Universidade do Minho
Escola de Medicina

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EVALUATION OF THE SYSTEMIC PRO-INFLAMMATORY RESPONSE ASSOCIATED WITH DIABETIC RETINOPATHY AS A LIMITING FACTOR ON THE EFFICACY OF CURRENT INTRAVITREAL TREATMENTS FOR DIABETIC MACULAR EDEMA

Setembro de 2020

Systemic Pro-inflammatory Response as a Limiting Factor on the Efficacy of Intravitreal Treatments for Diabetic Macular Edema

Pedro Brito

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Tese de Doutoramento em Medicina

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Setembro de 2020

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

We are what we repeatedly do. Excellence, therefore, is not an act, but a habit.

Will Durant, The Story of Philosophy (1926)

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Assinado por : **Pedro Nuno Ferreira Pacheco da Silva Brito**
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Data: 2020.09.09 23:44:43+01'00'

TÍTULO: AVALIAÇÃO DA RESPOSTA PRO-INFLAMATÓRIA SISTÉMICA ASSOCIADA À RETINOPATIA DIABÉTICA COMO FATOR LIMITANTE DA EFICÁCIA DOS ATUAIS AGENTES INTRAVITREOS PARA O TRATAMENTO DO EDEMA MACULAR DIABÉTICO

RESUMO: A retinopatia diabética (RD) é uma complicação microvascular do Diabetes Mellitus (DM) capaz de causar perda visual significativa devido a duas complicações: edema macular diabético (EMD) e retinopatia diabética proliferativa. O aparecimento dos agentes neutralizantes do fator de crescimento do endotelial vascular (VEGF), permitiu obter resultados clínicos sem precedentes no tratamento do EMD. No entanto, várias análises *posthoc* de estudos randomizados revelaram que uma percentagem significativa de doentes evoluirá para EMD persistente, mesmo após anos de tratamento anti-VEGF; acresce que cerca de um terço não atingirão melhoria significativa na acuidade visual. Existe claramente uma grande variabilidade na resposta ao tratamento com anti-VEGF, o que indica que existem múltiplos fatores relevantes na patogénese do EMD. Efetivamente, tem sido destacado o possível contributo do componente pró-inflamatório subclínico, subjacente à DM. Por conseguinte, o objetivo deste projeto, foi estudar o papel de fatores metabólicos e pró-inflamatórios sistémicos como possíveis moduladores da resposta ao tratamento com anti-VEGF. Os resultados revelaram associações significativas entre níveis séricos aumentados de proteína C-reactiva ultra-sensível (PCRus), ICAM-1 e MCP-1, e a ocorrência de EMD persistente, com resposta anatómica limitada a anti-VEGF. Curiosamente, o grupo com resposta anatómica mais limitada apresentou VEGF-A sérico significativamente mais baixo, sugerindo, que a disfunção da barreira hemato-retiniana não foi primariamente mediada pelo aumento de VEGF-A. Os resultados obtidos sugerem a existência de dois fenótipos de EMD: uma forma vasogénica mediada pelo aumento do nível de VEGF-A, e exibindo resposta precoce e robusta aos fármacos anti-VEGF; e um segundo fenótipo caracterizado por EMD persistente, associado a níveis aumentados de biomarcadores pró-inflamatórios e VEGF-A mais baixo. Mais relevante ainda, os resultados sugerem que os tratamentos intravítreos atuais não abordam especificamente a patogénese deste último fenótipo. Em conclusão, verificamos que o estado pró-inflamatório sistémico pode modular a ocorrência de EMD persistente com resposta clínica limitada ao tratamento com anti-VEGF. Será por isso necessário, continuar a investigação e desenvolvimento de agentes terapêuticos direcionados a esta forma essencialmente não-vasogénica de EMD.

Palavras-chave: Anti-VEGF, Edema Macular Diabético, Inflamação, PCRus

TITLE: EVALUATION OF THE SYSTEMIC PRO-INFLAMMATORY RESPONSE ASSOCIATED WITH DIABETIC RETINOPATHY AS A LIMITING FACTOR ON THE EFFICACY OF CURRENT INTRAVITREAL TREATMENT AGENTS FOR DIABETIC MACULAR EDEMA

ABSTRACT: Diabetic Retinopathy (DR) is a microvascular complication of Diabetes Mellitus (DM) capable of causing significant visual impairment due to two major complications: diabetic macular edema (DME) and proliferative diabetic retinopathy. The clinical introduction of vascular endothelial growth factor (VEGF) neutralizing agents, allowed unprecedented visual outcomes for patients with DME. However, several *posthoc* analyses of major clinical trials revealed that a significant percentage of patients will have persistent DME years after initiating anti-VEGF treatment, and about one third of cases will not achieve a significant improvement in visual acuity. There is clearly a variable response spectrum to anti-VEGF treatment, suggesting that there are multiple factors involved in the pathogenesis of DME. Recently, an important role has been attributed to a subclinical pro-inflammatory state associated with DM. Therefore, the major objective of this PhD project was to study the role of several systemic metabolic and pro-inflammatory factors, as possible modulators of clinical response to anti-VEGF treatment for DME. Our results revealed a significant association between increased serum markers of inflammation such as high-sensitivity C-reative protein (hsCRP), ICAM-1 and MCP-1 and the occurrence of persistent DME with limited anatomic response to anti-VEGF. Interestingly, the poorly responding group had significantly lower serum VEGF-A, suggesting that the disruption of the blood-retinal barrier was not primarily mediated by increased VEGF-A. Overall, the results suggest the existence of two main phenotypes of DME: a vasogenic form mediated by increased VEGF-A levels, exhibiting an early and robust response to anti-VEGF treatment; and a second phenotype, characterized by persistent DME associated with increased circulating pro-inflammatory biomarkers and lower levels of VEGF-A. More importantly, our findings emphasize that current intravitreal treatment strategies are not specifically addressing the pathogenesis of such phenotype. In conclusion our results are clinically relevant as they provide consistent evidence that the systemic pro-inflammatory status may modulate chronic, persisting DME with limited clinical response to anti-VEGF treatment. Continued research is necessary in order to develop improved treatment agents targeting this persistent non-vasogenic form of DME.

Keywords: Anti-VEGF, Diabetic Macular Edema, Inflammation, hsCRP

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ABBREVIATIONS

AGE – advanced glycosylation endproduct

BRB – blood-retinal barrier

CRP – C-reactive protein

CRT – Central retinal thickness (may also appearing in the literature as central subfield, foveal or central macular thickness)

CSME – clinically-significant macular edema

DM – Diabetes Mellitus

DME – diabetic macular edema

DR – Diabetic retinopathy

ELM – External limiting membrane

EZ – Ellipsoid zone

hsCRP – high sensitivity C-reactive protein

ICAM1 - Intracellular adhesion Molecule 1

IOP - intraocular pressure

MCP1 - Monocyte chemoattractant protein 1

MV - Macular Volume

NPDR - nonproliferative diabetic retinopathy

PDR - proliferative diabetic retinopathy

PRN – *pro re nata*

TA - triamcinolone acetonide

TNFα - Tumor necrosis factor alpha

VA - visual acuity

VEGF-A - Vascular endothelial growth factor A

μm - Micrometer

CHAPTER I – INTRODUCTION AND PURPOSE

CHAPTER I – Introduction and Purpose

1 - The Retina: fundamental anatomic and physiologic concepts

The retina is the innermost layer of the eye and is responsible for the conversion of light energy into a neural impulse that is transmitted to the brain, via the optic nerve, creating visual perception. Such process is named phototransduction and occurs in the light sensitive photoreceptor cells. The two types of photoreceptors in the human retina are the rods and the cones. Rods are responsible for dim light vision, while the cones provide color vision in daylight conditions with high spatial acuity. To accomplish its function, the retina evolved as a highly differentiated tissue including different cell types, organized into layers, forming the neuroretina which itself is adherent to a monolayer of hexagonal cells – the retinal pigment epithelium (RPE) which provides functional and metabolic support (Figure 1). Regarding the structural organization of the neuroretina, the nucleus of rod and cone photoreceptors are located in the outer nuclear layer. Their respective terminal projections (cone pedicles and rod spherules) synapse with the dendrites of bipolar and horizontal cells forming the outer plexiform layer (known as the Henle fiber layer in the macular region). The inner nuclear layer is formed by the nuclei and cell bodies of the amacrine cells, bipolar cells and horizontal cells. The axons of the bipolar cells synapse with the dendrites of ganglion cells in the inner plexiform layer. The nuclei of the ganglion cells are located in the ganglion cell layer, while the respective axons give rise to the nerve fiber layer and continue to form the optic nerve fibers.

Another important cellular element is the glial Müller cell, its nucleus is located in the inner nuclear layer but the cell extends to form the internal limiting membrane, as well as to connect with the apical portion of the RPE, stabilizing the structural organization of the retinal layers. In fact, Müller cell processes create an extensive network that surrounds the retinal capillaries, contributing to maintain the inner blood-retinal barrier integrity (BRB). Additionally, by regulating the extracellular potassium level, water content and the release of neurotransmitters, the Müller cell maintains extracellular homeostasis and the functional integrity of the neighboring retinal cells [1]. Overall, the 10 individual retinal layers, may be grouped as contributing to the 4 main stages regarding light perception: photoreception; transmission of the neural impulse to bipolar cells; then on to the ganglion cells, and finally transmission along the optic nerve. The optic nerve, composed of the ganglion cell axons, synapses with the lateral geniculate nucleus in the thalamus. Finally, the neural

impulse is conducted via the optic radiations to the occipital cortex, where it is interpreted as a visual image.

The central area of the retina is named macula and measures approximately 5.5 mm in diameter. In the center of the macular region, a circular area with 1500 μm in diameter named fovea, contains the highest density of cone photoreceptors (exceeding 140.000 cones/mm2), and it is responsible for the highest visual acuity. The photoreceptors are organized into four distinct segments: the outer segment (OS), the inner segment (IS), the nucleus, and the synaptic terminals. The OS is organized into discs, where the phototransduction process is initiated, with the absorption of light, resulting in the conversion of 11-*cis* retinal to the all-*trans* form. The IS is composed of the ellipsoid zone, which contains mitochondria and the myoid zone, which contains the cell's protein synthesis structures. During phototransduction, retinal splits from an opsin molecule and is transported to the RPE, where it is isomerized back to the 11-*cis* form and recombined with opsin. Due to its high metabolic rate, the retina is susceptible to oxidative stress which can damage the photoreceptors. To maintain functional integrity, the OS discs are continuously shed and renewed[2]. Alongside its metabolic support function, the RPE also contains melanin granules which act as free radical stabilizers, and also play a role in the absorption of stray light contributing to improved optical quality.

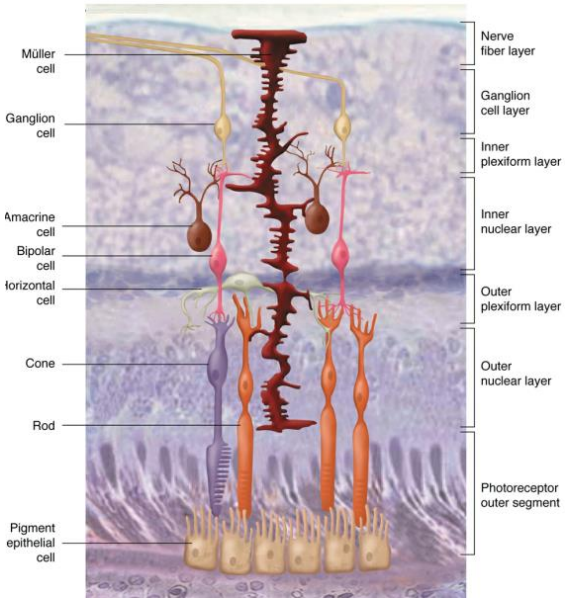

Figure 1. Schematic representation of the morphology and structural organization of the cell types found on the human retina. Adapted from Basic and Clinical Science Course – Section 2 – Fundamentals and principles of Ophthalmology. American Academy of Ophthalmology 2019

1.1 – Retinal Vasculature and the Blood-Retinal Barriers

The inner retina receives its blood supply from retinal capillaries which derive from branches of the central retinal artery. The photoreceptors and RPE are supplied by the choroidal circulation, which itself branches from the anterior and posterior ciliary arteries. The choroid is organized into three layers: the inner choriocapillaris layer is immediately adjacent to the Bruch membrane (an elastic membrane containing the basement membrane of the RPE and of the choriocapillaris), is composed of large diameter fenestrated capillaries, which allow the rapid passage of blood cells and molecules. The outer layer, known as the Haller layer includes the larger-caliber choroidal vessels, which branch into smaller-diameter vessels and precapillary arterioles in an intermediate layer known as the Sattler layer. The structural integrity of the choroid is essential for normal retinal function. Compromised choroidal blood flow may result in photoreceptor and retinal pigment epithelium (RPE) dysfunction[3].

To preserve retinal function, the neural cells need to be isolated from toxic substances or blood components, which under pathologic conditions may leak from the nourishing blood vessels. Therefore, the role of the inner and outer blood-retinal barriers (BRB) is critical to retinal function. The inner blood-retinal barrier if formed by tight and *adherens* junctional complexes between individual endothelial cells forming the retinal capillaries, while the outer BRB is created by the tight junctions found in the apical portion of the RPE cells. The combined action of tight junctions restricting paracellular fluid movements, and the endothelial and RPE cell walls regulating transcellular movements contribute to maintaining the optimal retinal microenvironment. Disruption of either of the BRBs may lead to fluid accumulation in the macula, causing significant vision loss.

2 - Diabetes Mellitus: current concepts and epidemiologic data

Diabetes Mellitus (DM) defines a group of metabolic disorders characterized by abnormally increased blood glucose levels (hyperglycemia). Classic symptoms include: polyuria, polydipsia, fatigue, blurred vision, frequent superficial infections and slow wound healing. The currently established diagnostic criteria are:

a) fasting blood glucose ≥ 126 mg/dl (or ≥ 7,0 mmol/l);
b) clinical symptoms plus random blood glucose level ≥ 200 mg/dl (or ≥ 11,1 mmol/l)
c) blood glucose ≥ 200 mg/dl (or ≥ 11,1 mmol/l) after 2 hours in the oral glucose tolerance test with 75g of glucose
d) glycated Hemoglobin A1c (HbA1c) ≥ 6,5 %.

Diabetes is typically classified as type 1 when it results from the destruction of pancreatic β-cells, leading to absolute insulin deficiency, occurring as an idiopathic or autoimmune phenomenon. It is more common in children or young adults, but may occur at any age. Treatment for this form of diabetes mandates insulin therapy. Type 2 diabetes mellitus refers to a more heterogeneous group of disorders characterized by insulin resistance, impaired insulin secretion or increased hepatic glucose production. Early type 2 DM may have a subtle clinical presentation which contributes to its delayed diagnosis. It is most commonly diagnosed above 40 years of age, frequently associated with obesity, although other factors such as increasing age, sedentary lifestyle and family history may contribute to disease onset. Many patients are diagnosed occasionally during routine blood tests or when serious complications develop in target organs[4]. Treatment usually starts with oral antidiabetic medication although in more severe cases insulin may be added to improve glucose control. An integral part of treatment also includes adopting a healthy diet and regular physical activity, as well as controlling blood pressure and blood lipid profile. Prolonged uncontrolled glucose will eventually result in target organ complications, the most commonly affected are the eye, kidney and peripheral nerve system (lower limbs) and there is also significant cardiovascular disease and stroke risk.

The prevalence of diabetes is increasing worldwide and is expected to reach 592 million people by the year 2035[5]. In our country the estimated prevalence in the population between 20 and 79 years of age is 13.3% corresponding to about 1 million individuals. There is a marked increase in

prevalence with increasing age (a quarter of all cases are found between the ages of 60 to 79 years). Another important association is with increased body mass index, in fact it is estimated that 90% of all cases with diabetes have excessive weight. Incidence of DM is increasing in our country, in fact the latest data reporting to the year 2015 indicates between 591-699 new cases per 100.000 inhabitants[6]. DM complications cause significant morbidity and increased mortality, making this disease one of the leading causes of death worldwide, according to the latest World Health Organization data[7].

4. Diabetic retinopathy

4.1 Definition and epidemiologic concepts

Diabetic retinopathy (DR) is a microvascular complication of diabetes affecting the eye and is a major cause of impaired vision and blindness, particularly in developed countries[8]. The most widely accepted classification of DR is the international clinical diabetic retinopathy and diabetic macular edema disease severity scales[9] (Table 1). Succinctly, DR may be classified as nonproliferative (NPDR), when retinal vascular changes are present, ranging from microaneurysms in the mild form to a combination of retinal hemorrhages, venous beading and intraretinal microvascular abnormalities in the severe form, however no neovascular lesions are identified; the most advanced level of DR is named proliferative diabetic retinopathy (PDR), and is defined by the presence of optic disc and / or retinal neovascularization, resulting from severe retinal ischemia. This stage of RD is considered vision-threatening due to the possibility of complications such as vitreous hemorrhage or tractional retinal detachment. Interestingly, increased microaneurysm turnover has been found to indicate progression of retinopathy, and may be predictive of future visual impairment[10]. Another major complication of DR is named diabetic macular edema (DME) which results from increased permeability of the inner BRB leading to leakage of plasma fluid into the retinal interstitial space. DME may occur at any level of DR severity and it is graded according to whether retinal thickening extends to the central foveal area (Table 2).

Table 1. International Classification of Diabetic Retinopathy

Diabetic Retinopathy severity	Findings Observable on Dilated Ophthalmoscopy
No retinopathy	No abnormalities
Mild nonproliferative diabetic retinopathy	Microaneurysms only
Moderate nonproliferative diabetic retinopathy	More than just microaneurysms but less than severe signs of disease
Severe nonproliferative diabetic retinopathy	Any of the following: more than 20 intraretinal hemorrhages in each of 4 quadrants; definite venous beading in 2 quadrants; Prominent intraretinal microvascular abnormalities in 1 quadrant. No signs of proliferative retinopathy
Proliferative diabetic retinopathy	One or more of the following: neovascularization, vitreous/preretinal hemorrhage

Table 2. International Clinical Diabetic Macular Edema Severity Scale

Diabetic Macular Edema Categories	Findings Observable on Dilated Ophthalmoscopy
Mild	Some retinal thickening or hard exudates in posterior pole but distant from the center of the macula
Moderate	Retinal thickening or hard exudates approaching the center of the macula but not involving the center
Severe	Retinal thickening or hard exudates involving the center of the macula

One of the most cited studies regarding prevalence of DR is the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR)[11]. In this study, a prevalence of DR was found in 71% of cases with type 1 DM and 47% of cases with type 2 DM. A direct association was found between increased prevalence of DR and longer duration of DM in both type 1 and type 2 patients. In fact, the results

indicate that after 20 years of DM, about 99% of patients with type 1 and 60% with type 2 will have signs of DR. Proliferative DR was found in 50% of type 1 patients who had 20 years' duration of disease and in 25% of type 2 patients who had 25 years' duration of disease. More recently, another study including data from 35 studies in the US, Australia, Europe and Asia, showed that the overall age-standardized prevalence of any DR was 34,6% and vision threatening DR was 10.2%, thus confirming a high worldwide prevalence of DR[12]. Another well-known risk factor for DR is uncontrolled hyperglycemia, in fact the DCCT study of type 1 diabetics showed that tight glucose control significantly reduced both the risk of developing DR and the rate of retinopathy progression[13]. The UKPDS study in type 2 diabetics also reported similar benefits of tight glucose control in progression of DR. Additionally, control of blood pressure had a favorable effect in slowing progression of retinopathy and reducing the risk of vascular complications of DM [14]. There is also evidence that hyperlipidemia may be associated with clinically significant macular edema [15] [16]. In our country, the RETINODIAB study evaluated data from the DR screening program in the Lisbon and Tagus Valley area and verified a prevalence of DR of 16.3% in a population of 52739 diabetic patients, of which (1,3%) had severe nonproliferative DR, (1,8%) had proliferative DR and (1.4%) had diabetic maculopathy [17]. Regarding incidence, national data indicates a cumulative incidence over a five-year follow-up period of 14.47% new cases of any grade DR, and more importantly the risk of DR was found to increase with increasing duration of disease and also earlier onset of DM[18]. Considering that DM prevalence is expected to continue to rise[19], the number of persons with DR-related vision impairment is also likely to continue to increase.

4.2 Pathogenesis of Diabetic Retinopathy and Diabetic Macular Edema

4.2.1 Histologic changes and dysfunction of the neurovascular unit

The complete sequence of events that lead to DR is not completely understood, yet there is evidence that prolonged uncontrolled hyperglycemia is the major determinant of the vasculopathic process[20]. The pathogenesis of DME seems to be related to three major histologic changes occurring in the inner BRB that lead to compromised function: endothelial cell dysfunction and apoptosis, pericyte loss, and thickening of the basement membrane[21].

The integrity of the endothelial monolayer is maintained by cell-to-cell tight junctional complexes and *adherens* junctions. In experimental models of diabetes, breakdown of these junctions occurs

through a decrease in occludin[22] and cadherin levels[23]. Capillary endothelial cells may also undergo apoptosis, resulting in the formation of acellular capillaries[24] which are prone to occlusion ultimately leading to retinal ischemia. There is also evidence that the endothelial glycocalyx, acting as a permeability barrier on the luminal side of endothelium, becomes significantly reduced during uncontrolled diabetes, further contributing to endothelial dysfunction[25]

The pericytes are modified smooth muscle cells that regulate retinal capillary blood flow. Pericyte loss is an early lesion of DR[26] and results in focal endothelial cell proliferation, contributing to the formation of dilated capillaries and microaneurysms. There is evidence that the relative surface coverage of pericytes on capillaries is positively correlated with endothelial barrier properties, possibly due to modulation of junctional integrity[27].

The basement membrane surrounds the endothelial cells, providing structural support and also acting as a filtration barrier. Thickening of the basement membrane may occur before the development of overt DR lesions, and is due to increased synthesis of collagen type IV, fibronection and laminin, under hyperglycemic conditions. These changes are thought to cause a relative decrease in anionic charges, contributing to increased permeability [28-30].

Alongside the retinal vascular changes, there is evidence that the biochemical changes induced by the diabetic milieu[31], may lead to apoptosis of ganglion cells[32] and dysfunction of Muller cells[33]. More specifically, a disturbance of the aquaporin-4 channels may contribute to increased retinal thickness[34]. Finally, the application of Starling's rules to retinal fluid movement[35], indicates that an increase in luminal hydrostatic pressure (hypertension, congestive heart failure, renal failure) can further damage the altered BRB in DR. Overall, while the complete sequence of events leading to DR and DME is not fully known, it seems that such complications result from an interplay of factors including prolonged hyperglycemia, accumulation of AGE leading to increased oxidative stress and release of pro-inflammatory factors, which lead to histological changes in the retinal neurovascular unit, ultimately resulting in disruption of the BRB.

4.2.2 Hyperglycemia

Hyperglycemia is the hallmark feature of DM and there is evidence from animal models that it may be sufficient to initiate the development of DR[36]. In fact, several pathways have been shown to be involved in intracellular glucose toxicity, including the polyol pathway, the hexosamine pathway, activation of protein kinase C (PKC) and perhaps the most relevant, the accumulation of advanced

glycation endproducts (AGEs). All of these pathways contribute to increased oxidative stress, inflammation, and vascular dysfunction compromising the BRB[20]. There is substantial evidence for the pathogenic effect of AGEs in the retina. In fact, glycated hemoglobin in red blood cells causes reduced oxygen delivery, leading to oxidative stress, and increasing reactive oxygen species, which are believed to lead to apoptosis of retinal endothelial cells[37]. Interestingly, AGE inhibitors, such as pyrodoxamine, were shown to inhibit BRB dysfunction in diabetic animals[38]. Also, the interaction of AGEs with receptors of AGE (RAGE) on endothelial cells, may cause increased expression of vascular cell adhesion molecule-1 (VCAM-1) or intercellular adhesion molecule-1 (ICAM-1), which promote adherence of leukocytes leading to capillary occlusion and endothelial cell apoptosis[39]. Furthermore, circulating endothelial progenitor cells, are reduced in number when exposed to high glucose levels[40]. Regarding the association of hyperglycemia and inflammation, a recent study verified that the increase in serum concentration of AGEs was a significant independent determinant of plasma C-reactive protein (CRP) levels[41]. Additionally, Müller cells exposed to hyperglycemic conditions produce increased levels of pro-inflammatory factors such as IL-1β and TNF-α[42]. A similar phenomenon occurs in retinal pericytes exposed to high glucose levels, resulting in increased expression of several pro-inflammatory factors, persisting even after restoration of normal glucose levels[43].

4.2.3 Hypoxia

Retinal hypoxia, is a major feature of advanced DR and is due to progressive capillary occlusion and consequent retinal nonperfusion. Physiological retinal hypoxia varies throughout the day, as dark-adapted rods require more oxygen than any other cell type[44]. Therefore, the threshold to develop pathological hypoxia in the retina is low comparing to other tissues. Increased retinal ischemia leads to expression of hypoxia inducible factor-1 (HIF-1), a transcription factor that regulates cellular oxidative metabolism, by activating various target genes (including VEGF, erythropoietin and nitric oxide synthase), in an attempt to restore oxygen supply or improve cell survival in hypoxic conditions[45]. It was experimentally shown that VEGF expression associated with oxidative stress, may be blocked by treatment with a HIF-1 inhibitor[46]. However, the potential clinical application is limited as other genes activated by HIF-1 have neuroprotective roles. Retinal hypoxia is also known to upregulate angiopoetin-2 (Ang-2) expression[47], which decreases the adhesion of

pericytes on endothelial cells[48] and may increase vascular permeability by interfering with the endothelial tight junctions[49].

