ECs	Endothelial cells
ELISA	Enzyme-Linked Immunosorbent Assay
ETDRS	Early treatment diabetic retinopathy study
Flk-1	Fetal liver kinase 1 or VEGFR-2
Flt-1	Fms-like tyrosine kinase 1 or VEGFR-1
Flt-4	Fms-like tyrosine kinase 4
FDA	Food and Drug Administration
ICAM-1	Intercellular adhesion molecule-1
IGF-1	Insulin-like growth factor 1
IVTA	Intravitreal triamcinolone acetonide
KDR	Kinase insert domain receptor or Flk-1 or VEGFR-2
MCP-1	Monocyte chemoattractant protein-1
MV	Macular volume;
NPDR	Non-proliferative diabetic retinopathy
NRP-1	Neuropilin-1
NRP-2	Neuropilin-2
OCT	Optical coherence tomography
PCs	Pericytes
PDGF-A	Platelet-derived growth factor A
PDGF-B	Platelet-derived growth factor B
PDR	Proliferative diabetic retinopathy

PlGF	Placental growth factor
PPV	Pars plana vitrectomy
ROP	Retinopathy of prematurity
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RVO	Retinal vein occlusion
SMCs	Smooth muscle cells
sFlt-1	Soluble fms-like tyrosine kinase-1 (sFlt-1 or sVEGFR-1)
Tie2	TEK receptor tyrosine kinase
VA	Visual acuity
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor 1
VEGFR-2	Vascular endothelial growth factor receptor 2
VEGFR-3	Vascular endothelial growth factor receptor 3
VEGF-A	Vascular endothelial growth factor A
VEGF-B	Vascular endothelial growth factor B
VE-PTP	Vascular endothelial-phosphotyrosine phosphatase
VMT	Vitreomacular traction

Chapter 1

Introduction

1. Structure of the Eye: anatomy and physiology

The human eye is a complex organ that serves as the core of our most treasured sense sight. The eye captures light and transmits information to the brain, and this information is transformed by the brain into the images that we see. The adult eye is a filled sphere about 2.5 cm in diameter. Only the anterior one-sixth of the eye can be seen. The remaining part of the eye is enclosed within the eye socket, protected by a cushion of fat and the walls of the skeletal orbit (Marieb, 2008), (Figure 1).

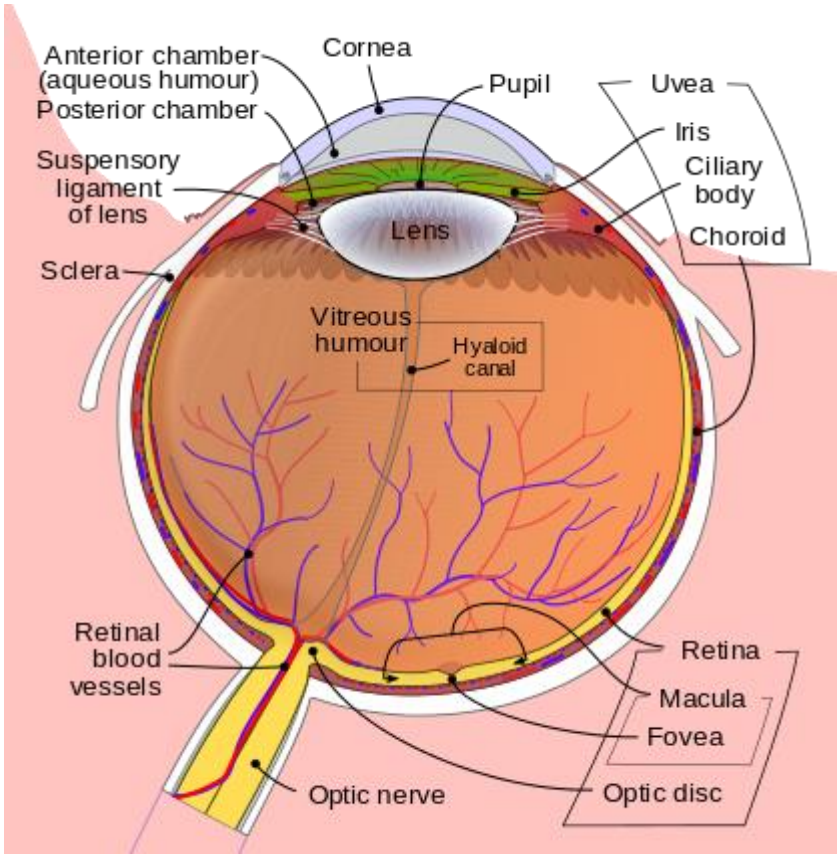


Figure 1: Illustrative diagram of the human eye. Horizontal section of the right eye. By RhCastilhos. (Public Domain).

There are 3 anatomical continuous layers of the eye:

1. The outer layer of the sclera and cornea;
2. The inner layer of the retina;
3. The middle layer of the choroid, ciliary body and iris, called uvea (Yorio et al., 2008).

It is usual to divide the eye in two segments: anterior and posterior (Snell & Lemp, 1998), which are protected by anatomical and biological barriers (Yorio et al., 2008).

The anterior segment

The main function of the anterior segment is to allow the passage of controlled quantities of light into the eye and refract this light so that it focuses on the retina. The cornea is a transparent and a vascular structure continuous with the sclera, observable as the white part of the eye. It is composed of five layers and joins the sclera at the limbus region. From the anterior to the posterior surface of the cornea these layers are surface epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium.

The cornea and the crystalline lens are mainly responsible for the focusing of incident light entering the eye. Light enters the eye through the cornea. Together the cornea and sclera enclose and protect the internal structures of the eye in a robust, fibrous coat.

The conjunctiva is a mucous membrane composed of non-keratinizing squamous epithelium that covers the external surface of the eye and inner surface of the eyelids. The majority of the conjunctiva is made up of specialized stratified squamous cells. Goblet cells are interspersed within this epithelium assisting in the production of mucin. This helps in nourishing the cornea and decreasing the friction and drying of the opposing palpebral and bulbar conjunctiva (Gray et al., 1991).

The lens is a transparent, elastic capsule of cells that changes shape to focus near and distant objects in the retina by a mechanism called accommodation. It is composed of concentric layers of cells, which form a biconvex structure which functions to focus images on the retina. It is located directly behind the posterior chamber and pupil and is enclosed in a fibrous, elastic capsule suspended from the ciliary body by suspensory ligaments. During accommodation, the ciliary muscle contracts and decreases tension in the suspensory ligaments leading the elastic lens to assume a curved shape (Gray et al., 2015). Accommodative power is gradually lost with age secondary to increased lens size and stiffness of the lens nucleus.

The iris is a circular rim structure in front of the lens, and the pupil is located at its center through which light passes into the lens. It is in the aqueous humor anterior to the lens and behind the cornea. The iris is mainly responsible for controlling the pupil diameter and size. Depending on the amount of light reaching the retina, iris muscles expand or contract the aperture at the pupil.

The anterior chamber varies in depth and is surrounded anteriorly by the cornea and posteriorly by the pupil and iris diaphragm. It consists of aqueous humor formed by the ciliary epithelium in the posterior chamber. Aqueous humor is formed from the blood's plasma by mechanisms of diffusion, ultrafiltration, and active transport (Goel et al., 2010). Although, the lens and the cornea have no blood vessels, they are tissues that absorb nutrients and secrete waste products. The circulating aqueous humor fulfills this function for the back of the cornea and lens. It is a filtrate of plasma secreted from capillaries in the ciliary body and

iris. The production of aqueous humor decreases with sleep, age, uveitis, retinal detachment and ciliochoroidal detachment (Goel et al., 2010).

The ciliary body, which is ring-shaped, has fibers named zonules that connect with muscles in the ciliary body. This mechanism helps to adjust the convexity of the lens. The ciliary body also secretes aqueous humor (Marieb, 2008).

The posterior segment

The posterior segment of the eye occupies two-thirds of the eye and includes the following structures: sclera, vitreous humor, retina, macula, choroid, and optic nerve.

The sclera is the opaque or white portion of the eye, which is fibrous in nature and forms the outer protective layer (Yorio et al., 2008).

The retina (Figure 2) is a thin layer of photoreceptor cells and associated neurons. It is the innermost layer of the eye and consists of a multi-layered sensory tissue. It contains millions of rod and cone cells, also known as photoreceptors. The cones are predominant in the macula, which is the retinal portion responsible for central vision. The retina is divided into two layers: retinal pigment epithelium (outer layer) and neural retina (inner layer). These 2 layers are separated by the fluid filled subretinal space (Yorio et al., 2008).

Light rays entering the eye converge at the cornea and the crystalline lens making them intersect at a point just behind the lens (in the vitreous humor). These rays then, pass through nine layers and diverge back to the outermost retinal layer (pigmented epithelium), which is reflected back to the rods and cones. Rods are responsible for night vision. Rod and cone cells capture light rays and translate them into electrical impulses. These impulses reach the brain via optic nerve and are converted into images (Yorio et al., 2008).

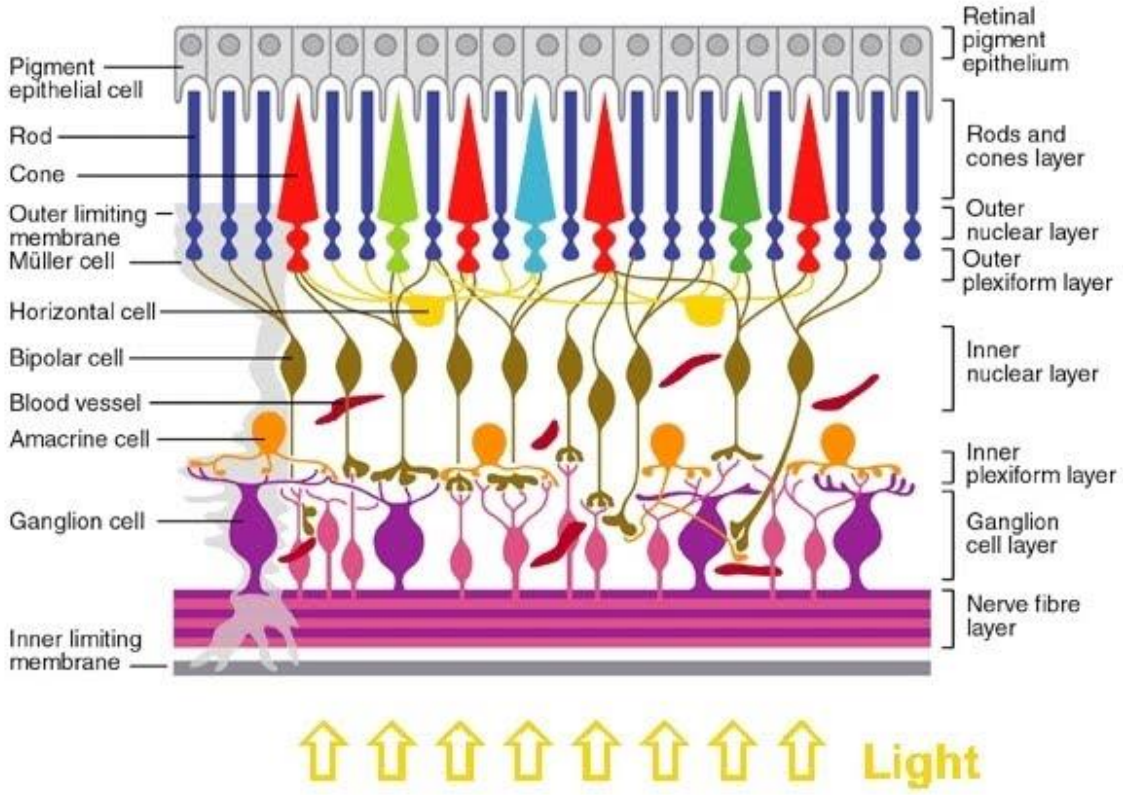


Figure 2: Structure of the retina. Adapted from Gray's Anatomy (public domain).

The retinal pigment epithelium (RPE) is a non-visual portion located between the neural portion of the retina and choroid. The RPE forms the outer blood-retinal barrier and has tight junctions that enable the epithelium to form a barrier by connecting neighboring cells (Rizzolo, 2007). RPE cells are well differentiated and regulate the trans-epithelial transport of various molecules. RPE expresses numerous efflux transporters, which prevents the entry of xenobiotics into the retina (Rizzolo, 2007).

Macula is a Latin word, which means "spot". It is an oval-shaped, yellow spot that is highly pigmented and is present in the center of the retina. It has a diameter of approximately 5 mm with two or more layers of ganglionic cells. The fovea is located at the center of the macula, and has the largest cone cell concentration making it responsible for the most visual acuity. The macular/foveal area is responsible for color discrimination. Moreover, the yellow color of the macula helps in the absorption of excess light entering the eye and thus acts as a natural form of protection (Marieb, 2008).

The choroid is a highly vascular tissue that supplies the sclera and the outer part of the retina with blood and nutrients. It is also rich in pigments that absorb light so that it does not scatter within the eye.

After entering the inner surface of the retina, light travels through and finally reaches the light-sensitive rods and cones at the outer retina, where the light is converted into electrochemical signals, which travel until the ganglion cells in the inner retina. The signals

are conducted via the optic nerve, out of the retina and eventually reach the visual cortex in the brain for processing, resulting in vision.

Photoreceptor cells are distributed over the entire retina, except where the optic nerve leaves the eyeball at the optic disc or 'blind spot' (Marieb, 2008).

There are around 100 million rods and 5 million cones in each retina (Fauci et al., 2009). As rods are responsible for night vision, anything that interferes with rod function causes night blindness (Marieb, 2008).

Cones are specialized for high spatial resolution and allow us to see in color in good light. There are three varieties of cones, each most sensitive to particular wavelengths of visible light (Marieb, 2008). The absence of the different cone types leads to color blindness: the lack of three cone types lead to total color blindness, while the lack of one cone type leads to partial color blindness (Marieb, 2008).

The blood–ocular barrier structure is mainly made of 2 barriers: the blood–aqueous barrier and the blood–retinal barrier (BRB) (Cunha Vaz, 1979). The blood-retinal barrier (BRB) forms a barrier between the retina and the blood supply (Cunha Vaz, 1979; Ehrhardt & Kim, 2008). It is formed by both the retinal blood vessels (inner BRB) and the retinal pigment epithelium (outer BRB) (Cunha Vaz et al., 1979; Yanoff & Duker, 2008), (Figure 3). Changes in the BRB lead to the development of retinal diseases. While in diabetic retinopathy (DR) we observe changes of the inner BRB, in age-related macular degeneration (AMD) there are changes in the outer BRB (Cunha Vaz, 2009); in both diseases we observe an accumulation of extracellular fluid.

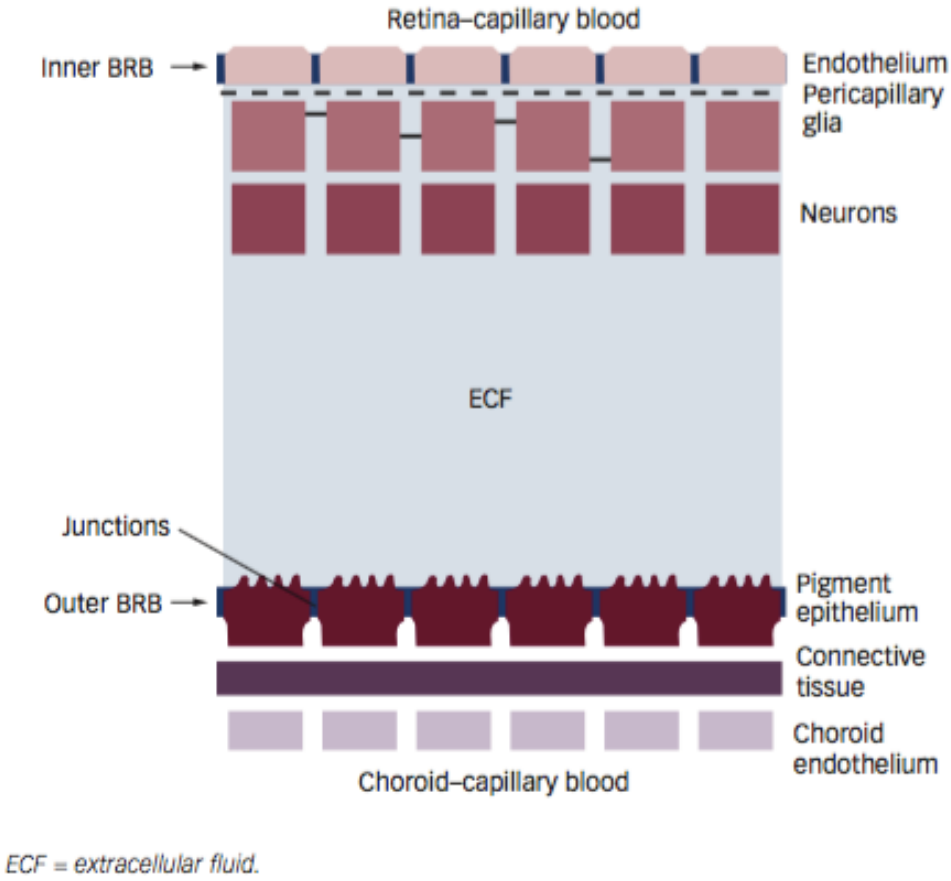


Figure 3: Schematic representation of the Inner and Outer Blood-Retinal Barriers. Adapted from Cunha Vaz, 2009.

The inner layer has tight junctions between retinal vascular endothelial cells, creating a barrier that is normally impermeable to proteins (Anand-Apte & Hollyfield, 2010). The exchange of metabolites and waste between the vascular lumen and the neural retina is controlled by the BRB. Damage to the retinal microvascular system may disrupt the tight junctions of the inner BRB. Consequently, the breakdown of the inner BRB allows the leakage of substances and liquids from capillaries into the extracellular space, causing accumulation of fluid. When this accumulation of fluid appears in the macula, it leads to the development of macular edema (Bressler & Wenick, 2012; Cunha Vaz, 1979).

Sometimes, abnormal new blood vessel formation takes place in the presence of inflammation or low oxygen levels (hypoxia), hence making these vessels susceptible to rupture. When this occurs, it allows blood and fluid to leak into the retinal tissue. This is called intraretinal hemorrhage, and the leaking fluids are called subretinal fluid or exudates (Wu & Acón, 2017).

The vitreous body or also called vitreous humor or simply vitreous (Figure 4) is a thin transparent gel, which helps transport metabolites to the lens and inner retina. Almost 80% of the volume of the eye is made up of this clear gel-like substance. This fluid fills up the space

between the lens and the retina. Vitreous humor consists of water (~99%), hyalocytes, hyaluronic acid, ascorbic acid, inorganic salts, sugar, and a mesh of collagen fibrils (Sebag, 1992). The network of non-branching collagen fibers with hyaluronic acid provides viscosity to the vitreous humor which is two to four times higher than pure water and has a refractive index of 1.336. Vitreous humor is stagnant, while aqueous humor is continuously replenished (Tolentino, 1974). The vitreous humor is in continuous contact with the retina and adheres to the retina at three places: the macula, optic nerve disc, and fovea (Tolentino, 1974).
The vitreous humor fills the center of the eye. In children, the vitreous has the consistency of a gel. With age, it gradually thins and becomes more liquid. As the vitreous thins, it separates from the parts, which is attached to the retina, causing retinal detachment.

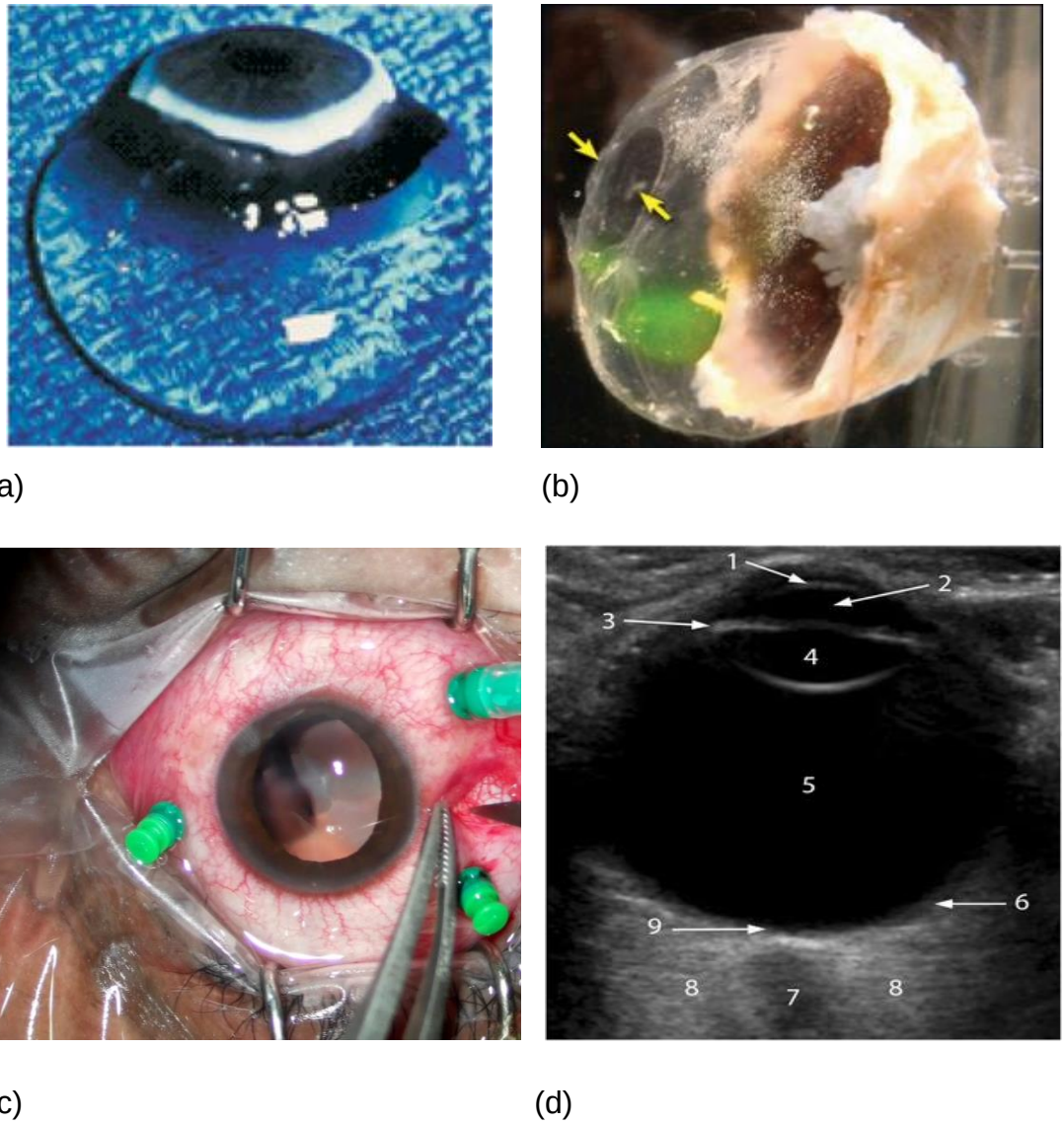


Figure 4: Schematic pictures of vitreous humor.
(a) Intact human vitreous gel (Sebag, 2014); (b) Human vitreous (Yannuzzi, 2014); (c) Vitreous humor removal from the eyeball during a vitrectomy (public domain); (d) Sonographic appearance of the

structures of the normal eye. The vitreous gel can be visualized in the number 5 of the image (De La Hoz Polo, 2016).

The optic disc is responsible for the conduction of visual information from the retina to the brain. It is composed of 1.2 million myelinated axons of retinal ganglion cells and has a varying diameter of approximately 1-10 μm. It is located within the orbit, and it extends from the eyeball to the optic foramen. The length of the optic nerve varies from 20-30 mm with a diameter of 5 mm (Snell & Lemp, 1998).

2. Eye diseases – overview of posterior eye diseases

The combination of an aging world population along with a dramatic decline in fertility, has led to a vast number of people over 65 years worldwide (West & Sommer, 2001). The loss of vision and blindness is increasing every year among the elderly and has become a major public health concern ("Age-related Macular Degeneration", 2017).
DR, diabetic macular edema (DME), AMD, retinal vein occlusion (RVO), uveitis, glaucoma and cataract, are the common diseases that if left undiagnosed and/or untreated lead to potential blindness. All these diseases except cataract, affect the posterior eye that is composed by the vitreous, the retina, the retinal pigment epithelium and the choroid. The anterior segment of the eye includes the cornea, the anterior chamber, the iris, the posterior chamber, the ciliary body, and the lens. The main eye diseases involving the anterior segment include dry eye or keratoconjuntivitis sicca, cataract, conjunctivitis (blepharo-conjunctivitis, kerato-conjunctivitis and episcleritis) (Fauci et al., 2009).

Diabetes mellitus (diabetes) results in considerable morbidity and premature mortality. Diabetes has profound effects on the structure and function of many tissues and organs in the body. Complications of diabetes afflict the macrovascular and microvascular structures. Macrovascular diseases include cardiovascular conditions such as stroke, myocardial infarction and peripheral vascular disease while microvascular diseases comprise of DR, diabetic neuropathy and diabetic nephropathy (Holt, 2010). Diabetes is one of the main public health issues in today's society. The incidence of blindness is 2-3 times greater in persons with diabetes when compared with the non-diabetic population (Hayward et al., 2002). Visual loss and blindness due to diabetes are preventable in the vast majority of people, through optimal management of diabetes, hypertension and hypercholesterolemia and also the implementation of a screening program to detect DR early.

Diabetic retinopathy is the most frequent microvascular complication of diabetes and the leading cause of blindness in the working population in developed countries worldwide. Approximately 75% of all diabetic patients show clinical signs of retinopathy within 15 years after the onset of diabetes and more than 10% of diabetic patients develop visual impairment

within this period (Klein et al. 1995). It is a chronic microvascular complication of diabetes, consisting of damage to the retina and capillaries caused by hyperglycemia and hypoxia and may lead to a significant loss of vision. Vision loss from DR is usually painless, progressive and bilateral (Bhagat et al., 2009). The initial alterations are manifested by biochemical signs of oxidative stress and cellular signs of subclinical inflammation (Bhagat et al., 2009). The earliest vascular progressions involve leukostasis, aggregation of platelets, modification of the blood flow, degeneration of pericytes and thickening of basement membranes. Blockage of the retinal capillaries may cause localized hypoxia, triggering increased tissue production of angiogenic factors, including VEGF-A. The release of VEGF-A and other angiogenic factors causes loosening of the vascular endothelial cell-cell junctions, leading to an increased vascular permeability. Despite the increased production of VEGF-A, a potent survival factor for endothelial cells (ECs), microvascular endothelial cells degenerate, leading to capillary closure and formation of a cellular, non-perfused capillaries. With disease progression, appear vascular alterations, such as micro-aneurysms, blot hemorrhages, cotton-wool spots, venous beading and vascular loops (Penn et al., 2008). The vascular leakage increases inducing blood and fluid accumulation within the retinal tissue and formation of exudative deposits. This condition is known as non-proliferative diabetic retinopathy (NPDR). Tissue swelling caused by the accumulation of excess interstitial fluid can alter metabolic processes and ion fluxes within retinal neurons and glia. When the edema affects the macula, it can result in neuronal distortion leading to decrease of visual acuity (Kent et al., 2000). In some patients, the NPDR progresses to proliferative diabetic retinopathy (PDR). PDR is characterized by the growth of new blood vessels on the surface of the retina. The new blood vessels are fragile and easily rupture, leaking blood into the neural retina and vitreous, thus clouding the vitreous and compromising vision. With advanced PDR, fibrovascular scar tissue grows from the retinal surface into the vitreous cavity. If untreated, this can cause retinal detachment resulting in blindness. Studies of clinical specimens have shown a strong correlation between increase in intraocular VEGF-A levels and the development of PDR (Duh & Aiello, 1999).

The condition of diabetic macular edema (DME) is more common in patients with type 2 diabetes (7.5%) than in patients with type 1 diabetes (5.9%), (Hirai et al., 2008). In DME there is accumulation of fluid within the intra-retinal layers of the macula due to exudation from the retinal microvasculature. DME may occur in any stage of DR and it is also a leading cause of blindness in the diabetic population (Bodansky et al., 1982).
DME mainly affects people with a history of diabetes mellitus. It is characterized by the swelling of the retina within the macula gradually leading to leakage of fluids from blood vessels and breakdown of the blood-retinal barrier (Cunha Vaz, 2009; Klein et al., 2009). Macula is a small structure present at the center of the retina that is rich in cones and specialized nerve endings. Visual impairment due to DME occurs when the macula is involved, as this is the area responsible for high-acuity vision. Disruption of the BRB leads to leakage

and therefore to retinal edema. Retinal edema affecting the macular region may lead to blurred vision, which is a most common symptom of visual impairment in DME (Aspelund et al., 2011).
Additional symptoms of visual impairment associated with DME include image distortion, floaters, altered sensitivity to contrast, photophobia, changes in color vision and scotomas (Bouhaimed et al., 2008).

There are several classifications for DR and DME as seen in Table 1, all classifications emerged in an attempt to obtain a consensus.

Table 1 - Classifications of DR and DME (Gangwani et al., 2016)

ETDRS	**NCS (UK)**	**AAO/ International**	**RCOphth**
10 None	R0 None	No apparent retinopathy	None
20 Microaneurisms only	R1 Background	Mild NPDR	Low risk
35 Mild NPDR		Moderate NPDR	-
43 Moderate NPDR	R2 Pre-proliferative	-	High risk
47 Moderately severe NPDR	-	-	-
53 A-D Severe NPDR	-	Severe NPDR	-
53 E Very severe NPDR	-	-	-
61 Mild PDR	R3 Proliferative	PDR	PDR
65 Moderate PDR	-	-	-
71,75 High risk PDR	-	-	-
81, 85 Advanced PDR	-	-	-

AAO – American Academy of Ophthalmology; ETDRS – early treatment diabetic retinopathy study; NCS (UK) – National Screening committee (United Kingdom); NPDR – non-proliferative diabetic retinopathy; PDR – proliferative diabetic retinopathy; RCOphth – Royal College of Ophthalmologists

DME classification according to the International Clinical DME Disease Severity Scale is shown in Table 2.

Table 2 - International Clinical DME Disease Severity Scale (Wilkinson et al., 2003)

Proposed Disease Severity Level	**Findings observable upon dilated ophthalmoscopy**
DME apparently absent	No apparent retinal thickening or hard exudates in posterior pole
DME apparently present	Some apparent retinal thickening or hard exudates in posterior pole
If DME is present, it can be categorized as follows:	
Mild DME	Some retinal thickening or hard exudates in posterior pole but distant from the center of the macula
Moderate DME	Retinal thickening or hard exudates approaching the center of the macula but not involving the center
Severe DME	Retinal thickening or hard exudates involving the center of the macula

DME – Diabetic Macular Edema

Fluorescein angiography (FA) is used to evaluate DME, providing information about the retinal perfusion, the BRB, and neovascularization. The angiographic classification of DME comprises of non-cystoid and cystoid macular edema (CME) and focal or diffuse DME (Lobo et al., 2012). Focal DME results from microaneurysms leakage. Diffuse DME results from the breakdown of BRB with leakage from microaneurysms, retinal capillaries, and arterioles (Wu et al., 2013).

The ETDRS study defined DME as focal or diffuse retinal thickening in the macular area and, when the thickening involves the fovea, it is defined as clinically significant macular edema (CSME) (Panozzo et al., 2004).
CSME was defined as "(1) thickening of the retina at or within 500 μm of the center of the macula; or (2) hard exudate at or within 500 μm of the center of the macula associated with thickening of adjacent retina; or (3) a zone of retinal thickening 1 disc area or larger, any part of which is within 1 disc diameter of the center of the macula" (Bandello et al., 2003).

Based on optical coherence tomography, DME can assume different morphologic patterns (Gangwani et al., 2016, Wu et al., 2013).

Overall, the classification of DME can be:

- Clinical: it is the standard classification to assess the severity of DME and it is based on the ophthalmoscopy observation. It may then be classified as: 1. Clinical significant macular edema (CSME), which is based on ETDRS or, 2. International clinical classification.
- Angiographic: it is the classification based on the angiographic observation and can be focal, diffuse, ischemic, exudative and a combination of multiple patterns.
- Based on vitreo-retinal interface alteration: It is a morphological classification and based on the existence of vitrogenic components.

Further investigations on the pathophysiological processes of DME demonstrated that VEGF-A is in part the mediator of the increase in retinal capillary permeability that occurs following the breakdown of the blood-retinal barrier (Aiello et al., 1994). VEGF-A has a key role in the pathogenesis of DME due to its action in increasing vascular permeability (Figure 5), (Bhagat et al., 2009).

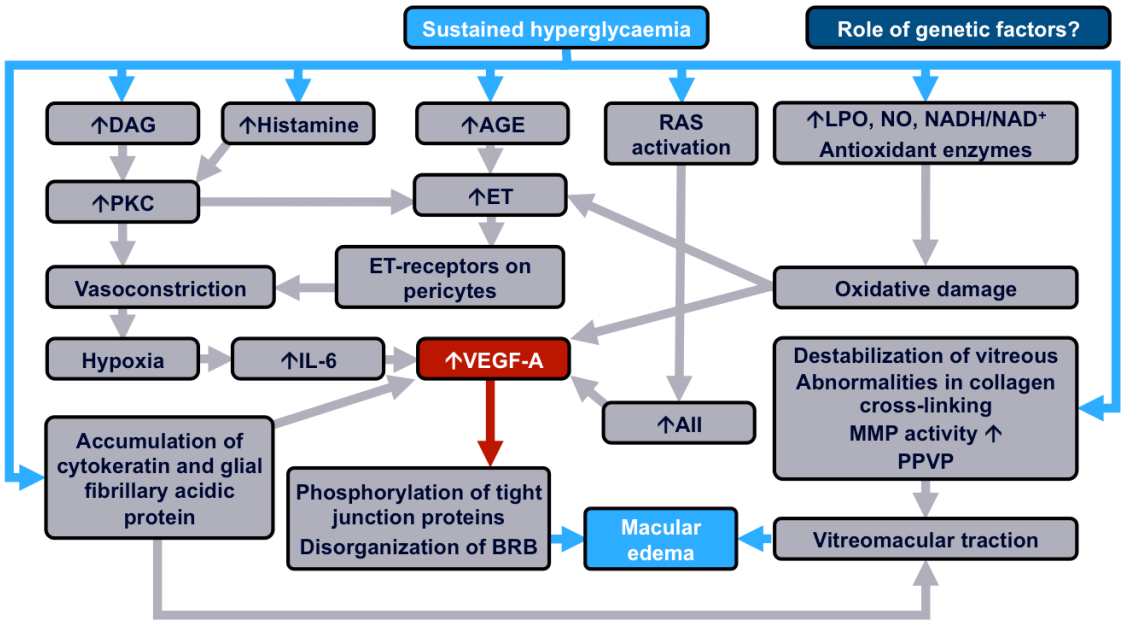


Figure 5: Pathogenesis of DME (adapted from Bhagat et al., 2009).
AII - angiotensin II, AGE - advanced glycation end, BRB - blood-retinal barrier, DAG - diacylglycerol, ET - endothelin, LPO - lipoxygenase, MMP - matrix metallo-proteinases, NO - nitric oxide, PKC - protein kinase C, PPVP - posterior pre-cortical vitreous pocket, RAS - renin-angiotensin system

Age-related Macular Degeneration (AMD) is another leading cause of vision loss worldwide (Rosenfeld et al., 2006). The neovascular form comprises only 10% of AMD but accounts for 90% of all cases of blindness due to AMD (Rosenfeld et al., 2006).
AMD is a medical condition affecting older adults, which results in loss of central vision. The center part of the retina (macula) is affected; hence, the person loses central vision. Central

vision is necessary to perform certain activities like reading and driving. The disease does not affect peripheral vision and therefore the person does not suffer from total vision loss. AMD occurs in two forms, wet or exudative or neovascular and dry or non-exudative or non-neovascular. Wet AMD is associated with the formation of new blood vessels under the retina. This abnormal growth of choriocapillaries results in leakage of blood and proteins, and the formation of scar tissue, and consequently damaging the photoreceptors of the retina. The wet form of the disease spreads rapidly and can result in complete vision loss if left untreated. Dry AMD, on the other hand, is a slow process of degeneration of the photoreceptors of the retina. It is characterized by the formation of drusen and deterioration (atrophy) of photoreceptors, RPE and the choriocapillaries in the macular area. Dry AMD progresses slowly with a generally better prognosis than wet AMD (Rosenfeld et al., 2006).
Age is considered as one of the major risk factors for AMD patients. Apart from age, the other risk factors for AMD development include smoking, obesity, race, gender and family history (Chakravarthy et al., 2010). Wet AMD patients are also characterized by high levels of VEGF-A (Aiello et al., 1994). Figure 6 represents a schema of the pathogenesis of neovascular AMD (Augustin & Kirchhof, 2009).

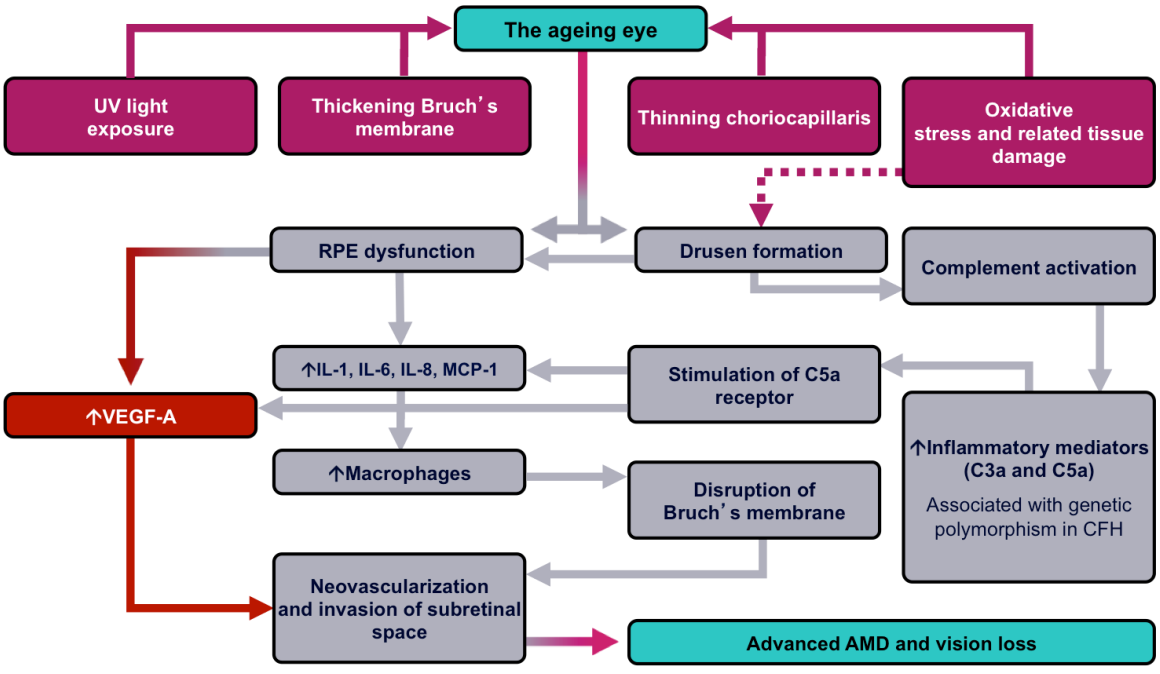


Figure 6: Pathogenesis of neovascular AMD (adapted from Augustin & Kirchhof, 2009)
CFH - complement factor H, IL – interleukin, MCP - monocyte chemoattractant protein, RPE - retinal pigment epithelium.

Retinal vein occlusion (RVO) is a potentially sight-threatening disease caused by blockage of the retinal vein that drains blood from the back of the eye. After DR, RVO is the second cause

of retinal vascular disease (Kiire & Chong, 2012). It is classified into branch RVO (BRVO) and central RVO (CRVO) based on the site of the venous occlusion. Branch retinal vein occlusion (BRVO) and central retinal vein occlusion (CRVO) affect 1.8% and 0.5% respectively, of the population aged 50 years or older (Klein et al., 2008). BRVO is typically caused by blockage of an arteriovenous crossing, whereas CRVO is caused by a blockage of the central retinal vein (Battaglia et al., 2009). Macular edema (ME) is also a complication of RVO that can lead to blindness. RVO causes increased secretion of cytokines such as VEGF-A and interleukin-6 that increase the permeability of the blood-retinal barrier. This generates fluid leakage into the retinal tissue, which results in ME.
Hemi-retinal vein occlusion (HRVO) affects the superior or inferior retinal hemisphere.
The two principal complications of RVO are retinal ischemia and ME, which may lead to iris neovascularization. Eyes with RVO contain some of the highest vitreous VEGF-A concentrations (Aiello et al., 1994).

