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FUNCTIONAL AND STRUCTURAL CHARACTERIZATION OF THE RESPONSE TO THE TREATMENT OF DIABETIC MACULAR EDEMA WITH INTRAVITREAL ANTI-VEGF THERAPY

Tese de doutoramento no âmbito do Programa de Doutoramento em Ciências da Saúde – ramo de Ciências Biomédicas, orientada pelo Professor Doutor Rufino Martins da Silva e apresentada à Faculdade de Medicina da Universidade de Coimbra

Janeiro de 2020

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“It always seems impossible until it’s done.”
Nelson Mandela

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Table of contents

List of abbreviations

AIBILI	Association for Innovation and Biomedical Research on Light and Image
AO	Adaptive Optics
AUC	Area Under the Curve
AV	*Acuidade Visual*
BCVA	Best Corrected Visual Acuity
BRB	Blood-Retinal Barrier
CCT	Central Choroidal Thickness
CFP	Colour Fundus Photography
CHUC	*Centro Hospitalar e Universitário de Coimbra*
CMT	Central Macular Thickness
CME	Cystoid Macular Edema
COST	Cone Outer Segments Tips
CORC	Coimbra Ophthalmology Reading Centre
CRT	Central Retinal Thickness
CSME	Clinically Significant Macular Edema
CT	Choroidal Thickness
CVD	Choroidal Vessel Density
CVV	Choroidal Vessel Volume
DM	Diabetes Mellitus
DME	Diabetic Macular Edema
DR	Diabetic Retinopathy
DRCR.net	Diabetic Retinopathy Clinical Research Network
DRIL	Disorganization of the Retinal Inner Layers
EDI	Enhance Deep Imaging
ELM	External Limiting Membrane
EMD	*Edema Macular Diabético*
ERM	Epiretinal Membrane
ETDRS	Early Treatment Diabetic Retinopathy Study
EUROCONDOR	European Consortium for the Early Treatment of Diabetic Retinopathy
EZ	Ellipsoid Zone
FA	Fluorescein Angiography
FAF	Fundus Autofluorescence
FAZ	Foveal Avascular Zone
FDA	Food and Drug Administration
GCL	Ganglion Cells Layer
HbA1c	Glycosylated Haemoglobin
ICAM-1	Intercellular Adhesion Molecule 1
ILM	Inner Limiting Membrane

INL	Inner Nuclear Layer
IPL	Inner Plexiform Layer
IRMA	Intraretinal Microvascular Abnormalities
IRC	Intra-Retinal Cysts
IS	Inner Segments
ISCEV	International Society for Clinical Electrophysiology of Vision
IVT	Intravitreal Treatment
MA's	Microaneurysms
MCP-1	Monocyte Chemoattractant Protein 1
mfERG	Multifocal Electroretinography
MLE	*Membrana Limitante Externa*
MP	Microperimetry
MS	Mean Sensitivity
NPDR	Non-Proliferative Diabetic Retinopathy
NSD	Neurosensorial Serous Detachment
LOR	Lower than normal Optical Reflectivity
OCT	Optical Coherence Tomography
OCTA	Optical Coherence Tomography Angiography
OCT-L	Optical Coherence Tomography Leakage
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
OR	Odds Ratio
OS	Outer Segments
PEDF	Pigment Epithelium Derived Factor
PDR	Proliferative Diabetic Retinopathy
PRN	*Pro Re Nata*
RD	*Retinopatia Diabética*
RBZ	Ranibizumab
ROC	Receiver Operating Characteristic
RPE	Retina Pigment Epithelium
SD	Standard Deviation
SD-OCT	Spectral Domain Optica Coherence Tomography
SDF-1	Stromal-cell Derived Factor 1
SRF	Subretinal Fluid
SS-OCT	Swept-Source Optical Coherence Tomography
TNF - α	Tumour Necrosis Factor Alfa
UWF-FA	Ultra-widefield Fluorescein Angiography
VA	Visual Acuity
VD	Vessel Density
VEGF	Vascular Endothelial Growth Factor
VMT	Vitreo-Macular Traction

Abstract

Diabetic Macular Edema (DME) is the most frequent cause of vision loss in patients with diabetic retinopathy (DR), resulting mainly from a failure of the blood-retinal barrier and consequent leakage to the retina, leading to macular thickness increase and visual acuity (VA) loss. Intravitreal anti-vascular endothelial growth factor (anti-VEGF) injections have proven efficacy in reducing macular thickness, but not always with functional improvement, requiring multiple injections and a tight follow-up to maintain its efficacy and avoid vision loss, representing a burden to both patients and clinicians.

Clinical parameters and new diagnostic features obtained from imaging with optical coherence tomography (OCT) have proven to be useful not only for disease staging but also for the identification of biomarkers of disease prognosis and treatment response. However, the heterogeneity of inclusion criteria, the inadequacy of evaluation methods and the lack of standardization with respect to DME classification may explain the difficulty to establish a good correlation between each possible factor and the treatment response. Baseline VA is, until now, the only confirmed predictor of treatment outcome. Nevertheless, VA has a limited value in the characterization of patients' functional vision state as it does not always reflect the difficulties on daily tasks, such as reading, cooking, driving or shopping. Therefore, there is a clear need to identify and validate additional functional methods to evaluate the impact of DME and its treatment on patients' visual performance.

The overall purpose of this thesis was to characterize DME treatment response to anti-VEGF therapy in a large and well identified group of DR patients undergoing the same treatment regimen. Several morphological and functional aspects were covered using the most recent techniques of OCT, mfERG and microperimetry, as well as customized computer tools. Our main goal was to identify DME characteristics with predictive value of good or poor treatment response and to evaluate the effects of this therapy in visual function. This information may be used in the development of visual prognosis metrics and may open new perspectives for the management of DME treatment, having a potential impact on clinical practice.

We started with a preliminary retrospective study in 51 naïve DME patients **(Chapter 2)**, to investigate different OCT features that could have a predictive value for the visual response

to anti-VEGF treatment. This study showed that OCT morphological DME patterns at baseline give important information regarding differences in good versus poor response to treatment, suggesting that the presence of cystoid spaces in the inner retina layers seems to be related with a poor functional response. A strong correlation between central retinal thickness (CRT) decrease and best corrected visual acuity (BCVA) improvement after treatment was also showed. A cut-off value of 20% of CRT decrease was identified as a robust discriminator between good or poor responders to anti-VEGF therapy.

With the results of this preliminary work, we designed a prospective observational study **(Chapter 3)**, including 71 naïve DME patients with indication for anti-VEGF treatment with ranibizumab that were submitted to the same treatment regimen (loading dose of 3 ranibizumab IVT + Pro Renata) and followed during 12 months. Several imaging techniques were performed on these patients, namely Spectral Domain OCT (SD-OCT) and Swept-Source OCT (SS-OCT). To explore potential biomarkers of anti-VEGF treatment response in DME, recently described morphological features, such as disorganization of retinal inner layers (DRIL) and integrity of the ellipsoid zone of the photoreceptors layer (EZ) and external limiting membrane, were evaluated on SD-OCT. Our results showed that presence of DRIL, and especially presence of damaged photoreceptors before treatment, are important indicators of a poor response to ranibizumab therapy, and hence, should be used as reliable prognostic factors for therapeutic decisions.

In **Chapter 4**, SD-OCT was used to extract retina fluid information in a completely noninvasive way, using a customized and in-house software – OCT-Leakage. By mapping lower than normal reflectivity sites (LOR) on structural OCT B-scans, we were able to identify the location of DME fluid accumulation within retina layers and to analyze its behavior after anti-VEGF treatment. Accumulation of fluid in the outer plexiform and outer nuclear layers showed an important predictive value of treatment response, discriminating, at baseline, patients that improve BCVA. This parameter showed to be a better discriminator than DRIL or photoreceptors disruption assessed in chapter 3.

Choroidal vasculature features were explored as possible discriminators of treatment response in DME patients **(Chapter 5)**. Considering that DR is mainly a microvascular pathology and that choroid is a highly vascularized structure, we hypothesized that vessel density and blood flow could be altered in the course of the disease. Using Swept-Source

OCT, a technology designed specifically to enhance choroidal visualization with higher resolution, it was possible to extract quantitative information about choroidal vessels density and volume. Our results suggested that patients with higher baseline values of central choroidal thickness, choroidal vessel density and volume, have a higher probability of improved BCVA after anti-VEGF treatment. Important differences between treatment response groups were also observed at the choroid level: poor responders increased choroidal vessels dilation after treatment, while good responders remained almost unchanged. Therefore, choroidal features can represent potential biomarkers of treatment response, and may contribute to the development of personalized care in DME patients.

Finally, after dissecting different morphological aspects of DME undergoing anti-VEGF treatment, patients' functional response was also explored using microperimetry and mfERG, apart from BCVA, with the purpose of obtaining a detailed characterization of this disease and its treatment impact in patients' quality of vision **(Chapter 6)**. The results showed that microperimetry and electrophysiology were able to detect visual response changes during the course of the treatment with higher sensitivity than BCVA. When treatment regimen changed from monthly injections to a Pro Re Nata scheme, macular luminous sensitivity and mfERG implicit time were affected while BCVA remained unchanged, supporting patients' dissatisfaction frequently reported about their vision performance, despite 20/20 of visual acuity. These results are particularly important as they highlight the need to find new functional methods to evaluate how different DME treatment strategies affect patients' ability to function in real life.

In conclusion, this work showed that OCT is an important tool not only for DME diagnosis but also to obtain important biomarkers of the disease, namely ellipsoid zone (EZ) and DRIL integrity, accumulation of fluid in the outer retinal layers and choroidal vasculature changes. This information has a crucial impact in DME treatment management, contributing for an individualized care, reducing treatment burden and improving visual recovery. Differentiated functional methods, besides BCVA, showed to be useful in patients' vision characterization and should be target of further studies to improve DME visual function understanding and to help monitoring patients undergoing anti-VEGF treatment. Avoiding visual loss, but specially leverage visual gain, should be the primary goal of any ophthalmic therapy.

Resumo

O edema macular diabético (EMD) é a causa mais frequente de perda de visão nos doentes com retinopatia diabética (RD). Deve-se principalmente a uma perturbação no funcionamento da barreira hemato-retiniana interna e consequente extravasamento de fluídos para a retina, levando ao aumento da espessura macular e a uma perda de acuidade visual (AV).

As injeções intravítreas de anti-fatores de crescimento vascular endotelial (anti-*VEGF*) têm uma eficácia comprovada na redução do fluido extracelular retiniano e consequente diminuição da espessura macular mas nem sempre são acompanhadas de uma melhoria funcional. Esta terapêutica exige múltiplas injeções e um acompanhamento rigoroso para manter a sua eficácia e evitar a perda de visão, o que representa um encargo económico e de tempo tanto para os doentes como para os médicos.

Parâmetros morfológicos do EMD obtidos através de imagens de tomografia de coerência óptica (*OCT*) provaram ser úteis não apenas no diagnóstico e estadiamento da doença, mas também na identificação de biomarcadores de prognóstico e resposta ao tratamento. No entanto, a heterogeneidade dos critérios de inclusão dos diversos estudos, a inadequação dos métodos de avaliação e a falta de padronização em relação à classificação do EMD, estão na origem da dificuldade de estabelecer uma boa correlação entre cada fator possível e a resposta ao tratamento. A AV na *baseline* é, até agora, o único fator preditor da resposta ao tratamento do EMD confirmado na literatura. No entanto, a AV tem um valor limitado na caracterização do estado funcional da visão destes doentes pois nem sempre reflete as reais dificuldades nas tarefas diárias, como ler, cozinhar, conduzir ou fazer compras, o que demonstra uma clara necessidade de identificar e validar outros métodos funcionais para avaliar o impacto do EMD e do seu tratamento no desempenho visual dos doentes.

Desta forma, o objetivo geral desta tese foi caracterizar de forma detalhada a resposta do EMD ao tratamento com anti-*VEGF* num grupo bem identificado de doentes com RD, submetidos ao mesmo regime de tratamento. Foram avaliados vários aspetos morfológicos e funcionais desta patologia utilizando técnicas recentes de *OCT*, mfERG e microperimetria, bem como ferramentas personalizadas não disponíveis na prática clinica atual, que permitiram extrair informação adicional das imagens retinianas.

O objetivo principal do presente trabalho foi a identificação de características estruturais do EMD que possam ter um valor preditivo para uma resposta funcional boa ou má ao tratamento e avaliar e caracterizar os efeitos dessa terapia na função visual, permitindo obter uma medida objetiva da recuperação funcional dos doentes com EMD. A informação recolhida poderá ser usada na construção de métricas e algoritmos para o prognóstico visual e poderá abrir novas perspetivas na gestão do tratamento do EMD, com potencial impacto na prática clínica.

Foi inicialmente realizado um estudo preliminar retrospetivo em 51 doentes com EMD sem tratamentos prévios **(capítulo 2)**, submetidos a tratamento intravítreo com ranibizumab com o objetivo de investigar diferentes parâmetros obtidos no *OCT* com potencial valor preditivo para a resposta visual ao tratamento com anti-*VEGF*. Os resultados deste estudo mostraram que a presença inicial de padrões morfológicos específicos de EMD fornece informações importantes sobre o tipo de resposta ao tratamento, sugerindo nomeadamente que a presença de espaços cistoides nas camadas internas da retina parece estar relacionada com uma fraca resposta funcional. Foi também demonstrada uma forte correlação entre a diminuição da espessura central da retina (*CRT*) e a melhoria da AV após o tratamento. Um valor de corte de 20% de redução da *CRT* foi identificado como um discriminador robusto entre bons e maus respondedores à terapia anti-*VEGF*.

A partir dos resultados deste trabalho preliminar, foi realizado um estudo observacional prospetivo **(capítulo 3)**. Foram incluídos 71 doentes com EMD sem tratamentos prévios e com indicação para tratamento intravítreo com ranibizumab. Todos os doentes foram seguidos durante 12 meses e submetidos ao mesmo regime de tratamento (dose de carga de 3 injeções intravítreas mensais de ranibizumab + retratamento em regime *Pro Re Nata*). Foram realizadas várias técnicas de imagem, incluindo *Spectral-Domain OCT* (*SD-OCT*) e *Swept-Source OCT* (*SS-OCT*), e avaliadas várias características morfológicas da retina recentemente descritas, a fim de explorar o seu valor como potenciais biomarcadores da resposta ao tratamento anti-*VEGF* no EMD, tais como: desorganização das camadas internas da retina (*DRIL*), integridade da zona elipsoide da camada de fotorreceptores (*EZ*) e integridade da membrana limitante externa. Verificou-se que a presença de *DRIL*, e principalmente a presença de disrupção na camada de fotorreceptores antes do tratamento, são indicadores importantes para uma má resposta funcional à terapia com

ranibizumab e, portanto, devem ser usados como fatores de mau prognóstico nas decisões terapêuticas.

No **capítulo 4**, utilizámos a técnica imagiológica de *OCT* para extrair, de forma completamente não invasiva, informação sobre a presença de fluido na retina, usando um *software* personalizado e desenvolvido internamente, o *OCT-Leakage*. Ao mapear locais de refletividade abaixo do normal (*LOR*) nos *B-scan* obtidos pelo OCT estrutural, foi possível identificar a acumulação de fluido causada pelo EMD e a sua localização nas várias camadas da retina, conseguindo analisar o seu comportamento antes e após o tratamento anti-*VEGF*. A acumulação de fluido (rácios de LOR) nas camadas plexiforme externa e nuclear externa mostrou constituir um importante valor preditivo para a resposta ao tratamento, discriminando, na *baseline*, pacientes que melhorariam a AV após o tratamento. Este parâmetro (rácios de LOR) mostrou-se melhor discriminador do que a presença de DRIL ou a disrupção dos fotorreceptores, avaliadas no capítulo 3.

A vasculatura coroideia foi também explorada como um possível fator discriminador da resposta ao tratamento em pacientes com EMD **(Capítulo 5)**. Considerando que a RD é principalmente uma doença microvascular e que a coróide é uma estrutura altamente vascularizada no olho, questionou-se se características vasculares desta estrutura, tais como a densidade dos vasos e o fluxo sanguíneo, poderiam estar alteradas no curso da doença e/ou tratamento. Utilizando imagens de *Swept-Source OCT*, uma tecnologia desenvolvida especificamente para melhorar a visualização da coroide com grande resolução, foi possível extrair informação quantitativa sobre a densidade e o volume dos vasos coroideus. Os resultados obtidos sugerem que doentes com valores basais mais altos de espessura central da coróide, e maior densidade e volume dos vasos coroideus, têm maior probabilidade de obter uma melhor AV após o tratamento anti-*VEGF*. Por outro lado, após o tratamento foram observadas diferenças importantes entre os diferentes grupos de resposta: observou-se um aumento da dilatação dos vasos coroideus nos doentes que obtiveram uma resposta funcional fraca, enquanto que nos doentes com uma boa resposta funcional o calibre dos vasos permaneceu quase inalterado. Tais resultados permitiram-nos concluir que parâmetros da vasculatura da coróide devem ser alvo de investigações futuras, podendo ser considerados potenciais biomarcadores de resposta ao tratamento e contribuir para o desenvolvimento de cuidados personalizados nos doentes com EMD.

Finalmente, após a avaliação de diferentes aspetos morfológicos do EMD, antes de após o tratamento com anti-*VEGF*, a resposta funcional dos doentes foi também ela explorada de forma detalhada. Para além de avaliar a variação da AV foram ainda aplicadas técnicas diferenciadas, tais como a microperimetria e o mfERG, tendo como objetivo obter uma caracterização pormenorizada da função visual nesta doença e perceber o impacto do tratamento na *performance* visual destes doentes **(capítulo 6)**. Os resultados obtidos mostraram que a microperimetria e a eletrofisiologia permitem detetar alterações na resposta visual durante o curso do tratamento com maior sensibilidade do que a AV. Ao alterar o regime de tratamento com ranibizumab, de injeções mensais para um esquema *Pro Re Nata*, a sensibilidade luminosa macular avaliada através da microperimetria, e o tempo de latência das respostas do mfERG, revelaram estar afetados enquanto que a AV permaneceu inalterada. Estes resultados vão de encontro ao que regularmente se observa na prática clinica: a insatisfação frequentemente relatada pelos doentes sobre o desempenho da sua visão, apesar de uma acuidade visual de 20/20 avaliada na consulta de rotina. Tais achados são particularmente importantes, não só para o EMD mas para várias outras doenças oculares, pois destacam a necessidade crescente de encontrar novos métodos funcionais que avaliem o impacto de diferentes estratégias de tratamento na capacidade visual dos doentes e no desempenho das suas tarefas na vida quotidiana.

Em conclusão, os conhecimentos obtidos durante este trabalho mostraram que o *OCT* é uma ferramenta importante não apenas para o diagnóstico do EMD mas também na obtenção de importantes biomarcadores da doença, nomeadamente o grau de integridade das camadas retinianas como os fotorreceptores (EZ) e as camadas internas (DRIL), a acumulação de fluido nas camadas externas da retina e as alterações visíveis ao nível do calibre dos vasos coroideus.

Estes dados têm um impacto crucial na gestão do tratamento do EMD, contribuindo para cuidados individualizados que permitam a melhor opção terapêutica para cada doente, reduzam o numero de sessões de tratamento e a consequente necessidade de visitas e deslocações frequentes aos cuidados médicos e, principalmente, diminuindo a perda funcional e maximizando a recuperação visual nesta doença.

Métodos funcionais diferenciados, para além da avaliação da AV, mostraram-se úteis na caracterização da visão destes doentes e devem ser alvo de novos estudos para aumentar o entendimento da função visual no EMD e ajudar no seguimento de doentes em tratamento com anti-*VEGF*. Evitar a perda, mas principalmente potenciar o ganho visual, deve ser o objetivo maior de qualquer terapia oftalmológica.

List of publications

1. **Degree of Decrease in Central Retinal Thickness Predicts Visual Acuity Response to Intravitreal Ranibizumab in Diabetic Macular Edema**

   Ana Rita Santos, Sara Cristina Gomes, João Figueira, Conceição Lopes Lobo, José Guilherme Cunha-Vaz

   Ophthalmologica 2014; 231:16–22

   Impact Factor 2014 (JCR): 1.927; Quartile 2014: Q1

2. **Optical Coherence Tomography Baseline Predictors for Initial Best Corrected Visual Acuity Response to Intravitreal Anti-Vascular Endothelial Growth Factor Treatment in Eyes with Diabetic Macular Edema. The CHARTRES Study**

   Ana R. Santos, Miguel Â. Costa, Christian Schwartz, Dalila Alves, João Figueira, Rufino Silva, José G. Cunha-Vaz

   Retina Journal 2018 Jun; 38(6):1110-1119

   Impact Factor 2018 (JCR): 4.013; Quartile 2018: Q1

3. **Measurements of Retinal Fluid By Optical Coherence Tomography Leakage in Diabetic Macular Edema: A Biomarker of Visual Acuity Response to Treatment**

   Ana R. Santos, Dalila Alves, Torcato Santos, João Figueira, Rufino Silva, José G. Cunha-Vaz

   Retina Journal 2019 Jan; 39(1):52-60

   Impact Factor 2018 (JCR): 4.013; Quartile 2018: Q1

4. **Swept Source OCT Choroidal Indices as Predictors of Visual Outcomes to anti-VEGF Treatment in DME patients**

   Ana Rita Santos, Dalila Alves, Patrícia Barreto, Inês Laíns, Jay C. Wang, John B. Miller, João Figueira, Rufino Silva

   Submitted to Retina Journal

   Impact Factor 2018 (JCR): 4.013; Quartile 2018: Q1

5. **mfERG and Microperimetry as Functional Measurements in Diabetic Macular Edema undergoing Intravitreal Ranibizumab Treatment**

   Ana Rita Santos, Miguel Raimundo, Marta Lopes, Sérgio Pestana, Rufino Silva, João Figueira, José Cunha-Vaz

   Submitted to Eye Journal

   Impact Factor 2018 (JCR): 2.366; Quartile 2018: Q1

Chapter 1

General Introduction

General introduction

Diabetes Mellitus (DM) is a major public health problem with significant socioeconomic implications due to the increased disease prevalence. Its incidence is expected to rise from 382 million people in 2013 to 592 million by 230[1]. Diabetic retinopathy (DR) is the most frequent complication in diabetic patients and remains the leading cause of legal blindness in working-age populations of industrialized countries, responsible for 10% of new cases of blindness each year[2,3]. DR rates are higher among people with type 1 diabetes, people with longer duration of diabetes, caucasian populations and among people of lower socioeconomic status[2].

Diabetic Macular Edema (DME) is currently the major vision-threating complication of DR, being the main responsible for the visual impairment in patients with this ocular disease[4]. Despite the demonstrated efficacy of the recent therapies for DME, almost 40% of the treated patients still have vision impairment[5,6].

Differentiating patients that will be responsive or not to a specific therapy, as well as predicting the functional and structural outcomes of a treatment, are of crucial importance in the management of the disease and can significantly contribute for a more efficient and personalized care. The innovative imaging modalities available nowadays for detection and follow-up of DME offer a variety of parameters that can be used as potential biomarkers, not only for disease progression but also, especially, as prognostic tools.

Therefore, the main focus of the present thesis is to identify imaging biomarkers that may have predictive value in DME treatment outcomes and, thus, be useful in the management of the disease, namely by choosing the most effective therapy and the right moment to treat each individual to maximize the functional gains.

A more detailed description of DME pathophysiology, diagnostic tools, available treatments, possible biomarkers and functional outcomes is included in the present chapter. Further, an outline of the thesis is given along with its specific objectives and methods.

DME epidemiology and risk factors

DME prevalence is estimated to be up to 8% in diabetic people aged 20-79 years old with an increasing tendency due to the global epidemic numbers of diabetes type 2 patients[2,7,8]. This means that about one in 15 people with diabetes has DME and more than 20 million people are affected worldwide[9,10].

As reported by the Wisconsin Epidemiological Study of Diabetic Retinopathy[10], the incidence of macular edema over a period of 10 years is around 20.1% in patients with type 1 diabetes and up to 25% in patients with type 2 diabetes.

The principal risk factors for DME development are mainly related to duration of diabetes and metabolic control, namely higher levels of HbA1C[9,11]. Other factors such as the type of diabetes, higher systolic blood pressure, dyslipidaemia, microalbuminuria, and even ethnicity, have shown some association with DME[9]. Diabetic retinopathy severity is the major ocular risk factor associated with DME, increasing its prevalence from 3% in mild non-proliferative DR to 38% in moderate to severe non-proliferative DR and 71% in eyes with proliferative DR[11]. Approximately 50% of DME patients will experience a loss of 10 letters of visual acuity (VA) after 2 years of follow-up[12] having a substantial impact in their quality of life and work capability. DME is a maculopathy of working-age people and these patients have higher rates of comorbidity and loss of work and personal time compared with diabetic patients without DME. As showed by Wallick et al.[13], working age patients with DME had an average of 25 annual days with healthcare visits compared with 14 days in patients with diabetes but no DME with the same age. The average medical cost ratio, adjusted for age, sex, race, geographic region and comorbidity, for patients with DME over 3 years was also 1.31 times superior to diabetic patients without DME[14].

DME pathophysiology

The pathogenesis of DME is complex and involves multiple factors[15]. It results mainly from inner blood-retinal barrier (BRB) disruption, which leads to increased accumulation of fluid within intraretinal layers and consequent increase in macular thickness with central vision loss[16–18]. From the several agents that cause the BRB breakdown, vasoactive factors, such as vascular endothelial growth factors (VEGF), are the major contributors for the vascular permeability alterations (figure 1).

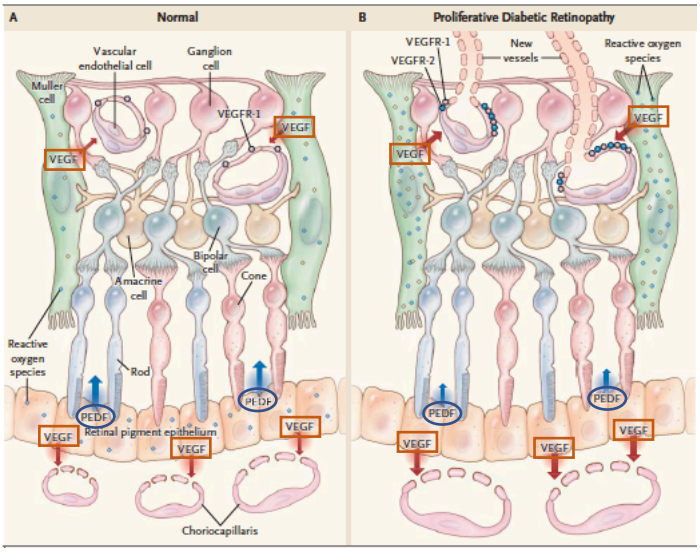

Figure 1: Role of VEGF and PEDF in the pathophysiology of Diabetic Retinopathy. A normal retina is shown in figure A and a retina with proliferative diabetic retinopathy in figure B. Over expression of VEGF is seen in diabetic retinopathy, not only in the inner retina but also at retinal pigment epithelium and choriocapillaris level. (Adapted from Frank R[15] and reproduced with author's permission)

VEGF is upregulated in hypoxic and hyperglycaemic environments and interferes in multiple fronts: (1) it has a disruptive effect on the endothelial zona occludens resulting in the opening of the endothelium "tight junctions" and affecting the integrity of retinal vessels, leading to a consequent extravasation of fluid, blood plasma proteins and lipids to the retinal tissue[19]; (2) it plays a role as proinflammatory agent in the retina[20] acting as an up-regulator of intercellular adhesion molecule-1 (ICAM-1)[21] on vascular endothelial cells, leading to leucocyte adhesion to the vascular endothelium, capillary occlusion and endothelial cell apoptosis, consequently also causing BRB disruption.

In addition to VEGF, inflammatory cytokines resulting from persistent hyperglycaemia, and other inflammatory mediators like MCP-1 and SDF-1[9], may also contribute to the breakdown of retina vessels endothelium and extracellular edema[22].

On the other hand, at an intracellular level, activation of monocytes that differentiate into macrophages increases the secretion of cytokines and other growth factors like angiopoietin-2, TNFα, interleukins and chemokines, creating a leaking environment and also exacerbating an inflammatory response that contributes to vasoregression and neurodegeneration occurring at several stages of DR[7].

Dysregulated metabolism secondary to hyperglycaemia is also associated with accumulation of intracellular osmotically active solutes that draw in water and cause cellular swelling[23]. The first and most affected retinal cells by intracellular fluid accumulation are Muller cells, followed by bipolar and ganglion cells[6]. The intracellular edema is also called cytotoxic edema. In DME patients, both forms of edema may be present: extracellular (also called vasogenic) and intracellular (cytotoxic). Some authors believe that cytotoxic edema is the first to happen, followed by the vasogenic edema with accumulation of fluid mainly in the extracellular space of external plexiform and inner and outer layers of the retina[6].

Retinal pigment epithelium (RPE) and choroid may also have a role in DME. Histologic studies have showed many choroidal vascular changes in diabetic patients, namely, dilatation and obstruction of the choriocapillaris, increased vascular tortuosity and beaded vessels, areas of vascular non-perfusion or decreased blood flow and choroidal neovascularization[24–26]. Some of these changes were found even in diabetic patients without clinical DR, giving a possible elucidation on the unexplained loss of visual acuity in diabetic eyes without clinical evidence of DR[27]. Changes in choroidal thickness (CT) have also been shown in DR and DME with several studies reporting a decrease of choroidal thickness with the increase of DR severity level or presence of DME[28–30].

RPE is in contact with the choriocapillaris layer of the choroid, which allows an exchange of molecules between the retina and choroid. This layer is also important for pumping fluid from neurosensorial retina into the choriocapillaris, preventing accumulation of fluid

inside retina layers and the formation of a macular edema. The presence of an hypoxic environment in choroid and choriocapillaris in DR can create an imbalance in the RPE and cause overexpression of VEGF by this structure[6,15]. This can lead to a consequent dilation and leaking of choroidal vessels and to an increase of the outer BRB permeability, which can contribute for DME. An anatomic sign of possible choroid and RPE involvement in DME pathophysiology is the presence of a neurosensorial detachment in the area of macular edema, very common in these patients[6,31], showing a clear incapacity of RPE/choroid elimination mechanisms[15] (figure 2).