4.2.4 Diabetic retinopathy and Inflammation

Inflammation is a nonspecific response of the innate immune system intending to protect the host after exposure to a pathogen. Inflammation is triggered when Toll-like or RAGE receptors become activated by binding to pathogen-associated molecular patterns (PAMPs). This process in turn activates nuclear factor kappa B (NF-κB), a transcription factor that increases the expression of pro-inflammatory cytokines, leading to mobilization of leukocytes[50]. While beneficial in the acute injury setting, inflammation becomes deleterious if persisting chronically. The hypothesis that inflammation could be associated with DR was first formulated based on reports that diabetic patients taking salicylates for rheumatoid arthritis had a lower-than-expected incidence of DR[51].In the specific case of DME, evidence from clinical studies has shown that treatment with intravitreal corticosteroids is effective in reducing macular edema[52] and this was interpreted as major evidence that inflammation plays an important role in DME. Additionally, there is evidence that inflammatory cytokines are increased in serum[53] and ocular samples (vitreous and aqueous humor) of patients with DR[54]. Experimental evidence showed that incubation of retinal cells in high glucose causes upregulation of proinflammatory factors such as iNOS, COX-2 and leukotrienes[55]. Also, macrophage depletion studies have shown that the presence of macrophages in the retina is necessary for the development of DR[56]. In fact, quantification by flow cytometry demonstrated a 2-fold increase monocyte/macrophage and microglia cells in retinas of diabetic animals in comparison with controls[57]. Leukocytes bind to ICAM-1 and VCAM expressed on the surface of endothelial cells, becoming adherent (leukostasis) and contribute to microvascular damage by releasing cytokines and also by physically occluding the capillaries, causing local ischemia[58]. However, the problem with uncovering the precise role of inflammation in DR, stems from the fact that no animal model can reliably replicate the effects of DR in the human retina. Nevertheless, recent advances in DME treatment, resulted from targeting inflammatory and angiogenic mediators. The fact that individual patient response to therapy varies, suggests that the vascular lesions of DR and the disruption of the inner BRB may result from a variable interplay of molecular factors, including:

a) Vascular Endothelial Growth Factor – A

The hypothesis that a soluble and diffusible growth factor was responsible for retinal neovascularization was first proposed by Michaelson in 1948[59]. It was not until 1989, when Ferrara and collaborators identified VEGF, a secreted pro-angiogenic factor regulated by hypoxia[60]. It was then shown that intraocular levels of such protein correlated with iris neovascularization *in vivo*[61]. Additionally, it was verified that VEGF injected into primate eyes caused vascular abnormalities similar to ischemic retinopathies, leading to breakdown of the BRB and increased fluorescein leakage[62]. Such findings ignited the research for therapeutic agents that could inhibit pathological intraocular VEGF expression. Nowadays it is well known that VEGF, produced in the Müller glial cells, promotes BRB permeability by increasing transcellular vesicular transport[63] as well as by downregulating occludin, a protein found in the endothelial tight junctions[64, 65]. Additionally, VEGF may also act as a proinflammatory cytokine, by increasing expression of ICAM-1 and TNFα[66, 67]. Furthermore, VEGF concentration is significantly increased the vitreous and the aqueous humor of DME patients[68-73]. In fact, Funatsu first reported that vitreous levels of VEGF correlated with central retinal thickness and had the strongest influence on the severity of DME[74]. However, another study reported a significant correlation between vitreous VEGF and OCT reflectivity of the subretinal fluid (SRF), but not with SRF height or width or even fluorescein angiography score[75]. Regarding aqueous VEGF concentration, a significant association with retinal macular thickness has also been reported[76]. Finally, regarding serum VEGF, higher concentrations have been reported in DR patients comparing with diabetics with no eye disease or healthy subjects[77]. In fact, a recent study verified that an increase of 100 pg/mL in serum VEGF concentration correlated with clinically-significant macular edema (CSME)[78]. Interestingly, a significant correlation has been reported between serum VEGF levels and the grade of external limiting membrane (ELM) and photoreceptor inner segment-outer segment disruption as assessed by SD-OCT[53]. Yet, as occurs with intraocular VEGF, there is conflicting evidence regarding the association of serum VEGF and DR[79]. Nevertheless, the contribution of VEGF to the pathogenesis of proliferative DR and DME led to the development and clinical use of various anti-VEGF treatment agents[80-82]. The groundbreaking efficacy of ranibizumab and aflibercept for both DME and proliferative DR[83, 84], is a testament to the crucial role of VEGF in DR severity.

b) Monocyte Chemoattractant Protein-1

MCP-1 is a pro-inflammatory chemokine that regulates tissue migration of monocytes and macrophages. Noticeably, MCP-1 is known to be produced by retinal endothelial cells and it is remarkably upregulated comparing with angiogenic factors, such as VEGF, angiopoietin (Ang)-2, and TNF-a[57]. Regarding the effect in the BRB, MCP-1 may contribute to increased phosphorylation of occludin[85], leading to dysfunction of the endothelial tight junctions. Furthermore, an indirect effect may result from increased monocyte trafficking into the retina, considering that activated monocytes differentiate into macrophages, which in turn secrete several cytokines and growth factors (VEGF, Ang-2, TNF-a, ILs, MMP-2, and MMP-9), all of which have been shown to alter the BRB[57].

Various studies have demonstrated a statistically significant correlation between the levels of vitreous or aqueous MCP-1 and the presence of DME[68, 71, 74, 86-88]. In fact, in experimentally induced diabetes, a significant reduction in retinal vascular leakage and monocyte infiltration was verified in MCP-1 knockout mice[57]. An important clinical consideration derives from a study by Sohn et al[86], revealing that aqueous MCP-1 was significantly elevated in patients with DME vs controls and the level of MCP-1 was significantly reduced following with intravitreal triamcinolone injection, resulting in a reduction of central macular thickness. However, bevacizumab had no effect on aqueous MCP-1 level. Serum MCP-1 level may also have an important association with complications of DR. In fact, a significant correlation was found between serum MCP-1 and both HbA1c and serum VEGF levels, more importantly a significant rise in MCP-1 level was associated with DR progression[77].

c) Intracellular Adhesion Molecule - 1

Intracellular Adhesion Molecule – 1 (ICAM-1) is a glycoprotein expressed on the surface of endothelial and immune system cells, which acts in the context of an inflammatory response to increase leucocyte migration and targeting in the extravascular space[89]. Soluble forms of adhesion molecules are detectable in the plasma and it is thought that their respective concentrations may reflect their level of expression on leucocytes and endothelial cells[90]. Increased ICAM-1 expression is potentiated by multiple aspects of DM pathogenesis. In fact, interaction of AGEs with RAGE on endothelial cells enhances the expression of various adhesion molecules (VCAM1, ICAM1 and E-selectin), stimulating leukocyte adherence to the endothelium[91]. Additionally, ICAM-1 is known to

be upregulated by VEGF, oxidative stress and dyslipidemia, at least in part via NF-κB activation[92]. Several studies have implicated ICAM-1 in the pathogenesis of DR[93-95]. In fact, ICAM-1 has been appointed as a critical mediator of retinal leukostasis[96], which is an early event in animal models of DR. Interestingly, diabetic mice deficient in ICAM-1 or its ligand (CD18) did not develop leukostasis or BRB breakdown[97]. Additionally, there is substantial evidence indicating an association between serum or intraocular ICAM-1 and the severity of DME. In fact, Jonas et al verified that while macular thickness was associated with aqueous level of various factors such as VEGF-A, MCP-1, IL-6, IL-8, in the multivariate analysis, ICAM-1 was the most significant factor[76]. A significant association has also been reported between submacular fluid height measured by OCT and concentration of aqueous ICAM-1[98]. Another study found that vitreous VEGF and ICAM-1 levels were the two factors most significantly associated with severity of DME[74]. Regarding serum ICAM-1, a significant independent association with HbA1c level was found in diabetic patients[99]. Additionally, an analysis of the DCCT trial population revealed an increased risk of retinal hard exudates with increasing quintiles of serum ICAM-1[100]. Van Hecke et al had previously demonstrated an increase in prevalence of DR with increasing terciles of plasmatic ICAM-1 level[101]. Additionally, the risk of DME is estimated to triple in individuals with a plasmatic level above 165pg/mL[102]. There is also a report indicating a significant correlation between disruption of the ELM and IS-OS junction with serum ICAM-1[53]. Interestingly, a recent meta-analysis on possible systemic biomarkers of DR, found that factors associated with leukocyte adhesion, namely ICAM-1, were consistently found to be significantly increased in patients with DR[103]. The possible association between systemic or ocular ICAM-1 and DR complications is relevant as it may have therapeutic implications. In fact, Tamura et al verified that intravitreal injection of dexamethasone in diabetic rats significantly decreased leukocyte accumulation by 31.6%, suppressed retinal vascular permeability and reduced ICAM-1 levels in the retina[104]. Years later, the first sustained-release intravitreal dexamethasone implant was found to be effective and therefore approved for treatment of DME[105].

d) TNF-a and other cytokines

Tumor necrosis factor alpha is a pro-inflammatory cytokine produced by activated macrophages and associated with the acute phase reaction. There is evidence that increased TNF-α plays a role in the development of insulin resistance and the pathogenesis of type 2 DM[106]. TNF-α has been shown to be elevated in the vitreous and serum of patients with PDR[107-109]. It was experimentally

verified that TNFα increases retinal endothelial permeability through downregulation of tight junction proteins[110]. Such observations led to the investigation of a possible association between TNF-α and DME. Interestingly, a recent study found that a 10 pg/mL increase in serum TNF-α, approximately doubled the risk of proliferative DR with clinically significant macular edema[78]. Another study, verified that aqueous levels of TNF-α were significantly elevated in the DME cases comparing with controls. Moreover, in the DME group, the serum and aqueous levels of TNF-α were found to be positively correlated[111]. Such observations led to a study evaluating the efficacy of intravitreal injections with TNFa-inhibitors (infliximab or adalimumab) for refractory DME, however, no significant therapeutic effects were seen[112].

Many other cytokines have been reported to be elevated in the fluid of eyes affected by DR compared to nondiabetic eyes. The most commonly reported include: interleukin-1β (IL-1β)[107], interleukin-6 (IL-6)[54], interleukin-8 (IL-8)[86, 113]and interferon-induced protein-10 (IP-10)[86, 113].

Elevated levels of IL-1β have been detected in the vitreous of diabetic patients with PDR[107]. However, a study on the efficacy of a selective IL-1β antibody, for treatment of proliferative DR showed stabilization but no regression of retinal neovascularization[114]. Interleukin 6 (IL-6) is known to induce acute phase reactions and increase vascular permeability. It has also been shown to promote angiogenesis specifically via induction of VEGF expression[115]. In fact, aqueous levels of IL-6 appear to increase with progression of DR from nonproliferative to active PDR[116]. There is also evidence that intraocular IL-6 may correlate with DME severity[117, 118,119]. Interestingly, recurrent DME has been associated with increased aqueous IL-6 but no effect was seen for VEGF level, suggesting that the pathogenesis leading to recurrent DME may be independent of VEGF[73]. Interleukin 8 (IL-8) is a potent chemoattractant, activator of neutrophils and T-lymphocytes[120]. It was verified to be produced by endothelial and glial cells in ischemic retina[121] and increased vitreous levels have been consistently associated with active PDR[113, 119, 122]. A number of studies have demonstrated that IL-8 is significantly elevated in the aqueous fluid of patients with DME[76, 123]. In this regard, it is important to note that aqueous levels of IL-8 seem to be unaffected by intravitreal injection of either bevacizumab or triamcinolone[86], suggesting that current treatments may not be addressing the contribution of IL-8 to the pathogenesis of DME. Regarding IP-10, there is evidence that it is significantly elevated in eyes of diabetic patients comparing with nondiabetics. Interestingly, IP-10 levels were significantly higher in eyes with PDR that had received PRP compared to eyes with PDR that had not yet been treated[119]. A previous report found that IP-10 was significantly elevated in the

vitreous of patients with inactive PDR, suggesting that this chemokine might be involved in the regression of PDR[113].

e) Angiopoietins

Angiopoietins are growth factors that bind to the endothelial receptor tyrosine kinase Tie-2 and regulate vascular function. Angiopoietin 1 (Ang-1) is a proangiogenic factor produced by pericytes and is responsible for the stabilization and maturation of growing blood vessels[124]. However, in a pathologic setting, endothelial cells produce angiopoietin 2 which competes with Ang-1 for binding to the Tie-2 receptor. When Ang-2 binds to Tie-2, vascular endothelium-cadherin, the protein responsible for the integrity of adherens junctions, becomes phosphorylated leading to increased vascular permeability[49]. There is also evidence that Ang-2 deficient mice fail to develop hyperglycemia-induced pericyte loss, whereas overexpression of Ang-2 induces pericyte migration and vascular pathology[48]. Also, in the presence of increased VEGF, Ang-2 may act as a promoter of angiogenesis[125]. In fact, production of Ang-2 seems to be upregulated by hypoxia, VEGF[126] and hyperglycemia[49]. There is also evidence that Ang-2 may promote adhesion of monocytes by modulating the expression of endothelial adhesion molecules[127]. The aforementioned actions are particularly relevant if we consider that concentrations of Ang-2 were reported to be twice as high as those of Ang-1, in the vitreous of patients with DME[128]. Therefore, the Ang-2 pathway has been targeted by means of a Tie-2 activator (AKB-9778) which may provide additional treatment benefit for DR and DME[129]. Recently the results of the multicenter trial on the efficacy of faricimab (a novel antibody targeting VEGF-A and angiopoietin2) for DME were revealed indicating statistically superior visual acuity gains versus ranibizumab, suggesting a benefit for simultaneous inhibition of angiopoietin-2 and VEGF-A in DME[130].

f) C-reactive protein

C-reactive protein (CRP) is an acute phase reactant produced in the liver and triggered by interleukin-6, secreted by macrophages in response to various inflammatory conditions. Its physiological role is to promote phagocytosis of necrotic or apoptotic cells and bacteria[131]. When inflammation occurs, CRP levels can rise up to 10,000-fold, however, once inflammation subsides, the concentration quickly falls indicating a plasmatic half-life of 19 hours. CRP concentrations

between 2 and 10 mg/L are considered representative of metabolic inflammation associated with arteriosclerosis and type 2 DM[132]. In fact, a high-sensitivity C-reactive protein (hsCRP) test was developed to measure low levels of CRP using laser nephelometry, and it is commonly used to assess risk of cardiovascular disease[133]. There has been increasing evidence of the association between CRP and DM. In fact, a large population study verified that the concentration of CRP was significantly higher in diabetic patients, and this result was maintained even after accounting for body mass index[134]. Another study found a significant association between increasing features of metabolic syndrome and CRP level[135]. CRP was also reported to be a powerful independent predictor of DM2 risk, even after adjustment for obesity and other clinical risk factors. Overall the evidence supports a possible role for inflammation in diabetogenesis[136]. In fact, Pickup and Crook hypothesized that DM could result from an ongoing acute phase response initiated by the innate immune system under the influence of stimuli such as overnutrition, occurring in predisposed individuals[137]. Finally, it is worth mentioning a meta-analysis which verified that the association between CRP and DM was more pronounced in Caucasians, particularly in the group carrying haplotype 4 of CRP. Thus, the authors concluded that genetic susceptibility to high serum CRP increases the risk of diabetes[138]. While the pathogenesis underlying the association of CRP with diabetes is not entirely clear, it has been shown that interaction of CRP with endothelial cells leads to decreased nitric oxide synthase expression and activity[139], contributing to compromised retinal endothelial function in terms of NO-mediated vasodilation[140]. Additionally, CRP may also contribute to a proatherogenic effect on vascular cells by upregulation of endothelial adhesion molecules ICAM 1 and VCAM1[141]. Interestingly, CRP level was found to be significantly elevated in diabetic patients with DR versus those without DR supporting an association between systemic inflammation and retinal microangiopathy[142] In fact, the Hoorn Study revealed that the prevalence of DR was associated with increasing terciles of CRP and soluble ICAM-1[101]. Regarding the incidence of CSME, an analysis of DCCT cohort indicated a significant association with increasing quintiles of baseline hsCRP and ICAM-1[100]. Additionally, there was a significantly increased risk of hard exudates with increasing quintiles of hsCRP and ICAM-1. The authors concluded that hsCRP could be a useful adjunct to other clinical information in order to predict the risk of developing CSME.

g) Homocysteine

Homocysteine is a potentially cytotoxic, nonprotein amino-acid, therefore requiring conversion to methionine. This process is regulated by an enzyme which uses folate and vitamin B12 as cofactors. An increase in plasma homocysteine can be caused by low folate intake, inadequate plasma concentrations of vitamin B or genetic factors. Hyperhomocysteinemia results in increasing production of oxidation products, which damage the vascular endothelium, through decreased bioavailability of nitric oxide, altered endothelial function, and enhanced thrombogenicity[143].

Elevated homocysteine levels have been associated with increased risk of DR in patients with DM2[144, 145]. In fact, a recent study even suggested that homocysteine could be a useful biomarker for severity of DR[146]. Recent evidence also suggests that plasma homocysteine concentration is independently associated with the occurrence of DME[147], and may even correlate with central macular thickness or average macular volume in DM2 patients without overt DME[148].

5 Complementary exams for staging and evaluation

5.1 Fluorescein Angiography

Fluorescein angiography (FA) is the gold-standard to assess the vascular integrity of the retina. It is based in the fluorescence phenomenon, that is the ability of a molecule to emit light of longer wavelength when stimulated by light of shorter wavelength. A fluorescein dye is used because it is approximately 80% bound to circulating proteins; meaning that in normal physiological conditions it does not diffuse through an intact inner BRB. The procedure requires intravenous injection of 5 mL of 10% fluorescein solution, normally in the antecubital vein. Fluorescence is first seen 15 seconds later, with the dye rapidly filling the choroid, optic disc and the retinal arteries. A few seconds later, fluorescein becomes visible in the retinal veins in a very characteristic laminar filling pattern. Peak fluorescence occurs at about 30 seconds (Figure 2). The foveal avascular zone appears as a dark circular shaped area due to the absence of retinal vessels, the presence of macular xanthophylls and melanin pigment from the RPE cells. The recirculation phase then follows, as the dye is removed from circulation, and the intensity of fluorescence decreases until about 10 minutes, when it becomes absent from the retinal vessels however, fluorescence may

still be observed due to staining of structures such as the optic disc, Bruch's membrane and the sclera. Abnormalities observed with FA are mainly grouped as hypofluorescent or hyperfluorescent. In the context of staging DR and evaluating DME the most relevant hypofluorescent findings are: vascular filling defects due to capillary closure resulting in retinal nonperfusion appearing as

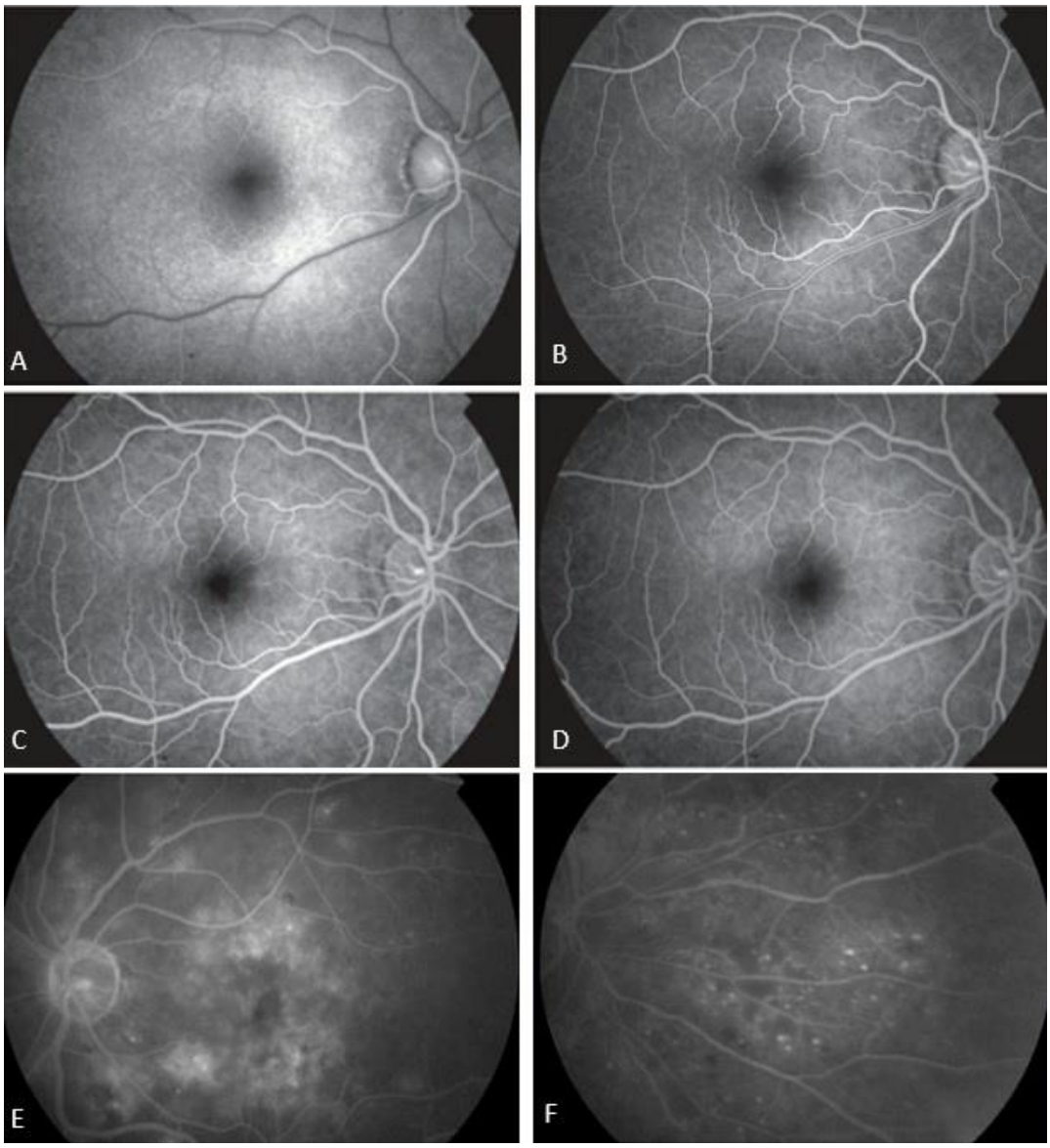

Figure 2. Fluorescein angiography (FA) of a healthy eye. A – Choroidal and retinal artery filling originate the first visible fluorescence. B - Vascular filling continues with characteristic laminar flow seen in the retinal veins. C - maximum fluorescence is seen at about 25 seconds. D - After a few minutes, fluorescence decreases as the dye is removed from the blood stream. E – FA of an eye with diabetic macular edema showing macular hyperfluorescence due to diffuse leakage of fluorescein dye. F – FA of an eye with severe nonproliferative diabetic retinopathy showing multiple areas of capillary dropout and nonperfusion, microaneurysms, blot hemorrhages and venous beading. Partially adapted from Basic & Clincal Science Course. Section 12 – Retina and Vitreous. American Academy of Ophthalmology 2018.

hypofluorescent areas of variable extent; hypofluorescence may also be due to blocked fluorescence caused by blot or flame-shaped retinal hemorrhages. Hyperfluorescence occurs due to leakage of fluorescein from incompetent blood vessels, creating a gradual increase of fluorescence with blurred margins over the affected area. It may appear in a focal pattern when due to leaking microaneurysms or retinal neovascularization sites; while diffuse macular leakage occurs when there is with extensive BRB breakdown. The combination of the aforementioned findings, namely the extent of retinal nonperfusion and the presence of retinal neovascularization will help the clinician assess the severity of DR, guiding the decision for subsequent panretinal LASER photocoagulation treatments. Despite being a valuable diagnostic modality, FA is not completely innocuous, in fact a few adverse reactions may occur varying from a temporary yellowing of the skin and conjunctiva, to nausea and vomiting (in approximately 5% of injections), urticaria (5% of injections) or

rarely severe anaphylactic reactions[149]. Considering the possibility of adverse effects, FA has become secondary to OCT for macular evaluation.

5.2 Spectral-Domain Optical Coherence Tomography

Optical coherence tomography (OCT) is a noninvasive diagnostic imaging modality which uses low-coherence infrared light scanned across the tissue and focused with an internal lens. An interferometer then combines the reflected light with the reference arm light, producing an interferogram, resulting in a bidimensional cross-sectional image of the retina. In modern devices the axial resolution is about 5–7 μm[150]. The addition of eye movement tracking, makes it possible to perform repeated scans at precisely coincident fundus locations, enabling rigorous monitoring of disease progression. In fact, the quality of retinal imaging provided by OCT is often referred to as an optical biopsy[151]. However, an actual correlation between OCT scans and histology of the retina has changed a few times. The currently accepted nomenclature (Figure 3) was proposed by The International Nomenclature of OCT Panel[152]. The use of OCT has greatly improved the diagnosis and characterization of several retinal diseases. In the case of macular edema certain morphological findings may provide insight into disease severity, such as: intraretinal cyst size, the integrity of both the external limiting membrane and the ellipsoid zone; disorganization of the retinal inner layers; presence of subretinal fluid; presence and number of hyperreflective foci and hard exudates, and finally the status of the vitreomacular interface[153]. Regarding DME, there has been

growing research into the possible role of OCT imaging biomarkers regarding clinical response to intravitreal anti-VEGF or steroid agents[154-157]. This theme will be further analyzed in the Discussion section of this doctoral dissertation.