Glaucoma affects the optic nerve head and results in the loss of retinal ganglion cells. The optic nerve head carries visual information to the brain. Increase in intraocular pressure is a major risk factor and damage to the optic nerve head causes progressive loss of peripheral vision. Untreated glaucoma can even result in loss of central vision. It is a common condition in persons over 40 years (Weinreb et al., 2014).

Uveitis means an inflammation occurring in the middle layer of the eye also known as the uvea (vascular layer present in between the retina and the sclera). The exact cause of uveitis is still not clear but studies indicate that may include infectious agents: viral (mumps and herpes), fungal (histoplasmosis) and bacterial (toxoplasmosis) play an important role in the establishment of this disease ("The Multicenter Uveitis Steroid Treatment Trial: Rationale, Design, and Baseline Characteristics", 2010). The cause of uveitis may also include trauma or autoimmunity (Forster, 2011). Based on the structures affected, uveitis can be categorized into anterior, intermediate, posterior and pan-uveitic forms. Anterior uveitis is the most common form of uveitis affecting the iris and anterior chamber while posterior uveitis affects mainly retina and choroid. Intermediate uveitis is characterized by inflammation in the vitreous cavity while pan-uveitis affects all layers of the uvea (Forrester, 2007)
Symptoms can include blurred vision, pain, photophobia and ‘floaters’ (cellular debris in the vitreous which floats across the field of vision). The mainstay of treatment in non-infectious uveitis is corticosteroids (Forster, 2011).

In this dissertation, we focus our attention on the treatment of posterior segment diseases, particularly AMD, RVO and DR, which are the leading causes of blindness and vision impairment.

3. Ocular drug delivery

One important topic is the treatment of eye diseases and consequently methods of delivering ocular therapeutics to the eye; although not subject to discussion in this dissertation, it will be briefly highlighted in this section (Figure 7).

The anatomy and physiology of the eye present unique challenges to ocular drug delivery. Most pharmacologic administrations to the eye use topical solutions, which are applied to the surface of the eye, such as drops. Depending on the physicochemical properties, the absorption of a drug following topical administration occurs by one or two pathways: corneal or non-corneal. A major fraction of the drug absorbed through the cornea enters through anterior ocular tissues, while the non-corneal absorption process primarily results in systemic drainage via the nasolacrimal duct (Geroski & Edelhauser, 2000). A typical eye drop volume ranges between 30–50 µL. Topical administration of an eye drop creates a rapid increase in the tear volume causing swift reflex blinking resulting in wash out of approximately 90% of the applied dose within 5-10 minutes. Furthermore, the impermeable nature of the cornea prevents permeant entry into the eye (Geroski & Edelhauser, 2000).

Nevertheless, topical administration is not successful in the treatment of posterior eye diseases. These diseases are treated by systemic, periocular, or intravitreal administration (Geroski & Edelhauser, 2000). Though the systemic administration is most widely indicated, only 1–2% of the dose reaches the vitreous (Cunha-Vaz, 1997). Moreover, entry of therapeutic agents following systemic and periocular administration is challenging due to the presence of blood-ocular barriers (BOBs). BOB is a physical barrier between the local blood vessels and ocular tissues. There are two types of BOBs: blood-aqueous barrier (BAB) and blood-retinal barrier (BRB).

This plays a major role in the pathophysiology of retinal drug delivery. It prevents the entry of large drug molecules from choriocapillaris into the retina (Cunha-Vaz, 1997). Additionally, the entry of xenobiotics following systemic or periocular (subconjunctival, retrobulbar, peribulbar and sub-tenon) administration is hindered by the BRB (Cunha-Vaz, 1997).

These barriers along with the complex structure of the eye result in poor ocular bioavailability (Gaudana et al., 2010).

However, remarkable advancements have been observed in ocular drug delivery over the last few decades, namely:

(a) Intravitreal Injections: Intravitreal injection is the choice of drug delivery for the treatment of retinal diseases such as AMD, DR, DME and RVO. It is capable of delivering high concentrations of drugs into the vitreous and retina. The elimination of drugs delivered by intravitreal injection are correlated with their molecular weight: molecules with higher molecular weight have longer retention times in the vitreous humor, generally being more associated with various complications, such as retinal detachment, cataracts, glaucoma,

increase in intraocular pressure, endophthalmitis, or intravitreal hemorrhages (Marmor et al., 1985; Patel et al., 2013; Velez & Whitcup, 1999).

(b) Periocular Injections: retrobulbar injection; peribulbar injection, sub-tenon injection and subconjunctival injection.

Furthermore, different and innovative drug delivery systems are being developed such as: nanoparticles, microneedles, liposomes, dendrimers, niosomes, nanosuspensions, ultrasound, iontophoresis and implants, which allow the control rate and duration of drug release. Ocular implants may be divided into two categories depending on the polymer used in the matrix:

- Non-biodegradable implants: These delivery systems are neither metabolized nor erodible once they are inserted. They are usually made of an inert material not harmful to the organism or organ where it remains (Patel et al., 2013).
- Biodegradable implants: They degrade in vivo into soluble components by enzymatic and/or non-enzymatic processes (Patel et al., 2013).

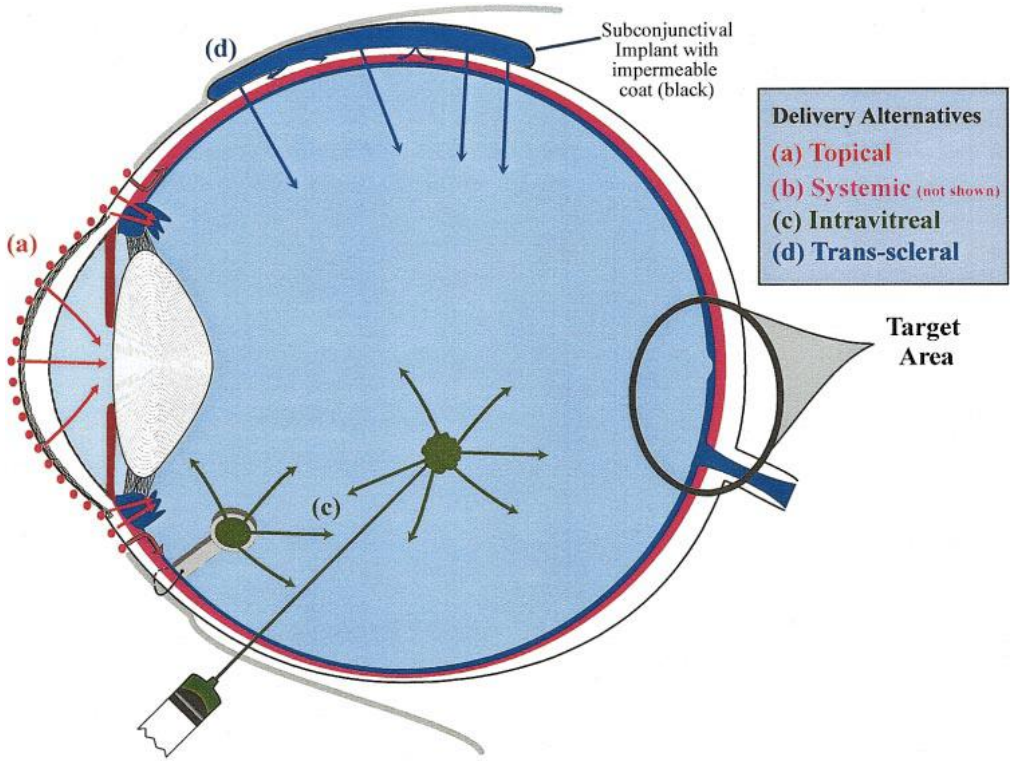


Figure 7: Drug delivery pathways for the treatment of posterior ocular diseases (adapted from Geroski & Edelhauser, 2000).

4. Ocular Angiogenesis and Retinal Diseases

Angiogenesis is a process that occurs in adults under specific conditions; in physiological situations such as wound healing and the female reproductive cycle or in pathological states such as cancer and retinal diseases. The counterbalance among proangiogenic and anti-angiogenic factors and proteins outline the occurrence of angiogenesis. This is the so-called "angiogenic switch" which is believed to be "on" when angiogenesis occurs (Hanahan & Folkman, 1996), (Figure 8).

The VEGF family growth factors are important not only in adult vascular remodeling but also as factors for growing blood vessels. ECs are responsible for new blood vessel formation, while other cells types are responsible for vessel maturation. Macrophages and smooth muscle cells (SMC) secrete angiogenic factors contributing to angiogenesis (Cleaver & Melton, 2003).

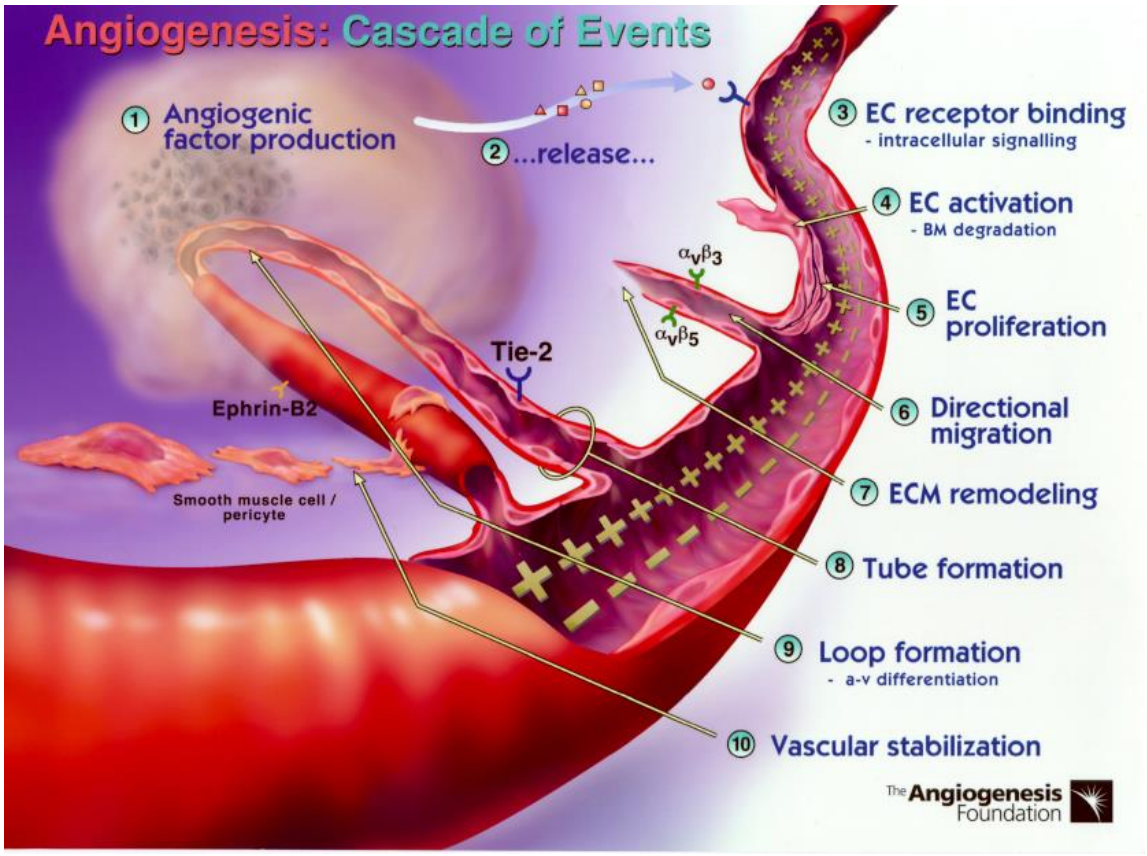


Figure 8: Cascade of events in angiogenesis process (adapted from https://angio.org/understanding/understanding.html, © 2000 The Angiogenesis Foundation, Inc. All rights reserved).

Neovascularization, the development of new blood vessels, is a crucial process for fetal development and several pathological states. Vasculogenesis and angiogenesis are the two mechanisms responsible for neovascularization (Yamazaki & Morita, 2005).

VEGFs represent a set of structurally and functionally related growth factors that modulate critical physiological functions of ECs playing a primary role in pathological angiogenesis in retinal disorders. Angiogenesis is critical for the progression of many different diseases, including ocular diseases, representing the main cause of irreversible vision loss in developed countries.

The blood vasculature forms by two processes: vasculogenesis, the differentiation of precursor cells (angioblasts) into ECs and the de *novo* formation of a primitive vascular network, and angiogenesis, the growth of new capillaries from pre-existing vessels (Olofsson et al., 1999). The development of blood vessels (angiogenesis) and the vascular supply is a requirement for organ development and differentiation during embryogenesis. In addition to normal physiological processes, such as wound healing, tissue regeneration, organ regeneration, and intervention in the reproductive cycle in women, pathological angiogenesis occurs in neovascular eye disorders and are recognized to be dependent on the development of new blood vessels. The process of blood vessel formation is complex and highly coordinated and dependent on a series of interactions between receptors and ligands (Nash et al., 2006).

VEGF-A is the most studied growth factor of the VEGF family and has been proven to have a substantial role in the process of vascularization and angiogenesis. Evidence shows that there are increased levels of VEGF-A in the retina and in the vitreous of patients with retinal diseases, supporting the existence of significantly higher levels of VEGF-A in diabetic patients, AMD and RVO patients (Aiello et al., 1994). VEGF-A promotes angiogenesis in each of the studied ocular conditions making it an exceptional therapeutic target (Ferrara et al., 2003). Contrary, VEGF-B is the least studied and the most enigmatic molecule of the VEGF family concerning its role (Li et al., 2009). Furthermore, and to the best of our knowledge, the contribution of PlGF in neovascularization has received little attention, but it may also represent a significant target (Rakic et al., 2003).

Intraocular neovascularization occurs in numerous ischemic retinal disorders, including DR, ischemic RVO and AMD. This proliferation often results in visual loss (Amadio et al., 2016).

Relative to the role of the VEGF family in ocular diseases, it is known that endothelial growth factors and their receptors provide important therapeutic pathways for the treatment of disorders characterized by an abnormal angiogenesis. VEGF-A is a critical angiogenic growth factor for vasculogenesis and angiogenesis in the healthy state and in the presence of pathological conditions.

However, not only VEGF-A has an important role in angiogenesis and consequently in the development of neovascular eye diseases. We believe that VEGF-B as well PIGF is strongly implied in this process.

In summary, there are three main reasons to identify other molecules of the VEGF family besides VEGF-A as important factors in the pathogenesis of intraocular diseases:

1) Pathological transformations of the retinal vasculature are associated with an increased expression of VEGF-A, VEGF-B, and PlGF as well other factors (Cabral et al., 2017);

2) VEGF-A alone is sufficient to trigger neovascularization, but other growth factors also act together in this process (Penn et al., 2008);
3) Inhibition of VEGF-A, VEGF-B and PlGF is associated with anatomical and functional improvements in the affected eye (Cabral et al., 2017).

5. Vascular Endothelial Growth Factor Family

The VEGF family consists of seven members: VEGF-A (sometimes referred to as only VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and PlGF (placental growth factor), (Figure 9a). Alternative splicing results in several VEGF variants (Penn et al., 2008).

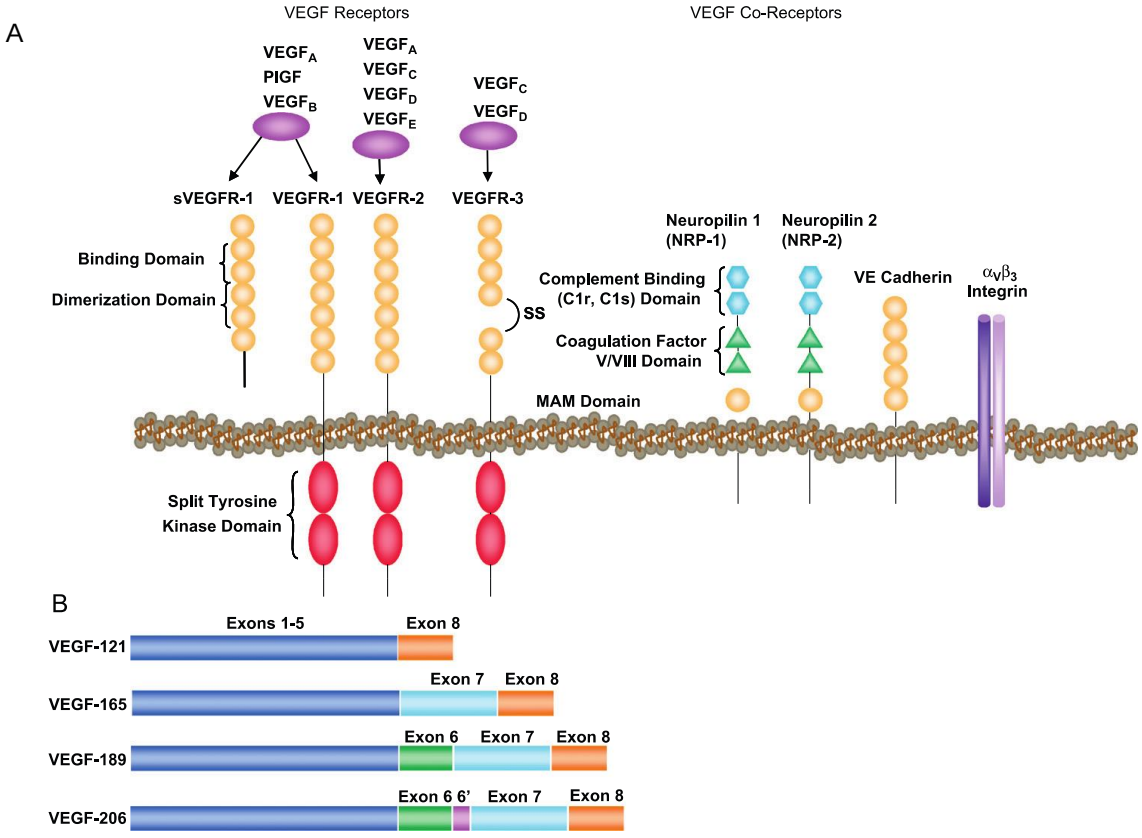


Figure 9: (a) Interactions between VEGF isoforms and VEGF receptors or between co-receptors; (b) VEGF-A isoforms formed by alternative splicing (adapted from Penn et al, 2008).

Although all VEGF structures are different, they are related and include a normal feature, called the VEGF homology domain as seen in Figure 9b.
The nomenclature used to designate the various isoforms refers to the number of amino acids that comprise the mature, secreted protein; human $VEGF_{165}$, for example, consists of 165 amino acids. All VEGF isoforms discovered till date contain exons 1 to 5, with various combinations of the remaining exons 6 to 8. Exons 3 and 4 contain the vascular endothelial

growth factor receptor 1 (VEGFR-1) and vascular endothelial growth factor receptor 2 (VEGFR-2) binding domains, respectively. All isoforms that are able to bind to these receptors are biologically active (Ng et al., 2006).

Alternative exon splicing generates VEGF-A, VEGF-B, and PlGF. Each VEGF subtype selectively binds to some of these receptors or to the co-receptors, neuropilins 1 and 2 (NRP-1 and NRP-2), often with different affinity and selectivity, demonstrating the diversity of their biological functions. They are dimeric glycoproteins that act through three cell surface receptor tyrosine kinases, known as the VEGF receptors (VEGFRs). VEGF-A, VEGF-B, and PlGF bind to VEGFR-1 (or Flt-1); VEGF-A and VEGF–E bind to VEGFR-2 (or Flk-1); VEGF-C and VEGF–D bind to VEGFR-3; and VEGF-F binds to both VEGFR-2 (or Flk-1) and VEGFR-1 (or Flt-1), (Olsson et al., 2006).

5.1 VEGF-A

Vascular Endothelial Growth Factor-A (VEGF-A) or as initially named, Vascular Permeability Factor, (VPF) was discovered nearly two decades ago as an angiogenesis stimulating and permeability factor (Leung et al., 1989). VEGF-A was the first VEGF to be discovered and is currently the most well characterized member of the VEGF family. While physiological angiogenesis due to VEGF-A is predominantly restricted to wound healing, bone formation, hematopoiesis, development and the female reproductive cycle, it also plays a key role in the pathogenesis of several human diseases, including cancer, eye diseases and inflammation among other disorders (Ferrara et al., 1998). VEGF-A is produced by several kinds of cells: tumor cells (Itakura et al., 2000), macrophages (Sunderkotter et al., 1994), platelets (Verheul et al., 1997), keratinocytes (Frank et al., 1995), and renal mesangial cells (Iijima et al., 1993). The VEGF-A gene is comprised of eight exons and seven introns. Alternative exon splicing generates six isoforms: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{165b}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$ (Tong & Yao, 2006). Each isoform is characterized by the respective number of amino acids after cleavage of the signal sequence variants (Tong & Yao, 2006), (Figure 10). VEGF-A is produced in various cell types, including ECs and leucocytes and binds to both VEGFR-1 and VEGFR-2 and neuropilins (Favier et al., 2006). Hypoxia is the most common inducer of VEGF-A synthesis. However, VEGF-A is not only regulated by hypoxia. Its function is also affected by Insulin-like growth factor 1 (IGF-1), which plays an important role in retinal vascularization (Penn et al., 2008).

VEGF-A is important for vasculogenesis (blood vessel growth from endothelial progenitor cells) and angiogenesis (blood vessel growth from pre-existing vessels). If there is inactivation of a VEGF-A allele, embryonic lethality occurs at embryonic day 11-12 in mice and tetraploid embryos, resulting in vascular defects and cardiovascular abnormalities (Carmeliet et al., 1996). VEGF-A expression is down-regulated after embryogenesis but is upregulated during physiological and pathological angiogenesis (Breier et al., 1992).

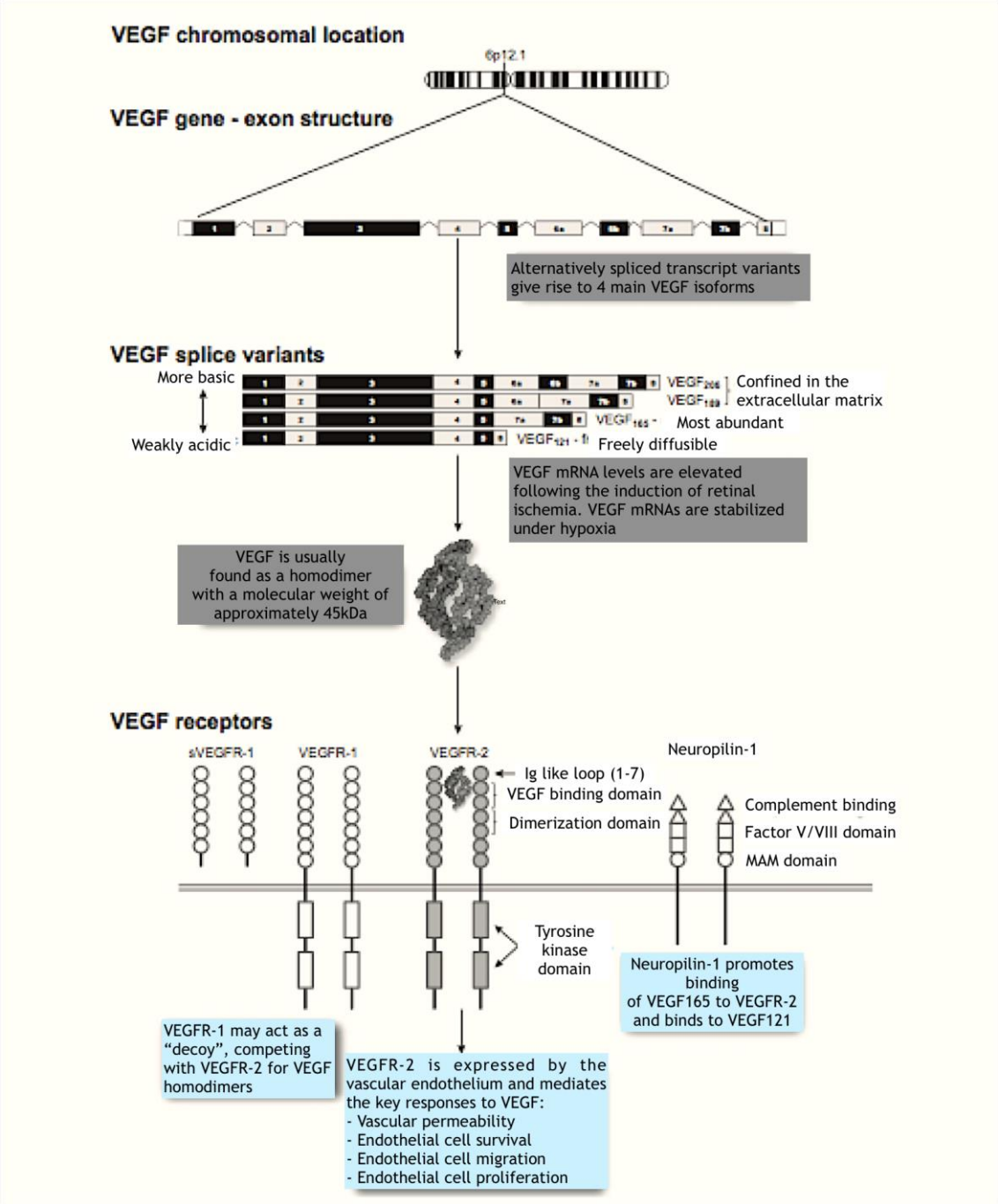


Figure 10: VEGF-A structure, splice variants and receptors (adapted from Miller et al., 2012)

Regarding its biological activity, VEGF-A induces proliferation and migration of ECs and vascular permeability, resulting in angiogenesis in vivo. Most of the major biological activities of VEGF-A are mediated by VEGFR-2, which shows stronger ligand-dependent tyrosine phosphorylation than VEGFR-1. VEGFR-1 is believed to function as a "decoy" receptor for secreted VEGF-A and its activation is also reported to result in inhibition of VEGFR-2 (Ferrara et al., 2003; Yamazaki & Morita, 2006).

To summarize, the *vegf-a* gene expression is regulated by:

1. Hypoxia through the hypoxia-inducible protein complex HIF-1;
2. Oxidative stress and the reactive oxygen species (ROS),
3. Hyperglycemia;
4. Advanced glycation end-products (AGEs),
5. Growth factors and cytokines, such as insulin growth factor (IGF), tumor necrosis factor – alpha, tissue growth factor-ß, epidermal growth factor and platelet-derived growth factor-BB.
6. Hormones: estrogens and progestins.

VEGF-A plays a key role in:

1. Stimulating angiogenesis (Penn et al., 2008);
2. Acting as a direct proinflammatory mediator. VEGF-A is a potent inducer of vascular inflammation. In vitro, VEGF-A treatment increases intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1). It is thought that $VEGF_{165}$ isoform is the most implicated in this process (Penn et al., 2008). Angiogenesis and inflammation are dependent processes (Ferrara et al., 2003). Inflammatory mediators have significant effects on angiogenesis and the inverse is also true (Oura et al., 2003);
3. Increasing vascular permeability (Penn et al., 2008);
4. Anti-apoptotic effect (Bachelder et al., 2001).

For example, in cancer, the stimulation of tumor cells by VEGF-A may protect the cells from apoptosis (Harmey & Bouchier-Hayes, 2002). It also, plays a key role in the support of neuronal and endothelial cell integrity in the survival of retinal neurons after ischemia. Moreover, it was described by Duh and Aiello (1999) that the function of VEGF-A was also as a promoter of ECs survival (Duh & Aiello, 1999). Therefore, Anti-VEGF therapies block two functions of VEGF-A: the pro-angiogenic activity and the anti-apoptotic/pro-survival action (Duffy et al., 2004).

VEGF-A is induced in various diseases of the central nervous system with a neuroprotective role. Also, in cardiovascular pathology, VEGF-A may be useful to increase collateral vessel formation during ischemic heart disease. However, its use as therapeutic agent may fail in cardiovascular diseases due to two reasons; first the VEGF-A driven angiogenesis in progressive ischemic disease may not be sufficient since the vessels formed may not be functional and second, the permeability function of VEGF-A will cause serious side effects (Tammela et al., 2005).

Moreover, the effects of VEGF-A appear to be dependent of local concentration (Figure 11).

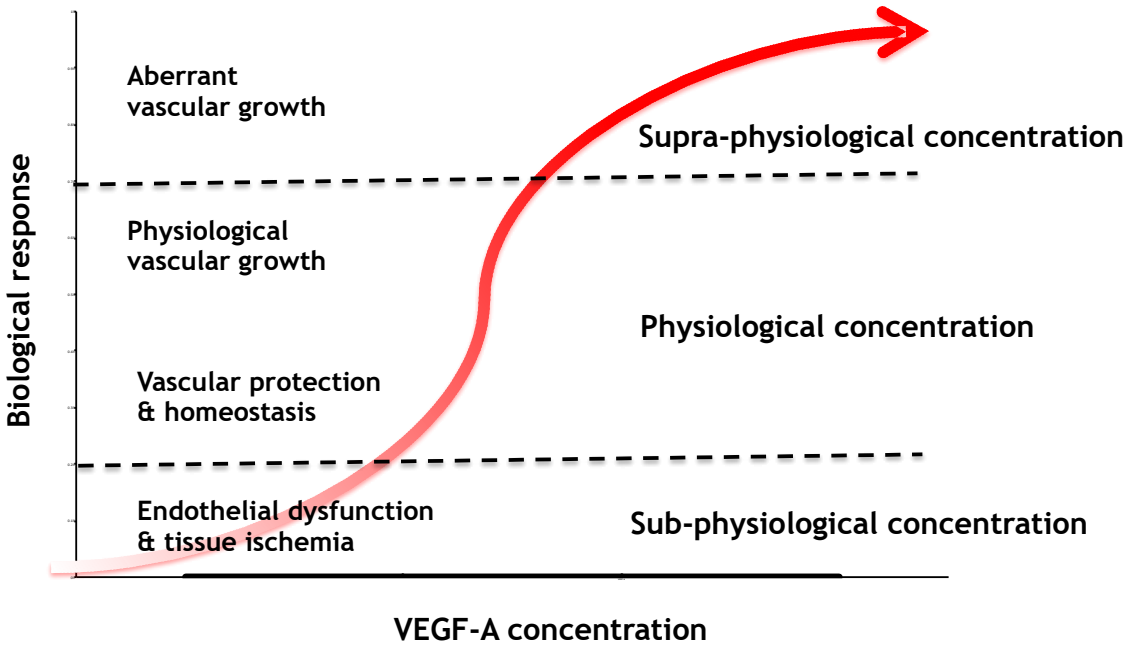


Figure 11: Biological response of VEGF-A (adapted from Ylae-Herttuala et al., 2007)

The influence of VEGF-A in retinal diseases is well known. Aiello et al. (1994) reported detectable concentrations of VEGF-A in ocular fluids from patients with active retinal neovascularization associated with several ocular diseases coupled with underlying retinal ischemia (Aiello et al., 1994). VEGF-A is associated with highly prevalent eye pathologies, such as AMD, DR, retinopathy of prematurity (ROP), RVO and neovascular glaucoma (Penn et al., 2008).

Taken together, we can conclude that overexpression of VEGF-A as a response to the presence of retinal ischemia, leads to intraocular proliferation, increased vascular permeability and inflammation and thus, consequently becomes an important target in the treatment of neovascular diseases.

5.2 VEGF-B

VEGF-B was discovered nearly 20 years ago as a factor related to VEGF-A, however, its function was unknown (Olofsson et al., 1996). It is by far the most charismatic and mysterious vascular endothelial growth factor of the VEGF family. It is now recognized to have an amino acid sequence of the VEGF homology domain of VEGF-B 47% and 37% equal to $VEGF_{165}$ and PlGF (placental growth factor), respectively (Li & Eriksson, 2001). Alternative splicing of exon 6 generates, two isoforms of VEGF-B, $VEGF\text{-}B_{167}$ and $VEGF\text{-}B_{186}$ (Figure 12).

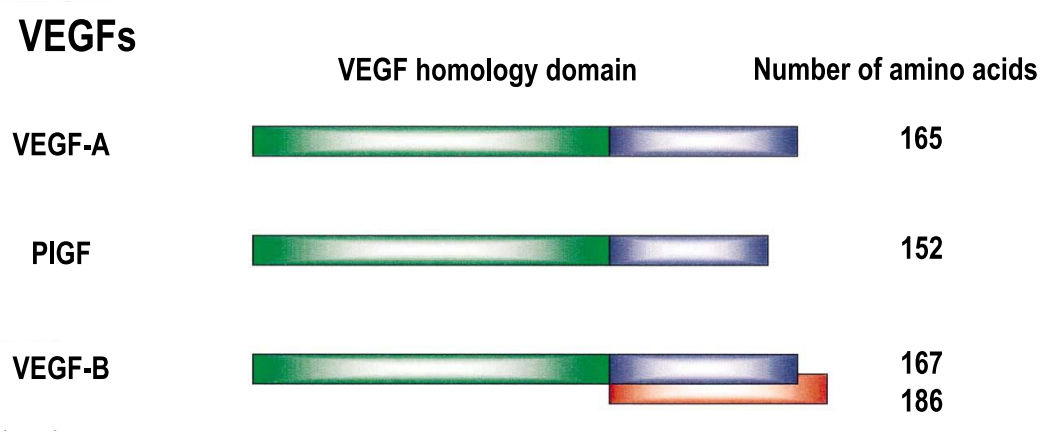


Figure 12: Schematic illustration of the domain structures of some members of the VEGF family, including VEGF-A, VEGF-B and PlGF. Adapted from Li and Eriksson, 2001.

VEGF-B specifically binds to VEGFR-1 with high affinity, but not to VEGFR-2 or VEGFR-3, competing with VEGF-A for VEGFR-1 binding (Olofsson et al., 1998). VEGF-B also binds to NRP-1 (Olofsson et al., 1998). VEGF-B_{167} has a molecular mass of 21 kDa while VEGF-B_{186}has a molecular mass of 26 kDa (Olofsson et al., 1996). VEGF-B_{167} and VEGF-B_{186} differ in their C-terminals. The C-terminal domain of VEGF-B_{167} is hydrophilic and this domain interacts with the co-receptor, NRP-1. The C-terminal domain of VEGF-B_{186} is hydrophobic and the binding to NRP-1 is regulated by limited proteolysis. VEGF-B isoforms can form heterodimers with VEGF-A_{165}. VEGF-A_{165}/VEGF-B_{167} heterodimers exist in the bound form, while the heterodimers VEGF-A_{165}/VEGF-B_{186} heterodimers are freely secreted. The presence of heterodimers will affect the equilibrium between homo- and heterodimers and consequently VEGF-A signaling. The expression of VEGF-B is not regulated by hypoxia, or other known stimuli including growth factors, cytokines, hormones, or oncogenes. To date, the mechanisms behind regulation of VEGF-B expression remains poorly understood.

VEGF-B is highly expressed in several tissues and organs. In adult tissues, VEGF-B expression is abundant in heart and skeletal muscles. The high expression in heart and brain indicate that the neovascularization in these organs is very extensive and that both VEGF-A and VEGF-B are active in this process (Bry et al., 2014).

VEGF-B_{167} is the most commonly existing isoform of VEGF-B with ample distribution among tissues and organs while VEGF-B_{186} is expressed less extensively than VEGF-B_{167} (Li et al., 2001). As mentioned previously, the function of VEGF-B is stable and it is not upregulated by factors that affect and regulate the production of VEGF-A. However, the expression of VEGF-B was found to be increased in tumor cells lines, making this molecule a potential target to treat cancer (Enholm et al., 1997; Li et al., 2001). The gain and loss of function studies suggest it plays a role in the functioning of the heart or in angiogenesis (Aase et al., 2001).

Moreover, the role of VEGF-B either in normal or in pathological situations is still unclear and needs to be studied further.

VEGF-B was reported as angiogenic in some studies but not in others. VEGF-B was shown to be involved in blood vessel growth under some situations while under other situations or in an environment where there is a high concentration of growth factors, VEGF-B inhibited tumor growth and angiogenesis (Li et al., 2012). In a review by Li et al., (2012) VEGF-B is referred to as multifunctional safeguarding molecule displaying a functional ambiguity role. For example, under degenerative conditions when cells are dying, this molecule acts as a survival factor to rescue the cells from death. In contrast, in the presence of high levels of potent growth factors, this molecule acts as a growth-inhibiting factor and prevents overgrowth, possibly through VEGFR-1 which acts as a decoy receptor suppressing angiogenesis (Li et al., 2012).

Karpanen and Mould demonstrated the role of VEGF-B in gain and loss-of-function studies using transgenic mice and showed that VEGF-B under normal conditions does not display activity (Karpanen et al., 2008; Mould et al., 2003). Interestingly, VEGF-B was the only member of the VEGF family that does not stimulate angiogenesis or lymphangiogenesis (Li et al., 2012).

Sands et al. (2011) reported that VEGF-B does not promote vascular endothelial cell proliferation and migration in cultured cells. In vivo administration of an intravitreal injection of VEGF-B_{167} recombinant protein in adult mouse did not induce ocular angiogenesis or other abnormalities. VEGF-B_{167} does not induce blood vessel permeability, unlike all the other VEGF family members (Li et al., 2012). Li and co-workers showed that the administration of an injection of VEGF-B_{167} recombinant protein into mouse brain or eye did not instigate blood vessel permeability (Li et al., 2008). However, there is a contradictory study, reported by Zhong et al (2011) where an intraocular injection of adeno-associated virus (AAV) encoding VEGF-B_{186}, but not VEGF-B_{167}, stimulated retinal vascular permeability. Looking at these data, we can conclude that VEGF-B_{167} does not induce blood vessel permeability while VEGF-B_{186} does (Zhong et al., 2011).

Li et al (2012) refer to VEGF-B as a survival factor. VEGF-B was shown to be a survival factor for vascular ECs, pericytes (PCs), and smooth muscle cells (SMCs) (Figure 13), (Li et al., 2012). In fact, increased apoptosis was observed in ECs and SMCs lacking VEGF-B when the cells were tested by oxidative stress or serum starvation (Zhang et al., 2009). In vivo, VEGF-B targeting inhibited both choroidal and retinal neovascularization, implicating VEGF-B as an important target in anti-angiogenic therapy (Li et al., 2012). The vascular survival effect of VEGF-B is achieved by regulating the expression of vascular prosurvival genes via both NRP-1 and VEGFR-1 (Li et al., 2012). The function of VEGF-B in the vascular system is defined as a ‘‘survival factor’’ instead of an ‘‘angiogenic’’ factor (Li et al., 2012). It has a potent survival/anti-apoptotic effect, while having a deficient angiogenic activity (except in the heart). Being abundantly expressed in the heart, VEGF-B has a specific role in the

cardiovascular system, with a critical function for cardiac blood vessel survival (Li et al., 2001; Olofsson et al., 1996). VEGF-B treatment increased cardiac blood vessel density in the ischemic myocardium (mouse model), where blood vessels suffered serious degeneration, (Claesson-Welsh, 2008). Besides, VEGF-B treatment reduced the stroke volume in an *in vivo* stroke model (Li et al., 2008). Additionally, VEGF-B plays a role in vessel growth in ischemic retinas (Li et al., 2012). Also, VEGF-B selectively promotes angiogenesis in the ischemic myocardium, warranting further investigations of the therapeutic potential of VEGF-B in promoting functional recovery of myocardial infraction and being described as having a cardioprotective effect. Apart from the survival effect of VEGF-B on blood vessels, VEGF- B may also have a direct protective effect on cardiac myocytes (Karpanen et al., 2008), suggesting that it could be used as a therapy in treating cardiac ischemic diseases.
VEGF-B has also been shown to be a powerful neuroprotective survival factor for several types of neurons, including brain cortical neurons, retinal neurons, and motor neurons in the spinal cord (Claesson-Welsh, 2008; Li et al., 2008; Sun et al., 2004). In a retina model, VEGF-B treatment increased the survival of retinal ganglion cells and protected neurons (Li et al., 2008), also suggesting its use as a therapeutic agent for neurodegenerative diseases.