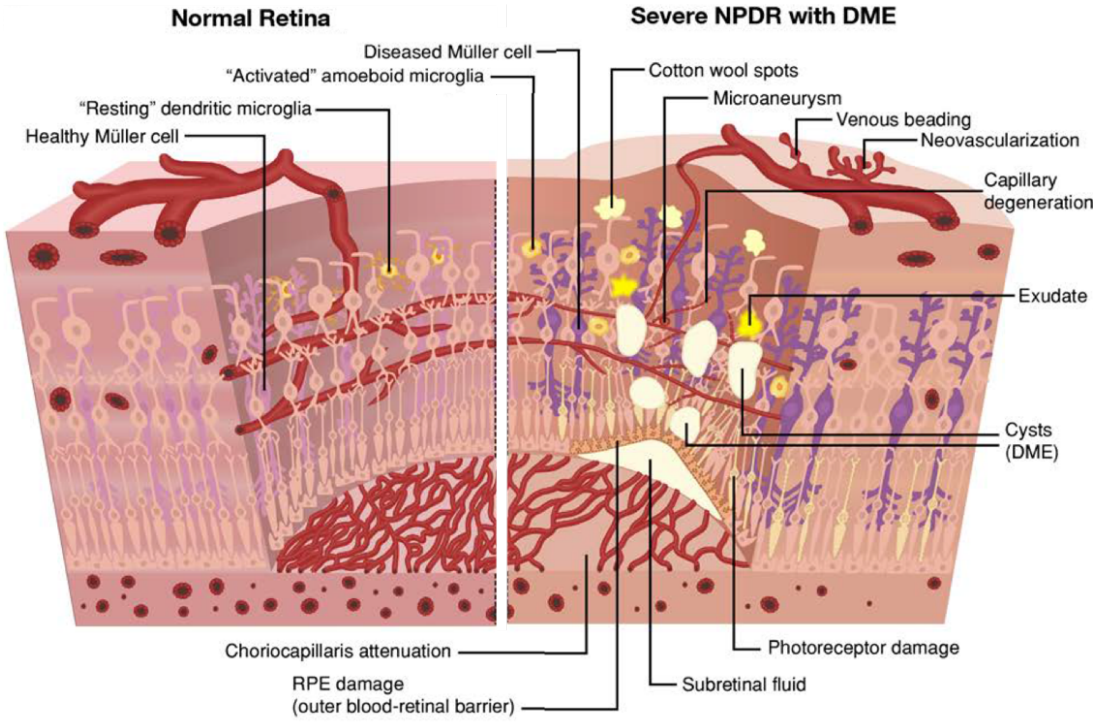

Figure 2: Schematic illustration of a normal retina compared with a diabetic retina with DME (Adapted from Duh et al.[32] and reproduced with author's permission)

DME pathophysiology appears to be a combination of several factors with both vascular and inflammatory agents playing a role, which means that a single treatment strategy may not be sufficient for this pathology. Loss of vision in this condition occurs only when at least 50% of neuronal cells in the macular area are affected, being a clearly late complication of the disease. With the rising number of DM people and consequent DR

prevalence, early detection and especially early treatment of DME with adequate and specific therapeutic agents are conditions that should be considered in order to reduce the visual impairment of this disease and also its burdens[2,33].

Clinical assessment and imaging

Diabetic Macular Edema is mainly defined as the increase of retinal thickness in macular area. It can be detected by slit-lamp examination or colour fundus photography, but unless these two techniques are performed stereoscopically, they lack in three-dimensional perception to actually confirm retinal thickening. Therefore, DME was classically defined by the presence of characteristic signals in the posterior pole, as hard exudates, microaneurysms and blot haemorrhages, visible in 2-dimensional observations.

The Early Treatment Diabetic Retinopathy Study Group introduced the concept of clinically significant macular edema (CSME)[34], based in stereoscopic images (figure 3). To be considered CSME, at least one of the following criteria needed to be fulfilled:

a. Retinal thickening at or within 500 µm of the centre of the macula;

b. Hard exudates at or within 500 µm of the centre of the macula with adjacent retinal thickening;

c. One disk area, or larger, of retinal thickening, any part of which is within one disk diameter of the centre of the macula.

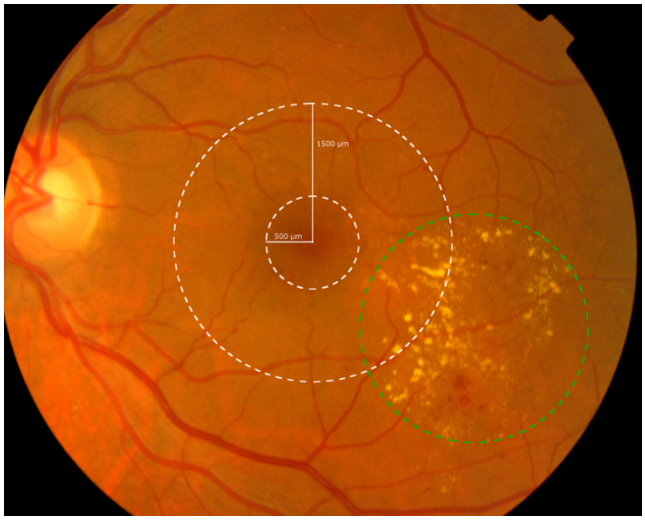

Figure 3: Clinically Significant Macular Edema with no central involvement defined according to the ETDRS criteria.[34] (Created by our research group)

Fluorescein angiography was a gold standard method for DME diagnosis as it made it possible to detect the breakdown of BRB and consequent accumulation of fluid in the macular area. FA remains the only approved modality that can define DME as focal or diffuse depending on the aetiology of the leakage that is present in the angiogram: Focal edema if the leakage arises from microaneurysms or diffuse edema if it comes from generally dilated and hyperpermeable capillaries throughout the macula[35]. Nevertheless, most cases of DME have mixed characteristics and aetiologies, making this distinction difficult, and the invasive character of this technique, in opposition to recent non-invasive ones, makes it less frequently used.

With the development of optical coherence tomography (OCT), detection and monitoring of DME were simplified and the importance of this imaging tool cannot be ignored. OCT is a non-invasive, non-contact technology, with high reproducibility, that currently can image retinal layers with a resolution of 2-5 μm[36]. It allows objective qualitative and quantitative assessments by generating maps of retinal thickness in 9 macular regions, similar to the ETDRS grid of nine areas (figure 4). Central macular thickness (CMT), in the 1mm area centred in the fovea, is the most used single measurement in DME management, not only for diagnosis but also for monitoring changes over time and assessment of treatment efficacy.

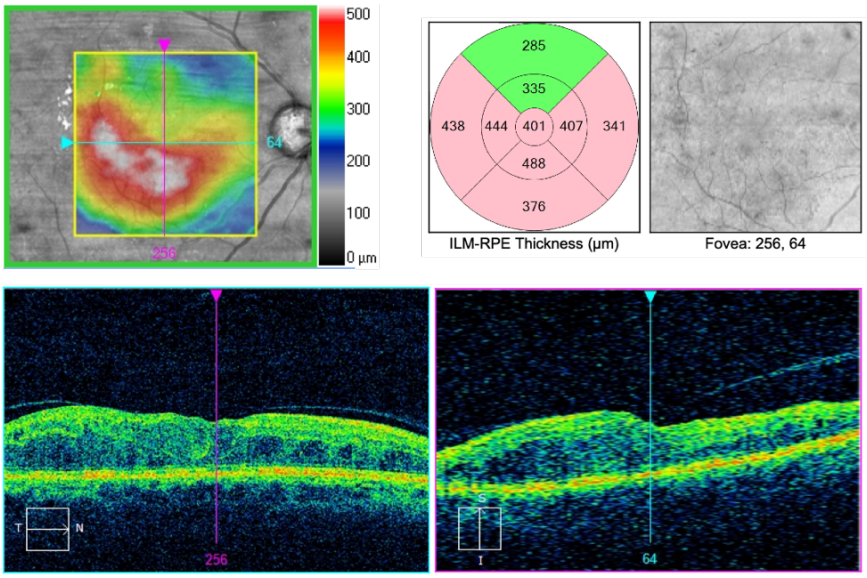

Figure 4: OCT Macular Thickness output from Cirrus 5000 (Zeiss Meditec, Dublin USA) of a patient with Diabetic Macular Edema. A colour retinal thickness map can be seen in the superior left corner. The retinal thickness values, from ILM to RPE, in microns, are displayed in the ETDRS grid in the superior right corner, and compared to normative data. OCT horizontal and vertical false colour B-scans passing through fovea are displayed in the black bottom windows. (Output from a study patient.)

In fact, the presence of DME is currently defined in the most important clinical trials as a macular thickening ≥ 300 μm in the OCT 1mm central subfield, together with the presence of fluid accumulation within retinal layers[37,38].

Several studies tried to categorize and classify DME based on OCT depending on the location of extracellular fluid accumulation, but a consensus is still missing[39–42]. Otani et al.[39] defined 3 types of DME: diffuse or spongiform-like edema, cystoid edema or serous-retinal detachment. The authors also defined a fourth type - tractional DME – if thickening of the retina occurs in the presence of epiretinal membranes or posterior hyaloid traction. The SAVE study[42] was another attempt to implement an OCT based grading protocol for clinically significant macular edema, separating it into several categories: presence of subretinal fluid (SRF), areas of affected retina by intra-retinal cysts (IRC), presence of vitreoretinal interface abnormalities and aetiology of leakage. However, it associates FA to help defining leakage aetiology, which is a drawback of this classification due to its invasive character.

OCT Angiography (OCTA) is a very recent technique that has revolutionized the imaging of DR and DME. It combines the classic structural OCT with the imaging of the different vascular networks of the eye (Figure 5).

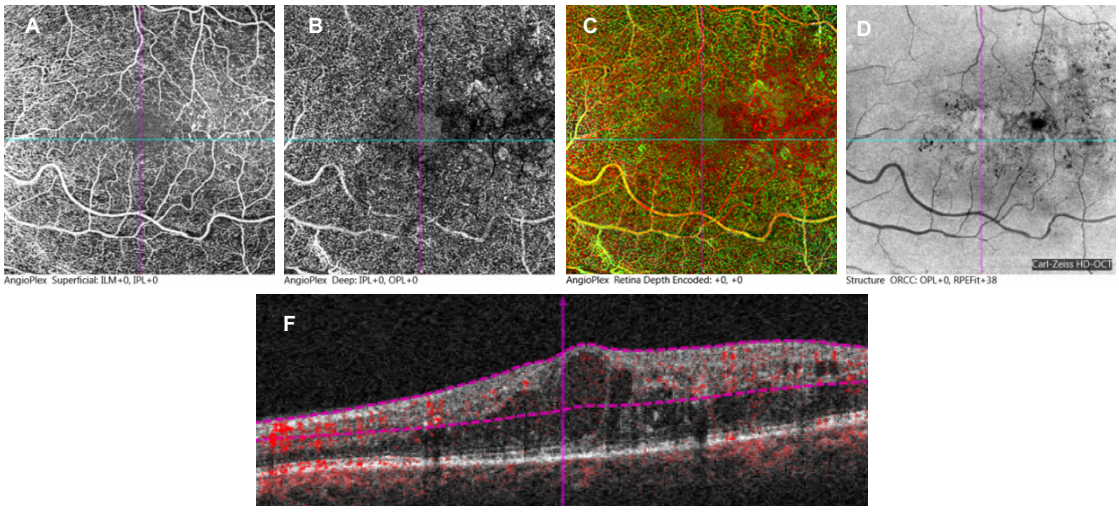

Figure 5: OCT Angiography of a patient with Diabetic Macular Edema. Foveal avascular zone (FAZ), microaneurysms and non-perfusion areas can be seen in the superficial vascular plexus (A). Cystoid spaces reflectivity can be seen in the deep vascular plexus (B) as well as when all retina plexus are analysed in the colour image (C). *En*-face image of the retina is also showed (D), where is possible to see the edema regions surrounded by microaneurysms and vascular tortuosity. Structural B-scan with flow information (red spots) can also be analysed (F). (Images from a study patient.)

OCTA provides the opportunity to observe the vascular structure of the superficial plexus (between the optic nerve fibre layer and ganglion cell layer) and deep plexus (between the inner and outer plexiform layers), as well as choroidal and choriocapillary vascular networks, in a completely non-invasive way using only light reflectivity and acquisition of clusters of images in the same location to detect the presence and movement of erythrocytes in retinal and choroidal vessels to reconstruct their vascular networks[43].

OCTA enables not only a qualitative assessment of retinal vascular network but also vascular quantitative data that can be compared and monitored during the course of DR or macular edema, namely vessel and perfusion density, which could be promising indicators for this condition management. However, the variety of available OCTA equipment's increase the discrepancy between measurements[44] and normative databases of vascular indices from normal and diseased eyes still lacks.

In DME, the use of OCTA is still relatively limited. As this technique is based on light reflectivity and penetration ability, in pathologies with severe fluid accumulation, as DME, the resolution is compromised and the presence of reflectivity artifacts (projection artifacts) is significantly high, especially in the deep layers[45]. Moreover, as location and integrity of retinal layers in the presence of DME are changed, segmentation algorithms of OCTA usually fail in the detection of vascular plexus boundaries, compromising not only the interpretation of features but also its reproducibility and repeatability.

Nevertheless, this technique will have special value in distinguishing patients with ischemic maculopathy that are usually refractory to any type of DME therapy. Up to the era of OCTA, evaluation of diabetic macular ischemia was only possible with FA. It also has the potential to identify several vascular abnormalities that usually are masked by extensive leakage in FA, as capillary dilations and intraretinal microvascular abnormalities (IRMA), associated with a vasogenic aetiology of DME[6].

An updated classification of DME is still needed as the existing attempts can rarely be used in the clinical practice due to its complexity or important limitations like the use of time-domain OCT images. Also, most of the published classifications[39,40,46,47] consider few

parameters in the categorization, focusing only on cysts size or location of retina thickening or presence of subretinal fluid/vitreomacular tractions, lacking the inclusion of therapeutic or prognostic outcomes.

With the broad number of treatment strategies and regimens, there is clearly the need for studies to validate structural features with potential prognostic value that can be easily identified in the imaging techniques available nowadays. This would help creating a DME classification comprising clinical decisions. Future studies to evaluate the relation between these features and retina function are, as well, of crucial importance for the management of this disease as it adds information about the treatment outcomes.

In view of the main purpose of this thesis, current biomarkers in DME will be covered in detail in the next chapter.

DME treatment

Developments and limitations

Several treatments of DME are available nowadays, which gives clinicians the opportunity of choice depending on the efficacy, adverse events or unresponsiveness to previous approaches.

Laser photocoagulation was demonstrated by the Early Treatment Diabetic Retinopathy Study (ETDRS) as capable of stopping the progression of the disease in 50% of the cases. The thermic action of laser therapy aims to close leaking microaneurysms and trigger endothelial repair, reducing leakage through the disrupted inner BRB[48,49]. It also stimulates the retinal pigment epithelium, leading to activation and upregulation of cytokines and growth factors, contributing to reabsorption of the retinal extracellular fluids in DME[49,50]. It was considered in the past the standard treatment for DME[48] but the results were not always satisfactory[3,19] due to it destructive nature and the broad spectrum of unwanted effects, including paracentral visual field defects, colour vision and contrast sensitivity impairment[51,52]. These adverse reactions can compromise its therapeutic effect and may result in different degrees of visual disability[19,53].

The most important agent in the pathophysiology of DME is the overexpression of VEGF in diabetic patients. Thus, blocking or inhibiting VEGF with anti-VEGF agents has shown to be the strategy with most favourable results in the DME treatment[54]. There are three commonly used anti-VEGF drugs with stablished efficacy by important clinical trials for the treatment of DME: bevacizumab, ranibizumab, and aflibercept. Bevacizumab is only approved for cancer treatment but is widely used off-label in this condition due to its substantial lower cost compared to the other drugs. Ranibizumab was the first anti-VEGF drug to get approval for DME treatment and has shown consistently superiority in improving and preserving vision, both in monotherapy or in combination with focal laser therapy, with a visual acuity increase of 10 or 12 letters and a substantial decrease of macular thickness[5,55]. Ranibizumab was also associated with an improvement in the level of retinopathy severity and in a slowing of its progression. The best functional and structural results with this agent are achieved with a monthly regimen of intravitreal injections, as it was showed by RIDE AND RISE randomized trials[56], at least during the first year, from which could then be reduced over time. Aflibercept is more recent than ranibizumab or bevacizumab but two prospective, randomized, comparative effectiveness trials of these three drugs for the treatment of DME (VISTA and VIVID-DME studies)[57] showed no differences between them in improving visual acuity in the course of 1 or 2 years of follow-up. Aflibercept was associated with a higher gain of vision among patients with worse baseline visual acuity ($\leq$ 20/50) and it is being suggested as an alternative therapy in patients with no or partial response to ranibizumab or bevacizumab[58].

Other management strategies, as pars plana vitrectomy in case of vitreo-macular tractions and intravitreal injections of corticosteroids to reduce the retina inflammatory response, as triamcinolone, dexamethasone, and fluocinolone, have been used to prevent or delay the decrease of visual function due to this condition[54,59,60]. However, the number of complications and risks related to both treatments[59,61,62] and the lack of long-lasting vision improvements[19,63] make these treatment options mainly as last-line strategies or in chronic persistent or recurrent DME.

Despite the variability of available therapies (figure 6) and the described structural and functional improvements, the results are usually short-term and most of the times still

reversible, requiring multiple laser sessions or intravitreal injections to maintain the efficacy. About only 38% of patients achieved the targeted increase of 10 or more letters and about 30% remain nonresponsive[6,64,65].

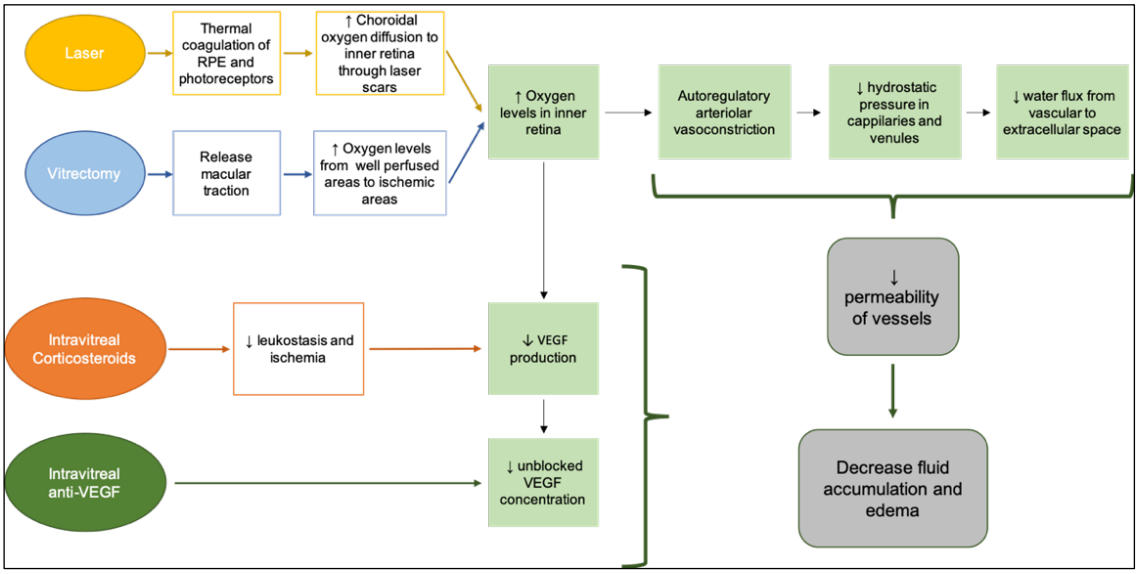

Figure 6: Schematic summarization of the several treatment strategies for diabetic macular edema and their mechanisms of action. (Based on Browning et al.[7])

More importantly, in several cases, a resolution of DME is not followed by the increase of visual function[66], presumably due to structural damage of other structures as photoreceptors or RPE, or due to the coexistent macular ischemia that is also frequently present in a chronic DME.

DME treatment biomarkers

To early identify eyes that will respond to a specific therapy and to distinguish the ones that may benefit a switch in treatment is still a gap, despite all the works that have being published. Identification of biomarkers and surrogate endpoints that can predict treatment outcomes is critical to reduce treatment failure or vision impairment and should be a target of current and future researches. Not only will patients benefit, but also will individualized and effective treatment decrease this disease health care burden.

A biomarker is, by definition, a characteristic that can be objectively measured and

evaluated as an indicator of normal or pathogenic biological processes, or pharmacological responses to a therapeutic intervention[67]. To be considered a valid biomarker, it should be quickly assessed, cost-effective and applicable in daily clinical decision-making[68].

Following these directions, the main aim of the present thesis was to identify potential imaging biomarkers that could be predictors or discriminators of responders and non-responders to anti-VEGF treatment, namely to ranibizumab. The innovative imaging modalities available nowadays for detection and follow-up of DME offer a variety of parameters that can be used not only for disease progression but especially as prognostic tools. OCT was the chosen imaging technology to explore these potential biomarkers of DME response. It is non-invasive and easy to operate, providing images with a histological level of resolution. Moreover, it is widely used in the clinical practice, which means that any development on this area could certainly be largely applicable with impact in this pathology management.

Different morphological features identifiable by OCT have being demonstrated to have prognostic relevance in DME[54,68–70]. For example, some authors reported that the presence and location of retinal fluid could be a predictor of visual response to anti-VEGF treatment in DME[71,72]. Retinal fluid in DME can be classified as intraretinal fluid, intraretinal cysts or subretinal fluid. Presence of subretinal fluid before treatment seems to be associated with a better functional recovery after anti-VEGF injections, whereas intraretinal cysts seem to be associated with a poorer response[40,73].

Pelosini et al.[74] presented a possible reason for that, describing that formation of cystoid spaces is caused by the stretch of Muller cells to accommodate extracellular fluid that starts to accumulate in the outer layers of the retina (Figure 7). As the extracellular fluid accumulation increases and becomes chronic, cystoid spaces expand to the inner retinal space, between the two plexiform layers, stretching cells axons until the limit and extending to all retinal layers. Because of that, a displacement and loss of bipolar and neighborhood cells occurs, leading to retina ischemia, atrophy and loss of function.

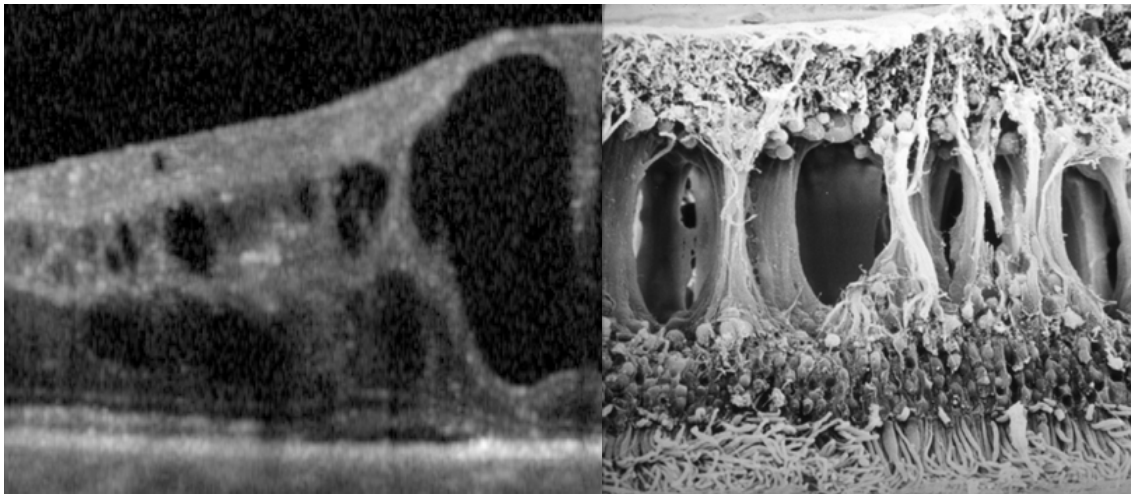

Figure 7: 2D OCT B-scan of a cystoid DME *vs* scanning electron microscopy of cystoid macular edema. Columns of tissue are standing up in a continuous space of fluid pooling. Retinal elements along the z-plane are represented by bipolar axons and Muller fibers. (Right image: OCT scan from a study patient. Left Image: Adapted from Pelosini et al.[74] and reproduced with author's permission)

Therefore, location and size of intraretinal cysts appear to play a role in visual function of DME patients[41]. Following this concept, a first step of this work was to perform an exploratory and retrospective analysis in a cohort of DME patients submitted to anti-VEGF treatment to explore the association between these features and treatment outcomes. This work originated a published article that is described in **Chapter 2 - Degree of Decrease in CRT Predicts BCVA Response to anti-VEGF in DME.**

Beyond cysts location or DME OCT patterns, some studies pointed that integrity of retinal layers could be a key factor in predicting functional response in DME patients. Presence of damaged photoreceptors, evaluated by either disruption of the ellipsoid zone, length of this interface[75] or the visibility of the cone outer segments tips (COST)[76], the integrity of external limiting membrane or RPE[77] as well the presence of hyperreflective foci[78,79] were explored by several authors, with different degrees of agreement (figure 8).

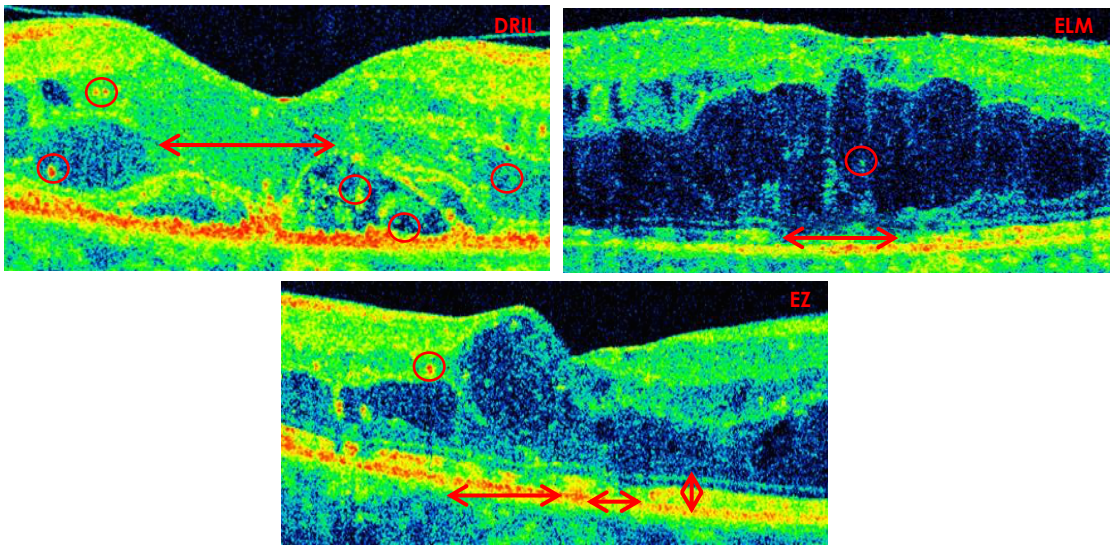

Figure 8: OCT B-scans with different features explored as predictive biomarkers. DRIL – disorganization of the retinal inner layers; ELM – external limiting membrane; EZ – ellipsoid zone. Horizontal arrows – disorganization/disruption; Vertical arrows – length of EZ layer; Circles – hyperreflective foci. (Adapted from Santos et al.[80])

Disorganization of the retinal inner layers (DRIL)[76,81] was other feature identifiable in OCT (figure 8) and explored as a predictive biomarker for visual outcomes with promising results. DRIL is defined by the inability to distinguish boundaries between any two of the inner retinal layers (including the ganglion cell-inner plexiform layer complex, the inner nuclear layer and the outer plexiform layer) in the foveal 1-mm zone[76] and showed a strong association with visual acuity in eyes with centre-involving DME[82]. Also, resolving DRIL seemed to be a good indicator of subsequent visual improvement. Patients with DME showed a significant gain in visual acuity if DRIL resolved compared with non-resolvers, whose visual acuity worsened[82]. The exact mechanisms of DRIL affecting VA are yet to be determined. As these layers are composed by the axons and nuclei of bipolar, amacrine, muller and horizontal cells, their disruption or disorganization caused by fluid accumulation can generate an alteration of the phototransduction process from photoreceptors to ganglion cells, affecting visual acuity[68].

All these features were mainly explored in retrospective cohorts or by including patients submitted to mixed treatment regimens, which can induce bias and confine the applicability of results. Therefore, it was clear for us the need to develop a prospective, randomized study in treatment *naïve* diabetic patients that were submitted to a single

anti-VEGF agent (ranibizumab), to provide information that could answer our research question: Why are functional and structural outcomes so different in DME patients submitted to the same treatment?

The original article that describes the results of this work is detailed **in Chapter 3 - OCT Baseline Predictors for BCVA Response to anti-VEGF treatment of DME.**

Despite OCT ability to quantify retinal thickness, to evaluate retinal layers integrity and to identify presence of fluid, it does not yet allow quantification of the amount of fluid. As described before, the presence of fluid within retina layers means leakage of the inner and outer BRB to the extracellular space, which was shown to be correlated with response to treatment[83].

The gold-standard method for the detection of leakage is Fluorescein Angiography. However, in addition to the invasive nature of this technique, it only provides two-dimensional information about the presence or absence of leakage. It does not permit quantification of the leaking fluid or give any information about its origin or the structures that are affected by its accumulation. Discrimination of extracellular fluid location and its quantification in specific retinal layers may be differentiating factors between patients that respond better or worse to anti-VEGF treatment, not only structurally but also functionally.

To explore a new non-invasive method of extracting leakage information without the need of injecting an intravenous dye, we used OCT images from our study patients and applied a new imaging processing technique named OCT Leakage[84]. With this technique was possible not only to detect the presence of extracellular fluid but also to discriminate its location and to quantify its amount in each of the retinal layers. Possible correlations between this feature and visual outcomes to anti-VEGF treatment were explored in a published article detailed in **Chapter 4 – OCT Leakage as a Biomarker of Visual Acuity Response to Treatment in DME.**

Recent works suggested that some choroidal features detected by OCT may also be used as biomarkers of treatment response in DME[30]. As it was already described, choroidal thickness (CT) and volume are affected by anti-VEGF agents[85,86]. CT has even been pointed

out as a possible biomarker of treatment response to anti-VEGF treatment with some studies showing that a thick sub-foveal choroid before treatment may be a predictor of good vision outcomes[30,86]. However, due to the fact that this parameter is dependent on so many factors as age, refractive errors, DM duration or even previous treatments as laser photocoagulation, none of the published works were able to demonstrate its validity as a predictor of treatment response in DME patients as reported by Campos et al. in recent works[86,87].

Choroid is a highly vascularized structure in the eye, and being DR mainly a microvascular pathology, maybe vessel density and blood flow could be altered in the course of the disease.[88,89] Also, the effects of anti-VEGF agents in these parameters remained unexplored despite reports that show that ranibizumab and bevacizumab molecules reach rapidly the choroid after an intravitreal injection in some animal models[90].

As conventional SD-OCT's own several limitations for choroidal visualization due to their limited wavelength in penetrating such deep structure, more recent swept source OCT's technology (SS-OCT) opened a new window to explore choroidal features possibly associated to DME pathophysiology and treatment response[91].