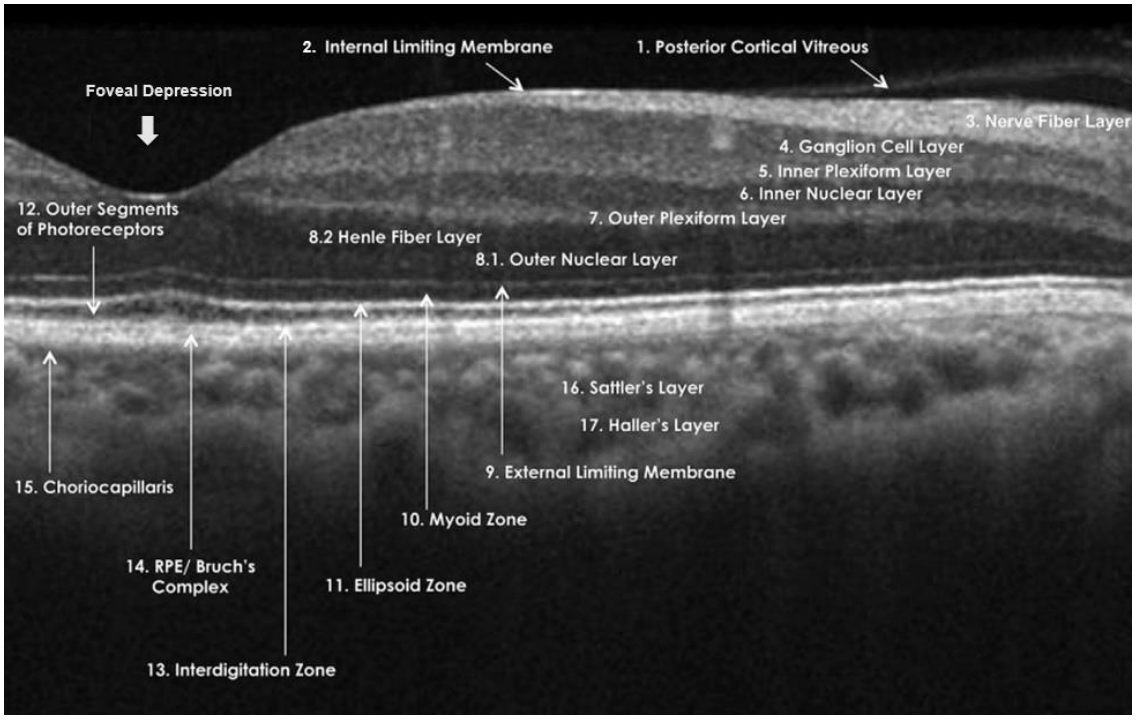

Figure 3. Currently accepted normal anatomical landmarks identified on spectral domain optical coherence tomography (SD-OCT). Adapted from the nomenclature proposed by the International Nomenclature for Optical Coherence Tomography Panel.

6. Treatment for Diabetic Macular Edema and Proliferative Diabetic Retinopathy

6.1 Systemic Medical Management for control of risk factors

Considering that DR is a local manifestation of a complex systemic disease, it is important to emphasize that good glycemic control is a crucial factor in the management of DR. In fact, both the DCCT Trial[13] and the UKPDS study[14] revealed that intensive glycemic control (HbA1c ≤ 7%) was associated with a reduced risk of retinopathy onset, as well as reduced progression of existing retinopathy. The DCCT results also indicated a reduced incidence of DME, and decreased need for panretinal or focal photocoagulation. Earlier initiation of intensive glycemic control was found to be more effective, particularly if complications had not yet developed. The benefits of intensive treatment persisted over at least 2 decades[158], such phenomenon has therefore been named

"metabolic memory". Based on the aforementioned results, diabetic patients are now treated with the goal of achieving an HbA1c level <7.0%. Another treatment regimen aiming to achieve HbA1c levels <6.0%, further slowed DR progression in patients with type 2 DM, however, this intensive regimen was associated with greater mortality rate therefore it is not recommended[159]. Interestingly, despite the importance of HbA1c levels in preventing or delaying progression of DR, the evidence regarding the effect of glucose control on clinical response to anti-VEGF treatment seems to be contradictory, as no significant effect was found in the *posthoc* analyses of the RIDE/RISE trials[160, 161] or protocol I[162], whereas in the recent analysis of Protocol T results, lower HbA1c levels were associated with greater improvement of visual acuity[163].

There is also evidence that hypertension is associated with a higher risk of progression of DR and DME. In fact, the UKPDS study showed a significant benefit of targeting a systolic blood pressure <150 vs. <180 mmHg, leading to reduced progression of DR and reduction of vision loss. Additionally, the FIELD Study, revealed that laser treatment was more frequent in patients with poor glycemic or blood pressure control. Also, regarding the effect of lipid profile, patients taking fenofibrate were significantly less likely to require an initial laser treatment[164]. Previously, ETDRS data had also suggested that lipid lowering may decrease the risk of hard exudate formation and vision loss in patients with DR[15]. As well as rate of progression to proliferative DR[165]. Therefore, in order to optimize treatment of DR it is important to account for the systemic component of DM, and patients should be managed by expert physicians in order to achieve the ideal glucose, lipid and blood pressure profiles.

6.2 LASER treatment for DME and PDR

Laser photocoagulation was the first approved treatment for both DME and proliferative DR. In fact, the results from the ETDRS trials revealed a significant reduction in the risk of moderate visual loss (>15 letters of VA) in cases with clinically-significant macular edema treated with focal LASER[166, 167]. The efficacy of LASER treatment has been attributed to the occlusion of leaking microaneurysms and destruction of ischemic retina, resulting in improved oxygenation of neighboring retinal areas and reduced proangiogenetic factors. However, only 3% of patients showed a VA improvement of at least 3 lines, while 12% lost 3 or more lines. Additionally, LASER treatment may be associated with impairment of color vision, night vision and contrast sensitivity, as well as visual-field sensitivity

deterioration[168, 169]. It is also important to consider the possible complications of LASER scars such as subretinal fibrosis[170] or choroidal neovascularization[171].

Nowadays, intravitreal anti-VEGF agents are considered the first-line treatment for DME, and there seems to be no benefit in combining injections with macular LASER treatment, in fact it seems that delaying the application of LASER as rescue therapy until 6 months may even allow for better visual acuity outcomes[172]. Therefore, LASER treatment has now mostly an adjunctive role mainly for treating non central edema. It still remains the standard of treatment for proliferative DR, particularly when not associated with active DME[173].

6.3 Anti-VEGF Agents for DME

Following the identification of VEGF-A as the main intraocular factor responsible for increased retinal neovascularization and BRB permeability, various anti-VEGF agents were researched for clinical use. The unmatched results regarding the improvement of VA and macular thickness led to the establishment of such drugs as first-line treatment for center-involving DME. There are currently 3 different VEGF inhibitors in clinical use. Their specific characteristics and clinical trial results are briefly described in the next subheaders:

6.3.1 Bevacizumab

Bevacizumab (Avastin®) is a 149 KDa full-length, monoclonal antibody that binds to and inhibits all VEGF isoforms. It is approved for systemic treatment of several cancer forms and is used "off label" for DME due to having a significantly lower cost comparing with the approved anti-VEGF agents. With the standardized dose of 1.25 mg in 0.05 mL, the reported mean half-life in human vitreous samples is 4.9 days[174], while in the aqueous is estimated at 9.82 days[175]. It is also important to consider the potential for systemic diffusion with intraocular bevacizumab leading to significantly reduced plasma levels of VEGF up to 4 weeks after a single intravitreal injection[176]. Additionally, systemic accumulation with repeated monthly dosing has been reported[177]. Such findings are relevant because despite the good safety profile associated with intravitreal anti-VEGF treatment, there are concerns about the possibility of serious cardiovascular events[178, 179]. The only randomized controlled trial evaluating bevacizumab for DME indicated a median improvement of 9 letters which was significantly higher than the 2.5 letters of improvement verified in the LASER group[180]. Recent

data, comparing the three currently used anti-VEGF agents, seems to indicate that bevacizumab provides slightly inferior outcomes while requiring a higher number of injections, when comparing with either ranibizumab or aflibercept[181, 182]. Therefore, despite the more favorable economic profile of bevacizumab, it is important to account for the possible lower efficacy in cases with worse initial VA.

6.3.2 Ranibizumab

Ranibizumab (Lucentis®) is a 48 KDa antigen-binding fragment (Fab) derived from bevacizumab, and developed specifically for intravitreal administration. Thus, ranibizumab has a higher affinity for all VEGF-A isoforms comparing with that of bevacizumab. There is no data regarding the vitreous half-life of ranibizumab in humans, however a value of 4.75 days has been derived from a mathematical model[183]. The measured half-life in human aqueous samples was determined to be 7.19 days[184]. Interestingly, no significant reductions were observed in the concentration of systemic VEGF following intravitreal ranibizumab[177]. Several studies reported the efficacy and superiority of ranibizumab versus laser photocoagulation for DME. In the RESTORE study, ranibizumab was given monthly during the first 3 months then *pro re nata* according to defined retreatment criteria. At month 12, the mean VA improvement in both ranibizumab groups (monotherapy and combined with laser) was significantly better than the laser monotherapy group (approximately +6 vs +0.8 letters). A 15 letter VA gain was seen in 22% of ranibizumab cases vs. 8.2% of laser treated cases. The RESTORE trial was also one of the first studies to verify that there was no advantage in combining LASER treatment and ranibizumab injections[185]. Similar results were found in the DRCR.net Protocol I study, with a VA gain of +9 letters in the ranibizumab arms comparing with +3 letters in the LASER group. Additionally, a VA improvement of at least 15 letters was verified in 30% of ranibizumab cases vs. 15% of LASER cases[186]. A significant observation in both RESTORE and Protocol I is that there seems to be a tendency for maintaining outcomes with significantly less injections in the third year of follow-up[187, 188]. The most significant VA outcomes were verified in the RIDE/RISE trials, which evaluated ranibizumab treatment in a fixed monthly regimen[80]. The results indicated a mean VA gain in the range of +10.9 to +12.5 letters; and a >15-letter gain was verified in about 40% of ranibizumab treated cases. It is important to notice that delayed initiation of ranibizumab treatment did not result in the same level of VA improvement as in promptly treated patients[189]. An open-label extension of the RIDE/RISE trial allowed patients to be followed with

ranibizumab treatment in a *pro re nata* (PRN) strategy. Interestingly, while on average 4.5 injections were required to maintain the 3-year VA outcomes, about 25% of patients required no injections whatsoever in this follow-up period[190], indicating significant variability in long-term response to anti-VEGF treatment. Despite the observation that PRN treatment regimens seem to provide inferior vision gains comparing to prolonged fixed monthly dosing, the current consensus suggests that fixed monthly injections, may not be required for a period of more than 3–6 months[172, 187, 189, 191]

6.3.3 Aflibercept

Aflibercept (Eylea®) is a 115 KDa recombinant fusion protein composed of key domains from human VEGF receptors 1 and 2 fused to the Fc domain of human immunoglobulin G1. It has approximately 100-fold greater binding affinity to VEGF-A than either bevacizumab or ranibizumab. Regarding pharmacokinetics, a vitreous half-life of 7.13 days has been calculated[183], whereas a study conducted in five patients with AMD reported an aqueous half-life of approximately 9 days[192]. It should be noted that as with bevacizumab, systemic accumulation of aflibercept occurs with repeated monthly injections. Additionally, in a comparative study including the three anti-VEGF agents, aflibercept caused the greatest reductions in plasma free-VEGF relative to baseline levels.[193] The efficacy of aflibercept for DME was documented in the VIVID / VISTA trials[194] in which aflibercept was given every 4 weeks or every 8 weeks after 5 initial monthly loading doses. The mean VA gain at 1 year for the combined studies was +11.6 letters for 4-weekly injections, and +10.7 letters for 8-weekly injections, VA was stable after 100 weeks in both groups[195]. Similar to what had been verified for ranibizumab, eyes initially treated with LASER who switched to aflibercept had a gradual increase in VA, but did not reach the same level of improvement as promptly treated cases. The DRCR.net Protocol T study which compared all 3 currently used anti-VEGF agents, indicated that at one year of follow-up, aflibercept provided significantly better VA results in cases with VA below 20/50 (69 letters)[181], however the difference towards ranibizumab was attenuated in the second year of follow-up[182]. It is also interesting to recall that in Protocol T, and for all anti-VEGF agents, about half of the injections given in the first year were needed in the second year of treatment. The evidence regarding aflibercept seems to suggest the possibility of significant VA gains with reduced injection frequency, particularly after the first year of treatment.

6.4 Steroids for DME

The rationale for the use of steroids in DME is associated with the increasing evidence that chronic inflammation plays a role in the pathogenesis of DR, namely the importance of leukostasis in the disruption of the inner BRB[196]. Whereas VEGF inhibitors act to reduce excess vascular permeability by acting only on VEGF-A, corticosteroids act on several pathways of DME pathogenesis, such as: decreasing leukostasis by inhibiting ICAM-1 expression[104], reducing the action of several inflammatory cytokines[86], and enhancing barrier integrity of retinal endothelial cells by upregulating claudin-5 and occludin expression[197]. Despite the robust anti-inflammatory action, there are significant concerns relating to intravitreal corticosteroid use, namely the high rates of cataract formation and increased risk of increased intraocular pressure (IOP). Such concerns mean that these agents are reserved for second-line use in cases poorly responsive to anti-VEGF treatment. Exceptionally, they could be considered as first-line treatment for cases with recent history of major cardiovascular event[198]. The currently used steroids are: the "off-label" triamcinolone acetonide, and the sustained delivery implants of dexamethasone and fluocinolone acetonide.

6.4.1 Triamcinolone Acetonide

Triamcinolone acetonide (TA) is a synthetic, water insoluble corticosteroid approved in the USA for intraocular inflammation not responsive to topical medication, but no commercially available formulation of TA was given approval in Europe. Pharmacokinetic data indicates that vitreous concentration is highest in the first day, then elimination occurs with an exponential decrease within the first 4 weeks, followed by a steady decline over the next few months[199]. The reported elimination half-life is 18.6 days in non-vitrectomized eyes, suggesting that measurable triamcinolone in the vitreous may last for up to 3 months (93 ± 28 days)[200]. The major study evaluating the effect of intravitreal TA was the DRCR.net Protocol B. Patients were randomized to laser therapy, 1 mg TA, or 4 mg TA and retreatment was given at 4-month intervals. By the end of the first year there were no significant differences among the groups regarding VA gains. However, from the 16th month onwards, VA was superior in the LASER group. Such result could be explained by the fact that the majority of TA-treated eyes developed cataract at the second year, additionally 40% of eyes in the 4 mg group had an ocular hypertension-related event, and 4 eyes required glaucoma surgery[201]. Interestingly, DRCR.net Protocol I data indicated that in the group of

pseudophakic patients treated with TA, VA gains were comparable to those of pseudophakic eyes treated with ranibizumab and superior to that of pseudophakic eyes treated with laser only. Yet once again, eyes treated with TA were significantly more likely to have a significant IOP elevation and 59% required cataract surgery[186].

6.4.2 Dexamethasone

Dexamethasone is a less potent corticosteroid than TA and has a higher solubility in water, therefore to achieve therapeutic doses it requires a slow release system. It is commercially available as an intravitreal bioerodible implant delivered by a 22-gauge injector (Ozurdex®). Pharmacokinetic studies indicate that this formulation releases with a significant burst for approximately 2 months, followed by an exponential decline in release. After 6 months, DEX was below the detection limits of the assay[202]. The approval of intravitreal dexamethasone implant for DME was based on the results of the MEAD trial, in which patients were randomized to receive Ozurdex (0.7 or 0.35 mg) with a follow-up of 3 years. Retreatment was possible at 6-month intervals according clinical criteria. At the end of 3 years, the percentage of patients with a ≥15-letter gain of VA from baseline was 22.2/18.4% in the Ozurdex 0.7/0.35 mg group versus 12% in the sham group. An IOP increase of ≥10 mm Hg occurred in 27.7/24.8% of patients in the 0.7/0.35 mg Ozurdex groups. There was a significant rate of cataract-related events in the Ozurdex groups (64.1-67.9)[105]. It is estimated that dexamethasone is released in the vitreous for up to 6 months[202], however re-injection may be needed before that time interval. In fact, in the CHROME study, which included patients with DME, retinal vein occlusion, and uveitis, the mean re-injection interval was 2.3–4.9 months[203]. The fact that steroids lead to decreased levels of several pro-inflammatory factors and the prolonged action of dexamethasone implant would make it ideally suitable for treating chronic and persistent DME. Such potential was evaluated in the DRCR.net Protocol U, in which cases with persistent DME were eligible for combined treatment with dexamethasone implant. No significant difference was found in mean improvement of VA and the authors concluded that combination did not provide additional benefit. Yet, it should be noted that patients in the combination group were significantly more likely to achieve a normal central subfield thickness (CST), also mean CST change was significantly superior in the combination group[204]. At least one other study on DME patients unresponsive to anti-VEGF injections, indicated a significant improvement in both VA improvement and macular thickness in cases switched to dexamethasone implant[205], therefore the hypothesis of a favorable

effect for the combination of anti-VEGF and steroid treatments in patients with persistent DME, remains viable. Ozurdex 0.7 mg was first approved by the EMA in July 2014 for the treatment of patients with DME who are pseudophakic and considered insufficiently responsive to noncorticosteroid therapy.

6.4.3 Fluocinolone Acetonide

Fluocinolone acetonide (FAc) is more potent than TA, but also has a higher solubility in water, therefore it requires a sustained delivery system for efficient therapeutic dosing. It is commercially available in the form of a nonbioerodible implant injected in the posterior segment via a 25G needle (Illuvien®). Pharmacokinetic data indicates that the overall burst concentration of fluocinolone in the vitreous is orders of magnitude lower than that verified with dexamethasone implant or TA injection (1–20 ng/g for FAc vs. >1100 ng/g for DEX, >10,000 ng/g for TA)[206], but drug release in the vitreous may last for up to three years[207]. Such characteristics make the FAc particularly suitable for chronic macular edema. The evidence for the use of the FAc implant is based on the results of the FAME trial, in which patients with DME, received an intravitreal insert releasing 0.2 or 0.5 μg of fluocinolone per day. Additional treatment was allowed after one year and according to predefined criteria. The percentage of patients with a VA gain of ≥15 letters after 2 years was 28% in the fluocinolone groups versus 16% in the sham group[208]. However, such result was mainly due to the effect seen in cases with chronic DME (considered as DME persisting ≥ 3 years from diagnosis), while in the cases with acute DME the difference with sham treatment was not significant. Such finding is particularly worrisome if we consider that the rate of glaucoma surgery in both treatment groups was high at 7.6/3.7% in the 0.5/0.2 μg groups, respectively. Cataract surgery was needed in 50.9/41.1% of patients in the 0.5/0.2 μg groups[209]. Illuvien was first approved by the EMA in April 2014, for the treatment of chronic DME considered insufficiently responsive to other available therapies. The FDA granted the approval in September 2014 for DME patients who were previously treated with corticosteroids and did not have a clinically significant elevation of IOP.

7. Limitations associated with current treatment options for DME

The introduction of anti-VEGF agents into clinical practice allowed unprecedented clinical outcomes. Nevertheless, in order to achieve optimal treatment benefit, as seen in clinical trials, serial intravitreal injections are required, often in a monthly basis. Such high frequency of treatments and follow-up visits, may not be readily applicable in a real-world clinical setting, due to variable logistical constraints or patient compliance, leading to outcomes substantially inferior to the ones seen in randomized clinical trials. In fact, a retrospective cohort study in the USA revealed that the mean number of bevacizumab injections received by DME patients over the first year of treatment is only 2 to 4[210]. Another study reported that 69% of eyes received 3 or fewer anti-VEGF injections over the 12-month study period, leading to a mean visual acuity change of +4.7 letters[211], which is quite lower than the vision gains reported in randomized studies. One of the most recent real-world studies[212], reported a mean of 5.7±2 injections given in the first year of treatment, resulting in +5 letters of VA improvement after one year, which again is inferior to the outcomes of clinical trials[80, 185, 186]. Additionally, in the aforementioned study, 18.8% of cases did not show a significant anatomic response during the entire study period. Even in the context of randomized trials it is worth noting that the proportion of ranibizumab-treated patients gaining at least 3 lines of VA was 36.8% to 51.2% in RIDE/RISE at 36 months[189], while in the VISTA and VIVID studies, 35.8% to 42.9% of aflibercept-treated patients obtained such outcome at week 148[213]. That is, less than 50% of patients will achieve 3 lines of VA improvement. Additionally, the anatomic response to anti-VEGF therapy is often incomplete, with 20% to 65% of eyes failing to achieve resolution of retinal thickening, depending on the treatment agent[214]. Considering the possible negative effects of prolonged DME on visual function[215], it is of the utmost importance to identify specific ocular or systemic characteristics that would allow timely consideration of additional treatment strategies in this subgroup of patients, for whom continued anti-VEGF therapy will likely result in limited improvement. In this regard, it is interesting to look at data from major randomized trials, in order to identify possible variables related to functional or anatomical outcomes. Indeed, a *posthoc* analysis of Protocol I data, verified that 39.7% of treated eyes showed < 5-letter improvement in VA at 12 weeks and there was a significant correlation between VA response at 12 weeks and that seen from 52 weeks onward. The group of patients with <5-letter improvement were older, had better baseline VA and a lower baseline CRT than those with ≥ 10-letter improvement in VA[216]. A similar analysis of the Protocol T population, revealed that, eyes with less VA gain at 12 weeks had

lower VA letter score at 2 years. However, only about 25-50% of the variability in vision outcomes was explained by the combined effects of baseline VA and CST. The authors concluded that a suboptimal response at 12 weeks did not preclude further vision gains[217]. When analyzing the possible factors associated with VA outcomes, a multivariable model revealed that younger age, lower hemoglobin A1c levels, and the absence of prior panretinal photocoagulation were associated with better VA gains at 2 years[163]. The effect of age was also verified in the RIDE/RISE trials, as for every 5-year increase in participant age, the odds of obtaining a 15 letter gain decreased (odds ratio=0.88)[160].

Regarding possible factors associated with anatomic response, a recent Protocol I analysis revealed a significant correlation between a limited third-month central retinal thickness (CRT) response (<20%), and the same outcome occurring at week 52. The group with a ≥ 20% CRT reduction at week 12 were found to be significantly younger (mean 61.5 vs. 65.0 years of age), and also had significantly lower baseline VA (mean 61.6 vs. 65.4 ETDRS letters), and higher CRT (433 vs 345 mm)[218]. On the other hand, a report on the RIDE / RISE trials, verified that delayed responders tended to have significantly thinner retinas, slightly better VA, and lower DR severity at baseline[219].

Regarding persistent DME (> 6 months despite treatment), a *posthoc* analysis of Protocol I revealed that of all eyes with persistent DME after 24 weeks of anti-VEGF therapy, 40.1% will maintain DME at 3 years. Such eyes had greater baseline CST and macular volume[220]. A similar analysis regarding the protocol T study verified that of all the eyes with persistent DME at 6 months, the cumulative probability for persistent DME at 2 years was 44.2% for aflibercept, 68.2% for a bevacizumab, and 54.5% for ranibizumab[214]. Such result means that even under rigorous clinical trial monitoring and treatment scheduling, eyes with persistent DME, tend to maintain their anatomic response profile. This finding is of clinical relevance, because it was also verified that at 24 weeks, mean improvement in visual acuity was greater in eyes without persistent DME comparing with those with persistent DME. It is also interesting to note that there seem to be four main anatomic response patterns verified in Protocol I, according to whether a significant macular response was observed at the 16 week and if such response was maintained at the later follow-up visits, the possible responses were classified as: early and consistent; early but inconsistent; slow and variable; and non-responder. However, the authors could not identify variables that were predictive of such response patterns. Considering that cases with early and consistent response had approximately six letters more improvement compared to the non-responder group[162], it may be of clinical relevance to understand the factors that are responsible for such clinical differences under

the same treatment protocol. The current consensus is that there is no clear way to identify on first presentation how a patient will respond to treatment in terms of anatomic or functional clinical response. However, if that were possible, these patients could be promptly selected for an optimized treatment strategy, which could include close monthly follow-up, prolonged monthly treatment or even adopting a combination of treatment agents. Considering the growing evidence that the pathogenesis of DME exhibits features of a chronic inflammatory process, and patients with severe manifestations of DR have increased systemic or ocular levels of pro-inflammatory factors, it is possible that different biochemical profiles are associated with different response patterns to anti-VEGF treatment. Therefore, a thoughtful investigation of such hypothesis has the potential to provide further insight into the management of DME and possibly lead to optimized treatment strategies.

8. Rationale and Aims

Considering the variability in individual patient response to anti-VEGF treatment for DME, it is likely that certain ocular or systemic factors may modulate disease aspects such as severity, recurrence and chronicity. Additionally, there is ample evidence regarding increased levels of angiogenic and pro-inflammatory factors found in the serum, aqueous and vitreous humor of patients with DR and DME. Interestingly, the most consistently reported factors (ICAM-1, MCP-1, TNFAα and CRP) are related to leukocyte differentiation, migration and accumulation, which in the context of diabetic microangiopathy, leads to increased leukostasis in the retinal capillaries. However, there was a distinct lack of studies analyzing the possible associations between such molecular factors and clinical response of DME patients under treatment with anti-VEGF. While the intraocular concentration of such factors may be more accurate representation of DR pathogenesis than systemic levels, there are obvious ethical concerns regarding the collection of ocular fluid samples. Considering the previously cited studies suggesting a correlation between serum levels of certain molecular factors (VEGF-A, ICAM-1, MCP-1, TNF-α) and manifestations of DR, the main objective of the present dissertation was to study the role of metabolic and pro-inflammatory biomarkers associated with DR, as possible limiting factors of the clinical response to intravitreal anti-VEGF agents for DME. To accomplish the main objective the research process aimed to provide answers to the following questions:

1. Is there any significant association between systemic metabolic or pro-inflammatory factors with the occurrence of an early limited anatomic response to anti-VEGF treatment?
2. Are there any significant correlations between systemic pro-inflammatory factor levels and intravitreal injection patterns, namely is there any significant difference among patients achieving early stability with anti-VEGF monotherapy versus those requiring either continued treatment or steroid combination treatment?
3. Is there an association between baseline serum pro-inflammatory factors and long term (≥ 1 year) qualitative or quantitative indicators of functional and anatomic response to anti-VEGF treatment for DME?