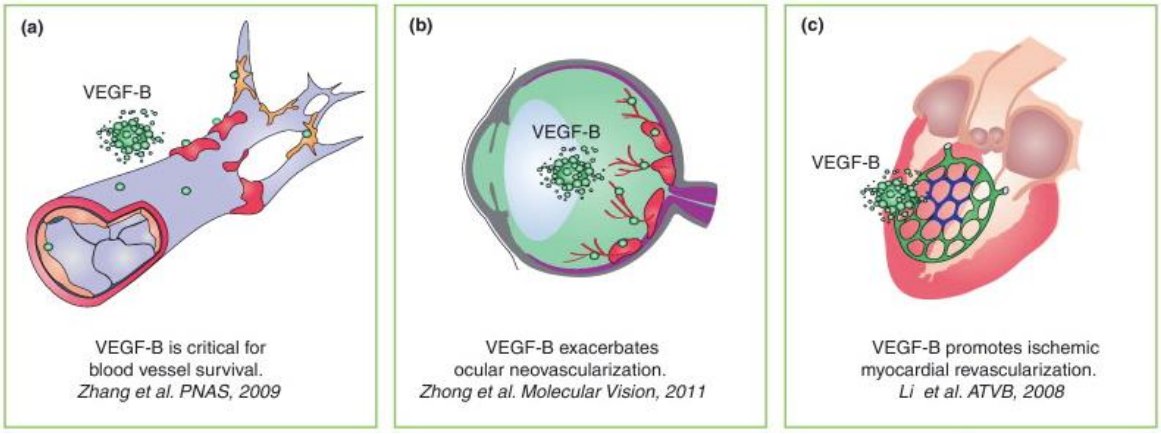


Figure 13: Illustrations showing the pro-angiogenic activity of VEGF-B under pathological conditions. Adapted from Trends in Molecular Medicine, (Li et al., 2012).

VEGF-B is expressed in the eye, and its expression can be upregulated in a pathological retina (Li et al., 2008). Subretinal injection of adeno-associated viruses encoding VEGF-B_{167} or VEGF-B_{186} increased retinal and choroidal neovascularization (Zhong et al., 2011). Another study demonstrated that targeted inhibition of VEGF-B by shRNA (short hairpin RNA) and intravitreal injection of a neutralizing antibody suppressed choroidal and retinal neovascularization in mice (Zhang et al., 2009). Therefore, VEGF-B targeting inhibited retinal neovascularization.
It seems that the 'angiogenic' activity of VEGF-B during ocular neovascularization is probably due to its potent survival effect on vascular and non-vascular cells. Also, it is known that both NRP-1 and VEGFR-1 play a role in mediating the vascular survival effect of VEGF-B. Therefore, despite VEGF-B having minimal angiogenic activity in blood vessel growth, the vascular

survival activity of VEGF-B, which protects the neo-vessels from apoptosis, may play a significant role in enhancing ocular neovascularization. Thus, targeted VEGF-B inhibition may also have therapeutic implications in the treatment of ocular neovascular disorders (Zhang et al., 2009).

In conclusion, several studies indicate that VEGF-B is critically required for blood vessel survival under pathological conditions. As a result of its antiapoptotic effect or potent survival effect and the fact that it is capable of remaining inactive under normal conditions, VEGF-B appears to be a valuable therapeutic option for the treatment of neurodegenerative and cardiovascular diseases with an attractive safety profile. Once the expression of VEGF-B is altered in neovascular diseases, the administration of a VEGF-B antagonist may result in promising outcomes either in monotherapy or in combination with other drugs.

5.3 PlGF

Another member of the VEGF family of growth factors is the PlGF. It is 53% identical to VEGF-A and binds to VEGFR-1 (Park et al., 1994). PlGF like VEGF-A is a dimeric glycoprotein structurally related to the platelet-derived growth factors A and B (PDGF-A and PDGF-B). Compared with VEGF-A, the mitogenic or permeability-enhancing activities of PlGF are weak but PlGF is able to potentiate the actions of VEGF-A (Park et al., 1994).

Although primarily expressed in the placenta, PlGF is also detected in significant levels in the heart and lungs (Persico et al., 1999). PlGF binds specifically to VEGFR-1 and to NRP-1 and NRP-2 (Maglione et al., 1991). Alternative splicing of the human primary transcript generates four isoforms: PlGF-1 ($PlGF_{131}$), PlGF-2 ($PlGF_{152}$), PlGF-3 ($PlGF_{203}$), and PlGF-4 ($PlGF_{224}$) (Maglione et al., 1991). PlGF- 2 is able to bind to both NRPs (NRP-1 and NRP-2) and heparin, while PlGF-1 does not (Hauser & Weich, 1993).

Gene targeting revealed that PlGF is dispensable for physiological angiogenesis during development and reproduction in mice but genetic loss of PlGF consistently impairs pathological angiogenesis, as well as other disorders such as ischemia, inflammation, and cancer, suggesting its role in several pathological conditions (Carmeliet et al., 2001).

The overexpression of PlGF in the skin results in increased vessel formation and permeability (Oura et al., 2003).

PlGF plays a significant role in pathophysiological neovascularization and in EC growth and migration (Rakic et al., 2017). PlGF is a pleiotropic factor affecting different types of cells and regulating several biological responses, as illustrated below (Figure 14), (Dewerchin & Carmeliet, 2012).

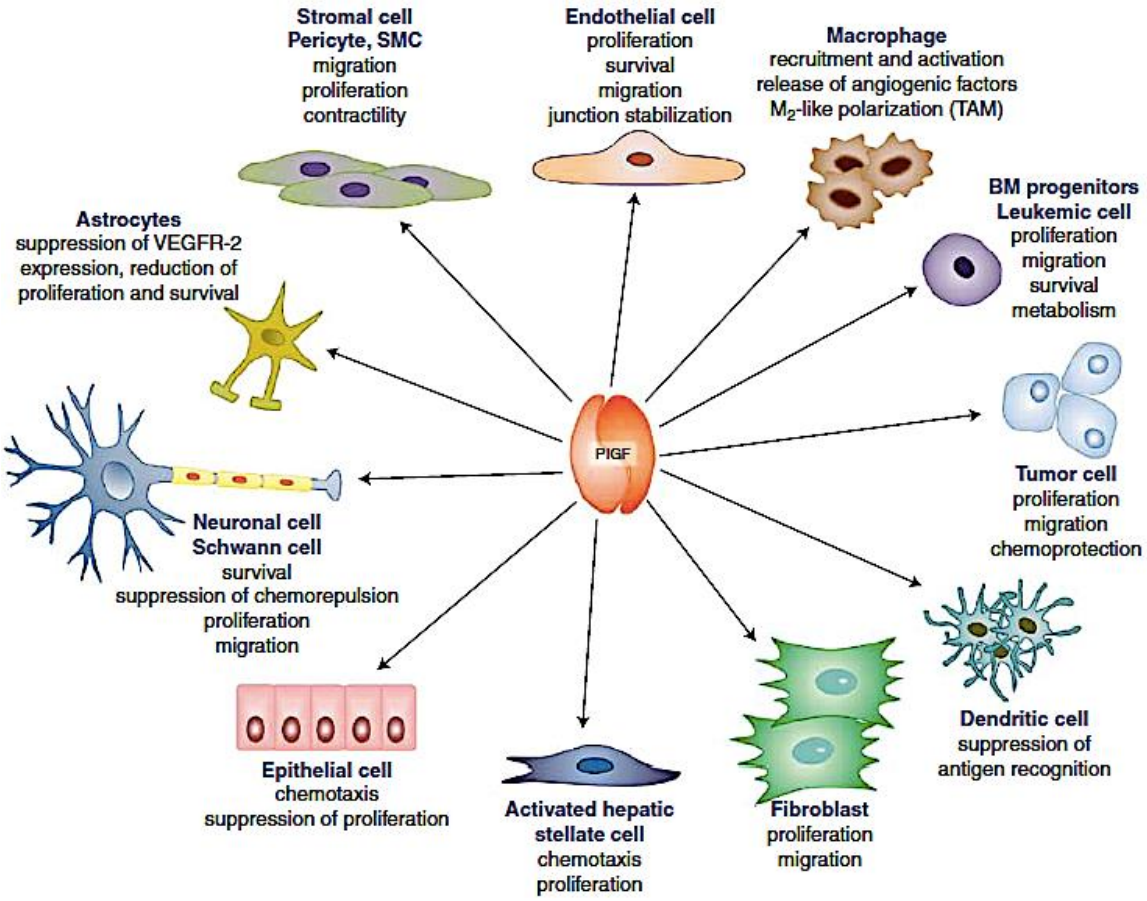


Figure 14: Scheme illustrating the pleiotropic activity of PlGF: survival, migration, proliferation, metabolism and activation effects on vascular (endothelial cells, pericytes/smooth muscle cells) and nonvascular cells (macrophages, bone marrow–derived progenitors, tumor cells, dendritic cells, fibroblasts, hepatic stellate cells, epithelial cells, neurons, Shwann cells, astrocytes), (adapted from Dewerchin & Carmeliet, 2012).

BM - Bone marrow, SMC - smooth muscle cell, TAM - tumor-associated macrophage

There are several proposed mechanisms for PlGF function (Dewerchin & Carmeliet, 2012):

1. PlGF may stimulate EC growth indirectly by the displacement of VEGF-A from VEGFR-1 and releasing VEGF-A to bind to VEGFR-2. It acts as a co-factor potentiating the activity of VEGF-A (De Falco, 2012);

2. PlGF may up-regulate the expression of other angiogenic factors such as VEGF-A, basic fibroblast growth factor, platelet derived growth factor B, and matrix metalloproteinases;

3. The release of PlGF leads to VEGFR-1 activation which induces an intermolecular cross-talk between VEGFR-1/VEGFR-2, amplifying VEGF-A/VEGFR-2 signaling;

4. The heterodimers formed between VEGF-A/ PlGF are thought to have pro-angiogenic and not anti-angiogenic activity. VEGF-A and PlGF form a heterodimer that binds to VEGFR-1, activating this receptor (Cao et al., 1996). However, this heterodimer's activity is not clear and only becomes evident when PlGF is overexpressed.

PlGF is induced in response to ischemic, inflammatory, and malignant conditions and is increased in pathological angiogenesis in the systematic vascular system by binding to VEGFR-1, (Autiero et al., 2003; Fischer et al., 2008).

In the retina, PlGF expression is not upregulated by hypoxia but in a model of retinopathy of prematurity (ROP) PlGF deficiency reduced the extent of retinal neovascularization by 60%. Additionally, PlGF was considered as proangiogenic on retinal ECs (Carmeliet et al., 2001; Khaliq et al., 1998; Simpson et al., 1999). Finally, PlGF was detected in the vitreous and in neovascular membranes in PDR (Khaliq et al., 1998).

When conditions comparable to the hypoxic-ischemic retinopathy were induced, the loss of PlGF protects mice against intravitreous neovascularization (Carmeliet et al., 2001). Thus, the low concentration of PlGF is still able to support these pathological events. In addition, the hypoxic conditions highly upregulate VEGF-A, but not PlGF. Therefore, PlGF can be a selected target for the inhibition of angiogenesis, in comparison with VEGF-A (Carmeliet et al., 2001).

Recent work from Inoue and colleagues (2013) investigated the effects of PlGF on retinal neuronal cells. It is known that PlGF is present in the retina; however, the neuroprotective role of the PlGF in the retina is still unclear. Their work suggested that PIGF may be an important protective factor in the retina (Inoue et al., 2013).

Sandro De Falco and colleagues (2012), described the role of PlGF in cardiovascular diseases and suggested three main functions for PlGF in the cardiovascular system: 1) its role in myocardial angiogenesis that seems to be synergistic with that of VEGF-A; 2) its role as a mediator of macrophage chemotaxis, being involved in the regulation of vascular growth in pathological conditions; 3) its selective action as a modulator of pathological rather than physiological vascular development making this molecule an attractive candidate for therapeutic angiogenesis (De Falco, 2012).

There are two contradictory requirements for the therapeutic regulation of angiogenesis:

1) To inhibit angiogenesis in pathological conditions such as tumors, inflammatory disorders, and diabetic retinopathy;

2) To stimulate angiogenesis in ischemic cardiovascular disease. One of the main limitations in using VEGF-A as a therapeutic stimulation of angiogenesis is its potent activity on vascular permeability (that leads to significant inflammation). PlGF could be an alternative therapeutic option for ischemia. The advantage would be that PlGF works by amplifying the physiological concentration of VEGF-A but as PlGF is less potent than VEGF-A, it would lead to potentially fewer side effects.

Additionally, the important role of PlGF/VEGFR-1 in the activation and sustainment of inflammation strongly prompts investigations in the search of an inhibitor of PlGF not only for ocular pathologies but also for other diseases (De Falco, 2012).

Further research on the suppression of PlGF is needed in order for it to be useful as an alternative target for anti-angiogenic strategies in ocular neovascularization.

5.4 VEGF-C

VEGF-C (also known as VEGF Related Protein, VRP) was discovered in 1996 and does not have splice variants. It is expressed in the heart, placenta, ovary, small intestine and thyroid gland (Joukov et al., 1996). Its expression is not increased by hypoxia and many oncogenes; however, interleukin 1 and tumor necrosis factor do upregulate VEGF-C levels. VEGF-C binds to VEGFR-2 and VEGFR-3, but not to VEGFR-1. It has a significant role in the formation of venous and lymphatic vessels during embryogenesis. It stimulates the growth of lymphatic vessels and blood vessels by activation of VEGFR-2 and VEGFR-3 (Jeltsch, 1997). VEGF-C is required not only for the initial sprouting of lymphatic vessels but also for the survival of lymphatic ECs (Karkkainen et al., 2003). Furthermore, loss-of-function studies demonstrated that *vegf-c*$^{-/-}$ mice die and *vegf-c*$^{+/-}$mice have flaws in lymphatic vascular development. VEGF-C is expressed in numerous types of cancer, namely breast cancer, cervix and prostate cancer, lung cancer, colon and stomach cancer, and is associated with metastases, vascular invasion, and therefore with a poor prognosis and often related to poor patient survival (He et al., 2004).

5.5 VEGF-D

VEGF-D (or c-Fos-induced growth factor) is the most closely related member to VEGF-C, both structurally and functionally. VEGF-D is able to bind to both VEGFR-2 and VEGFR-3 and capable activating these receptors (Stacker et al., 1999). Like VEGF-C it does not bind to VEGFR-1. Loss and gain of function studies demonstrated that *vegf-d*$^{-/-}$ mice are normal, displaying normal lymphangiogenesis and functional lymphatic vessels during embryogenesis, development and in adult life (Baldwin et al., 2005).It is a mitogen for ECs and plays a role in EC growth, lymphangiogenesis and angiogenesis. It also alters vascular permeability. It is expressed in the colon, heart, lung, skeletal muscle, small intestine and also in small amounts in the ovary, prostate and spleen (Achen et al., 1998). VEGF-D promoted tumor metastasis in lymph nodes and it is expressed in human melanoma in vessels near tumor cells (Achen et al., 2001; Stacker et al., 2001).

5.6 VEGF-E

VEGF-E (Orf Virus), a gene encoding a VEGF homologue was discovered in the genome of parapoxvirus Orf virus (OV) (Lyttle et al., 1994). Similar to VEGF-A, VEGF-E binds to VEGFR-2 with high affinity, but does not bind to VEGFR-1 nor VEGFR-3. It is a potent angiogenic, able to stimulate angiogenesis. Like VEGF-A, VEGF-E stimulates proliferation of the ECs and vessel permeability (Lyttle et al., 1994).

6. Vascular Endothelial Growth Factor Receptors and Neuropilins

The VEGF receptors are cell surface tyrosine kinase receptors (Figure 15). They can be membrane-bound (mbVEGFR) or soluble (sVEGFR). VEGFR-1, also called Flt-1, acts as a decoy receptor by sequestering VEGF-A and regulates the interaction between VEGF-A and VEGFR-2 (Flk-1) (Wirostko et al., 2008). VEGFR-1 plays an important role not only for blood vessel development during embryogenesis but also in pathological angiogenesis and wound healing by interaction with VEGFR-2 (Cébe-Suarez et al., 2006). VEGFR-1 has poor catalytic activity compared to VEGFR-2 and the downstream signaling pathways upon its activation are not well known. VEGFR-2 is involved in permeability, angiogenesis, vasculogenesis, and proliferation (Wirostko et al., 2008).

VEGFR-3 is important for wound healing, and sFlt-1 (soluble VEGFR-1) search for available VEGF-A (Wirostko et al., 2008). VEGFR-2 and VEGFR-3 are also involved in lymphatic angiogenesis (Cébe-Suarez et al., 2006). There are several VEGFR inhibitors under investigation for the treatment of different types of cancer. Actually, Pazopanib and Regorafenib are approved and are being used in the treatment of renal cell carcinoma and colorectal cancer respectively-

The neuropilin receptors are transmembrane glycoproteins important for axonal guidance, angiogenesis, tumorigenesis, and are involved in the immunologic response (Sulpice et al., 2008). NRP-1 and NRP-2 act as co-receptors for class III semaphorins and growth factors of the VEGF family. The neuropilin receptors are involved in both physiological and pathological angiogenesis (Sulpice et al., 2008).

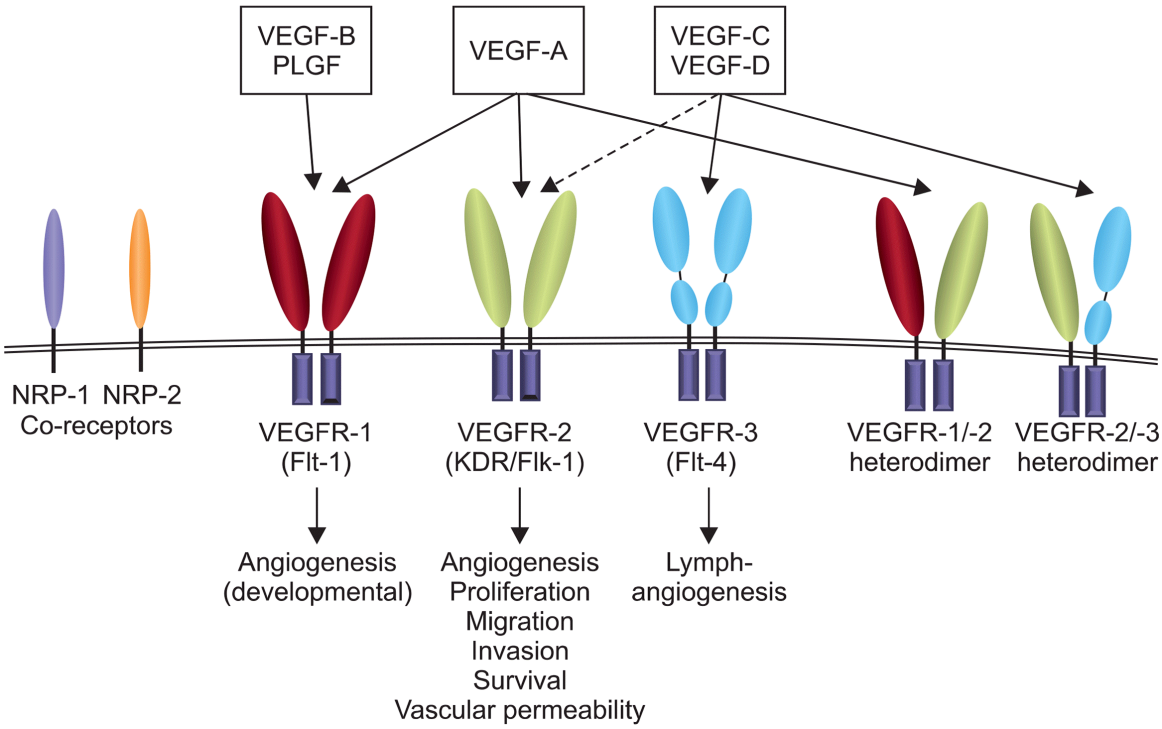


Figure 15: Interactions between VEGF and VEGF receptors, and their biological functions.
Flt - fms-like tyrosine kinase, Flk - fetal liver kinase, NRP – neuropilin, KDR - kinase insert-domain containing receptor, PIGF - placenta growth factor, VEGF - vascular endothelial growth factor, VEGFR - vascular endothelial growth factor receptor (adapted from Bae et al., 2015).

6.1 VEGFR-1

VEGFR-1 otherwise known as Flt-1 (Fms-like tyrosine kinase-1) is a receptor for VEGF-A, VEGF-B and PlGF. It is known to play a role in vascular development and homeostasis (Li et al., 2012). VEGFR-1 acts as a decoy receptor for other growth factors. Its function in angiogenesis is to act as a VEGF-trap to modulate VEGFR-2 function (Rahimi, 2006).

6.2 VEGFR-2

VEGFR-2 also called Flk1 (fetal liver kinase 1), or KDR (kinase insert domain containing receptor) is a receptor for VEGF-A, VEGF-C, VEGF-D and VEGF-E and the principal receptor responsible for angiogenesis. VEGFR-2 upregulate tumor angiogenesis and ocular angiogenesis, making it an interesting target for tumor growth and ocular disorders involving neovascularization (Campochiaro & Hackett, 2003; Cross et al., 2003).

6.3 VEGFR-3

VEGFR-3, also known as Flt-4 (Fms-like tyrosine kinase 4) or tyrosine-protein kinase receptor FLT4, is a receptor for VEGF-C and VEGF-D playing a critical role in blood vessel and lymphatic vessel development. VEGFR-3 is required for lymphatic vessel survival and maintenance during human embryogenesis (Mäkinen et al., 2001). It is capable of forming heterodimers with KDR/ VEGFR-2. It promotes proliferation, survival and migration of ECs. It is also able to regulate angiogenic sprouting, playing a significant role in tumor angiogenesis. It is upregulated in the blood vessels of many tumors even though it is normally expressed only in lymphatic vessels (Partanen et al., 1999; Zarkada et al., 2015).

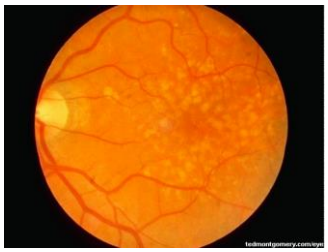
6.4 Neuropilins

NRP-1 and NRP-2 are co-receptors for semaphorins and VEGFs. NRP-1 is expressed at the endothelium of arteries while NRP-2 is expressed in veins and lymphatic vessels (Herzog et al., 2001; Yuan et al., 2002). NRP-1 is a co-receptor capable of regulating angiogenesis, arteriogenesis and vascular permeability during development and in pathological conditions. It acts essentially through the binding to other receptors. However, Roth and co-workers reported NRP-1 as self-sufficient to mediate vascular permeability alone without binding to VEGFR-2 (Roth at al., 2016). Expression of NRP-1 is upregulated in several tumor types, namely, brain, prostate, breast, colon, and lung cancers. Levels of NRP-1 are also known to be positively correlated with the presence of metastasis (Parikh et al., 2004). NRP-2 plays a role in cardiovascular development. In cancer, it is considered to be involved in tumorigenesis. Therefore NRP-1 can mediate angiogenesis, and malignant tumor progression (Chen et al., 1997).

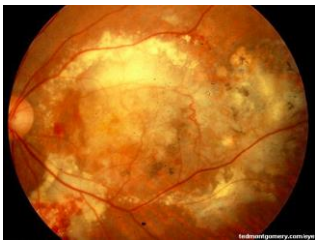
7. Treatment of retinal diseases

There is a variety of retinal diseases (Figure 16), some involve only the macula such as AMD, macular hole, or macular pucker (epiretinal membrane) while others can impact the entire retina, as for example retinal detachment or DR.

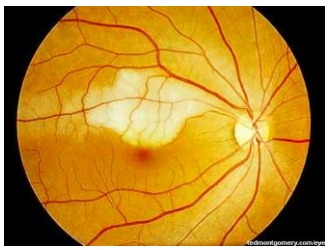
a) Age-related macular degeneration



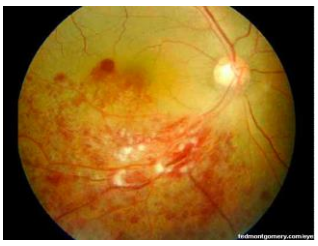
b) Age-related macular degeneration



c) Branch retinal artery occlusion



d) Branch retinal vein occlusion

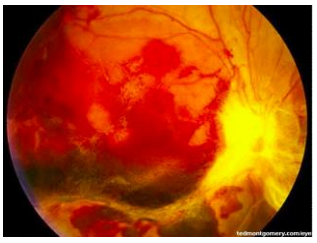


e) Central retinal vein occlusion

f) Epiretinal membrane

g) Diabetic retinopathy

h) Diabetic retinopathy



i) Retinal detachment

j) Macular hole

Figure 16: Visualization of images of retinal pathologies. (Adapted from Montgomery, 2017).

The main goals of the treatment of retinal diseases are to stop or slow down disease progression and to preserve, improve or restore vision. In many cases, the damage that has already occurred cannot be reversed, making early detection an important factor in the treatment of these pathologies. Treatment of retinal diseases may be complex and sometimes

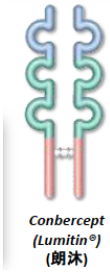
urgent. Visual impairment is a considerable health problem for older adults and has a significant impact on functional status and quality of life. For example, it limits the ability to read, to safely drive a car, to have an independent way of life and is also associated with increased rates of falls and hip fractures in patients suffering from these diseases (Lord & Dayhew, 2001).

There is a wide range of treatment options for retinal diseases, from administration of eye drops to surgical procedures such as intraocular injections, laser therapy, and vitreoretinal surgery. In all instances, the aim is to maintain the integrity of the retina, especially the macula. However, despite the development of innovative therapeutic options for retinal diseases, published data of many pivotal studies, clinical knowledge and the real-world experience, treatment of these patients remains a challenge (Amandio et al., 2016).

Neovascular (wet) AMD treatment:

Ten years ago, before injections for wet macular degeneration became available, most of the affected patients lost their central vision. Later, verteporfin (Visudyne®) was made available. Actually, due to extensive medical research, patients can be treated with anti-VEGF intravitreal injections such as ranibizumab (Lucentis®), aflibercept (Eylea®), conbercept (Lumitin®) or with the off-label drug, bevacizumab (Avastin®), as summarized in table 3.

Table 3 – Main characteristics of current anti-VEGF agents used for the treatment of diabetic retinopathy and wet age-related macular degeneration (adapted from Horton and Guly, 2007; Lu and Sun, 2015).

Anti-VEGF therapy	Class	MW	Mode of action	Binding affinity to $VEGF_{165}$	Key relevant clinical trials	Targets
Bevacizumab (Avastin®) 	Monoclonal Ab*	149 kDa	Humanized full-length monoclonal antibody derived from the same antibody as ranibizumab. Bind to all VEGF-A as ranibizumab with different affinity.	58 pM	AMD studies: CATT and IVAN (non-inferiority studies against ranibizumab) DME studies: Protocol T from DRCRnet.	All VEGF-A isoforms
Ranibizumab (Lucentis®) 	Fab**	48 kDa	Humanized Fab fragment of a monoclonal antibody that binds to and inhibits the action of all isoforms of VEGF-A.	46 pM	AMD studies: MARINA, ANCHOR (superiority studies against sham injection and verteporfin, respectively). DR / DME studies: RESOLVE, RESTORE, RETAIN	All VEGF-A isoforms
Aflibercept (Eylea®) 	VEGF-Trap/ Fusion protein	115 kDa	Fusion protein that inhibits all isoforms of VEGF-A, VEGF-B and PIGF. Fusion protein: domain 2 of VEGFR-1 and domain 3 of VEGFR-2 fused with IgG1 Fc	0.5 pM	AMD studies: VIEW 1/ VIEW 2 (non inferiority studies against ranibizumab) DME studies: VIVID and VISTA.	All VEGF-A isoforms, VEGF-B and PIGF
Conbercept (Lumitin®) 	Fusion protein	143 kDa	Fusion protein that inhibits all isoforms of VEGF-A, VEGF-B, VEGF-C and PIGF Fusion protein: domain 2 of VEGFR-1 and domains 3 and 4 of VEGFR-2 fused with IgG1 Fc	0.5 pM	AMD studies: Phase II AURORA and the phase III PHOENIX RVO studies: currently recruiting participants.	All VEGF-A isoforms, VEGF-B, VEGF-C and PIGF

*Ab - antibody; **Fab - antibody fragment; MW - molecular weight.

For several patients with wet AMD, the anti-VEGF treatments offer good outcomes; however, these drugs are frequently injected into the eye leading to a significant burden of injections and visits not only for the patient, but also for caregivers, physicians, and the healthcare system. Long-term and continued release medications are required and are now in clinical trials for wet AMD in order to decrease the number of intravitreal injections needed to obtain and maintain functional as well structural outcomes (Horton & Guly, 2017):

1. Sustained Release Lucentis: it is in phase II clinical trials. The study is called LADDER. It has received "fast track" label from the FDA, in order to obtain a faster approval. The device is implanted into the eye and can be refilled every 4-6 months.
2. Sustained Release Eylea: it is in development but not yet in clinical trials. In this system, aflibercept is incorporated into a gel and after intravitreal injection releases the drug slowly.
3. Other therapies are in research for wet AMD, such as Abicipar Pegol, which is being developed by Allergan. This protein remains for longer periods in the eye. During the phase II studies, the study medication was injected quarterly. It is now in phase III trials, a comparative study of Abicipar Pegol with ranibizumab with final data collection date for primary outcome measure aimed at May 2019 ("Safety and Efficacy of Abicipar Pegol in Patients With Neovascular Age-related Macular Degeneration - Full-Text View - ClinicalTrials.gov", 2017).

RVO treatment

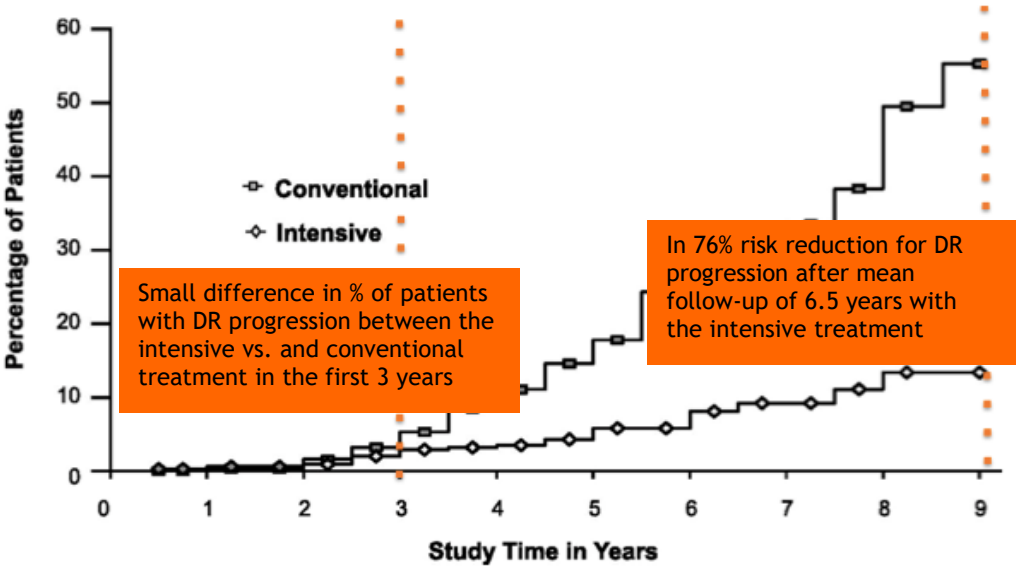
There are several reported methods for the clinical treatment of RVO (Sivaprasad, Amoaku, Hykin & Guideline Group, 2016):

1. Laser photocoagulation;
2. Anti-VEGF agents are used to treat macular edema due to CRVO or BRVO, once VEGF-A has shown to be increased in this pathology mediating vascular leakage and therefore causing macular edema. The most common anti-VEGFs used are ranibizumab, aflibercept and bevacizumab (off-label).
3. Intravitreal steroids. Corticosteroids are potent anti-inflammatory drugs that reduce retinal capillary permeability and inhibit the expression of VEGF-A. The Score clinical trial demonstrated that patients treated with intravitreal triamcinolone (IVTA) showed anatomical and functional improvements of macular edema related to CRVO or BRVO, but due to the short half-life of this drug, the effects are short and patients may experiment a rebound of the edema. Another drug used to treat macular edema associated with RVO is dexamethasone. Although the rationale for the use of intravitreal dexamethasone is similar to that of IVTA, dexamethasone is a more potent corticosteroid. Nevertheless, the free form of intravitreal dexamethasone also has a short half-life. To overcome this issue, the pharmaceutical company, Allergan, developed a biodegradable implant, containing 0.7 mg of dexamethasone (Ozurdex®) that was investigated in the GENEVA study program. Based on this program, Ozurdex® was approved by the FDA and EMA for the treatment of macular edema following CRVO or BRVO.

DR and DME treatment:

The current treatment for DR and DME undergoes through a strict systemic and ocular control (Bhavsa et al., 2017). Concerning the systemic treatment, this may include the control of glucose, blood pressure, and blood lipid, besides other multifactorial interventions. The maintenance of HbA1c levels between of 6-7% is one of the goals for an ideal management of diabetes and their complications. The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study (DCCT/EDIC) included approximately 1,441 subjects, 726 had no DR at the baseline (primary prevention cohort) and 715 had mild DR at baseline (secondary intervention cohort). One of the objectives was to evaluate whether intensive or traditional treatment aiming to achieve glucose blood levels as near as possible to non-diabetic patients decrease the risk of progression of DR (Figure 17a& 17b). Indeed, it was concluded that in patients capable of preserving HbA1c values at a low and sustained level, the progression of diabetic retinopathy was reduced (Nathan, 2013). Moreover it was shown that the progression of DR was associated with HbA1C levels (Figure 18).

a)



b)

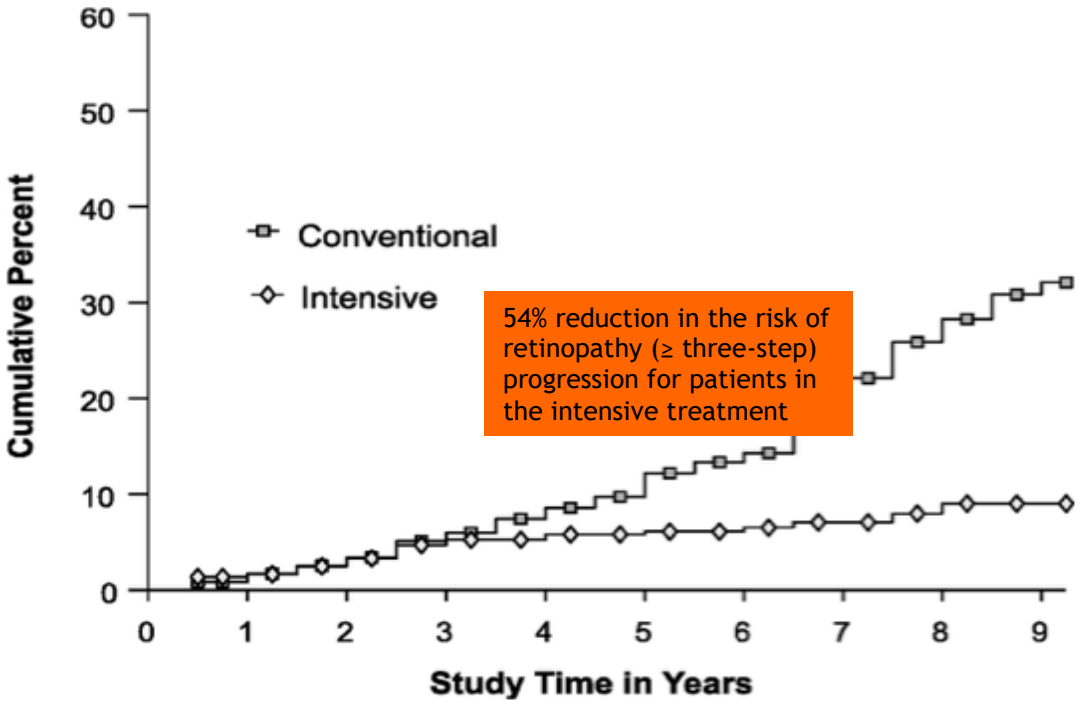


Figure 17: a) Cumulative incidence of DR progression (three-step or greater) in the primary prevention cohort of the DCCT; b) Cumulative incidence of DR progression in the secondary intervention cohort of the DCCT (adapted from Aiello, 2014).

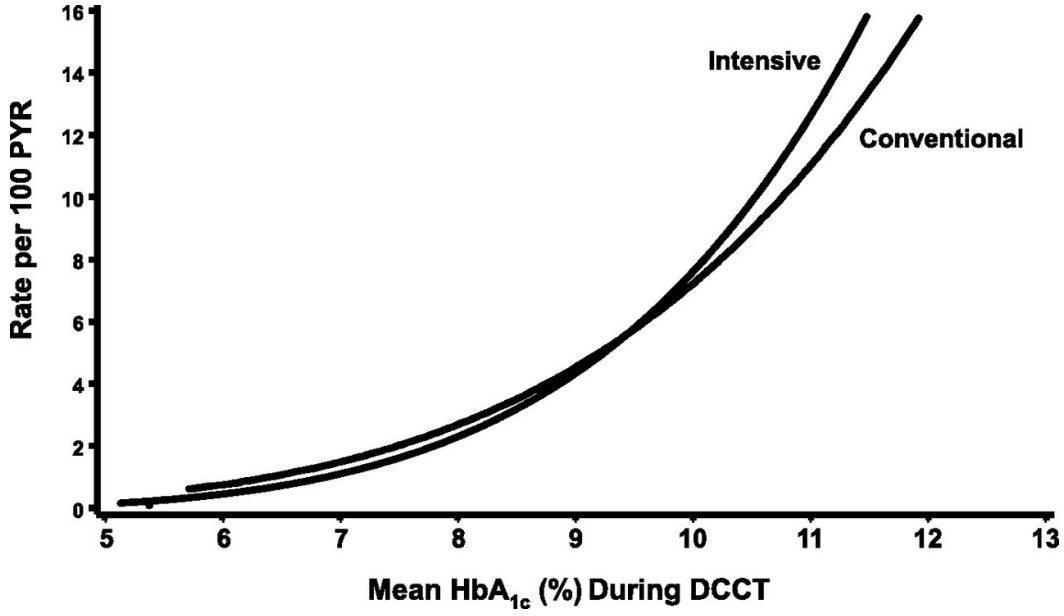


Figure 18: Relation between the risk of progression of DR and Hba1c (adapted from Aiello, 2014).
DCCT - Diabetes Control and Complications Trial; HbA1c – glycosylated hemoglobin; PYR – patient-years

Regarding the ocular treatment, there are currently various drug and non-drug treatments, although none of them treat the diseases, DR or DME, but only ameliorate symptoms and decrease disease progression. The most current and available treatment options are laser photocoagulation, pharmacological agents, and vitrectomy.

Laser photocoagulation was one of the first non-invasive treatments with relatively low rates of associated complications and with great accomplishment. The Early Treatment Diabetic Retinopathy Study (ETDRS) was a memorial clinical trial and achieved a milestone demonstrating the benefit of the focal macular laser in the treatment of DR and DME ("Early Treatment Diabetic Retinopathy Study", 1981). This study defined the criteria for the treatment of "clinically significant macular edema" (CSME) used to select which patients should be treated with macular laser. The results from the ETDRS demonstrated that laser photocoagulation decreased the risk of moderate vision loss by approximately 50% and improved the vision of approximately 30% of the patients. However, a percentage of the patients still suffer from vision loss despite treatment photocoagulation (Early Treatment Diabetic Retinopathy Study Research Group., 1985; Jampol et al., 2014).

The rationale for the use of photocoagulation is based on the fact that the photoreceptors use more oxygen than any other cells of the organism and so destroying them would be an effective way to reduce the oxygen consumption by the retina. The mechanism of action of the traditional laser photocoagulation destroys the photoreceptors of the retina, decreasing the oxygen consumption and restoring the balance between the supply and the demand of oxygen (Figure 19).

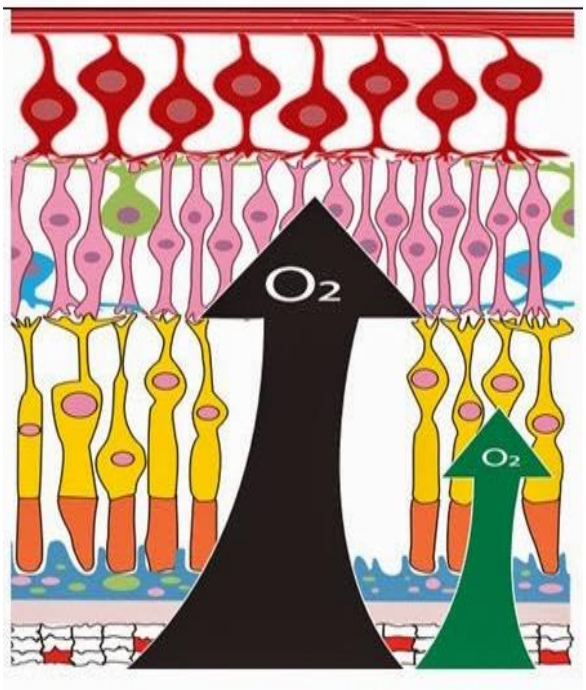


Figure 19: Schematic drawing showing the oxygen flow from choroid through laser scar into the inner retina (adapted from Stefánsson, 2009).