Tan et al.[88], using enhance deep imaging (EDI) technology of a standard SD-OCT, was able to show a decreased Choroidal Vessel Index in patients with DM, compared with healthy subjects, independent of changes in choroidal thickness on these patients. Wang et al.[92], explored swept source OCT *en*-face images to extract choroidal vessel density and volume information and found significant differences of these features between diabetic patients and healthy volunteers and also a significant reduction of vessel density and volume with the increased severity of DR. However, the effects of anti-VEGF agents in these parameters remained unexplored as well as its value as biomarkers of treatment response.

Therefore, we applied this image technique in our naïve DME study patients, to investigate the effects of ranibizumab on the choroidal structure, and to explore the validity of choroidal vessel parameters (density and volume) as possible discriminators of treatment response and, consequently, as indicators of therapy efficacy. This work is presented in **Chapter 5 – Choroidal Indices as Predictors of Visual Outcomes in DME** and is under

review waiting for publication**.**

Recent optical coherence tomography angiography (OCTA) imaging is an advance in retinal microvascular evaluation. Despite the limitations of the technique in the presence of DME, as obscuration of retinal vascular networks due to shadow effects, displacement of the capillaries due to cystic spaces occupation or presence of projection artifacts[45], some OCTA features have been proposed as useful biomarkers of DME treatment[93]. Less microaneurysms (MA's) detected in both superficial and deep capillary plexus are associated with a better response of DME to anti-VEGF therapy, vessel density (VD) was also reported to predict DR severity with a relatively high sensitivity and specificity, and patients with DME and higher VD before treatment have shown a better response to anti-VEGF therapy[93]. In another study, Toto et al. were able to demonstrate that the diameter of blood vessels, qualitatively assessed by OCTA, decreased in the deep capillary network after DME treatment with intravitreal dexamethasone implant[94].

In summary, OCTA reveals alterations in density and morphology of the microvasculature in the superficial and deep capillary plexuses, which improves understanding of the pathophysiology behind the edema. It also detects several features as non-perfusion areas, FAZ alterations, cystic changes, and MA's[95] which therefore, may help clinicians to better assess DME severity, choosing the best therapies, and following up the treatment efficacy. Despite the utility and advantages of this imaging modality, it became commercially available in the course of this thesis and was not object of study in our patients. However, we are currently exploring it capabilities in ongoing[96,97] and future projects.

Technological advances in imaging of the posterior segment of the eye have enabled ophthalmologists to develop hypotheses about pathological mechanisms of DME, monitor disease progression, and assess response to treatment. Emerging imaging modalities include fundus autofluorescence, wide-field imaging technology and adaptive optics.

Fundus autofluorescence is a non-invasive imaging modality that maps naturally or pathologically occurring fluorophores in the retina. The main fluorophore detected by FAF is lipofuscin, mostly composed of peroxidation products of lipids and proteins[98]. In eyes

with cystoid DME, several patterns of blue hyper-autofluorescence have been described[99]. In general, the intraretinal cysts are seen as oval or round hyper-autofluorescent lesions that are surrounded by a dark rim. Some authors[100] found that eyes with DME had a lower blue FAF signal intensity in the parafoveal areas than eyes with DR but without DME. The parafoveal blue FAF signal intensity correlated indirectly with retinal thickness in the corresponding subfield and visual acuity[100]. A good correlation among blue FAF, FA, optical coherence tomography (OCT), and microperimetry was also described by other authors[101,102].

Ultrawide-field imaging permits visualization of most of the fundus in a single image and ultra-widefield fluorescein angiography (UWF-FA) makes possible the evaluation of retinal vascular integrity in the central area together with retina periphery. Some authors quantified the area of retinal ischemia visible on UWF-FA on treatment-naive eyes with DME and found that eyes with peripheral retinal ischemia had higher odds of having DME when compared with those without peripheral ischemia supporting the hypothesis that ischemic peripheral retina may be the source of VEGF in DME[103].

Adaptive optics (AO) is a technology that measures and corrects ocular aberrations caused by the cornea, pupil diameter or lens allowing visualization of individual cones and rods and assessing photoreceptor cell spacing, cell density, and mosaic regularity[104]. Authors reported an increasing irregularity of cone spacing with increasing severity of DR and DME[105]. However, this imaging technology has important limitations that restrain its massified use. It only allows very small scan areas (generally 1°×1.2°) turning acquisitions of posterior pole zones extremely limited or time consuming. It has a lateral resolution of only 2.5 µm making it impossible to evaluate the cones localized in fovea, as they have a diameter with less than that size. Finally, it is a very expensive technology, only available in few research centres, which constitutes a barrier for the exploration of these features as biomarkers of any ocular disease.

Functional outcomes in DME treatment

It was also of particular interest since the beginning of this study, to improve the characterization of the functional vision of these patients. Although best corrected visual acuity (BCVA) is considered the gold standard in clinical practice for vision testing, it only represents foveal function and it often does not adequately reflect functional vision. The pathophysiology of DME is complex and BCVA may underestimate the impact of this condition on patients quality of life as they often report difficulties with reading and other vision-related tasks, despite good visual acuity[106]. Also, it was shown that visual acuity in diabetic patients with DME can even fluctuate with blood glucose levels variation and the time of day[107,108] meaning that this parameter can fail in the detection and characterization of visual impairment.

Previous research[109,110] have identified several other disturbances of visual performance in DME patients, including changes in contrast sensitivity and colour vision, presence of photophobia and metamorphopsia. Standard treatments such as panretinal or focal laser photocoagulation can also permanently reduce retinal sensitivity, causing relative or absolute scotomas by destruction of tissue and consequent neuroretinal damage[111,112]. Presence of central scotomas induce eccentricity and fixation instability slowing down patient's reading speed[111].

Therefore, a gap was identified in our study: the need to evaluate visual impairment caused by DME using additional and specialized tests to fully characterize different parameters of visual function, besides BCVA.

Due to the subjective character of functional vision and because it was our purpose not only to characterize visual function but also to evaluate the changes along anti-VEGF treatment course, two objective and highly reproducible methods were chosen: Microperimetry and multifocal electroretinography (mfERG).

Microperimetry

Microperimetry (MP) has been increasingly used for the evaluation of functional impairment in DME. It is an automated technique that quantifies retinal sensitivity in several macular points while is simultaneously imaging the retina, enabling a direct correlation between structure and function[113].

Microperimetry uses a background luminance of 10 cd/m^2, maximum stimulus intensity of 125 cd/m^2, white color stimulus size of 0.11-1.73 degrees (Goldmann I-V) and a dynamic range of intensity of 0-20 dB. It can test several retinal locations in a field of view of 30º (figure 9).

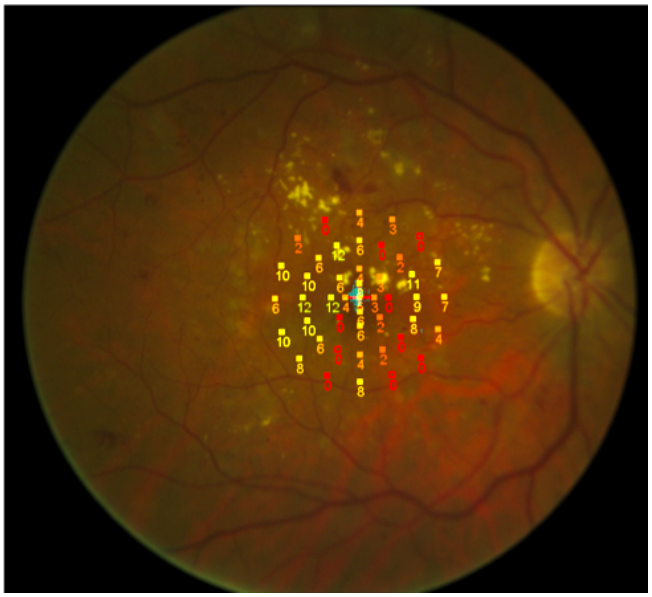

Figure 9: Microperimetry examination used in the present thesis patients. A macular strategy of 12º, centred on fovea, with a stimulus size of Goldmann III and a 4-2 threshold strategy was used. A cloud of blue points can be seen in the centre corresponding to the fixation stability of the patient. (Images from a study patient).

Due to an eye-tracking system that compensates for eye movements and fixation losses, MP is also able to evaluate patient's fixation stability and to test luminous sensitivity in identical points in the retina through several visits allowing an exact evaluation, point by point, of the functional impairment or recovery in the course of a disease or treatment.

Mean macular sensitivity seems to correlate significantly with BCVA and may provide additional information about macular function in patients with DME. Some works[114]

demonstrated that MP is a useful adjunct to OCT and BCVA in assessing DME, as it is more closely related to visual function, independently of macular thickness. It was also shown that disruption of the photoreceptors layer, identified by OCT, is correlated with a significant decrease in MP sensitivity these eyes[115] and a progressive loss of function with the structural alterations seems to occur during the course of this maculopathy[116].

Therefore, microperimetry seems to be a good diagnostic but especially a good assessment tool to evaluate the efficacy of a therapy in DME.

Multifocal ERG

Multifocal electroretinography (mfERG) is an objective test that identifies functional changes of the retina by objectively recording electric responses from many regions of the posterior pole in a topographic way[117], as opposed to full-field flash electroretinography that records mass responses from the whole retina. Its main clinical use is to detect localized variations in mfERG responses identifying retinal damage in discrete regions of the central retina: macula, paramacula or distinct peripheral areas. With this technique, small scotomas in the posterior pole can be mapped and the degree of retinal dysfunction quantified.

Multifocal ERG responses are biphasic waves with a negative trough (N1) followed by a positive peak (P1) and a second negative wave named N2[118] (figure 10). It is believed that N1 is generated by photoreceptors and P1 is generated by Müller and bipolar cells.[117,119,120] Bipolar cells are the first-neuron cell to process the electrical stimulus coming from the photoreceptors before transmitting it to the ganglion cells. Therefore, these cells are responsible for the electrical response of the retina as objectified in multifocal electroretinography.

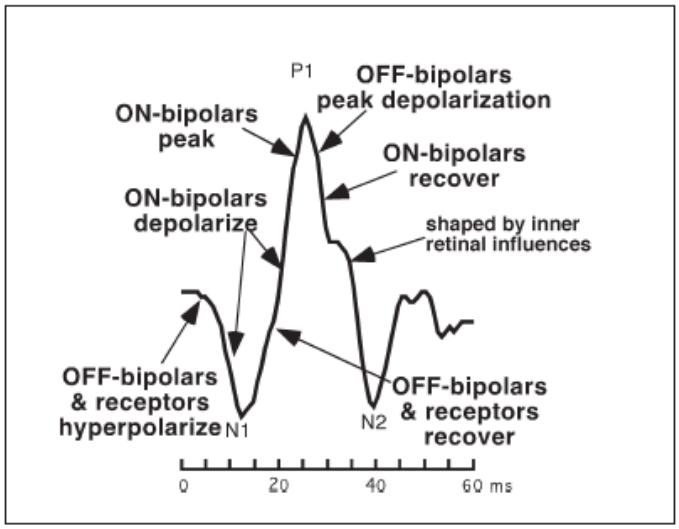

Figure 10: A model of how the different retinal cell types contribute to produce the mfERG waveform in the macular region. (Adapted from Hood et al.[117] and reproduced with author's permission)

The amplitude of mfERG waves give information about the function and integrity of specific retina cells in distinct retinal layers (figure 11). Damage at or before the bipolar cells (middle retina) will substantially decrease the amplitude of the mfERG while retinal damage in amacrine and/or ganglion cells (inner retina) can affect the waveform but with subtle effects in its amplitude[121,122].

On the other hand, mfERG implicit time (latency) is mainly influenced by damage in the outer cells (photoreceptors/outer plexiform layer). Changes in the bipolar cells or beyond generate relatively small changes in implicit time and may even shorten it[117].

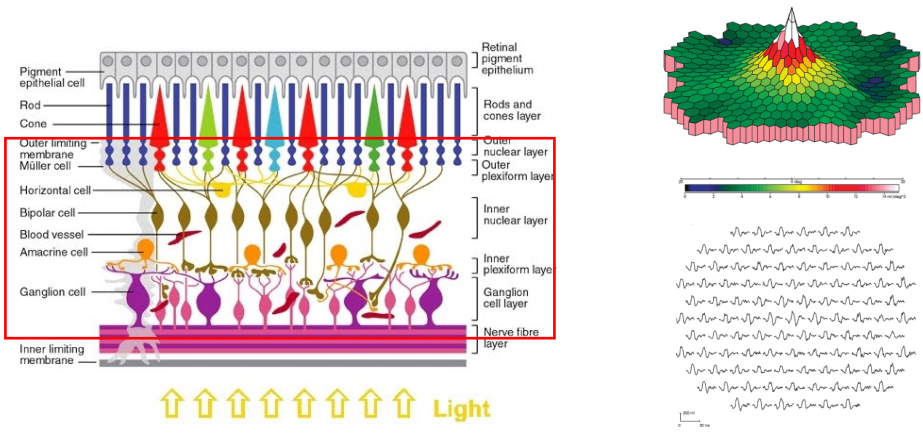

Figure 11: Retina layers scheme and mfERG response plots.
Adapted from www.memorangapp.com/flashcards/110876/Neuro+2.1/. Accessed on April 13, 2019.

Some studies have reported impaired function in the retina middle and inner layers of diabetic patients, even before vascular complications[123,124]. This function is severely impaired in the presence of advance forms of the disease, as DME. The increase in central retinal thickness secondary to extracellular fluid accumulation in these patients cause stretching or even rupture of several retinal layers. The inner nuclear layer (INL) that contains the nuclei of bipolar cells is one of the most affected by DME.

Therefore, early identification of functional changes in middle and inner retinal layers could be very helpful for treatment management and follow-up in diabetic patients.

Following these concepts, microperimetry and mfERG were performed in our study patients, before and after treatment, to characterize visual function changes in DME patients and to evaluate the effects of anti-VEGF therapy. This work is described in **Chapter 6 – Microperimetry and mfERG as functional measurements in DME**, having originated a manuscript that is currently under revision at an international peer-reviewed journal.

Aims of the thesis

The main goal of the present thesis was to identify DME characteristics with predictive value that can be used as biomarkers of good or poor treatment response to anti-VEGF therapy. We were mainly focused on imaging biomarkers, easily assessible by non-invasive techniques and possible to be evaluated in the daily clinical practice with Spectral Domain or Swept Source OCT.

It was also our goal to characterize visual function in diabetic patients with DME, using differentiated methods, such as microperimetry and mfERG, to have detailed information about the impact of this disease not only on vision but also on the quality of life of these patients. Functional evaluation before and after anti-VEGF treatment adds important information about the effects of this therapy in different visual outcomes besides visual acuity that represents only central foveal function. This information is particularly important as we are dealing with a working age disease and different degrees of vision recovery can change patients' ability to work or perform tasks of daily life.

We expect to provide new information than can contribute to open new perspectives for the management of DME treatment and the improvement of visual prognosis, having a potential impact on these patients care.

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Chapter 2

Degree of Decrease in Central Retinal Thickness Predicts Visual Acuity Response to Intravitreal Ranibizumab in Diabetic Macular Edema

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Abstract

Purpose: To characterize factors that may be associated with optimal or suboptimal response to ranibizumab intravitreal injections in diabetic macular edema (DME).

Methods: Fifty-nine eyes with DME treated with ranibizumab were included. All underwent best-corrected visual acuity (BCVA) assessment and optical coherence tomography (OCT) at baseline, 3 and 6 months. Central retinal thickness (CRT) was assessed at each visit, and OCT images were classified according to their morphological patterns.

Results: A mean BCVA increase of 4.78 and 5.52 letters, and a CRT decrease of 80.25 µm and 106.12 µm were found after 3 and 6 months of treatment ($p<0.001$). BCVA improvement was found to be dependent on baseline BCVA and the degree of CRT decrease. Twenty-six eyes (44%) showing a CRT decrease ≥ 20% improved BCVA by 10.3 ± 13.0 letters, whereas 33 eyes (56%) with a CRT decrease <20% had BCVA improvement of 1.8 ± 7.2 letters (odds ratio=3.31).

Conclusions: The degree of CRT decrease obtained by spectral-domain OCT identifies well the optimal responders to intravitreal ranibizumab and predicts BCVA improvement after treatment.

Introduction

Diabetic retinopathy (DR) is the most frequent cause of blindness in Europe and North America in people between 20 and 74 years old[1]. Diabetic macular edema (DME) is a vision threatening complication of DR that causes loss of central vision in the course of the disease when involving the center of the macula. Chronic DME can be associated with cystic degeneration of the macular retina and is called cystoid macular edema[2,3]. It has been demonstrated that in DR there is production by the retina of growth factors such as the vascular endothelial growth factor (VEGF), also known as permeability factor, stimulating vascular leakage and causing breakdown of the blood-retina barrier. The resulting increase in the vascular permeability leads to accumulation of fluid and proteins on the macula causing DME[3–6].

Anti-VEGF drugs, such as ranibizumab, have shown their efficacy in treating DME. Approval of ranibizumab by the European Medicines Agency to treat visual impairment caused by DME fulfils the previously unmet medical need for a treatment that can improve best corrected visual acuity (BCVA) in these patients. Many studies showed an improvement of BCVA with significantly superior benefit over standard-of-care photocoagulation in patients with visual impairment due to DME (even if recurrent and persistent)[7–14]. These results were sustained for at least 2 years and are generally well tolerated and with minimal ocular or systemic adverse events. Although the effectiveness of intravitreal anti-VEGF was well demonstrated by these studies, not every patient responds to treatment with improvement of BCVA. It is, therefore, of clear interest to analyze the factors that may be associated with an optimal (BCVA improvement of 10 letters) or suboptimal (BCVA improvement of less than 10 letters) response to treatment[15–18].

Optical coherence tomography (OCT) allows a precise evaluation and quantification of retinal thickness and shows the changes in the morphology of the macular edema and the vitreomacular interface as well as the presence of subretinal fluid or foveal microstructural changes[19,20]. In this work, we address the issue of identification and characterization of response to intravitreal ranibizumab in patients with DME, in an effort to identify characteristics that may be associated with different visual outcomes to intravitreal ranibizumab therapy[18,21–24].

Methods

Patient Eligibility and Study Design

This is a retrospective study of patients with DME in at least 1 eye treated with intravitreal injections of ranibizumab (Lucentis®, Novartis Pharma AG, Basel, Switzerland) at the Association for Innovation and Biomedical Research on Light and Image and the Centro Hospitalar Universitário de Coimbra in Coimbra, Portugal. The clinical records of 80 consecutive patients (95 eyes) with clinically significant macular edema as defined by the Early Treatment Diabetic Retinopathy Study (ETDRS)[25], treated with a loading dose of 3 monthly intravitreal injections of ranibizumab (0.5 mg) were reviewed. The study adhered to the tenets of the Declaration of Helsinki and was approved by the institutional review board/ethics committee of the Association for Innovation and Biomedical Research on Light and Image. An inform consent form was obtained before collecting and reviewing patient records. Eyes with the following exclusion criteria were not considered for this study: intravitreal injections of steroids within a period of 18 months and/or focal or pan photocoagulation of the retina less than 6 months before the first injection of ranibizumab, previous injection of any anti-VEGF drug, macular edema unrelated to DR, history of ocular hypertension or glaucoma with concomitant retinal or choroidal disorder other than DR, significant central lens opacities and/or conditions that limit the view of the fundus and decreased vision due to other causes in the investigator's opinion (at visit 1). Thirty of the 95 eyes were excluded for one of these reasons. From the other 65 eyes, 6 were also excluded due to an insufficient number of OCTs or follow-ups. A final number of 59 eyes (51 patients) was considered for this study.

Ophthalmological Examination

Patients received 3 initial consecutive monthly injections of ranibizumab (months 0-1-2; loading phase), performed according to the physician usual routines, and both pre and post injection topical antibiotics. Further ranibizumab treatment was given according to physician retreatment criteria; 1 injection per month was to be continued if stable BCVA was not reached. Treatment was suspended if either one of the following criteria were met: (1) if the physician's opinion was that no (further) BCVA improvement was

attributable to treatment with intravitreal injection at the last 2 consecutive visits, or (2) a score of >85 BCVA letters (>20/20 Snellen equivalent) was observed at the last 2 consecutive visits. After suspension, injections were resumed if there was a decrease in BCVA due to DME progression, confirmed by clinical evaluation and/or OCT or other anatomical and clinical assessments, in the opinion of the physician. Patients were treated at monthly intervals until stable BCVA was reached again.

All patients performed BCVA measurements using the ETDRS protocol at baseline, 3 and 6 months after initial injection. Baseline central retinal characteristics were analyzed by spectral-domain OCT (Cirrus HD-OCT, Carl Zeiss, Dublin, USA) using the macular cube acquisition protocol (512 A scans x 128 A scans). The retinal thickness in the 1-mm central retina (central subfield area) was obtained from the macular thickness map and used as the OCT central retinal thickness (CRT). Patients were included in this consecutive series only if there was a minimum of 6 months of follow-up.

Evaluation and Classification of OCT Scans

Based on previous reports[26–32], OCT scans from each visit were graded and classified according to the following categories (figure 1): type 1 = diffuse DME without cystoid spaces (hyporeflective area of retinal thickening, sponge-like DME), type 2 = inner cystoid DME (macular edema with presence of cystoid hyporeflective empty spaces in the inner layers of the retina), type 3 = outer cystoid DME (macular edema with presence of cystoid hyporeflective empty spaces predominantly in the outer layers of the retina), type 4 = overall cystoid DME (macular edema with presence of large cystoid spaces involving the entire retina), type 5 = presence of serous retinal detachment. Presence of epiretinal membrane and vitreoretinal traction was also evaluated. In cases where more than one type coexisted, all of them were registered. The OCT classification was performed by 2 trained specialists, and consensus was achieved in cases of disagreement.

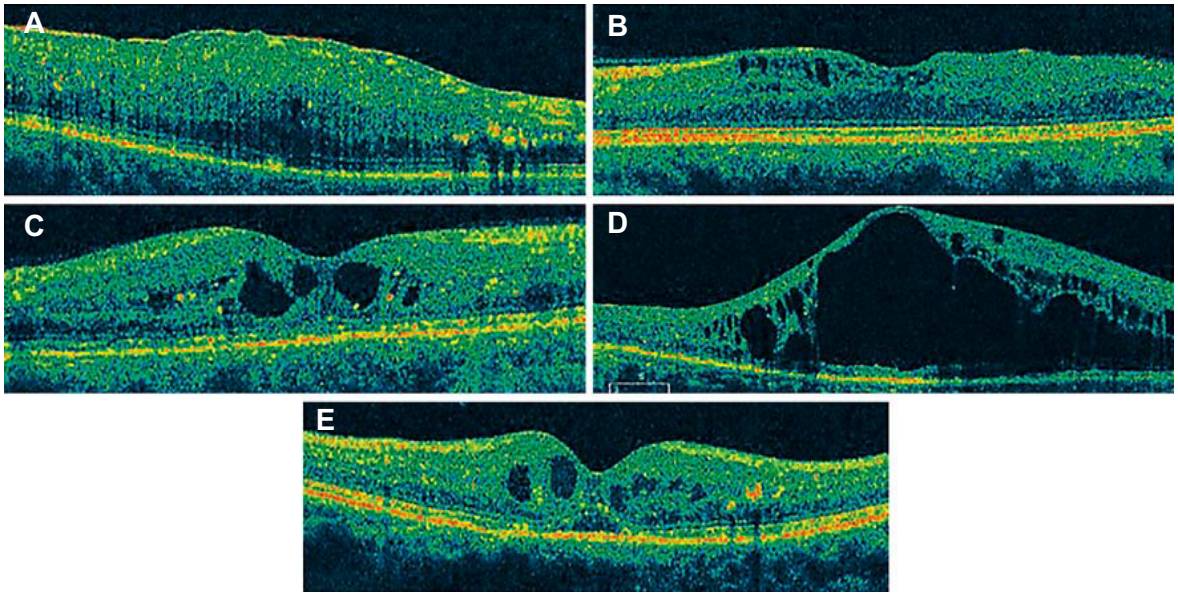

Figure 1: Patterns of macular edema (epiretinal membrane and vitreoretinal traction are not shown in this figure. A) Type 1 – diffuse DME without cysts. B) Type 2 – inner cystoid DME. C) Type 3 – outer cystoid DME. D) Type 4 – overall cystoid DME. E) Type 5 – serous retinal detachment.

Statistical Analysis

Main outcome measures included changes from baseline to month 6 in BCVA and CRT as measured by OCT. Data were analyzed at baseline, 3 and 6-month follow-up visits. Statistically significant differences between visits were tested using the Friedman and the Wilcoxon tests for CRT and BCVA values. The degree of CRT decrease, i.e. the percentage of CRT changes from baseline to month 6, was correlated with BCVA improvement. Different cutoff values for the degree of CRT decrease were tested, and for the one that showed a statistically significant difference for the BCVA response, the odds ratios were computed. Statistically significant differences between groups were tested using the Mann-Whitney test. Statistical analyses were performed using STATA (Stata Corp LP, V8A) software version 12.1. Statistically significant results were considered for p-values <0.05.

Results

Fifty-one patients (59 eyes) with a minimum of 6 months of follow-up were included for analysis with a mean age of 69.02 ± 7.75 years. Thirty-three were male (64.71%) and 18 female (35.29%).

BCVA Analysis

At the baseline visit, the mean BCVA for the 59 eyes was 49.97±20.88 letters. The mean BCVA increased at the 3-month follow-up to 54.75 ± 17.61 letters (p< 0.001), and continued to increase in the following 3 months to 55.49 ± 19.84 letters (p = 0.668). A statistically significant increase of 5.52 letters was observed between the baseline visit and the 6-month follow-up (p< 0.001). Twenty-two eyes had an initial BCVA <49 letters (<2/10) and 37 eyes an initial BCVA ≥ 49 letters (≥ 2/10). The group initiating the treatment with lower letter scores (<49 letters) was the group with major improvement of BCVA at the end of the 6-month follow-up (+9.4 *vs* +3.2 letters in the group of BCVA ≥ 49 letters; figure 2). Because of the very different baseline BCVA values, it was not possible to identify better from worse responders using only the BCVA score.

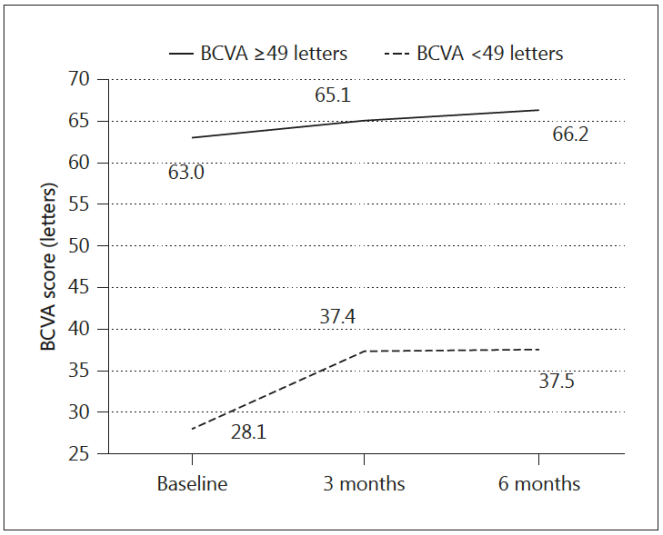

Figure 2: Mean BCVA score at baseline, 3- and 6-month follow-up, by baseline BCVA ≥ 49 letters and <49 letters.

CRT Analysis

The mean CRT for all patients was 507.61 ± 147.36 µm at baseline. By the 3rd month of follow-up, the mean CRT decreased to 427.36 ± 154.33µm (–15.8%), a difference that was statistically significant (p < 0.001). CRT continued to decrease in the following 3 months, from 427.36 ± 154.33 to 401.49 ± 153.20 µm (–6.05%; p = 0.190), being statistically different from baseline (p < 0.001).

Correlation between BCVA and CRT

Analysis of the correlation between BCVA response and CRT response to the treatment at 6 months of follow-up (figure 3) shows that eyes with a decrease in CRT ≥ 20% had a much better BCVA response, showing a good correlation with the degree of decrease in CRT. On the other hand, eyes with a decrease in CRT <20% had no significant improvement of BCVA. The higher the percent CRT decrease from baseline values, the better was the BCVA response (figure 3), indicating that the percentage of CRT decrease from baseline after anti-VEGF treatment may be a predictive biomarker of BCVA response in DME.

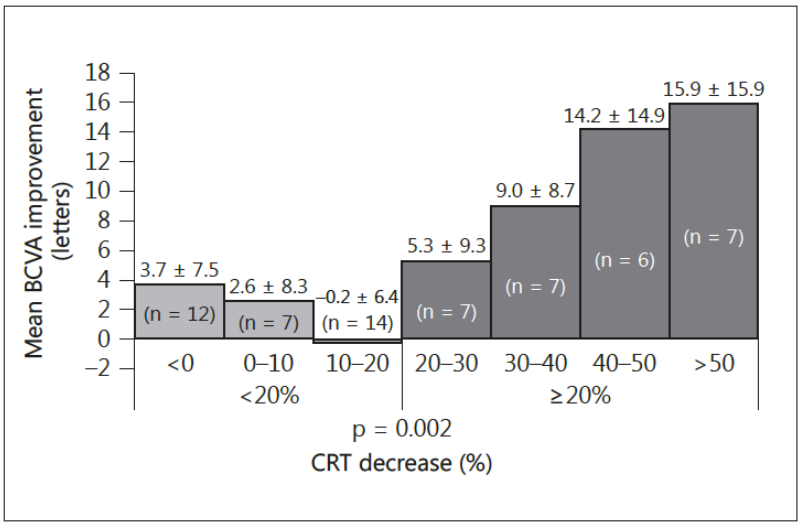

Figure 3: Relationship between mean BCVA improvement (mean ± SD) and CRT decrease after treatment (from baseline to month 6).

As we can see in figure 4, the group with a decrease in CRT ≥ 20% (n = 26; 44%) showed a mean CRT decrease of 42.9% ($p < 0.001$) between baseline and 6 months, while the group with a CRT decrease <20% (n = 33; 56%) had no significant difference in CRT in the same period (1.35%; $p = 0.929$).

Analyzing the BCVA (figure 4) by these two groups, there was a significant improvement of mean BCVA from baseline to 6 months of follow-up of 10.3±13.0 letters in the group with a decrease in CRT ≥ 20% (optimal responders), while the group with a decrease in CRT <20% showed an improvement of only 1.8±7.2 letters during the 6 months of follow-up (suboptimal responders; $p = 0.002$).

The results showed that patients with a CRT decrease ≥ 20% after treatment are 3 times more likely to have an improvement of BCVA of 10 letters or more than patients with a CRT decrease <20% (odds ratio = 3.31; 95% confidence interval = 1.02–10.71).