CHAPTER II – RESULTS

9. SEROLOGICAL INFLAMMATORY FACTORS AS BIOMARKERS FOR ANATOMIC RESPONSE IN DIABETIC MACULAR EDEMA TREATED WITH ANTI-VEGF

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SEROLOGICAL INFLAMMATORY FACTORS AS BIOMARKERS FOR ANATOMIC RESPONSE IN DIABETIC MACULAR EDEMA TREATED WITH ANTI-VEGF

Systemic biomarkers in diabetic macular edema

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Abstract

Purpose: To study the relationship between systemic pro-inflammatory factors and macular structural response to intravitreal bevacizumab for diabetic macular edema (DME)

Methods: Prospective study including 30 cases with DME, treated with bevacizumab and a minimum follow-up of 6 months. All cases underwent baseline laboratory testing for cardiovascular risk (high sensitivity C-reactive protein (hsCRP), homocystein), dyslipidemia, renal dysfunction and glucose control. Serum levels of VEGF, soluble ICAM-1, MCP-1 and TNF-α were assessed by enzyme-linked immunosorbent assay kits. Significant associations between systemic factors and quantitative and qualitative spectral-domain optical coherence macular features were analyzed

Results: A mean of 4.82±0.56 intravitreal injections was performed, resulting in significant improvement of central foveal thickness (CFT) ($p<0.001$). A significant association with third month CFT decrease < 10% was found for hsCRP (3.33±2.01 vs 1.39±1.15 mg/L, $p=0.007$) and ICAM1 (975.54±265.49 vs 727.07±336.09 pg/ml, $p=0.012$). ROC curve analysis indicated hsCRP and ICAM1 as significant biomarkers for 3rd month reduced anatomic response (area under the curve (AUC)=0.807, $p=0.009$ for hsCRP; AUC=0.788, $p=0.014$ for ICAM1). ROC curve analysis revealed hsCRP as a significant biomarker for 6th month CFT decrease < 10% (AUC=0.903, $p<0.001$, cutoff value=1.81 mg/L). A significant association with 6th month CFT decrease ≥ 25% was found for serum MCP1 (244.69±49.34 pg/ml vs 319.24±94.88 pg/ml, $p=0.017$) and serum VEGF (90.84±37.33 vs 58.28±25.19, $p=0.027$). The combined model of serum VEGF and LDL-cholesterol was found to be predictive of 6th month hard exsudate severity ($p=0.001$, $r2=0.463$)

Conclusions: Increased levels of hsCRP and ICAM1 were found to be significant biomarkers for early reduced anatomic response to anti-VEGF treatment. Cases with higher serum levels of such factors had increased CFT values, despite treatment, suggesting inner blood-retinal barrier breakdown that is not adequately responsive to anti-VEGF monotherapy.

Keywords

Anti-VEGF, Bevacizumab, C-Reactive Protein, Diabetic Macular Edema, Inflammatory biomarkers

Introduction

Diabetic macular edema (DME) is a major cause of visual impairment in industrialized countries due to increasing prevalence of Diabetes Mellitus[1]. Pivotal trials, revealed that monthly intravitreal injections of vascular endothelial growth factor (VEGF) inhibitors, allowed DME cases to achieve significant gains in VA[2, 3], therefore such agents have become the gold standard treatment for DME. However, a significant percentage of patients won't achieve a sustained response to anti-VEGF injections[4, 5]. Additionally, there is a wide variability in the frequency of injections and duration of treatment required to achieve clinical stability[6, 7]. Therefore, there is increased interest in finding significant predictors of clinical response. While the pathogenesis of DME is not fully understood, it is known that prolonged hyperglycemia leads to activation of pro-inflammatory cascades that in turn cause structural damage to the capillary endothelium[8]. The end result is capillary obliteration leading to retinal ischemia[9] and inner blood-retina-barrier (iBRB) breakdown causing intraretinal fluid accumulation[10]. Indeed, the pioneering studies DCCT[11] and UKPDS[12] identified the importance of metabolic control in delaying DR. More recently, attention shifted to identifying the molecular agents underlying the pathogenesis of DR. It is now well known that VEGF plays a crucial role in retinal angiogenesis and vasopermeability[13, 14]. Additionally, there seems to be increasing evidence that insulin resistance occurring in DM2 is associated with a subclinical pro-inflammatory state identified by increased levels of C-reactive protein and Interleukin-6 (IL6)[15-17]. In the case of diabetic eye disease, there is evidence of increased levels of VEGF, IL6[18], Intracellular Adhesion Molecule 1 (ICAM1)[19] and Monocyte chemoattractant protein-1 (MCP1)[20] in the aqueous or vitreous of patients with advanced DR. Despite, the increasing evidence of pro-inflammatory activity occurring in DR, there is little research on how such biochemical factors can potentially interfere with macular response to current intravitreal treatments. Considering DME is a local manifestation of a complex systemic disease, it is possible that different metabolic and pro-inflammatory profiles portend different diabetic eye disease patterns. In this regard, we evaluated several systemic factors encompassing metabolic, renal, cardiovascular and inflammatory functions known to be associated with the pathogenesis of DM and/or DR, in order to study the possible associations with tomographic features of DME, and to assess their value as possible biomarkers for anatomic response to anti-VEGF treatment.

Methods

This was a prospective interventional study including cases diagnosed and treated for DME at the Ophthalmology Department of Hospital de Braga, Braga, Portugal. Study protocols were submitted to and approved by the Hospital Ethics Committee. The research procedures followed the tenets of the declaration of Helsinki and all patients provided written informed consent for the inclusion in the study. The recruited cases had Diabetes Mellitus type 2 (DM2) and nonproliferative diabetic retinopathy (NPDR) with central-involved diabetic macular edema (DME). All cases underwent complete ophthalmological examination and diagnosis was confirmed by fluorescein angiography and spectral-domain-optical coherence tomography (SD-OCT). The severity of NPDR and foveal involvement was graded based on fluorescein

angiography findings, according to the international clinical diabetic retinopathy and diabetic macular edema disease severity scales[21]. The following inclusion criteria were considered: cases with DM2, NPDR and central DME with CFT > 330 µm and intraretinal cysts in the foveal area. The exclusion criteria included: history of any other vision impairing ocular disease, history of retinal laser treatment or intravitreous injection, as well as those previously submitted to vitreoretinal surgery were excluded. Regarding systemic history, the could be no record of cardiovascular events in the preceding year, no known history of chronic infectious, inflammatory or malignant disease, and no surgical procedures or hospital admissions of any kind in the preceding 6 months.

All recruited cases underwent treatment with intravitreous bevacizumab (BVZ) injections (1.25 mg/0.05 cc) following the strategy of 3 monthly injections plus *pro re nata* treatment according to tomographic criteria, namely persistent central subfield thickness > 330 µm, with identifiable intraretinal cystic lesions and / or subretinal fluid.

Blood samples were taken at baseline to evaluate the following systemic markers: cardiovascular risk (high sensitivity C-reactive protein, serum homocysteine), renal dysfunction (blood urea nitrogen (BUN), serum creatinine), hypercholesterolemia (low-density lipoprotein (LDL), high-density lipoprotein (HDL) and total cholesterol (T.Chol)) and diabetic profile (glycated hemoglobin, blood glucose level). Additionally, for each case a blood sample was obtained and centrifuged at 1000G to isolate the serum fraction which was then immediately stored at -80ºC, for posterior dosing of the following pro-inflammatory cytokines: VEGF, sICAM-1, MCP-1 and TNF-α, by enzyme-linked immunosorbent assay (ELISA) using the specific ELISA kits, procured from Sigma-Aldrich®. All procedures were performed according to the manufacturer's protocol. Samples were diluted accordingly, to comply with the detection range of the relevant assay. Color intensities were determined using a microplate reader. Duplicate samples were used in all assays. The level of each factor in serum was within the detection range of the relevant assay.

SD-OCT images were obtained with the Spectralis OCT (Heidelberg Engineering, Heidelberg, Germany) using the following acquisition protocol: a 30º horizontal foveal scan resulting from the averaging of 100 frames, and a 20x20º macular square consisting of 25 individual horizontal scans, resulting from the averaging of a minimum of 20 frames. The value obtained in the 1mm central ring of the macular thickness map was considered the central foveal thickness (CFT). In cases with bilateral DME, the eye with highest CFT was chosen for this study.

The main outcome measures were: change from baseline in CFT (µm) and macular volume (mm^3), obtaining a CFT < 330 µm, achieving a CFT decrease ≥ 25% or a CFT decrease < 10% of baseline CFT. Also, regarding OCT analysis, three of the authors (PB, JC, RS) blinded to clinical records, registered and/or graded the following qualitative findings in the 5 central scans: subretinal fluid, largest intraretinal cyst (IRC) height (Figure 1), disruption of the ellipsoid zone (EZ) and presence of hyperreflective spots (HRS). The presence of HRS was graded based on the most common tomographic patterns, according to the following criteria: Grade 1 – few small clearly spaced HRS; Grade 2 – larger rounded HRS clumped closer together, some could cause faint shadowing in the underlying retinal layers; Grade 3 – even larger rounded HRS forming plaque like hyperreflective lesions with significant shadowing of the underlying retinal layers (Figure 2). Disruption of the EZ band was classified as grade 0 if there was continuous reflectivity in the 5 central scans; grade 1 if minor disruption (≤200 µm) was present; and grade 2 if at least one large (>200 µm) disruption of the EZ was found[22].

The systemic blood/serum concentrations of measured metabolic and pro-inflammatory factors were assessed for predictive associations with the previously listed anatomic response parameters at the 3rd month and 6th month of follow-up. Such time points were chosen because they correspond respectively to the moment in which all cases had received the mandatory loading dose of BVZ (3rd month) and the moment at which persistent DME is considered to be poorly responding to anti-VEGF treatment (6th month)[4, 23]. All recorded results were subject to statistical analysis using the IBM SPSS Statistics software (SPSS version 23, IBM Corporation). Numerical values are expressed as mean±standard deviation. The study of possible interactions between variables was performed by Spearman 2-tailed correlations. Predictive associations were studied by ordinary least squares regression and bivariate logistic regression. The value of significant systemic factors as biomarkers was further studied by ROC curve analysis. Categorical variables were assessed using chi-square test and numerical variables were compared by Mann-Whitney-U or Kruskal Wallis tests. Statistical significance was considered for $p < 0.05$.

Results

The study included 30 consecutive patients diagnosed with DME requiring treatment with intravitreal anti-VEGF injections. Mean patient age was 66.76±9.36 years and mean duration of DM2 was 17.20±6.85 years. Thirteen patients were on insulin therapy for a mean of 7.23±6.91 years. All cases had NPDR, classified as moderate in 17 cases and severe in 13 cases. There was a significant association between NPDR severity and serum VEGF level; cases with severe NPDR had higher circulating VEGF than those with moderate NPDR (92.25±37.70 vs 59.42±25.87 pg/ml, p=0.014) (Table 1). The mean baseline CFT value was 519.63±119.26 µm, improving to 401.53±167.40 µm at the 6th month (p<0.001) (Figure 3). The mean CFT change from baseline was -135.31±139.76 at the 3rd month and -113.50±131.11 at the 6th month. Mean macular volume followed a similar improvement profile (11.08±2.22 mm^3 at baseline improving to 9.83±2.00 mm^3 at the 6th month, p<0.001). Between the 3rd and 6th month visit, 8 cases had an increase in CFT, but the difference did not reach statistical significance (p=0.089 for CFT and p=0.061 for MV). A favorable anatomic response (≥ 25% decrease in CFT) was found in 13 cases (43.3%) at the 6th month. On the other end, a limited anatomic response (< 10% decrease in CFT) was found in 10 cases (33.3%) at the 6th month. Finally a CFT < 330 µm was verified in 10 cases at the 6th month (33.3%). Such results were obtained with a mean of 4.82±0.56 intravitreal bevacizumab injections.

Systemic factors and quantitative macular outcomes

No significant associations were found between systemic factors and baseline CFT or MV. At the third month of follow-up significant associations were found between both baseline hsCRP (3.33±2.01 vs 1.39±1.15 mg/L, p=0.007) and ICAM1 (975.54±265.49 vs 727.07±336.09 pg/ml, p=0.012) with obtaining a CFT decrease < 10% (Table 2). Logistic regression revealed a predictive association between hsCRP and CFT decrease < 10% (p=0.014, R^2=0.249, odds ratio=2.17, confidence interval 95%, 1.17-4.04) and such association was independent of DM related variables such as duration of disease, blood glucose level and HbA1c percentage. On the other end of the anatomic response spectrum, a lower serum MCP1 level was significantly associated with a CFT decrease ≥ 25% (242.42±48.96 vs 315.93±93.14, p=0.015). ROC curve

analysis indicated hsCRP and ICAM1 as significant biomarkers for 3rd month reduced anatomic response (area under the curve (AUC)=0.807, p=0.009, cutoff value=1,54 mg/L for a sensitivity of 78.0% and a specificity of 61.9% for hsCRP; AUC=0.788, p=0.014, cutoff value=900.08pg/ml for sensitivity of 78.0% and specificity of 85.7% for ICAM1).

At the sixth month of follow-up (Table 3), significant Spearman correlations with 6th month CFT were found for hsCRP (p=0.02, 0.423) and MCP1 (p=0.024, r=0.440). Mean 6th month CFT change correlated with hsCRP (p=0.007, r=0.468), MCP1 (p=0.015, r=0.472) and VEGF (p=0.034, r= - 0.416). Further exploring these results, we verified a significant association between higher hsCRP level and obtaining a CFT decrease < 10% (3.36±1.65 mg/L vs 1.28±1.24 mg/L, p<0.001). Such association was found to be predictive by logistic regression analysis (p=0.009, R^2=0.312, odds ratio=2.59, confidence interval 95%, 1.26-5.28) and such result was again independent of DM related variables (DM duration, blood glucose level and HbA1c percentage). ROC curve analysis of hsCRP as a biomarker for 6th month reduced anatomic response, indicated an AUC of 0.903, p<0.001, and a value of 1.81 mg/L for a sensitivity of 90% and specificity of 85%. A significant association with 6th month CFT decrease ≥25% was found for lower serum MCP1 (244.69±49.34 pg/ml vs 319.24±94.88 pg/ml, p=0.017) and higher serum VEGF (90.84±37.33 vs 58.28±25.19, p=0.027). By logistic regression serum VEGF was predictive of a 6th month significant anatomic response (p=0.029, R^2=0.219, odds ratio=1.035, CI95%, 1.00-1.06) and such association was independent from DM related variables. Finally, a significant association was found between obtaining a 6th month CFT < 330 µm and hsCRP (1.10±0.96 vs2.48±1.83 mg/L, p=0.021) and MCP1 (238.96±46.47 vs 318.47±91.81 pg/ml, p=0.004), the latter was found to be a negative predictor of such outcome (logistic regression, p=0.035, r^2=0.305, odds ratio=0.97).

Systemic factors and qualitative macular outcomes

The mean baseline intraretinal cyst height was 350.04±144.00 µm, improving to 221.68±211.80 µm at the 6th month (p=0.002). A significant correlation was found between hsCRP and 6th month IRC height (p=0.004, r=0.588). Regarding HRS severity, at presentation 14 cases were classified as grade 3, while 7 cases had grade 1 small HRS. The classification of HRS improved significantly at the 6th month (p=0.008, Wilcoxon signed rank) with grade 3 HRS persisting in only 8 cases. Stepwise linear regression revealed that only VEGF was predictive of baseline HRS grade (p=0.002, r^2=0.340, beta=0.583). Regarding 6th month HRS grade, the statistical model including LDL-cholesterol and serum VEGF level, was found to be the most significantly predictive (p=0.001, r^2=0.463) (Table 4). Cases with improved HE severity at the 6th month were significantly younger (58.88 vs 67.23 years-old, p=0.019), but no significant association was found between systemic factor levels and improvement of HE severity. Subretinal fluid (SRF) was present at baseline in 10 cases. Cases with SRF had significantly higher serum homocysteine levels (16.58 vs 13.24 µmol/L, p=0.032). There was a significant improvement in SRF at the 6th month, persisting but with reduced vertical height in only 3 cases (p=0.02, Wilcoxon signed-rank).

Finally, grade 2 EZ disruption was found at baseline in 17 cases, while at the 6th month such defects persisted in 14 cases (p=0.096). No significant associations were found for baseline EZ disruption grade, but cases with persistent grade 2 EZ band disruption had significantly higher serum VEGF when comparing with cases with continuous EZ band (50.42±23.30 vs 82.65±31.39, p=0.031).

Discussion

Our results revealed that systemic inflammatory factors such as hsCRP, ICAM1 and MCP1 may have an important role in the identification of anti-VEGF nonresponders. In fact, higher hsCRP levels were consistently associated with more severe cystoid macular edema (higher 6th month CFT, lower CFT change and larger intraretinal cysts), both hsCRP and ICAM1 were associated with early reduced anatomic response (CFT change < 10%) and both hsCRP and MCP1 were significantly lower in cases which obtained a sixth month favorable anatomical outcome (CFT < 330 µm). These results suggest a prominent role for pro-inflammatory factors causing persistent cystoid edema despite treatment with anti-VEGF. In this regard, it is interesting to note that hsCRP, an inflammatory protein associated with cellular apoptosis, was found by ROC curve analysis, to be a statistically significant biomarker of reduced foveal response to BVZ, particularly at the 6th month of follow-up when for suitable cases treatment was administered according to *pro ne nata* strategy, which resulted in a small increase in CFT in 8 cases. Such result suggests that despite the anatomic response to the initial anti-VEGF loading dose, cases with higher hsCRP values, may benefit from a more prolonged monthly treatment dosing. Alternatively, considering the inhibitory effect of corticosteroids in leukocyte migration[24], if OCT imaging reveals severe macular edema with large intraretinal cystic spaces after the loading dose of anti-VEGF, the patient could benefit from early switching to a corticosteroid implant. Such point is particularly important as it is well known that chronic cystoid macular edema may lead to permanent neuronal retinal damage thereby limiting the potential for visual acuity recovery[25]. It is therefore of the utmost importance to identify as early as possible those cases that exhibit limited anatomic response to anti-VEGF treatment in order to promptly optimize treatment strategies. A possible interpretation is that in cases with higher circulating pro-inflammatory proteins, the cystoid edema is probably more related to cellular iBRB breakdown rather than increased vasopermeability from hypoxia-induced VEGF expression. Indeed, leukostasis is considered to have a prominent role in the pathogenesis leading to iBRB breakdown, with upregulated ICAM1 playing a role in increased leukocyte adhesion to the capillary endothelium, while MCP1 seems to be an important biomarker for increased leukocyte mobilization to the retina[26]. Previous studies had demonstrated a role for elevated CRP in DR and DME[27-30], but to our knowledge, this is the first study identifying a relation between hsCRP level and macular outcomes in DME cases having completed a treatment course of at least 4 BVZ injections.

Also of note was the finding of a significant correlation between systemic VEGF level and the stage of NPDR. Previous studies demonstrated a correlation between serum VEGF and occurrence of DR[31-33] and DME[34], but the results are not unanimous. In fact, a recent meta-analysis reviewed the literature on circulating biomarkers of diabetic retinopathy and verified that VEGF was not consistently elevated comparing to diabetic patients without DR[35]. The previous referred studies did not provide detail on the composition of the NPDR group, also the immunoassay technique varied among studies, which adding to the inherent complexity of studying a systemic disease such as DM may at least partly explain the different reports. In order to minimize the effect of unrelated systemic conditions, we did not include any cases with any other known infectious, inflammatory or malignant disease, additionally we excluded all cases with hospital admissions due to cardiovascular events in the prior 12 months. Also, we included two biomarkers currently in use to assess cardiovascular risk (hsCRP and homocystein), which provided and objective indicator to guide the review of systemic history records. Nevertheless, experimental models reveal that VEGF expression increases in the hypoxic retina[36]. Considering VEGF is a mediator of angiogenesis[37] it is theoretically feasible

that as RD progresses there is increased stimulus for retinal VEGF expression. Prolonged DM increases the risk of vascular damage affecting the eyes, kidneys and peripheral nerves and is also a major risk factor for acute ischemic events affecting the brain and the heart, we could therefore hypothesize that the correlation of serum VEGF to NPDR stage suggests either a tendency of ocular disease to mirror the overall systemic vascular state or that ocular expression of VEGF is reliably detected in the systemic circulation. In this regard, it is worth noticing that a significant association was found between higher serum VEGF levels and persistent photoreceptor EZ disruption. Such result suggests that in this study population the higher serum VEGF could indicate a more advanced hypoxic ocular state leading to impaired photoreceptor metabolism and decreased reflectivity of the EZ. Also of interest in our study was the relation of serum VEGF with hard exudate grade on OCT imaging. It is known that VEGF, a potent mediator of vascular permeability, is secreted by the retinal Muller cells under hypoxic conditions[38], facilitating leakage of plasma protein and lipid into the retinal space. It is therefore possible that patients with higher VEGF levels may be more prone to pronounced exudation as the increased capillary permeability may supersede the capacity of the retinal pigment epithelium to remove the accumulating plasmatic molecules. In agreement with the ETDRS report[39], we also found dyslipidemia to be an important factor for HRS severity, namely patients with higher LDL-cholesterol had significantly more severe HRS. In fact both VEGF and LDL- cholesterol were associated with HE severity after 6 months of treatment, meaning that while BVZ is effective at significantly reducing macular thickness, the complete reabsorption of lipoproteinaceous material may take much longer to occur if at all. Such results suggest that in patients with DME and pronounced hard exudates visible on fundoscopy, optimum control of blood lipids may be beneficial in order to mitigate the leakage of potentially inflammatory lipoproteins[34, 40].

Finally, it is also important to notice that despite the importance of glycemic control in delaying the progress of DR, in this study glycemic variables (blood glucose level and HbA1c) had no correlation with either baseline or 6th month macular features. A previous study evaluated the role of metabolic factors on the clinical response to BVZ[41]. The authors verified that cases with better glycemic control (HbA1C <7%) had more significant improvements in central subfield thickness (CST), but there was no significant difference in final CST between groups. In this regard, a study by Bressler et al[42] reviewed several systemic and ocular variables in cases with DME treated with ranibizumab, but after 1 year of follow-up, no significant difference in CST was found according to HbA1c value (<7.5% vs >7.5%). While glycemic control is a crucial factor in delaying the onset of clinical DR, glucose parameters may not be reliable markers for DME clinical response. In fact, glycated hemoglobin translates the mean plasmatic glucose concentration of the preceding 3 months, such value may rapidly change with dietary adjustment and oral antidiabetics or insulin treatment. Considering the risk of DR increases with duration of diabetes[43], the most recent HbA1c value may not reflect the longstanding hyperglycemia that led to ocular disease. The largest studies evaluating the role of systemic factors on clinical response to DME treatment are the post hoc analysis of the RIDE/RISE trials[44, 45]. In such studies, no systemic variables were found to be correlated with macular outcomes, but components of systemic state such a cardiovascular status were based on medical records, and not on reliable laboratory markers, additionally no serum or ocular cytokines were studied, which limits the insight on the evaluation of inflammatory status in the pathogenesis of DME. Our study has limitations, namely the fact that the study population is small and systemic biomarkers where only measured at baseline. Nevertheless we believe our results are relevant as in addition to a rigorous effort to exclude possible confounding factors, we performed an extensive study of all major systemic variables related to DM by

quantification of corresponding biochemical factors, with particular emphasis on pro-inflammatory status (hsCRP, VEGF, ICAM1, MCP1 and TNFα). Additionally, the results provided relevant insight regarding the possible effect of systemic inflammation in the pathogenesis of persistent DME and may lead to improvement of current clinical practices, namely in guiding implementation of DME treatment strategies according to inflammatory profiles.

Conclusions

In conclusion, our study revealed a significant effect of systemic inflammatory status on DME treatment, verified in a real-world practice setting. Indeed, the levels of serum VEGF, LDL-cholesterol, MCP-1 and hsCRP revealed to be significantly associated with tomographic features of DME. More importantly, we verified that elevated systemic inflammatory factors such as hsCRP and MCP1, were associated with increased CFT, six months after commencing BVZ treatment, indicating a possible role for identification of anti-VEGF nonresponders. A longer follow-up and larger study population will be crucial to identifying the true role of such systemic biomarkers as prognostic factors for clinical response in DME, and eventually lead to the optimization of current treatment guidelines.