Funatsu and co-workers proposed the following mechanism of action for the laser photocoagulation:

1. The oxygen provided to the retina comes from the choriocapillaris and disseminates into the outer retina. The outer retina photoreceptors have high oxygen consumption.
2. If oxygen is consumed by the photoreceptors of the outer retina it will not reach other parts of the retina where it is needed.
3. The laser will cause the destruction of the photoreceptors of the outer retina, opening a path that allows the penetration of oxygen, without being consumed by the outer retina photoreceptors, reaching the inner retina.
4. This action decreases the metabolic function of the outer retina and decreases the oxygen consumption, ultimately leading to an improvement of hypoxia and re-establishing of the balance between oxygen demand and supply to the inner retina.
3. An improvement of hypoxia will decrease the production and secretion of neovascular growth factors (as VEGF-A).

Hypoxia stimulates VEGF-A production, and neovascularization, therefore laser treatment is an efficient process to correct this hypoxia, (Figure 20), (Funatsu et al. 1996, Stefánsson, 2009).

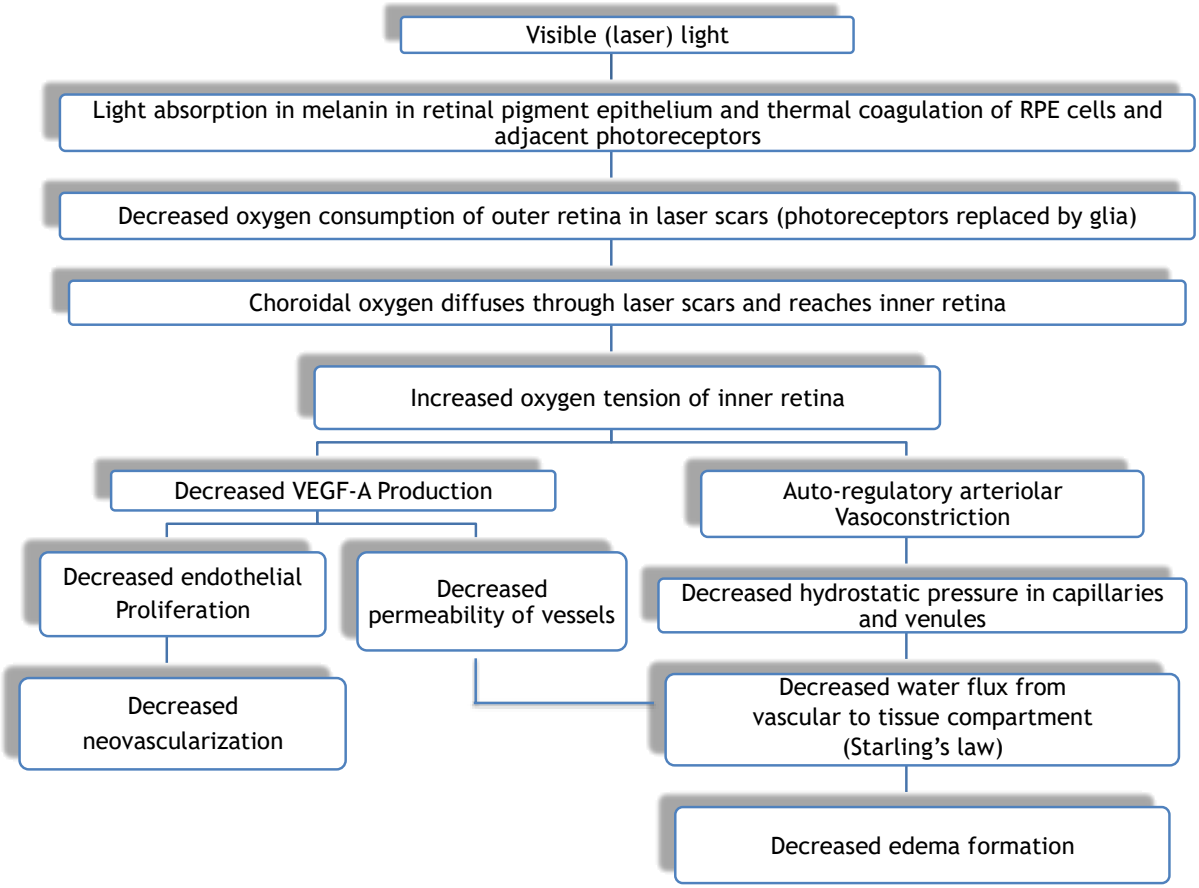


Figure 20: Flow diagram explaining the mechanism of effect of retinal photocoagulation on retinal neovascularization and macular edema (adapted from Stefánsson, 2009).
RPE - retinal pigment epithelial; VEGF-A = vascular endothelial growth factor A.

To date, dramatic evolutions in the type of laser treatments from the conventional lasers to more advanced technologies, such as the micropulses, has been observed, thus increasing the treatment benefits associated with laser usage and decreasing retinal damage.

The two-year outcomes of the protocol I, a "Randomized Trial Evaluating Ranibizumab Plus Prompt or Deferred Laser or Triamcinolone Plus Prompt Laser for Diabetic Macular Edema", from the Diabetic Retinopathy Clinical Research Network (DRCRnet), showed that the ranibizumab groups in combination with prompt or deferred laser treatment achieved greatest structural (OCT) and functional (visual acuity) outcomes than laser monotherapy (Elman et al., 2010). Both ranibizumab groups showed an improvement of 10 or more letters in approximately 50% of eyes and a vision gain of 15 or more letters in 30% of the studied eyes (Bandello, 2012). The IVTA study arm in combination with laser treatment did not demonstrate superior visual acuity outcomes compared with laser alone whereas in a subgroup of pseudophakic eyes, the results achieved were similar between corticosteroids and anti-angiogenics (Elman et al., 2011). This is due to the fact that corticosteroids accelerated the progression of cataracts,

apparently worsening the vision. However, it is known today from the results of the FAME study with fluocinolone acetonide 0.2 μg/day that patients recover vision gains after surgically removal of cataracts (Campochiaro et al., 2012).

Another conventional treatment is vitrectomy. A vitrectomy is a surgical procedure that is performed to remove the vitreous gel. The Diabetic Retinopathy Vitrectomy Study (DRVS) aimed to compare early versus delayed pars plana vitrectomy (PPV) in patients with PDR, active neovascularization, and vitreous hemorrhage (Chang & Sarraf, 2008). The study group endorsed early PPV for vitreous hemorrhage in patients with diabetes mellitus type I or for eyes with advanced PDR and compromised visual acuity. Even though medical practice varies, a vitrectomy is commonly performed in patients with vitreous hemorrhage, tractional retinal detachment or mixed tractional and rhegmatogenous retinal detachment, epiretinal membrane and macular dragging. The goal of surgical treatment is: 1) to remove the blood, 2) release traction, 3) repair retinal detachment, and 4) to eliminate the scaffold to avoid the development of neovascular complexes.

VEGF inhibition has been vastly used and become the gold standard for the treatment of DR and DME. The anti-VEGFs agents bevacizumab, ranibizumab and aflibercept are commonly used in the treatment of retinal diseases (Table 3).
The similar properties between the monoclonal antibodies, bevacizumab and ranibizumab with respect to VEGF-A binding are due to the fact that the two molecules originated from the same precursor, the murine monoclonal antibody, however it should be noted that they are not the same molecule:

- Bevacizumab is indicated for the treatment of patients with metastatic cancer of the colon, rectum or breast, and patients with non-small cell lung cancer or metastatic renal cell cancer. Bevacizumab vials are intended for single use as an intravenous infusion but when used off-label in ophthalmology, vials are split into multiple doses increasing the potential for contamination (particulate or microbial), for human error and incorrect dose or incorrect drug, during preparation of the intravitreal injections.
- Bevacizumab has two VEGF-A binding sites, whereas ranibizumab has only a VEGF-A binding site.
- Bevacizumab is a full-length recombinant humanized monoclonal antibody (3 times larger than ranibizumab), with both Fc and Fab regions. It is produced in mammalian expression system (glycosylated molecule) and has a greater serum and vitreous half-life than ranibizumab. The Fc antibody domain has a role in immune activation. Moreover, it is also known that glycosylated proteins have a higher immunogenic potential compared with non-glycosylated proteins.
- Ranibizumab is a recombinant humanized monoclonal antibody fragment (Fab), produced in an Escherichia coli expression system (and thus not glycosylated). It was genetically engineered to increase its affinity for binding and inhibition of VEGF-A.

- Further, the two molecules differ in size (149 kDa for bevacizumab vs. 48 kDa for ranibizumab), in affinity for binding to VEGF-A (bevacizumab being lower affinity) and plasma half-life (which is 17 to 21 days for bevacizumab vs. 9 days for ranibizumab).
- Ranibizumab is a drug designed specifically to be used intravitreally, having been approved by the FDA and EMA for the treatment of various diseases of the retina.

3. Aflibercept or VEGF-Trap is a fusion protein that combines the ligand-binding components from the extracellular domains of VEGFR-1 and VEGFR-2 merged to the Fc portion of IgG. This anti-angiogenic abolishes tumor growth and vascularization, resulting in almost completely avascular tumors. Moreover, this anti-VEGF (Zaltrap® and Eylea®, from Regeneron) binds to all isoforms of VEGF-A, VEGF-B, and PlGF. In this case, the molecule aflibercept, used for the treatment of cancer is the same as that used in the treatment of neovascular eye diseases (Simó et al., 2014).

In the DRCRnet clinical trials, specifically in the T protocol, which compared the 3 anti-angiogenic drugs, Eylea® (aflibercept), Lucentis® (ranibizumab), and Avastin® (bevacizumab) in the treatment of DME, patients who initially at the start of the study had a visual acuity of 20/50 or worse, had better outcomes with aflibercept at the end of the first year. However, in patients who had a baseline vision of 20/40 to 20/32, there were no differences in the results between the three drugs at the end of the first year. Likewise, at the end of the second year of protocol T, there was no significant difference in the outcomes among the three drugs. In addition, regarding drug safety, there were no major differences observed between bevacizumab, ranibizumab and aflibercept in the treatment of DME (Baker et al., 2016). Nevertheless, not all patients responded sufficiently to anti-VEGF therapy, leading to new research approaches in the fight against DR and DME.

Corticosteroids have a significant role in the treatment of DME. Despite the large benefits of steroids therapy, all steroids are associated with the risk of adverse events such as the occurrence of increased intraocular pressure and cataract (Chang & Sarraf, 2008). The most well-known corticosteroids for this indication are triamcinolone acetonide (off-label), dexamethasone, and fluocinolone acetonide. The role of corticosteroids in the treatment of DME is multifactorial. Corticosteroids act not only as powerful anti-inflammatory drugs but also antagonize the action of VEGF-A, inhibit leukostasis and decrease inflammatory cytokines. All the above three corticosteroids have an elimination half-life inside the vitreous of 2–3 hours in animal models (Schwartz, Flynn & Scott, 2013), consequently being swiftly cleared from the eye. In order to extend the half-life and duration of the corticosteroids in the eye, researchers developed diverse strategies. In the case of triamcinolone acetonide, it was found that it could be dissolved slowly from a crystal structure. For dexamethasone and fluocinolone acetonide, drugs were deposited in a specific matrix and inserted in a particular slow release device that is intravitreally injected into the eye.

IVTA suspension is a powerful and effective drug for remaining for approximately 3 months in a non-vitrectomized eye, so it is necessary to repeat injections to preserve the treatment effect (Schwartz et al., 2013). In order to reduce the burden of injections, and the associated risks with the frequent intravitreal injections, pharmaceutical companies investigated extended release steroid implants. There are two steroid implants for the treatment of DME: a shorter acting steroid implant for dexamethasone, lasting up to 6 months according to the MEAD study and a longer acting steroid implant for fluocinolone acetonide lasting for at least 3 years (Bonfiglio et al., 2017, Boyer et al., 2014, Campochiaro et al., 2012, Cunha-Vaz et al., 2014). Besides, according to Schwartz and co-authors, a long acting and continuous release of low corticosteroids dosage is more effective than a discontinuous and sporadic bolus delivery of high doses. Also in general, non-bioerodable implants are associated with more precise control of drug release than the bioerodable implants (Schwartz et al., 2013).

Similar to AMD and RVO, future treatments behind anti-VEGF therapy and besides the ones currently used are being investigated for the treatment of DR and DME (Shamsi et al. 2013), namely:

1. Anti-tumor necrosis factor alpha (or tumor necrosis factor antagonist), such as the monoclonal anti-TNF antibody infliximab used for inflammatory arthritic conditions and Crohn's disease. In a first small phase III study conducted with infliximab in patients at risk of vision loss secondary to DME refractory to laser photocoagulation, an improvement of visual acuity was observed and the treatment was well tolerated (Sfikakis et al., 2010; Wu et al. 2011). However, the outcomes obtained with additional studies were not encouraging. The role of TNF inhibition in patients with DME is unknown and still needs to be studied. Adalimumab (Humira®, Abbvie) is another TNF-inhibitor and an anti-inflammatory agent approved for the treatment of non-infectious intermediate, posterior, and panuveitis in adult patients. It binds to tumor necrosis factor-alpha reducing the inflammatory response. It is administered by a subcutaneous injection. Adalimumab received CHMP positive opinion in July 2017 for the treatment of pediatric patients with chronic non-infectious anterior uveitis (HUMIRA EPAR, 2017). Still, its benefits in DME patients are uncertain.

2. Protein Kinase-C beta-isoform inhibitors (PKC-b or ruboxistaurin) regulate EC permeability and triggers VEGF-A (Tsilimbaris et al., 2007). Ruboxistaurin is an oral PKC-b inhibitor that demonstrated inconsistent results in the treatment of DME. In animal models, ruboxistaurin improved diabetic retinopathy and DME (Suzuma et al., 2002). In a phase II study with oral ruboxistaurin tested in DR patients, it increased visual acuity in NPDR patients, decreased moderate visual loss, macular edema progression and the need for laser treatment for macular edema. Ruboxistaurin was well tolerated but did not receive approval from the FDA (Aiello et al., 2006).

3. Nicotinic acetylcholine receptor antagonist (mecamylamine). The topical ocular formulation of mecamylamine was tested in a multicenter phase I/II clinical trial in patients with chronic DME. The results showed an improvement of visual acuity with no safety problems

(Campochiaro et al., 2010). Further clinical trials comparative with anti-VEGFs are needed in order to establish its efficacy.

4. AKB-9778 (Aerpio Therapeutics) is a small molecule inhibitor of vascular endothelial-phosphotyrosine phosphatase (VE-PTP) that is administered by a subcutaneous injection. VE-PTP is a member of protein tyrosine phosphatase (PTP) family and targeting VE-PTP activates TEK receptor tyrosine kinase (Tie2) stabilizing the ocular vasculature (Shen et al., 2014). The TIME-2 study provided proof-of-concept for treatment of diabetic eye disease, both DME and DR, by activation of Tie2 with AKB-9778. The TIME-2 is a phase 2 double-masked, placebo-controlled study to assess the safety and efficacy of subcutaneously administered akb-9778 15mg once daily or 15mg twice daily for 12 months in patients with moderate to severe NPDR. This study has an estimated primary completion date for May 2019 (final data collection date for primary outcome measure ("The TIME-2b Study: A Study of AKB-9778, a Novel Tie2 Activator, in Patients with Non-Proliferative Diabetic Retinopathy (NPDR) - Full Text View - ClinicalTrials.gov", 2017).

5. ARP-1536 is a novel humanized monoclonal antibody that targets the extracellular domain of VE-PTP. With a mechanism of action that activates Tie2, this intravitreal drug can be administered stand-alone or in combination with anti-VEGF therapy to target wet AMD and DME. Pre-clinical studies established a similar biologic activity to AKB-9778 (Frye at al., 2015; "Pipeline – Aerpio Pharmaceuticals", 2017).

6. Nesvacumab (Regeneron) is a fully human monoclonal antibody that was designed for the treatment of cancer. It targets the protein angiopoietin 2. The phase II clinical trial RUBY (anti-vascular endothelial growth factor plus anti-angiopoietin 2 in fixed combination therapy: evaluation for the treatment of diabetic macular edema) was completed in July 2017 ("Anti-vascular endothelial growth factor plus Anti-angiopoietin 2 in Fixed combination therapy: Evaluation for the Treatment of Diabetic Macular Edema - No Study Results Posted - ClinicalTrials.gov", 2017).

7. Adeno-associated virus based platform is a method that will make use of AAV vector to induce anti-VEGF production by transduced cells.

8 Implantation of encapsulated modified cells.

9. Stem cell therapy.

Several algorithms and guidelines have been proposed for the treatment of this multifactorial disease to improve patients' vision and quality of life for all involved in this disease. There is a resilient need to understand the disease, its treatments, regimens available and convenience for all those involved to propose an adequate algorithm for the treatment of DR and DME in an individualized regimen and probably taking advantage of the combination therapy in the near future (Figure 21), (Henriques et al., 2017).

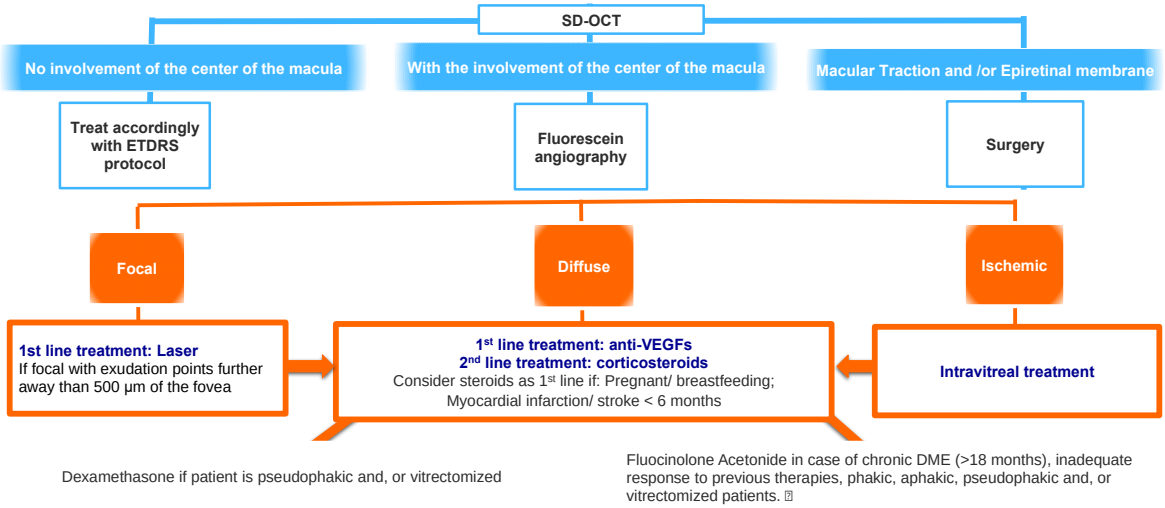


Figure 21: Algorithm of DME treatment, proposed therapeutic schema. Guidelines of Diabetic Macular Edema (DME) the GER perspective towards a more personalized treatment (used with permission).

Despite the emergence of consensus guidelines as well as algorithms, DR and DME remain challenging to treat. Further understanding of the biochemical, genetic and environmental, factors that contribute to the development and progression of DR should continue to bring new discoveries and promising new targets for more effective and better-tolerated treatments.

Paper I

Vascular endothelial growth factors and placenta growth factor in retinal vasculopathies: current research and future perspectives

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Vascular endothelial growth factors and placenta growth factor in retinal vasculopathies: Current research and future perspectives

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Vascular endothelial growth factor B (VEGF-B)

ABSTRACT

Vision loss due to disease or degeneration of the eye (retina, choroid, retinal veins, or macula) is a leading cause of blindness worldwide. In most cases, vision-threatening ocular diseases are accompanied by abnormal changes in the vasculature of the eye, especially the retina, and these conditions are collectively referred to as retinal vasculopathies. Impaired blood supply or hypoxia stimulates angiogenesis in the vascular and non-vascular sections of the eye, which results in neovascularization, leading to conditions such as diabetic retinopathy or age-related macular degeneration. Studies show that vascular endothelial growth factors: VEGF-A, VEGF-B, and placental growth factor (PlGF) are elevated in these diseases, and hence, these factors could be used as markers for disease prognosis and therapy. In this review, we discuss the function of these growth factors in normal development and disease, with focus on ocular disorders and emphasize the importance of accurately determining their levels in the vitreous and serum of patients for correct diagnosis and therapy.

1. Introduction

Growth factors are naturally occurring molecules, usually proteins or steroids, which stimulate cellular growth, proliferation, and differentiation [1]. Typically, growth factors act as signaling molecules between cells and bind to cell surface receptors to initiate signaling cascades that affect gene expression and cell fate. For example, bone morphogenetic protein induces bone differentiation [2], whereas fibroblast growth factor [3] and vascular endothelial growth factors (VEGF) stimulate angiogenesis, i.e., growth of blood vessels [4,5].

For the last two decades, growth factors are also being extensively used in medicine for treating hematologic, oncologic, and cardiovascular diseases [6–9]. In particular, the VEGF family of growth factors are finding increasing use in the treatment of cardiac [10], renal [11], and bone-related diseases [12] as agonists and in ophthalmic diseases [13,14] and cancer as antagonists [15]. Angiogenesis plays important roles in normal and pathological proliferative processes. It is involved in normal growth, wound healing [16–18] as well as in tumor growth and metastases [19], which makes regulators of angiogenesis critical biomedical molecules.

The VEGF family consists of seven secreted dimeric proteins, namely, VEGF-A (or VEGF), B, C, D, E (or viral VEGF), F (snake venom VEGF) and the placental growth factor (PlGF) [20]. VEGF-A has several isoforms that arise via alternative splicing of the eight-exon VEGF-A gene. All VEGFs function by binding to cell surface-bound tyrosine kinase receptors called vascular endothelial growth factor receptors (VEGFRs), causing them to dimerize and be activated through transphosphorylation, albeit with different specificities. VEGF ligands bind to three main transmembrane endothelial receptors namely, VEGFR-1, VEGFR-2, and VEGFR-3 [21]. In addition, neuropilins, neuropilin 1 (NRP-1), and neuropilin 2 (NRP-2) provide co-receptor function in endothelial cells [22]. Among the growth factors of the VEGF family, VEGF-A is well-studied and PlGF less studied in terms of function and clinical application, whereas the physiological role of VEGF-B was ambiguous until recently and is just beginning to be understood. Nonetheless, the effect of these three factors in ophthalmic development and diseases is relatively less investigated. In this review, we would first discuss the roles of VEGF-A, VEGF-B, and PlGF in general development, followed by discussion of their emerging functions in ophthalmic disorders, as well as targeted molecules.

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2. Vascular endothelial growth factors in development and disease

2.1. VEGF-A

VEGF-A, which signals via VEGFR-1 and 2 and NRP-1 and 2 [23,24], is essential for vascular development. Mice lacking any of the VEGF allele die during embryogenesis due to impaired angiogenesis [25]. Alternative exon splicing produces seven VEGF-A isoforms: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{165b}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$. Each isoform is characterized by the respective number of amino acids after cleavage of the signal sequence variants [26]. Initial in vitro studies showed that capillary endothelial cells proliferate and form tubular structures in the presence of VEGF-A [27]. VEGF-A expression is downregulated after embryogenesis [28], whereas it is upregulated during physiological and pathological angiogenesis [29] and also during exercise and muscle contraction [30]. Increased blood flow during exercise along with VEGF-A also stimulates the production of its receptors, which initiates a massive signaling cascade leading to the production of nitric oxide (for vessel permeability), FGF (for cell proliferation), and ICAM/VCAM/matrix metalloproteases (for migration) all of which contribute to the formation of new blood vessels. In addition, VEGF and VEGFR-1 are upregulated in hypoxic conditions via hypoxia inducible factor (HIF) 1-α-dependent [31] and independent pathways [32], highlighting the close relationship between blood oxygen levels, angiogenesis, and tissue metabolism. Similarly, the levels of VEGF-A and its corresponding receptors arise immediately after a traumatic injury to the central nervous system and decline with time, which corresponds to endogenous post-injury revascularization [33].

Rheumatoid arthritis (RA) is an autoimmune disease, and synovial angiogenesis is critical for inflammation and immune activation associated with RA pathogenesis. A recent study showed that serum VEGF-A levels and VEGF-A polymorphisms were strongly associated with incidence of RA in the Polish population [34]. Cancer cells require high nutrient and oxygen supply for rapid proliferation and dissemination (metastasis), which necessitates de novo angiogenesis inside the tumor; thus, cancer may be categorized as an "angiogenic" disease. Studies have shown that VEGF-A overexpression is implicated with poor prognosis of breast cancer, which acts as an angiogenic switch that induces metastasis [35]. Similarly, VEGF-A has been found to be upregulated in hepatic, gastric, pancreatic, ovarian, bladder, colorectal, myeloid, and thyroid cancers and medulloblastoma [7]. Thus, VEGF-A is a good candidate for anti-cancer treatment, and an anti-VEGF-A monoclonal antibody called bevacizumab was clinically approved for cancer treatment in 2004. VEGF-A is also involved in renal and pulmonary disorders. Patients with pulmonary emphysema have decreased VEGF-A levels in pulmonary arteries [36]. VEGF-A is also a biomarker of asthma and compulsive obstructive airway disease [37]. In contrast, increased VEGF-A expression is associated with glomerular hypertrophy and proteinuria [38] although physiological levels of VEGF-A are required for renal development and maintenance of glomerular capillary structure.

2.2. VEGF-B

VEGF-B is highly related to VEGF-A, although it signals via the VEGFR-1 receptor unlike that of VEGF-A, which uses both VEGFR-1 and 2, thereby competing with VEGF-A for VEGFR-1 binding [39]. VEGF-B also binds with NRP-1 [39]. Despite similarity in structure, the physiological role of VEGF-B is ambiguous. VEGF-B has two isoforms: $VEGF\text{-}B_{167}$ and $VEGF\text{-}B_{186}$. Both VEGF-B isoforms can form heterodimers when co-expressed with $VEGF\text{-}A_{165}$ in cells; however, whether heterodimers exist in nature is not known. Despite being cell-bound, the $VEGF\text{-}A_{165}/VEGF\text{-}B_{167}$ heterodimers can be freely secreted by endothelial cells. The balance between homo- and heterodimers can affect VEGF-A signaling if heterodimers are formed [40].

VEGF-B possesses low angiogenic potential; hence did not induce vessel formation or sprouting when delivered in muscle or periadventitial tissue [41], whereas transgenic overexpression of VEGF-B minimally increased vasculature. In contrast, VEGF-B overexpression was reported to potentiate and not initiate angiogenesis in endothelial cells of transgenic mice [42]. VEGF-B was therefore considered as a survival factor of endothelial cells that regulated the expression of pro-survival genes via VEGFR-1 and NRP-1 signaling [43].

Unlike VEGF-A, VEGF-B is not induced by hypoxia, other growth factors, cytokines, hormones, or oncogenes [44,45]. Although a recent study has reported that hypoxia induces VEGF-B in the retina [46]. However, VEGF-B is expressed in a wide range of tissues; being most abundant in tissues with high metabolic activity, such as the myocardium, skeletal muscle, vascular smooth muscle, brown adipose tissue, kidney, brain, and parietal cells of the stomach [47,48]. This indicates a role of VEGF-B in coordinating angiogenesis with metabolism. A study demonstrated that VEGF-B is highly expressed in metabolically active tissues, such as the heart and skeletal muscle, suggesting its function in maintaining oxidative metabolic and contractile function in these tissues [49]. VEGF-B is expressed on the cell surface of cardiomyocytes, which are released for downstream signaling after cleavage with endothelial cell-secreted heparanase. The bidirectional interaction between endothelial cells and cardiomyocytes could provide the diabetic heart protection against cell death and may be a critical tool for delaying or preventing cardiomyopathy. However, VEGF-B and heparanase production decline under sustained hyperglycemic conditions and VEGF-B signaling declines albeit upregulation of VEGFR-1, which results in diabetic cardiomyopathy [49]. Another study identified VEGF-B to be a coronary growth factor in rats where it induced cardiac hypertrophy via the endothelium [50]. VEGF-B also induced angiogenesis and arteriogenesis in myocardium of patients via VEGFR-1 and NRP-1 signaling [51]. Moreover, VEGF-B selectively promotes angiogenesis in ischemic myocardium. Several studies have shown that VEGF-B has a specific role in the revascularization of ischemic myocardium in different disease models of mice, pigs, and rabbits. Therefore, VEGF-B might have a cardioprotector effect and might harbor therapeutic potential for ischemic heart diseases [42,48].

Studies in VEGF-B knockout mice show that VEGF-B is dispensable for embryonic growth and survival, unlike VEGF-A knockouts that die during embryogenesis. However, the hearts of the VEGF-B knockout mice were smaller and displayed vascular dysfunction after coronary occlusion and impaired recovery from experimentally-induced myocardial ischemia, indicating a role of VEGF-B in coronary vasculature development [52].

Diabetic kidney disease (DKD) is a severe renal disease that is characterized by defects in glomerular filtration, proteinuria, and steatosis. DKD tissues show high VEGF-B expression. Since VEGF-B controls muscle lipid accumulation through regulation of endothelial fatty acid transport [53], therefore therapeutic reduction in VEGF-B levels ameliorates symptoms of DKD, such as renal lipotoxicity and insulin insensitivity [54].

VEGF-B also has a crucial role in neuroprotection. VEGF-B-deficient mice showed impaired recovery from cerebral ischemic injury and neurogenesis was seen to be stimulated in adult mice on administration of VEGF-B [55]. VEGF-B is also required for nerve regeneration, sensory recovery, and trophic functions of injured corneal peripheral nerves, but is not present in a mouse model having no nerve injury [56]. In addition, VEGF-B was shown to increase angiogenesis in a mouse model of arthritis as Vegfb-/- mice exhibited decrease in inflammation-associated synovial angiogenesis [57].

The role of VEGF-B in cancer remains unclear. However, Yang and colleagues demonstrated that VEGF-B promotes cancer metastasis through remodeling of tumor microvasculature and a VEGF-A–independent mechanism [58]. The function of VEGF-B is multifaceted, thus it was considered by Li and co-workers as a survival factor rather than an angiogenic molecule [59].

2.3. PlGF

PlGF is a pleiotropic factor that affects different cell types and regulates various biological processes via signaling through VEGFR-1. PlGF has four splice variants that are generated via alternative splicing: PlGF-1 ($PlGF_{131}$), PlGF-2 ($PlGF_{152}$), PlGF-3 ($PlGF_{203}$), and PlGF-4 ($PlGF_{224}$) [60]. The PlGF-2 and PlGF-4 isoforms also bind to neuropilins. PlGF can also form heterodimers with VEGF-A [61]. One of the primary functions of PlGF is regulation of vessel growth and maturation, and therefore, this cytokine is associated with pro-angiogenic activities similar to VEGF-A. PlGF recruits myeloid progenitors to growing sprouts and collateral vessels; it attracts macrophages, which release angiogenic and lymphangiogenic factors. In addition, PlGF regulates ossification, wound healing, retinal pigment cell chemotaxis, and survival of cortical neurons, etc. [62] PlGF overexpression in murine epidermal cells elicited severe inflammatory response associated with pronounced edema, inflammatory cell infiltration, and vascular enlargement indicating direct role of PlGF in cutaneous inflammatory response [63].

Sandro De Falco and colleagues described the role of PlGF in cardiovascular diseases and suggested three major functions for PlGF in the cardiovascular system: 1) myocardial angiogenesis, 2) mediating macrophage chemotaxis, 3) selective action in modulating pathological rather than physiological vascular development, which makes this protein an excellent candidate for therapeutically modulated angiogenesis [64].

PlGF levels are low in normal adult tissues; however, it was upregulated in 4 out of 16 menangioma tumors, whereas VEGF-A level was upregulated in 3 out of 16 samples. There was no significant correlation between PlGF and VEGF-A expression levels. VEGF-B was uniformly expressed in all tumor samples. In a PlGF-positive tumor sample, immunoreactive VEGFR-1 and VEGFR-2 were detected in endothelial cells of the blood vessels and PlGF was detected in most tumor capillaries. Thus, PlGF might be yet another marker for tumor angiogenesis in human menangiomas [65]. Interestingly, supra physiological levels of PlGF have been documented to inhibit angiogenesis of tumors co-expressing VEGF-A. This possibly occurs because of heterodimerization of PlGF with VEGF-A, which outnumbers the biologically active VEGF-A homodimer [66]. Indeed, Yang et al. demonstrated that PlGF can affect tumor angiogenesis in both positive and negative ways in a VEGF-A-dependent manner. In one tumor model, PlGF remodeled tumor vasculature to a normalized phenotype, whereas ablation of VEGF-A in a PlGF-positive tumor accelerated tumor angiogenesis and growth [67].

3. Vascular endothelial growth factors in ophthalmic diseases

3.1. VEGF-A

The eye possesses a special anatomy where completely avascular and highly vascular structures lie in close apposition. Stringent regulation of the balance between vascular growth and quiescence maintains this structure. Vascular growth occurs mainly during embryonic development and is almost absent in the adult eye. Therefore, ophthalmic diseases associated with angiogenesis represent cases where this delicate balance has been disturbed by external conditions, such as hyperglycemia, oxidative stress and other factors (Fig. 1) [68].

Vitreous levels of VEGF-A were high in several retinal diseases, such as diabetic retinopathy (DR), age-related macular degeneration (AMD), and retinal vein occlusion (RVO) [69]. Among diseases contributing to ocular anomalies, diabetes has been studied extensively owing to its manifold effects on healthcare [70]. The deleterious effects of diabetes mellitus on macro and microcirculation underline the morbidity and mortality associated with this disease. Research on pathological factors related to diabetes mellitus have highlighted the involvement of VEGF-A in conditions, such as DR, diabetic macular edema (DME) and AMD, which are the major causes of blindness worldwide [68]. DR is

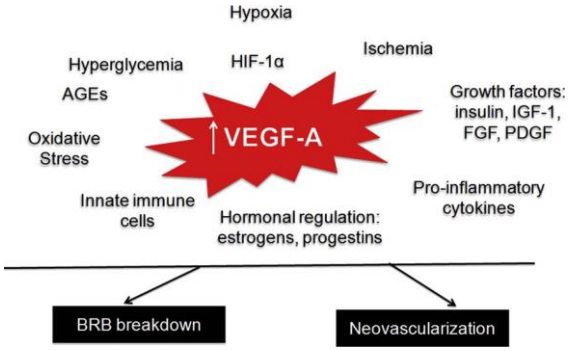

Fig. 1. Factors involved in upregulation of VEGF-A and main repercussions. Hypoxia is one the most important through HIF-1, followed by hyperglycemia; AGEs and pro-inflammatory cytokines are other stimulating factors that increase VEGF-A production. The main consequences for retinal diseases are the BRB breakdown with increasing vascular permeability and neovascularization.
AGEs – Advanced glycation end products; BRB – Blood retinal barrier; HIF-1α – Hypoxia-inducible factor 1-alpha; IGF-1–Insulin–like growth factor 1; FGF – Fibroblast growth factor; PDGF – Platelet-derived growth factor.

accompanied by loss of the retinal barrier function and increased vascular permeability. Several blood-borne proteins might contaminate the vitreous and show an elevated level; therefore, simultaneous testing of protein levels in both serum and vitreous is crucial for correctly interpreting whether the increased vitreal expression is due to upregulation of gene expression or vascular leakage. An accurate interpretation of vitreal pro-angiogenic protein levels is important for the development of correct biomarkers and therapies for retinal diseases [71].

Proliferative DR (PDR) is characterized by progressive loss of retinal capillaries, followed by hypoxia and hypoxia-induced VEGF-A expression, which stimulates neovascularization of the retina, disc, angle, and iris. Several clinical studies have confirmed the correlation between ischemic retinopathies and RVO and VEGF-A levels [69,72]. Elevated VEGF-A levels were observed in the aqueous and vitreous samples of 143 patients with proliferative retinopathies undergoing intra-ocular surgery [73]. On the other hand, laser surgery considerably reduced the intraocular VEGF-A levels [74]. VEGF-A levels were considerably lower in individuals with non-neovascular disease or diabetes without retinopathy. Similar results were reported by Aiello et al. [69], who detected high levels of VEGF-A in 69/136 ocular fluid samples from patients with DR, 29/38 iris samples with neovascularization, and 3 of 4 samples from patients with ischemic occlusion of the central retinal vein, compared to 2 of 31 samples from patients with no neovascular disorders. Other studies showed that advanced glycation end products and decreased anti-oxidant status correlated with DR pathogenesis via VEGF-A induction [75]. A quantitative proteomic study using the vitreous of patients with PDR and non-PDR and those treated with anti-VEGF-A therapy revealed 230 proteins involved in inflammation, complement activation, cell adhesion, and the coagulation cascade, and apolipoproteins, immunoglobulins, etc. to be overexpressed in PDR than in non-PDR [76]. This reflects the multifactorial nature of DR and suggests new possibilities for developing therapeutics. Elevated levels of various cytokines, such as interleukin- (IL-) 1β, IL-2, IL-4, IL-5, IL-6, IL-10, interferon-γ, tumor necrosis factor-α, and VEGF were observed in the aqueous humor of patients with DR, and the levels increased with the severity of the disease [77].

Neovascularization of the angle leads to neovascular glaucoma (NVG), which is currently being treated with anti-VEGF-A therapy in clinical trials [78] as VEGF-A is a marker for NVG in the aqueous humor and serum of such patients [78]. However, it is noteworthy that VEGF-A expression is observed in both normal and diabetic retina photoreceptor and ganglion cells, indicating a physiological role of this growth factor in ocular angiogenesis [79]. A proteomic analysis showed that VEGF-A was upregulated in AMD [80] and that vitrectomy, followed by retinal

photocoagulation, decreased VEGF-A levels [81].

DME is a vision-threatening complication of DR, with a prevalence rate of 20% and 25% in patients with type I and type II diabetes, respectively. DME is caused by macular thickening and cyst formation post retinal-blood barrier breakdown, increased vascular permeability, and fluid accumulation in hyperglycemic condition. Optical coherence tomography (OCT) and enzyme-linked immunosorbent assay (ELISA) of the vitreous of 71 patients with DME revealed a concentration gradient of VEGF-A in DME from the macula to the periphery and from the posterior to the anterior globe [82]. Laser treatment as well as intravitreal anti-angiogenics, such as ranibizumab, aflibercept, and bevacizumab (off-label) have been widely used for the treatment of diseases targeting VEGF-A and are considered as the gold standard for treatment of angiogenic disorders. Intravitreal corticosteroid administration is also used to reduce VEGF levels and angiogenesis in patients with DME [83]. Moreover, other revolutionary modes of treatment, such as dexamethasone and fluocinolone acetonide corticosteroid implants are being used for the treatment of angiogenic and inflammatory diseases [84].

In addition to DR and AMD, VEGF-A causes multiple age-related eye diseases such as cataracts and neovascular and non-exudative AMD-like pathologies. High VEGF-A levels induce age-related opacifications in the lens, which is accompanied by ERK activation, inflammation, and oxidative damage. Targeting of inflammasome components considerably downregulated VEGF-A-stimulated cataract formation. Elevated VEGF-A also causes choroidal neovascularization [85].

It is noteworthy that VEGF-A is the most studied growth factor till date. However, other growth factors are also involved in these angiogenesis-associated pathologies, such as VEGF-B and PlGF, which are gradually gaining attention in basic and clinical research.

3.2. VEGF-B

Previously, the role of VEGF-B in ophthalmic development and diseases was obscure; however, recent studies have shed light on the function of this enigmatic molecule. For example, the overexpression of VEGF-B promoted pathological retinal and choroidal neovascularization and blood-retinal barrier disruption without inflammation, unlike VEGF-A [85]. Thus, VEGF-B could be involved in the progression of DR and AMD in an inflammation-independent way and could therefore be used for developing anti-angiogenic therapies [86]. Interestingly, Reichelt et al. [87] reported that VEGF-B was not required for the development of retinal vasculature under normal conditions or in oxygen-induced retinopathy.

In a study performed by our research group, the measurement of VEGF-B levels in patients with DR and rhegmatogenous retinal detachment revealed that VEGF-B levels were significantly higher ($p = 0.006$) in the vitreous of diabetic patients with ocular disease, and the levels increased in advanced stages of DR [88]. In another study, we estimated VEGF-A and VEGF-B levels using ELISA in the vitreous and serum of patients with proliferative ocular disorders (POD), which included patients with DR, AMD, and retinal vein occlusion ($n = 10$), and compared it with a control group of patients with non-proliferative ocular disorders (NPOD) ($n = 4$). Similar to the results of earlier studies, we observed that VEGF-A and B levels were elevated in POD than in NPOD mainly because of DR, and that the serum and vitreous levels of VEGF-A and B showed high correlation [89].