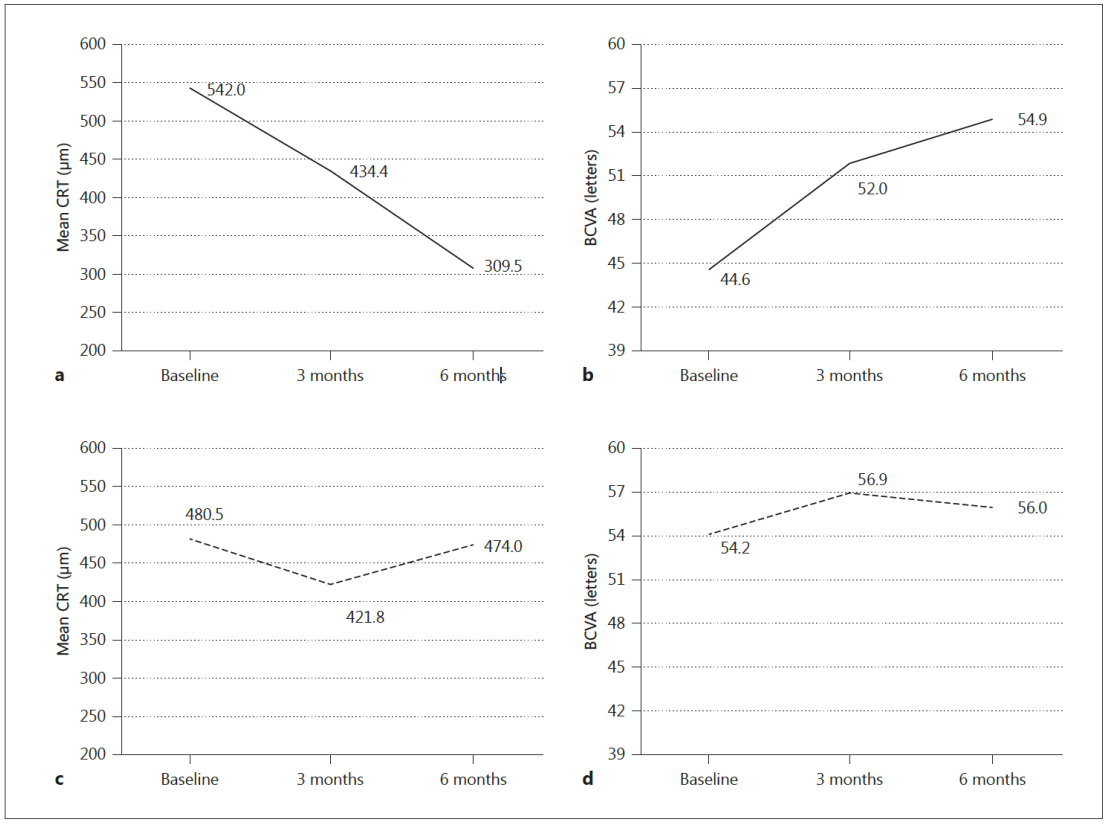

Figure 4: Mean BCVA score and mean CRT by groups of CRT percent decrease at baseline, 3- and 6-month follow-up. a) Thickness in the group of CRT decrease ≥ 20%. b) BCVA in the group of CRT decrease ≥ 20%. c) Thickness in the group of CRT decrease <20%. d) BCVA in the group of CRT decrease <20%.

Morphological Patterns of DME in OCT and Response to Treatment

Considering the different retinal OCT morphological patterns at baseline, diffuse DME (type 1) was seen in 47.5% of 59 eyes. The outer retinal layers appeared to be the privileged site for tissue swelling which is consistent with previous findings [6, 28] . In our study, cystoid DME was present in 54.2% of the 59 eyes, the majority (52 eyes; 88.1%) involving the outer retinal layers – outer cystoid DME (type 3). Thirty-six eyes (61.0%) were classified with inner cystoid DME (type 2) and 28 eyes (47.5%) with both inner and outer cystoid DME. Presence of overall cystoid DME was observed in 31 eyes (52.5%). Serous retinal detachment (type 5) was present in 11 eyes (18.6%), epiretinal membrane in 20 eyes (33.9%) and vitreoretinal traction in 10 eyes (16.9%).

Analyzing the OCT morphological patterns by CRT response to treatment (CRT decrease ≥ 20% and <20%), presence of outer cystoid DME and overall cystoid DME were more frequent at baseline in the optimal responder group (93.9 and 60.6%, respectively; figure 5). In contrast, diffuse DME and inner cystoid DME seem to be related with a suboptimal response to the treatment (53.8 and 69.8%, respectively). Presence of serous retinal detachment, vitreoretinal traction and epiretinal membrane was also more frequent at baseline in this group and can be associated with a suboptimal response to the treatment. However, these results did not reach statistical significance.

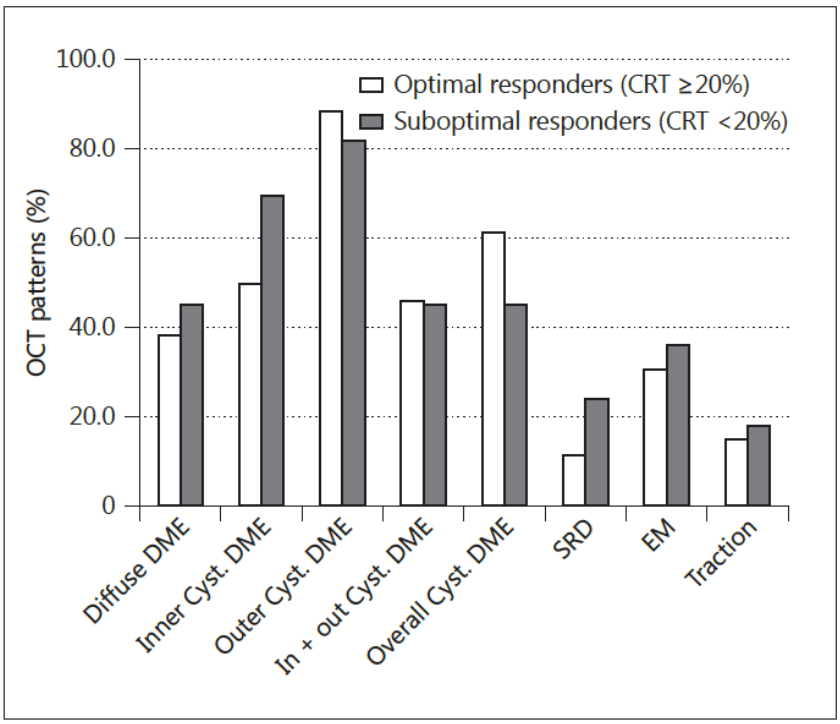

Figure 5: Presence of the different morphological patterns at baseline, by response group. SRD=Serous retinal detachment; EM=epiretinal membrane; Cyst.=cystoid

Discussion

The goal of treatment of DME with laser photocoagulation was to achieve BCVA stabilization[25]. Immediate focal photocoagulation of DME reduced moderate visual loss by 50% but 12% of treated eyes still lost ≥ 15 ETDRS letters after 3 years of follow-up and 24% of immediately treated eyes had thickening involving the center of the macula at 36 months. The anti-VEGF drug ranibizumab was approved for the treatment of visual impairment due to DME and fulfilled the unmet medical need for a therapy that can

improve visual acuity in patients with visual loss[7–14].

In this retrospective study, the BCVA of the 59 eyes treated with ranibizumab increased a mean of 4.78 letters after 3 months of treatment, and a mean of 5.52 letters after 6 months, in comparison with the baseline mean (49.97±20.88 letters). Our BCVA results are similar to the values reported in other studies like READ-2[9] that showed an improvement at month 6 of 7.4 letters for the group of patients that had ranibizumab (0.5 mg) monotherapy, and of 7.7 letters after 2 years. The RESOLVE study[7] , using the same loading dose of 3 monthly injections of ranibizumab (0.3 or 0.5 mg), showed a gain of 10.3 letters in BCVA after 12 months, and RESTORE[8] showed an increase in BCVA of 6.8 letters in the same period.

In our study, the group which initiated the treatment with a lower letter score (<49 letters) was the group with more improvement of BCVA at the end of the 6-month follow-up (+9.4 *vs* +3.2 letters in the group of BCVA ≥ 49 letters). Confirming previous studies[33], patients with worse BCVA had a better visual benefit. Characterization of response to treatment based on BCVA clearly depends on baseline BCVA, making it very difficult to compare response to treatment between eyes with very different baseline BCVA values.

In the 59 eyes enrolled in this study, CRT decreased by a mean of 80.25 µm after the 3 month and 106.12 µm after the 6-month follow-up in comparison with the baseline (507.61 ± 147.3µm). Our results are similar to the previous studies RESOLVE, RESTORE and READ, but an analysis by CRT response to treatment was not reported.

In this study, when analyzing the available data by CRT response, 26 eyes had a decrease in baseline CRT higher than 20% (mean of – 42.9%, i.e. 232.5 µm) after anti-VEGF treatment, whereas 33 eyes had a decrease in baseline CRT inferior to 20% (1.35%, i.e. 6.5µm; p=0.002). The eyes showing a CRT decrease ≥ 20% had a BCVA improvement of 10.3 letters after the 6 months of follow-up while the eyes with a CRT decrease <20% had an increase in BCVA of only 1.8 letters (p = 0.002). This occurred even considering that baseline CRT was not statistically different between groups (541.96 ± 153.92 and 480.5 ± 138.3 µm, respectively; p = 0.111), thus allowing for comparative analysis of the CRT decrease after treatment.

This study shows that the response to anti-VEGF treatment can be better characterized by

a decrease in CRT. This observation should be expected as the primary effect of anti-VEGF drugs is the stabilization of the blood-retina barrier with a resulting decrease in the accumulation of fluid in the retina[22–24]. Furthermore, it may indicate that other factors than VEGF may play a more important role in the development and maintenance of DME in eyes that do not respond with an important CRT decrease after anti-VEGF treatment.

Our study shows also that OCT scans are a very important tool to follow the DME response to anti-VEGF treatments and may give relevant qualitative information of treatment response. We classified the OCTs as described in the Methods, based in several previous studies[26–29] with the purpose of characterizing the possible different patterns of DME and analyzing the usefulness of these OCT patterns in predicting response to treatment. We hypothesized that detailed interpretation of OCT images in each follow-up visit of patients with DME may be an additional tool to determine the prognosis of DME and the response to treatment helping to understand some of the discrepancies found in the correlation between CRT and BCVA in these patients. The various morphological patterns of DME seen in OCT may represent different levels of severity or chronicity of the disease.

Our study shows that OCT morphological DME patterns at baseline may give important information regarding differences in optimal versus suboptimal response to treatment between patients. Of major relevance is the observation that the response to treatment of DME by intravitreal ranibizumab is best characterized by the degree of decrease in CRT from baseline. The degree of decrease in CRT correlates well with BCVA improvement, is independent of baseline values and identifies better the optimal and suboptimal responders to intravitreal anti-VEGF treatment.

However, these results have the limitation of being based on a retrospective analysis of a relatively small number of patients treated according to prevalent clinical practice. Further studies planned in a prospective manner and with a larger cohort of patients are needed.

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Chapter 3

Optical Coherence Tomography Baseline Predictors for Initial Best Corrected Visual Acuity Response to Intravitreal anti-VEGF Treatment in Eyes with Diabetic Macular Edema - The CHARTRES study

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Abstract

Purpose: To identify baseline OCT morphological characteristics predicting the visual response to anti-VEGF therapy in Diabetic Macular Edema.

Methods: Sixty-seven patients with diabetic macular edema completed a prospective, observational study (NCT01947881-CHARTRES). All patients received monthly intravitreal injections of Lucentis® for 3 months followed by PRN treatment and underwent best corrected visual acuity measurements and spectral domain optical coherence tomography at Baseline, Months 1, 2, 3, and 6. Visual treatment response was characterized as good (≥10 letters), moderate (5–10 letters), and poor (<5 or letters loss). Spectral domain optical coherence tomography images were graded before and after treatment by a certified Reading Center.

Results: One month after loading dose, 26 patients (38.80%) were identified as good responders, 19 (28.35%) as Moderate and 22 (32.83%) as poor responders. There were no significant best-corrected visual acuity and central retinal thickness differences at baseline (P = 0.176; P = 0.573, respectively). Ellipsoid zone disruption and disorganization of retinal inner layers were good predictors for treatment response, representing a significant risk for poor visual recovery to anti-vascular endothelial growth factor therapy (odds ratio = 10.96; P = 0.001 for ellipsoid zone disruption and odds ratio = 7.05; P = 0.034 for disorganization of retinal inner layers).

Conclusions: Damage of ellipsoid zone, higher values of disorganization of retinal inner layers, and central retinal thickness decrease are good predictors of best-corrected visual acuity response to anti-vascular endothelial growth factor therapy.

Introduction

Diabetic Macular Edema (DME) is the major cause of visual acuity impairment in patients with Diabetic Retinopathy (DR)[1]. Vascular Endothelial Growth Factors (VEGFs) play an important role in the alterations of vascular permeability and development of DME. It interferes with the "tight junctions" of the endothelium of the retinal vessels leading to a breakdown of the BRB and consequent leakage to the retinal tissue[2]. Based on this concept, the administration of intravitreal (IVT) anti-VEGFs in DME has been widely demonstrated to be efficient in macular thickness improvement and consequent increase of BCVA[3,4], although these results may not be permanent and multiple injections may be required to maintain treatment efficacy. Furthermore, in some cases, the resolution of DME is not followed by recovery of visual function. According to Elman et al[5], after 24 months of treatment with ranibizumab and deferred laser, 49% of the subjects had a BCVA gain ≥10 letters (good responders), 22% had a BCVA gain between 5-10 letters (responders), and 29% had a BCVA gain <5 letters or a decrease in BCVA (poor responders). Massin et al[6] and Mitchell et al[7] refer that after 12 months of treatment with ranibizumab in monotherapy, 40 to 60% of the subjects had a BCVA gain ≥10 letters, 30% had a BCVA gain between 5-10 letters, and 10 to 30% had a BCVA gain <5 letters or a decrease in BCVA. Moreover, Gonzalez et al[8], in a post hoc analysis of Diabetic Retinopathy Clinical Research Network's Protocol I data, showed that the mean change in BCVA from Month 3 to Month 12 is lower than 5 letters indicating that the response to the loading dose (3 initial monthly injections) appears to determine the final visual recovery at 1 and 3 years.

It is, therefore, of major importance to characterize the baseline features that may identify the different visual outcomes observed in different eyes after the initial 3 monthly injections of anti-VEGF in DME and if any of these characteristics can predict poor response to treatment.

Damage in the inner/outer segments of the photoreceptors layer (IS/OS), currently termed as Ellipsoid Zone (EZ)[9], or in the retinal pigment epithelium (RPE) have been reported to predict the visual response to treatment with anti-VEGF injections, as well as the extent of disorganization of the retinal inner layers (DRIL)[10-12]. However, most of these studies were

performed retrospectively or in patients previously treated with intravitreal corticosteroids or anti-angiogenic drugs.

In this study, we sought to analyse and quantify the DME morphological features that could correlate with BCVA response in the initial stage of anti-VEGF treatment response (after the loading dose) and up to 6 months, in a prospective study of well characterized naïve patients with DME that have clinical indication for ranibizumab treatment. Using a detailed grading of spectral domain optical coherence tomography (SD-OCT) images, we assessed not only the central retinal thickness (CRT) response to therapy but also the baseline morphological characteristics of outer and inner retinal layers, as well as size and location of cystoid spaces, and their relationship with visual acuity outcomes.

Methods

Study Design

A prospective, exploratory and observational study (NCT01947881-CHARTRES) was conducted in diabetic type 2 patients receiving the same interventional treatment following clinical practice guidelines. The study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional review board and ethics committee of AIBILI, Coimbra, Portugal. Written informed consent was obtained from all study patients. Patients were treated and followed according to the standard practice for DME treatment with ranibizumab intravitreal injections as described in the Summary of Product Characteristics (SmPC): Loading dose of 3 monthly injections followed by PRN regimen.

Sample Calculation

The previously mentioned authors[5-7], showed that it is expected to have 40% to 60% of good responders, 20% to 30% of responders, and 20% to 30% of poor responders after 12 and 24 months of intravitreal treatment with ranibizumab for DME. Therefore, focusing on the initial 3 months of treatment (the loading dose of 3 monthly IVT injections), and considering that at least one of the 3 groups may only represent 20% of the sample, the inclusion of 70 subjects was considered appropriate to cover the extreme situation of 42 good responders (60%), 14 responders (20%) and 14 poor responders (20%).

Study Participants

Naive patients with indication for treatment with ranibizumab injections for DME in the investigator's opinion and fulfilling the following inclusion criteria: (1) type 2 Diabetes Mellitus; (2) Center-involved DME, confirmed by OCT and defined as a baseline SD-OCT central subfield retinal thickness ≥300μm[13,14]; (3) visual impairment due to DME with BCVA ≥ 20/160 and ≤ 20/40 ((≥39 letters and ≤73 letters); (4) glycated haemoglobin (HbA1C) ≤12% at screening visit. Exclusion criteria: (1) presence of proliferative diabetic retinopathy (PDR); (2) previous laser photocoagulation (panretinal or focal) in the study eye within 6 months prior to study inclusion; (3) previous treatments with IVT injections of triancinolone or anti-VEGF drugs in the study eye; (4) prior vitrectomy surgery; (6) other chorioretinal diseases like central serous chorioretinopathy, high myopia, chorioretinitis or any other fundus disease associated with morphological or functional changes.

Study Procedures

All included patients performed an initial visit (V1) with the following procedures: clinical history (medical history, demographics and concomitant medications); vital signs, metabolic analysis; biomicroscopy; Intraocular pressure with Goldmann tonometry; ophthalmoscopy; BCVA (using ETDRS method); colour fundus photography– CFP (7 ETDRS fields); SD-OCT (HD-OCT Cirrus, Zeiss Meditec) and fluorescein angiography–FA (Topcon TRC 50DXTM).

After baseline visit (V1), all patients were treated with 3 monthly IVT injections of anti-VEGF ranibizumab during 3 months (loading dose – V2, V3 and V4) and monitored at each visit before injection with BCVA and OCT measurements. One month after the last injection of the loading dose period (V5), i.e., 3 months after the first injection, BCVA, OCT, CFP and FA procedures were repeated and patients received more monthly injections following a PRN regimen if the central retinal thickness remained ≥300μm. Patients were monitored monthly with BCVA and OCT examinations and the final visit (V6) was performed 6 months after the first injection. BCVA, OCT, CFP, and FA were performed as well. Optical Coherence Tomography, CFP and FA images were graded by certified graders in a reading centre – CORC (Coimbra Ophthalmology Reading Centre). Quality control of

CFP and FA grading was ensured by double grading in 8% of all cases with an observed agreement of 93.8% between graders.

OCT Acquisition and Grading:

A Macular Cube 512x128 scan and two macular 5 HD lines (at 180º and 90º) were acquired in all patients using HD-OCT Cirrus 5000 (Zeiss Meditec, Dublin). A detailed OCT double grading was done in all visits by two CORC independent graders. The observed agreement between the two graders was 93.6%. All disagreement cases were resolved by mutual agreement. Central retinal thickness, parafoveal and perifoveal retinal thicknesses were quantified using Macular Cube maps. DME was classified as diffuse or cystoid (CME)[15]; CME was also classified according the location of cystoid spaces in the retinal layers (inner, outer or overall cystoid spaces) and severity (mild, moderate or severe cystoid spaces)[16]. The integrity of both inner and outer retinal layers was also accessed (Figure 1). Disorganization of the Retinal Inner Layers (DRIL) was defined as the horizontal extent in microns for which any boundaries between the ganglion cell–inner plexiform layer complex, inner nuclear layer, and outer plexiform layer could not be identified[11]. The DRIL extent was measured using the equipment software calliper in each of the 5 horizontal HD B-scans, and these measurements were averaged across 5 scans to derive a global DRIL area for each eye at baseline. Disruption of External Retinal Membrane (ELM), Ellipsoid Zone (EZ) and Retinal Pigment Epithelium complex (RPE) were defined as the horizontal extent with loss of the hyper-reflective signal that characterizes each layer[17]. The disruption of these layers was also measured in the central 1mm of the 5 consecutive horizontal scans of 5 HD Line protocol. Presence of Neurosensorial Serous Detachment (NSD), Vitreo-Macular Traction (VMT) and Epiretinal Membrane (ERM) was also analysed.

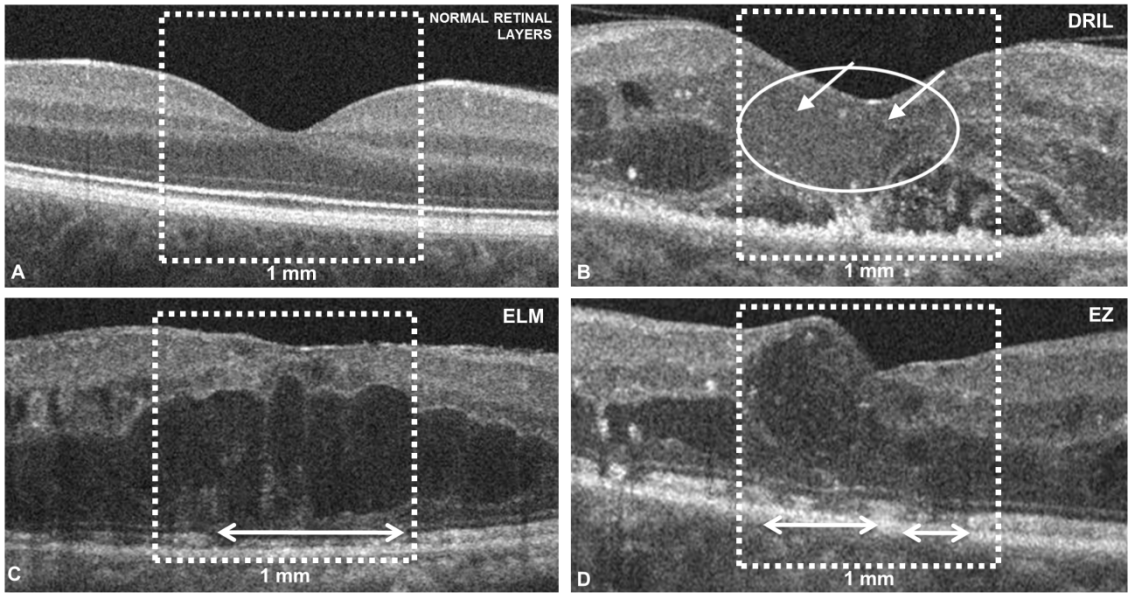

Figure 1: Representative images of the presence and extension of DRIL and disruption of EZ and ELM layers, measured in the 1-mm central area (white box): (A) Normal retinal layers without disruption; (B) Severe DRIL–retina inner layers boundaries cannot be identified in almost half of the 1-mm central area (white circle and white arrow); (C) Severe ELM disruption showed by absence of reflectivity in the ELM layer (white arrow); (D) Severe EZ disruption showed by the presence of several areas with no hyperreflective signal (white arrows).

Statistical Analysis:

One month after the loading dose, at Visit 5, patients were categorized according to their BCVA evolution from baseline and were stratified in 3 treatment response groups: Good responders (≥10 ETDRS letters gained), Moderate Responders (≥5 & <10 ETDRS letters gained) or Poor responders (<5 ETDRS letters gained or loss of visual acuity). Morphologic SD-OCT characteristics were compared between treatment response groups by univariate analysis carried out with ANOVA or Kruskal-Wallis test and Fisher's exact test. Subsequently, multinomial logistic regression was performed to identify possible treatment response predictors. The multinomial logistic regression generates an Odds Ratio (OR) for each category of the dependent variable in relation to the reference category.

The OR value includes the confidence interval (CI 95%) allowing estimating the degree of accuracy. To analyze associations between variables, Spearman's correlation coefficient and the respective statistical significance were computed. A receiver-operating characteristic (ROC) analysis was performed to identify the best predictors for a more than 5 ETDRS letters improvement in BCVA. All tests were two sided and significance was set at

0.05. Statistical analysis was performed with Stata 12.1 SE (College Station, TX: StataCorp LP).

Results

A total of 71 patients were included in this study and 67 were considered for analysis. Four patients (5.6%) discontinued the study, 2 voluntarily and 2 because of health complications unrelated to the study (Figure 2).

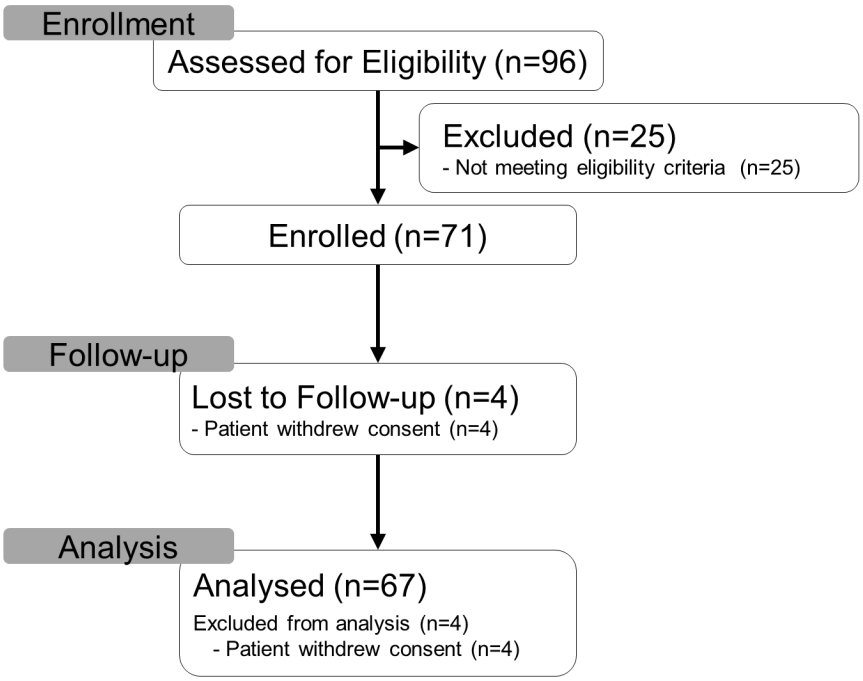

Figure 2: Flow chart of the study.

Response to anti-VEGF Treatment according to Final BCVA

According to BCVA changes from baseline to Visit 5 (after 3 monthly injections of ranibizumab), 26 patients (38.81%) were considered good responders, 19 patients (28.35%) were considered moderate responders, and 22 patients (32.84%) were considered poor responders.

Baseline Characteristics of Study Patients by Treatment response

Baseline characteristics (demographics, vital signs, metabolic factors, diabetes duration, BCVA, CRT and ETDRS DR level) for all study population, and by treatment response, are summarized in table 1. No significant differences were found at baseline between treatment response groups, even after using age and diabetes duration as adjusting factors.

Table 1 - Baseline Characteristics of Patients

	Study population (n=67)	Good Responders (n=26)	Moderate Responders (n=19)	Poor Responders (n=22)	*P*
Demographics					
Age, mean ± SD (years)	64.4±10.3	64.9±7.5	60.5±15.7	67.2±5.4	0.106
Females, n (%)	26 (38.8)	7 (26.9)	6 (31.6)	13 (59.1)	0.056
Vital signs					
Heart rate, mean ± SD (bpm)	73.5±10.6	74.3±9.9	74.1±12.2	72.0±10.1	0.702
Systolic blood pressure, mean ± SD (mmHg)	142.5±10.3	141.9±9.4	139.4±10.5	145.7±10.6	0.177
Diastolic blood pressure, mean±SD (mmHg)	72.6±9.0	72.7±9.5	74.1±8.5	71.3±9.0	0.608
Metabolic factors					
HbA1C, mean ± SD (%)	7.8±1.5	7.9±1.7	8.1±1.2	7.5±1.5	0.385
Total cholesterol, mean ± SD (mg/dL)	189.7±50.9	191.8±42.3	197.8±69.5	179.7±40.7	0.701
HDL cholesterol, mean ± SD (mg/dL)	47.4±10.8	48.2±11.7	46.5±11.6	47.2±9.3	0.586
LDL cholesterol, mean ± SD (mg/dL)	127.1±39.1	129.0±35.8	134.7±51.6	117.9±28.7	0.542
Triglycerides, mean ± SD (mg/dL)	155.1±92.2	155.5±76.7	163.5±135.8	146.9±106.1	0.682
Disease characteristics					
Diabetes duration, mean ± SD (years)	18.1±7.7	18.1±7.6	15.4±5.7	20.5±8.8	0.333
DME duration, median (IQR) (months)	7.94±23.62	7.81±28.57	6.47±15.81	9.36±23.74	0.744
CRT, mean ± SD (µm)	427±107	421±101	463±144	404±64	0.573
BCVA, mean ± SD (letters)	(63.4±9.2)	(63.6±8.5)	(65.7±9.0)	(61.3±10.0)	0.176
Snellen Equivalent	20/63	20/50	20/50	20/63	
DR level (ETDRS scale), n(%)					
35 (C-F)	48 (71.6)	17 (65.4)	11 (57.9)	20 (90.9)	
53 (A-B)	14 (20.9)	6 (23.1)	7 (36.8)	1 (4.6)	0.062
57 (A-D)	5 (7.5)	3 (11.5)	1 (5.3)	1 (4.6)	

HbA1C=Glycated Haemoglobin; HDL=High Density Lipoprotein; LDL=Low Density Lipoprotein; DME=Diabetic Macular Edema; CRT=Central Retinal Thickness; BCVA=Best Corrected Visual Acuity; DR=Diabetic Retinopathy. P=p-value; Age and Diabetes duration were adjusted in this analysis.

CRT Decrease as a Predictor for BCVA Treatment Response

Despite no significant CRT differences between groups at baseline, a higher and significant CRT decrease was found in the treatment groups with better response at Visit 5 (1 month after loading dose) and Visit 6 (6 months after initiating treatment), respectively (p<0.001) (Figure 3).

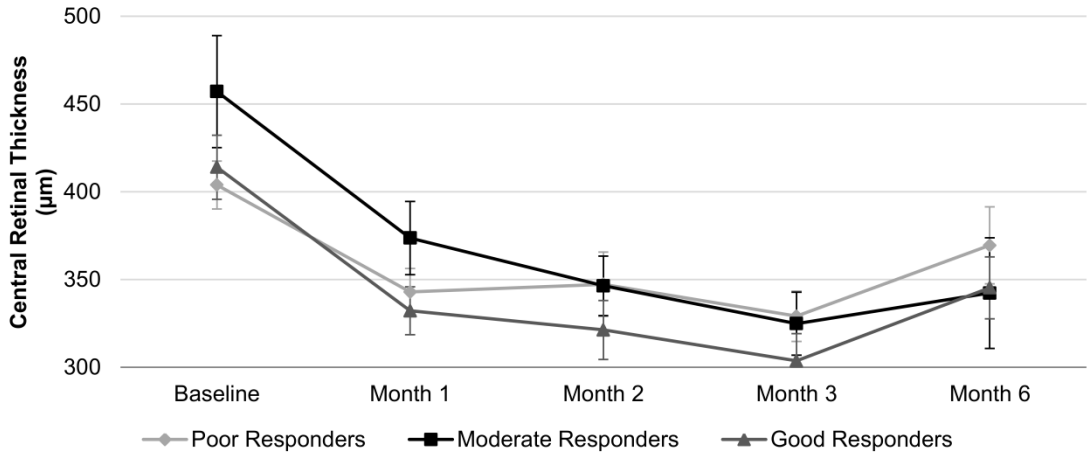

Figure 3: Decrease of retinal thickness by treatment response from Baseline to Month 1 (after first injection), Month 2 (after second injection), Month 3 (after third injection), and Month 6.