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Tables

Table 1 – demographic and laboratory data according to diabetic retinopathy type

	Moderate NPDR	Severe NPDR	P value
Age	63.00±10.09	66.71±17.52	0.476
DM2 years	18.50±8.59	21.92±17.73	0.497
Glucose	177.50±58.11	178.64±34.59	0.072
HbA1c	8.10±1.07	7.80±1.08	0.660
Creatinin	1.18±1.04	0.89±0.20	0.448
B.U.N	38.81±14.95	53.21±17.16	**0.019**
LDL	106.87±37.36	107.68±27.11	0.702
HDL	48.60±12.05	53.71±21.25	0.790
Total-Chol	184.18±51.52	185.92±25.33	0.111
hsCRP	1.94±1.81	2.00±1.60	0.886
Homocystein	13.91±3.46	15.07±4.71	0.257
VEGF	59.42±25.87	92.25±37.70	**0.027**
MCP1	308.71±102.86	272.27±33.77	0.164
ICAM1	821.61±384.29	778.76±274.97	0.984
TNFα	0.078±0.075	0.058±0.012	0.237

Table 2 – Mean value of systemic factors and macular outcomes at the third month of follow-up

Systemic factors	CFT decrease < 10%		P Value	CFT decrease ≥ 25%		P Value	CFT < 330 µm		P Value
	Yes	No		Yes	No		Yes	No	
Glucose	184,60	174,75	0.321	189,16	170,61	0.152	177,77	178,14	0.705
HbA1c %	8,51	7,79	0.921	8,02	7,92	0.310	8,17	7,87	0.395
Creatinine	0,95	1,09	0.657	1,21	,89	0.518	1,35	,91	0.722
B. U. N	53,66	44,04	0.328	44,00	46,55	0.545	44,00	46,19	0.563
LDL-Chol	93,71	113,05	0.720	115,66	101,64	0.153	113,60	104,53	0.060
HDL-Chol	48,62	52,00	0.625	48,50	52,64	0.884	50,33	51,26	0.790
Total-Chol	162,11	194,80	0.235	191,66	180,55	0.207	192,00	182,00	0.226

Homocyst.	15,80	13,88	0.413	14,45	14,46	0.819	12,98	15,09	0.397
hsCRP	3,33	1,39	**0.007**	1,55	2,25	0.368	1,60	2,13	0.372
VEGF	60,66	77,97	0.188	82,44	66,62	0.281	74,01	72,94	0.868
MCP1	347,71	261,66	0.094	242,42	315,93	**0.015**	266,57	294,50	0.597
ICAM1	975,54	727,07	**0.012**	687,08	877,97	0.172	717,98	837,49	0.533
TNFa	0.051	0.076	0.866	,055	,080	0.919	,10	,054	0.874

Table 3 – Mean value of systemic factors and macular outcomes at the sixth month of follow-up

Systemic factors	CFT decrease < 10%		P Value	CFT decrease ≥ 25%		P Value	CFT < 330 µm		P Value
	Yes	No		Yes	No		Yes	No	
Glucose	184,60	174,75	0.350	182,92	174,29	0.711	178,63	177,68	0.933
HbA1c %	8,51	7,79	0.053	7,78	8,10	0.385	7,86	8,02	0.641
Creatinine	1,01	1,07	0.120	1,18	,95	0.408	1,22	,95	0.350
B. U. N	54,90	40,85	0.120	38,23	51,12	0.059	36,45	46,79	0.062
LDL-Chol	102,36	109,70	0.713	119,80	97,65	0.094	114,74	102,91	0.145
HDL-Chol	58,80	47,08	0.131	48,15	53,15	0.805	51,45	50,71	0.420
Total-Chol	187,60	183,70	0.713	198,15	174,94	0.300	194,18	179,68	0.268
Homocyst.	16,41	13,48	0.074	13,15	15,45	0.103	13,02	15,29	0.094
hsCRP	3,36	1,28	**<0.001**	1,44	2,38	0.072	1,10	2,48	**0.021**
VEGF	53,95	80,44	0.094	90,84	58,28	**0.027**	77,61	70,16	0.646
MCP1	291,33	282,44	0.427	244,69	319,24	**0.017**	238,96	318,47	**0.004**
ICAM1	952,87	725,98	0.183	746,47	843,78	0.742	665,02	880,69	0.102
TNFa	0.052	0.076	0.988	,079	,062	0.311	,0942	,052	0.919

Table 4 – mean total cholesterol and serum VEGF levels according to hyperreflective (HRS) spots grade at the 6th month of follow-up

6.Month HRS grade		N	Mean	Std. Deviation	Minimum	Maximum	p value
Chol. Total	1,00	9	169,11	40,15	99,00	228,00	0.052
	2,00	13	182,53	33,16	131,00	240,00	
	3,00	8	206,87	47,68	132,00	304,00	
	Total	30	181,3000	40,73391	99,00	304,00	
VEGF	1,00	7	45,57	17,51	23,94	78,70	0.032
	2,00	12	75,63	35,97	20,91	153,41	
	3,00	7	97,07	28,72	50,34	132,01	
	Total	26	73,86	36,67764	3,94	153,41	

Figure Legends

Figure 1

Intraretinal cyst height was measured using the caliper in the Heidelberg Eye Explorer software (HeyEX), by placing the cursor in the superior limit of the cyst hyporeflectivity and dragging along the shape of the cyst until the lower hyporreflective limit

Figure 2

The presence of hyperrreflective spots (HRS) corresponding to hard exsudates was classified according to severity in 3 grades. A – only a few, small, clearly spaced HRS are identifiable (grade 1); B - larger rounded HRS clumped closer together (grade 2); C - larger rounded HRS, some of which become coalescent forming hyper reflective plaque-like deposits with significant shadowing effect of the underlying retinal layers (grade 3).

Figure 3

The boxplot graph represents the mean central foveal thickness during follow-up. The numeric values correspond to the the mean 50th percentile for CFT at each time point, indicating a significant change from baseline to the third month and a nonsignificant increase at the 6th month

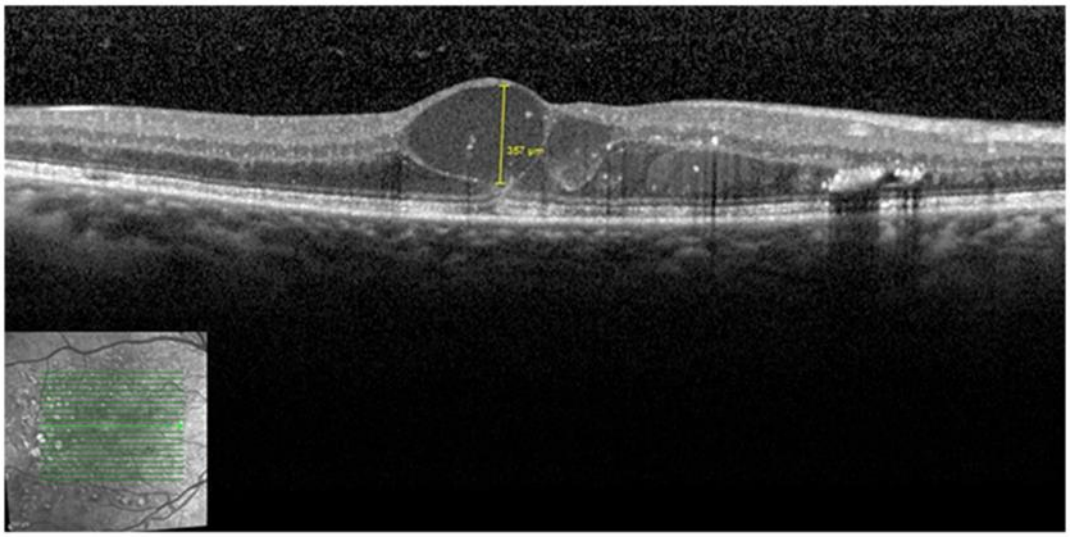

Figure 1

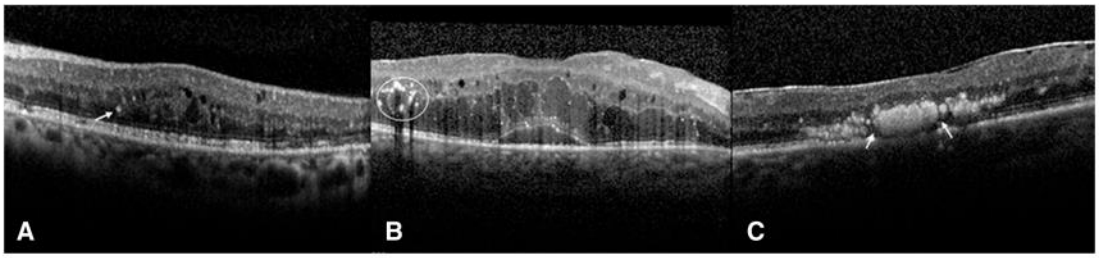

Figure 2

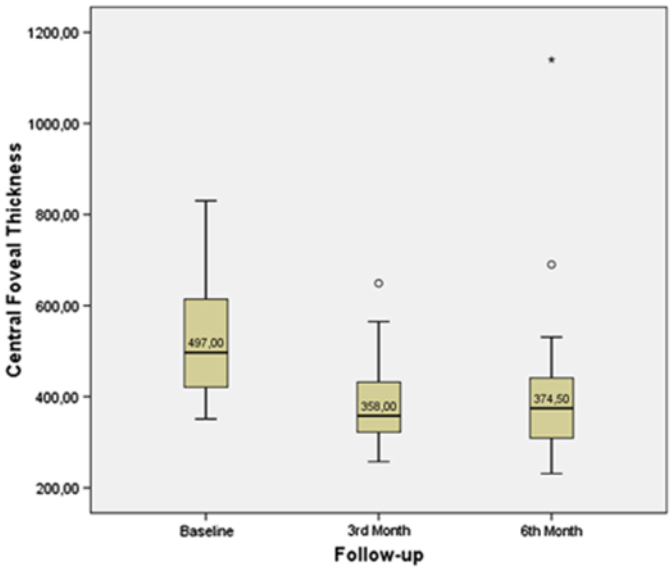

Figure 3

10. SERUM PRO-INFLAMMATORY FACTORS AS PREDICTORS OF PERSISTENT DIABETIC MACULAR OEDEMA WITH LIMITED ANATOMIC RESPONSE TO ANTI-VEGF: ASSOCIATION WITH INTRAVITREAL INJECTION TREATMENT PROFILES IN REAL-WORLD SETTING

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Serum pro-inflammatory factors as predictors of persistent diabetic macular oedema with limited anatomic response to anti-VEGF: association with intravitreal injection treatment profiles in real-world setting

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ABSTRACT.

Purpose: **To study the role of serum levels of pro-inflammatory factors in the identification of persistent diabetic macular oedema (DME) cases with limited anatomic response to anti-VEGF. Additionally, possible predictive associations between serum factors and intravitreal treatment profiles were analysed.**

Methods: **Cases with DME were treated with monthly bevacizumab (BVZ). After the sixth month of follow-up, if the change in central foveal thickness (CFT) was <20% of baseline, combination treatment with triamcinolone was initiated. All cases underwent a baseline laboratory workup including inflammatory, metabolic and prothrombotic factors. The following outcome parameters were evaluated: percentage of CFT change from baseline, occurrence of persistent DME with <20% change in CFT, achieving CFT <330 μm with ≤6 BVZ injections, total number of intravitreal injections (IVI), number of IVI after the 6th month and number of triamcinolone acetonide (TCA) injections.**

Results: **A total of 58 cases were included receiving a mean of 7.23 ± 1.55 IVI in 12 months, resulting in a significant improvement of visual acuity (VA) and CFT. No significant differences were found for baseline CFT, baseline LogMAR VA, diabetic retinopathy grade, age or duration of DM2 between cases initiating TCA and those treated only with anti-VEGF. Significant correlations were found between total number of IVI and the following serum factors: high-sensitivity C-reactive protein (hsCRP) ($p = 0.004$, $r = 0.395$), creatinine ($p = 0.023$, $r = 0.338$) and homocysteine ($p = 0.037$, $r = 0.309$). Regression analysis revealed that hsCRP was a significant predictor of TCA treatment ($p = 0.028$, $r^2 = 0.350$). Cases requiring ≤6 IVI had significantly lower values of hsCRP (1.33 ± 1.07 versus 2.46 ± 2.18 mg/l, $p = 0.016$) and creatinine (0.71 ± 0.28 versus 0.94 ± 0.19 mg/dl, $p = 0.003$).**

Conclusions: **Serum markers of microvascular damage (hsCRP, homocysteine and creatinine) were associated with a higher frequency of IVI due to persistent DME, suggesting a role for such biomarkers in the identification of limited responders to anti-VEGF monotherapy.**

Key words: anti-VEGF – C-reactive protein – diabetic macular oedema – triamcinolone

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Introduction

Randomized clinical trials demonstrated the efficacy of intravitreal anti-vascular endothelial growth factor (anti-VEGF) agents (Rajendram et al. 2012; Brown et al. 2013; Brown et al. 2015) in eyes with centre-involved diabetic macular oedema (DME). Nevertheless, a significant number of patients will not achieve sustained or significant improvement in VA (Gonzalez et al. 2016). In fact, a *post hoc* analysis of the DRCR.net Protocol I data revealed that after 3 years of intravitreal ranibizumab, 34.2% of cases had non-significant improvement in VA (<5 letters) and such outcome could be predicted as early as 3 months into treatment (Gonzalez et al. 2016). Additionally, depending on the chosen anti-VEGF agent, it is estimated that up to 65% of cases may have persistent DME, despite six monthly intravitreal injections (IVI; Bressler et al. 2018). Such results suggest that, notwithstanding the prominent role of VEGF in vasopermeability, other pathogenic pathways may contribute to persistent disruption of the inner blood-retinal barrier (BRB). Considering that persistent retinal oedema may lead to poor visual outcomes (Sun et al. 2014), early identification of cases who are partial or nonresponders to anti-VEGF is of the utmost importance in order to allow timely consideration of alternative treatment strategies. While the pathogenesis of DME is not fully understood, it is important to consider the increasing evidence that insulin resistance occurring in DM2 is associated with a subclinical systemic pro-inflammatory state (Pradhan et al. 2001; Dehghan et al. 2007). Additionally, there are several reports of increased pro-inflammatory cytokines in the aqueous and vitreous of patients with DR and DME (Funatsu et al. 2005, 2009; Funk et al. 2010; Jonas et al. 2012). Also of note is the fact that pseudophakic cases in Protocol I treated with intravitreal triamcinolone acetonide (TCA) achieved gains in VA comparable to cases treated with ranibizumab (Diabetic Retinopathy Clinical Research N et al. 2010), suggesting that intravitreal steroid treatment may be an important therapeutic alternative to anti-VEGF agents. Considering that diabetic retinopathy is a local manifestation of a complex systemic disease and that there seems to be a preponderant role for leukostasis leading to inner BRB disruption (Miyamoto et al. 1999), it is possible to hypothesize that systemic levels of pro-inflammatory factors such as C-reactive protein, VEGF, MCP1 and ICAM1 may act as biomarkers for parameters of clinical response to intravitreal treatments.

Therefore, the objective of this study was to analyse several systemic factors which have been associated with DM2, encompassing renal function, lipid profile, cardiovascular risk and glycemic profile, as well as serum levels of VEGF, MCP1 and ICAM1, in order to identify significant associations persistent DME and the respective intravitreal injection treatment patterns, namely total injection volume, number of injections after the 6th month of follow-up and the use of TCA combination therapy for persistent DME with limited anatomic response to anti-VEGF injections.

Methods

This prospective interventional study included cases diagnosed and treated for centre-involved DME at the Ophthalmology Department of Hospital de Braga (Braga, Portugal). Study protocols were submitted to and approved by the Hospital Ethics Committee. The research procedures followed the tenets of the Declaration of Helsinki, and all patients provided written informed consent for the inclusion in the study. The recruited cases had Diabetes Mellitus type 2 (DM2) and nonproliferative diabetic retinopathy (NPDR) with centre-involved DME. All cases underwent complete ophthalmological examination, and diagnosis was confirmed by fluorescein angiography and spectral-domain optical coherence tomography (SD-OCT). The severity of NPDR and foveal involvement was graded based on fluorescein angiography findings, according to the international clinical diabetic retinopathy and DME disease severity scales (Wilkinson et al. 2003). The following inclusion criteria were considered: cases with DM2, NPDR and DME with central foveal thickness (CFT) >330 μm and intraretinal cysts in the foveal area. The exclusion criteria included: history of any other vision-impairing ocular disease, history of retinal laser treatment or intravitreous injection, as well as previous vitreoretinal surgery. Regarding systemic history, there could be no record of cardiovascular events in the preceding year, no known history of chronic infectious, inflammatory or malignant disease, and no surgical procedures or hospital admissions of any kind in the preceding 6 months.

All recruited cases underwent treatment with intravitreous bevacizumab (BVZ) injections (1.25 mg/0.05 cc) following the strategy of 3 monthly injections plus *pro re nata* treatment according to tomographic criteria, namely persistent central subfield thickness >330 μm, with identifiable intraretinal cystic lesions and/or subretinal fluid. After 6 months of follow-up, if the change in CFT was <20% of baseline, combination treatment with intravitreal TCA 4 mg was initiated due to persistent DME considered to be poorly responsive to anti-VEGF treatment (Bressler et al. 2016). Such strategy entailed alternating IVI of 1.25 mg BVZ with 4 mg TCA on a monthly basis until a stable macular thickness was achieved, or a significant increase in intraocular pressure (>10 mmHg) was noted in two consecutive visits. The same PRN criteria were applied when a case was started on combination treatment. Cases achieving a significant macular response (≥20% decrease in CFT) at the 6th month were always retreated with BVZ monotherapy when necessary. Follow-up visits were scheduled between 8 and 12 days after the last intravitreal injection. In case of treatment being withheld due to clinical stability, the following visit was scheduled to 1 month after the last injection. In cases with bilateral DME, the eye with highest CFT was chosen for this study.

Blood samples were taken at baseline to evaluate the following systemic markers: cardiovascular risk (high-sensitivity C-reactive protein, serum homocysteine), renal dysfunction (blood urea nitrogen (BUN), serum creatinine), hypercholesterolaemia (low-density lipoprotein (LDL), high-density lipoprotein (HDL) and total cholesterol (T.Chol)) and diabetic profile (glycated haemoglobin, blood glucose level). Additionally, for each case a blood sample was obtained and centrifuged at 1000G to isolate the serum fraction which was then immediately stored at −80°C, for posterior dosing of the following pro-

inflammatory cytokines: VEGF, sICAM-1, MCP-1, by enzyme-linked immunosorbent assay (ELISA) using the specific ELISA kits, procured from Sigma-Aldrich® (Merck KGaA, Darmstadt, Germany). Such biochemical assays were performed in the appropriate laboratories of the Life and Health Sciences Research Institute (Braga, Portugal) under the supervision of one of the investigators (SC). Succinctly, all sampling procedures were performed according to the manufacturer's protocol. Samples were diluted accordingly, to comply with the detection range of the relevant assay. Colour intensities were determined using a microplate reader (Multiskan™ FC, Thermo Fisher Scientific, Waltham, MA, USA). Duplicate samples were used in all assays. The level of each factor in serum was within the detection range of the relevant assay.

The main outcome measures were as follows: central subfield thickness, percentage of CFT change from baseline, probability of persistent DME with limited anatomic response (<20% change CFT) to BVZ at the 6th month, probability of achieving a CFT <330 μm with 6 or less BVZ injections, total number of IVI, number of IVI after the 6th month of follow-up and number of TCA injections due to persistent DME in the last 6 months of follow-up.

The blood/serum concentrations of measured metabolic and pro-inflammatory factors were assessed for predictive associations with the previously listed intravitreal treatment profiles. The results were subject to statistical analysis using the IBM SPSS Statistics software (SPSS version 23; IBM Corporation, Armonk, NY, USA). Numerical values are expressed as mean ± standard deviation. Boxplot graphs were used to display minimum, maximum and percentile (25, 50 and 75) values for LogMAR VA and CFT during follow-up. The study of possible interactions between variables was performed by Spearman 2-tailed correlations. Predictive associations were studied by ordinary least squares regression or bivariate logistic regression, as applicable. Categorical variables were assessed using chi-squared test, and numerical variables were compared by Mann–Whitney U or Kruskal–Wallis tests. Statistical significance was considered for $p < 0.05$.

Results

The study included 58 patients diagnosed with centre-involved DME requiring treatment with intravitreal anti-VEGF injections. Mean patient age was 66.76 ± 9.36 years, and mean duration of DM2 was 17.20 ± 6.85 years. Thirteen patients were on insulin therapy for a mean of 7.23 ± 6.91 years. All cases had NPDR, classified as moderate in 26 cases and severe in 32 cases. Regarding phakic status a total of 17 cases had previously undergone cataract surgery. The mean baseline CFT value was 508.19 ± 120.17 μm, improving to 354.84 ± 79.19 μm at the 12th month ($p < 0.001$) (Fig. 1), corresponding to a mean 12th month CFT decrease of 151.62 ± 128.54 μm. Persistent centre-involved DME (CFT ≥ 330 μm) at the 6th month was verified in 36 cases (62.0%), of which 25 (43.1%) had a CFT reduction of <20% baseline; therefore, combination treatment with TCA injections was initiated in these cases (16 of which were phakic). At the end of follow-up, both BVZ monotherapy and TCA combination treatment groups achieved a significant reduction in CFT comparing to baseline values ($p < 0.001$) (Fig. 2). However, of the total 25 cases in combination treatment, 11 (44%) maintained a limited (<20% CFT) anatomic response at the 12th month. Of the 22 (37.9%) cases that achieved DME resolution at the 6th month, 16 (27.5%) required no additional BVZ injections in the next 6 months of follow-up.

Regarding VA, baseline mean LogMAR VA was 0.62 ± 0.21, improving to 0.42 ± 0.21 at the 12th month ($p < 0.001$) (Fig. 3), corresponding to an improvement of at least two lines of VA in 32 cases (53.1%) and a final VA of ≤0.3 LogMAR was achieved in 23 cases (39.6%). Cases requiring TCA treatment had worse LogMAR VA at all points during follow-up but the difference was not significant (Fig. 4), and both groups achieved significant improvement from baseline values.

Such results were obtained with a mean of 7.23 ± 1.55 IVI, corresponding to a mean of 6.16 ± 1.12 BVZ injections in the monotherapy group and 6.35 ± 0.98 BVZ plus 1.88 ± 0.72 TCA injections in the combination treatment group. On the final 6 months of follow-up, a mean of 2.11 ± 1.12 injections was performed.

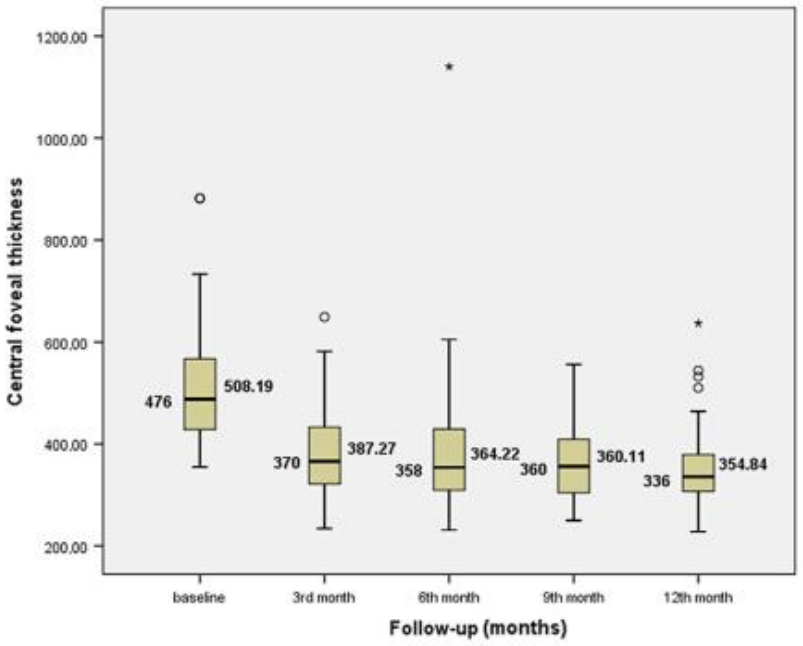

Fig. 1. Boxplot graph of central foveal thickness (CFT) (μm) during follow-up month. Numeric values to the left indicate the arithmetic mean while values to the right indicate the median value (percentile 50). Mild outliers are displayed as circle and extreme outliers as asterisk. At baseline, more than 75% cases had CFT values above 400 μm. A significant improvement in median CFT was seen at the 3rd month and thereafter was somewhat maintained, albeit with reduction in the upper limits of CFT verified at the 12th month.

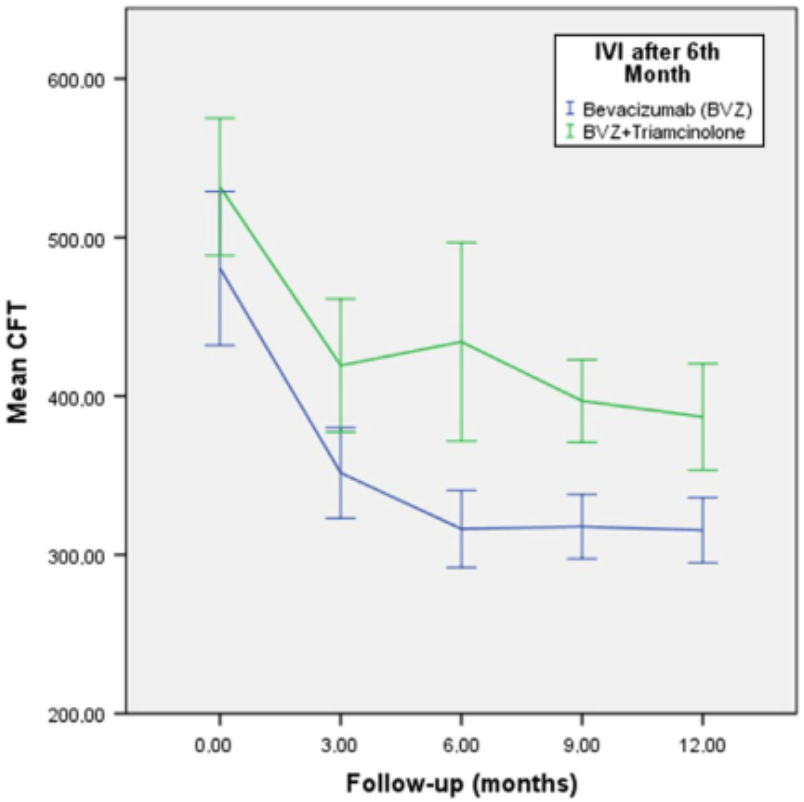

Fig. 2. Line graph representation of mean central foveal thickness (CFT) (μm) values in two groups according to whether or not triamcinolone combination treatment was required. Error bars indicate 95% confidence interval. Cases treated with bevacizumab monotherapy had improvements in CFT until the 6th month, whereas cases which required triamcinolone acetonide (TCA) had a slight increase in CFT at the 6th month (434.17 μm), and therefore initiated combination treatment which resulted in a further decrease of CFT to 386.82 μm at the 12th month (p = 0.156).