We also performed ELISA to estimate the levels of VEGF-B and PlGF in the vitreous of 42 patients with DR undergoing vitrectomy. OCT was used to estimate macular volume (MV) and central retinal thickness (CRT). The results showed elevated VEGF-B levels in these patients, which showed moderate ($p < 0.05$) and robust ($p < 0.01$) correlation with CRT and MV, respectively. PlGF, however, did not show any statistically significant correlation. Thus, VEGF-B targeting in these patients might offer beneficial therapeutic outcomes [90].

VEGF-B is expressed in the eye and its expression is upregulated after pathological challenge of the retina [48]. A recent study showed that subretinal injection of adeno-associated viruses encoding VEGF-B_{167} or VEGF-B_{186} increased ischemia and laser injury-induced retinal and choroidal neovascularization, respectively [86]. Another study showed that targeted inhibition of VEGF-B by shRNA (short hairpin RNA) or intravitreal injection of neutralizing antibody suppressed choroidal and retinal neovascularization in mice [43]. Thus, it can be concluded that VEGF-B targeting inhibited retinal neovascularization.

It appears that the 'angiogenic' activity of VEGF-B during ocular neovascularization is probably because of its potent survival effect on vascular and nonvascular cells. In addition, both NRP-1 and VEGFR-1 participate in mediating the vascular survival effect of VEGF-B. Therefore, even though VEGF-B has a minimal role during the initial phase of blood vessel growth, the vascular survival activity of VEGF-B, which protects the neovessels from apoptosis may play a significant role in enhancing ocular neovascularization. Thus, targeted VEGF-B inhibition may also have therapeutic implications for the treatment of ocular neovascular diseases [43]. VEGF-B is currently receiving attention because of recent exciting advances in VEGF-B biology. Owing to its antiapoptotic and potent survival effect, and its ability to remain inactive under normal conditions, VEGF-B appears to possess valuable therapeutic potential for the treatment of degenerative diseases with an attractive safety profile.

3.3. PlGF

PlGF, originally isolated from the human placenta, participates in pro-angiogenic processes not only by direct signaling through VEGFR-1, but also indirectly by amplifying VEGF-A angiogenesis through regulation of the VEGFR-1 and VEGFR-2 cross-talk [91]. Although PlGF and VEGF-A are both expressed during neonatal retinal development, they have different modulatory influences on retinal vascular development [92]. Studies show that PlGF synergizes with VEGF-A for angiogenesis-associated eye diseases. Indeed, PlGF levels were elevated in the vitreous [93] and aqueous humor of patients with DR and NVG [94]. Another comparative study of vitreal PlGF levels in patients with proliferative DR with or without bevacizumab (anti-VEGF therapy) treatment showed that PlGF level was high in DR patients irrespective of the status of bevacizumab therapy and that it correlated strongly with VEGF-A levels. Thus, PlGF is implicated in DR pathogenesis in parallel to the involvement of VEGF-A, and use of aflibercept (anti-PlGF) might be beneficial in such cases [95]. Interestingly, PlGF deletion in a diabetic mouse model inhibited Akt signaling and HIF1 alpha-dependent VEGF-A activation, indicating that PlGF is required for VEGF-A-mediated DR pathogenesis [96]. Furthermore, intraocular injection of PlGF gene or protein causes retinal vessel disorganization, dilatation, microaneurysm formation, retinal-blood barrier disruption, and edema [97].

Although high VEGF-A levels is one of the main risk factors for angiogenic eye diseases, more factors are currently indicated in the development of subretinal angiogenic pathogenesis. Consequently, Rakic et al. showed that PlGF levels were elevated in cases of choroidal neovascularization, whereas a VEGF-A isoform was present in the early stages of angiogenesis [98]. Huo et al. [99] used a laser burn mouse model of choroidal neovascularization to show that both PlGF and VEGF-A levels were elevated in mouse eyes and that these two factors coordinated to regulate choroidal neovascularization during ocular injury; anti-PlGF therapy alone did not stem the increase in vessel density post laser burn, but it augmented the anti-angiogenic function of anti-VEGF-A in their model.

We analyzed vitreous and serum PlGF levels in diabetic and non-diabetic patients undergoing vitrectomy ($n = 17$ for diabetic and $n = 21$ for non-diabetic) using ELISA. Results showed that vitreous PlGF levels were higher in the diabetes group and that it increased with severity of the disease, i.e., levels were higher in proliferative DR and in non-proliferative DR. Serum PlGF levels were also elevated in the

diabetes group, although the mean difference was not statistically significant. In this work, we did not observe any correlation between vitreous and serum PlGF levels [100].

4. Targeting VEGF in retinal diseases

4.1. Current drugs

Ocular angiogenesis is a cause of severe visual loss. The treatment of ocular neovascular diseases is challenging and has improved dramatically in the last few years with the development of anti-VEGFs, which transformed the treatment of eye disorders. However, currently there is no cure, only therapies that slow down the progression of the disease.

According to a review by Tah and colleagues [101], anti-VEGF started its appearance approximately in 1948, however only after several years later the vascular permeability factor (VPF) was described and in 1989 it was named, as we know, VEGF.

Despite not being an angiogenic agent, one of the first pharmacological therapies to treat AMD was verteporfin (Visudyne®, Novartis Pharmaceuticals Corporation, East Hanover, New Jersey), an angiooccclusive drug that in 2000 changed the course of AMD [102]. The aim of verteporfin therapy was to occlude vessels within the choroidal neovasculature while preserving the overlying retinal tissue.

A short time after, anti-angiogenic agents clinical trials began with powered outcomes of VEGF inhibition. VEGFs were recognized as angiogenic regulators of neovascularization and promoters of vascular permeability reasons for key targets for treating neovascular diseases. They become the standard care therapy for conditions involving neovascularization. The first phase I clinical trials in colon cancer with an anti-angiogenic drug were performed in 1997 by Genentech with the drug called bevacizumab (Avastin®, Genentech, Inc., San Francisco, California, USA), which was granted approval by U.S. Food and Drug Administration (FDA) in 2004 for the treatment of colon cancer as adjuvant to chemotherapy [102].

Additionally, in December 2004, FDA approved pegaptanib sodium (Macugen®, OSI Pharmaceuticals, Inc., Melville, New York) that was the first anti-VEGF therapy for neovascular AMD [102]. However, after bevacizumab approval for cancer therapy, ophthalmologists began to use this molecule intravitreally to treat ocular neovascularization (off-label). Furthermore, Genentech designed a new molecule from the same precursor as bevacizumab, generating a different molecule, specifically for intravitreal use. This new molecule, ranibizumab (Lucentis®, Genentech Inc., San Francisco, California, USA) is believed to penetrate better into the retina [101]. Additionally, to decrease systemic adverse events, the portion Fc of the monoclonal antibody was removed from the original precursor. This new molecule showed to be safe and effective in the first clinical trials: MARINA (Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular Age-Related Macular Degeneration) and ANCHOR (Anti-VEGF Antibody for the Treatment of Predominantly Classic Choroidal Neovascularization in Age-Related Macular Degeneration) [102].

In November 2011, another anti-angiogenic agent, aflibercept (Eylea®, Regeneron Pharmaceuticals Inc., Tarrytown, New York) developed by Regeneron was approved by FDA [102] based on the VIEW studies, which showed to be a safe and an effective drug like ranibizumab. Currently, there are four therapies involving VEGF inhibition [103]:

1. Pegaptanib sodium intravitreal injections, (Macugen®), a pegylated VEGF aptamer. A single strand of nucleic acid that binds with specificity to the 165 isoform of VEGF-A was approved in 2004 by FDA for the treatment of neovascular AMD [102].
2. Bevacizumab (Avastin®) is a humanized anti-VEGF-A monoclonal IgG antibody developed as an anti-angiogenic agent for colon-rectal cancer, lung cancer, glioblastoma, and renal-cell carcinoma. It was approved for medical use in the United States in 2004 [102]. Bevacizumab has been used intravitreally (off-label) in the treatment of proliferative eye diseases.
3. Ranibizumab (Lucentis®) is a fully humanized monoclonal antibody fragment targeted against human VEGF-A, with high affinity for all isoforms of VEGF-A. Until the arrival of ranibizumab, the primary endpoint in clinical trials was the proportion of subjects losing < 15 letters. Ranibizumab changed the landmark of treatment with an unexpectable vision gains and a turnover of the clinical trials primary endpoint to the proportion of subjects gaining ≥ 15 letters. The binding of ranibizumab to VEGF-A at the receptor-binding site prevents the interaction of VEGF-A with its receptors VEGFR-1 and VEGFR-2 on the surface of the endothelial cells, inhibiting the cascade of events that leads to increased vascular permeability, increased activity and proliferation of endothelial cells and inflammation. Ranibizumab granted FDA approval in June 2006 [102].
4. Aflibercept (Eylea®) is a fusion protein of key domains from the human VEGFR-1 and VEGFR-2 and the human immunoglobulin G (IgG) Fc domain that was originally developed for oncology use. It binds to all isoforms of VEGF-A, to VEGF-B and PlGF. Aflibercept was approved for ocular use as Eylea®, and for metastatic colorectal cancer as Zaltrap®[102]. Eylea®was approved in 2011 by the FDA.

VEGF inhibitors are promising drugs in the treatment of neovascular eye diseases, however, should be of noted that there are some limitations of its usage [103]:

1. The unknown duration of anti-neovascular effects. Anti-angiogenics reduce regression of neovascularization after treatment and improve structural and functional parameters, but the duration of the effect is limited to a short period of time.
2. The mode of drug delivery, an intravitreal injection. There are complications after intravitreal injections, such as endophthalmitis, intraocular inflammation, rhegmatogenous retinal detachment, acute intraocular pressure elevation and ocular hemorrhage.
3. Anti-VEGF therapy requires frequent injections and assessments to determine patient response to a treatment. This fact leads to a significant burden of injections and visits for all involved in the treatment of those patients. The solution for this problem would be a medication that improves visual and structural outcomes, simultaneously increasing drug effectiveness and lengthening the durability of the treatment.
4. The frequent anti-angiogenic therapy may be associated with the progression of geographic atrophy.
5. Another issue is the systemic safety. Several pharmacovigilance reports have been generated with the administration of either systemic or ocular anti-VEGFs: thromboembolic events, myocardial infarction, stroke, hypertension, gastrointestinal perforations, and kidney disease which lead to the inclusion of a black box in the summary of product characteristics. Moreover, intravitreal anti-angiogenic drugs were found at detectable levels in the systemic circulation, capable to suppress VEGF-A systemic levels. This fact suggests a rationale for the cardiovascular reports of serious adverse events. Although the death rates did not seem to be increased due to the use of angiogenic drugs, the long-term consequences are still unknown.

Furthermore, VEGF-A, VEGF-B, and PlGF play critical roles in neuroprotection and cardioprotection [103]. Therefore, their blockage may also have consequences in the long run. Nevertheless, not all patients respond sufficiently to anti-VEGF intravitreal injections despite frequent treatments. About 50% of patients have an insufficient response to angiogenic therapy and accordingly with an analysis performed by Gonzalez and colleagues, a percentage of patients would benefit from an early therapy switch as shown in the EARLY study [104]. Therefore, and despite not being an angiogenic therapy,

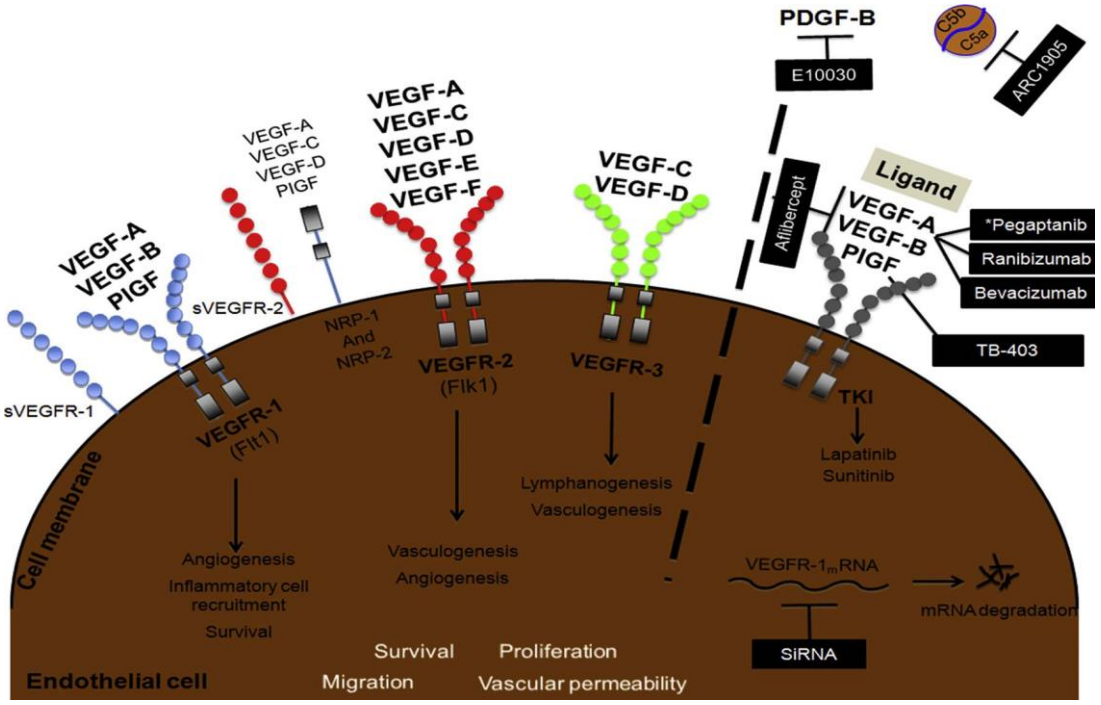

Fig. 2. The VEGF family, receptors and current anti-angiogenic drugs that target VEGF/VEGFR signaling.

corticosteroids treatments are invaluable and one of the oldest treatments available in ophthalmology for the treatment of persistent or recurrent diseases. Steroids have proven to be powerful and effective in suppressing inflammation and also playing a significant role in inhibition of several cytokines inclusively antagonizing the action of VEGF-A.

The best-studied steroids are triamcinolone acetonide (off-label), dexamethasone, and fluocinolone. Dexamethasone implant (Ozurdex®, Allergan, Dublin, Republic of Ireland) is a bioerodable, extended-release of 700 μg of dexamethasone in a solid, bioerodable polymer [84]. Fluocinolone acetonide (Iluvien®, Alimera Sciences, Alpharetta, Georgia) is the smallest, non-bioerodable, slow extended-drug release implant lasting 3 years of duration [84]. Notwithstanding the well-known side effects caused by steroids, cataract formation and increase of intraocular pressure, efficacy and benefits usually outweighed risks. Moreover, there is an enormous advantage of corticosteroids once systemic side effects of locally administered steroids occur rarely. Although laser photocoagulation, anti-VEGFs and steroids pathways established as successful target treatments, new therapeutical enhancements are being developed, holding promises in the improvement of eye pathologies.

4.2. Drug discovery – searching for promising molecules

Several diseases are accompanied by dysregulated angiogenesis and by excessive formation of blood vessels, such as in cancer, RA, AMD, RVO, and PDR or by deficiency in blood vessels, such as in heart and limb ischemia. These diseases can be life-threatening as they cause severe pain and reduce the quality of life. They are also considered to be a burden to the society, not only because of costs incurred but also due to the time-consumed by the physicians, patients, and respective families.

It is therefore of great importance with regard to improve and develop better treatments. The VEGF family and the anti-angiogenics are fascinating molecules that had been receiving interest and research efforts all over the world. Researchers and pharmaceutical companies have been involved in the search for the critical need for new therapies to be given early on in the disease to cure or delay its progression or to prevent. However, there is still a lack of therapeutically viable options for intervention.

Regardless, a remarkable selection of compounds has been described in recent years, targeting different molecules in the signaling pathway, from VEGF-A, VEGF-B, PlGF, and platelet derived growth factor (PDGF), and tyrosine kinase inhibitors (TKIs) to VEGFRs and tyrosine kinase receptor (TKR) inhibitors (Fig. 2). These compounds comprise not only the above described pegaptanib, bevacizumab, ranibizumab, and aflibercept, but also other new molecules such as abicipar pegol, various siRNA, avancincaptad pegol, brolucizumab, multi VEGF-PDGF DARPin, TB-403, and 5D11D4 and TKIs such as lapatinib, sunitinib, sorafenib, axitinib, and pazopanib. Some of those drugs are approved and are used against retinal diseases; however, others are in the clinical or pre-clinical stage. Table 1 summarizes the anti-angiogenic drugs currently approved and available, the molecules on pre and post clinical trials, and possible drugs never studied in ophthalmology that may be considered in the future as targeted molecules.

Significant progress has been made in the understanding of the molecular pathogenesis of retinal neovascular disorders and new targets have been investigated for therapeutic interventions.

5. Outcome and future perspectives

We reviewed literature regarding the involvement of VEGF growth factors, especially VEGF-A, VEGF-B, and PlGF in normal development and diseases with special emphasis on ocular disorders. We showed that accurate estimation of vitreous/aqueous and serum levels of these factors are critical for elucidating their roles in ocular disease pathogenesis. Studies are mainly focused on the involvement of vitreous and serum VEGF-A levels with the prognosis of AMD, DR, and RVO, whereas the role of VEGF-B and PlGF as biomarkers for these conditions is beginning to be understood (Table 2). Table 2 shows that elevated vitreous and/or serum levels of VEGF-A were detected in patients with different ocular disorders, and the levels increased with the severity of the disease [108,110]. The angiogenic potential of VEGF-A aggravates the pathology of the diseases and anti-VEGF-A therapy is often beneficial for ameliorating or regressing the symptoms. However, certain

Table 1
Summary of anti-angiogenic agents [105–107].

Generic drug name	Trade name	Type of molecule	Target	Clinical stage in ophthalmology	Therapeutic Indications	Route of administration	Commercialized by
Pegaptanib	Macugen®	RNA aptamer	VEGF-A $_{165}$	Commercialized	Wet AMD	Intravitreal injection	OSI Pharmaceuticals/ Pfizer/ Bausch & Lomb
Bevacizumab	Avastin®	Recombinant humanized full monoclonal antibody	All VEGF-A isoforms	Not commercialized for ocular use	Off-label usage	Intravitreal injection	Genentech in U.S. and Roche in Europe
Ranibizumab	Lucentis®	Recombinant humanized monoclonal antibody fragment	All VEGF-A isoforms	Commercialized	Wet AMD, macular edema following RVO, DME, DR with DME, and myopic CNV	Intravitreal injection	Genentech in U.S. and Novartis in Europe
Aflibercept	Eylea®	Fusion protein	All VEGF-A & VEGF-B isoforms and PlGF	Commercialized	Wet AMD; ME following RVO, DME and DR in Patients with DME	Intravitreal injection	Regeneron in U.S. and Bayer in Europe
Conbercept/ KH902	Lumitin	Fc fusion protein	All VEGF-A & VEGF-B isoforms, VEGF-C and PlGF	Commercialized in China	Wet AMD	Intravitreal injection	Chengdu Kanghong Biotechnology Co.
AGN-150998; Abicipar Pegol	–	DARPin	VEGF-A	Phase III	Wet AMD, DME	Intravitreal injection	Allergan
E10030; Pegpleranib	Fovista®	DNA aptamer	PDGF-BB	Phase III	Wet AMD	Intravitreal injection	Ophthotech/ Novartis
ESBA-1008; RTH258 (brolucizumab)	–	Humanized single chain antibody fragment	VEGF-A	Phase III	Wet AMD, DME	Intravitreal injection	Alcon Research
TB-403; THR 317	–	Monoclonal antibody	PlGF	Phase II – DME Pre-clinical – DR	DME, DR	Intravenous infusion	ThromboGenics/ Roche
Sunitinib maleate-Sorafenib	Sutent®(oral) Nexavar®	TKI TKI	Small molecules Small molecules	– Phase I/ II AMD	[a] Hepatocellular and Renal cell carcinoma; Thyroid cancer	Intravitreal Oral	GrayBug Inc. Bayer
Axitinib/ Axitinib ophthalmic	Inlyta®Oral formulation/ Pfizer)	TKI, PDGF, VEGF-A	Small molecules	Preclinical DME, RVO	Renal cell carcinoma	Oral	Clearside Biomedical

Note: CNV – Choroidal neovascularization, DARPin – Designed Ankyrin Repeat Proteins, DDIT4 mRNA- DNA-damage-inducible transcript 4 mRNA, DME – diabetic macular edema, DR – diabetic retinopathy, KSP mRNAs – Kinesin spindle protein mRNAs, PDGF – Platelet-derived growth factor, PlGF – placental growth factor, RVO – retinal vein occlusion, siRNA– Small interfering RNA, VEGF – vascular endothelial growth factor, VEGFR-1 – vascular endothelial growth factor receptor 1, Wet AMD – Wet age related macular degeneration. TKI- tyrosine kinase inhibitors.
[a] Sunitinib licensed for Pfizer is marketed for gastrointestinal stromal tumors, pancreatic cancer; renal cell carcinoma.

Table 2
Comparative list of VEGF-A, VEGF-B, and PlGF levels in vitreous, aqueous and serum samples of patients with various ocular diseases.

Number of patients	Mean vitreous/aqueous VEGF-A/VEGF-B/PlGF levels (study and control)	Mean serum VEGF-A/VEGF-B/PlGF levels study and control	Comments of significance	Reference
57 PDR vs 16 controls vs 6 NPDR	1135.2 pg/mL VEGF-A vs 19.3 pg/mL VEGF-A ($p < 0.0001$) vs 49.9 pg/mL VEGF-A ($p < 0.0001$).	ND	Vitreous levels of VEGF-A were significantly higher in patients with active PDR than in those with quiescent PDR ($p < 0.0001$).	[108]
20 PDR vs 20 controls	1340 pg/mL VEGF-A vs 9 pg/mL VEGF-A (median) ($p < 0.0001$).	177 pg/mL VEGF-A vs 170 pg/mL VEGF-A (median), (no signficant difference).	Direct correlation between VCAM-1 and VEGF-A, suggest connection between cellular adhesion and neovascularization.	[109]
11 PDR vs 23 control	7200 pg/mL VEGF-A vs 1800 pg/mL VEGF-A ($p < 0.001$).	ND	Vitreous VEGF-A levels are associated with DR. VEGF-A plays a critical role in the DR progression.	[110]
23 PDR vs 17 controls	1420 pg/mL VEGF-A vs 9 pg/mLVEGF-A ($p < 0.001$).	120 pg/mL VEGF-A vs 150 pg/mL VEGF-A (ns).	VEGF-A is increased in the vitreous of diabetic patients with PDR, suggesting intraocular production as the main factor for the intravitreous enhancement of VEGF-A.	[111]
41 PDR vs 18 controls	812 ± 1.108 pg/mL VEGF-A vs 1.7 ± 4.4 pg/mL VEGF-A ($p < 0.0001$).	ND	VEGF-A levels in eyes with active PDR were significantly higher than in those with inactive PDR.	[112]
39 PDR vs 11 controls	1134 pg/mL VEGF-A vs < 50 pg/mL VEGF-A ($p < 0.001$), (median values).	ND	Controls and patients without PDR had low and comparable VEGF-A levels (medians < 50 pg/mL). Patients with PDR had high vitreous VEGF-A concentrations (median 1134 pg/mL).	[113]
16 NPDR vs 13 controls	192.7 pg/mL VEGF-A vs < 31.2 pg/mL VEGF-A (median values), ($p < 0.001$).	414.3 pg/mLVEGF-Avs332.7 pg/mL VEGF-A (ns).	No significant correlations were found between concentrations of VEGF-A in serum and vitreous.	[114]
37 PDR vs 21 controls	1380 pg/mL VEGF-A vs 9 pg/mLVEGF-A ($p < 0.0001$)	130 pg/mL VEGF-A vs 160 pg/mL VEGF-A.	No correlation between serum and vitreous in diabetic patients nor in control group ($r = 0.06$, $p =$ ns and $r = -0.15$, $p =$ ns, respectively)	[72]
70 PDR vs 25 NPDR vs 41 quiescent PDR vs 31 controls	3600 ± 6300 pg/mL VEGF-A vs 100 ± 100 pg/mL VEGF-A ($p = 0.008$) vs 200 ± 600 pg/mL VEGF-A ($p < 0.001$) vs 100 ± 200 pg/mL VEGF-A ($p = 0.003$).	ND	VEGF have an important role in mediation active intraocular neovascularization in patients with ischemic retinal diseases, such as DR and RVO.	[69]
27 PDR vs 14 controls	410 pg/mL VEGF-A vs 17 pg/mL VEGF-A (median values), ($p < 0.001$).	VEGF-A levels in PDR (median values) 190 pg/mL lower than vitreous VEGF-A ($p < 0.05$).	Vitreous levels of VEGF-A were higher in PDR, and not influenced by its serum concentrations. VEGF-A plays an important role in neovascularization of PDR.	[115]
22 PDR vs 28 controls	1759 ± 1721 pg/mL VEGF-A vs 27 ± 65 pg/mL VEGF-A ($p < 0.001$).	ND	Correlation of glutamate and GABA levels with high VEGF-A levels providing biochemical support for ischemia-induced neovascularization in PDR.	[116]
19 PDR vs 7 controls	5660 pg/mL VEGF-A vs 350 pg/mL VEGF-A ($p < 0.05$).	ND	VEGF-A vitreous levels in PDR were elevated, and play a role in its pathogenesis.	[117]
30 PDR vs 35 controls	383.1 ± 107.48 pg/mL VEGF-A vs 24.81 ± 1.85 pg/mL VEGF-A ($p \leq 0.005$) respectively.	515.12 ± 44.8 pg/mL VEGF-A vs 343.58 ± 46.41 pg/mL VEGF-A ($p \leq 0.005$), respectively.	Positive correlation between serum and vitreous VEGF-A levels ($p = 0.012$ and $r = 0.453$).	[118]
20 PDR vs 13 controls	1.75 ng/mL VEGF-A vs 0.009 ng/mL VEGF-A (median values).	No differences in serum VEGF-A between groups.	High vitreous VEGF-A levels in PDR patients were not attributed to serum levels.	[119]
20 PDR vs 12 controls (MH)	833.7 ± 281.3 pg/mL VEGF-A vs 32.9 ± 18.1 pg/mL VEGF-A ($p > 0.001$).	30.2 ± 11.8 pg/mL VEGF-A vs 22.1 ± 9.2 pg/mL VEGF-A ($p > 0.05$), respectively.	Vitreous VEGF-A significantly higher vs serum in diabetic patients.	[120]
46 PDR vs 49 NPDR vs 31 without DR vs 28 healthy subjects	ND	149.12 pg/mL VEGF-A vs 153.07 pg/mL VEGF-A vs 125.37 pg/mL VEGF-A vs 98.20 pg/mL (median values).	No correlation between macular thickness and serum VEGF-A levels ($p > 0.05$).	[121]
31 PDR vs 15 controls	Vitreous VEGF-A significantly increased in the PDR vs controls.	No differences were evident in serum VEGF-A.	No correlation between the vitreous and serum levels of VEGF in patients with PDR.	[122]
42 PDR vs 48 without DR	ND	219 ± 99 pg/mL VEGF-A vs 139 ± 98 pg/mL VEGF-A ($p < 0.001$).	Serum VEGF-A was significantly higher in patients with PDR than in those without DR.	[123]

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Table 2 (continued)

Number of patients	Mean vitreous/aqueous VEGF-A/VEGF-B/PlGF levels (study and control)	Mean serum VEGF-A/VEGF-B/PlGF levels study and control	Comments of significance	Reference
15 PDR vs 15 NPDR vs. no DR vs 15 normal controls	ND	616 ± 301 pg/mL VEGF-A ($p < 0.05$) vs 787 ± 476 pg/mL VEGF-A ($p < 0.05$) vs 508 ± 262 pg/mL VEGF-A vs 323 ± 8 pg/mL VEGF-A.	No correlation between serum VEGF-A and severity of DR. Serum VEGF-A levels in NPDR are higher than in PDR patients. Circulating VEGF-A is involved in progression of DR.	[124]
45 PDR vs 28 controls	723.21 pg/mL VEGF-A vs 20.81 pg/mL VEGF-A respectively ($p < 0.001$).	ND	Vitreous VEGF-A significantly higher in active PDR than in eyes with inactive PDR ($p = 0.008$).	[125]
20 samples (PDR and controls)	Vitreous VEGF-A levels were significantly higher in eyes with PDR than in eyes without PDR ($p = 0.006$).	ND	VEGF-A functions as a physiologically angiogenic factor in PDR.	[126]
22 NVG patient vs20 controls (aqueous humor)	3037 ± 2387 pg/mL VEGF-A in NVG patients 1078 ± 712 pg/mL PlGF ($p < 0.001$ with respect to control) in NVG patients.	Serum levels of VEGF-A and PlGF were low in the patients and controls.	High concentrations of VEGF-A correlated with high levels of PlGF in patients with NVG ($r = 0.593$, $p = 0.004$). Concentrations of VEGF-B in aqueous humor and serum remained unchanged ($p > 0.05$). Positive correlation between VEGF-A and PlGF aqueous humor.	[127]
30 NVG vs 30 control eyes (aqueous humor)	832.88 ± 96.44 pg/mL VEGF-A vs 206.5 ± 45.84 pg/mL VEGF-A.	356.88 ± 68.45 pg/mL VEGF-A vs 112.54 ± 65.13 pg/mL VEGF-A.	Aqueous VEGF-A significantly different between patients and control ($p < 0.001$). Positive correlation between serum and aqueous VEGF-A levels in the NVG group ($r = 0.638$, $p = 0.001$).	[128]
71 patients with DME	VEGF-A in the pre-macular vitreous (1386.2 ± 2134.1 pg/mL) vs the peripheral cortical vitreous (1169.7 ± 1840.3 pg/mL, $p = 0.0216$) vs mid-vitreous (1080.9 ± 1534.1 pg/mL; $p = 0.0017$). Controls VEGF-A concentrations in the premacular vitreous, peripheral cortical vitreous and mid-vitreous were all below the detection limit (< 20 pg/mL).	ND	Vitreous VEGF-A concentration followed a gradient. VEGF-A was higher in premacular vitreous than in mid-vitreous and peripheral cortical vitreous, suggesting diffusion from the macular region to the periphery, and from the posterior to the anterior globe.	[82]
37 diabetic vs 8 controls	Mean PlGF levels in diabetics 103 pg/mL vs non-detectable in control samples.	ND	PlGF was present in all diabetic vitreous samples but non-detectable in controls. The results demonstrated a role for PlGF in the pathogenesis of PDR.	[129]
10 IR vs 26 controls (aqueous humor) plus vitreous samples from: 11 eyes with PDR, 2 eyes with non-diabetic IR (central RVO, acute retinal necrosis) and 7 eyes without IR	PlGF was detected in 1 out of 36 aqueous samples with severe PDR at a very high concentration (2270 pg/mL). Vitreous PlGF concentration ($n = 13$) from 11 eyes with PDR was 360 ± 272 pg/mL. PlGF concentration in the 2 eyes with non-diabetic IR was 458 pg/mL. PlGF not detected in the eyes without IR.	ND	The concentration of PlGF was significantly correlated with that of VEGF-A ($n = 19$, $r = 0.526$, $p = 0.019$)	[130]
50 PDR vs 19 control	Vitreous PlGF and vitreous VEGF-A levels (median range) in PDR (PlGF, 100.6 pg/mL, range 7.6–1,038.6; VEGF-A 653.9 pg/mL, 9.0–5, 423.8) were significantly higher ($p > 0.0001$) than in the control (PlGF 7.0 pg/mL, 7.0–12.1; VEGF-A 9.0 pg/mL, 9.0–10.0).	ND	The ratio of vitreous PlGF and vitreous VEGF-A to protein in active PDR was significantly higher than that in quiescent PDR (PlGF 33.5, 2.7–250.7 vs 11.1, 1.5–35.8, $p = 0.0039$; VEGF-A 130.1, 7.8–904.0 vs 73.9, 2.0–150.3, $p = 0.0328$). Intravitreous PlGF levels significantly correlated with intravitreous VEGF levels in both PDR patients ($r = 0.824$, $p < 0.0001$) and total subjects ($r = 0.857$, $p < 0.0001$).	[92]
50 PDR vs 56 controls	Vitreous VEGF-A was 585.67 ± 57.40 pg/mL in the PDR vs 123.85 ± 109.42 pg/mL in controls.	VEGF-A concentration (plasma) was 410.07 ± 74.70 pg/mL in the PDR vs 114.41 ± 110.99 pg/mL in healthy controls.	Vitreous and plasma VEGF-A levels were significantly elevated in PDR patients than those in controls (p vitreous < 0.001, p plasma < 0.001). Both vitreous and plasma VEGF-A levels were significantly higher in PDR progression group than in stable group (p vitreous < 0.001; p plasma = 0.004). Vitreous VEGF-A was positively associated with plasma VEGF-A in PDR patients ($p < 0.001$).	[131]
20 PDR (DMI) vs 16 PDR (DMII) vs control	Vitreous VEGF-A in diabetics with PDR and DMI was 432.23 pg/mL, in diabetics with PDR and	ND	Mean vitreous VEGF-A levels of diabetic patients were significantly higher than in controls.	[132]

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Table 2 (continued)

Number of patients	Mean vitreous/aqueous VEGF-A/VEGF-B/PlGF levels (study and control)	Mean serum VEGF-A/VEGF-B/PlGF levels study and control	Comments of significance	Reference
	DMII was 147.5 pg/mL and in controls was 63.26 pg/mL ($p < 0.005$).		Mean vitreous VEGF-A levels were significantly higher in diabetics with PDR and DMI than in diabetics with PDR and DMII.	
25 diabetic patients with PDR (n = 19) or NPDR (n = 6) vs control group of 8 non-diabetic patients	Vitreous VEGF-B was higher in the diabetic group (18.82 ± 1.44 pg/mL) vs control group (17.90 ± 0.32 pg/mL), ($p = 0.006$).	ND	Mean vitreous VEGF-B was higher in PDR (19.03 ± 1.52 pg/mL) vs NPDR (18.18 ± 0.96 pg/mL). VEGF-B was significantly increased in DR, and this increase is significantly higher as the DR is at a more advanced stage.	[88]
21 diabetic (4 NPDR and 17 PDR) vs 17 non-diabetic	Mean vitreous PlGF was 70.0 vs 46.47 pg/mL, $Z = -2847$, $p = 0.004$.	Mean serum PlGF was 50.5 vs 48.8 pg/mL ($Z = -1196$, $p = 0.232$).	PDR patients had significantly elevated vitreous PlGF vs. NPDR patients, (76.5 vs. 42.5 pg/mL, $Z = -2.612$, $p = 0.009$). PlGF is overexpressed in the vitreous of diabetic patients and levels increase with the severity of the disease. No correlation between vitreous and serum levels of PlGF.	[100]
14 patients with POD: 8 DR, 1 RVO, 1 AMD vs 4 NPOD patients with VMTS vitreomacular traction syndrome	Mean vitreous VEGF-A and B was 603.65 ± 688.60 and 368.46 ± 451.46 pg/mL in POD, respectively vs non-detectable values in NPOD.	Mean serum VEGF-A and B in POD was 101.28 ± 60.68 and 53.72 ± 42.39 pg/mL vs 81.82 ± 64.97 ($p = 0.604$) and 41.55 ± 35.62 pg/mL ($p = 0.777$) in NPOD.	There was a strong and positive statistically significant correlation between VEGF-A and B in vitreous and serum samples.	[89]

Note: DMI: Diabetes mellitus I, DMII – Diabetes mellitus II, DR – Diabetic retinopathy, IR – Ischaemic retinopathy, ND – not done, NPDR – Non-PDR, ns – not statistical significant, MH – Macular Hole, NVG – Neovascular glaucoma, POD – proliferative ocular disease, PDR – Proliferative diabetic retinopathy, RVO – retinal vein occlusion, VCAM-1–vascular cell adhesion molecule 1, VEGF – vascular endothelial growth factor, VMTS – vitreomacular traction syndrome. Results are provided as mean ± SD (standard deviation) when available.

cases are also characterized by high VEGF-B and PlGF levels in the vitreous of the diseased eye. Our work and few other studies have revealed the importance of screening patient samples for VEGF-B and PlGF levels as future prognostic markers or targeted therapies [88–90,100]. Reports show that PlGF often synergizes VEGF-A signaling in retinal pathologies and therefore, targeting PlGF should be critical for completely containing the symptoms [62,66]. Similarly, VEGF-B is an emerging druggable candidate for the treatment of angiogenic ocular disorders [59]. Finally, caution should be exercised while interpreting the vitreous and serum levels of these growth factors as high vitreous levels may sometimes be due to increased vascular permeability, which allows serum proteins to leak inside the vitreous and result in false positive results. [124]. Contrary to this, some authors describe that vitreous levels are independent of serum levels as it is not easy to find a positive and robust correlation between them [119]. Further, caution must be exercised during interpretation of serum levels and growth factors as other systemic diseases may increase VEGF levels. Therefore, robust correlation between vitreous and serum levels of these growth factors would increase the confidence level of the approach.

Multiple challenges in the treatment of ocular diseases include: 1) identification of biomarkers before diagnosis and appearance of clinical symptoms, which would enable treatment of early stages of the diseases, 2) identification of markers to monitor disease progression, and 3) identification of new targeted therapies. More clinical trials in ophthalmology are required to test the efficacy and safety of new therapies targeting VEGF-B and/or PlGF, either in combination with existing therapies or in monotherapy.

Future research should continue to focus on new anti-VEGF strategies in the treatment of ocular diseases linked to abnormal vascularization. Discovery of new isoforms of the VEGF family reveal an increased biological complexity and has faced many obstacles that must be overcome while exploring new targets. The blockage selectivity is one of the most important factors to be considered when testing the new anti-VEGF molecules.

Understanding the detailed molecular mechanisms underlying angiogenesis and physiology is of vital importance for the future development of drugs, which can regulate angiogenesis. However, there are many unanswered questions that need to be explored as well as new and innovative molecules to be discovered before the full potential of these strategies can be understood, which would result in better outcomes for patients with pathological ocular angiogenesis.

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Conflicts of interest

None of the authors have any conflicts of interest to declare.

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Chapter 2

Global aims

The aims of this study were to investigate the levels of VEGF-A, VEGF-B and PlGF in vitreous humor and serum among different ocular pathologies: neovascular pathologies (diabetic retinopathy, age-related macular degeneration and retinal vein occlusion) and compare with non-neovascular pathologies (rhegmatogenous retinal detachment or vítreo macular traction syndrome of idiopathic etiology) by:

1. Comparing serum and vitreous VEGF-A, VEGF-B and PIGF levels between ocular diseases
2. Comparing serum and vitreous VEGF-A, VEGF-B and PIGF levels with disease progression
2. Correlating growth factors levels in vitreous or in serum;
3. Correlating growth factors between vitreous and serum
4. Correlating vitreous growth factors with structural (central retinal thickness and macular volume) and functional (visual acuity) parameters

A better understanding of these cytokines and their correlation in serum and vitreous humor may lead us to:

1. Better-targeted precise and specific therapies
2. Early intervention in disease prevention before clinical symptoms appear and before the occurrence of ocular neovascularization
3. Effective therapeutic interventions

Ultimately,

1. Leading to the prevention of blindness
2. Avoiding unnecessary blindness

Chapter 3

Paper II

VEGF-B Levels in the Vitreous of Diabetic and Non-Diabetic Patients with Ocular Diseases and Its Correlation with Structural Parameters

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Article

VEGF-B Levels in the Vitreous of Diabetic and Non-Diabetic Patients with Ocular Diseases and Its Correlation with Structural Parameters

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Abstract: Vascular endothelial growth factor B (VEGF-B) is one of the enigmatic members of the VEGF family. The knowledge gap about VEGF-B expression and how its levels are altered in diabetic eyes were the focus of this investigation that was addressed by comparing and correlating vitreous VEGF-B between diabetic and non-diabetic patients. VEGF-B levels were measured by enzyme-linked immunosorbent assay in vitreous samples (n = 33) from diabetic (n = 25) and non-diabetic (n = 8) patients. Results were compared between groups. Optical coherence tomography from diabetic patients was evaluated for central retinal thickness (CRT) and macular volume (MV). Mean vitreous VEGF-B concentration was higher in diabetic (18.82 ± 1.44 pg/mL) vs. non-diabetic patients (17.90 ± 0.32 pg/mL) (p = 0.006), and in proliferative diabetic retinopathy (PDR) (19.03 ± 1.52 pg/mL) vs. non-PDR (NPDR) patients (18.18 ±0.96 pg/mL) (p = 0.025). In diabetic retinopathy (DR) patients, correlation between VEGF-B and CRT (µm) was positive and moderate: r_s = 0.441 ($p \leq 0.05$) and the correlation between VEGF-B and MV (mm^3) was positive and robust: r_s = 0.716 ($p \leq 0.01$). VEGF-B levels are overexpressed in vitreous of diabetic patients, and the levels are higher in developed stages of DR. Correlation results show that CRT and MV increase with increased levels of VEGF-B. Targeting VEGF-B inhibition may have therapeutic beneficial implications.