On a ROC analysis, CRT decrease 1 month after the first injection was not a statistically significant predictor for treatment response. However, using a threshold of 8.7% for CRT decrease, we were able to distinguish 73.3% of patients that recovered more than 5 BCVA letters after 3 monthly injections despite a high percentage of false negatives (sensitivity 73.3%, specificity 50.0%, ROC AUC 0.581) (Figure 4).

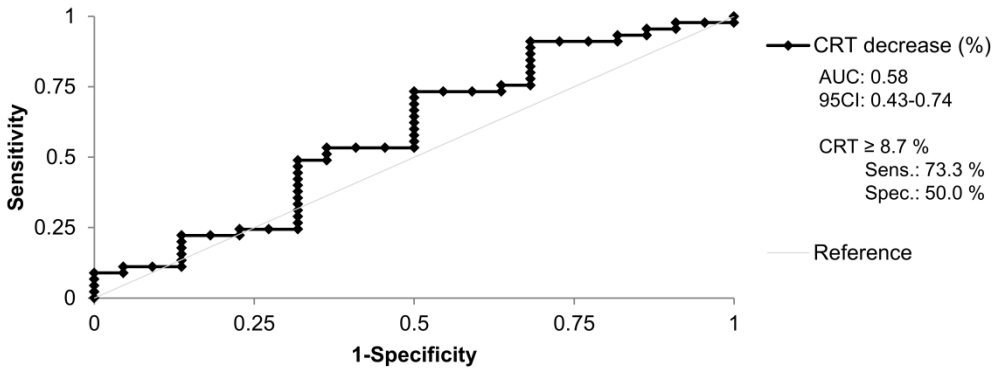

Figure 4: ROC analysis for CRT decrease as a threshold for BCVA improvement.

Baseline OCT Morphological Features by Treatment BCVA Response

As described above, baseline morphological features of DME were analysed on OCT, as well as the degree of disruption and disorganization of inner and outer retinal layers to evaluate the possibility of predicting different treatment responses. Significant differences were found among treatment response groups regarding DRIL area (p=0.021) and EZ and ELM disruption area (p=0.006 and p=0.003, respectively), at baseline. Likewise, cystoid spaces severity appears also to be associated with a poor response to anti-VEGF treatment. Poor responders group have higher percentage of moderate cystoid spaces (57.14%) while good responders group showed higher percentage of mild cystoid spaces (23.08%). However, these differences were not statistically significant (p=0.252) (Table 2).

Table 2 - Baseline OCT Morphological Features of DME for the Study Population and by Treatment Response

	Study population (n=67)	**Good Responders (n=26)**	**Moderate Responders (n=19)**	**Poor Responders (n=22)**	***P***
OCT morphologic features,					
Diffuse Macular Edema	58 (86.57)	22 (84.62)	17 (89.47)	19 (86,36)	0.999
Cystoid Macular Edema	67 (100)	26 (100)	19 (100)	22 (100)	-
Outer Cystoid Spaces	64 (96.97)	25 (96.15)	18 (94.74)	21 (100)	0.745
Inner Cystoid Spaces	63 (95.45)	25 (96.15)	18 (94.74)	20 (95.24	0.999
Overall Cystoid Spaces	20 (30.3)	7 (26.92)	9 (47.37)	4 (19.05)	0.159
Severity of Cystoid Spaces,					
Mild	14 (21.21)	6 (23.08)	5 (26.32)	3 (14.29)	
Moderate	31 (46.97)	14 (53.85)	5 (26.32)	12 (57.14)	0.252
Severe	21 (31.82)	6 (23.08)	9 (47.37)	6 (28.57)	
Cystoid Spaces Size, n (%)					
Small (<250 µm)	6 (9.09)	4 (15.38)	1 (5.25)	1 (4.76)	
Medium (≥250µm & <500µm)	39 (59.09)	12 (46.15)	10 (52.63)	17 (80.95)	0.107
Large: ≥500 µm	21 (31.82)	10 (38.46)	8 (42.11)	3 (14.29)	
Disruption of Retinal					
DRIL, mean±SD	367.8±211.0	278.6±191.7	404.4±158.9	441.6±240.5	**0.021**
EZ, mean±SD	314.7±249.2	196.1±201.2	327.3±219.1	444.0±266.2	**0.003**
ELM, mean±SD	103.4±158.8	30.2±64.7	135.8±177.9	161.9±189.6	**0.006**
RPE, mean±SD	31.2±74.2	14.2±57.6	16.4±46.0	63.9±99.2	0.148
Other OCT features, n (%)					
NSD	22 (32.84)	6 (23.08)	7 (36.84)	9 (40.91)	0.394
Epiretinal membrane	22 (32.84)	8 (30.77)	4 (21.05)	10 (45.45)	0.263
Vitreo-retinal traction	3 (4.48)	1 (3.85)	2 (10.53)	0	0.224

DRIL=Disorganization of Retinal Inner Layers; EZ=Ellipsoid Zone; ELM=External Limiting Membrane; RPE=Retina Pigment Epithelium; NSD = Neurosensorial Serous Detachment; *P* = p-value

Poor Responders presented, at baseline, a greater extent of EZ and ELM disruption (*p*=0.003 and 0.006, respectively). Extension of DRIL area was also significantly higher in this group (*p*=0.021) (Figure 5).

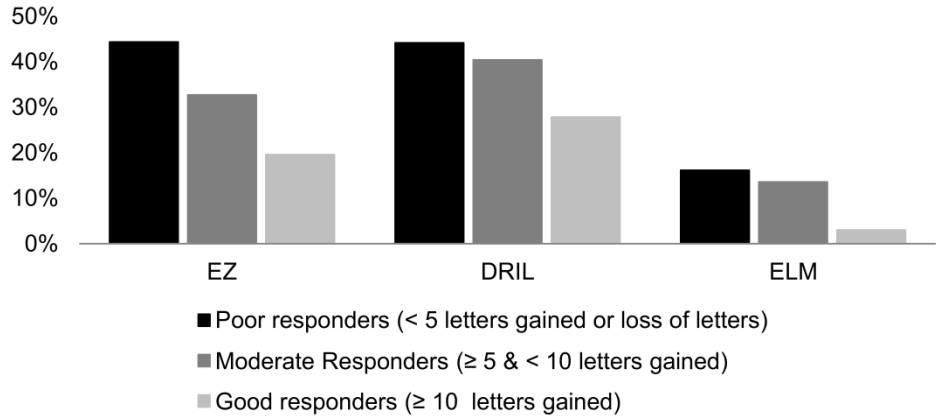

Figure 5: Disruption of retinal inner layers (DRIL) area and disruption of EZ and external limiting membrane (ELM) retinal layers by treatment response group.

Moreover, a correlation was found between EZ and ELM disruption area and DRIL area with the treatment response. The presence of EZ disruption was the morphologic characteristic with a stronger relation to a poor response to treatment (Table 3).

Table 3 - Correlation between retinal layers disruption and BCVA treatment response

	Correlation	***P***
EZ disruption area	r_s = -0.56; CI: -0.70 to -0.36	P<0.001
ELM disruption area	r_s = -0.52; CI: -0.67 to -0,32	P<0.001
DRIL area	r_s = -0.39; CI: -0.58 to -0.17	P=0.001

r_s = Spearman Correlation Coefficient; *P* = p-value; DRIL=Disorganization of Retinal Inner Layers; EZ=Ellipsoid Zone; ELM=External Limiting Membrane

OCT Predictors for Treatment BCVA Response

To identify the best OCT morphological predictors for an improvement of more than 5 letters in BCVA after treatment, a ROC analysis was performed (Table 4), showing that the

best predictor for this visual improvement was the EZ disruption area (ROC AUC 0.71- sensitivity 59%, specificity 80%).

Table 4 - Area under the curve and sensitivity and specificity for the DRIL area and EZ and ELM disruption areas

	ROC AUC	Sensitivity at 80% Specificity
DRIL area	0.65 (CI 95%: 0.50-0.81)	55%
EZ disruption area	0.71 (CI 95%: 0.56-0.85)	59%
ELM disruption area	0.64 (CI 95%: 0.50-0.78)	45%

CRT and RPE disruption area did not showed acceptable accuracy to discriminate poor responders
AUC=Area Under the Curve; CI=Confidence Interval; DRIL=Disorganization of Retinal Inner Layers; EZ =Ellipsoid Zone; ELM=External Limiting Membrane; CRT=Central Retinal Thickness; RPE=Retinal Pigment Epithelium

The predictive value of OCT morphological features to treatment response after the loading dose was analysed with univariate multinomial logistic regression. Then, multivariate multinomial logistic regression was carried out with the following parameters: EZ disruption area and DRIL area since these two variables showed statistically significant differences between good responders and poor responders in the univariate logistic analysis. Being the primary treatment outcomes, BCVA and CRT were also analysed (Table 5).

Table 5 - Univariate and Multivariate Multinomial Logistic Regression Analysis of baseline OCT features with influence on BCVA treatment outcome

	Univariate Analysis				**Multivariate Analysis**			
	Moderate Responders		**Poor Responders**		**Moderate Responders**		**Poor Responders**	
Variables	OR (95%CI)	*P*	OR (95% CI)	*P*	OR (95%CI)	*P*	OR (95% CI)	*P*
BCVA	1.03 (0.96-1.11)	0.421	0.97 (0.92-1.03)	0.399	1.09 (0.99-1.20)	0.076	1.03 (0.93-1.13)	0.589
CRT	1.00 (1.00-1.01)	0.230	1.00 (0.99-1.00)	0.526	1.00 (1.00-1.01)	0.512	0.98 (0.97-1.00)	**0.001**
DRIL area	1.03 (0.20-5.26)	0.970	**4.58 (1.18-17.79)**	**0.028**	0.88 (0.15-5.03)	0.886	**7.05 (1.16-42.89)**	**0.034**
EZ disruption area	1.93 (0.84-4.40)	0.120	**3.93 (1.72-8.94)**	**0.001**	2.72 (0.86-8.64)	0.090	**10.96 (2.94-40.8)**	**<0.001**
Cystoid Spaces Severity	1.50 (0.65-3.48)	0.336	1.32 (0.59-2.94)	0.501	-	-	-	-
NSD	1.94 (0.53-7.17)	0.318	2.31 (0.66-8.03)	0.189	-	-	-	-
Epiretinal Membrane	0.60 (0.15-2.39)	0.469	1.88 (0.57-6.12)	0.297	-	-	-	-

(-): Variables not included in the multivariate analysis.
OR = Odds Ratio; CI = Confidence Interval; *P* = p-value; BCVA = Best Corrected Visual Acuity; CRT = Central Retinal Thickness; DRIL = Disorganization of Retinal Inner Layers; EZ = Ellipsoid Zone; NSD = Neurosensorial Serous Detachment

EZ disruption area appears to have an important contribution to a poor functional outcome to anti-VEGF treatment, with an OR of 10.96 (CI: 2.94-40.8; *p*<0.001) for Poor Responders versus Good Responders, which means that the higher the EZ disruption area, the worst is expected to be the functional treatment recovery. DRIL area appears also to be a risk factor for BCVA response to treatment with an OR of 7.05 (CI: 1.16-42.89; p=0.034) for Poor Responders versus Good Responders. These results are similar at Month 6 (V6), with an OR of 7.86 (CI: 2.10-29.43; p=0.002) for EZ and an OR of 8.05 (CI:1.20-54.01; p=0.032) for DRIL.

A sub-analysis was performed excluding patients who received laser therapy 6 months before inclusion (32.8%) to evaluate the possible impact of this treatment in the present biomarkers. No significant changes were found and DRIL and EZ remained the major predictors of poor treatment response.

Discussion

Regular intravitreal treatment with ranibizumab in patients with DME decreases CRT and improves BCVA[7,18]. In the present study, we prospectively observed 67 patient eyes with naïve DME after initiating a loading dose of three monthly intravitreal ranibizumab injections followed by PRN treatment for up to 6 months. A mean CRT decrease of 107.93 µm (-25%) was obtained immediately after the loading dose (three monthly injections), with a significant recovery of BCVA (+6.78 letters; p<0.001). These results are in accordance to other clinical trials like RESTORE, RISE and RIDE[7,18], where significant CRT decreases and BCVA increases were achieved after the same regimen of ranibizumab therapy.

Although CRT is widely used to evaluate and follow eyes with DME, it has been shown to be only moderately correlated with BCVA outcomes[19]. The available clinical trials data have shown that only 50-60% of eyes treated with anti-VEGF for DME respond with complete retinal thickening resolution or have improvement of VA to 20/20 or better[20].

In our study, we analysed the potential role of CRT decrease after the first injection as a predictor for BCVA. CRT decrease does not reach statistical significance as a predictor for treatment response, but a threshold of CRT decrease of 8.7% immediately after the first

anti-VEGF injection (at 1 month) was able to distinguish 73.3% of patients that recovered more than 5 BCVA letters at 3 months, with a modest specificity. Larger sample size may, in the future, help to clarify this finding. It is noteworthy that other studies[21] have based their re-treatment criteria in a similar percentage of CRT decrease (10% between visits).

However, whereas some patients have an excellent visual outcome after treatment, some others maintain a substantial visual disability. Robust predictive biomarkers for treatment response in eyes with DME are still lacking despite the large number of studies and reports dedicated to this subject.

A number of studies[10,22,23] have suggested modest associations between OCT parameters, such as presence of intraretinal cysts, hyperreflective foci, subretinal fluid, disruption of ELM and photoreceptors layer (EZ), with BCVA in eyes with DME, but these correlations have not been strong enough to predict visual acuity reliably and most of the reported studies were done retrospectively in mixed treatment cohorts. Other studies[11,24] have identified DRIL on OCT as a parameter indicating highly associated with current and future vision in eyes with DME. These authors found that DRIL affecting 50% of the 1mm central retinal zone was the only parameter consistently associated with worse BCVA in eyes with current DME and resolved DME after treatment. They also found that increasing of DRIL in the course of the treatment was associated with reduction in BCVA. But again, data from these studies were obtained retrospectively, as part of routine clinical care rather than part of a research protocol and included eyes that underwent different DME treatments before and during the study follow-up and with different DME durations.

In this prospective study, we were able to confirm that presence and extent of DRIL before treatment is correlated with BCVA outcomes to anti-VEGF therapy after the loading dose and, most important, the presence of these morphological changes appears to be a good predictor of treatment response, representing a risk of almost 8 times higher for poor visual recovery than patients without DRIL. The mechanisms by which DRIL affect BCVA are yet to be determined, although it likely represent signs of anatomic interruption in the visual transmission pathway from the photoreceptors to the ganglion cells[11,25].

On the other hand, Maheshwary et al[17] found a statically significant correlation between the percentage of photoreceptors IS/OS disruption and visual acuity, which means that EZ disruption may be another significant predictor of VA in patients with DME. However,

patients that gained normal vision after treatment were excluded from the analysis, which can compromise the predictive value of this feature. In our study, a significantly higher percentage of EZ and ELM disruption was found at baseline in the Poor Responders group compared with Good Responders, and a moderate correlation was found between the presence of these features and the response to treatment. Our data have also showed that more EZ disruption at baseline represents a higher risk for a poor visual recovery (OR: 10.96; $p<0.001$), when comparing to the presence of DRIL (OR: 7.05; $p=0.001$).

Macular cystoid spaces have been proposed as another indicator of retinal damage. Raafay et al[10] found that their presence predicted a reduction in BCVA letter score and that the presence of large cystoid spaces seems to be more disruptive than small ones. Likewise, in our work, cystoid spaces severity appears also to be a predictor of poor response to anti-VEGF treatment.

Our study validates the importance of determination of morphological patterns on SD-OCT and shows that the integrity of both inner (DRIL) and outer retinal layers (EZ and ELM) can be good predictive biomarkers of future BCVA in patients with DME undergoing anti-VEGF therapy.

This study is considered particularly relevant since it was done prospectively, in treatment naïve patients that were submitted to the same regimen of ranibizumab treatment and followed-up with several examinations before, during and after therapy. Diabetes duration and DME duration were not significantly different between treatment response groups ($p=0.333$ and $p=0.444$, respectively) and most of the patients (70%) presented a DR severity ETDRS level of 35 at baseline which means that it is a population of relatively mild to moderate DR and, thus, ideal to study such detailed morphological retinal changes as DRIL, EZ and ELM disruption with accuracy. It also means that these changes are already present in early stages of DR and, therefore, could provide a significant contribution to counselling, management and treatment of diabetic macular edema.

It is noteworthy that in our study, previous laser treatment performed 6 months before inclusion did not show any significant influence in the characterization of the OCT nor any identifiable impact in treatment response to anti-VEGF treatment.

Study limitations include the fact that the study deals with a relatively small population, although chosen according to sample size calculation, and the chosen focus on the initial stage (loading dose) of intravitreal anti-VEGF, not allowing the evaluation of long-term effects of the anti-VEGF therapy. Larger and longer prospective studies are needed to evaluate these aspects. However, despite the focus on initial treatment response (after the loading dose), there were no significant changes in the predictive value of these biomarkers for the BCVA response at 6 months, even after PRN regimen, an observation which is in accordance with previously described studies[5,6,7].

In conclusion, SD OCT provides useful information for determining visual prognoses and outcomes in DME treatment. In naïve cases of DME, DRIL and specially EZ are confirmed as useful structural markers to evaluate retinal tissue integrity, and are closely associated with final BCVA after treatment.

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Chapter 4

Measurements of Retinal Fluid by Optical Coherence Tomography Leakage in Diabetic Macular Edema. A Biomarker of Visual Acuity Response to Treatment

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Abstract

Purpose: To evaluate the effects of anti-VEGF treatment on retinal fluid in patients with Diabetic Macular Edema (DME) by using optical coherence tomography leakage (OCT-L), a new method of quantifying sites of lower than normal optical reflectivity (LOR) in OCT, and to correlate these findings with best-corrected visual acuity (BCVA) response.

Methods: Prospective analysis of 21 eyes with DME, naïve to anti-VEGF treatment. Macular Cube 512x128 and OCT angiography 6x6mm scans (CIRRUS AngioPlex (Zeiss Meditec, Dublin, California, USA)) were acquired in all eyes before the first ranibizumab injection (V1) and 1 week after treatment (V2). OCT-L analysis was performed with Angioplex raw scan data used to calculate lower than normal optical reflectivity (LOR) maps and ratios. LOR ratios at baseline and differences from V1 to V2, and other OCT morphological features such as central retinal thickness (CRT) measurements, disorganization of the inner retinal layers (DRIL) and disruption of ellipsoid zone (EZ), were compared with BCVA response 1 month after the first intravitreal injection.

Results: After the first intravitreal injection of ranibizumab, 8 patients (38.1%) were identified as good responders, 5 (23.8%) as moderate and 8 (38.1) as poor. There were no significant BCVA differences at baseline (p=0.06). Significant differences were found in LOR ratios changes between the different treatment response groups after 1 week of treatment, especially in OS and OPL (OS - good responders: -53.2%, responders: -12.1% and poor responders: 6.5% (p=0.026); OPL - good responders: -48.8%, responders: 17.5% and poor responders: 5.1% (p=0.010)).

LOR ratios differences after 1 week of treatment predict better the BCVA treatment response at 1 month than changes of CRT, DRIL or EZ disruption, especially in the OS and OPL (AUC = 0.82 and 0.73, respectively).

Conclusions: OCT-Leakage changes after anti–VEGF treatment of diabetic macular edema, identifying the degree of decrease in retinal fluid in the outer layers of the retina is a more robust biomarker of BCVA recovery than CRT, DRIL, or EZ disruption changes.

Introduction

Diabetic Macular Edema (DME) is the major cause of visual acuity impairment in patients with Diabetic Retinopathy (DR) affecting up to 21 millions of people worldwide[1]. Breakdown of the Blood Retina Barrier (BRB) and consequent leakage of abnormal fluid into the retinal tissue[2] due to Vascular Endothelial Growth Factors (VEGF) proliferation are understood to be the major factor in the development of DME. Consequently, intravitreal (IVT) administration of anti-VEGF drugs is considered the most efficient therapy for DME resolution and visual acuity improvement[3–5]. This response to intravitreal injections of anti-VEGF for the treatment of DME has been shown to be generally determined quite early in the treatment process, immediately after the first injections[6].

However, not all DME patients show a good response to anti-VEGF agents[6] and the identification of factors that can be responsible for unresponsive or only partly responsive patients to anti-VEGF therapy remains an important goal.

Fluorescein angiography (FA) is the imaging technique most frequently used to document the changes occurring in the BRB in DR[7]. However, it is an invasive technique that is not without dangers and does not permit the precise visualization of the retina vasculature. Several studies have proposed different spectral domain optical coherence tomography (SD-OCT) morphological features that may be correlated with best-corrected visual acuity (BCVA) response to anti-VEGF therapy, such as retinal thickness (RT)[8], disorganization of the inner retinal layers (DRIL)[8,9] and ellipsoid zone (EZ) disruption[10].

In the present work, we tested the possibility of using a new non-invasive imaging technique, OCT-Leakage (OCT-L), in naïve patients with DME that have clinical indication for anti-VEGF treatment, to identify the location and to quantify the abnormal extracellular fluid accumulation before and after treatment. Our aim was to correlate the retinal fluid changes with BCVA outcome in the initial stages of anti-VEGF therapy response (after one IVT) to compare its value with other previously proposed biomarkers of anti-VEGF treatment response and to evaluate its predictive value as a prognostic biomarker in the management of DME treatment with anti-VEGF.

Methods

Study Design

A prospective, consecutive case series analysis was conducted in diabetic Type 2 patients receiving the same interventional treatment following clinical practice guidelines. The tenets of the Declaration of Helsinki were followed and approval from the ethics committee of the clinical site was obtained. Written informed consent was collected from all included patients. Patients were treated and followed according to the standard practice for DME treatment with ranibizumab intravitreal injections as described in the Summary of Product Characteristics (SmPC): loading dose of 3 monthly injections followed by *Pro Re Nata* (PRN regimen).

Participants

Naive patients with indication for treatment with ranibizumab injections for DME in the investigator's opinion and fulfilling the following inclusion criteria: (1) Type 2 diabetes mellitus; (2) central-involved DME, confirmed by OCT and defined as a baseline SD-OCT central subfield retinal thickness (CRT) ≥300µm[11,12]; (3) visual impairment due to DME with BCVA ≥ 20/320 and ≤ 20/40 ((≥25 letters and ≤73 letters) and (4) glycated haemoglobin (HbA_{1C}) ≤12% at screening visit. Exclusion criteria were as follows: (1) presence of Proliferative Diabetic Retinopathy (PDR); (2) previous laser photocoagulation (panretinal or focal) in the study eye within 6 months prior to study inclusion; (3) previous treatments with IVT injections of steroids or anti-VEGF drugs in the study eye; (4) previous vitrectomy surgery; (6) other chorioretinal diseases like central serous chorioretinopathy, high myopia, chorioretinitis or any other fundus disease associated with morphological or functional changes.

Study Procedures

All included patients performed an initial visit (V1) before their first IVT ranibizumab injection with the following procedures: clinical history (medical history, demographics and concomitant medications); biomicroscopy; best corrected visual acuity – BCVA (using

ETDRS method) and SD-OCT Angiography (HD-OCT Cirrus 5000 Angioplex, Zeiss Meditec, Dublin, California). Patients repeated the same procedures 1 week after the first injection (V2) and 1 month after the first injection (V3).

OCT Acquisition and Processing

OCT scans of 6x6 mm^2 were acquired in all patients using HD-OCT Cirrus 5000 Angioplex (Zeiss Meditec, Dublin, California, USA). Raw data from the AngioPlex system were exported and processed using the OCT-Leakage (OCT-L) software for the full A-scan and for each individual segmented retinal layer. The OCT-L software identifies sites of lower than normal optical reflectivity (LOR) and depicts them as 2-dimensional *en*-face images of the retina by assigning a simple representative value to each A-scan. These representative values register the existence of optical reflectivity values falling below a predefined threshold obtained from the analysis of A-scans gathered from a healthy control population[13]. The white areas depicted in the LOR maps represent the location of the A-scans having reflectivity values below the predefined threshold, and black areas are above the threshold.

Extracellular fluid distribution of given areas of the retina can be measured by LOR ratios, which represent the number of A-scans with optical reflectivity values below the threshold divided by the total number of A-scans within the considered area. To identify LOR sites in the different retinal layers, a segmentation algorithm was implemented to identify 8 retinal interfaces, namely, the vitreous to inner limiting membrane, retinal nerve fibre layer to ganglion cell layer (GCL), inner plexiform layer (IPL) to inner nuclear layer (INL), INL to outer plexiform layer (OPL), OPL to outer nuclear layer (ONL), inner segment (IS) to outer segment (OS), OS to RPE, and RPE to choroid[14.] All segmented examinations were reviewed by experienced graders. Maps of the LOR sites are obtained not only for the full retina, but also layer by layer as *en*-face images.

Macular Cube 512x128 scan was also acquired in all included eyes to obtain CRT, perifoveal and parafoveal retinal thicknesses. The presence and horizontal extent of DRIL and EZ disruption were also measured, according to previous works[9,15] using the equipment software calliper in 5 horizontal B-scans in the central 1mm, and these

measurements were averaged across the 5 scans to derive global DRIL and EZ areas for each eye at baseline.

OCT images and segmentation were graded by two independent graders. The observed agreement between the two graders was 93.6%. All disagreement cases were resolved by mutual agreement.

Data Analysis

Patients response to treatment was categorized according to their BCVA evolution from baseline to one month after the first injection (V3), and were stratified in 3 treatment response groups: good responders (≥8 ETDRS letters gained), moderate responders (≥5 & <8 ETDRS letters gained) or poor responders (<5 ETDRS letters gained or loss of visual acuity).

To test statistically significance differences at baseline between treatment response groups a univariate analysis was performed. The Fisher's exact test was used for categorical variables and the Kruskal-Wallis test was used for continuous variables.

Central retinal thickness, DRIL, EZ disruption and LOR ratios changes after 1 week of anti-VEGF treatment were compared between treatment response groups by univariate analysis performed with the Kruskal-Wallis test. These changes were calculated as the difference between V2 and V1 in relation to the baseline value (V1).

To analyse associations between variables, Spearman correlation coefficient and the respective statistical significance were computed. A receiver operating characteristic (ROC) analysis was performed to identify the best predictors for more than seven ETDRS letters improvement in BCVA (good responders).

All tests were two sided and significance was set at 0.05. Statistical analysis was performed with Stata 12.1 SE (StataCorp LP, College Station, TX).

Results

A total of 21 eyes of 18 consecutive patients were included in this analysis. Baseline characteristics of all eyes are presented in Table 1.

Table 1 - Baseline Characteristics of Patients

	Study population (n=21)	Good Responders (n=8)	Responders (n=5)	Poor Responders (n=8)	*P*
Demographics					
Age, mean±SD (years)	66.5±7.1	67.9±6.3	65.4±9.6	65.8±7.0	0.874
Females, n (%)	11 (52.4)	3 (27.3)	4 (36.4)	4 (36.4)	0.461
Disease characteristics					
BCVA, mean±SD (letters)	65.4±12.5	61.6±16.3	60.0±7.1	72.5±7.5	0.062
CRT, mean±SD (µm)	432.7±139.4	517.2±172.5	442.4±83.1	342.0±64.8	**0.026***
LOR ratio, mean±SD	0.59±0.23	0.73±0.23	0.64±0.12	0.42±0.16	**0.029***
DRIL, mean±SD (μm^2)	259.3±183.1	235.3±177.9	236.8±183.6	297.4±205.4	0.686
EZ, mean±SD (μm^2)	293.9±204.7	260.7±230.9	339.1±219.9	298.9±189.8	0.600

*Bold text indicates a statistically significant difference. Abbreviations: BCVA=Best Corrected Visual Acuity; SD=Standard Deviation; CRT= Central Retinal Thickness; LOR= Low Optical Reflectivity; DRIL=Disorganization of Retinal Inner Layers; EZ =Ellipsoid Zone.

According to BCVA changes from baseline to 1 month after the first injection, 8 patients (38%) were considered good responders (≥8 ETDRS letters gained), 5 patients (24%) were considered moderate responders (≥5 & <8 ETDRS letters gained), and 8 patients (38%) were considered poor responders (<5 ETDRS letters gained or loss of visual acuity). These different responses to treatment were independent of the BCVA at baseline, which was not statistically different between the three different response groups.

Significant differences at baseline were found between treatment response groups for the CRT and LOR ratios ($p<0.030$), with higher values registered at baseline in the good responders group.

Central Retinal Thickness decrease after 1 week of anti-VEGF treatment by BCVA treatment response groups

Considering the full retina thickness, a CRT decrease was observed after 1 week of treatment in all patients (-12%), with greater decreases of thickness in the good responders group (-17%) in comparison with moderate responders (-12%) and poor responders (-6%) groups, but without statistical significance between the three groups.

When analysing the CRT by segmented layers, the ONL+IS layer showed the larger thickness decreases. The good responders showed a decrease of -24%, moderate responders -6%, and poor responders -4%, but the differences did not reach statistical significance.

EZ disruption and DRIL areas decrease after 1 week of anti-VEGF treatment by BCVA treatment response groups

A decrease in EZ disruption associated with BCVA response was observed after 1 week of treatment. The good responders showed a decrease of - 41%, moderate responders - 17%, and poor responders - 7%, but these differences did not reach statistical significance.
The DRIL area after 1 week of anti-VEGF treatment was not associated with BCVA improvement. The DRIL showed an increase of 12% in the good responders group, whereas moderate responders decreased - 16% and poor responders increased 41%.

LOR ratios decrease after 1 week of anti-VEGF treatment BCVA treatment response

Considering the full retina after 1 week of treatment, larger LOR ratio decreases were observed in the good responders group (-21%) in comparison with moderate responders (-20%) and poor responders (+1%) groups.

When analysing the LOR ratios by segmented layers, the OS and OPL showed the larger LOR ratios decreases (-21% and -11%, respectively). Comparing the LOR decrease with the BCVA treatment response, a statistically significant association was achieved in OS (good responders: -49%, responders: 18% and poor responders: 5% (p=0.026)) and in OPL (good responders: -53%, responders: -12% and poor responders: 7% (p=0.010)). The LOR ratios decrease after 1 week of anti-VEGF treatment is represented in the OCT-L maps of a good responder patient before and after treatment (Figure 1).