Clinical variables and intravitreal treatment profile

No significant differences were found for baseline CFT, baseline LogMAR VA, NPDR grade, age or duration of DM2 between cases requiring TCA and those treated only with anti-VEGF, but significantly more cases on the combination treatment group were on insulin therapy (p = 0.003) (Table 1). Cases requiring ≤6 IVI to achieve resolution of DME had significantly better baseline VA (0.51 ± 0.25 versus 0.67 ± 0.24 LogMAR, p = 0.026), but no significant difference in baseline CFT (470.67 ± 139.14 versus 528.05 ± 105.68 μm, p = 0.102). A significant positive correlation was found between total number of IVI and baseline CFT (p = 0.014, r = 0.339). When considering the number of IVI received in the last 6 months of follow-up, a significant correlation was also found with baseline CFT (p = 0.04, r = 0.285). No correlations were found for number of TCA injections.

Serum metabolic and inflammatory factors and intravitreal treatment profile

Analysing possible interactions between systemic factors and intravitreal treatment profile, we verified that cases started on TCA combination treatment due to persisting DME had significantly higher values of high-sensitivity C-reactive protein (hsCRP) (2.84 ± 2.20 versus 1.19 ± 0.93 mg/l, p = 0.020) and creatinine (0.94 ± 0.21 versus 0.76 ± 0.27 mg/dl, p = 0.016) but exhibited lower values of serum VEGF (62.03 versus 97.81 pg/ml, p = 0.018; Table 2). Binary logistic regression revealed hsCRP was a significant predictor of poorly responsive DME (<20% CFT change) requiring TCA treatment (p = 0.010; odds ratio = 2.01), and such result was independent of baseline VA or CFT or duration of DM2 or glycemic control (p = 0.042, odds ratio = 2.00). Cases requiring ≤6 IVI for DME regression had significantly lower values of hsCRP (1.33 ± 1.07 versus 2.46 ± 2.18 mg/l, p = 0.016) and creatinine (0.71 ± 0.28 versus 0.94 ± 0.19 mg/dl, p = 0.003) than those with >6 injections. Binary logistic regression revealed that serum creatinine was a negative predictor of achieving DME resolution with 6 or less IVI, even when accounting for baseline VA, CFT or glycemic control variables (p = 0.010, odds ratio = 0.005).

A significant Spearman correlation was found between total number of IVI and serum values of creatinine (p = 0.023, r = 0.338), homocysteine (p = 0.037, r = 0.309) and hsCRP (p = 0.004, r = 0.395). Stepwise linear regression revealed hsCRP was the most significant predictor of total number of injections (p = 0.002, r^2 = 0.200), and such effect was maintained when accounting for baseline VA, baseline CFT, duration of DM2 or HbA1c values (p = 0.036, r^2 = 0.293). Significant correlations with number of IVI in the last 6th month of follow-up were found for the following variables: homocysteine (p = 0.031), creatinine (p = 0.011) and hsCRP (p = 0.005). Stepwise linear regression revealed that the combined model of creatinine and hsCRP was significantly predictive of injection frequency after the 6th month (p = 0.001, r^2 = 0.285) and such result was independent of baseline VA, CFT or glycemic variables (p = 0.019, r^2 = 0.240). For number of TCA injections, significant correlations were found with homocysteine (p = 0.032, r = 0.317) and hsCRP (p = 0.001, r = 0.441). Linear regression revealed that hsCRP was a significant predictor of TCA treatment frequency, even when accounting for confounding variables such as baseline VA, CFT, duration of DM2 or HbA1c values (p = 0.028, r^2 = 0.350).

Discussion

Our results reveal important considerations regarding the impact of systemic inflammatory and metabolic factors as having a limiting effect on the efficacy of anti-VEGF treatment for DME. More specifically we verified that cases with higher levels of hsCRP, homocysteine and creatinine seem to require a higher number of IVI treatments. Additionally, cases with higher values of hsCRP

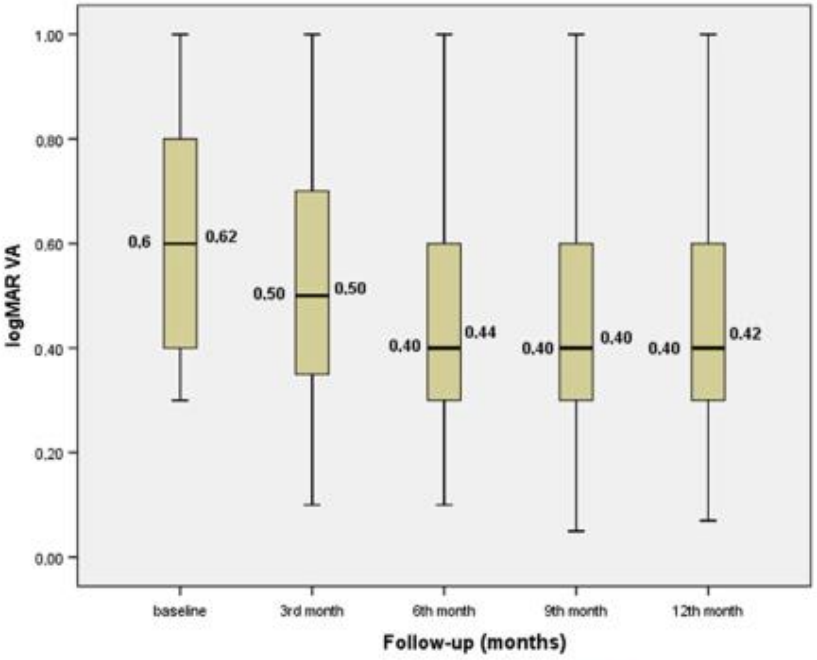

Fig. 3. Boxplot graph of logMAR visual acuity during follow-up (months). Numeric values to the left indicate the arithmetic mean while value to the right indicates the median value (percentile 50). At baseline, more than 50% of cases had 0.6 LogMAR VA or worse. A significant improvement in median LogMAR VA was seen at the 3rd and 6th months.

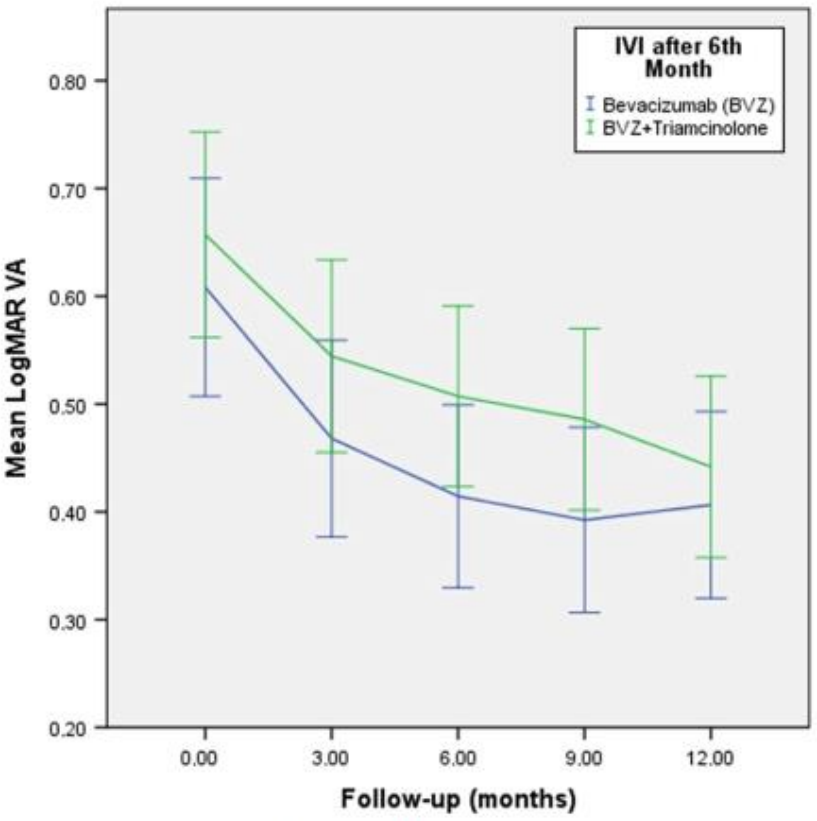

Fig. 4. Line graph representation of mean LogMAR visual acuity values according to whether or not triamcinolone injection was required. Error bars indicate 95% confidence interval. Both groups had significant improvements in LogMAR VA, nevertheless cases requiring triamcinolone acetonide (TCA) always maintained higher mean values of LogMAR VA.

were found to have a significantly higher probability of persistent DME poorly responsive to anti-VEGF monotherapy (<20% change from baseline in CFT). Also, it is interesting to note that such cases also had significantly lower values of serum VEGF, while there is wide variability in mean serum and intraocular values of VEGF, such result supports the hypothesis that in poor responders to anti-VEGF injections for DME, other pathogenic mechanisms are preponderant over VEGF in causing persistent inner BRB dysfunction. It is known that C-reactive protein (CRP) levels increase via IL-6 secreted from macrophages, T cells and adipocytes in the context of tissue inflammation, and by activation of the complement system, this protein facilitates cellular apoptosis. Increased levels of CRP have been reported to be an independent risk factor for DM2 (Pradhan et al. 2001; Dehghan et al. 2007) as well as cardiovascular disease risk, in fact the high-sensitivity CRP analysis has been widely used to define risk groups for coronary heart disease (Sabatine et al. 2007). More interestingly, there is experimental evidence that increased CRP leads to dysfunction of the retinal vascular endothelium, by interfering with nitric oxide-mediated vasodilation (Nagaoka et al. 2008). In this context, a recent analysis of the DCCT trial verified that cases with hsCRP in the highest quintile were 83% more likely to have clinically significant DME versus those in the lowest quintile (Muni et al. 2013). While the results of such study were verified in type 1 diabetic patients and therefore may not translate the pathogenic mechanism in the general diabetic population, there is further evidence of a significant association between increased CRP and DME (Kocabora et al. 2016). Additionally, our group previously reported a significant association between early (3rd month) limited anatomic response to anti-VEGF and higher serum hsCRP and ICAM-1 levels. Interestingly, hsCRP remained a significant predictor of limited anatomic response at the 6th month (Brito et al. 2018). Therefore, we could hypothesize that similarly to what is verified for cardiovascular risk, cases with increased levels of hsCRP may be more prone to advanced inflammatory damage to the inner BRB, increasing the risk of chronic DME and therefore

Table 1. Mean values for clinical variables comparing cases requiring triamcinolone combination treatment versus cases treated with bevacizumab monotherapy.

	Triamcinolone treatment		
Clinical variables (mean values)	Yes (n = 25)	No (n = 33)	p value
Age	66.96 ± 7.94	65.29 ± 10.67	0.521
DM2 duration	17.60 ± 6.13	16.08 ± 8.12	0.445
Insulin treatment	9	4	0.003
Baseline VA	0.66 ± 0.24	0.56 ± 0.24	0.172
Baseline CFT	525.17 ± 116.96	488.37 ± 123.29	0.275
Mod NPDR	15	12	1.00
Severe NPDR	15	16	

CFT = central foveal thickness; NPDR = nonproliferative diabetic retinopathy.

Table 2. Mean values for serum factors comparing cases requiring triamcinolone combination treatment versus cases treated with bevacizumab monotherapy.

	Triamcinolone treatment		
Serum variables (mean values)	Yes (n = 25)	No (n = 33)	p value
HbA1c	7.85 ± 1.14	7.71 ± 1.13	0.685
Glucose (mg/dl)	173.00 ± 46.65	161.00 ± 63.03	0.486
Total Chol.	186.50 ± 40.51	163.95 ± 34.13	0.052
LDL Chol.	106.24 ± 34.60	90.07 ± 29.76	0.105
HDL Chol.	50.90 ± 18.21	52.93 ± 21.23	0.732
Creatinine	0.94 ± 0.21	0.76 ± 0.27	**0.016**
B.U.N	49.88 ± 18.52	43.15 ± 20.96	0.361
Homocysteine	14.94 ± 4.69	13.35 ± 5.19	0.080
hsCRP	2.84 ± 2.25	1.19 ± 0.94	**0.020**
ICAM1	841.15 ± 353.64	770.20 ± 255.13	0.540
MCP1	301.53 ± 96.82	253.52 ± 61.09	0.057
VEGF	62.03 ± 28.52	97.81 ± 33.74	**0.018**

HDL = high-density lipoprotein; hsCRP = high-sensitivity C-reactive protein; LDL = low-density lipoprotein.Values in bold were found to be statistical significant (p value <.05)

a limited anatomic response to anti-VEGF monotherapy. Alternatively, it is possible that hsCRP could be an indirect biomarker of chronic diabetic maculopathy perhaps by acting as an accurate indicator of DM duration. In this regard, we verified that cases requiring less than 6 IVI to achieve DME resolution had significantly better baseline VA, but no difference in baseline CFT, which suggests that in these cases DME onset was probably more recent. Such cases also had significantly lower values of hsCRP and creatinine, indicating that cases more responsive to BVZ, could have less advanced inflammatory microvascular damage. In this regard, a recent *post hoc* analysis (Wykoff et al. 2016) of the RIDE/RISE studies analysed the impact of several variables on treatment frequency, and verified that HbA1c level was not correlated with number of PRN injections, yet shorter duration of both DM and DME correlated with fewer injections. Previous studies evaluated the role of glycemic variables on DME treatment efficacy, but no significant effect was found (Bressler et al. 2012; Matsuda et al. 2014). While glycemic control is of the utmost importance in delaying the onset of microvascular diabetes complications (Diabetes et al. 1993; King et al. 1999), it is possible that the severity and persistence of retinal vascular disease are more dependent on the individual susceptibility to inflammatory response than on blood glucose value *per se*. Additionally, it is important to note that while our results were achieved following a treatment strategy applied in a real-world clinical setting, our rate of persistent DME after 6 months of BVZ treatment (62% of cases) was very similar to that reported in a *post hoc* analysis of Protocol T (65.6% of cases) (Bressler et al. 2018). Such DRCR.net analysis revealed that there is a tendency for persistent DME to improve with continued anti-VEGF treatment, yet it is important to notice that with BVZ, 68.2% of cases still had macular thickening after 24 months of treatment and while aflibercept fared better, there was still a very significant percentage of these cases with persistent oedema (44.2%) at 24 months. Such results indicate that there is clearly a subset of patients which are slow/poor responders to anti-VEGF. Additionally, it is important to consider DRCR.net Protocol U results which revealed a significant improvement of macular thickness in cases treated with combination of anti-VEGF plus dexamethasone implant when comparing with a control group treated with ranibizumab alone (Maturi et al. 2018). Another recent study revealed anatomic and functional benefits of early switching to DEX implant in refractory DME (Iglicki et al. 2019); therefore, we believe once a limited or slow pattern of anatomic response to anti-VEGF is verified at the 6th month of monthly injections, it may be more advantageous to initiate combination treatment with intravitreal corticosteroid. Due to hospital administrative policies, we had to start with TCA in order to verify a favourable clinical response to steroid treatment. Our results in the combination treatment group are encouraging, as of the 25 cases with persistent oedema and <20% CFT response at 6 months, only 11 (44%) maintained the same response profile at 12 months. Such favourable outcome in our study is possibly explained by the effect of combining an anti-VEGF agent with a potent intravitreal steroid, allowing sustained VEGF suppression while providing a wider anti-inflammatory effect on the inner BRB. We chose to alternate BVZ and TCA on a monthly basis because of the well-known risk of ocular hypertension associated with intraocular steroids (Diabetic Retinopathy Clinical Research N 2008; Boyer et al. 2014) and also because pharmacokinetic data suggests that for TCA intraocular concentrations are expected to fall below therapeutic range in well under 90 days (Beer et al. 2003; Yang et al. 2015). Considering the possible consequences for VA recovery of persistent DME, it is of the utmost importance to identify early clinical or laboratory variables that may be predictors of treatment response phenotypes. To our knowledge, this is the first study to verify a limiting effect of higher levels of hsCRP and other peripheral blood factors on the clinical response to intravitreal treatment for DME. Indeed, our results

suggest that cases with elevated biomarkers of microvascular damage, namely hsCRP, homocysteine and creatinine, may be worse candidates to anti-VEGF monotherapy. Interestingly, in a *post hoc* analysis of the RIDE/RISE studies, renal disease was found to be associated with worse visual outcomes in patients treated with macular laser (Sophie et al. 2015). Considering increased serum creatinine suggests advanced renal injury, it is possible that the worse treatment outcomes may indicate a similar course between renal and retinal microvascular disease which may become apparent with PRN BVZ treatment. Finally regarding homocysteine, increased levels of this amino acid have been associated with macular oedema (Klein et al. 2009; Li et al. 2014). While the pathogenesis of this association is not clear it is known that hyperhomocysteinemia leads to vascular injury by a combination of oxidative stress, decreased nitric oxide availability, endothelial cell apoptosis and enhanced thrombogenecity; therefore, high levels of homocysteine could theoretically promote inner BRB disruption(Lai & Kan 2015).

In conclusion, our results indicate that cases with elevated serum hsCRP, homocysteine and creatinine required a higher frequency of IVI to manage DME, more specifically hsCRP was consistently associated with persistent DME with limited anatomic response after 6 months of BVZ treatment, suggesting a possible role in the early identification of cases refractory to anti-VEGF monotherapy. Further studies of such biomarkers could potentially lead to optimized treatment strategies for persistent DME.

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11. ASSOCIATION OF SERUM VASOGENIC AND PRO-INFLAMMATORY FACTORS WITH CLINICAL RESPONSE TO ANTI-VEGF FOR DIABETIC MACULAR EDEMA

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ASSOCIATION OF SERUM VASOGENIC AND PROINFLAMMATORY FACTORS WITH CLINICAL RESPONSE TO ANTI–VASCULAR ENDOTHELIAL GROWTH FACTOR FOR DIABETIC MACULAR EDEMA

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Purpose: To study the role of serum biomarkers as prognostic factors for qualitative and quantitative response to anti–vascular endothelial growth factor injections for diabetic macular edema (DME).

Methods: Sixty-seven eyes with DME were treated with intravitreal bevacizumab during a 12-month follow-up period. All cases underwent a baseline workup consisting of 12 inflammatory, metabolic and prothrombotic factors. The following outcomes were evaluated at 3-month intervals until 1 year of follow-up: visual acuity, central subfield thickness (CST), macular volume (MV), % of change from baseline in CST, occurrence of a CST change < 10%, a CST change >20%, and a CST <330 μm, achieving an improvement ≥2 lines of visual acuity, achieving visual acuity ≥20/40.

Results: A significant improvement in CST and visual acuity was seen from third month onwards. Twenty-eight (48.1%) cases were classified as "early responders," 24 (35.8%) as "late responders", and 15 (22.4%) as "poor responders." Serum vascular endothelial growth factor–A levels were significantly lower in "poor responders" (P = 0.006). C-reactive protein (hsCRP) was associated with a limited anatomic response (<10% CST change) (P = 0.002, OR = 1.845, cutoff value of hsCRP = 1.84 mg/L). hsCRP was also negatively associated with obtaining a final CST <330 μm (P = 0.04, r^2 = 0.112, OR = 0.643). Baseline visual acuity was significantly associated with 12th month visual acuity (P < 0.001, r^2 = 0.602) and also with an improvement ≥2 visual acuity lines (P = 0.009, OR = 20.54).

Conclusion: Increased high-sensitivity C-reactive protein was associated with limited anatomic response to anti–vascular endothelial growth factor treatment and persistent DME. Poor responders had significantly lower values of serum vascular endothelial growth factor–A, suggesting an alternative pathogenic pathway for persisting DME.

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Anti-VEGF agents are the mainstay of treatment for diabetic macular edema (DME), on account of major randomized trials revealing significant gains in visual acuity and improved macular thickness.[1,2] Nevertheless, post hoc analyses of protocol T data indicated that between 31.6% and 65.6% of cases had persistent central DME even after 6 of monthly anti-VEGF treatments.[3] In addition, it is estimated that more than half of cases will be limited to <10 letters of visual acuity improvement after 3 years of ranibizumab treatment.[4] Such results indicate a wide variability in response patterns when treating DME with current anti-VEGF agents. It is also important to consider that the methodology of a clinical trial setting is not readily applicable in real-world practice due to a combination of logistic and scheduling constraints, meaning that the real-world results may be even less favorable.[5,6] Although the pathogenesis of DME is not fully known, it is undoubtedly associated with prolonged hyperglycemia,[7,8] yet studies on the role of

glycated hemoglobin as predictive factor for DME have revealed contradictory results.[9–11] Interestingly, there is increasing evidence for a subclinical proinflammatory state occurring in DM Type 2 patients, verified by increased serum levels of C-reactive protein and other inflammatory cytokines[12,13] Also, recent studies on the efficacy of corticosteroid implants have revealed encouraging results,[14] particularly in chronic DME,[15] further emphasizing the role of inflammation in persistent DME. It is possible that certain metabolic and proinflammatory profiles lead to different outcomes in diabetic eye disease. To optimize treatment strategies, it is of the utmost importance to identify variables that may act as significant factors modulating the clinical response to anti-VEGF. In this regard, the purpose of this study was to analyze potential associations between systemic and/or ocular variables with macular thickness response pattern and improvement in visual acuity in DME cases treated with anti-VEGF in a real-world clinical setting.

Methods

This was a prospective interventional study including cases diagnosed and treated for center-involved DME at the Ophthalmology Department of a single referral center (Hospital de Braga, Braga, Portugal). Study protocols were submitted to and approved by the Hospital Ethics Committee. The research procedures followed the tenets of the declaration of Helsinki, and all patients provided written informed consent for the inclusion in the study. The following inclusion criteria were considered: age ≥18 years with Type II diabetes mellitus and nonproliferative diabetic retinopathy with DME involving the foveal center, optical coherence tomography (OCT) revealing increased central subfield thickness (CST) >320 μm[16], and cases should be intravitreal treatment naïve. Exclusion criteria were history of macular or other vitreoretinal disease besides diabetic retinopathy as well as previous vitreoretinal surgery, macular grid LASER, or any ocular surgery in the previous 3 months.

Evaluation Procedures

All patients underwent a complete ophthalmologic examination, completed with spectral domain OCT (Spectralis; Heidelberg Engineering, Heidelberg, Germany) and fluorescein angiography. Visual acuity was assessed using Snellen visual charts and then converted to logMAR units for statistical analysis. SD-OCT images were obtained using a standard acquisition protocol. The value obtained in the 1-mm central ring of the macular map was considered the CST. In cases with bilateral DME, the eye with highest CST was chosen for this study. The following OCT variables were registered at baseline and each follow-up visit: CST (μm), macular volume (MV) (mm^3), and CST change from baseline (negative or positive variation then converted to percentage). The severity of NPDR was graded based on fluorescein angiography findings, according to the international clinical diabetic retinopathy and DME disease severity scales.[17]

Blood samples were taken at baseline to evaluate the following systemic markers: cardiovascular risk (high-sensitivity C-reactive protein and serum homocysteine), renal dysfunction (blood urea nitrogen and serum creatinine), hypercholesterolemia (low-density lipoprotein, high-density lipoprotein, and total cholesterol [T.Chol]), and diabetic profile (glycated hemoglobin and blood glucose level). In addition, a blood sample was obtained and centrifuged at 1,000g to isolate the serum fraction, which was then immediately stored at −80°C, for posterior dosing of the following proinflammatory cytokines: VEGF, sICAM-1, and MCP-1 by enzyme-linked immunosorbent assay using specific enzyme-linked immunosorbent assay kits, procured from Sigma-Aldrich. Color intensities were determined using a microplate reader (Multiskan; Thermofisher Scientific). Duplicate samples were used in all assays.

Treatment and Follow-Up Protocol

Treatment was initiated with intravitreal 1.25 mg/0.05 mL bevacizumab, following a protocol of at least three monthly injections and thereafter continued monthly if there was persistent central DME. If at

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the third month or beyond CST change was <20%, treatment was switched to aflibercept, if CST change was <20% at the sixth month or beyond, combination treatment with triamcinolone (alternating with aflibercept) was allowed to assess possible favorable response to steroid agents. A pro re nata regimen was adopted if there was OCT evidence of DME recurrence after a period of stable macular thickness of at least two consecutive monthly visits or decreased visual acuity with persistent intraretinal cysts. The objective was to achieve a CST <330 with no evidence of intraretinal cysts in the foveal area or at least structural stability not deemed to improve with further injections and with best stable visual acuity. Follow-up visits were scheduled monthly whenever treatment was stopped or at 3-month intervals (after the first 6 monthly visits) when under consecutive injections. Focal/grid laser macular treatment was not allowed in the first 12 months. PRP was allowed if fluorescein angiography revealed extensive peripheral ischemia with intraretinal microvascular abnormalities.

The primary objective of the study was to analyze and report significant associations between baseline systemic factors and DME clinical response, considering the following quantitative and qualitative outcome variables: CST, MV, and visual acuity during follow-up, CST reduction <10%, CST reduction ≥ 20%, CST <330 μm, visual acuity ≥20/40 (LogMAR ≤ 0.3), and visual acuity improvement of at least two lines (equivalent to 10 ETDRS chart letters). In addition, the following anatomic response patterns were considered: "early responder" if a stable (two consecutive visits) CST decrease >20% was verified at the third month of follow-up, "late responder" if a CST change >20% was not verified at the third month but occurred until the sixth month or beyond, and "poor responder" if at the 12th month there was persistent DME with CST change <20% during all follow-up.

Statistics

All results were subjected to statistical analysis using IBM SPSS Statistics Version 23 software. The Shapiro–Wilk test was used to test continuous variables for normality. The independent-sample *t*-test or the Mann–Whitney test was used to compare mean values according to response categories. Linear correlations between systemic factors and all quantitative and qualitative outcomes were assessed at each follow-up checkpoint, using the appropriate Pearson or Spearman correlation coefficients (r). Significant correlations were then further studied by multivariate linear regression or binary logistic regression. A *P*-value < 0.05 was considered statistically significant. To protect against false-positive type errors, all significant *P*-values were subject to the Benjamin–Hochberg statistical procedure, which adjusts the significance values according to the expected false-discovery rate.