Keywords: angiogenesis; diabetic retinopathy; enzyme-linked immunosorbent assay; vascular endothelial growth factor-B; VEGF inhibition; vitreous humor

1. Introduction

Vascular endothelial growth factor A (VEGF-A) is the most studied member of the VEGF family and is an important regulator of angiogenesis and vascular permeability in physiological and pathological conditions. It induces proliferation and migration of endothelial cells and vascular permeability, resulting in angiogenesis in vivo [1]. Aiello [2] demonstrated that the levels of VEGF-A levels are increased in ocular fluids in patients with active retinal neovascularization associated with several ocular diseases in diabetic patients. Since it promotes angiogenesis in pathological

conditions [3–5] VEGF-A is considered an important therapeutic target and a focus of considerable ophthalmologic research.

Besides VEGF-A, there are other members of this family that may be of relevance for ocular disease. In particular, vascular endothelial growth factor B (VEGF-B) has been described in cardiovascular disease, diabetes, and cancer; however, its function in ophthalmology remains poorly understood, as it is the most controversial molecule of the VEGF family [3,6,7]. VEGF-B has been investigated in different tumor types, and its expression levels were found to be upregulated in cancer tissue [6]. Yang et al. studied the role of VEGF-B in promoting cancer metastasis and tumor invasion, referring to this molecule as a vascular remodeling factor and a target for cancer treatment [8].

VEGF-B was discovered in 1996 as a VEGF homolog [9,10]. It is expressed in two different isoforms: a heparin binding VEGF-B_{167} and diffusible VEGF-B_{186} [11], as seen in Table 1 [6,7,12–14]. VEGF-B binds selectively to vascular endothelial growth factor receptor-1 (VEGFR-1) [9] and neuropilin-1 (NRP-1), exerting its effects through binding to those receptors. VEGF-A, VEGF-B, and PIGF (placental growth factor) bind to VEGFR-1 via D2 domains while VEGF-A binds to VEGFR-2 through the domains D2 and D3 [15]. Platania and colleagues studied the interactions of VEGF antagonists/VEGF-A complexes in silico and showed the differences in the interactions and affinities, although higher affinities alone do not always translate to better efficacy [16].

Table 1. Summary of the characteristics of Vascular endothelial growth factor B (VEGF-B).

Characteristics	
Exons	7
2 Isoforms	VEGF-B_{167}—heparin binding isoform, carboxyl terminus VEGF-B_{186}—diffusible isoform, hydrophobic carboxyl terminus
Binding	VEGFR-1 and NRP-1
Molecular masses of homodimers	VEGF-B_{167} = 42 kDa VEGF-B_{186} = 60 kDa
Expression	In various tissues, as the heart and skeletal muscle, vascular smooth muscles, endothelium cells, mural cells, pericytes, smooth muscle cells, vascular stem/progenitor cells. Overall, it is expressed in tissues with high metabolic activity such as the myocardium.
Role	VEGF-B was described as playing roles in angiogenesis, however its ability to stimulate directly angiogenesis is poor. VEGF-B has an important role in the heart (cardioprotection), neurologic diseases (inducing neuroprotection), cancer and metabolic diseases.

VEGFR:-1: vascular endothelial growth factor receptor 1 ; NRP-1: neuropilin-1 . [6,7,12–14].

Furthermore, Lyer [17] studied the structure of VEGF-B(10–108)•VEGFR-1D complex and demonstrated that the domain 2 of VEGFR-1 was the minimal binding domain needed for VEGF-B interaction. It was also showed that the topology of the studied dimer did not alter when bound to the receptor. The fact that VEGF-B interacts with VEGFR-1 but not with VEGFR-2 is explained by electrostatic surface potentials: VEGF-B has a negative charge showing an affinity for the basic interface of VEGFR-1 and no affinity for VEGFR-2 [17]. In addition, VEGF-B may form heterodimers with VEGF-A and PIGF, regulating the bioavailability and the activity of those growth factors to bind VEGFR-1 and NRP-1 [17]. VEGF-B is not stimulated by hypoxia, cytokines, hormones, or oncogenes [18].

VEGF-B was studied mainly in animal models; however, the expression of VEGF-B in human eyes and the changes in its levels in certain eye diseases, such as diabetic retinopathy (DR), need to be elucidated. A better understanding of the molecular pathological events triggered by the increase in VEGF expression, and specifically in VEGF-B, may be of fundamental importance for the development of new treatments for these pathologies. This is because the anti-angiogenic therapy is particularly promising for the treatment of neovascular diseases. Although the anti-angiogenic properties of VEGF-B are poor, VEGF-B promotes cell survival through its binding to VEGFR-1 and NRP-1, playing

an important role as an anti-apoptotic molecule. It also suppresses growth through VEGFR-1 when it acts as a decoy for VEGF-A [12,19].

This project aims to provide a better understanding of the VEGF-B expression in human vitreous humor and its relationship with DR. It may contribute to the development of improved targeted anti-VEGF therapies for VEGF-driven eye diseases, with a lower occurrence of serious adverse events and ultimately for better effective therapeutic interventions. Thus, the main objective of this study was the quantification and comparison of VEGF-B levels in vitreous samples of diabetic and non-diabetic patients. Additionally, VEGF-B levels were compared between patients with non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) and correlated with central retinal thickness (CRT) and macular volume (MV). In addition, the glucose levels in the vitreous were measured and compared between the study groups: diabetic versus non-diabetic patients and NPDR versus PDR.

2. Materials and Methods

2.1. Participants

The study enrolled 33 patients: 25 diabetic patients with PDR (n = 19) or NPDR (n = 6) (13 women and 12 men) and a control group of 8 non-diabetic patients (2 women and 6 men) with rhegmatogenous retinal detachment (RRD). To the best of our knowledge, there are no reports on the measurement of the VEGF-B levels in vitreous humor of patients with ocular disease, making it difficult to use it as a reference value. Rhegmatogenous retinal detachment patients with no reports of other eye diseases or disorders were selected to serve as a control sample, minimizing the bias caused in the interpretation of the results. Only one eye from each patient was studied.

2.2. Collection of Samples and Data from Patients

Undiluted vitreous humor samples were collected from patients who were referred to the hospital for pars plana vitrectomy (PPV).

These samples were collected at the beginning of the PPV (core vitrectomy). Tubes from the vitrectomy were disconnected and connected to a syringe (in coordination with vitrectomy aspiration at the beginning of the surgery). Before turning on the intravitreal infusion, an undiluted sample of vitreous was obtained by aspiration into a 2 mL syringe attached to the vitreous cutter. The sample was transferred to sterilized tubes, placed immediately on ice, and frozen at −80 °C until further analysis.

Approximately 500 samples were collected from vitrectomized patients with several pathologies; of those, 33 samples were chosen for this study based on the following criteria: (1) patients with a confirmed diagnostic of DR (for the diabetic patient's study arm), either with PDR or NPDR; (2) patients naïve to aflibercept treatment; and (3) patients with a sample volume that would allow the confirmation of the results through repeated enzyme-linked immunosorbent assay (ELISA) tests (Wuhan, China) for the determination of VEGF-B levels.

Quantitative analysis of the optical coherence tomography (OCT) scans was performed to evaluate CRT (µm) and MV (mm^3) from DR patients using SPECTRALIS OCT (Heidelberg Engineering, Heidelberg, Germany).

Moreover, patient clinical history was investigated to confirm patient diagnosis, baseline characteristics, and associated concomitant diseases. Information about concomitant medications or non-therapeutic drugs that may influence the results was collected, including the use of previous anti-angiogenic drugs.

2.3. Measurement of Vitreous Vascular Endothelial Growth Factor B Levels

Quantification of vitreous VEGF-B was performed using ELISA Kit for VEGF-B for human samples (product SEA144Hu, USCN Life Sciences, Wuhan, China). Detection range was between

15.63–1000 pg/mL, and the sensitivity or the minimum detectable level was less than 5.5 pg/mL. The assay was conducted according to the protocol specified by the kit manufacturer.

2.4. Measurement of Vitreous Glucose Levels

The enzymatic UV test (hexokinase method) was used for specific quantitative determination of glucose in vitreous using a Beckman Coulter analyzer AU2700 (Brea, CA, USA) and Glucose reagent OSR6521. The results were reported in mmol/L.

2.5. Statistical Analysis

Statistical analysis was performed with SPSS (Statistical Package for Social Sciences) version 20.0 for Windows (Microsoft, Armonk, NY, United States of America). Student's *t*-test and Mann–Whitney tests were used to analyze differences. Values of vitreous VEGF-B and glucose levels are reported as mean ± standard deviation (pg/mL or mmol/L, respectively). For all statistical analyses, a p-value ≤ 0.05 was considered to indicate statistically significant differences. Spearman's ordinal correlation coefficient was used to analyze the relationship between quantitative variables because the variables had no normal distribution.

2.6. Ethical Statement

The study was approved by the Comissão de Ética para a Saúde (CES)/Ethics Committee for Health of the Centro Hospitalar de Leiria (Code: CHL-15481). All patients included in this study, which adhered to the tenets of the Declaration of Helsinki, gave their informed consent.

3. Results

3.1. Study Population—Baseline Characteristics

The baseline characteristics of both groups are shown in Table 2. The mean age of the diabetic and control groups was 68.21 ± 9.59 years and 61.50 ± 14.87 years, respectively. Six patients (24%) had NPDR, and the other 19 diabetic patients (76%) had PDR. Of the 25 patients in the diabetic group, 19 had prior laser treatment; of which 2 patients received a prior intravitreal injection of triamcinolone acetonide and 1 received previous treatment with anti-VEGF therapy, namely ranibizumab. None of the diabetic patients included in the study had been previously treated with aflibercept.

Table 2. Patient baseline clinic, demographic characteristics, and previous treatments.

Patient Number	Gender	Eye Submitted to PPV	Diagnosis and/or Stage of Severity of Diabetic Retinopathy	Previous Treatment for Diabetic Retinopathy (before Vitrectomy)
1	F	OD	NPDR	No treatment
2	M	OS	PDR	Laser therapy
3	M	OS	PDR	Laser therapy
4	M	OD	NPDR	No treatment
5	M	OD	PDR	Laser therapy plus intravitreal triamcinolone acetonide
6	F	OS	PDR	Laser therapy
7	F	OD	PDR	Laser therapy
8	M	OD	NPDR	No treatment
9	F	OS	PDR	Laser therapy plus ranibizumab intravitreal injection
10	F	OD	PDR	Laser therapy
11	F	OS	NPDR	No treatment
12	M	OS	PDR	Laser therapy
13	F	OD	PDR	Laser therapy
14	M	OS	PDR	Laser therapy
15	M	OS	NPDR	Laser therapy
16	F	OD	PDR	Laser therapy
17	M	OD	PDR	Laser therapy

Table 2. *Cont.*

Patient Number	Gender	Eye Submitted to PPV	Diagnosis and/or Stage of Severity of Diabetic Retinopathy	Previous Treatment for Diabetic Retinopathy (before Vitrectomy)
18	F	OD	PDR	Laser therapy
19	M	OD	PDR	Laser therapy plus intravitreal triamcinolone acetonide
20	F	OD	PDR	Laser therapy
21	F	OD	NPDR	No treatment
22	M	OD	PDR	Laser therapy
23	F	OD	PDR	Laser therapy
24	F	OD	PDR	Laser therapy
25	M	OS	PDR	Laser therapy
26	M	OD	RRD	Not applicable
27	M	OS	RRD	Not applicable
28	M	OS	RRD	Not applicable
29	M	OS	RRD	Not applicable
30	M	OD	RRD	Not applicable
31	M	OS	RRD	Not applicable
32	F	OS	RRD	Not applicable
33	F	OD	RRD	Not applicable

F: Female; M: Male; PPV: Pars plana vitrectomy; OD: right eye; OS: left eye; PDR: Proliferative diabetic retinopathy; NPDR: Non-proliferative diabetic retinopathy; RRD: Rhegmatogenous retinal detachment.

3.2. Comparison of Vascular Endothelial Growth Factor B Levels in Vitreous Humor between Diabetic and Control Groups

The mean VEGF-B concentration in vitreous humor was higher in the diabetic patients' group (18.82 ± 1.44 pg/mL) in comparison with the control group of non-diabetic patients (17.90 ± 0.32 pg/mL) (Figure 1). This difference was considered statistically significant ($p = 0.006$).

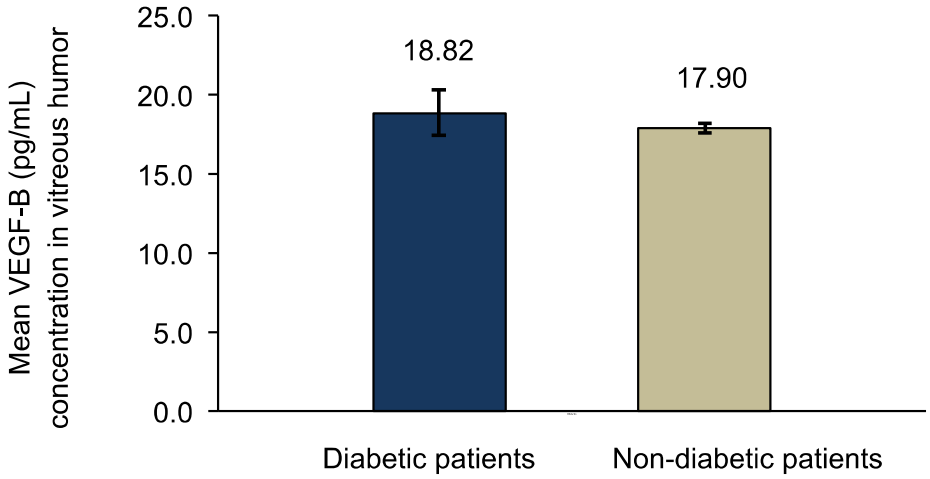


Figure 1. Comparison of VEGF-B vitreous levels between diabetic patients ($n = 25$) and non-diabetic patients ($n = 8$), analyzed with independent samples, t-test ($p = 0.006$).

3.3. Comparison of Vascular Endothelial Growth Factor B Levels in Vitreous Humor between Patients with Proliferative Diabetic Retinopathy vs. Non-Proliferative Diabetic Retinopathy

Mean VEGF-B measurements were higher in vitreous humor of patients with PDR (19.03 ± 1.52 pg/mL) vs. NPDR (18.18 ± 0.96 pg/mL), and this difference was statistically significant ($p = 0.025$) (Figure 2).

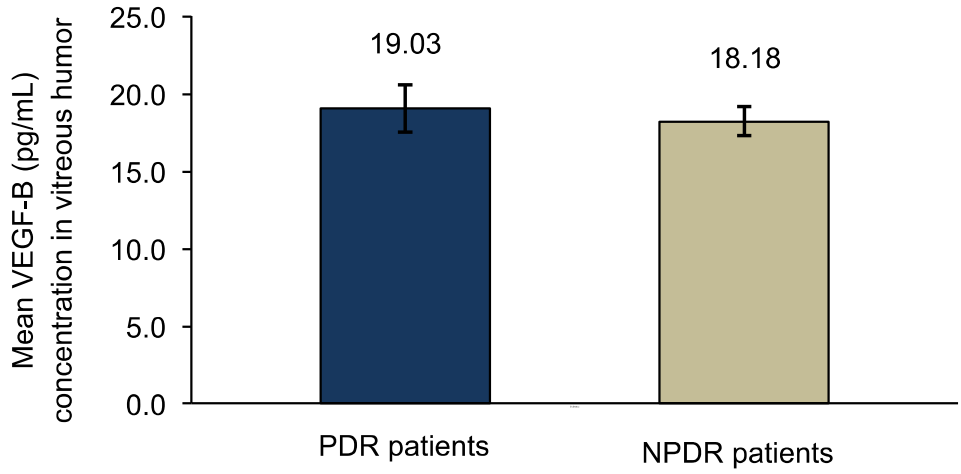


Figure 2. Comparison of VEGF-B levels in vitreous between PDR (n = 19) and NPDR patients (n = 6), analyzed with Mann–Whitney test (p = 0.025).

3.4. Comparison of Glucose Vitreous Humor Levels between Patients with Diabetic Disease and the Control Group

Diabetic patients showed increased levels of glucose in vitreous humor (4.57 ± 2.07 mmol/L) when compared with non-diabetic patients (3.65 ± 2.39 mmol/L), but this difference was not statistically significant (p = 0.300) (Figure 3).

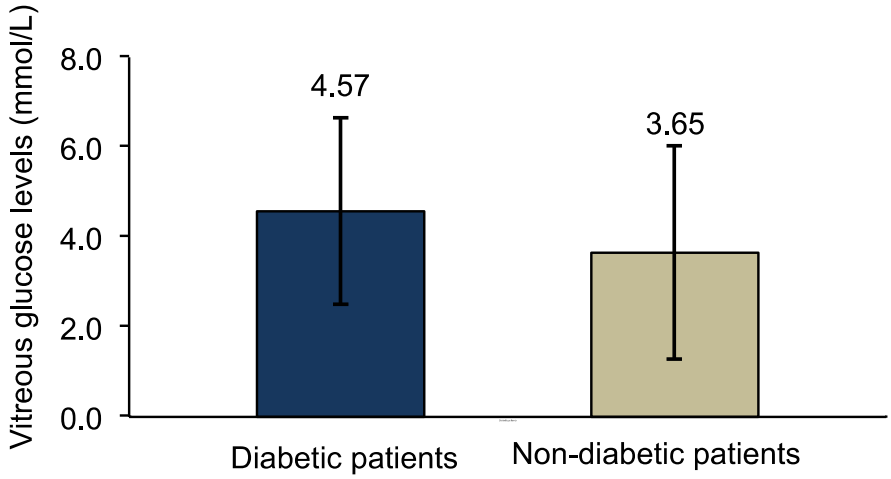


Figure 3. Comparison of glucose vitreous levels between diabetic (n = 25) and non-diabetic control group (n = 8) analyzed with independent-samples, t-test (p = 0.300).

3.5. Comparison of Glucose Vitreous Humor Levels Between Patients with Proliferative Diabetic Retinopathy vs. Non-Proliferative Diabetic Retinopathy

In this analysis, patients with PDR showed to have statistically higher values of glucose in vitreous when compared to NPDR group of patients (5.06 ± 1.89 mmol/L vs. 3.01 ± 1.98 mmol/L; p = 0.032) (Figure 4).

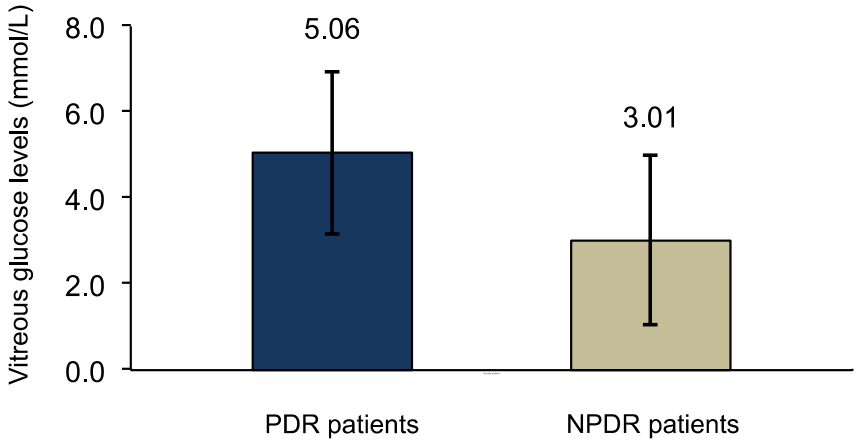
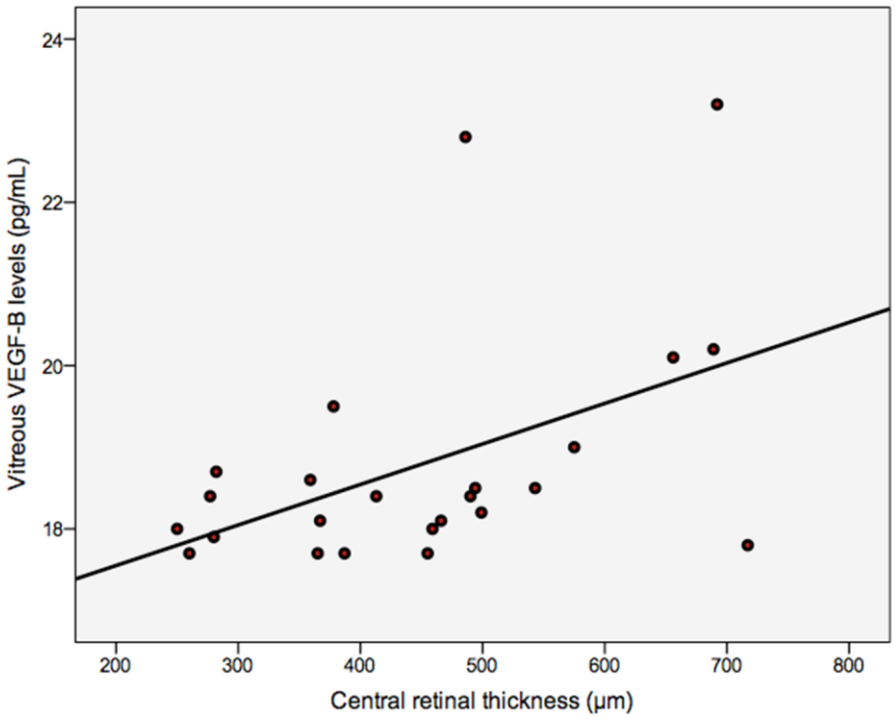


Figure 4. Comparison of glucose vitreous levels between NPDR (n = 6) and PDR patients (n = 19), analyzed with independent-samples, t-test (p = 0.032).

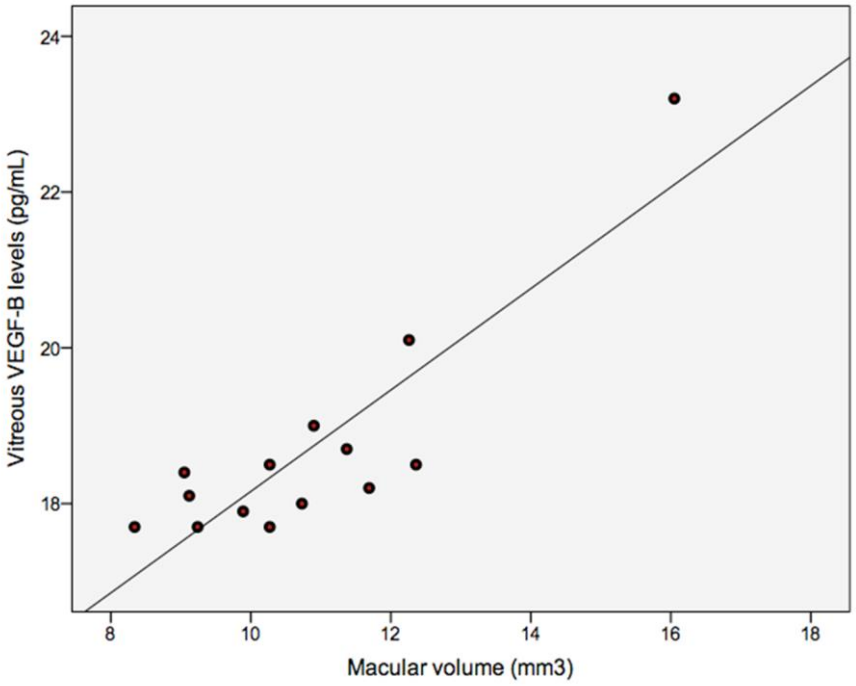
3.6. Correlation between Vitreous Vascular Endothelial Growth Factor B in Diabetic Retinopathy Patients and Quantitative Measurements by Optical Coherence Tomography

The correlation between vitreous VEGF-B of DR patients and CRT was statistically significant, positive, and moderate: r_s = 0.441 ($p \leq 0.05$), as seen in Figure 5a. Moreover, the correlation between vitreous VEGF-B and MV in these patients was statistically significant, positive, and robust: r_s = 0.716 ($p \leq 0.01$), as observed in Figure 5b.



(**a**)

Figure 5. *Cont.*



(**b**)

Figure 5. Correlation between vitreous VEGF-B (pg/mL) levels in DR patients (**a**) and central retinal thickness (CRT; µm), $r_s = 0.441$ ($p \leq 0.05$); or (**b**) and macular volume (MV; mm^3), $r_s = 0.716$ ($p \leq 0.01$), analyzed using Spearman's correlation coefficient.

4. Discussion

VEGF-B is a proangiogenic cytokine [20] that has been implicated in several diseases. Contrary to VEGF-A, the prototypical member of the family that was found increased in the vitreous humor of patients with retinal diseases such as DR, the biological role of VEGF-B in the eye has not been sufficiently studied. Moreover, its function remains controversial and needs to be elucidated, specifically in pathological events in eye disease.

The present study demonstrated that VEGF-B is significantly increased in diabetic patients with ocular disease in comparison with non-diabetic patients. This is an interesting finding as the VEGF-B levels have not been measured in the vitreous humor of human eyes. Moreover, the levels of VEGF-B were found increased in PDR in comparison to NPDR, and this difference was statistically significant. In addition, we found a positive correlation between VEGF-B vitreous levels and the CRT or MV measured by OCT, suggesting that overexpression of VEGF-B may influence these quantitative parameters in DR patients.

It is uncertain whether the inhibition of VEGF-B is beneficial and improves patients' visual acuity as well as structural outcomes, given the protecting and survival action of VEGF-B.

Reports on the role of VEGF-B have provided controversial findings. Silvestre [21] demonstrated that VEGF-B in mice had angiogenic properties, in part through VEGFR-1. Also, Wafai et al. [22] showed that VEGF-B under certain conditions promoted angiogenesis in the tibidis anterior muscle of rabbits with bilateral hind limb ischemia. Aase et al. [23] revealed that VEGF-B was necessary for heart function but not required for heart development or the angiogenesis process in the adult mice. Reichelt et al. [24] demonstrated that under normal conditions, the retinal vasculature of the mice was not affected by the lack of VEGF-B. Rissanen [25] also showed, with his experience with skeletal muscles of rabbits, that VEGFR-1 ligand VEGF-B did not induce angiogenesis. Bhardwaj

et al. [26] studied the angiogenic responses of the different members of the VEGF family in rabbit carotid arteries and verified that VEGF-B did not display angiogenic activity. Li et al. [27] suggested a specific role of VEGF-B in the revascularization of ischemic myocardium, playing an important role as cardioprotector. In this specific case, VEGF-B was described as having angiogenic activity. In the same light, Mould [28] proposed VEGF-B therapy for ischemic heart disease due to its role in promoting vascular growth and, therefore, revascularization. An interesting finding by Li et al. [14] was that VEGF-B was a potent inhibitor of apoptosis, rescuing neurons in the retina from apoptosis in a mice model in ocular neurodegenerative diseases and the brain of a mice model of stroke. They also showed that the treatment with VEGF-B using an effective dose for the survival of neurons did not cause angiogenic activity.

VEGF-B therapy may lead to vessel survival and rescue of blood vessels from apoptosis; thus, it is being referred as a possible therapy in neurologic diseases, such as Parkinson's disease. Confirming these findings, Poesen et al. [29] showed the function of VEGF-B_{186} in the nervous system in an animal study. Specifically, the neuroprotective effects of VEGF-B in combination with its very low angiogenic and permeability action. They reported its role as a neuroprotector, turning it into an appealing treatment for neurodegenerative diseases. Based on the findings of the above studies, it can be stated that VEGF-B plays a role in response to pathological conditions, and its upregulation may demonstrate benefits.

However, other studies reported that VEGF-B plays a role as an inhibitor of choroidal and retinal neovascularization through VEGFR-1 and NRP-1. In his work, Zhang et al. [30] referred to VEGF-B as a survival factor instead of an angiogenic factor, suggesting that the survival factor was mediated by NRP-1 and VEGFR-1 complexes. Li et al. [19] reported that VEGF-B targeting inhibits pathological angiogenesis by abolishing blood cell survival, confirming the apoptotic effect of VEGF-B. Li et al. [12] also referred that antigrowth and anti-angiogenic effects of the VEGF-B are mediated through VEGFR-1, which acts as a VEGF-A decoy, suppressing angiogenesis.

Based on the above studies describing the different functions of VEGF-B and the results of our investigation where VEGF-B appears to be overexpressed in DR in comparison with RRD, we suggest that VEGF-B targeted therapy by an anti-angiogenic drug may have a therapeutic effect on neovascular diseases. Due to the inhibition of VEGF-B, the treatment of vascular diseases of the retina may improve outcomes and have positive effects on the patients. It is important to acknowledge that VEGF-B does not act under normal physiological conditions, but instead in pathological conditions, which makes VEGF-B an attractive and safe therapeutic molecule.

Lundquist O. and Österlin [31] demonstrated in their study that vitreous glucose concentration was in general (with some exceptions) lower than in blood but was higher in the diabetic group than in the non-diabetic group of patients. In our study, glucose concentration was also assayed in vitreous humor samples obtained from patients of the two study groups. The diabetic patients showed higher glucose levels in the vitreous than the non-diabetic group; however, the difference was not statistically significant. Moreover, vitreous glucose concentration was lower in NPDR patients than in the PDR patients, suggesting that vitreous glucose concentration increases with disease severity.

5. Conclusions

VEGF-B was found significantly increased in DR patients, and this increase is significantly higher as the DR is at a more advanced stage. We suggest VEGF-B can offer an alternative and challenging therapeutic target in the treatment of neovascular conditions, such as diabetic eye disease. Randomized clinical trials are needed to be conducted in order to confirm the efficacy of anti-VEGF-B therapy. Furthermore, due to the cardioprotective and neuroprotective effect of this molecule, and taking into account that diabetic patients have multiple disease risk factors, studies targeting VEGF-B inhibition must be conducted carefully to determine its safety, particularly in these patients.

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Author Contributions: Joana Mesquita managed the experiment, conducted data collection, and wrote the manuscript. Joana Mesquita, João Paulo Castro-Sousa, and Cândida T. Tomaz conceived and designed the experiment. João Paulo Castro-Sousa and Arminda Neves collected vitreous and serum samples during PPV. Joana Mesquita, Sara Vaz-Pereira, João Paulo Castro-Sousa, and Cândida T. Tomaz analyzed the data and experiment and reviewed all manuscript versions. Paulo Tavares-Ratado and Fátima M. Santos contributed to laboratory analysis, revisions, and analysis tools; Luís A. Passarinha, João Paulo Castro-Sousa, and Cândida T. Tomaz oriented the work. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Paper III

Serum and vitreous placental growth factor in diabetic retinopathy patients. Relationship with disease severity and optical coherence tomographic parameters

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Title Page

Serum and vitreous placental growth factor in diabetic retinopathy patients: Relationship with disease severity and optical coherence tomographic parameters

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Abstract

Purpose: This study aimed to evaluate serum and vitreous placental growth factor (PIGF) levels in diabetic retinopathy (DR) vs. non-DR patients. Correlation between serum and vitreous PIGF levels and between vitreous PIGF levels in DR patients and optical coherence tomography (OCT) parameters was also performed.

Methods: Serum and vitreous samples were collected from patients undergoing vitrectomy (n=38): 21 DR and 17 non-DR, and PIGF analyzed by ELISA. OCT scans from DR patients were evaluated to measure central retinal thickness (CRT) and macular volume (MV).

Results: Mean vitreous PIGF was significantly higher in DR vs. non-DR patients: 70.0 ± 39.2 vs. 46.47 ± 9.7 pg/mL (mean ± standard deviation), p=0.004. Mean serum PIGF level was higher in DR vs. non-DR patients but result was not statistically significant. Comparison between retinopathy stages showed that proliferative DR patients had significantly higher PIGF vitreous levels compared to non-proliferative DR patients: 76.5 ± 41.0 vs. 42.5 ± 5.0 pg/mL, p=0.009, respectively. There was no correlation between vitreous and serum PlGF levels. Vitreous PIGF vs. OCT parameters showed a tendency for positive correlation.

Conclusions: PIGF plays an important role in neovascularization and it is overexpressed in vitreous of DR patients; moreover PIGF levels increase with the severity of the disease. The absence of correlation between vitreous and serum PIGF may demonstrate dissociation between the eye and other organs and also suggest intravitreal synthesis of PIGF in case of neovascular eye disease. Therefore, targeting PIGF may offer an additional strategy for ocular pathologies with a neovascular component.

Keywords: Diabetic retinopathy; Placental Growth Factor; Vascular endothelial growth factor; Vitreous humor, Serum PIGF

1. Introduction:

The incidence of diabetes mellitus is increasing every year. A common complication linked with the onset of diabetes is diabetic retinopathy (DR), which is a microvascular complication and a major cause of blindness in the working-age population [1]. DR can be characterized clinically as non-proliferative DR (NPDR) or proliferative DR (PDR). NPDR presents microaneurysms, retinal hemorrhages, hard exudates, cotton wool spots, venous beading and intra-retinal microvascular abnormalities (IRMA), whereas the presence of retinal neovascularization due to retinal ischemia, hypoxia and vascular endothelial growth factor A (VEGF-A) stimulation is the hallmark of PDR. The new blood vessels proliferate, leak and bleed, and can lead to vitreous hemorrhage, tractional detachment or neovascular glaucoma, which may result in in irreversible visual loss [2].

VEGF-A is the most studied and influential molecule in the DR process. However, there are other growth factors, such as the placental growth factor (PlGF), besides VEGF-A that seem to be implied in this process [3,4,5].

PlGF was discovered after VEGF-A, and it was considered the second member of the VEGF family [6]. It's alternative splicing generates four isoforms (PlGF-1, PlGF-2, PlGF-3 and PlGF-4). PlGF binds to vascular endothelial growth factor receptor 1 (VEGFR-1), soluble fms-like tyrosine kinase-1 (sFLT1) and neuropilins (NRP) -1 and -2 [7,8].

PlGF binds specifically to VEGFR-1; however, it may activate VEGFR-2 through other mechanisms; for example, PlGF may bind VEGFR-1 dislocating VEGF-A from VEGFR-1 and freeing VEGF-A and making it available to bind to VEGFR-2 [9]. Or else, PlGF and VEGF-A may produce heterodimers capable to bind and activate VEGFR-1 [10].

PlGF seems to have a significant role in pathologies involving ischemia, malignancy, inflammation, and increase in vascularization. In fact, the role of PlGF has been observed in pathological states rather than in physiological states [11]. Several studies showed that PlGF is a superfluous molecule during normal vascular development and maintenance but has an important role in the angiogenic and inflammatory switch of some diseases [10].

Pharmacological studies focused on loss-of-function and gain-of-function that led to the characterization and identification of therapeutic needs in delivering and blocking PlGF [8].

Some pathological conditions may improve due to delivering of PlGF: (a) in the cardiovascular system: to preserve cardiac performance after an infarction, PlGF induces revascularization of ischemic myocardium and vessel enlargement, playing a significant role in myocardial angiogenesis, regulation of vascular growth in pathological states and a selective action in modulating pathological rather than physiological vascular development; (b) in the nervous system: during cerebral ischemia, PlGF is upregulated in neurons and vascular cells having neuroprotective properties; and (c) in the skin: PlGF is overexpressed during wound healing. The increased PlGF levels lead to an increase in angiogenesis, thus, improving wound healing

and ultimately skin regeneration. Also during bone fracture repair, colitis, sepsis and preeclampsia, where healing angiogenesis is present, PIGF therapy delivery may help to restore normal functions [8]. While some pathological conditions improve with upregulation of the PIGF, others, such as ocular neovascularization, become worse [12]. In fact, PIGF deficiency or PlGF receptor neutralization leads to a decrease in choroidal neovascularization in animal models [13]. Moreover, the intraocular delivery of PIGF promotes progression of DR. The pharmacological inhibition of PIGF as suggested by Van de Veire et al., (2010) inhibits neovascularization by inhibiting angiogenesis and vascular leakage and improving ocular inflammation [14].

The role of PIGF has been documented controversially, but after studies, it is now possible to confirm its role not only in angiogenesis but also in modulating inflammation [5,10].

Huo et al. (2015) suggested that the effect of PIGF on choroidal neovascularization is case-dependent, through a mechanism of co-inhibition of PIGF that reinforces the effect of anti-VEGF-A inhibition [15].

There has been a great improvement in the treatment of ocular diabetic disease and consequent outcomes due to the use of anti-VEGF therapies and use of corticosteroids (not subject of discussion here). However, disease recurrence is common within these patients. The long-term consequences of anti-VEGF therapy, despite seeming safe, are still not completely known. Given the increasing incidence of diabetes mellitus and consequent DR, and taking into account the enormous disease burden associated with DR treatments, focusing on other contributory molecules as well as developing new targeted therapies is critical to fight vision loss.

In our study, we evaluated serum and vitreous PIGF levels by enzyme-linked immunosorbent assay (ELISA) in diabetic patients (with DR) and compared them with a control group of non-diabetic patients (with rhegmatogenous retinal detachment). The study aimed at finding correlations between this parameter and the severity of the disease. We also correlated PIGF levels between vitreous and serum and PIGF vitreous levels with structural measurements (central retinal thickness and macular volume) performed by optical coherence tomography (OCT). A better understanding of the expression and behavior of this molecule in eye diseases and its correlation with functional and structural outcomes will contribute to the development of better-targeted therapies.

2. Materials and Methods

2.1 **Participants:**

Undiluted samples of vitreous humor and serum were collected from patients who were submitted to pars plana vitrectomy (PPV) due to different ocular pathologies.

Samples from patients were selected for analysis and included for PlGF quantification based on the following criteria:

1. Sufficient sample volume collected to allow the confirmation of the results through repeated ELISA tests;
2. Patients with a confirmed diagnosis of DR;
3. Patients last treated (anti-VEGF, corticosteroid or laser) for their eye condition more than 3 months from PPV surgery and;
4. Naïve patients to aflibercept, either systemically or intravitreally.

Hemolyzed samples were also excluded from analysis.

At the end of the selection, a total of 38 patients were included: 21 with DR (12 female and 9 male patients), and 17 with rhegmatogenous retinal detachment (5 female and 12 male patients). Rhegmatogenous retinal detachment patients with retinal tears secondary to posterior vitreous detachment or trauma and with no reports of other eye disease or disorders that may confound the results were selected to serve as a control sample, minimizing the bias caused in the interpretation of the results. For the correlation of vitreous PlGF levels with central retinal thickness (CRT) and macular volume (MV), only patients with DR and completed data were included in this analysis. Only one eye from each patient was studied.

2.2 Collection of samples and data from patients

The collection of undiluted vitreous humor samples, as well as the serum samples, was performed in a public hospital to patients submitted to PPV.

Serum samples were collected in an appropriate serum sterile tube just before the surgery and prepared to be frozen at -80°C for further analysis. Vitreous humor was collected at the beginning of the PPV (core vitrectomy). Tubes from the vitrectomy were disconnected and connected to a syringe (in coordination with vitrectomy aspiration at the beginning of the surgery). Before turning on the intravitreal infusion, an undiluted sample of vitreous was obtained by aspiration into a 2 mL syringe attached to the vitreous cutter. The sample was transferred to sterilized tubes and placed immediately on ice and frozen at -80°C until it was required for analysis. All collection procedures, either serum or vitreous humor, were performed in a room attached to the surgery room to minimize sample damage.

Additionally, patients' clinical history was investigated to re-confirm patient diagnosis, baseline characteristics and associated concomitant diseases. Patients under concomitant medications that could influence the results, such as drugs that bind to PlGF (for example: aflibercept) were removed from the study. All information about additional drugs to treat patients' eye condition even performed 3 months from vitrectomy and although not interfering with the analysis were collected and are available in table 1.

2.3 Measurement of vitreous and serum placental growth factor levels

Quantification of vitreous and serum PIGF levels was performed by ELISA kit for human samples (ABIN1379954, Assay Biotechnology, San Francisco, California, United States of America), according to the protocol specified by the manufacturer. Detection range was between 32-2000 pg/mL, and the sensitivity or the minimum detectable level was less than 32 pg/mL.