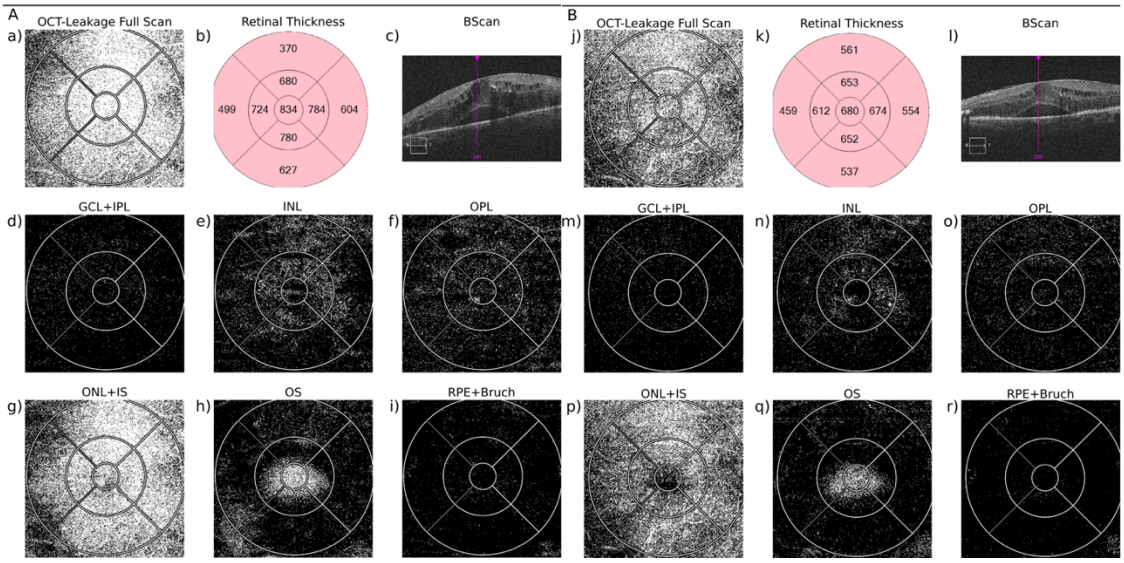

Figure 1: OCT-L maps of a good responder patient before treatment (A) and after treatment (B). Decrease of LOR is observed in all layers, especially in OPL, ONL+IS and OS.

Correlation between LOR ratios after 1 week of anti-VEGF treatment and BCVA after 1 month of anti-VEGF treatment

LOR ratios decreases after 1 week of anti-VEGF treatment showed a moderate to strong correlation with decreases in BCVA after 1 month of anti-VEGF treatment that was borderline significant for the full retina (r_s = - 0.42 [95% confidence interval: -0.72 to 0.01]; p=0.060) and achieved statistical significance in inner nuclear layer, OPL, and OS. By contrary, CRT decreases after 1 week of anti-VEGF treatment did not show a significant correlation with BCVA after 1 month of anti- VEGF treatment in any layer (Table 2).

Table 2 - Correlation between LOR ratios and CRT decrese after 1 week of anti-VEGF treatment with BCVA after 1 month of anti-VEGF treatment

Layer	Correlation Coefficient	95% CI
LOR Ratio		
INL	**r_s=-0.47; p=0.031***	(-0.75;-0.05)
OPL	**r_s=-0.59; p=0.048***	(-0.81;-0.21)
ONL+IS	r_s=-0.38; p=0.087	(-0.70;0.06)
OS	**r_s=-0.65; p=0.001***	(-0.85;-0.31)
RETINA	r_s=-0.42; p=0.060	(-0.72;0.02)
CRT		
INL	r_s=-0.11; p=0.644	(-0.52;0.34)
OPL	r_s=-0.19; p=0.409	(-0.58;0.26)
ONL+IS	r_s=-0.33; p=0.141	(-0.67;0.12)
OS	r_s=-0.07; p=0.755	(-0.49;0.37)
RETINA	r_s=-0.38; p=0.088	(-0.70;0.06)

*Bold text indicates a statistically significant correlation. CI, confidence interval; INL, inner nuclear layer; ONL + IS, outer nuclear layer + inner segments; P, P-value; rs, Spearman correlation coefficient.

The layers showing a stronger correlation between LOR ratio decreases and BCVA after anti-VEGF treatment were OPL and OS (r_S= -0.47, p=0.031; r_S= -0.59, p =0.005; and r_S= -0.65, p=0.001; respectively) (Figure 2).

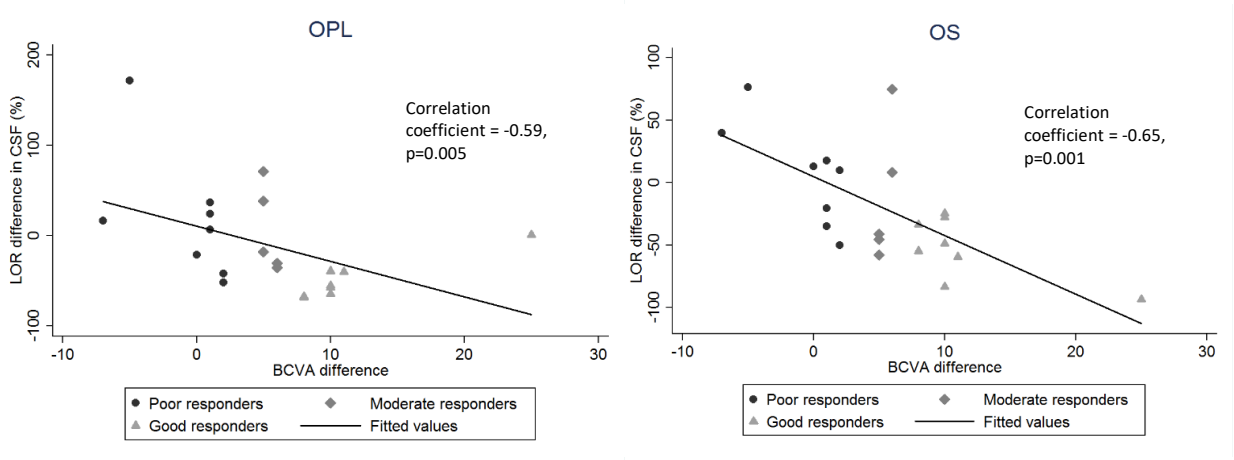

Figure 2: Correlation between differences in the LOR ratios in the CSF and BCVA after 1 month of anti-VEGF treatment.

Correlation between LOR ratios and CRT decrease after 1 week of anti-VEGF treatment

LOR ratios decreases showed a moderate to strong correlation with decreases in CRT achieving statistically significance only in OPL, ONL+IS and for the full retina (Table 3).

Table 3 - Correlation between LOR ratios and retinal thickness decrease in CSF after 1 week of anti-VEGF treatment

Layer	Correlation Coefficient	95% CI
INL	r_s=0.29; p=0.199	(-0.16;0.64)
OPL	**r_s=0.46; p=0.037***	(0.03;0.74)
ONL+IS	**r_s=0.75; p<0.001***	(0.48;0.90)
OS	r_s=0.31; p=0.179	(-0.15;0.65)
RETINA	**r_s=0.57; p=0.008***	(0.18;0.80)

*Bold text indicates a statistically significant correlation. Abbreviations: INL= Inner Nuclear Layer; OPL=Outer Plexiform Layer; ONL+IS=Outer Nuclear Layer + Inner Segment; OS=Outer Segment; r_s=Spearman Correlation Coefficient; p=*P*-value.

Comparison between the different candidates after 1 week of anti-VEGF treatment for predicting BCVA response after 1 month of anti-VEGF treatment

LOR ratios are the best predictor of good BCVA response in comparison with CRT, DRIL and EZ disruption to anti-VEGF treatment (more than 7 ETDRS letters improvement in BCVA).

Analysing the LOR ratios by segmented layers, the presence of fluid in OPL presents the best discriminating ability for the treatment response (ROC AUC=0.90, sensitivity 85%, specificity 80%), with OS showing almost similar predictive capabilities (ROC AUC=0.83, sensitivity 62%, specificity 80%).

EZ disruption presented a reasonable discriminating ability for the treatment response (ROC AUC=0.65), although with low sensitivity, 23%, at 80% specificity.

CRT and DRIL failed to show similar ability to predict BCVA recovery after anti-VEGF treatment. In fact, confidence intervals suggests that CRT and DRIL change after 1 week of anti-VEGF treatment is no better than a coin toss in predicting BCVA response (Table 4 and Figure 3).

Table 4- Area under the curve and sensitivity and specificity for the LOR ratio, RT and for the DRIL area and EZ and ELM disruption areas

	ROC AUC	Sensitivity at 80% Specificity
	LOR ratio	
INL	0.68 (CI 95%: 0.41-0.95)	39%
OPL	0.90 (CI 95%: 0.77-1)	85%
ONL+IS	0.65 (CI 95%: 0.40-0.91)	23%
OS	0.83 (CI 95%: 0.65-1)	62%
	CRT	
INL	0.47 (CI 95%: 0.20-0.75)	8%
OPL	0.70 (CI 95%: 0.43-0.98)	23%
ONL+IS	0.72 (CI 95%: 0.49-0.96)	38%
OS	0.42 (CI 95%: 0.15-0.69)	15%
	DRIL and EZ disruption	
DRIL area	0.41 (CI 95%: 0.32-0.50)	15%
EZ disruption area	0.65 (CI 95%: 0.55-0.75)	23%

Abbreviations: AUC=Area Under the Curve; CI=Confidence Interval; INL= Inner Nuclear Layer; OPL=Outer Plexiform Layer; ONL+IS=Outer Nuclear Layer + Inner Segment; OS=Outer Segment; DRIL=Disorganization of Retinal Inner Layers; EZ =Ellipsoid Zone.

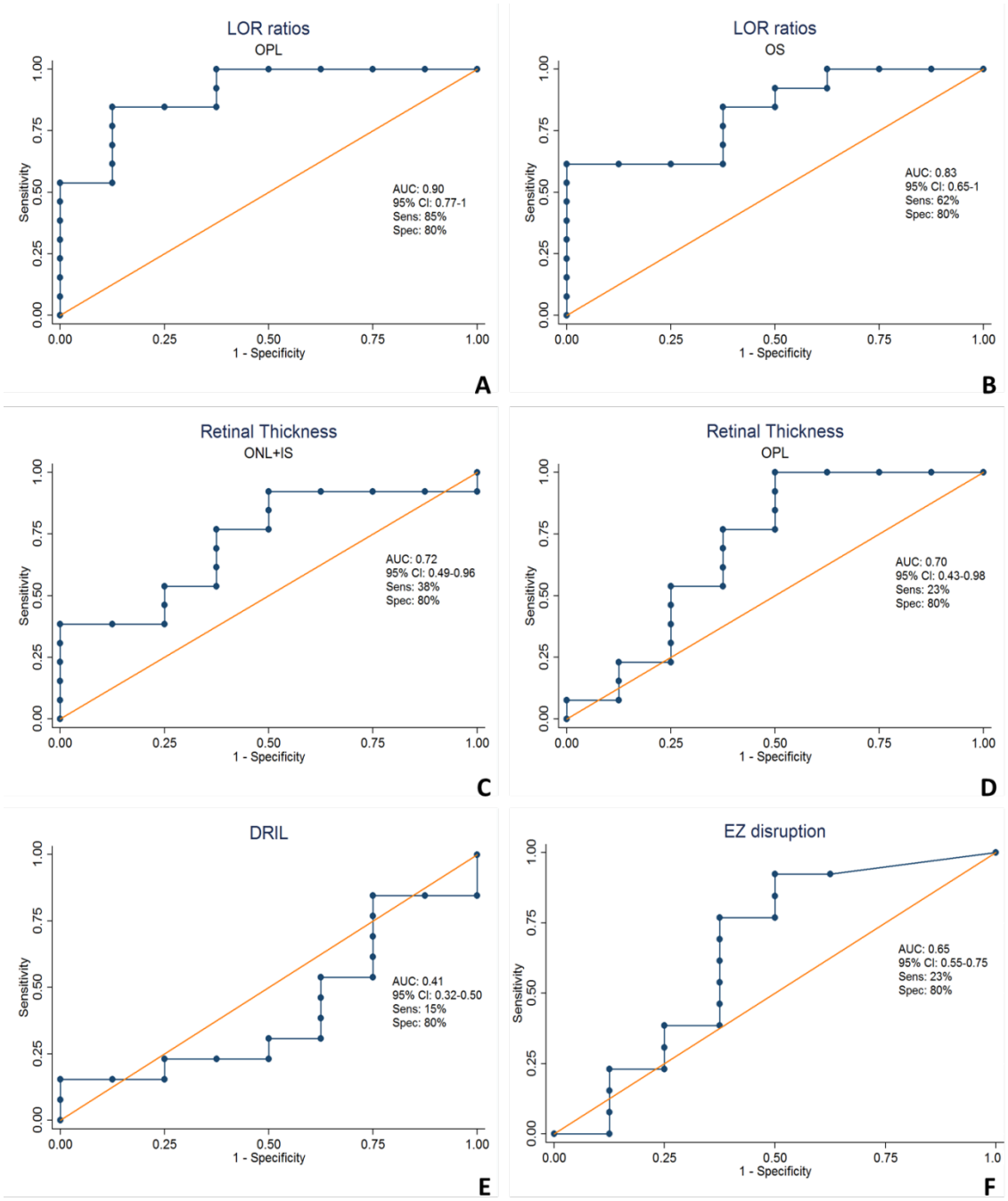

Figure 3. ROC analysis for LOR ratios (a and b) and Retinal Thickness in the CSF (c and d), DRIL (e) and EZ disruption (f) changes after 1 week of anti-VEGF treatment as predictors for BCVA response after 1 month of anti-VEGF treatment.

Discussion

Intravitreal administration of anti-VEGFs in DME has been widely demonstrated to result in improvement and consequent increase in BCVA. However, not every eye responds in the same way. Furthermore, in some cases, the resolution of DME is not followed by recovery of visual function[16,17]. Massin et al[18] and Mitchell et al[5] refer that after 12 months of treatment with ranibizumab in monotherapy, 40 to 60% of the subjects had a BCVA gain ≥ 10 letters (good responders), 30% had a BCVA gain between 5-10 letters (responders) and 10-30% had a BCVA gain < 5 letters or a decrease in BCVA (poor responders). These studies also showed that the improvement in BCVA from Month 3 to Month 12 is lower than 4 letters indicating that the initial response to intravitreal injections of anti-VEGF determines the final visual recovery.

It is, therefore, of major relevance to characterize the features that are present at baseline and/or immediately after the initial IVT injections of anti-VEGFs that may identify the different visual outcomes observed in different eyes after initiating treatment and if any of these features can predict good or poor response to treatment.

A number of studies have reported variable results but reduction in retinal thickness[8], disorganization of the inner retinal layers (DRIL)[9] and disruption of the ellipsoid zone (EZ)[19] have been proposed as prognostic biomarkers of visual acuity response after anti-VEGF treatment of DME.

DME represents, mainly, increased accumulation of fluid in the retina[20,21]. Therefore, the availability of OCT-Leakage, a new OCT-based method to identify and quantify abnormal retinal fluid offers the possibility of examining eyes with DME before and after anti-VEGF treatment.

In this study we examined prospectively using OCT-Leakage, a series of eyes with naïve DME looking for correlations with BCVA response to anti-VEGF treatment and comparing its efficacy with other previously proposed prognostic biomarkers of BCVA treatment response.

We have shown recently in a relatively large prospective study that degree of CRT decrease, DRIL and EZ disruption were acceptable predictors of BCVA response to anti-

VEGF therapy[8]. In this report, LOR ratios appear to be a better indicator of BCVA response after treatment than CRT changes.

The OCT-Leakage results reported here show that the location of abnormal retinal fluid in DME and the degree of its elimination after anti-VEGF treatment are more robust biomarkers of BCVA response to treatment than degree of CRT reduction or degree of DRIL or EZ disruption.

Our results suggest that the BCVA response may be dependent on the degree of decrease in abnormal retinal fluid present in the outer layers of the retina, particularly in the OPL and OS layers, as a result of treatment. It is as if the visual acuity improvement depended on the degree of decrease in the retinal fluid around the photoreceptors. The administration of anti-VEGF stabilizes the blood–retina barrier breakdown stopping the abnormal entry of fluid, thus allowing the retinal pigment epithelium to pump out the remaining abnormal retinal fluid[21]. This is a relatively simplistic view but it opens promising perspectives. The major goal of treatment in DME is to achieve good visual acuity recovery, and this is apparently related to rapid decrease in abnormal retinal fluid in the outer retina retina[19]. Macular edema is directly associated with an abnormal accumulation of fluid in the retina[21]. The photoreceptors are the power centre for good visual acuity and it is to be expected that poor visual acuity is linked with abnormal permanence of large accumulation of fluid around them. This situation can be compared with flooding of the machine room in a steam boat resulting in rapid sinking of the boat. This may occur through to inactivation of the NA^{+}/Ca^{2+}, K^{+} exchanger[22]. Efficient drying of the outer retinal layers and the photoreceptors environment may be the determining factor for visual acuity recovery in the treatment of DME[19].

This study has several limitations that must be considered, the more important being the small number of eyes studied and its focus in the initial response to one anti-VEGF intravitreal injection. It must be also taken into account that the group of good responders to the treatment had worse vision and increased thickness at baseline, factors that have been associated with larger improvements of BCVA response[6]. It has, however, the advantage of being prospective including only eyes naive to previous treatments, laser, or IVT injections.

In conclusion, we reveal that the degree of retinal fluid elimination and drying of the retina, particularly in the OPL and OS retina layers, resulting from intravitreal administration of an anti-VEGF drug, and the degree of its decrease appear to determine the degree of BCVA recovery when treating DME with intravitreal anti-VEGF injections.

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Chapter 5

Swept Source Optical Coherence Tomography Choroidal Indices as Predictors of Visual Outcomes to anti-VEGF Treatment in DME patients

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Abstract

Purpose: To evaluate changes in choroidal vasculature features with anti-angiogenic therapy in patients with diabetic macular edema (DME), and their association with visual outcomes, using swept source optical coherence tomography (SS-OCT).

Methods: Prospective, longitudinal study, including consecutive patients with treatment-naive DME. All patients received monthly intravitreal injections of ranibizumab for 3 months (loading dose), followed by a treat-and-extend regimen for a total of 12 months. For all participants, best corrected visual acuity (BCVA) (ETDRS) and 3D horizontal volume macular SS-OCT scans were obtained before the first injection (M0), 1 month after the loading dose (M3), and at 6 (M6) and 12 months (M12). Central Choroidal Thickness (CCT) was obtained using automated software, as the mean value in the central 1mm of the ETDRS grid. *En-face* SS-OCT images of the choroidal vasculature were binarized to calculate choroidal vessel density (CVD) and volume (CVV). CVD was defined as the percent area occupied by choroidal vessels in the entire posterior pole (12x 9mm area) and in the central macular region (6-mm diameter circle centered on the fovea). CVV was also was calculated in the same area by multiplying the average CVD by the macular area and choroidal thickness. Treatment visual outcome was defined as BCVA improvement after the loading dose (M3), and was categorized into two groups: good responders (≥ 5 letters) and poor responders (< 5 letters).

Results: Twenty-three eyes with naïve DME (n=23 patients) were included, mean age 66.2±5.3 years, 30% (n=7) females. After receiving the loading dose of ranibizumab (M3), 17 eyes (74%) were considered good responders and 6 eyes (26%) were considered poor responders. At baseline, good responders showed a thicker choroid compared with poor responders (199.7 ± 79.6µm *vs* 182.5 ± 60.4µm; p=0.134). A significantly higher macular CVD (0.26±0.06 *vs* 0.21±0.03; p=0.026), as well as higher CVV (1.73 ± 0.95 *vs* 1.28 ± 0.48; p=0.151) were also observed in good responders.

After treatment, two distinct behaviors were observed: a significant decrease of CCT in good responders (- 11%, p=0.014), and an increase in poor responders that did not reach statistical significance (+ 9%; p=0.576). CVD and CVV showed analogous changes with statistically significant reductions in good responders (CVV= - 14%; p=0.008) and increases

in poor responders (CVD= + 16%; p=0.006 and CVV= + 34%; p=0.134). CVD at baseline was a good predictor of good response to anti-angiogenic treatment (ROC AUC=0.74; p=0.030).

Conclusions: Baseline choroidal indices, such as CVD and CVV, discriminate good and poor responders to anti-angiogenic therapy in DME patients, and may represent good predictors of treatment response. These results highlight the potential of SS-OCT for contributing to the management of DME.

Introduction

Diabetic retinopathy (DR) is a major cause of blindness in working-age populations[1], and diabetic macular edema (DME) is one of the main causes of visual impairment in this disease. The pathogenesis of DR and DME is primarily attributed to a hyperpermeability of the retinal vasculature, and a deregulation and breakdown of the blood retinal barrier[2]. However, choroidal dysfunction has also been implicated[3]. Choroidal changes include loss of choriocapillaris, dilation of vessels and increased tortuosity, among other abnormalities[4,5]. Moreover, the choroid may represent a pro-inflammatory environment in DR and it is possible that the choroidal vasculature may modulate and determine the absorption rate of intraretinal fluid, thus having an important impact in the development of DME[6].

Several studies[7–14] have also shown that DR is associated with choroidal thickness changes. Most authors[8,10–12] reported that patients with diabetes present a thinner choroid compared to controls, particularly in the advanced stages of the disease. Despite our knowledge of choroidal changes in DR, few studies have assessed the effects of anti-angiogenic therapy on choroidal vascular parameters[12,13,15–17]. Anti-angiogenics are currently the gold standard treatment for DME and proliferative diabetic retinopathy (PDR)[18–21]. Additionally, most of the studies performed so far were retrospective and included patients that received different anti-angiogenic agents[13,15,17], and were performed using the enhanced depth imaging (EDI) modality of the spectral domain OCT (SD-OCT), which has several limitations. Namely, unreliable detection of the choroidal-scleral boundary due to the relatively short wavelength of SD-OCT, and the lack of

automated segmentation software for choroidal assessment, leading to greater variability in measurements of choroidal thickness[13].

Swept-source OCT (SS-OCT) has important advantages over the EDI technique. These include its wavelength-tunable laser of 1050nm, thus improving penetration and enabling lower scattering at the retinal pigment epithelium (RPE). Additionally, the Atlantis/Topcon DRI SS-OCT (Topcon, Tokyo, Japan) enables a dense scanning of the eye fundus at a higher image speed allowing a three-dimensional image reconstruction, and it provides automatic segmentation of the choroidal-scleral boundary, generating automatic thickness maps and high-resolution *en-face* images[14]. *En-face* images were recently used by our group to explore other choroidal features that may play a role in DR pathogenesis[22], namely, central choroidal vascular density (CVD) and choroidal vascular volume (CVV), which may add information about the vascular integrity of the choroid in patients with different stages of DR[22].

This study aimed to evaluate changes in choroidal vasculature parameters with anti-angiogenic therapy over a period of 12 months in patients with DME, and their association with visual outcomes, exploring the features of SS-OCT.

Methods

Study design

This was a prospectively designed, longitudinal, observational study conducted in the clinical unit of the Association for Innovation and Biomedical Research on Light and Image (AIBILI), Coimbra, Portugal, in collaboration with the Ophthalmology Department, Centro Hospitalar e Universitário de Coimbra (CHUC), Coimbra, Portugal, and the Department of Ophthalmology of the Massachusetts Eye and Ear (MEE), Harvard Medical School, Boston, United States. The study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional review board and ethics committee of AIBILI, Coimbra, Portugal. Written informed consent was obtained from all included participants. The Portuguese National Committee of Data Protection (CNPD) also approved this study.

Study Participants

We identified consecutive patients with type 2 diabetes and naive DME who were coming to their regular visits and had indication to initiate treatment with ranibizumab intravitreal injections, according to their ophthalmologist. If deemed appropriate, the ophthalmologist referred them as potential participants to this study. A study investigator assessed the eligibility criteria, and those that fulfilled them were considered. Inclusion criteria: (1) Type 2 diabetes mellitus; (2) center-involved DME, confirmed by OCT and defined as a baseline SS-OCT (Topcon® DRI OCT-1 Atlantis) central subfield retinal thickness ≥300µm[23]; (3) visual impairment due to DME with BCVA ≥ 20/160 and ≤ 20/40 (≥39 letters and ≤73 letters); (4) glycated hemoglobin (HbA1C) ≤12%. Exclusion criteria: (1) presence of proliferative diabetic retinopathy (PDR); (2) previous laser photocoagulation (panretinal or focal) in the study eye within 6 months prior to study inclusion; (3) previous treatments with injections of triamcinolone or any anti-VEGF drugs in the study eye; (4) Prior vitrectomy surgery; (6) other chorioretinal diseases (including central serous chorioretinopathy, high myopia, chorioretinitis or any other fundus disease associated with morphological or functional changes); (7) systemic diseases that might affect CT, such as uncontrolled hypertension, systemic lupus erythematosus, anemia, leukemia and obstructive sleep apnea; and decreased media transparency that precluded appropriate OCT imaging. Only one eye was selected for the study. If both eyes were eligible, the eye with worse visual acuity was selected as the study eye.

For patients fulfilling all eligibility criteria, the study investigator explained the character and duration of the study, namely that it was an observational study with a duration of 12 months, it included 4 visits, and followed the standard of care treatment for DME condition (i.e. anti-angiogenic agents). For those who agreed to participate, written informed consent was obtained.

Study Procedures

All patients were treated and followed according to the standard practice for DME treatment with ranibizumab intravitreal injections as described in the summary of product characteristics (SmPC): loading dose of 3 monthly injections followed by a treat-and-extent regimen. For the purposes of this study, all included patients agreed to be followed for 12

months and attend 4 clinic visits during that period. The baseline visit (M0) included: medical history; demographics; vital signs, HbA1c assessment; biomicroscopy; intraocular pressure with Goldmann tonometry; ophthalmoscopy; best corrected visual acuity (BCVA) (ETDRS scale); color fundus photography (CFP) and fluorescein angiography (7 ETDRS fields, Topcon® TRC- 50DX, Topcon Medical Systems, Tokyo, Japan); and a 3D horizontal volume macular scan (12 x 9mm, 512 x 256 resolution) on SS-OCT (Topcon® DRI OCT-1 Atlantis, Topcon Medical Systems, Tokyo, Japan). After M0, all patients received the loading dose of 3 monthly injections. One month after the loading dose period, a second study visit (M3) was performed including assessment of BCVA and SS-OCT. These procedures were also repeated 6 months (M6) and 12 months (M12). During this 12 month period, patients were evaluated with BCVA and SD-OCT every month and received further injections following a treat-and-extend regimen,[24] i.e. treatment was extended by 1 month if BCVA stability was achieved and/or central retinal thickness was <300 µm. The maximal length of an inter-treatment interval was confined at 3 months.

SS-OCT data and grading

Retinal thickness (RT) and choroidal thickness (CT) were obtained with the automatic built-in software of the SS-OCT device (Topcon® FastMap, version 9.30.003.02). User-independent thickness maps were created according to the conventional ETDRS grid (comprised of a circular grid of 6 mm of diameter with one central field of 1mm, 4 quadrants between 1mm and 3mm and 4 quadrants between 3mm and 6mm). For all subjects, an experienced investigator (ARS, SNS or MCL) confirmed the position of the grid as well as the retinal and choroidal segmentations for all the obtained volume scans. Manual corrections were performed if the automated position of fovea or segmentation of the layers were not accurate, as described by Laíns et al[14]. Finally, the obtained RT and CT values in the nine different fields of the ETDRS grid were registered.

Additionally, *en-face* images of the choroidal vasculature were obtained to assess choroidal vascular density (CVD) and choroidal vascular volume (CVV). These were obtained *by* flattening using the Bruch's membrane (BM) as a reference, using the *en-face* tool included in the DRI OCT visualization software. The *en-face* images were exported every 2.6 µm from the BM to the choroidal-scleral interface (CSI) and subsequently

imported to ImageJ® (National Institutes of Health, Bethesda, Maryland, USA) as an image stack. The image stack was converted to binary images in order to distinguish the choroidal vasculature from the choroidal stroma. The image analysis procedure is described by Wang et al[22] and represented in figure 1. Images with substantial motion artifacts were excluded from analysis (n=1).

The average CVD was calculated as the average of the choroidal vascular densities of all image slices between Bruch's membrane and corresponding maximal CT. It was calculated throughout the posterior pole (12 x 9 mm) and in the central macular region (6 mm diameter circle centered on the fovea) to access both overall and macular CVD, respectively. The choroidal vascular volume (CVV) was calculated in the central macular region by multiplying the average CVD by the macular area and average CT (Figure 1).

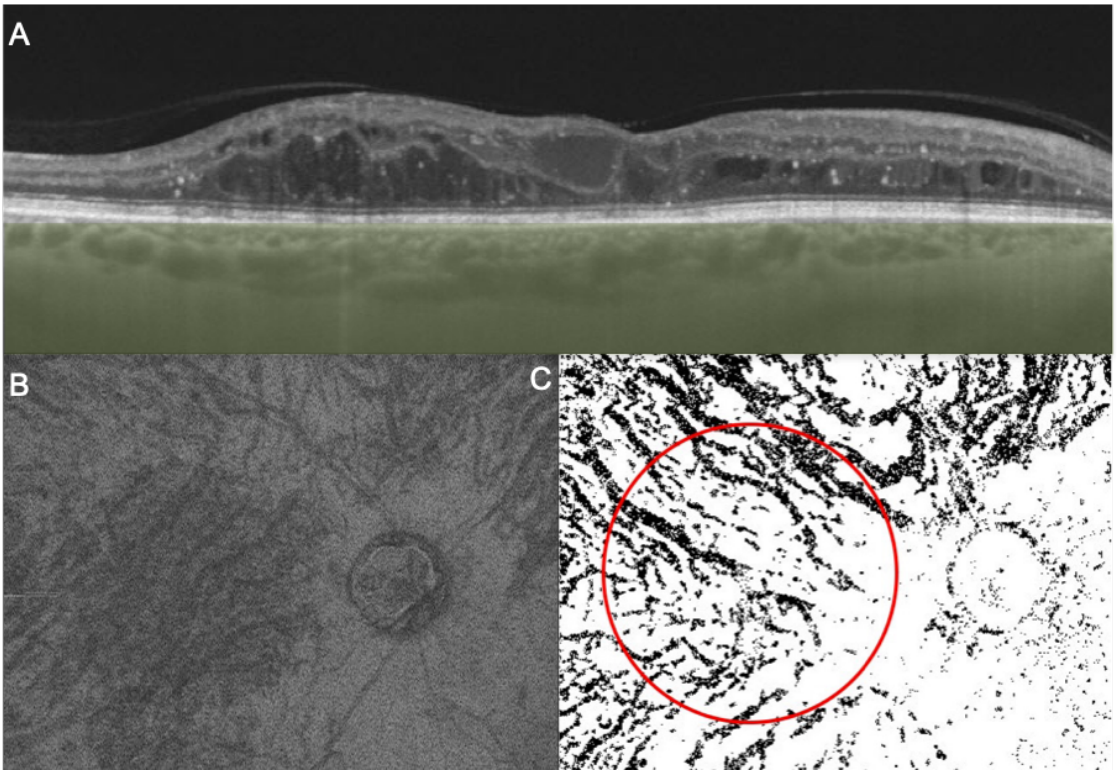


Figure 1: Representative example of swept source optical coherence tomography (SS-OCT) b-scan, *en*-face image and image processing.