Results

Of the original 71 recruited cases, 3 were excluded due to diagnosis of infectious or oncologic systemic illness, and one other was excluded due to missing more than two visits after 6 months of follow-up. The remaining 67 patients with center-involved DME completed all the intended treatment and visit appointments. Baseline characteristics of the study population are shown in Table 1. Mean patient age was 63.95 ± 13.81 years, and mean duration of DM2 was 18.23 ± 9.63 years. All cases had NPDR, classified as moderate in 30 cases and severe in 37 cases. A mean of 7.28 ± 1.51 injections were performed during the study follow-up period.

The mean baseline CST value was 500.97 ± 108.08 μm, improving to 360.46 ± 98.20 μm at the 12th month ($P < 0.001$). Mean MV followed a similar improvement profile (10.90 ± 1.89 mm^3 at baseline improving to 9.51 ± 1.32 mm^3 at the 12th month, $P < 0.001$).

A favorable anatomic response (≥20% reduction in CST) was found in 28 cases (48.1%) at the third month, improving to 44 cases (65.6%) at the 12th month. A limited anatomic response (<10% decrease in CST) was found in 20 cases (29.8%) at the third month, decreasing to 11 cases (16.4%) at the 12th month. Finally, a CST < 330 μm was found in 17 cases (25.3%) at the third month improving to 28 cases (41.7%) at the 12th month. Regarding the anatomic response pattern, 28 (48.1%) cases were "early responders," 24 (35.8%) were "late responders," and 15 (22.4%) were "poor responders." Table 2 lists the laboratory data according to anatomic response categories.

Regarding visual acuity, there was a significant improvement from 0.58 ± 0.24 logMAR visual acuity (approximately 20/80 on the Snellen chart) at baseline to 0.40 ± 0.20 logMAR (20/50) at the 12th month ($P < 0.001$). A visual acuity ≥20/40 was found in 11 (16.4%) cases at baseline, improving to 32 (47.7%) cases at the 12th month. An improvement in visual acuity of at least 2 lines was seen in 19 cases (28.4%) at the third month improving to 31 (46.2%) cases at the 12th month. Table 3 lists the laboratory data according to qualitative visual acuity outcomes.

Table 1. Baseline Demographic and Clinical Characteristic of Study Population According to Anatomic Response Patterns

	Early Resp.	Late Resp.	Poor Resp.	Total	*P*
N	28 (41.7%)	24 (35.8%)	15 (22.4%)	67	
Age	70.72 ± 8.21	61.86 ± 8.99	61.48 ± 16.91	64.09 ± 13.88	0.244
Duration of DM2	18.00 ± 8.96	15.40 ± 7.43	19.63 ± 10.90	18.22 ± 9.71	0.849
Insulin treatment	8 (11.9%)	6 (8.9%)	7 (10.4%)	21 (31.3%)	0.716
Male	10 (14.9%)	11 (16.4%)	9 (13.4%)	30 (44.7%)	0.777
Female	18 (26.8%)	13 (19.4%)	6 (8.9%)	37 (55.2%)	
Moderate NPDR	8 (11.9%)	11 (16.4%)	11 (16.4%)	30 (44.7%)	**0.019**
Severe NPDR	20 (29.8%)	13 (19.4%)	4 (5.9%)	37 (55.2%)	
Previous PRP	10 (14.9%)	8 (11.9%)	8 (11.9%)	26 (38.8%)	0.417
Previous macular laser	7 (10.4%)	6 (8.9%)	6 (8.9%)	19 (28.3%)	0.525
Pseudophakic	6 (8.9%)	8 (11.9%)	5 (7.45)	Y: 19 (28.3)	0.566

Association of Systemic Factors and Intravitreal Injection Profiles

Of the 67 included cases, 39 (58.2%) were switched to aflibercept due to persistent DME with <20% change in CST after the third month (24 late responders and 15 poor responders). An additional 8 cases (early responders) were treated with aflibercept due to recurrent DME after the sixth month. The mean number of aflibercept injections was 3.20 ± 0.87, and the switch occurred beyond the fourth month of follow-up (mean 4.94 ± 0.81 months). Cases switched to aflibercept had significantly higher hsCRP values (2.22 ± 1.78 vs. 0.91 ± 0.57, $P = 0.001$) (Table 4) comparing with cases treated with bevacizumab. At least one triamcinolone injection was given to 33 (49.3%) cases (mean 1.78 ± 0.78) due to persistent DME beyond the sixth month of follow-up (mean 7.91 ± 1.02 months); of these 33 cases, 15 were poor responders and 18 were late responders. Such cases had significantly higher hsCRP (2.49 ± 1.93 vs. 1.17 ± 0.88, $P = 0.002$) and MCP1 (231.89 ± 101.59 vs. 181.62 ± 63.25, $P = 0.032$) comparing with cases treated only with one or both anti-VEGF agents (Table 4).

Association of Systemic Factors and Macular Outcomes

Significant correlations were found between baseline hsCRP concentration and CST at all follow-up checkpoints: third month ($P = 0.002$, r = 0.386), sixth month ($P = 0.003$, r = 0.360), ninth month CST ($P < 0.001$, r = 0.423), and 12th month ($P = 0.001$, r = 0.410). No correlations were found for MV. Multivariate regression analysis revealed that baseline hsCRP value was the only factor significantly associated with CST during follow-up, even when accounting for baseline CST, age, DM duration, or glycemic parameters (Table 5). Regarding the percentage of CST decrease from baseline (%CST.var), a significant positive correlation (higher VEGF-A, higher CST decrease) was found between serum VEGF-A and sixth month %CST.var ($P = 0.014$, r = 0.344). In addition, a significant inverse correlation (higher

Table 2. Systemic Baseline Laboratory Data According to Anatomic Response Patterns

	Early Resp.	Late Resp.	Poor Responder	Total	
	28	24	15	67	*P*
Blood glucose (mg/dL)	190.60 ± 63.03	153.68 ± 50.22	179.30 ± 47.96	174.28 ± 56.39	0.115
HbA1c (%)	8.33 ± 1.51	7.48 ± 0.97	8.23 ± 1.23	7.96 ± 1.36	0.057
Creatinine (mg/dL)	0.87 ± 0.23	0.89 ± 0.19	0.84 ± 0.18	0.87 ± 0.20	0.777
BUN (mg/dL)	50.52 ± 15.52	48.10 ± 17.10	44.61 ± 14.99	48.42 ± 15.89	0.573
LDL (mg/dL)	104.46 ± 21.07	87.01 ± 39.76	97.92 ± 29.13	96.25 ± 31.66	0.066
HDL (mg/dL)	49.63 ± 13.34	51.08 ± 19.87	54.33 ± 22.19	51.34 ± 18.09	0.980
Total Chol (mg/dL)	174.78 ± 29.22	164.30 ± 43.14	180.33 ± 37.94	172.19 ± 37.07	0.398
hsCRP	1.54 ± 1.52	2.11 ± 1.90	1.95 ± 1.21	1.84 ± 1.63	0.247
Homocysteine (μmol/L)	14.15 ± 4.02	14.09 ± 5.44	14.05 ± 4.15	14.10 ± 4.51	0.860
VEGF-A (pg/mL)	95.57 ± 53.37	114.34 ± 76.29	54.96 ± 22.52	89.21 ± 57.89	**0.009**
MCP-1 (pg/mL)	208.79 ± 78.95	195.79 ± 115.54	220.29 ± 85.08	207.17 ± 92.63	0.416
sICAM-1 (pg/mL)	796.98 ± 222.61	823.07 ± 287.20	859.25 ± 410.62	820.81 ± 294.62	0.995

HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table 3. Systemic Baseline Laboratory Data According to Qualitative Visual Acuity (VA) Outcomes

	Final VA Improv ≥ 2 Lines			Final VA ≥ 20/40 Snellen		
	Y:31	N: 36	*P*	Y:32	N:35	*P*
Blood glucose (mg/dL)	173.28 ± 61.51	175.45 ± 51.07	0.891	174.28 ± 57.11	174.29 ± 56.82	0.999
HbA1c (%)	7.91 ± 1.57	8.02 ± 1.03	0.415	8.07 ± 1.43	7.86 ± 1.24	0.683
Creatinine (mg/dL)	0.89 ± 0.19	0.85 ± 0.21	0.478	0.82 ± 0.17	0.92 ± 0.22	0.101
BUN (mg/dL)	48.11 ± 17.01	48.78 ± 14.84	0.815	46.30 ± 16.73	50.22 ± 15.22	0.254
LDL (mg/dL)	100.88 ± 35.01	91.90 ± 28.05	0.234	94.75 ± 36.60	97.65 ± 26.77	0.515
HDL (mg/dL)	46.24 ± 10.09	56.10 ± 22.36	0.103	50.13 ± 18.02	52.47 ± 18.38	0.813
Total Chol (mg/dL)	175.82 ± 40.71	168.90 ± 33.76	0.471	170.55 ± 43.60	173.68 ± 30.63	0.745
hsCRP	1.61 ± 1.51	2.05 ± 1.76	0.207	1.44 ± 1.36	2.21 ± 1.82	0.056
Homocysteine (μmol/L)	13.99 ± 4.39	14.20 ± 4.67	0.919	13.32 ± 4.07	14.86 ± 4.84	0.139
VEGF-A (pg/mL)	82.37 ± 36.95	94.37 ± 73.06	0.749	86.93 ± 73.49	90.15 ± 41.14	0.079
MCP-1 (pg/mL)	214.60 ± 95.32	183.09 ± 82.47	0.191	207.10 ± 112.35	210.50 ± 73.87	0.405
sICAM-1 (pg/mL)	722.72 ± 183.12	910.09 ± 345.05	**0.025**	821.11 ± 337.15	826.72 ± 266.76	0.800

HDL, high-density lipoprotein; LDL, low-density lipoprotein.

PCR lower CST decrease) was found between hsCRP and third month %CST.var ($P = 0.035$, $r = -0.270$) as well as 12th month %CST.var ($P = 0.039$, $r = -0.259$); however, statistical significance for the latter two correlations was lost after the BH statistical procedure. In a multivariate linear regression model regarding the effect on %CST.var at all follow-up checkpoints, and including all systemic factors as well as baseline CST, a significant effect was found only for third month %CST.var and the combined model of baseline CST and hsCRP ($P < 0.001$, $r^2 = 0.405$, β(baseline CST) = 0.559, β(hsCRP) = −0.403) ($P = 0.003$, $r^2 = 0.585$) (Figure 1).

Regarding qualitative outcomes, the only significant association with obtaining decrease in CST≥ 20% was for DR grade, namely cases with severe NPDR were more likely to achieve such outcome at the third (Fisher test, $P = 0.013$) and sixth months of follow-up (Fisher test, $P = 0.024$), but significance was lost when accounting for baseline CST ($P = 0.076$ for third month and $P = 0.062$ for sixth month). Concentrations of sICAM1 (959.77 ± 324.54 pg/mL vs. 765.87 ± 266,280,736, $P = 0.032$) and hsCRP (1.46 ± 1.31 vs. 2.72 ± 1.97 mg/L, $P = 0.014$) were significantly higher in cases with a limited anatomic response (<10% change of CST) at the third month of follow-up (Figure 2). A similar trend was maintained for higher hsCRP and <10% CST change at the sixth month (3.39 ± 1.84 vs. 1.49 ± 1.38, $P < 0.001$). In fact, binary logistic regression confirmed that the significant association between hsCRP and such outcome at the sixth month was independent of DM2 duration, age, baseline CST, or other analytic variables ($P = 0.004$, $r = 0.560$, OR = 2.46). Receiver-operating characteristic curve analysis for such outcome revealed an area under the curve of 0.838, $P < 0.001$, and an optimal cutoff value of hsCRP = 1.84 mg/L. Regarding the outcome of obtaining a CST <330 μm, a significant association was seen between hsCRP and such outcome at the sixth month of follow-up (1.08 ± 0.76

Table 4. Systemic Proinflammatory and Metabolic Factors According to Treatment Agents Used During Follow-Up

	Bevaciz. Mono	Aflib. Switch Total	Aflib/No TCA	TCA/Aflibcombin.	*P* (Kruskal–Wallis)
N	20 (29.8%)	47 (70.2%)	14 (16.4%)	33 (49.3%)	
VEGF-A	79.41 ± 39.75	94.11 ± 65.10	123.58 ± 84.62	86.46 ± 58.58	0.226
ICAM-1	760.63 ± 189.05	846.60 ± 328.35	816.15 ± 340.63	856.11 ± 329.42	0.77
MCP-1	201.25 ± 67.46	209.65 ± 101.96	152.17 ± 44.03	231.89 ± 101.59	**0.033**
hsCRP	0.91 ± 0.57	2.22 ± 1.78	1.56 ± 1.11	2.49 ± 1.93	**0.002**
Homocysteine	12.98 ± 4.21	14.61 ± 4.60	13.51 ± 5.13	15.00 ± 4.42	0.128
HbA1c	8.17 ± 1.79	7.85 ± 1.03	7.71 ± 0.80	7.90 ± 1.10	0.897
Glucose	184.35 ± 74.78	169.40 ± 45.43	149.16 ± 40.47	173.58 ± 45.91	0.446
Creatinine	0.82 ± 0.19	0.89 ± 0.21	0.83 ± 0.18	0.92 ± 0.21	0.323
BUN	51.53 ± 15.19	47.32 ± 16.19	39.28 ± 9.37	49.20 ± 16.97	0.193
LDL Chol	89.80 ± 28.94	98.52 ± 33.39	82.92 ± 27.94	105.22 ± 32.75	0.102
HDL Chol	50.46 ± 20.62	51.43 ± 17.20	54.20 ± 13.98	50.95 ± 18.07	0.276
Total Chol	162.31 ± 35.80	176.66 ± 37.19	158.09 ± 20.69	183.25 ± 39.70	0.054

Aflib., aflibercept; Bevaciz., bevacizumab; BUN, blood urea nitrogen; Chol., cholesterol; HDL, high-density lipoprotein; TCA, triamcinolone.

Table 5. Multivariate Regression Results Considering Relevant Systemic Factors and CST During Follow-Up

	3rd Month CST		6th Month CST		9th Month CST		12th Month CST	
Parameter	B	Sig.	B	Sig.	B	Sig.	B	Sig.
DM years	−0.708	0.711	1.415	0.358	0.813	0.488	2.004	0.279
Age	−0.985	0.510	−1.060	0.424	−0.443	0.660	1.211	0.446
hsCRP	23.752	**0.006**	17.710	**0.023**	16.904	**0.005**	28.848	**0.003**
Homocyst	−2.823	0.369	1.830	0.498	−0.559	0.785	−6.812	0.040
HbA1c	−14.347	0.376	−12.994	0.257	−6.592	0.449	−11.297	0.410
Glucose	0.105	0.768	0.024	0.937	−0.024	0.918	−0.160	0.662
Creatinine	4.406	0.949	10.818	0.858	20.598	0.654	106.753	0.145
Baseline CST	0.334	**0.017**	0.399	**0.001**	0.288	**0.002**	0.295	**0.036**

vs. 2.26 ± 1.83 mg/L, $P = 0.007$). Logistic regression confirmed that hsCRP was the only factor significantly associated with such outcome, and this result was independent of DM2 duration, age, baseline CST values, or any other analytic variables ($P = 0.005$, r = 0.489, OR = 0.270). A similar trend of lower hsCRP in cases with CST <330 μm was maintained until the 12th month (1.30 ± 1.39 vs. 2.21 ± 1.70, $P = 0.005$), but at this timepoint, significance was lost in the logistic regression model ($P = 0.058$, OR = 0.624).

Among anatomic response patterns, we verified that early responders were more likely to have severe NPDR comparing with poor responders (Fisher test, $P = 0.009$); in addition, a significant difference was found in serum VEGF-A levels, which were significantly lower in the poor responder group ($P = 0.009$)

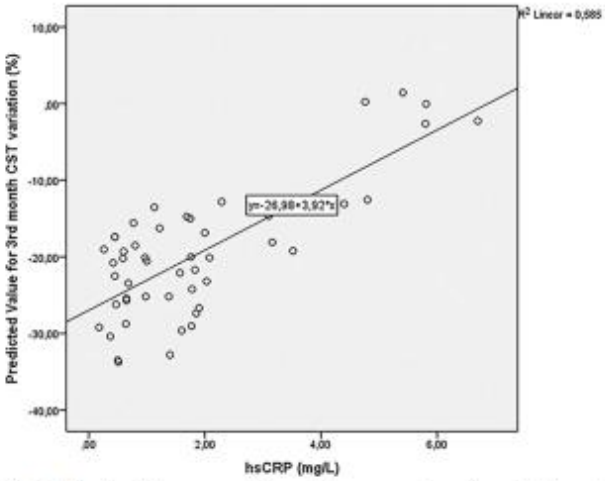

Fig. 1. Scatter/dot representation of the percentage of central foveal thickness change at the third month according to baseline serum high-sensitivity C-reactive protein level (hsCRP). The statistically predicted value was obtained from a multivariate linear regression model considering the effect of hsCRP ($P = 0.001$), patient age ($P = 0.661$), duration of diabetes ($P = 0.674$), baseline blood glucose level ($P = 0.048$), glycated hemoglobin percentage ($P = 0.637$), and baseline CST value $P < 0.001$). The best-fit regression line and equation are indicated.

(Table 2). Early responders had significantly worse baseline CST ($P < 0.001$) but achieved a significantly higher percentual decrease in CST from the 3th month onwards, when comparing with the late and poor responder groups (−37.9% vs. −8.5% vs. −9.8%, $P < 0.001$), even when accounting for baseline CST as a covariate ($P = 0.034$). By the 12th month, the difference between "early responder" and "late responder" was no longer significant (−34.8% vs. −27.9%, $P = 0.168$), but both were significantly better than poor responders (−8.9%, $P < 0.001$) (Table 6).

Association of Systemic Factors and Visual Outcomes

A significant correlation was found between baseline visual acuity and serum VEGF-A ($P = 0.047$, r = 0.282). Significant correlations between systemic factors and logMAR visual acuity during follow-up were seen only for hsCRP and sixth month visual acuity ($P = 0.016$, r = 0.301). However, the aforementioned results lost statistical significance after the BH statistical correction. The stepwise linear regression revealed that a combined model of hsCRP and baseline visual acuity was the most significantly associated with sixth month visual acuity ($P < 0.001$, $r^2 = 0.676$, β.hsCRP = 0.238, β.baseline visual acuity = 0.753). Baseline visual acuity was the only variable significantly associated with 12th month visual acuity ($P < 0.001$, $r^2 = 0.602$, β = 0.776).

Regarding qualitative visual acuity outcomes, no significant associations were seen between systemic factors and obtaining a final visual acuity ≥20/40. However, considering demographic and clinical variables, age ($P = 0.042$, $r^2 = 0.109$, OR = 0.95), baseline CST ($P = 0.007$, $r^2 = 0.172$, OR = 0.99), baseline MV ($P = 0.002$, $r^2 = 0.246$, OR = 0.56), and severe NPDR (chi-square, $P = 0.015$, OR = 0.28) were significantly negatively associated with such outcome.

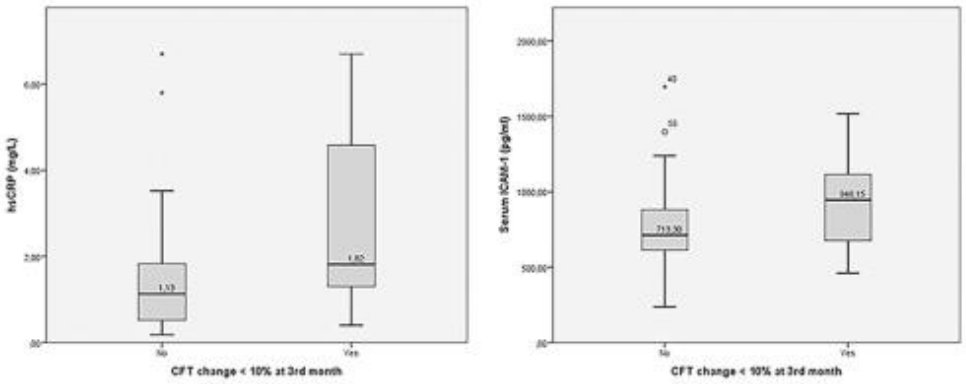

Fig. 2. Boxplot graphical representation of median and quartile values for high-sensitivity C-reactive protein and serum ICAM-1 according to the outcome of obtaining a percentage of CFT change from baseline <10% or ≥10% at the third month of follow-up. Cases with a limited anatomic response at the third month had significantly higher values of both serum factors.

As for an improvement of at least two visual acuity lines, cases achieving such outcome had significantly lower sICAM1 concentrations (722.72 ± 183.12 vs. 910.09 ± 345.05, $P = 0.025$). Logistic regression revealed that sICAM1 was negatively associated with such outcome, independently of baseline CST or baseline logMAR visual acuity ($P = 0.035$, $r^2 = 0.257$, OR = 0.97), but the effect was lost after BH statistical procedure. Regarding clinical and demographic variables, only baseline visual acuity was a significant factor for such outcome ($P = 0.009$, $r^2 = 0.146$, OR = 20.54).

Discussion

We analyzed several analytic and clinical factors to study associations with anatomic and functional outcomes in patients treated with anti-VEGF injections in a real-world clinical setting. In concordance with previous studies, we verified a significant variability in response to treatment with anti-VEGF for DME.[11,18,19] Namely, under our practice conditions, the majority (58.2%) of cases still had significant center-involved DME after 12 months of treatment. Such result is somewhat similar to a *post hoc* analysis of protocol T,[3] revealing that 68.2% of eyes with persistent DME at the sixth month would still have DME after 2 years. Our results suggest a role for systemic inflammation in the pathogenesis of persistent DME. In fact, we verified that such cases had higher baseline values of hsCRP, and both sICAM-1 and hsCRP were significantly associated with a limited anatomic response (≤10% CST) at the third month. In addition, serum VEGF-A was significantly lower in "poor responders" when comparing with the other two categories. A possible interpretation is that in cases with poor anatomic response to anti-VEGF, the pathogenesis of DME may be predominantly due to inflammatory breakdown of the inner blood retinal barrier, rather than retinal hypoxia leading to higher intraocular VEGF expression and increased capillary permeability. The latter process may be predominant in cases with more severe NPDR or proliferative DR. In this regard, Ma et al[20] verified a significant association between vitreous and plasma VEGF level in PDR patients. Also, a recent meta-analysis verified that serum VEGF levels were significantly higher in proliferative DR patients than in those with nonproliferative DR, and the authors concluded that serum VEGF could be a reliable biomarker for monitoring DR progression.[21] There is evidence that intravitreal injections of bevacizumab or aflibercept cause a significant decrease in plasma-free VEGF level measured at least 4 weeks after injection.[22,23] Therefore, it is theoretically possible that increased ocular levels of VEGF can also cross the BRB and accumulate in the systemic circulation. We could hypothesize that increased serum VEGF-A levels may portend a better potential for anatomic response to anti-VEGF monotherapy. However, biomarkers of cellular inflammation such as CRP and ICAM-1 (and perhaps even MCP1) may indicate propensity for persistent DME and less favorable outcomes. In fact, increased serum ICAM-1 levels had been previously reported in cases with diabetic retinopathy,[24,25] but to the best of our knowledge, this is the first study to identify significant associations between serum biomarkers of inflammation and parameters of clinical response to anti-VEGF treatment for DME. Our findings are clinically relevant because the early identification of anti-VEGF "poor responders" would allow for the timely consideration of other treatment strategies such as corticosteroid implants.[14,26]

Regarding functional outcomes, we verified a visual acuity improvement of at least 2 lines in 28.4% of cases at the third month, which is in agreement with a Protocol T analysis revealing that 32.9% of cases treated with bevacizumab achieved ≥ 10 letters of visual acuity improvement at the third month of follow-up.[27] Interestingly, we found a negative effect of sICAM1 on the probability of obtaining two lines of

Table 6. Characterization of Macular Outcomes According to Anatomic Response Patterns

	CST (μm) and Percentage of Decrease from Baseline Value					
Anatomic Response Pattern	CST Baseline	CST 3. M (%CST.var)	CST 6. M (%CST.var)	CST 9. M (%CST.var)	CST 12. M (%CST.var)
Early (n = 28)	554.50 ± 115.26	336.84 ± 70.67 (−37.9%)	337.14 ± 94.60 (−38.3%)	336.50 ± 73.76 (−38.0%)	354.39 ± 129.43 (−34.8%)
Late (n = 24)	487.00 ± 91.71	444.30 ± 94.96 (−8.5%)	377.87 ± 72.66 (−21.9%)	358.20 ± 58.38 (−25.7%)	348.39 ± 58.13 (−27.9%)
Poor (n = 15)	423.40 ± 55.89	387.14 ± 65.81 (−9.8%)	385.00 ± 60.56 (−9.2%)	377.73 ± 49.01 (−10.6%)	384.80 ± 62.93 (−8.9%)

visual acuity improvement, considering the role of this molecule on retinal leukostasis, and the fact that in our study ICAM1 was also associated with an early limited anatomic response, it is possible that this result indicates a propensity for irreversible damage to the neuroretina. In addition, hsCRP was found to be associated with sixth month visual acuity, considering that 6 months is typically the timeframe in which a significant response to anti-VEGF is expected to have occurred,[3,11,26,28] it is possible that such association indicates persistent DME as the cause for limited visual acuity recovery. Finally, in agreement with previous reports,[11] we found that baseline visual acuity was strongly associated with final visual acuity, and a negative effect was seen from advancing age and NPDR severity.[29] The effect of baseline visual acuity could be explained by a ceiling effect, in that patients with worse initial visual acuity have more potential to improve. The effect of age may be related to variables not analyzed in this study such as grade of lens sclerosis, neurologic status, or even education level. We also found that DR grade had a limiting effect on visual acuity, which may either result from increased retinal ischemia or perhaps and indirect association with systemic comorbidities leading to an unaccounted effect on visual acuity. Our combined results suggest that DME results from an interplay of factors consisting of retinal hypoxia and increased VEGF expression leading to vasogenic retinal edema and increased inflammatory activity leading to persistent inner BRB damage. In this regard, a study by Cunha-Vaz et al[30] identified three mild NPDR phenotypes with different risk for progression to DME. Cases with increased CST have higher risk of progression to DME, followed by those with increased MA turnover. Considering increased MA turnover as an indicator of retinal ischemia, such phenotype is probably characterized by increased retinal VEGF expression, and theoretically, these patients would experience more favorable prognosis when treated with VEGF agents.