2.4 Quantitative analysis of optical coherence tomography

OCTs were evaluated to measure CRT (μm) and MV (mm^3) from DR patients through the interpretation of the macular map. The same Spectralis OCT (Heidelberg Engineering, Heidelberg, Germany) was used for the duration of the study.

2.5 Statistical analysis

Statistical analysis was performed with SPSS (Statistical Package for the Social Sciences) version 22.0 for Windows (Armonk, New York, United States of America). The Mann-Whitney test was used because variables did not have a normal distribution (analyzed with the Shapiro-Wilk test). For ease of interpretation, the values of the means and not the values of the mean orders were presented in the descriptive statistics. A level of significance (α) ≤ 0.05 was set to accept or reject the null hypothesis. Spearman's ordinal correlation coefficient was used to analyze the relationship between the quantitative variables.

2.6 Ethical Statement

The study was approved by the Institutional Review Board, Ethics Committee for Health of Centro Hospitalar de Leiria (reference - CHL-15481). All patients included in this study, which adhered to the tenets of the Declaration of Helsinki, gave their informed consent.

3. Results

3.1 Study population

This analysis included serum and vitreous samples from 38 patients. Concerning the baseline characteristics, the mean age between diabetic (n=21) and control group (n=17) was 60.00±25.35 and 68.65±9.69 years, respectively. From the total of 21 DR patients, 17 (80.9%) had PDR and the remaining 4 diabetic patients (19.1%) had NPDR. A summary of the demographic and clinical characteristics of the study population, as well as the concomitant medications and non-drug therapies performed by patients 3 months before vitrectomy are presented in Table 1.

Table 1 - Baseline demographic and clinical characteristics of subjects. Previous treatments.

	DR patients	Non-DR patients
Sample size (%)	21 (55.3%)	17 (44.7%)
Mean age±SD	60.00±25.35	68.65±9.69
Other Characteristics of DR patients		
PDR patients % (n)	80.9 % (17)	Not applicable
NPDR patients % (n)	19.1% (4)	Not applicable
Previous treatments for diabetic ocular disease performed more than 3 months from PPV and therefore from collection of samples date Laser % (n) Ranibizumab % (n) Triamcinolone acetonide % (n)	 100 % (21) 14.3% (3) 14.3% (3)	 Not applicable Not applicable Not applicable

NPDR-non-proliferative diabetic retinopathy; PDR-proliferative diabetic retinopathy; PPV-Pars Plana Vitrectomy, SD-standard deviation

3.2 Comparison of vitreous and serum placental growth factor levels between diabetic retinopathy and control group

Vitreous samples of DR patients had significantly higher concentration values of PlGF in comparison with non-diabetic patients: 70.0 ± 39.2 pg/mL vs. 46.47 ± 9.7 pg/mL, respectively (Z = -2.847, p=0.004), as seen in Figure 1.

Concerning the serum samples, DR patients had higher PlGF concentration levels than non-diabetic patients: 50.5 ± 2.18 pg/mL vs. 48.8 ± 6.0 pg/mL, (Z = -1.196, p=0.232), although this difference was not statistically significant, as seen i n Figure 1.

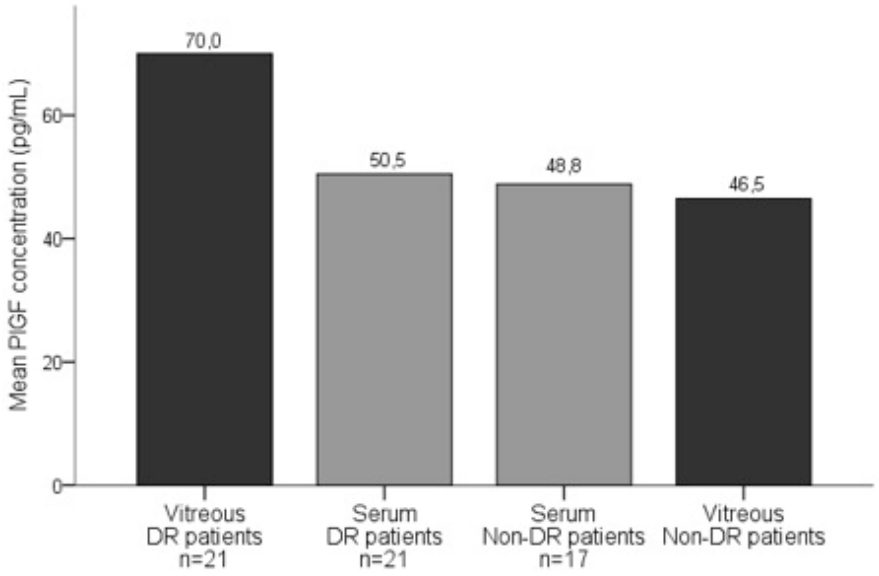

Figure 1 – Comparison of placental growth factor (PIGF) levels in vitreous and serum between diabetic retinopathy (DR) patients (n=21) and a control group of non-diabetic patients, (n=17), (p=0.004 and p=0.232, respectively), analyzed with Mann-Whitney test. DR: diabetic retinopathy; Non-DR: Non-diabetic retinopathy; PIGF: placental growth factor

3.3 Comparison of vitreous and serum placental growth factor levels between patients with proliferative diabetic retinopathy and non-proliferative diabetic retinopathy

PDR presented a significantly higher concentration value of PIGF when compared to patients with NPDR in vitreous samples: 76.5 ± 41.0 pg/mL vs. 42.5 ± 5.0 pg/mL, respectively (Z = -2.612, p=0.009), as seen in Figure 2.

Regarding serum samples, PDR patients showed PIGF concentration levels lower than NPDR patients: 50.0 ± 0.0 pg/mL vs. 52.5 ± 5.0 pg/mL, respectively (Z = -2.062, p=0.039); this difference was considered as statistically significant (Figure 2).

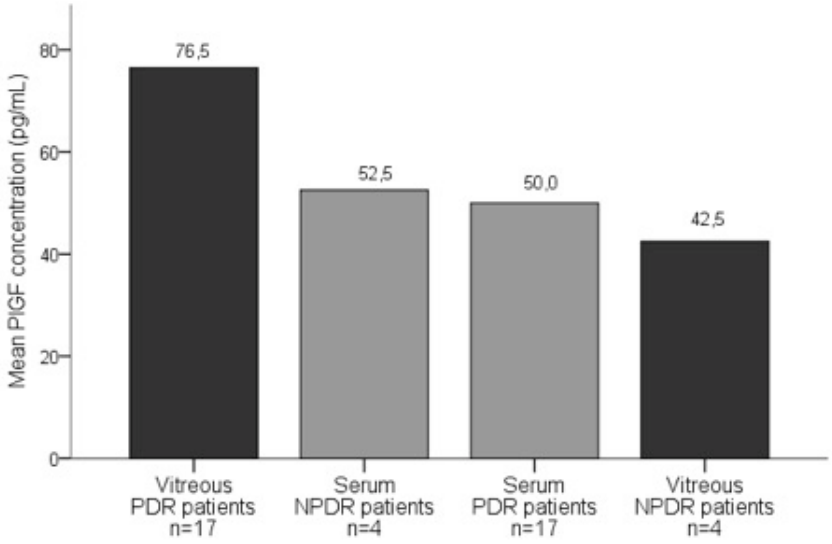

Figure 2 – Comparison of placental growth factor (PIGF) levels in vitreous and serum samples between proliferative diabetic retinopathy (PDR) patients (n=17) and non-proliferative diabetic retinopathy (NPDR) patients (n=4), (p=0.009 and p=0.039, respectively), analyzed with Mann-Whitney test.

3.4 Correlation coefficient between vitreous and serum placental growth factor samples

The correlation coefficient between vitreous and serum PIGF levels (n=38) was not statistically significant (p=0.645), and the result obtained was near zero (r_{sp} = 0.077). Moreover, the correlation coefficient of PIGF levels between vitreous and serum samples in the DR patients group (n=21) and non-diabetic patient group (n=17) was not statistically significant (p=0.614; r_{sp} = -0.117 and p=0.354; r_{sp} = 0.240, respectively).

3.5 Correlation between vitreous placental growth factor levels in diabetic retinopathy patients and quantitative measurements by optical coherence tomography

The correlation coefficient between vitreous PIGF levels from DR patients and CRT (µm) was weak (r_{sp} = 0.175, p= 0.488). Concerning the MV (mm^3), the correlation was also weak (r_{sp} = 0.288, p=0.262), however, a tendency of a positive correlation was observed between PIGF and MV (Figure 3).

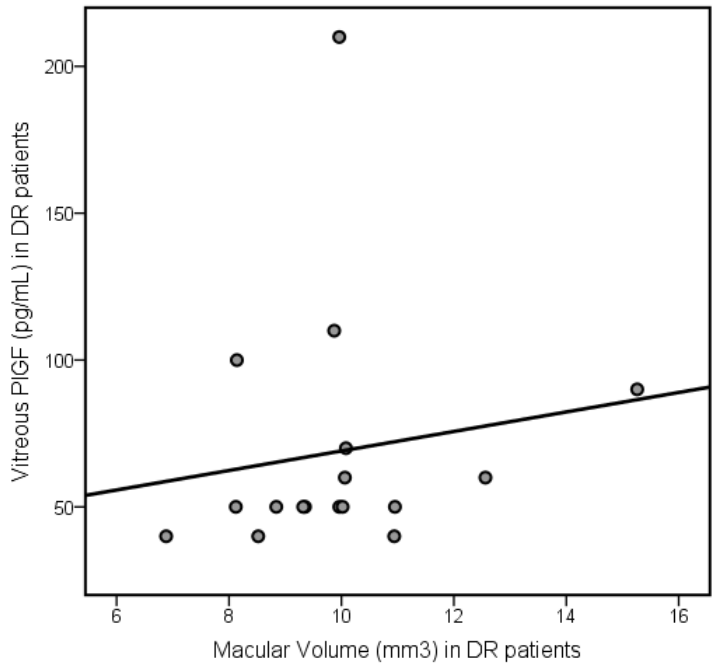

Figure 3 – Correlation between placental growth factor (PlGF) vitreous levels and macular volume (MV) (mm^3), in diabetic retinopathy (DR) (n=21) patients, analyzed with Spearman's correlation coefficient.

4. Discussion

DR is the most frequent complication of diabetes and the leading cause of blindness in the working-age population in developed countries worldwide. Most of the patients diagnosed with diabetes show clinical signs of retinopathy, approximately 15 years after the onset of diabetes, and more than 10% develop visual impairment [16].

PlGF is a growth factor important for vasculogenesis but indispensable for angiogenesis during ischemia, inflammation, wound healing, cancer and certain ocular diseases involving neovascularization. It appears to be inert in health; however, in pathological conditions, it has an active function. Oura et al. (2003) reported a critical role for PlGF in the induction of cutaneous inflammation and vascular permeability, and consequently, edema formation [17]. However, the vascular permeability reported by this molecule showed that PlGF was less potent than VEGF-A alone or VEGF-A/PlGF heterodimers. Nevertheless, it was suggested that for this cutaneous condition, inhibition of PlGF could be considered as a potential therapeutic approach acting as an anti-inflammatory drug. It must be emphasized that the under or overexpression of PlGF did not affect normal vascular development or function, suggesting that the conception of an anti-PlGF therapy would be safer than other anti-angiogenic molecules. However, concerning the role of PlGF in pathological neovascularization in cancer,

Sheibani (2013) noted that inhibition activity of PlGF may be tumor specific and not all anti-PlGF have antagonist activity due to an unknown and still to be discovered reason; if this fact is extrapolated to ocular disease, it may suggest that not all anti-PlGF may have an effective action [12].

In our study, we observed a significant increase in vitreous PlGF concentration values in DR patients when compared to a control group of non-diabetic patients. This fact confirms the studies of Khaliq et al. (1998) that found high levels of PlGF in diabetic vitreous samples, suggesting a role in the pathogenesis of PDR [18].

Additionally, the comparison of vitreous PlGF levels among DR patients, specifically between patients with PDR and NPDR, showed higher levels of PlGF in the vitreous humor of the former. Confirming our finding, Mitamura et al. (2002) also referred to significantly higher intravitreous levels of PlGF in active PDR than in quiescent PDR, suggesting involvement of this molecule in the developing stages of PDR [19]. Thus, it might be suggested that PlGF values are high in patients with DR and these values tend to increase with the severity of the disease, so an efficient PlGF antagonist would be desirable in all disease stages but indispensible for late stages.

In serum samples, there was no difference between DR and non-diabetic patients, however an interesting finding was the higher serum concentration of PlGF in NPDR patients than in PDR patients. There are several hypotheses why this may happen: 1. Concurrent systemic diseases not diagnosed or reported by the patients; 2. Different diabetic therapies may interfere with the obtained serum PlGF values; 3. Systemic levels may not be related to the production of vitreous cytokines by the retina [20].

Another interesting revelation was the lack of correlation between vitreous and serum samples of PlGF, which may indicate dissociation between the eye and other systems. This finding in PlGF was similar to the ones regarding the lack of serum vs. vitreous correlation between VEGF-A and VEGF-B, suggesting that the administration of a drug intravitreally may have minimally effects systemically [5,21]. Moreover this dissociation maybe the result of an intravitreal synthesis of PlGF.

PlGF plays an important role in neovascularization; it is found to be increased in the vitreous of DR patients. In addition, its levels were higher in more advanced disease states. Therefore, targeting PlGF should offer an additional treatment strategy for ocular pathologies with a neovascular component.

Treatment with anti-angiogenic agents for the ocular pathologies arose a few years ago, initially with pegaptanib, then bevacizumab (off-label) followed by ranibizumab and aflibercept intravitreal injections. These anti-angiogenic therapies rapidly became the gold standard for the treatment of neovascular eye diseases. The efficacy of the new drugs inhibiting VEGF, and consequently angiogenesis, is not in debate. However, in order to

improve the outcomes in treating those pathologies, avoid resistance and minimize toxicity, particularly due to the lack of available of long-term safety data of VEGF inhibition and possible side effects that may occur, additional therapeutic agents should be investigated. In light of this, the inhibition of PlGF is a possible intervention once PlGF regulates angiogenesis and vascular permeability in pathological conditions, and deletion of PlGF leads to the suppression of diabetic complications [22]. Also, according to Mitamura et al. (2002), PlGF acts indirectly by potentiating the activity of VEGF in pathological angiogenesis, suggesting the cooperative role of these two molecules in the progression of DR [19].

Additionally, not all anti-PlGF antibodies are functional and demonstrate antagonistic activity [8]. Several anti-PlGF have been tested for cancer treatment. Van de Veire and colleagues (2010) demonstrated in an animal experiment that the monoclonal antibody 5D11D4, which specifically targets PlGF, inhibits choroidal neovascularization, ocular angiogenesis, and inflammation by blocking PlGF [14]. Another advantage is that 5D11D4 do not have to be injected directly into the eye, as in the case of other VEGF inhibitors, which are too toxic for systemic administration. Therefore, this avoids the increased risk of adverse events that may occur due to the need of frequent intravitreal administrations.

TB-403 or THR 317 is another monoclonal antibody that binds to PlGF and blocks the binding to VEGFR-1. The results of the phase I clinical trials showed that TB403 was well tolerated without increased risk of adverse effects in healthy volunteers and terminal cancer patients [14,23]. The phase II study in pediatric subjects with relapsed or refractory medulloblastoma is currently recruiting, and the results are expected by December 2017 at the time of primary end point completion (clinicaltrials.gov) [5,24]. This anti-PlGF monoclonal antibody is in Phase II for diabetic macular edema and it is at preclinical stage for diabetic retinopathy (http://adisinsight.springer.com/drugs/800019736) [5,25], thus new efficacy and safety outcomes are expected.

Clinical trials are needed to assess and confirm the efficacy and safety of monoclonal antibodies against PlGF in the treatment of ocular pathologies.

In contrast to the essential role of VEGF-A in physiological and pathological angiogenesis, the role of PlGF is restricted to pathological conditions being considered for this reason a specific target for therapy. Of all evidence presented here, PlGF may represent a preferred target for the inhibition of angiogenesis with respect to VEGF-A. The safety profile of anti-PlGF needs preclinical studies and human clinical trials to confirm the possibility of systemic delivery of anti-PlGF monoclonal antibodies and also its effectiveness and safety, in combination with an anti-VEGF therapy or as a partial replacement of anti-VEGF.

A better and deep understanding of all mechanisms, interactions and specific functions between the members of the VEGF family and its receptors, in both normal states and diseases is needed. Also, new experimental and clinical trials targeting therapies against PlGF

will allow development of more specific, precise, safe and effective therapies, resulting in better outcomes for patients suffering from diseases with pathological angiogenesis.

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Paper IV

Evaluation of the growth factors VEGF-A and VEGF-B in the vitreous and serum of patients with macular and retinal vascular diseases

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Submitted for publication

Evaluation of the growth factors VEGF-A and VEGF-B in the vitreous and serum of patients with macular and retinal vascular diseases

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Abstract

VEGF-A and VEGF-B are proangiogenic factors and key regulators of blood vessel growth. This study aimed to evaluate serum and vitreous VEGF-A and VEGF-B levels in patients with neovascular pathology vs. a control group with non-neovascular pathology. Our findings showed that vitreous VEGF-A and VEGF-B are consistently overexpressed in patients with neovascular disease, with higher levels of expression of VEGF-A compared to VEGF-B ($p \leq 0.05$). In the neovascular group, higher vitreous VEGF-A or VEGF-B levels were found in proliferative diabetic retinopathy (PDR) than in non-PDR. An important finding was the strong correlation found between VEGF-A vs. VEGF-B that suggests a simultaneous pathological increase in those cytokines ($p<0.001$). There was no correlation between vitreous vs. serum VEGF-A or VEGF-B. However there was a correlation between vitreous (VEGF-A or VEGF-B) vs. macular volume ($p<0.05$) in DR patients. Targeting VEGF-A and VEGF-B in macular and retinal vascular diseases, involving neovascularization, may improve treatment outcomes.

Keywords: ELISA, neovascular, retinal diseases, VEGF-A, VEGF-B, vitreous humor

Introduction

Angiogenesis is one of the mechanisms responsible for neovascularization, in which new blood vessels sprout from the pre-existing (Roy, Bhardwaj & Ylä-Herttuala 2006). Neovascularization is a crucial process for embryonic development and several pathological states. While angiogenesis is a normal and an essential physiological process, it is also a pathological process involved in tumor growth, rheumatoid arthritis, psoriasis and neovascular ocular diseases such as age-related macular degeneration (AMD), retinal vein occlusion (RVO) and diabetic retinopathy (DR) (Ferrara, Gerber & LeCouter 2003). The resulting proliferation often ends in visual loss (Mesquita et al., 2017a).

The vascular endothelial growth factor (VEGF) family consists of seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and placental growth factor (PlGF) (Li et al., 2009). Alternative splicing results in several VEGF variants (Penn et al., 2008).

VEGF-A is implicated in the angiogenesis of pathological eye conditions and is, thus, a highly significant therapeutic target (Witmer, 2003). Carmeliet et al. (1996) and Ferrara et al (1996) showed the essential role of VEGF-A in embryonic vasculogenesis and angiogenesis. VEGF-A induces an angiogenic response. It also plays a significant role in inflammation due to its capacity to increase vascular permeability and induce vascular leakage (Amadio, Govoni & Pascale, 2016). VEGF-A exists in four different isoforms formed due to alternative exon splicing: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$; $VEGF_{165}$ is the most predominant isoform (Houck et al., 1991). VEGF-A binds to two receptor tyrosine kinases, i.e., VEGF receptor 1 (VEGFR-1) and VEGF receptor 2 (VEGFR-2), and also binds to neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2). VEGFR-1 and VEGFR-2 regulate physiological and pathological angiogenesis and are responsible for signaling angiogenesis (Shibuya, 2006). Binding to VEGFR-1 promotes tumor growth, metastasis, and inflammation (Shibuya, 2006).

Contrarily, VEGF-B, a closely related molecule to VEGF-A, is one of the least studied members and the most controversial protein of the VEGF family (Li et al., 2009). The biological function of VEGF-B is enigmatic. Li and co-workers (2012) referred to VEGF-B as a multifunctional safeguarding molecule having a functional ambiguity role. VEGF-B plays a specific role in angiogenesis during the repair process after brain injury and myocardium revascularization after myocardial infarct (Li et al., 2008; Nag et al., 2002). In vivo, VEGF-B targeting inhibited choroidal and retinal neovascularization (Zhang et al., 2009). The 'angiogenic' activity of VEGF-B during ocular neovascularization is due to its vascular survival effect rather than a genuine angiogenic effect, mediated by both NPR-1 and VEGFR-1, which safeguards the neovessels from apoptosis (Zhang et al., 2009). Therefore, direct VEGF-B inhibition may be a potential therapy for the treatment of ocular neovascular diseases (Zhang et al., 2009). Alternative splicing of exon 6 resulted in two isoforms of VEGF-B: $VEGF\text{-}B_{167}$ and $VEGF\text{-}B_{186}$. VEGF-B binds with high affinity to VEGFR-1 but not to VEGFR-2 or VEGF receptor 3

(VEGFR-3), competing with VEGF-A for VEGFR-1 binding (Olofsson et al., 1998). This selective binding is due to electrostatic surface potentials (Iyer, Darley & Acharya, 2010).

Moreover, VEGF-A may also generate heterodimers with VEGF-B, affecting the formation of VEGF-A homodímers and bioavailability of VEGF-A to bind to its receptors and exert its activity (Olofsson et al., 1996).

Evidence shows that patients with retinal diseases, such as DR, AMD, and RVO, have increased levels of vitreous VEGF-A (Aiello et al., 1994). Our research group has shown increased vitreous levels of VEGF-B in DR and its association with disease severity (Mesquita et al., 2017b).

The main role of VEGF-A and VEGF-B in both normal and pathological angiogenesis, including the diverse isoforms and receptors, is beginning to be clarified. With the growing interest in the use of anti-angiogenics in the treatment of different diseases related to anomalous angiogenesis, inflammation, and vessel hyperpermeability, more consideration must be paid to understand the accurate roles of the molecules of the VEGF family in vascular pathologies (Li et al., 2009).

In the present study, we investigated two cytokines (VEGF-A and VEGF-B) in vitreous and serum associated to neovascular eye pathologies: DR, AMD and RVO. Quantitative results of VEGF-A and –B and baseline clinical characteristics were then compared between 2 groups of patients (neovascular vs. non-neovascular). For further exploration, the serum and vitreous concentration levels of VEGF-A or VEGF-B were correlated between each other and between neovascular vs. non-neovascular groups with respect to neovascular pathologies, stage of diabetic retinopathy for diabetic patients, previous treatments and changes assessed by optical coherence tomography (OCT) and visual acuity (VA) measurements. A better knowledge of these cytokines and their correlation can lead us to better-targeted precise and specific therapies. This may also lead to early intervention in disease prevention through detention of ocular neovascularization before the appearance of clinical symptoms, subsequently leading to the prevention of blindness.

Materials and Methods

Participants

This retrospective study involved 20 patients divided in two groups: (1) A neovascular group (n=17) comprising RVO (n=2), AMD (n=2) and DR (n=13) patients and (2) A non-neovascular group (or control group), which included patients with vitreomacular traction (VMT) syndrome (n=3) of idiopathic etiology but without any other ocular disorder (including neovascular disease).

The neovascular ocular disease was defined for this study as a retinal disease associated with abnormal blood vessel growth, which may lead to blindness however, may differ in the local of neovascularization.

The DR group was classified into patients with proliferative diabetic retinopathy (PDR) (n=8) and non-proliferative diabetic retinopathy (NPDR) (n=5).

For the DR group, the diabetes mellitus duration was defined as the duration from the first diagnosis of diabetes mellitus to the time of vitrectomy and sampling collection. None of the patient samples selected to be studied performed either pharmacological (intravitreal injections) or laser treatments within the 3 months of vitrectomy. Moreover, diabetic therapy remained unchanged for at least 3 months before vitrectomy.

A treatment naïve patient was defined as a patient that has never been treated before with any drug or non-drug therapy to treat their ocular disease, including laser photocoagulation, bevacizumab, ranibizumab, aflibercept or triamcinolone.

Only one eye of each patient was studied.

Collection of samples and storage

Whole blood samples were collected from patients before the surgery. After allowing the blood to clot at room temperature for 30 minutes, the clot was removed by centrifugation at 3,000 rpm for 10 minutes. After serum withdrawal, it was immediately frozen at -80 °C. Each patient underwent Pars Plana Vitrectomy (PPV) for treatment of the current disease. The undiluted vitreous samples were collected at the beginning of the PPV in sterilized tubes, placed immediately on ice and frozen at -80 °C until it was required for analysis.

The inclusion criteria for the selection of samples were as follows:

Patients with a confirmed diagnosis of neovascular ocular disease (for the neovascular group);

For the control group, nondiabetic patients with no PDR and no signs of neovascularization, retinal vascular occlusion or other ocular disorder, except VMT syndrome, were included;

4. For the diabetic population, all samples selected included patients with diabetic treatments stable or unchanged for at least 3 months before the vitrectomy procedure;

Patients with a sample volume that would allow the confirmation of the results through repeated enzyme-linked immunosorbent assay (ELISA) for the determination of VEGF-A and VEGF-B levels simultaneously in the same patient sample, both in vitreous and in serum.

Patient samples were excluded from the analysis if:

The patients performed any intravitreal treatment (with anti-VEGFs and/or corticosteroids) or laser therapy less than 3 months of vitrectomy.

Collection of retrospective data from patients

Clinical history of the patients was collected to confirm the diagnosis and concurrent diseases as well as baseline and clinical characteristics. Despite the exclusion of patients from the study, information on previous concomitant treatments administered for their eye condition was collected.

Measurement of vitreous and serum VEGF-A and VEGF-B levels

ELISA was performed to measure the vitreous and serum levels of VEGF-B (VEGF-B ELISA Kit; Ref, E-EL-H2164; Elabscience; Wuhan, Hubei, China) and VEGF-A (VEGF-A ELISA Kit; Ref, E-EL-H0111; Elabscience; Wuhan, Hubei, China). Both analyses were conducted in accordance with the manufacturer instructions.

Measurement of structural parameters

OCT examinations were performed before PPV using the Heidelberg equipment (Heidelberg Engineering, Heidelberg, Germany). Thickness mapping of retinal layers was used to measure central retinal thickness (CRT) and macular volume (MV) associated with retinal pathologies.

Measurement of functional parameters

Best Corrected Visual Acuity (BCVA) was measured with early treatment diabetic retinopathy study (ETDRS) charts (Precision Vision, La Salle, Illinois, United States of America) at 4-meters.

Statistical analysis

Statistical analysis was performed using IBM SPSS version 24.0 (IBM Corp., Armonk, New York, United States of America) for Windows. A level of significance (α) ≤ 0.05 was set to reject the null hypothesis. Pearson test was used to correlate quantitative variables with a normal distribution while Spearman's correlation coefficient test was used to correlate quantitative variables without normal distribution. The Mann-Whitney was used to compare two groups in quantitative dependent variables, without normal distribution.

Ethical Statement

The study was approved by the Institution Review Board, named Ethics Committee for Health of Centro Hospitalar de Leiria (CHL-15481) and Portuguese National Data Protection Commission. All patients included in this study, which adhered to the tenets of the Declaration of Helsinki, gave their informed consent after appropriate explanation.

Results

Study Population – baseline characteristics

In this retrospective study, samples from 20 patients (5 women and 15 men) with a mean age of 70.3 years were selected to be analyzed based on specific inclusion and exclusion criteria. Two of the patients with RVO submitted to PPV surgery had active neovascularization. From the 2 AMD patients included, one had active neovascularization and the other had reminiscent neovascularization at the time of surgery, confirmed by fluorescein angiography. The patient presented with reminiscent neovascularization was previously treated (despite more than 3

months before surgery) with 8 bevacizumab intravitreal injections (before approval of the license drug ranibizumab), 4 ranibizumab injections (after approval of ranibizumab by health authorities) and 5 aflibercept injections.

Moreover from the 13 diabetic patients analyzed: 8 had PDR and 5 had NPDR.

The majority of the diabetic patients had DM type 2, with exception of 1 patient who had DM type 1. This patient (with DM type 1) had PDR and was under insulin treatment to control diabetes. The other 12 patients with DM type 2 were under insulin therapy (n=2); oral therapy (n=8) or both insulin and oral therapy (n=2).

Concerning the previous treatments performed by the patients (3 months before the PPV), in the neovascular group, 8 patients were naïve to treatment: 1 RVO, 1 AMD and 6 DR (2 patients had PDR and 4 had NPDR), and 9 patients were non-naïve to treatments. The non-naïve patients include 1 RVO, 1 AMD and 7 DR patients (6 patients with PDR and 1 with NPDR). The control group did not have any ocular neovascular pathology besides VMT syndrome.

Characterization of the baseline demographic and clinical characteristics are summarized in Table 1.

Table 1 - Patient baseline clinical and demographic characteristics

	Total (n=20)	RVO (n=2)	AMD (n=2)	DR (n=13)	Control group (n=3)
Demography Age (yrs) (Mean, SD)	70.3 ± 10.7	78.0 ± 5.6	77.0 ± 7.0	68 ± 11.9	71.0 ± 6.2
Other variables					
BCVA (ETDRS letters) at baseline (Mean, SD)	-	65.0 ±1.5	48.0 ± 3.5	62.1 ± 19.2	57.5 ± 3.5
CRT at baseline (Mean, SD)	-	328.5 ± 30.4	480.5 ± 140.7	413.8 ± 65.7	-
MV at baseline (Mean, SD)	-	8.07 ± 0.06	11.79 ± 3.08	9.59 ± 1.67	-
Diabetes Mellitus Type (n; %) in DR patients group					
DM 1 (n, %)	-	-	-	1, 8.3%	-
DM 2 (n, %)	-	-	-	12, 91.7 %	-
Diabetes treatment (n; %) stable for at least 3 months before vitrectomy procedure					
Insulin therapy	-	-	-	3 27.3	-
Oral therapy	-	-	-	8 54.5	-
Insulin and oral therapy	-	-	-	2 18.2	-
Other DR characteristics					
Duration of DR (yrs) (Mean, SD)	-	-	-	1.6 ± 1.5	-
PDR (n; %)	-	-	-	8, 38.5	-
NPDR (n; %)	-	-	-	5, 61.5	-

Previous treatments for ocular condition (performed more than 3 months before vitrectomy)					
Laser photocoagulation (n)	-	1	0	7	-
Bevacizumab (n)	-	0	1	0	-
Ranibizumab (n)	-	0	1	3	-
Aflibercept (n)	-	0	1	0	-
Triamcinolone (n)	-	0	0	2	-

VEGF-A - vascular endothelial growth factor A; VEGF-B - vascular endothelial growth factor B; BCVA – best corrected visual acuity; CRT – central retinal thickness; MV – macular volume; RVO – retinal vein occlusion; AMD – age macular degeneration; DR – diabetic retinopathy; PDR – proliferative diabetic retinopathy; NPDR – non-proliferative diabetic retinopathy; DM1 – diabetes mellitus type 1; DM2 – diabetes mellitus type 2; Control group – patients with vitreomacular traction syndrome of idiopathic etiology and without other ocular disorders; yrs – years; SD – standard deviation

After characterization, this pool of patients was re-grouped into the neovascular group and non-neovascular group as defined previously.

Comparison of vitreous and serum VEGF-A and VEGF-B levels between patients with neovascular disease vs. non-neovascular disease

A statistically significant difference was observed between vitreous VEGF–A in the neovascular group (343.30 ±543.82pg/mL), (mean ± standard deviation) vs. vitreous VEGF-A in the non-neovascular group (7.04 ± 1.35 pg/mL), (Z=-1.958, p=0.050). Likewise, the mean difference of vitreous VEGF–B concentration was significantly higher in the neovascular group (208.83 ± 353.58 pg/mL) vs. control group of non-neovascular disease (1.94 ± 0.61 pg/mL) (Z=-2.170, p=0.030) (Figure 1).

Regarding the analysis of the serum, there were differences between the two groups (neovascular and non-neovascular), although these differences were not statistically significant (Figure 1).

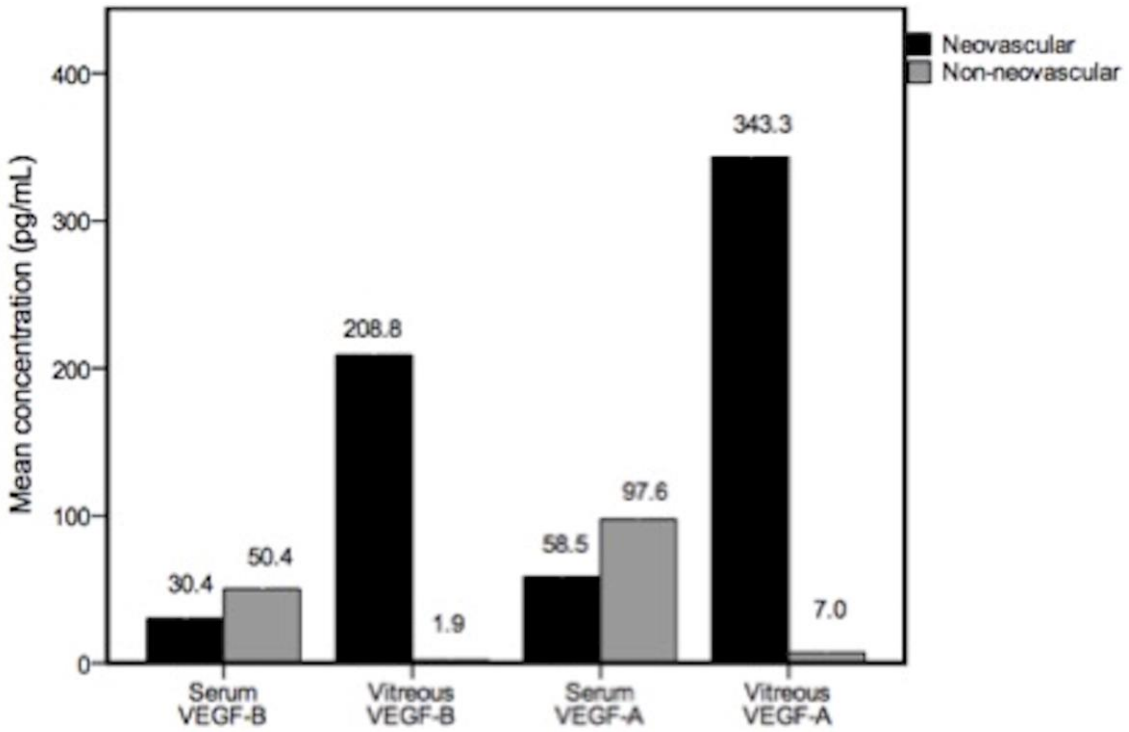

Figure 1 - Mean serum and vitreous VEGF-A and VEGF-B concentrations between the neovascular (n=17) and non-neovascular (n=3) study arm, analyzed with Mann–Whitney test (p<0.05).

Comparison of VEGF-A and VEGF-B levels in vitreous and serum of studied pathologies

The comparison between vitreous VEGF-A and vitreous VEGF-B levels showed increased levels in both DR and RVO patients. AMD patients revealed lower vitreous levels of both cytokines (VEGF-A and VEGF-B), suggesting that this result is due to one patient that has reminiscent neovascularization (Figure 2).

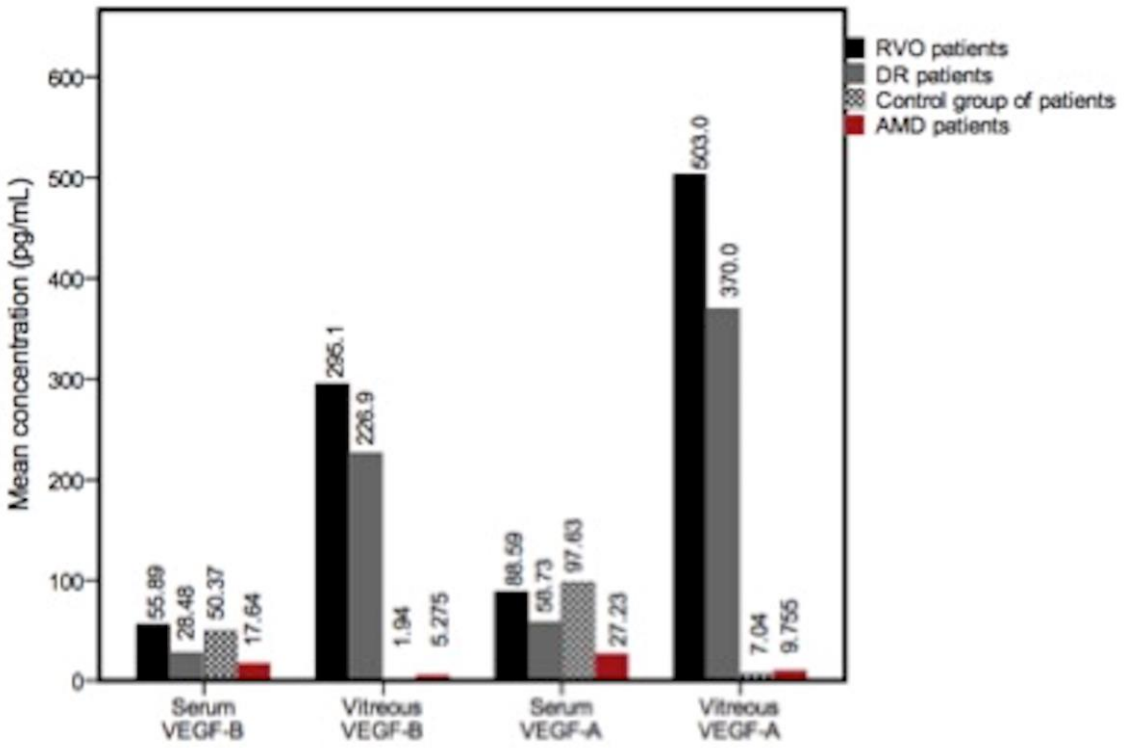

Figure 2 - Mean vitreous and serum levels of VEGF-A and VEGF-B among different studied pathologies vs. control group of patients, analyzed with Mann-Whitney test ($p>0.05$). VEGF-A – vascular endothelial growth factor; VEGF-B – vascular endothelial growth factor B; RVO – retinal vein occlusion (n=2); DR – diabetic retinopathy (n=13); AMD – age macular degeneration (n=2). The control group included patients with VMT syndrome with no other ocular disorders (n=3).

Serum levels of VEGF-A and VEGF-B did not reveal any significant difference between pathologies and control group.

Comparison of vitreous and serum levels of VEGF-A and VEGF-B between PDR and NPDR patients

Analysis of the DR patients (PDR vs. NPDR patients) revealed statistical differences in VEGF-A and VEGF-B levels: a) Vitreous VEGF-A levels were higher in PDR (576.88 ± 681.15 pg/mL) than in NDPR (39.11 ± 71.13 pg/mL), Z=-2.342, p=0.019; b) Vitreous VEGF-B levels were also higher in PDR (357.55 ± 453.22 pg/mL) than in NDPR (17.75 ± 34.92 pg/mL), Z=-2.342, p=0.019; c) Serum VEGF-A levels were increased in NPDR (95.08 ± 47.37 pg/mL) compared to PDR (36.02 ± 28.05pg/mL), Z=-2.196, p=0.028; d) Serum VEGF-B levels were higher in NPDR (46.38 ± 22.35 pg/mL) than in PDR (17.29 ± 14.74 pg/mL), Z=-2.049, p=0.040 (as seen in Figure 3).