(A) SS-OCT B-scan with choroid interface highlighted in the green area

(B) *En*-face SS-OCT image 70 µm below Bruch's membrane. The optic disc and retinal vessels are visible in addition to the choroidal vasculature.

(C) The same image after binarization and after removing optic nerve and retinal blood vessels. The red circle is the 6 mm diameter area centered on the fovea used for macular analysis of the choroidal parameters.

Data and Statistical analysis

The study population demographics, clinical and baseline structural features (CRT, CCT, CVD and CVV) were summarized with traditional descriptive methods. After the first 3 monthly injections (M3), patients were subdivided in two groups according to their BCVA changes from M0 to M3: good responders were considered those that had ≥ 5 ETDRS letters gained, and poor responders those with <5 ETDRS letters gained or with loss of vision. This stratification was chosen based on the definition of optimal and suboptimal response to anti-VEGF from Protocol I,[25] and was performed at M3 for two reasons: (i) this was the study point at which all patients had received the same treatment (i.e. three ranibizumab intravitreal injections), and (ii) because a strong association has been described[25,26] between response to the loading dose and long term visual recovery (at 1-3 years).

Comparisons between treatment response groups at each study visit (M0, M3, M6 and M12), were performed using the non-parametric Mann-Whitney U test, after checking for normality with the Shapiro-Wilk test, and Fisher exact test.

To study the effects of anti-VEGF therapy in CCT, CVD and CVV along time, e.g. changes of these features between M0 and each of the following visits (M3, M6, M12), the non-parametric Wilcoxon test was used, considering all patients and each treatment response groups.

A multivariate robust regression was performed to analyze associations between the study variables (CCT, CVD and CVV) at baseline, and possible confounders,[27] e.g. age, sex, DM duration, DME duration, HbA1c and BCVA. The number of injections performed during the study, was also considered in the model when looking for associations between changes in these with time and the confounders.

Because the choroidal vasculature is a very dynamic and changeable structure, it is known that CCT has high variability between patients.[28–31] To compare the inter-subject variability of the study variables (CRT, CCT, CVD and CVV) the coefficient of variation for each of them was calculated.

Finally, to assess the predictive value of CCT, CVD and CVV for the visual outcome after anti-VEGF treatment, a receiver operating characteristic analysis (ROC) was performed to

identify which of the above features would be the best discriminator at baseline for an improvement of at least five ETDRS letters in BCVA (good responders) after treatment.

All performed tests were two sided, and significance was set at 0.05. Statistical analyses were performed with Stata 12.1 SE (StataCorp LP, College Station, TX).

Results

Subject's demographic and clinical characteristics

A total of 26 eyes of 26 patients with naïve DME were recruited for this study; 3 patients were excluded due to loss of follow-up (Figure 2) and thus 23 were considered for analysis. From now on, all the described results refer to these patients. As shown, 17 eyes (74%) were considered good responders (≥5 ETDRS letters gained) and 6 eyes (26%) poor responders (<5 ETDRS letters gained or loss of visual acuity).

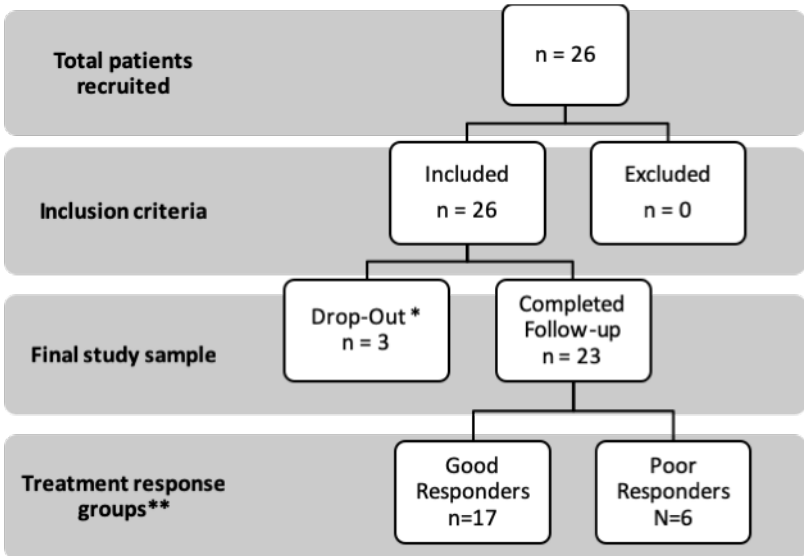


Figure 2: Flow chart of the study. * Exclusion of 3 patients due to loss of follow-up during the 12 months of the study **Treatment response groups according to BCVA response after loading dose (M3) - good responders (≥5 ETDRS letters gained) and poor responders (<5 ETDRS letters gained or loss of visual acuity).

The demographic and baseline clinical characteristics of the included patients are presented in Table 1.

Table 1: Baseline characteristics of study groups

	Study Population (n=23)	Poor Responders (n=6)	Good Responders (n=17)	p-value
Age, years (mean ± SD)	66.2 ± 5.3	69.7 ± 1.6	65.0 ± 5.6	0.005
Female sex, n (%)	7 (30%)	4 (67%)	3 (18%)	0.045
DM duration, years (mean ± SD)	17.8 ± 8.6	22.8 ± 9.0	16.0 ± 8.0	0.145
DME duration, years (mean ± SD)	4.1 ± 7.9	5.7 ± 6.4	3.6 ± 8.5	0.543
HbA1c (mean ± SD)	7.8 ± 1.3	7.3 ± 1.2	8.0 ± 1.3	0.229
BCVA, letters (mean ± SD)	65.3 ± 9.5	64.7 ± 11.5	65.5 ± 9.1	0.873
CRT, µm (mean ± SD)	397.7 ± 119.5	372.1 ± 84.8	406.7 ± 130.6	0.474
CCT, µm (mean ± SD)	195.2 ± 74.2	182.5 ± 60.4	199.7 ± 79.6	0.595
Overall CVD (mean ± SD)	0.20 ± 0.02	0.19 ± 0.01	0.20 ± 0.03	0.072
Macular CVD (mean ± SD)	0.25 ± 0.06	0.21 ± 0.03	0.26 ± 0.06	0.026
Macular CVV (mean ± SD)	1.61 ± 0.9	1.28 ± 0.48	1.73 ± 0.95	0.151

Legend: DM = diabetes mellitus, DME = diabetic macular edema, HbA1c = Hemoglobin A1C, BCVA = best corrected visual acuity, CRT = central retinal thickness, CCT = central choroidal thickness, CVD = choroidal vascular density, CVV = choroidal vascular volume, Significant p-values (p <0.05) are highlighted as bold.

Baseline (M0) Choroidal Vasculature Parameters

As shown in Table 1, CCT was not significantly different between groups (p=0.474). However, despite not reaching statistical significance, good responders showed a thicker choroid than poor responders (199.7 ± 79.6µm *vs* 182.5 ± 60.4µm, respectively, p=0.449). Regarding choroidal vascular indexes, macular CVD was significantly higher in good responders compared with poor responders (0.26 ± 0.06 vs 0.21±0.03; p= 0.026). Despite not reaching statistical significance, CVV was also superior in good responders (1.73 ± 0.95 vs 1.28 ± 0.48 in poor responders; p=0.151). None of these choroidal vascular parameters (CCT, CVD and CVV) were associated with any possible confounders (age, sex, DM or DME duration, HbA1c or BCVA) (p=0.137).

Choroidal vascular parameters after anti-angiogenic treatment

Table 2 and Figure 3 present the evolution of choroidal vascular parameters after anti-angiogenic treatment, for the entire study population and also separately for good and poor responders. As shown, for the entire population, after three monthly ranibizumab injections (M3), there was a decrease in CCT (p=0.002), which remained significant at 6 months (M6) (p=0.016) and 12 months (M12) (p=0.011) (Figure 3).

Table 2 - Results after anti-VEGF treatment in all DME patients and by treatment response groups.

	Baseline results				3 Months results				6 Months results				12 Months results			
	Total (n=23)	Poor Responders (n=6)	Good Responders (n=17)	p-value	Total (n=23)	Poor Responders (n=6)	Good Responders (n=17)	p-value	Total (n=23)	Poor Responders (n=6)	Good Responders (n=17)	p-value	Total (n=23)	Poor Responders (n=6)	Good Responders (n=17)	p-value
Age, years (mean ± SD)	66.2 ± 5.3	69.7 ± 1.6	65.0 ± 5.6	**0.005**		–	–	–		–	–	–		–	–	–
Female sex, n (%)	7 (20%)	4 (57%)	3 (43%)	–		–	–	–		–	–	–		–	–	–
DM duration, years (mean±SD)	17.8 ± 8.6	22.8 ± 9.0	16.0 ± 8.0	0.145		–	–	–		–	–	–		–	–	–
DME duration, years (mean±SD)	4.1 ± 7.9	5.7 ± 6.4	3.6 ± 8.5	0.543				–		–	–	–		–	–	–
HbA1c (mean ± SD)	7.8 ± 1.3	7.3 ± 1.2	8.0 ± 1.3	0.229	7.8 ± 1.7	7.4 ± 1.4	8.0 ± 1.8	0.459	8.1 ± 1.3	7.2 ± 1.2	8.4 ± 1.3	0.087	7.8 ± 1.4	7.4 ± 1.2	8.0 ± 1.5	0.335
BCVA, letters (mean ± SD)	65.3 ± 9.5	64.7 ± 11.5	65.5 ± 9.1	0.873	72.7 ± 10.8	62.5 ± 14.4	76.2 ± 6.6	0.067	72.3 ± 11.3	64.2 ± 14.3	75.2 ± 8.9	0.124	70.5 ± 13.0	62.3 ± 14.9	73.4 ± 11.4	0.072
N° of IVT after LD (mean ± SD)	–	–	–	–	–	–	–	–	1.48 ± 1.93	1.33 ± 1.97	1.53 ± 1.97	0.836	0.78 ± 0.99	0.50 ± 0.55	0.88 ± 1.11	0.433
CRT, µm (mean ± SD)	397.7 ± 119.5	372.1 ± 84.8	406.7 ± 130.6	0.474	289.6 ± 65.4	290.4 ± 43.1	289.3 ± 72.8	0.964	328.0 ± 107.0	302.8 ± 67.1	336.9 ± 118.3	0.403	332.2 ± 115.1	318.1 ± 95.9	337.1 ± 123.4	0.737
CCT, µm (mean ± SD)	195.2 ± 74.2	182.5 ± 60.4	199.7 ± 79.6	0.595	181.7 ± 69.8	193.4 ± 61.9	177.6 ± 73.8	0.619	184.5 ± 66.4	191.8 ± 47.5	182.0 ± 73.0	0.715	182.3 ±746.4	188.1 ± 56.3	180.3 ± 81.3	0.800
Overall CVD, (mean ± SD)	0.20 ± 0.02	0.19 ± 0.01	0.20 ± 0.03	0.072	0.20 ± 0.03	0.20 ± 0.02	0.20 ± 0.03	0.758	0.20 ± 0.03	0.21 ± 0.02	0.20 ± 0.03	0.615	0.21 ± 0.02	0.20 ± 0.03	0.21 ± 0.02	0.810
Macular CVD, (mean ± SD)	0.25 ± 0.06	0.21 ± 0.03	0.26 ± 0.06	**0.026**	0.26 ± 0.05	0.25 ± 0.03	0.26 ± 0.06	0.467	0.26 ± 0.05	0.26 ± 0.01	0.26 ± 0.05	0.712	0.26 ± 0.05	0.25 ± 0.03	0.27 ± 0.05	0.253
Macular CVV, (mean ± SD)	1.61 ± 0.9	1.28 ± 0.48	1.73 ± 0.95	0.151	1.51 ± 0.74	1.57 ± 0.44	1.49 ± 0.83	0.759	1.57 ± 0.78	1.60 ± 0.55	1.56 ± 0.86	0.884	1.59 ± 0.81	1.52 ± 0.71	1.62 ± 0.86	0.785
N° of IVT after LD (mean ± SD)	–	–	–	–	–	–	–	–	1.48 ± 1.93	1.33 ± 1.97	1.53 ± 1.97	0.836	0.78 ± 0.99	0.50 ± 0.55	0.88 ± 1.11	0.433

Legend: DM = Diabetes Mellitus, DME = diabetic macular edema, HbA1c = Hemoglobin A1C, BCVA = best corrected visual acuity, IVT = intravitreal anti-VEGF injections, LD = loading dose, CRT = central retinal thickness, CCT = central choroidal thickness, CVD = choroidal vascular density, CVV = choroidal vascular volume.

When analyzing by treatment response, two distinct patterns were observed: a significant decrease of CCT was seen in good responders (- 11% at M3, p=0.001); whereas in poor responders CCT increased, despite not reaching statistical significance (+ 9% at M3, p=0.917). These results were maintained along the 12 months follow-up of the study (Figure 3).

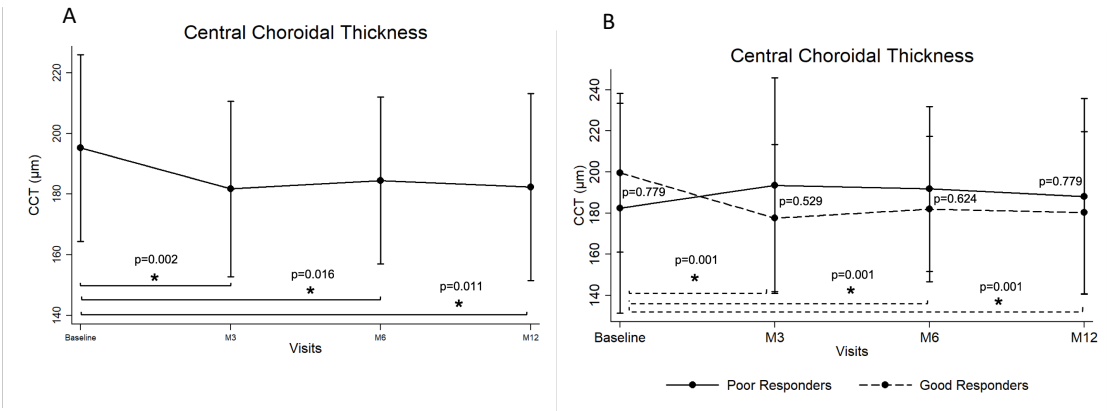


Figure 3: Central Choroidal Thickness during the 12 months follow-up, considering all DME patients (A) and by treatment response groups (B). * Significant p-values (p ≤0.05)

For the remaining choroidal indexes, there was a slight non-significant increase in CVD after the loading dose of anti-VEGF (M3) (+5% on macular CVD, p=0.301), which persisted and became significant at 12 months of follow-up (+7%, p=0.072 on macular CVD). On the other hand, macular CVV non-significantly decreased after the loading dose (M3) (-1%, p=0.143) and at 12 months (M12) (-0.7%; p=0.648).

Similar results were found for CVD regarding treatment response: good responders presented a slight decreased or maintenance of their CVD values throughout the study, while poor responders had a significant increase of CVD in the macular area, especially at M3 and M6, but also during the 12 months follow-up (+16%, at M3, p=0.046) (Figure 4). CVV was also significantly decreased in good responders after M3 (-14%, p=0.008), whereas substantially increased in poor responders (+34%, p=0.134). This tendency was maintained for the 12 months of follow-up (-7%, p=0.124 in good responders *vs* +18%, p=0.116 in poor responders).

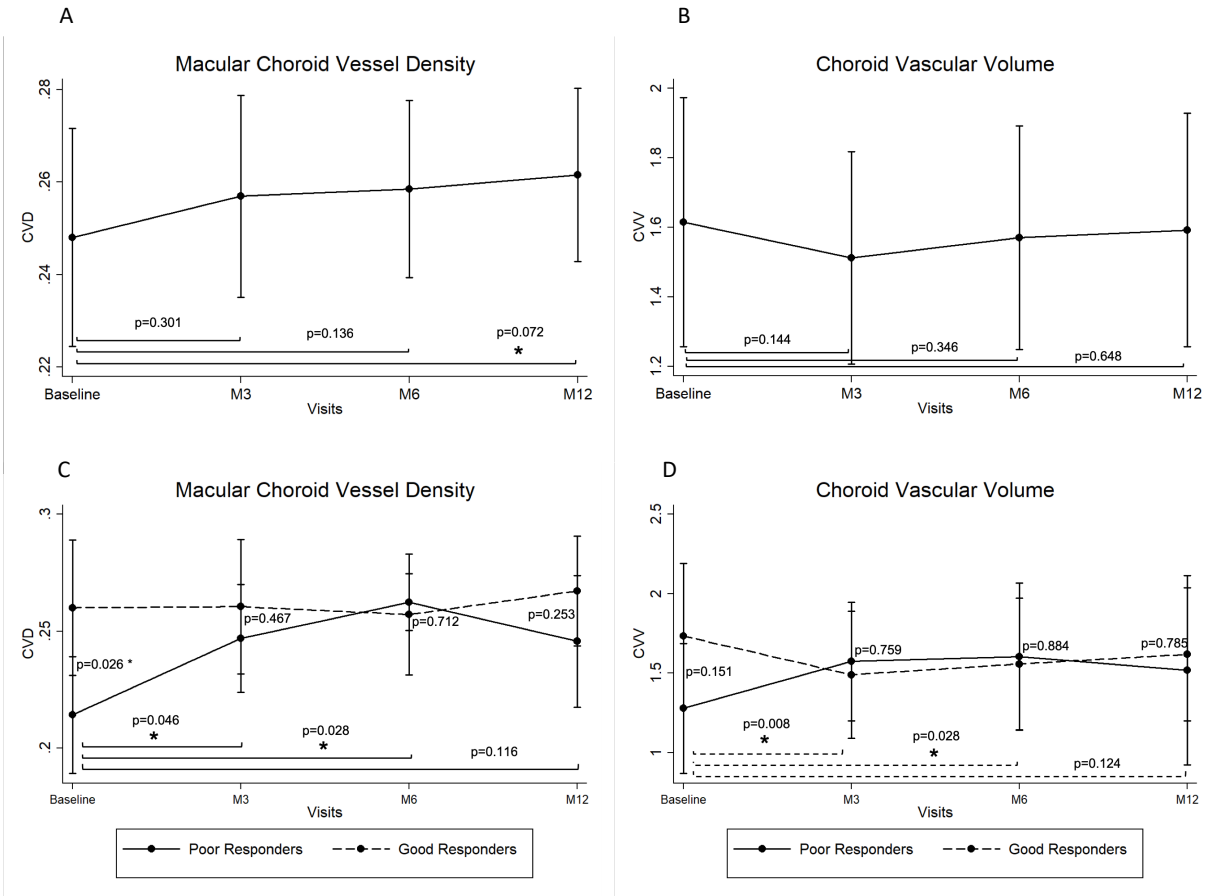


Figure 4: Choroidal Vessel Density and Choroidal Vascular Volume during the 12 months follow-up considering all DME patients (A,B) and by treatment response groups (C,D). * Significant p-values (p ≤0.05)

Factors associated with Choroidal Thickness and Choroidal Vascular Indexes

To better understand the relationships between choroidal indexes and the performed treatment, we assessed if potential confounders could be playing a role in the observed results. Namely, we performed a multivariate analysis to assess if CCT, CVD and CVV

changes during the 12 months study period were associated with age, sex, DM duration, DME duration, HbA1c or with the number of injections given after the loading dose. None of these analyses were statistically significant (age p=0.659; sex p=0.726; DM duration p=0.680; DME duration p=0.195; HbA1c p=0.929; number of injections p=0.071).

Table 3 – Coefficient of Variation for all retinal and choroidal features

	Mean	**SD**	**C_V**
CRT	397.7	119.5	41.0%
CCT	195.2	74.2	38.0%
Overall CVD	**0.20**	**0.02**	**12.2%**
Macular CVD	**0.25**	**0.06**	**23.0%**
CVV	1.61	0.9	53.5%

Legend: SD= Standard Deviation; C_V=Coefficient of Variation; CRT= Central Retinal Thickness; CCT=central choroidal thickness, CVD=choroidal vascular density, CVV=choroidal vascular volume.

Additionally, considering the large rage of values that choroidal index can present, which has been extensively described for choroidal thickness[30,31] we also assessed their inter-subjects variability. Our results showed that choroidal vascular indexes, mainly CVD, had substantially lower variability between subjects than central retinal thickness and central choroidal thickness (Table 3).

Choroidal Thickness and Choroidal Vascular Indexes as Predictors of functional response to anti-VEGF treatment

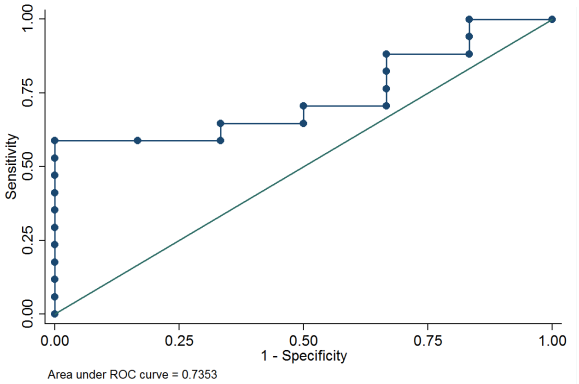
As described, our results suggest that patients with higher baseline values of CCT, macular CVD and CVV, have a higher probability of improved BCVA after anti-VEGF treatment. To verify how accurately these features could predict treatment response to anti-VEGF therapy, a ROC analysis was performed. This revealed that macular CVD was the best baseline discriminator of a good visual response, with an AUC of 0.74 (95%CI: 0.52 - 0.95) (p=0.030). Indeed, macular CVD differentiated good or poor responders with 60% of sensitivity and 80% of specificity (Table 4 and Figure 5).

Table 4 - Area under the curve for CCT, Overall and Macular CVD, and CVV

	ROC AUC	CI 95%	p-value
CCT	0.54	0.29 - 0.80	0.768
Overall CVD	0.70	0.48 - 0.91	0.076
Macular CVD	**0.74**	**0.52 - 0.95**	**0.030**
CVV	0.62	0.37 - 0.86	0.347

Legend: AUC=area under the curve; CI=Confidence Interval; CCT=central choroidal thickness, CVD=choroidal vascular density, CVV=choroidal vascular volume, Significant p-values ($p \leq 0.05$) are highlighted as bold.

Figure 5: Area under the curve (ROC) for Macular CVD



Discussion

We present a prospective, longitudinal study analyzing choroidal vascular parameters and their association with response to treatment to ranibizumab injections in patients with DME. Our results revealed that, when looking at the entire study population, CCT decreased after ranibizumab treatment, not only after the loading dose (M3) but also during the 6 and 12 months. However, when considering response to treatment, we observed that good responders presented significantly decreases in CCT over time, while poor responders the opposite changes. Choroidal vascular density and volume (CVD and CVV) presented analogous results, with significant reductions in good responders, and increases in poor responders. CVD at baseline showed to be a good predictor for gain of vision after anti-angiogenic treatment.

To the best of our knowledge, this is the first study analyzing the impact of anti-VEGF therapy on choroidal vessel density (CVD) and choroidal vessel volume (CVV) using Swept-Source OCT technology in patients with treatment naïve DME, and to correlate these findings with functional response to treatment. Our results are consistent with prior studies using SD-OCT that showed that anti-angiogenic therapy can contribute to decreased choroidal thickness[17,32,33]. Our study has the advantage of using SS-OCT, which enables a better visualization of the choroid, and of not relying on CCT manual segmentations, which increases data extrapolation and variability.

CT has also been proposed in the literature as a possible biomarker of treatment response[15,17], with some authors[17] showing that a thicker choroid at baseline could predict

a positive functional recovery after treatment. Our results suggest, however, that CCT at baseline is not able to discriminate visual response after treatment (ROC AUC=0.54; p=0.768). The limited number of patients included in our study might have influenced these results. However, we did observe that baseline CVD was a significant predictor of a good BCVA response with this sample size.

Differences among our study and the aforementioned studies may also affect the observed different results. Namely, our study was prospectively designed, we used automated CCT calculations, and we assessed during 12 months. The prior cited studies were both retrospective and used manual segmentation. Rayess et al.[17] considered patients that had received several anti-VEGF agents (ranibizumab or bevacizumab) and used Snellen acuity charts to access VA changes after treatment, which could possibly contribute to variations. Yui et al.[15] analyzed data from patients with a nonuniform treatment regimens and considered visual response only at 6 months after the first injection.

We explored other choroidal features as potential biomarkers of functional response to DME treatment with anti-angiogenic agents.[22] Indeed, our results revealed that CVD and CVV were higher in good responders at baseline and a ROC analysis showed that macular CVD was the best parameter to discriminate patients that increase more than 5 BCVA letters after treatment.

As the choroid is highly vascularized, we hypothesized that it could be easily used to detect the effects of anti-VEGF drugs, being more sensitive to its effects than retinal vasculature. It has already been demonstrated in animal models[34–36] that anti-VEGF agents injected intravitreally can reach the choroid and affect its structure, blocking VEGF expression with effects on vascular permeability, blood flow and angiogenesis. Laíns et al.[33] demonstrated it clinically in DR patients submitted to 3 different types of anti-angiogenic therapy. Our study adds that CCT, CVD and CVV seem to change differently after treatment depending on functional responses to anti-angiogenic therapy. Patients that decreased BCVA after treatment (poor responders) significantly increased choroidal thickness, vessel density and volume while the choroid of patients with a good visual response remained almost unchanged.

Considering that choroidal vessel density (CVD) in this work was calculated as the area occupied by vessels and CVV is derived from both the CVD and CT, the fact that CVD and CVV appear to be increased in poor responders after treatment could be suggestive of choroidal vessels dilation and increased choroidal vasopermeability. These changes, associated with a weak functional response of this group to the therapy, may be related to a higher expression of VEGF in circulation on these patients or maybe an inadequate blockade of VEGF by the treatment. These hypotheses are supported by a recent study[16] describing that anti-VEGF can affect choroidal vasculature and blood flow significantly. Okamoto et al[16], observed an increased luminal area in patients with DME when compared with controls, being even more pronounced in those not previously treated with panretinal photocoagulation (PRP). These results suggest that patients with a higher expression of VEGF have vessel dilation, leading to an increase of choroidal thickness, which is also supported by pathogenic mechanisms already reported in DR[37].

On the other hand, it is known that pro-inflammatory agents play also an important role in DME pathogenesis[38]. Increased concentrations of pro-inflammatory cytokines and other inflammatory mediators like leucocytes and adhesion molecules are also involved in vasodilation of vessels and breakdown of the BRB[39]. Our results could suggest the hypothesis that if these patients are not responding to anti-VEGF therapy, maybe they could benefit from switching therapies early on, namely, to anti-inflammatory agents.

New image modalities and analyses are needed to better explore the contribution of choroidal vessel disease to DME pathogenesis, prognosis and treatment response. Our study showed that choroidal vascular indexes present lower variability between subjects than choroidal or retinal thicknesses, the most common parameters evaluated, and may thus be more reliable indicators of disease progression or treatment changes. Therefore, we believe CVD and CVV could be very useful in DME, not only in diagnosis, but also in the evaluation of treatment efficacy, both structurally and functionally. In the era of OCT angiography (OCTA), information about vessel density or perfusion of the choroid seems to be important to explore in this disease and this technology could be an alternative approach for subsequent studies. However, its described imaging artifacts and penetration limitations constitute some of the challenges to be addressed in the study of choroidal vasculature[40].

Despite the prospective design of this study, the small number of included patients probably had an impact on our results. Although we were able to identify important differences between groups and to establish CVD and CVV as possible biomarkers of treatment response, further studies with larger samples are needed and essential to clarify the relationship between diabetic retinopathy and choroidopathy. It may also be important to explore the differences among the choroidal expression of VEGF among larger treatment response groups and in patients submitted to different anti-VEGF agents.

This study highlights important differences between DME treatment response groups at the choroid level. Choroidal features can represent potential biomarkers of treatment response, and this may contribute to the development of personalized care for patients with diabetic macular edema.

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Chapter 6

Microperimetry and mfERG as Functional Measurements in Diabetic Macular Edema undergoing Intravitreal Ranibizumab Treatment

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Abstract

Purpose: To evaluate Microperimetry (MP) and multifocal electroretinogram (mfERG) as whole-macula functional markers of treatment response in eyes with diabetic macular edema (DME) undergoing ranibizumab treatment, during a 12 months period of follow-up.

Methods: Prospective study in treatment-naïve DME patients. All patients underwent a course of three-monthly injections of ranibizumab (loading dose) followed by Pro Re Nata regimen (PRN) during one year of follow-up. At baseline, during and after the treatment course (Months 0, 3, 6 and 12), every subject was tested using best corrected visual acuity (BCVA), OCT, MP and mfERG. MP was performed in the central 12º, and retinal sensitivity was measured overall (mean sensitivity (MS)), and specifically in three concentric rings (R1-R3). The P1 amplitude and implicit time of the mfERG were measured and averaged over six concentric rings (R1-R6). Group comparisons (DME vs age-matched control group) and paired comparisons (at baseline, months 3, 6 and 12) were conducted, as well as subgroup analysis according to BCVA response after the loading dose (poor responders – decrease or increase < 5 ETDRS letters; responders – increase between ≥ 5 and < 10 letters; and good responders – increase ≥ 10 letters).

Results: Thirty-two eyes of 32 subjects were enrolled. MP mean sensitivity and rings sensitivity was significantly lower in DME versus controls ($p<0.001$). After the 3 monthly injections, a significant improvement in retina sensitivity was observed, particularly in good BCVA responders (MS +4.6 dB; R1= +4.9 dB, R2= +4.7 dB, R3= +4.6 dB; $p<0.001$). Overall retinal sensitivity was significantly correlated with BCVA improvement ($r=0.54$; $p=0.026$) and inversely correlated with OCT central subfield thickness improvement ($r= -0.39$, $p=0.026$). mfERG amplitude and implicit time were likewise lower in DME versus controls ($p<0.011$). After a 3-injection course of ranibizumab, an improvement of mfERG P1 amplitude and implicit time in R1 was noted in good responders only (+16.49nV/deg^2; $p=0.013$ and -0.005ms; $p=0.048$, respectively). When treatment with RBZ changed from monthly injections to a PRN regimen, visual function decreases were detected with MP and mfERG, despite maintenance of BCVA.

Conclusion: Microperimetry and mfERG were able to demonstrate: (a) baseline outer and inner retina dysfunction in DME and (b) functional improvement after loading dose treatment, followed by a loss in retinal function when changing to PRN regimen.