Our study has limitations, namely the fact that our results derive from a single clinical center, which may not be fully representative of treatment practices around the world. Also, we measured systemic factors only at baseline due to logistical constraints regarding acquisition of ELISA kits for repeated cytokine dosing. Nevertheless, this study provides real-world evidence for favorable results with intravitreal anti-VEGF treatment for DME in cases with certain serum biomarker profiles. It is important to consider that we performed an extensive analysis of systemic and ocular factors pertinent to the pathogenesis of DME, emphasizing the possible effect on treatment response to anti-VEGF. In conclusion, our results indicate that cases with lower serum VEGF-A and

increased proinflammatory factors such as hsCRP and ICAM-1 may be more likely to exhibit a limited anatomic response to anti-VEGF monotherapy and progress to persistent DME. Serum inflammatory factors may have a role in modulating anti-VEGF treatment response, and further research will possibly lead to continued optimization of current treatment strategies.

Key words: anti-VEGF, bevacizumab, C-reactive protein, diabetic macular edema, ICAM-1, serum, prognostic, response.

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CHAPTER III – DISCUSSION AND CONCLUSIONS

12. Discussion

The introduction of anti-VEGF agents into clinical practice for the management of DME, has yielded unprecedented clinical outcomes but also led to new challenging questions requiring further research. Namely, the high frequency of intravitreal injections and clinical monitoring visits, typical of randomized trials such as RIDE/RISE or VIVID/VISTA, is difficult to replicate in a real-world clinical setting. In order to circumvent such challenge, the most widely known strategy is to employ a *pro re nata* treatment regimen, that is, after a fixed "loading dose" of about 3-6 injections, treatments are further scheduled according to certain clinical criteria. Yet as major real-world analyses revealed, the outcomes with such modality are inferior to fixed monthly treatment regimens[211, 212]. Even if we examine the results obtained in the major clinical trials it becomes apparent, that there is a wide variability in clinical response to anti-VEGF treatment. In fact, while about 40% of cases will obtain at least 15 letters of VA improvement, most cases will be under that benchmark, more precisely about a third of cases will not achieve 10 letters of VA improvement[189, 213, 216]. Similarly, while most patients will achieve significant reductions in the macular thickness parameters, there is clearly a subset of patients with persistent DME even after 2-3 years of treatment[214]. Overall, if we consider that DME may result from a variable interplay of pathologic events, creating an extended spectrum which may include different levels of disease duration, clinical severity and morphologic and/or functional changes in the retina, it would be of paramount importance to identify which of these underlying factors lead to such variable responses to anti-VEGF agents. This question is certainly the most relevant paradigm regarding DME treatment, and the main goal of this thesis was to analyze possible systemic factors associated with the variability in clinical response to anti-VEGF.

Even before the appearance of anti-VEGF agents, it was known that the rate of progression and severity of RD varies even among patients with similar demographic or metabolic profiles. Indeed, while it has been reported that nearly all type 1 and about 80% of type 2 diabetics will have DR after 20 years[11], a study of patients who survived more than 50 years of type 1 diabetes, indicated that only about 50% of those presented with DR[221]. Additionally, not all patients with DR progress to sight-threatening phenotype complications such as DME or PDR[222]. While there is undeniable benefit in controlling the major risk factors for diabetic vascular complications, in the case of DR, the WESDR study showed that HbA1c, cholesterol and blood pressure only account for 10% of the risk for developing retinopathy[223]. Such result is corroborated by a follow-up analysis of the DCCT Trial

revealing that the glycemic exposure (consisting of duration of diabetes and HbA1c level) explained only 11% of retinopathy risk[224]. Such variability in DR severity and even regarding clinical response to anti-VEGF could possibly be explained by the contribution of other factors either environmental or genetic, playing a crucial role in modulating the DR phenotypes. In favor of genetic factors, there is evidence of familial clustering of severe DR among first-degree relatives of subjects in the DCCT study[225]. Additionally, many candidate gene studies have identified several associations with DR, such as: VEGF[226], aldose reductase, nitric oxide synthase 3, erythropoietin, receptor for advanced glycation end product (RAGE) and ICAM1 among others[227, 228]. However, these studies have yielded variable results[229, 230]. Recently, a genome-wide association study (GWAS) identified genetic variation near the GRB2 gene (chromosome 17q25.1) to be associated with sight-threatening DR[231]. Such results were confirmed in independent cohorts; however, it should be noticed that the discovery cohort included cases with nonproliferative DR, proliferative DR and clinically significant DME. Considering that clinically significant DME may occur at any grade of DR severity, and conversely proliferative DR may occur without central macular edema, the results of the aforementioned study are not readily applicable to the problematic different patterns response to anti-VEGF agents for DME. In fact, a recent review study not only confirmed the contradictory results regarding genetic analysis and DR, but also verified that very few studies have addressed potential genetic associations with the specific diagnosis of DME[232]. Therefore, most studies investigating the risk and severity of DR complications have focused on analysis of systemic or ocular variables associated with DR. In this regard, most *posthoc* analyses of major randomized suggest that patients with worse baseline DR severity, lower baseline VA scores or persistent DME, tend to achieve lower anatomic or visual acuity outcomes in the long term. Yet such observations do not provide further insight as to why such patients have worse DR or persistent DME to begin with. In the specific case of DME, it has been suggested that DME should be considered a distinct phenotype regarding the study of DR complications[232]. In this regard, we should take notice of the pioneering work by Cunha-Vaz and collaborators in which three distinct mild nonproliferative DR phenotypes were identified, each with a different risk of progression to DME[233]. In fact, increased baseline CRT was associated with increased risk of progression to macular edema, particularly when associated with MA turnover rate > 6 in the macular region. Interestingly increased CRT was more significant than MA turnover rate as a risk factor for DME. Additionally, the authors found that HbA1c was significantly higher in patients with high MA turnover[233]. However, no factors were found to be associated with the phenotype characterized by increased CRT and low MA turnover rate

(phenotype B). Interestingly, a candidate gene study, designed with the goal of identifying genetic biomarkers of DR progression, revealed the single nucleotide polymorphism (SNP) corresponding to ICAM1 rs1801714 was significantly associated with phenotype B. Such genetic profile indicates susceptibility to inflammation and leukostasis leading to BRB disruption and increased CRT in phenotype B. While in the phenotype associated with increased microaneurysm turnover (phenotype C), the associated SNPs suggest the occurrence of endothelial damage and ischemia-induced VEGF expression. Such results are noteworthy as they were verified in clearly defined DR phenotypes, indicating significant differences in genetic profiles. However, as the authors point out the results must be interpreted with caution due to the relatively small population for a genetic association study. Even considering that such studies represent a step in the right direction, there are still drawbacks regarding the clinical application of this information. Considering that both DM and DR are highly prevalent diseases it would be logistically difficult to perform genetic testing in every patient with DM and mild NPDR. Additionally, even if we could clearly identify the genetic risk for DME, there still would be no indication of how a certain high-risk patient would respond to anti-VEGF treatment once DME developed. Therefore, it seems that the most practical biomarkers for the clinical response to DME should either be identified by noninvasive or minimally invasive routine procedures. Therefore, after a revision of the available literature, we selected the major systemic metabolic and pro-inflammatory factors known to be associated with DR, we also used SD-OCT to obtain reliable serial measurements of central retinal thickness, macular volume as well as to identify some recently reported biomarkers such as hyperreflective foci, subretinal fluid, intraretinal cyst size and disorganization of retinal inner layers. Regarding OCT imaging biomarkers, there has been an increased number of publications evaluating their potential role as predictors of clinical outcomes. More specifically serous retinal detachment (SRD) has been associated with increased vitreous levels of IL6 suggesting increased inflammatory activity in such cases[72]. However, the relevance of such finding regarding response to anti-VEGF agents is not straightforward. In fact, Vujosevic et al in a study comparing OCT biomarkers among patients treated with ranibizumab or dexamethasone, reported that there was no significant difference in rate of resolution of SRD[157]. Additionally, posthoc analysis of both RESTORE and RIDE/RISE revealed that subretinal fluid responds well to ranibizumab treatment[156] and in the case of RIDE/RISE the presence of such imaging sign was predictive of a final VA >20/40 as well as an improvement ≥ 15 letters[160]. Considering that in a previous report Sohn et al reported that bevacizumab had no effect on aqueous IL-6 level, while triamcinolone caused a significant decrease of such interleukin [86], the role

of subretinal fluid as reliable indicator of increased retinal inflammation is of difficult interpretation. Regarding the presence of hyperreflective retinal foci (HRF), such sign was first described by Bolz and found in OCT images of DME patients[234]. The author interpreted this sign as resulting from extravasated lipoproteins, indicating BRB breakdown[234]. In fact, HRF are reportedly more frequent in cases with DR versus those with DM but no DR[235] and are more commonly found in the inner retinal layers. The fact that HRF were identified in cases without visible hard exudates or tomographic signs of DME, led to the hypothesis that such sign is probably secondary to early microglia activation, which is consistent with low-grade chronic inflammation contributing to diabetic retinal dysfunction. However, it has been shown that both anti-VEGF and intravitreal steroid treatments lead to a decrease of HRF[157], but no significant association with VA improvement was found[236, 237]. Additionally, currently there is no widely available OCT technology that allows reliable automated quantification of HRF in a selected macular area, meaning that there is still a subjective component associated with grading this sign. While potentially interesting, as of today it is difficult to interpret the association of this biomarker with intravitreal treatment response patterns. Another recently described OCT biomarker is disorganization of the retinal inner layers (DRIL), defined as the extent for which the boundaries between the ganglion cell-inner plexiform layer complex, inner nuclear layer, and outer plexiform layer could not be identified in a 1mm diameter of the 7 central OCT B-scans[238]. While baseline DRIL extent was not significantly associated with final VA, the authors reported that for every DRIL increase of about 300 μm from baseline to 4 months, VA was reduced by 1 line at 8 months of follow-up. The effect of DRIL has also been verified by other studies[155, 239]. However as with HRF, there is a subjective component to grading the extent of DRIL, as it has to be manually measured in individual OCT B-scans, in fact in a recent study the interrater agreement for DRIL measurement was only slight to moderate, perhaps due to confounding features such as generalized blurring of retinal layers, cystoid spaces that alter retinal boundaries or variable optical intensity[240]. The final OCT biomarkers worthy of mention are the disruption of the ELM, and of the ellipsoid zone band, which are found to be increased in poor responders[155]. In fact, Maheshwary was the first to report a significant correlation between increasing disruption of the then named photoreceptor IS/OS junction and decreased VA[241]. The most widely used quantitative and reproducible OCT variable is central retinal thickness (CRT), however, correlation with VA outcomes is moderate and seems to decrease with continued treatment[155, 242, 243]. Notwithstanding the contribution of imaging biomarkers in predicting of DME clinical response, we have to consider that DR and DME are local manifestations of a complex systemic metabolic disease with significant

risk for multiorgan vascular disease, meaning that is a possibility that other systemic variables may play a role in the overall diabetic vascular pathogenesis. In fact, it has long been known that improved glucose control leads to decreased progression of DR. Additionally, the possible contributions of serum lipid profile[16] and renal function[244, 245] place emphasis on the fact that systemic state may alter the course of DR and its complications such as DME. Therefore, a comprehensive approach to understanding the different patterns of DME clinical response, requires and thoughtful analysis of systemic and ocular parameters.

One hypothesis could be that certain ocular or systemic parameters interfere with the severity of DME and consequently the effectiveness of anti-VEGF therapy. Regarding hbA1c level, there is conflicting evidence towards its effect on DME treatment[162, 163]. The accumulating evidence that diabetic patients have increased serum pro-inflammatory factors such as CRP[134, 136, 138] and TNFα[111, 246, 247], and that cases with DR and DME also have increased VEGF, MCP1, ICAM1 and interleukins in the aqueous[76, 86, 117, 119, 248], vitreous humor[71, 74] or serum[53, 77, 100, 103], further emphasizes the need to investigate the contribution of the inflammatory component for DME severity. Therefore, after a thorough revision of the available literature we selected the major biochemical and pro-inflammatory factors known to be consistently associated with DR. Regarding the specific case of DME and inflammatory factors, most studies analyze ocular fluids, however obtaining such samples is not clinical straightforward due to ethical questions regarding invasive maneuvers. In order to obtain aqueous humor the only feasible scenario is to perform an anterior chamber tap during cataract surgery, while the collection of a vitreous sample typically requires a pars plana vitrectomy, which in the case of DR is mostly due to severe complications associated with proliferative DR. Fortunately, not all patients with DME will develop cataract, and we chose not to include cases with proliferative DR, due to the fact that the pathogenesis in this extremely severe form of DR can be mostly due to extensive retinal ischemia and neovascularization, more so than macular BRB breakdown. Ideally, predictive factors for DME response, should be easily obtained with safe noninvasive or minimally invasive routine procedures. To procure such clinically valuable biomarkers, we collected baseline blood samples for all recruited patients and serum was isolated to measure the 4 of the most consistently reported factors known to be associated with DME – VEGF-A, MCP-1, ICAM-1 and TNF-α. In early result analysis TNF-α did not reach statistical significance or any appreciable tendency towards the evaluated outcomes, so due to the costs associated with cytokine dosing, we decided to eliminate TNF-α from further investigation. The

remaining demographic, metabolic and pro-inflammatory factors were considered on the analysis of several markers of qualitative and quantitative clinical response to anti-VEGF treatment.

In order to identify biomarkers for a certain phenotype of known disease, we first have to define the major characteristics of such phenotype. Our main focus was the group of patients with persistent DME despite treatment with anti-VEGF. According to the recent randomized protocols from DRCR.net, persistent DME is defined as the presence of central retinal thickening above 305 µm for men and 320 µm for women (reference for the Heidelberg Engineering Spectralis® SD-OCT[249]) after at least 4 monthly anti-VEGF injections and persisting until 24 weeks after the first injection[204, 214]. Therefore, or first goal was to identify if systemic factors as well as tomographic quantitative and qualitative signs could be associated with a limited anatomic response to anti-VEGF. To our knowledge, we first reported in 2018[250] that patients with DME treated with anti-VEGF and exhibiting a central retinal thickness decrease < 10% of baseline after 3 injections, had significantly higher serum levels of hsCRP and ICAM-1. Such result was keystone because of the following reasons: firstly both CRP and ICAM-1 are associated with inflammatory response, with CRP favoring cellular apoptosis and ICAM-1 leading to increased adhesion of leukocytes to endothelial cells thus increasing leukocyte migration into tissues and capillary occlusion; secondly while CRP is known to be associated with insulin resistance and type 2 DM[251, 252], the association we verified was independent of DM duration, blood glucose level or HbA1c values, meaning increased hsCRP may be an independent factor contributing to the pathogenesis of DME; lastly, it is known that in DR there is upregulation of ICAM-1 expression on endothelial cells [96], while we cannot affirm that there is correspondence between systemic and intraocular ICAM-1 level, it is theoretically possible that patients with increased circulating pro-inflammatory factors may be more prone the deleterious effects of increased ICAM-1 in the retinal capillaries. Another interesting result we first reported was that patients with a significant anatomic response to anti-VEGF had significantly lower serum values of MCP-1, further contributing to the hypotheses of systemic inflammation favoring persistent DME. Finally, patients with severe NPDR and grade 2 ellipsoid zone disruption had significantly higher circulating VEGF-A which seems to suggest that increased retinal ischemia may lead to increased serum VEGF-A. Interestingly, such cases, with higher VEGF-A levels also seemed to have more significant anatomic response to anti-VEGF which could be readily understandable in a cause-effect logic. Overall, such results also seem to parallel the phenotypes of progression to DME described by Cunha-Vaz[233]. Essentially, we verified that patients with limited anatomic response had significantly higher baseline values of pro-inflammatory factors and increased CRT

persisting at the 6th month of follow-up[250]. Such subgroup would correspond to phenotype B, that is patients with increased CRT and low MA turnover. While patients with better macular outcomes not only had lower pro-inflammatory factors but seemed to have higher baseline circulating VEGF-A, which was also significantly higher in cases with severe NPDR[253]. Considering that MA turnover indicates retinal ischemia such subgroup would correspond to phenotype C. While our study population is relatively small, the results are important as there seems to be a molecular basis for different phenotypes of DME. Such findings potentiated further research directed to understanding if such associations would be maintained with longer follow-up time and would correlate with different intravitreal treatment patterns. Therefore, we then procured significant associations with total injection number, number of injections after the 6th month of follow-up, necessity of TA combination therapy for persistent DME and the possibility of resolving DME with 6 or less anti-VEGF injections[254]. We verified that cases initiating combination treatment with TA due to persistent DME, not only had higher hsCRP levels but there was also a correlation between baseline hsCRP and number of TA injections. Additionally, such cases also had higher levels of creatinine and lower circulating VEGF-A, favoring the hypothesis that in such patients, inflammatory mediated BRB breakdown is preponderant over VEGF-induced vasopermeability. Conversely, cases requiring ≤ 6 anti-VEGF injections for DME regression had significantly lower values of hsCRP and creatinine. In fact, serum creatinine was a negative predictor of achieving DME resolution with 6 or less injections. In the RIDE/RISE studies, renal disease was found to be associated with worse visual outcomes in patients treated with macular laser[160]. Our results suggest the possibility of a similar time course between renal and retinal microvascular disease which may become apparent under PRN anti-VEGF treatment. Interestingly, cases requiring less than 6 injections to achieve DME resolution also had significantly better baseline VA indicating that DME onset was probably of more recent.

Having verified that certain systemic pro-inflammatory factors were associated with early limited anatomic response to anti-VEGF and such associations could be extended to different patterns of intravitreal treatment, it was then important to understand if meaningful associations between systemic state and DME phenotypes would be found over the long term (at least 1 year of follow-up). Therefore, in our final analysis the overall functional and anatomical response to treatment was evaluated. Indeed, in agreement with our previous analyses, higher hsCRP was associated with persistent DME and a consequent switch to combined treatment beyond the 6th follow-up month. A very significant correlation between CRT and hsCRP was verified during all follow-up

checkpoints until the 12th month[253]. Such correlation was independent of glycemic control variables or baseline CRT. As with our previous study there was a tendency for patients with higher baseline VEGF-A to obtain a higher percentual decrease in CRT, however the only significant model predictive of percentual CRT change was the combination of baseline CRT and hsCRP level which were found to be predictive of 3rd month CRT change. In fact, serum ICAM-1 and hsCRP were significantly increased in patients with limited (<10%) anatomic response at the 3rd month, and this association was maintained for hsCRP and 6th month limited anatomic response. It seems that patients with a hsCRP value ≥ 1.84 mg/L, may be more likely to experience a limited anatomic response to anti-VEGF monotherapy. Additionally, hsCRP was the only variable predictive of obtaining a stable CRT< 330μm, that is cases with higher hsCRP were less likely to achieve an acceptable resolution of macular edema, however while such tendency was maintained until the 12th month of follow-up, lower hsCRP was no longer predictive of 12th month CST < 330 μm[253]. Such result could be interpreted by the fact that continued treatment with anti-VEGF or even introduction of intravitreal steroids, eventually leads to a further decrease of CRT even in cases with persistent DME, thereby attenuating the difference in macular outcomes irrespectively of pro-inflammatory profiles. Regarding the types of macular response categorized as "early responders", "late responders" or "poor responders", we verified that an early response pattern was more common in cases with severe NPDR, while serum VEGF-A was significantly lower in the poor responder group. Continuing the analysis of macular response patterns, we verified that by the 12th month of follow-up the percentage decrease in CRT was similar between early and late responders, however both continued to perform significantly better than the poor responder group which at the 12th month achieved only a mean of 8.9% decrease in CRT[253]. Such result is of paramount importance because, adding to the fact that such subgroup had lower baseline serum VEGF-A, such findings clearly suggest that in patients with a poor macular response to anti-VEGF, the pathogenesis of DME is not dependent on VEGF mediated pathways and therefore currently established treatment guidelines are not adequately addressing such subtype of DME. Regarding VA, the only variable that was consistently associated with final vision outcomes was baseline VA, however it is interesting to note that a combined model of baseline VA and hsCRP was found to be the most significantly associated with 6th month VA, that is patients with higher hsCRP and worse baseline VA tended to have a worse VA outcome at the 6th month. Additionally, regarding the qualitative outcome of obtaining an improvement of 2 lines of VA, we verified that cases which achieved such outcome had significantly lower levels of ICAM-1. Considering that both ICAM-1 and hsCRP were

consistently found to be associated with a limited anatomic response, it is possible that the association of such factors with VA outcomes is related to the negative effect of prolonged macular edema on the potential for vision recovery.

Overall, our results provide a theoretical background for the variability in clinical response to anti-VEGF treatment. By thoroughly studying systemic and tomographic biomarkers, the results reported in this thesis provide previously unreported findings that seem to suggest that there are two main pathways leading to DME. More specifically, there seems to be an ischemic type of DME characterized by severe NPDR, high serum VEGF-A and low pro-inflammatory factors (hsCRP, ICAM-1, MCP-1). This phenotype is suitable for treatment with anti-VEGF agents exhibiting an early and significant anatomic response, while vision recovery may be conditioned by increased patient age and perhaps retinal ischemia causing irreversible macular damage, which would explain the strong effect of baseline VA on most DME clinical trials. On the other end of the spectrum we have a phenotype of DME characterized by higher circulating pro-inflammatory factors, lower VEGF-A and a very limited (<10%) anatomic response to anti-VEGF agents, with DME persisting at 12 months of intravitreal treatment. Interestingly such cases are not necessarily associated with severe NPDR which favors the hypothesis that the pathogenesis of BRB breakdown in such phenotype in not primarily mediated by VEGF or related to DR severity. In this group BRB disruption is probably mediated by cellular migration and inflammatory damage affecting several components of the neurovascular unit. This would explain the persistent or recurring behavior of DME in such patients as well as the fact that dexamethasone implant seems to result in improved anatomic outcomes in such cases of persisting DME despite monthly anti-VEGF injections[204, 205]. Therefore, according to the results presented herein, patients with DME and elevated serum pro-inflammatory factors, should be considered for early introduction of combined treatment with anti-VEGF and intravitreal steroid, particularly if fluorescein angiography indicates absence or only limited peripheral retinal ischemia.

11. Conclusions

Regarding the main objective of the present dissertation, namely to study the role of systemic pro-inflammatory biomarkers associated with DR, as possible factors associated with the clinical response to intravitreal anti-VEGF agents for DME, the major conclusions are as follows:

- There is a significant association between elevated circulating biomarkers of inflammation and early limited anatomic response to anti-VEGF. Namely patients with a less than 10% change in CRT have consistently higher levels of hsCRP and ICAM-1.
- Patients achieving DME resolution with 6 or less anti-VEGF injections have significantly lower hsCRP and creatinine levels. While cases that required continued injections namely with triamcinolone combination treatment, had significantly higher hsCRP and creatinine. In fact, hsCRP correlated with number of TCA injections. These results underline a significant association between the systemic status, namely regarding risk factors for microangiopathy (hsCRP, creatinine, and possibly homocysteine) and the occurrence of persistent DME requiring a high treatment burden.
- The effect of increased hsCRP on limiting the macular response to anti-VEGF treatment, persists for at least a year despite continued intravitreal injections. In fact, the cases exhibiting a poor anatomic response during the entire follow-up, not only had higher inflammatory markers but also had significantly lower serum VEGF-A, comparing with early or late responders. The overall interpretation is that there is a clearly a subset of patients in which VEGF is not the major factor leading to persistent DME. There was also a tendency for a limiting effect of increased CRP and ICAM-1 on indicators of VA recovery, suggesting that persistent DME associated with inflammation may lead to irreversible retinal damage and limited functional recovery.

Overall, our results provide further evidence favoring the hypothesis of a chronic inflammatory component being associated with diabetic microvascular complications, such as DME. To our knowledge this dissertation provides the first reports of a statistically significant association between increased markers of inflammation and various parameters of clinical response to anti-VEGF treatment for DME. Namely, increased values of factors associated with inflammatory vascular damage (hsCRP, MCP-1, ICAM-1) may indicate a less favorable clinical response to anti-VEGF and consequently a higher treatment burden. The clinical application of the results reported herein may lead to a further optimization of treatment strategies for DME and contribute to research of further treatment agents.

12. Future perspectives

Anti-VEGF treatment represented a clinical breakthrough in the treatment of DME, however there is clearly a subgroup of DME patients which are not adequately treated by anti-VEGF monotherapy. An optimized treatment regimen is necessary for the subset of patients characterized by increased systemic inflammatory factors and low circulating VEGF-A. Also, the research reported in this dissertation warrants the possibility of validating a DME phenotype screening test including systemic levels of CRP, ICAM-1 and VEGF-A. Regarding the treatment of poor responders to anti-VEGF, a possible strategy could be the early introduction of the available steroid implants. In this regard, further research is needed into the best possible regimen of corticosteroid treatment, namely combination with anti-VEGF or a full treatment switch to steroid implant alone. In either case it would be necessary to identify the ideal timing of reinjection of either dexamethasone or fluocinolone implant in order to obtain maximum clinical benefit while minimizing the known side effects, namely vision-threatening IOP increase. Another possible direction is to further guide the research of new drugs, specifically targeting the agents of leukostasis and retinal leukocyte migration. In fact, a recent phase 2 report of a novel antibody targeting both angiopoietin-2 and VEGF-A has already shown significantly better outcomes comparing with ranibizumab treatment[130]. It will be interesting to see how it will perform in cases with persistent DME despite anti-VEGF treatment. Future research into the components of inflammation associated with DR, will undoubtedly contribute to further improve the clinical outcomes of DME patients.

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