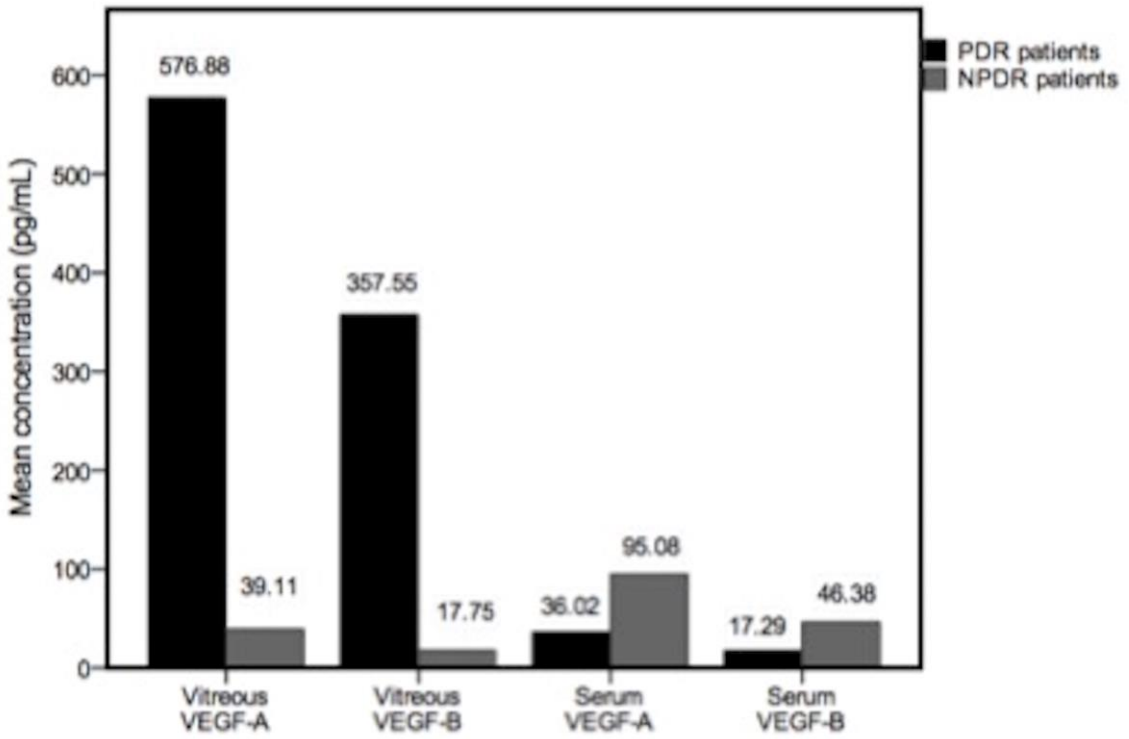

Figure 3 - Comparison of the mean vitreous and serum levels of VEGF-A and VEGF-B between NPDR (n=5) and PDR (n=8), analyzed with Mann-Whitney test (p<0.05). PDR – proliferative diabetic retinopathy, NPDR - non-proliferative diabetic retinopathy.

Comparison of vitreous VEGF-A vs. VEGF-B levels between naïve and non-naïve patients with neovascular pathology

For this analysis, as defined previously, a non-naïve was a patient that never underwent any drug or non-drug therapy to treat their ocular disease, including laser photocoagulation, bevacizumab, ranibizumab, aflibercept or triamcinolone.

Although the analyzed patients had performed intravitreal treatments and /or laser therapy 3 months before vitrectomy, a comparison of the mean values between naïve (n=8) and non-naïve (n=9) patients in the neovascular group (n=17) demonstrated a statistically significant difference (p<0.05) between vitreous VEGF-A and VEGF-B levels with higher levels of vitreous VEGF-A and VEGF-B in the group of non-naïve patients (Table 2).

Table 2 - Comparison of the mean concentration levels of vitreous VEGF-A and vitreous VEGF-B in the neovascular group between naïve and non-naïve patients ($p<0.05$).

	Patients in the neovascular group (n=17)					
	Non-naïve (n=9)		Naïve (n=8)		Z	p
	Mean	SD	Mean	SD		
Vitreous VEGF-B	321.48	441.11	82.09	169.42	-1.925	0.054
Vitreous VEGF-A	516.26	665.50	148.71	296.79	-2.021	0.043

VEGF-A – vascular endothelial growth factor A, VEGF-B – vascular endothelial growth factor B, SD – Standard deviation

Analyze of vitreous VEGF-A and VEGF-B levels in the group of patients with neovascular disease per treatment group

For this analysis, we grouped the non-naïve neovascular patients (n=9) per treatment groups into 5 groups, describing the treatments performed since they were diagnosed until vitrectomy (note that the selected samples for the neovascular group did not perform any treatment 3 months before the surgery): Group A – patients who performed only laser; Group B – patient who performed bevacizumab, ranibizumab and aflibercept; Group C – patients who performed ranibizumab and laser; Group D – patients who performed laser and triamcinolone; Group E – patients who performed laser, triamcinolona and ranibizumab.

Overall, the results revealed high vitreous levels of the either growth factors as seen in Figure 4.

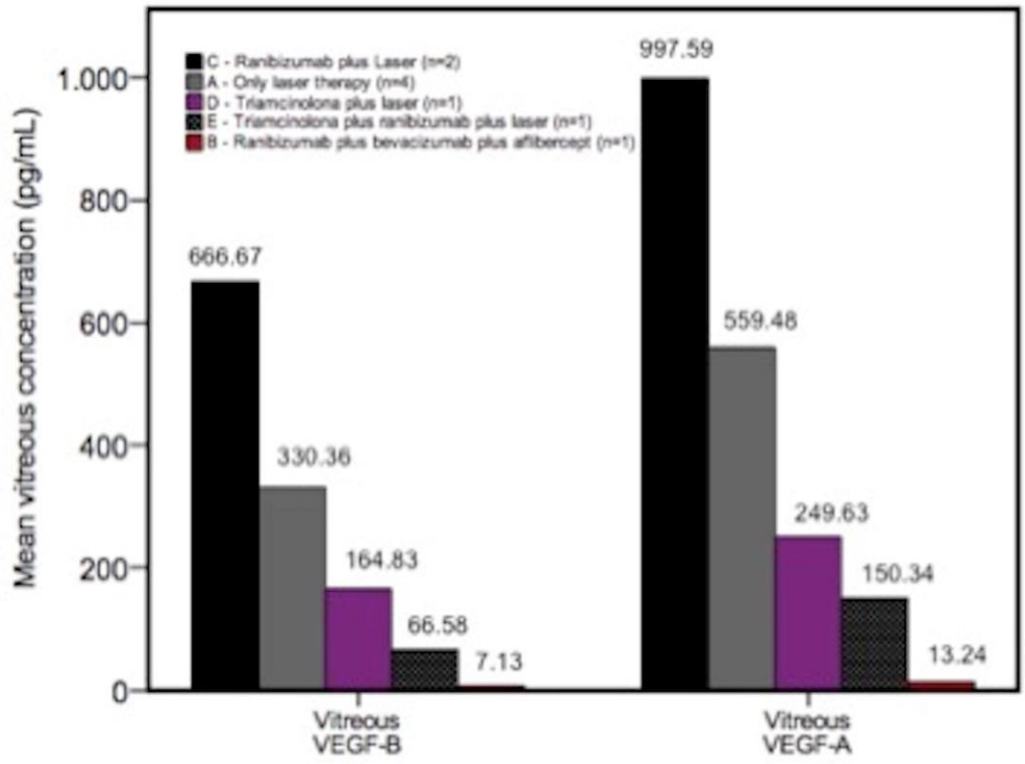

Figure 4 - Mean vitreous concentration levels of VEGF-A and VEGF-B among the 5 treatment groups in the neovascular patients group (non-naïve patients, n=9), using descriptive analysis. VEGF-A – vascular endothelial growth factor; VEGF-B – vascular endothelial growth factor B; Group A – Patients that only performed laser treatment; Group B - Patients that performed either or, ranibizumab, bevacizumab, aflibercept; Group C – Patients treated with ranibizumab plus laser; Group D – Patients treated with triamcinolone and laser; Group E – patients treated with ranibizumab plus triamcinolone plus laser; SD – standard deviation.

Correlation between vitreous VEGF-A vs. vitreous VEGF-B and serum VEGF-A vs. serum VEGF-B in the neovascular group of patients

There was a statistically significant, positive and strong correlation between vitreous VEGF-A vs. vitreous VEGF-B in patients with neovascular pathology (n=17), i.e., r_{sp}=0.983, p<0.001. Likewise, there was a positive and strong correlation between serum VEGF-A vs. serum VEGF-B (*r*=0.970, p<0.001) in the neovascular group (Figure 5).

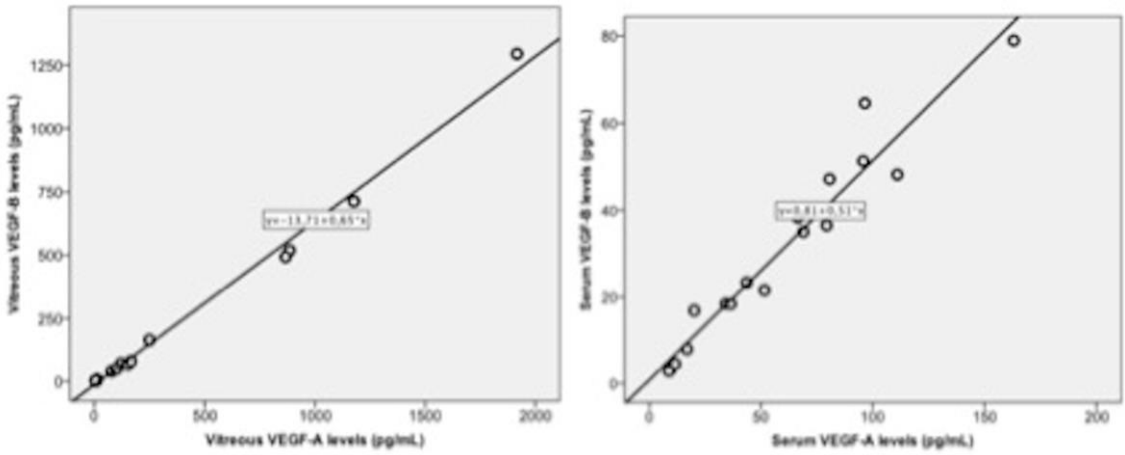

Figure 5 - Correlation between a) vitreous VEGF-A vs. vitreous VEGF-B and b) serum VEGF-A vs. serum VEGF-B (in patients with neovascular pathology, n=17), analyzed by Spearman's correlation (in vitreous samples) and by Pearson correlation coefficient (in serum samples) ($p<0.001$).

Similarly, the correlation coefficient between VEGF-A and VEGF-B in vitreous and serum for DR patients (n=13) was statistically significant, positive and strong; $r_{sp}=0.984$, $p<0.001$ and $r_{sp}=0.973$, $p<0.001$, respectively (Figure 6).

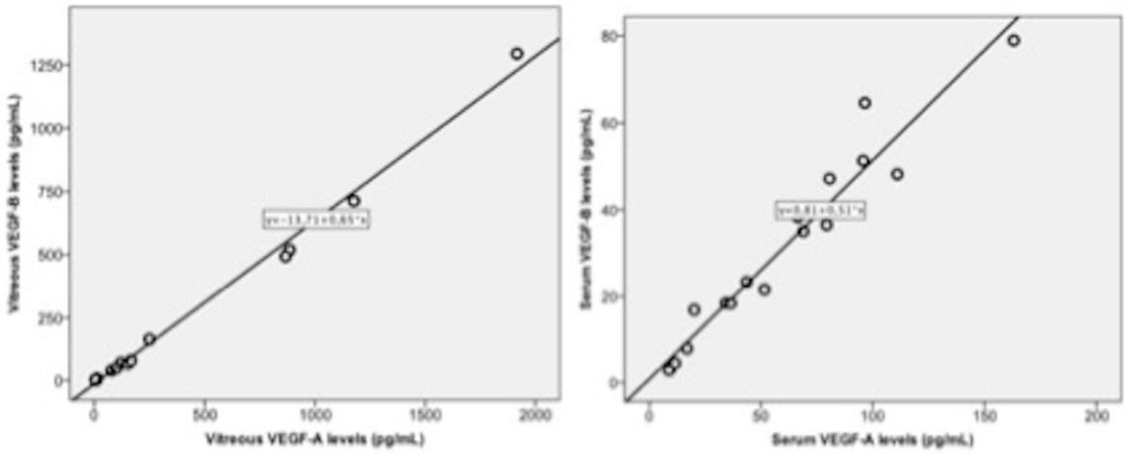

Figure 6 - Correlation between: a) vitreous VEGF-A vs. vitreous VEGF-B and b) serum VEGF-A vs. serum VEGF-B, in DR patients, (n=13), analyzed by Spearman's correlation coefficient ($p<0.001$).

Correlation between vitreous vs. serum VEGF-A and between vitreous vs. serum VEGF-B in the neovascular group of patients

The correlation coefficient between vitreous VEGF-A vs. serum VEGF-A for the neovascular group was not statistically significant (n=17; $r_{sp}=-0.051$; $p=0.844$). Also, there was no correlation between vitreous VEGF-A vs. serum VEGF-A in the DR patients group (n=13; $r_{sp}= -0.209$; $p=0.494$).

Similarly, the correlation coefficient between vitreous VEGF-B vs. serum VEGF-B levels was not statistically significant for neovascular patients' population (n=17; r_{sp}= 0.027; p=0.918). Relatively to DR patients, there also was no correlation between vitreous VEGF-B and serum VEGF-B (n=13; r_{sp} = - 0.110; p=0.721).

Correlations of vitreous VEGF-A or vitreous VEGF-B vs. structural and functional parameters in DR patients

In the DR patients group, a statistically significant, positive and moderate correlation was observed between vitreous VEGF-A and MV (r_{sp}=0.560, p=0.046) and between vitreous VEGF-B and MV (r_{sp}=0.588, p=0.035). No statistical significant correlation was found either for the visual acuity or CRT (Table 3).

Table 3 - Correlations between structural (CRT and MV), functional parameters (VA) vs. concentration levels of vitreous VEGF A and B in a group of DR patients (n=13), analyzed by Spearman's correlation coefficient (p<0.05).

	CRT	MV	BCVA
Vitreous VEGF-B levels (pg/mL)	0.008	0.588*	-0.103
Vitreous VEGF-A levels (pg/mL)	-0.041	0.560*	-0.118

*$P \leq 0.05$

VEGF-A - vascular endothelial growth factor A; VEGF-B - vascular endothelial growth factor; CRT – central retinal thickness; MV – macular volume; BCVA – best corrected visual acuity

Discussion

VEGF-A is the prototype angiogenic factor and is a mediator of angiogenesis and vascular permeability in inflammatory disorders (Amadio, Govoni & Pascale, 2016). VEGF-B, unlike VEGF-A, does not play a significant role in angiogenesis or vascular permeability (Li et al., 2012). Its role is to be a survival factor in pathological processes in several body systems, such as the eye, brain, heart and others (Zhang et al., 2009).

Our results demonstrated that vitreous VEGF-A and VEGF-B are increased in patients with neovascular diseases in comparison with a control group of patients with non-neovascular diseases. The pathologies with higher vitreous concentrations of VEGF-A and VEGF-B were RVO and DR, supporting the assumptions of these cytokines as important

contributors for the pathogenesis of neovascular eye diseases and their increased levels in the presence of active neovascularization (Li et al., 2009).

Although an increased concentration of these cytokines was expected in AMD patients, those results were not observed. This was probably due the fact that one AMD patient was in a reminiscent phase of the disease, despite being treated more than 3 months before the PPV, providing an explanation for the observed low vitreous VEGF-A and VEGF-B levels in this group of patients. Moreover, the greater increase of VEGF-A levels in comparison with VEGF-B levels demonstrated that the development of new drugs are in the right direction when inhibiting this growth factor for the treatment of neovascular pathologies. Nevertheless, the combined inhibition of VEGF-A with a drug specifically targeting VEGF-B should be beneficial due to the apoptotic proprieties of the growth factor B (Li et al., 2012).

Relatively to the analysis of diabetic patients in different stages of DR, the results of our investigation demonstrated statistically significant and high values of vitreous VEGF-A and VEGF-B in PDR patients. Similarly, Funatsu et al. (2002), Gao et al. (2016) and Watanabe et al. (2005), and showed a significantly higher concentration of vitreous VEGF-A in eyes with active PDR.

Intriguingly, the results obtained with serum samples in the DR patients showed higher VEGF-A and VEGF-B levels in NPDR than in PDR. Serum VEGF-A levels have been determined in other studies without any significant differences between PDR and control groups or between PDR and NPDR patients, however revealing higher serum values for NPDR in comparison with PDR (Burgos et al., 1997; Hernandez et al., 2001; Krizova et al., 2015; Lee, Chae & Kim, 2005; Praidou et al., 2009; Simó et al., 2002). Nevertheless, Baharivand and colleagues (2012) demonstrated statistically significant differences between PDR and NPDR patients and contrarily to reports from other investigators, serum PDR levels were higher than NPDR. However, while Lee, Chae and Kim (2005) attributed the progression of DR not only to identified factors, cells and modulators but also to the circulating systemic VEGF-A, Burgos et al. (1997) considered intravitreous levels of VEGF-A in PDR patients to be due to intraocular synthesis, excluding the mechanisms of serum diffusion.

There is a hypothesis that the high serum concentration levels in the NPDR are due to unknown concurrent systemic diseases. It is known that several concurrent diseases besides cancer may alter serum VEGF-A and VEGF-B values, especially in the analyzed aged population (Ferrara, Gerber & LeCouter, 2003). Another hypothesis is due to diabetic therapy, the stage of DR or simply due to the fact that systemic levels of VEGF-A or VEGF-B were not related to the production of those vitreous cytokines by the retina. Baharivand and co-workers (2012) showed that serum VEGF-A levels are lower in diabetic patients with oral therapy, well-controlled diabetes, and early stages of DR, which in another case may partially explain these results. Moreover, Burgos et al. (1997) and Funatsu et al. (2002) suggested that the intraocular synthesis of VEGF, and not serum diffusion, was the principal contributing factor for the high VEGF-A levels observed in the vitreous humor of PDR patients.

Most of the research reports from several investigators demonstrated increased levels of vitreous VEGF-A in PDR in comparison with a control group. However, the serum levels results were described by some investigators with no serum differences among distinctive groups of diabetic patients (Aiello et al., 1994; Burgos et al., 1997; Hernandez et al., 2001, 2002; Krizova et al., 2015; Praidou et al., 2009; Simó et al., 2002).

There is evidence that intravitreal levels of VEGF-B are increased in diabetic patients and vary dependently with disease severity from NPDR to PDR (Mesquita et al., 2017b). In the present study, higher vitreous VEGF-A and VEGF-B were associated with an increase in severity of DR.

The analysis performed in the neovascular group showed that non-naïve patients had higher levels of vitreous VEGF-A and VEGF-B. This may be because there are more PDR patients in the non-naïve group, confirming our supposition that vitreous levels of the studied growth factors are related with disease severity, being higher in more developed stages.

The comparison of vitreous VEGF-A and vitreous VEGF-B (in non-naïve patients) with the 5 treatment groups in the neovascular population suggested the existence of treatment recurrence. Anti-VEGFs are the standard gold treatment for neovascular and potentially blinding diseases, although there are limitations in the use of such therapies. One limitation is the unknown duration of anti-neovascular effects. Anti-angiogenics reduce regression of

neovascularization after treatment, but the duration of the effect is limited to a short period of time. In addition, the patients require several intravitreal injections to achieve successful treatment and stabilization of the disease. Moreover, some patients respond insufficiently even with several angiogenic treatments (Singer et al., 2016). Another limitation is the detectable levels of antiangiogenic drugs in the systemic circulation that may cause an increase in thromboembolic and cardiovascular events. Although the death rates did not seem to be increased due to the use of angiogenic drugs, the long-term consequences of its use are still unknown (Singer et al., 2016). Lastly, VEGF-A, as well as VEGF-B, plays an important role in neuroprotection, including the retina neurons, and promotion of cell survival. Therefore, VEGF-A and VEGF-B blockage may cause significant adverse events (Singer et al., 2016).

An interesting finding was the positive and robust correlation between VEGF-A and VEGF-B either in serum or in vitreous demonstrating that those cytokines might increase simultaneously. This may support the statement that VEGF-B, besides VEGF-A, is another contributor to the pathogenesis of neovascular diseases.

We tried to determine a correlation between serum VEGF-A vs. vitreous VEGF-A or between serum VEGF-B vs. vitreous VEGF-B. However, no correlation coefficient was found for VEGF-A or VEGF-B. Other researchers measured the levels of VEGF-A in vitreous and serum of patients and tried to correlate those growth factors (Abdel, Fahmy & Elsergani, 2008; Aiello et al., 1994; Ambati, Chalam & Chawla, 1997; Baharivand et al., 2012; Burgos et al., 1997; Celik et al., 2005; Deng, Wu & Gao, 1999; Funatsu et al., 2002; Gao et al., 2016; Hernandez et al., 2001, 2002; Hogeboom et al., 2002; Krizova et al., 2015; Lee, Chae and Kim, 2005; Malik et al., 2005; Ozturk et al., 2009; Praidou et al., 2009; Simó et al., 2002; Watanabe et al., 2005; Zhou & Zhang, 1997). While most of the researchers did not found a correlation, Baharivand and colleagues (2012) found a positive correlation between serum and vitreous VEGF-A.

The lack of correlation between the vitreous and serum may suggest that there is dissociation between the eye and other organs. It also suggests that vitreous VEGF-A and VEGF-B are synthetized intraocularly.

A statistically significant and positive correlation was found between vitreous VEGF-A vs. MV or between vitreous VEGF-B vs. MV in the diabetic population, demonstrating the effect that those two molecules may have in the establishment of edema.

Overall, all results suggest an overexpression of vitreous VEGF-A and VEGF-B as well a strong correlation between these two growth factors, confirming the interesting finding that they simultaneously increase in neovascular eye pathologies. It was also confirmed the increase of vitreous VEGF-A and VEGF-B with stage of DR with higher levels in PDR.

There are several pharmaceutical agents and non-drug procedures available to battle these multifactorial neovascular diseases. Despite laser and anti-angiogenics being the leading treatments for neovascular diseases, it is known that about 50% of patients have an insufficient response to angiogenic therapy and some would benefit from an early therapy switch as shown in the EARLY study (Gonzalez et al., 2016). Corticosteroids demonstrated their capability to lower inflammation and decrease VEGF levels (Gonzalez et al., 2016). Nevertheless, the combination therapy may be the rational approach to fight neovascular diseases by targeting VEGF factors while combating multiple and complex factors in the inflammatory and angiogenic cascade.

Only with a profound knowledge of pathophysiology, vascular, inflammatory and biochemical mechanisms as well targeted molecules, it will be possible to develop a new treatment approach. Thus, targeting VEGF-A or and VEGF-B as additional treatment options for neovascular ocular diseases might provide better outcomes, sustained duration of action, and an increased efficiency.

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Chapter 4

General Discussion

In this dissertation, VEGF-A, VEGF-B, and PIGF were quantified in the serum and vitreous of patients with neovascular diseases and compared with a group of patients with non-neovascular diseases. This was done to clarify the role of these three growth factors involved in the pathogenesis of DR, RVO and AMD. The effects of the overexpression of VEGF-A, VEGF-B, and PIGF were evaluated by the correlation:

1. Between the three growth factors,
2. Between serum and vitreous measurements within the same growth factor;
3. Between growth factors and structural parameters measured by the OCT (central retinal thickness and macular volume);
4. Between growth factors and functional parameters, such as visual acuity.

For DR patients and to determine if the levels of growth factors increase with disease progression, its concentrations were related and compared with the stages of DR.
Finally, the growth factors, VEGF-A, VEGF-B and PIGF levels were evaluated and compared with therapies performed by the patients to treat their eye condition before vitrectomy.

The results showed an increase in the vitreous concentration of VEGF-A, VEGF-B, and PIGF in patients with neovascular diseases in comparison with patients with non-neovascular diseases. The concentration of these cytokines in the serum did not show significant differences between patients. However, there was a correlation between VEGF-A and VEGF-B, in vitreous and in serum of patients with neovascular pathology, which means a coordinated increase, i.e., when vitreous VEGF-A increases, vitreous VEGF-B also increases; the same is also true for serum samples.
Moreover, between diabetic patients, a vitreous increase in VEGF-A, VEGF-B and PIGF was observed in patients with PDR in comparison with NPDR. Our research showed that the intravitreal levels of VEGF-A, VEGF-B and PIGF were increased in diabetic patients and were dependent on disease severity from NPDR to PDR. As expected, the levels of those growth factors were higher in the more severe stages of the DR, since they increase angiogenesis and consequently neovascularization and vascular permeability, thus, leading to macular edema and inflammation. This fact was supported by the finding that there was a positive correlation between vitreous VEGF-A or vitreous VEGF-B and MV, demonstrating the effect of those molecules in the establishment of edema. In the case of PIGF, the correlation was weak probably due to the patient population included in this study; however, a tendency of a positive correlation was observed between PIGF and MV.

Contrarily and unexpectedly, an increase in the studied VEGFs was detected in the serum of NPDR patients in comparison with PDR patients. There are several hypotheses why this may happen:

1. Concurrent systemic diseases, i.e., not only cancer but also several other diseases that are influenced by growth factors levels.
2. Concurrent diseases that may not be diagnosed or reported to the physicians by the patients. The difficulty in finding patients free of systemic health issues among this patient population is a reality and a limitation of this research.
3. Intravitreal concomitant medications and non-drug therapies administered to the patients are other limitations of the study, even though the interval between intravitreal injections and vitrectomy was greater than 3 months.
4. Diabetic therapy and stage of DR. Baharivand and co-workers showed that serum VEGF-A levels were lower in diabetic patients with oral therapy, well-controlled diabetes, and early stages of DR. Likely, lower serum results were also obtained in our research study (Baharivand et al., 2012).
5. Systemic levels of VEGF-A or VEGF-B may not be related to the production of vitreous cytokines by the retina. Some researchers advocate that intraocular synthesis of VEGF-A (and not diffusion) is the responsible mechanism for the high VEGF-A levels observed in the vitreous humor of PDR patients. Other investigators attributed the progression of DR to the circulating systemic VEGF-A to the high levels of VEGF-A (Abdel et al., 2008; Burgos et al., 1997; Funatsu et al., 2002; Lee et al., 2005)

Also, there was no correlation between vitreous and serum samples for VEGF-A, VEGF-B and PlGF, indicating dissociation between the eye and some organs. This suggests that the application of specific drugs to the eye could be relatively safe.

Furthermore, in our study, higher levels in the vitreous VEGF-A were constantly observed in comparison to vitreous VEGF-B and vitreous PlGF. This shows that despite additional and needed investigations to suppress other growth factors than VEGF-A and other pathways, the current pathway inhibiting VEGF-A is appropriate.

The treatment of ocular neovascular diseases is challenging but improved dramatically in the last years. However, currently, there is no cure but only therapies that slow down the progression of the disease.

One of the first therapies that appeared for the treatment of neovascular diseases was verteporfin (Visudyne®), an angio-occlusive that changed the course of AMD. The aim of Visudyne® therapy was to occlude vessels within the choroidal neovasculature while

preserving the overlying retinal tissue. Anti-angiogenic agents were discovered, and clinical trials showed powered outcomes of VEGF inhibition. VEGFs are angiogenic regulators of neovascularization and promoters of vascular permeability and are, thus, the key targets for treating neovascular diseases.

Currently, there are five therapies involving VEGF inhibition for the treatment of retinal diseases. Pegaptanib sodium intravitreal injections, (Macugen®), it is a pegylated VEGF aptamer, a single strand of nucleic acid that binds with specificity to the 165 isoform of VEGF-A, approved in 2004 by the U.S. Food and Drug Administration (FDA) being the first therapy available for the treatment of neovascular AMD. However, it is currently not in the market, being succeeded by more innovative molecules, such as ranibizumab and aflibercept. Bevacizumab (Avastin®), is humanized anti-VEGF-A monoclonal IgG antibody developed as an anti-angiogenic agent for colon-rectal cancer, lung cancer, glioblastoma, and renal-cell carcinoma that was approved for medical use in the United States in 2004. However, it has been used by ophthalmologists (off-label) intravitreally for the treatment of proliferative eye diseases.

Nevertheless, the drug that becomes the gold standard of treatment in these diseases it was ranibizumab. Ranibizumab (Lucentis®) is a fully humanized monoclonal antibody fragment targeted against human VEGF-A, with high affinity for all isoforms of VEGF-A. Until the appearance of ranibizumab, all medications used for treating AMD had the endpoint of maintaining vision or determine the percentage of patients who did not lose 15 or more letters. Ranibizumab changed the landmark of treatment being the first drug that recovered vision lost, with a turnover of the endpoint of the clinical trials to the percentage of patients who gained 15 or more letters. The binding of ranibizumab to VEGF-A at the receptor-binding site prevents the interaction of VEGF-A with its receptors VEGFR-1 and VEGFR-2 on the surface of the endothelial cells. This inhibits the cascade of events that leads to increased vascular permeability, increased activity, and proliferation of endothelial cells and inflammation. It is currently approved for the treatment of neovascular (wet) AMD, visual impairment due to choroidal neovascularization, visual impairment due to DME, and visual impairment due to macular edema secondary to RVO (BRVO or CRVO).

Recently a new therapy emerged as a new light for the treatment of retinal diseases, aflibercept. Aflibercept (Eylea®) is a recombinant fusion protein consisting of VEGF binding portions from the extracellular domains of human VEGFR-1 and VEGFR-2 fused to the Fc portion of the human IgG1 immunoglobulin. It binds to all isoforms of VEGF-A, VEGF-B, and PlGF. It was approved in the United States and Europe for the treatment of neovascular (wet) AMD, visual impairment due to macular edema secondary to RVO (BRVO or CRVO), visual impairment due to DME, and visual impairment due to myopic choroidal neovascularization

(myopic CNV); and for metastatic colorectal cancer (Zaltrap). For ocular use, the first approved indication (wet AMD) was granted in 2011 by the FDA and in 2012 by EMA.
Ultimately a new therapy it was developed being only available in some parts of Asia. Conbercept (Lumitin®) is an anti-VEGF approved drug for the treatment of wet AMD in China by the China State Food and Drug Administration in December 2013. It has a higher binding affinity, lower VEGF dissociation rate, and a longer clearance time. It is a recombinant fusion protein that binds to all VEGF-A isoforms, VEGF-B, VEGF-C and PIGF. The structural differences between this drug and other anti-VEGFs may pose a longer effect in vitreous. Conbercept has a therapeutically satisfactory effect even with a quarterly regimen.

VEGF inhibitors are promising drugs for the treatment of neovascular eye diseases; however, it should be of note that there are some limitations of its usage:
1. The unknown duration of anti-neovascular effects: Anti-angiogenics reduce regression of neovascularization after treatment and improve structural and functional parameters, but the duration of the effect is limited to a short period of time.
2. The mode of drug delivery: there are complications after intravitreal injections, such as endophthalmitis, intraocular inflammation, rhegmatogenous retinal detachment, acute intraocular pressure elevation and ocular hemorrhage.
3. Anti-VEGF therapy currently requires frequent injections and assessments to determine if a patient is responsive to a treatment. This circumstance leads to a significant burden of injections and visits for all involved in the treatment of those patients, namely, patients, physicians, caregivers, and other health providers. The solution for this problem would be the administration of a medication that would improve visual and structural outcomes and simultaneously increase drug effectiveness and lengthen the durability of the treatment.
4. Another issue is the systemic safety. Several serious and non-serious adverse events have been reported with the systemic administration of anti-VEGFs: thromboembolic events, myocardial infarction, stroke, hypertension, gastrointestinal perforations, and kidney disease, which lead to the inclusion of a black box in the summary of product characteristics of bevacizumab, ranibizumab, and aflibercept. Moreover, intravitreal antiangiogenic drugs were associated with detectable levels in the systemic circulation that may significantly suppress systemic VEGF levels, suggesting a rationale for the occurrence of potential cardiovascular adverse events. Although death rates observed in clinical trials did not seem to be increased due to the use of angiogenic drugs, the long-term consequences are still unknown.
Furthermore, VEGF-A, VEGF-B, and PIGF have an important role in neuroprotection and cardioprotection. Therefore, the blockage may also have consequences in the long run.
5. Nevertheless, not all patients respond sufficiently to anti-VEGF intravitreal injections despite frequent treatments. Descriptive and exploratory subanalyses conducted in our study by group therapies suggest an insufficient response of neovascular patients treated previously

with one or more of the following: laser, bevacizumab, ranibizumab, aflibercept or triamcinolone. Laser and anti-angiogenics are the standard gold treatment for neovascular diseases. However, it is known that about 40-50% of patients have an insufficient response to angiogenic therapy and that a significant percentage of patients would benefit from an early therapy switch as shown in the EARLY study.

Corticosteroids are used alone or in conjunction with other pharmacologic or surgical treatments. Steroid treatments are invaluable in ophthalmology and one of the oldest treatments available for the treatment of persistent or recurrent diseases. Steroids have proven to be powerful and effective in suppressing inflammation and also playing a significant role in the inhibition of several cytokines inclusively antagonizing the action of VEGF-A. The best-studied steroids are triamcinolone acetonide, dexamethasone, and fluocinolone acetonide.

Notwithstanding the well-known side effects caused by corticosteroids, such as the cataract formation and the increase in intraocular pressure, the efficacy already demonstrated that the benefits outweighed the risks. Moreover, there is an enormous advantage of the usage of intravitreal corticosteroids since the systemic side effects of locally administered steroids occur rarely.

In table 4, the anti-angiogenic drugs currently available are summarized. Clinical trials and other studies should focus on other possible drugs that have not been studied in ophthalmology for their consideration as future targeted molecules.

Table 4 – Summary of anti-angiogenic agents (DRUGS 2017; Steeghs et al., 2007; U.S. National Library of Medicine 2017)

Generic drug name	Trade name	Type of molecule	Target	Clinical stage in ophthalmology	Therapeutic Indications	Route of administration	Commercialized by
Pegaptanib	Macugen®	RNA aptamer	VEGF-A 165	Commercialized	Wet AMD	Intravitreal injection	OSI Pharmaceuticals/Pfizer / Bausch & Lomb
Bevacizumab	Avastin®	Recombinant humanized full monoclonal antibody	All VEGF-A isoforms	Not commercialized for ocular use	Off-label usage	Intravitreal injection	Genentech in U.S. and Roche in Europe
Ranibizumab	Lucentis®	Recombinant humanized monoclonal antibody fragment	All VEGF-A isoforms	Commercialized	Wet AMD, macular edema following RVO, DME, DR with DME, and myopic choroidal neovascularization	Intravitreal injection	Genentech in U.S. and Novartis in Europe
Aflibercept	Eylea®	Fusion protein	All VEGF-A & VEGF-B isoforms and PIGF	Commercialized	Wet AMD; ME following RVO, DME and DR in Patients with DME	Intravitreal injection	Regeneron in U.S. and Bayer in Europe
Conbercept	Lumitin®	Fusion protein	All VEGF-A isoforms, VEGF-B, VEGF-C and PIGF	Commercialized in China; Not commercialized in US or EU	Wet AMD	Intravitreal injection	Kanghong Biotech
AGN-150998; Abicipar Pegol	Not available	DARPin	VEGF-A	Phase III	Wet AMD, DME	Intravitreal injection	Allergan
Multi VEGF-PDGF DARPin	Not available	Multi-DARPin	VEGF-A	Pre-clinical	Wet AMD	Not available	Allergan
Bevasiranib	Not available	siRNA	VEGF-A mRNA	Phase III, Discontinued	Wet AMD	Intravitreal injection	Opko Health
AGN211745, formerly SIRNA-027	Not available	siRNA	VEGFR-1 mRNA	Phase I, Discontinued	Wet AMD	Intravitreal injection	Allergan
PF-04523655	Not available	siRNA	DDIT4 mRNA	Phase II, Terminated	Wet AMD and DME	Intravitreal injection	Quark Pharmaceuticals /Pfizer
ALN-VSP02	Not available	Dual siRNA	VEGF-A and KSP mRNAs	Phase I	–	–	Alnylam
E10030; Pegplenarib	Fovista®	DNA aptamer	PDGF-BB	Phase III Discontinued	Wet AMD	Intravitreal injection	Ophthotech/ Novartis

ARC1905; Avacincaptad pegol	Not available	RNA aptamer	C5 complement	Phase I	Dry AMD, Wet AMD	Intravitreal injection	Ophthotech
ESBA-1008; RTH258 (brolucizumab)	Not available	Humanized single chain anti-body fragment	VEGF-A	Phase III	Wet AMD, DME	Intravitreal injection	Alcon Research
TB-403; THR 317	Not available	Monoclonal antibody	PIGF	Phase II – DME Pre-clinical - DR	DME, DR	–	ThromboGenics /Roche
5D11D4	Not available	Monoclonal antibody	PIGF	Pre-clinical	_	Systemic	ThromboGenics
Lapatinib	Tyverb®	TKI	Small molecules	_	Breast Cancer	Oral	GlaxoSmithKline
Sunitinib maleate-	Sutent® (oral)	TKI	Small molecules	Phase I/II AMD Preclinical DME, RVO	**	Intravitreal	GrayBug Inc.
Sorafenib	Nexavar®	TKI	Small molecules	Phase I Acute	Hepatocellular carcinoma; Renal cell carcinoma; Thyroid cancer	Oral	Bayer
Axitinib/ Axitinib ophthalmic	Inlyta® Oral formulation /Pfizer)	TKI, PDGF, VEGF-A	Small molecules	Preclinical Age-related macular degeneration	Renal cell carcinoma	_	Clearside Biomedical
Pazopanib	Votrient®	TKI	Small molecules	Discontinued AMD	Renal cell carcinoma; Sarcoma	Oral	GlaxoSmithKline
Regorafenib/ BAY 73-4506	Stivarga	TKI	Small molecule		Colorectal cancer; advanced gastrointestinal stromal tumors	Oral	Bayer

Note: DARPin - Designed Ankyrin Repeat Proteins, DDIT4 mRNA- DNA-damage-inducible transcript 4 mRNA, DME – diabetic macular edema, DR – diabetic retinopathy, KSP mRNAs - Kinesin spindle protein mRNAs, PDGF - Platelet-derived growth factor, PIGF - placental growth factor, RVO – retinal vein occlusion, siRNA– Small interfering RNA, VEGF - vascular endothelial growth factor, VEGFR-1 - vascular endothelial growth factor receptor 1, Wet AMD – Wet age related macular degeneration. TKI- tyrosine kinase inhibitors

** Sunitinib licensed for Pfizer is marketed for gastrointestinal stromal tumors; pancreatic cancer; renal cell carcinoma.

Although laser, anti-VEGFs and steroids were established as successful target treatments, many other new therapies and approaches are arising in the pipeline, holding promises in the improvement of eye pathologies. Regardless of the future discoveries, significant progress has been made in understanding the molecular pathogenesis of retinal neovascular disorders and investigating new targets for therapeutic interventions.

Chapter 5

Concluding remarks and future trends

Overall from our research:

1. We have proven and measured for the first time vitreous VEGF-B levels and reported also for the first time their overexpression in ocular neovascular diseases. Also, vitreous PlGF levels were not much studied until date. Contrarily, vitreous and systemic VEGF-A have widely been the subject of research. VEGF-A facilitated the beginning of our investigation as a "control," becoming more important as a comparator and as a correlation factor for other growth factors. Moreover, it showed to be the growth factor that has higher concentration levels in retinal diseases, demonstrating the accurate direction of current drugs targeting VEGF-A for the treatment of neovascular diseases.

2. The VEGF-B overexpression confirms the possibility as a target. VEGF-B is a strong apoptotic molecule that can offer an alternative and challenging therapeutic target in the treatment of neovascular conditions. However, clinical trials are needed to confirm the efficacy of anti-VEGF-B therapy as well safety due to its neuroprotective and cardioprotective effects.

3. PlGF may be a favorite target for the inhibition of angiogenesis in relation to VEGF-A. The safety profile of anti-PlGF needs also preclinical studies and human clinical trials to confirm the possibility of systemic delivery of anti-PlGF monoclonal antibodies and also its effectiveness and safety, in combination with an anti-VEGF therapy or as a partial replacement of anti-VEGF.

Contrary to VEGF-A, the VEGF-B is inert under normal conditions and PlGF are restricted to pathological conditions, making those molecules high targets for therapy.

4. We tried to identify serum VEGF-A, VEGF-B, and PlGF as markers in the early detection of neovascular disease and we also tried to find a correlation between serum and vitreous VEGF-A or between serum and vitreous VEGF-B, or between serum and vitreous PlGF. However, no correlation coefficient was found between serum and vitreous VEGF-A, VEGF-B or PlGF, which can be a sign of the dissociation between eye and other organs.

Future research should continue focusing on new anti-VEGF strategies in the treatment of ocular diseases linked to abnormal vascularization. The discovery of new isoforms of the VEGF family revealed an increased biology complexity and faced many obstacles that must be overcome while exploring new targets. The blockage selectivity is as far one of the most important factors to be considered when testing new anti-VEGF molecules.

A better and deep understanding of all mechanisms, interactions and specific functions between the members of the VEGF family and its receptors in both normal states and diseases is crucial for the development of new drugs. Also, new experimental and clinical trials

targeting therapies for the newly discovered and innovative molecules will allow the development of more specific, precise, safe and effective therapies, resulting in better outcomes for patients with pathological ocular angiogenesis.

Finally, new serum and vitreous studies measuring VEGF family growth factors with a high number of patients should be continued in order to find out a correlation between disease characteristics and vitreous or serum levels of growth factors.
Only the early intervention in disease through an adequate screening of patients and the treatment with the specific drugs at the right time will lead to considerably vision improvements and a decrease in worldwide blindness.

Chapter 6

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