Introduction

Diabetic retinopathy (DR) and namely diabetic macular edema (DME) is a major cause of vision loss in people of working age, with significant personal, social and economic impact[1].

The use of anti-vascular endothelial growth factor (anti-VEGF) agents, such as bevacizumab, ranibizumab and aflibercept, has revolutionized treatment in DME[2–4]. However, most randomized clinical trials to date use best-corrected visual acuity (BCVA) as a primary endpoint, and central retinal thickness reduction as a secondary endpoint to evaluate treatment response. However, the given numbers on a distant vision test may not reflect the patient's ability to maintain an independent lifestyle which has a significant impact in vision-related quality of life. Moreover, the cutoff values of BCVA improvement that are frequently considered as clinically significant in the course of a therapy have been widely discussed by Food and Drugs Administrations[5,6] and investigators[7,8] due to several limitations including subjectivity of the method. These facts lead to the need of validating other functional evaluations as possible methods for assessing diseases progression or treatment efficacy.

While classically DR is mainly seen as a microvascular disease[9], there is increased recognition that neural changes occur in diabetes[10–12]. Indeed, psychophysiological and electrophysiological measurements of retinal function might address this issue, evaluating the neural component of DR in a larger retinal area and avoiding the subjectivity of BCVA. Microperimetry (MP) has proven to be an effective and useful functional method in the examination of retina sensitivity changes in DR and DME[13–16]. By presenting multiple luminous stimuli of different intensity in several locations of central retina, it objectively measures the achromatic luminance threshold in foveal and parafoveal regions. By a built-in eye tracking system and simultaneous imaging of the posterior pole, it allows a direct and precise association between retinal function and localized structural alterations. Multifocal electroretinography (mfERG)[17], is also able to concurrently extract retinal responses generated at multiple retinal locations, enabling topographic mapping of retinal function in the central 40-50º of the retina and improving the functional evaluation of retinal diseases. Studies have shown that mfERG is able to show neuroretina changes in

diabetic patients without retinopathy[18,19], with retinopathy[12,20] and with DME[21–24]. In DME, the most consistent changes seem to be amplitude decrease and implicit time increase of P1 (the positive peak that follows a focal flash)[24].

To the best of our knowledge, no previous studies have quantitatively evaluated improvement or lack of thereof after anti-VEGF treatment with these two methods and have related these changes to improvements in BCVA or central retinal thickness. Therefore, it is the aim of this study, to evaluate functional vision changes determined by microperimetry and mfERG in eyes with DME at baseline and after 3 monthly doses of ranibizumab (RBZ), as well as after 6 and 12 months of follow-up, and to investigate possible associations with visual acuity (VA) and optical coherence tomography (OCT) changes.

Methods

Study Design and Participants

Observational, longitudinal, prospective, single-center study. All research and data collection adhered to the tenets of the Declaration of Helsinki. Informed consent to participate in this research study was obtained from all patients before screening and after an explanation of the nature and possible consequences of participation. The study was approved by the local ethics committee. Adult patients with type 2 diabetes and treatment-naïve center-involving DME were enrolled, as defined by a central subfield thickness of 300 µm or more in the study eye, evaluated by spectral-domain OCT (Spectralis OCT, Heidelberg Engineering GmbH, Heidelberg, Germany), and with a BCVA below 79 Early Treatment Diabetic Retinopathy Study (ETDRS) letters. Exclusion criteria were (1) previous anti-VEGF or macular laser treatment (in both eyes), (2) other causes of macular edema (in the study eye), (3) cataract precluding fundus observation, (4) proliferative diabetic retinopathy, either active or treated in the previous 3 months, (5) aphakia, (6) uncontrolled glaucoma, (7) arteriothrombotic event in the previous 6 months, (8) pregnancy and breastfeeding and (9) glycosylated hemoglobin higher than 11.0%.

A sample of 62 age-matched control subjects was used for comparative purposes (mean age 64.31 ± 7.26 years; age was not significantly different between control and DME subjects, p=0.330).

Study Protocol

All participants were submitted to a baseline full ophthalmic evaluation, including BCVA, dilated fundus examination, color fundus photography and spectral-domain OCT (Spectralis OCT, Heidelberg Engineering GmbH, Heidelberg, Germany). BCVA was measured and recorded as letters read at 4 m on ETDRS charts. If less than 20 letters were read at 4 meters, the BCVA was evaluated at 1 meter. The final BCVA letter score was calculated by adding the number of letters read at 4 meters plus 30 (or the number of letters read at 1 meter). Patients who consented to participate in the study received a course of monthly intravitreal injections of ranibizumab (Lucentis, 0.5 mg in 0.05 mL; Novartis Europharm Limited, Camberley, UK) for 3 months, followed by a period of 12 months of PRN, according to the standard practice for DME treatment and the Summary of Product Characteristics (SmPC), and underwent mfERG and Microperimetry (MP) before the first injection (M0). One month after the third injection (M3), patients repeated BCVA measurements, OCT, mfERG and MP, as well as at months 6 and 12 (M6 and M12).

Microperimetry

MP was performed using the MP1 Microperimeter (Nidek, Gamagori, Japan) in the central 12º, and measured overall (MS - mean sensitivity) and specifically in three central rings (R1 - 2º; R2 - 4º; R3 - 6º), covering approximately 1 mm and 3 mm of the central retina area. A customized radial grid of 45 stimuli covering the central 12º was used with stimuli size of Goldman III and 200 msec of projection time. The fixation target was a red cross and stimulation was performed in a white, monochromatic background at 4 asb. The starting stimulus light attenuation was set at 10 dB and a 4-2 double staircase strategy was used with an built in automatic eye tracker that compensates for eye movements. Pretest training was performed and five-minute mesopic visual adaptation was allowed before starting the test. All subjects underwent microperimetry with dilated pupils. Fixation stability and location were also evaluated by the Fuji et al classification (stable, relatively unstable, and unstable; central, relatively eccentric, and eccentric fixation)[25].

Multifocal ERG

The mfERG was recorded monocularly using a CRT monitor (Retiscan; Roland Consult, Wiesbaden, Germany), according to the guidelines of the International Society for Clinical Electrophysiology of Vision (ISCEV). The projected stimulus consisted of 103 scaled hexagons. The recordings were performed under room light conditions in previously room light adapted subjects. The pupil of the study eye was fully dilated. The fellow eye was occluded by a pad. Summed responses from six concentric ring/annuli defined as R1 <3º, R2 3-7.8º, R3 7.8-15º, R4 15-24º, R5 24-31º and R6 31-42º, were used for analysis. These were described by the P1 amplitude density (nV/deg^2) and implicit time (ms). The P1 amplitude was measured from N1 trough to P1 peak, whereas the P1 implicit time was the time from the onset of the light stimulus until the P1 peak.

Statistical Analysis

Baseline (M0) mfERG P1 amplitude and implicit time, and baseline (M0) microperimetry mean sensitivity were compared between the enrolled DME patients and a sample of 62 age-matched control subjects. Repeated measures ANOVA was performed to analyze changes at four timepoints (M0, M3, M6 and M12) of mfERG P1 amplitude and implicit time, and microperimetry mean sensitivity in DME patients, for all patients and subdivided by BCVA response to anti-VEGF treatment categories: poor responders (decrease/increase < 5 letters), responders (increase ≥ 5 and < 10 letters) and good responders (increase ≥ 10 letters). OCT central retinal thickness (CRT) at M0, M3, M6 and M12 was also used for correlation analysis between retinal structure and functional mfERG and MP parameters. Continuous variables were described by mean and standard deviation (SD). Categorical values are described by absolute frequencies and percentages. The two-independent samples t-test and the paired samples t-test were used, after checking for normality with the Shapiro-Wilk test. Spearman correlation analysis was performed. A receiver operating characteristic (ROC) analysis was performed to identify the best predictors (mfERG P1 amplitude and implicit time and microperimetry mean sensitivity) for a more than ten ETDRS letters improvement in BCVA. All analyses were performed using STATA®, version 13.1 (StataCorp, College Station, EUA). A p-value of less than 0.05 was considered statistically significant and all tests were two-sided.

Results

Study Sample Characterization

We included 32 eyes of 32 subjects with treatment-naïve center-involving DME that underwent a course of monthly intravitreal injections of ranibizumab for 3 months and were followed for a total of 12 months in a PRN regimen. Enrolled subjects had a mean age of 65.76 ± 5.47 years and 63% (n=20) were male. At baseline (M0), mean BCVA was 62.58 ± 9.50 letters (minimum 38 letters, maximum 75 letters), and mean central subfield thickness was 406.35 ± 122.61 µm (minimum 245 µm, maximum 708 µm).

DME vs Controls –Baseline (M0) evaluation by Microperimety and mfERG

Microperimetry overall mean sensitivity (MS) and rings sensitivity was significantly lower in DME patients compared to controls (Table 1: mean difference DME – controls, MS: -9.76 dB; R1: -19.98 dB; R2: -13.22; R3: -9.44 dB; $p<0.001$). Retinal sensitivity was gradually increased from the central ring (R1- radii 2º) to the peripheral rings (R2- radii 4º, R3- radii 6º).

mfERG P1 amplitude was significantly lower in DME subjects in all studied rings (Table 1: mean difference DME – controls, R1: -69.78 nV/deg^2; R2: -32.43 nV/deg^2; R3: -19.98 nV/deg^2; R4: -13.22 nV/deg^2; R5: -9.34 nV/deg^2 and R6: -7.34 nV/deg^2; all $p < 0.001$). Unlike microperimetry, mfERG P1 amplitude gradually decreased from the center to the periphery in both groups. P1 implicit time was only significantly different between groups from R4 to R6, though only a small difference was observed (R4: +0.97 ms, p=0.023; R5: +1.07 ms, p=0.011 and R6: +1.40 ms, p=0.001).

Table 1 – Microperimetry and mfERG findings in controls vs baseline DME patients

	MEAN SENSITIVITY, dB					**AMPLITUDE (P1)**, *nV/deg²*				**IMPLICIT TIME (P1)**, *ms*			
	Control	**DME**	Difference (DME-Control)	*P*		**Control**	**DME**	Difference (DME-Control)	*P*	**Control**	**DME**	Difference (DM- Control)	*P*
MS	19.45 ± 0.5	9.69 ± 5.52	-9.76	*<0.001 **	**R1**	124.21 ± 24.90	54.43 ±24.5	-69.78	*< 0.001 **	36.38 ± 3.34	37.40 ± 6.96	+1.02	*0.408*
R1	19.36 ± 0.76	8.07 ± 5.58	-19.98	*<0.001 **	**R2**	61.89 ± 14.12	29.47 ± 8.64	-32.43	*< 0.001 **	36.29 ± 2.35	36.16 ± 3.90	-0.13	*0.854*
R2	19.51 ± 0.55	9.42 ± 0.5	-13.22	*<0.001 **	**R3**	41.64 ± 8.22	21.66 ± 5.89	-19.98	*< 0.001 **	35.38 ± 2.04	35.67 ± 2.73	+0.28	*0.605*
R3	19.04 ± 0.5	9.60 ± 5.52	-9.44	*<0.001 **	**R4**	28.97 ± 6.19	15.75 ± 3.88	-13.22	*< 0.001 **	34.49 ± 1.50	35.46 ± 2.15	+0.97	*0.023 **
					R5	22.81 ± 5.09	13.48 ± 3.38	-9.34	*< 0.001 **	34.54 ± 1.43	35.61 ± 2.11	+1.07	*0.011 **
					R6	18.37 ± 4.33	11.03 ± 2.58	-7.34	*< 0.001 **	34.84 ± 1.51	36.25 ± 2.15	+1.40	*0.001 **

* – significant at p< 0.05, two-sided independent samples t-test
R = ring; MS = mean sensitivity

DME – 3 months evaluation (M3), immediately after the loading dose (monthly intravitreal RBZ)

After three monthly ranibizumab injections, both BCVA (mean intra-subject improvement, 7.27 ± 9.99 letters, p<0.001, paired samples t-test) and OCT CRT improved significantly (mean intra-subject improvement, -120.28 ± 130.80 µm, p<0.001, paired samples t-test). BCVA change was further categorized by range of change in poor responders (decrease/increase < 5 letters), responders (increase ≥ 5 and < 10 letters) and good responders (increase ≥ 10 letters). In our sample, at M3, 25% (n=8) were classified as poor responders, 31% (n=10) as responders and 44% (n=14) as good responders.

MP overall sensitivity (MS) and rings sensitivity significantly improved after M3 treatment, in good responders only (Table 2; mean intra-subject improvement: MS= + 2.72 dB; p=0.049; rings sensitivity = R1 + 2.33 dB, R2 + 2.20 dB and R3 + 2.25 DB; p=0.049; data not shown). No changes were seen in responders or poor responders (p > 0.05).

In the same way, mfERG P1 amplitude in R1 was significantly increased after M3 treatment in good responders only (Table 2; mean intra-subject improvement: + 16.49 nV/deg^2; p = 0.013). A small but significant change was also seen in P1 implicit time, again in good

responders only (mean intra-subject improvement: -1.12 ms; p = 0.048). No changes in P1 amplitude or implicit time were seen in R2, R3, R4, R5 and R6, regardless of clinical response category (all p > 0.05).

BCVA improvement was moderately and significantly correlated with mfERG P1 amplitude and implicit time improvement in R1 (correlation coefficient, r= 0.36; p= 0.041; r= 0.45; p= 0.009, respectively) but specially with overall retinal sensitivity (correlation coefficient, r= 0.54; p= 0.026). No correlations were found between BCVA and mfERG or microperimetry for the peripheral rings. Conversely and as expected, a moderate inverse correlation between CRT thickness change and overall retinal sensitivity and mfERG P1 amplitude improvements was found (r= -0.39, p=0.026 and r= -0.38 p=0.074, respectively).

Table 2 – mfERG and MP1 findings (R1) in DME patients at baseline (M0) and after three RBZ monthly loading doses (M3), by clinical (BCVA) response category

	OVERALL MEAN SENSITIVITY, *dB*				**AMPLITUDE (P1)**, *nV/deg²*				**IMPLICIT TIME (P1)**, *ms*			
	M0	**M3**	Difference[a] (M3 – M0)	*P*	**M0**	**M3**	Difference[a] (M3 – M0)	*P*	**M0**	**M3**	Difference[a] (M3 – M0)	*P*
Poor Responders (n=8) Decrease/increase < 5 letters	7.50 ±4.38	8.57 ±1.71	*+1.07*	*0.282*	54.63 ±25.22	57.97 ±26.35	+3.34	*0.578*	37.84 ±6.22	37.14 ±6.97	-0.71	*0.499*
Responders (n=10) Increase ≥ 5 and < 10 letters	8.83 ±4.99	10.08 ±3.64	*+1.25*	*0.062*	65.27 ±26.37	51.06 ±19.52	-14.21	*0.132*	38.12 ±7.34	38.67 ±6.83	+0.55	*0.846*
Good Responders (n=14) Increase ≥ 10 letters	11.06 ±6.12	13.20 ±3.66	+2.72	***0.049****	42.84 ±21.04	59.32 ±27.38	*+16.49*	***0.048 ****	37.23 ±5.23	36.11 ±5.15	-1.12	***0.048 ****

[a] – mean intra-subject difference (M3 – M0, for each subject)
* – significant at p< 0.05, paired samples t-test

DME – 6 months and 1- year functional changes under PRN regimen

After the loading dose of 3 first monthly injections (M3) patients received intravitreal injections under a PRN regimen and were followed at months 6 and 12 (mean nº of injections during 12 months of PRN, 1.78 $\pm$ 1.53 IVT). Despite maintenance of BCVA results, all other functional parameters gradually decreased when treatment regimen was changed to PRN (Figure 1).

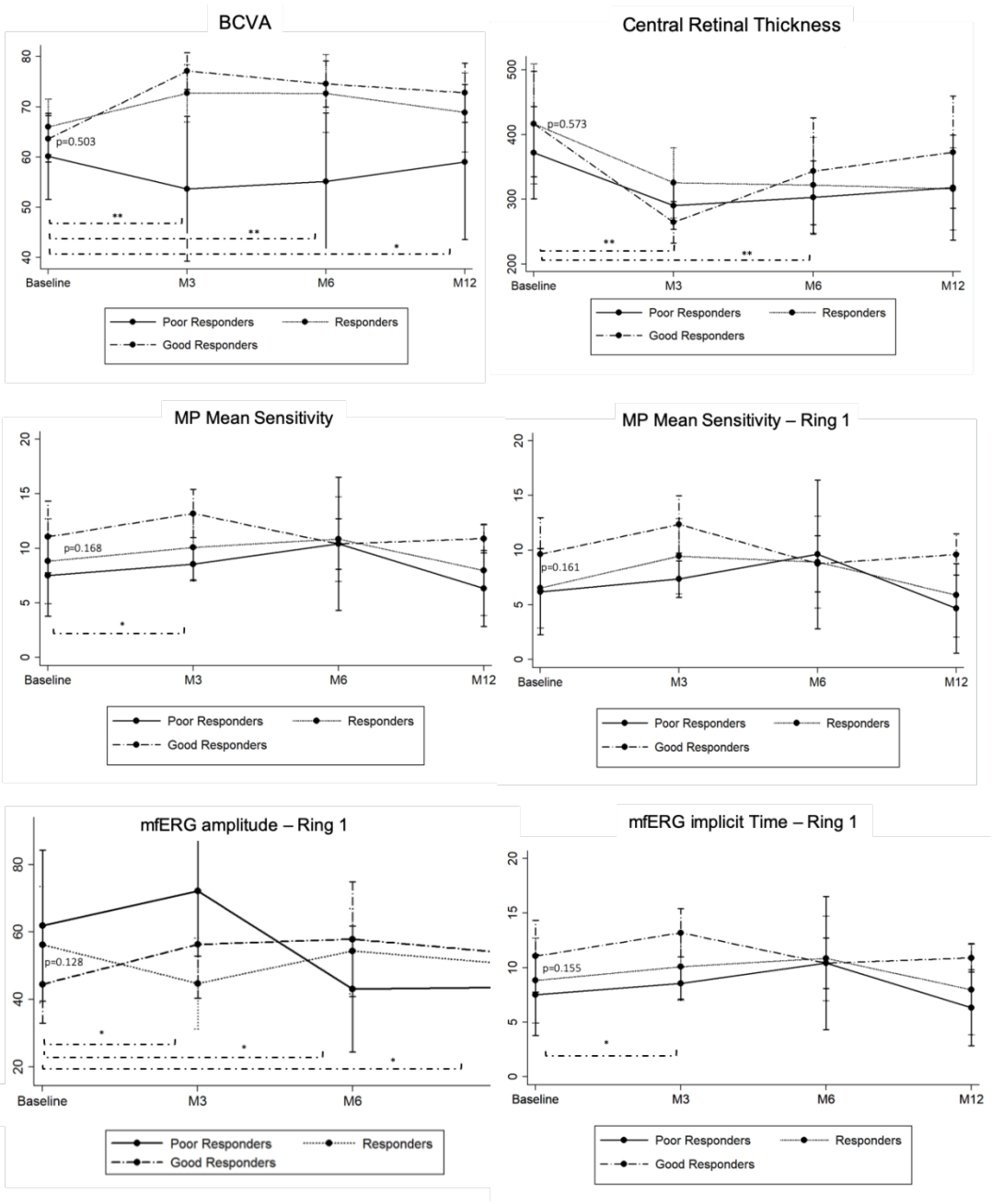

Figure 1: Functional and structural parameters along the 12-months of follow-up, by treatment response groups. BCVA= best corrected visual acuity; **=p-value<0.001; *=p-value<0.05

Microperimetry and mfERG as Predictors of functional response to anti-VEGF treatment

Response to treatment was defined as the improvement in number of letters after 3 monthly injections of ranibizumab. Nevertheless, analyzing graphs on figure 1, we can observe that even before treatment, patients with higher MP sensitivity and mfERG implicit time were the ones that had better BCVA outcomes after the therapy. To explore the value of microperimetry and mfERG as baseline discriminators of a good visual response to anti-VEGF therapy, a ROC analysis was performed revealing that both microperimetry sensitivity and mfERG implicit time at R1 were good baseline discriminators of BCVA response, despite not reaching statistical significance (Table 4). mfERG amplitude did not show any discriminative power.

Table 4 - Area under the curve for mfERG amplitude and implicit time of R1 and Microperimetry mean sensitivity and R1 sensitivity

	ROC AUC	CI 95%	p-value
Amplitude R1	0.31	0.10 - 0.52	0.079
Implicit time R1	0.68	0.48 - 0.89	0.079
Mean sensitivity	0.63	0.40 - 0.84	0.073
Sensitivity R1	0.65	0.43 - 0.86	0.184

Legend: AUC=area under the curve; CI=Confidence Interval.

Discussion

In the first step of our analysis we compared our cohort of DME patients with an age-matched control cohort regarding MP and mfERG findings. We found that retina sensitivity, evaluated by microperimetry (MP), was significantly lower in DME subjects compared to controls, in all studied areas. These results are in agreement with previous works showing that increased retinal thickness severely impairs luminous sensitivity[26,27] and validate MP as a method to demonstrate functional inner retinal impairment in DME. We also found that mfERG P1 amplitude was likewise significantly and markedly lower in DME subjects, in all studied rings, with an increase of the implicit time, showing functional outer retina impairment[28]. These results confirm published literature as a recent study by

Therani et al. showed that P1 amplitudes was significantly decreased in DME patients[21] and Weiner et al, using focal ERG reported decreased amplitudes and longer implicit times also in DME[29,30].

In the second part of our analysis we prospectively evaluated changes in MP and mfERG produced by RBZ loading dose on DME patients. We evaluated these changes in three clinical (BCVA) response categories: poor responders, responders and good responders. We have shown that after RBZ treatment, MP mean sensitivity and sensitivity in R1 – fovea, R2 – parafoveal area, and R3 – perifoveal area, as well as, mfERG P1 amplitude in R1 were significantly and clearly increased in good responders. These results show that RBZ treatment of DME is responsible for a functional recovery not only limited to the fovea, as measured by BCVA, but also of the entire macular area as shown by improvements in both MP and mfERG. In the daily practice, as well as in several clinical trials for evaluation of the safety and effectiveness of medical products, BCVA is the only used indicator of functional recovery[31]. In fact, it is frequently discussed if BCVA improvements of 1 or 2 or even 5 letters, with its individual variability, is significant enough to consider the efficacy of a therapy in improving visual function[6,7] as it does not always reflect the visual experience of the patient[8]. The entire process of testing BCVA yields information not only associated with a patient's ocular health but also with his neurological health[32] and requires a certain degree of mental capacity and compliance, which constitutes perhaps the biggest disadvantage of this method and raises the need to validate objective and reliable methods for functional evaluation. In our study, when correlating BCVA with MP and mfERG before and after treatment, BCVA improvement was moderately and significantly associated with these two functional parameters reinforcing their potential usefulness as functional biomarkers with clinical utility. Moreover, even before treatment (at baseline), when no BCVA significant differences exist between patients, microperimetry and mfERG appear to discriminate patients that are going to be good or poor responders after treatment.

Interestingly, a significant and negative correlation between central subfield thickness in OCT and both MP sensitivity and mfERG P1 amplitude was found in our results, confirming the value of OCT as surrogate for visual response in DME[33]. Therefore, a combined evaluation of functional and structural examinations in DME would appear to be appropriate for treatment assessments.

Our study also confirmed that better visual function improvement occur when patients receive monthly injections, and that these improvements decrease when treatment regimen changes to PRN. Studies like the RISE and RIDE[4] have shown that monthly RBZ injections were capable of a sustained improvement of vision during 24 months of follow-up, reducing the risk of further vision loss. In an attempt to reduce the burden and costs of this treatment plan, other regimens as PRN[34] or treat-and-extend[35] have been evaluated with non-inferior results. DRCR.net Protocol V[36] recently suggested that patients with center-involved DME and good vision can confidently be managed just by observation, scheduling anti-VEGF injections only if vision deteriorates. However, all these studies and treatment regimen based their retreatment decision on BCVA number of letters. Our data show a maintenance of BCVA results during the PRN regimen but a deterioration of macular function evaluated with both MP and mfERG. Baget-Bernaldiz et al[37] reached similar conclusions as well as Reinsberg et al[38] using similar methods but in AMD, which emphasizes that BCVA and OCT CRT may not be sufficient criteria to sustain medical decisions related to re-treatment, since patients quality of vision may suffer a significant impairment not adequately assessed by visual acuity only, and possibly loose the opportunity for future recovery.

The major strength of our study is the fact of being one of few studies that quantitatively evaluated the effects of anti-VEGF treatment in DME visual function, using mfERG and MP and correlating them to BCVA and CRT. While another study has evaluated the effects on RBZ therapy in DME using mfERG, it used arbitrary categories of improvement and failed to report any inferential statistics or correlations with other markers[39]. It also used pattern ERG for evaluation in DME, which by the authors own admission, might be an inadequate exam in DME since it covers a large retinal area and therefore is not sensitive enough for localized changes in the central macular/foveal region. Regarding microperimetry, Malagola R. et al[40] have also established a positive correlation between anti-VEGF therapy effects on CRT and functional outcomes (BCVA and Microperimetry), but included non-treatment *naïve* patients with persistent DME and previous laser therapy, compromising retinal sensitivity values. Other strengths of our study include the prospective, self-controlled design and the comparative baseline evaluation versus age-matched controls. This approach contributes to a thorough evaluation of microperimetry and mfERG changes in DME, both at baseline and after different stages of treatment, allowing the evaluation of functional/structural correlations.

Despite the demonstrated value of both functional methods, mfERG can be a longstanding and complex examination, with wide variability, expensive equipment and specifically-built normative databases[41], adding more burden to anti-VEGF therapy which already represents a huge burden for clinicians, particularly considering the increasing number of patients to be evaluated monthly. That said, of both used methods, microperimetry show clear advantages to be applied to clinical practice as it is less time consuming and more reliable and reproductible, giving immediate information about the functional status of the macula that can be compared along the treatment course[42].

Microperimetry and mfERG show clear differences between patients with DME and normal age-matched subjects. Monthly anti-VEGF injections improve not only foveal visual acuity but also macular function as shown by MP and mfERG, while a PRN regimen led to a decrease in these parameters, particularly in retina sensitivity, despite the maintenance of BCVA results. In summary, a combined evaluation of functional and structural parameters is relevant for determining the best treatment regimens to improve vision recovery.

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Chapter 7

General Discussion and Final Considerations

General discussion

DME is the major vision-threatening complication of diabetes mellitus and despite the growing evolution in therapeutic agents, there are still patients that lose visual function or require endless treatment sessions.

Ongoing research is helping to identify and validate a wide range of traditional and novel biomarkers that can prove to be useful in monitoring disease activity and response to treatment. Identification of DME biomarkers can help us to answer some fundamental questions relating to the current treatment strategies. It can shed light on why a patient responds to one specific anti-VEGF agent or steroid and not to another, and what the level of response might be. It also might improve methods for evaluating potential new therapies.

Although baseline visual acuity, central retinal thickness (CRT) and other OCT identifiable characteristics have demonstrated potential value as biomarkers of DME treatment, contradictory results remain.

OCT has demonstrated to be useful in identifying a variety of imaging findings in DME which seem to be related to or have impact in DME treatment response. Among them, central retinal thickness was the first to be identified but have been shown to be an imperfect predictor of outcomes for this patients as there is an inexact correlation between CRT and visual acuity[1]. There are patients where the edema resolves over the course of anti-VEGF therapy but the vision remains the same or worsens, and patients that have persistent or even worsening edema, with excellent visual acuity outcomes.

Many other structural parameters evaluated to this point (such as presence or absence of epiretinal membranes, presence and extent of intraretinal cysts, hyper-reflective foci, presence of microaneurysms, extent of subretinal fluid, and measures of retina layers reflectivity or thickness)[2] have not been shown, so far, to be strongly correlated with or predictive of vision. The majority of these studies were performed retrospectively, in pre-treated patients, submitted to different treatment agents.

Therefore, the main goal of this thesis was to characterize the DME patients' response to anti-VEGF treatment, using non-invasive imaging techniques based on OCT, in order to identify and validate imaging biomarkers that can be useful in the management of this disease.

For this purpose, the present work was divided in two steps: 1) Performing a retrospective study in DME patients that received ranibizumab treatment, to explore the relationship of retinal thickness changes with DME OCT patterns and BCVA recovery after treatment; 2) conducting a prospective randomized study in naïve DME patients, submitted to the same treatment regimen and followed for 12 months, with the objective of evaluating the contribution of different structural features as potential biomarkers of treatment response.

However, as previously described, structural changes are not determinants for functional outcomes in these patients and in a vast number of cases, structural recovery is not followed by a measurable functional gain.

BCVA is, until now, the only functional measurement used to demonstrate the efficacy of therapies in DME. However, improvement in the number of letters that patients can see is not frequently correlated with an increase in quality of vision[3]. There are multiple functional assessments that are possible with current biomarkers, such as microperimetry, contrast sensitivity, visual field, etc., many of which lack validation in controlled trials. Thus, an extensive and detailed characterization of visual function on our study patients was also a goal of this thesis. Using differentiated methods as microperimetry and mfERG, we were able to evaluate not only the vision impairment caused by this disease but also to characterize the functional recovery over the course of the treatment.

The main structural and functional findings obtained in this work are summarized as follows:

Structural characterization of the DME response to anti-VEGF treatment

1. **THE DEGREE OF CRT DECREASE APPEARS TO PREDICT BCVA INCREASE AFTER TREATMENT. PATIENTS THAT SHOWED A CRT DECREASE ≥ 20% AFTER 3 MONTHLY RZB, ARE 3 TIMES MORE LIKELY TO HAVE A BCVA RESPONSE > 10 LETTERS COMPARED WITH PATIENTS WITH A CRT DECREASE OF <20% (P=0.002).**

Despite its global use in all DME clinical trials, CRT is frequently considered a poor marker of treatment response[1]. CRT decrease does not happen in all DME patients submitted to ranibizumab intravitreal injections and it has been shown to be poorly correlated with visual function or treatment prognosis[4,5]. However, CRT is still a measurement that is easy to obtain in the daily clinic in a reproductible manner, and still represents a sign of recovery of retinal tissue with potential impact in visual function. The predictive power of CRT decrease for a functional improvement in DME patients was shown for the first time in the literature with the work described in **Chapter 2**.

2. **DIFFERENT PATTERNS OF DME BEFORE TREATMENT, NAMELY CYSTOID DME WITH DIFFERENT SIZES AND LOCATIONS OF CYSTS IN THE RETINAL LAYERS, ARE ASSOCIATED WITH DISTINCT FUNCTIONAL RESPONSES AFTER TREATMENT.**

Our first work described in **chapter 2** showed that presence of cystoid spaces in the inner retinal layers appears to be associated with a suboptimal response to anti-VEGF treatment (decrease of CRT less than 20%). In our second work, in **chapter 3**, this parameter was also explored by analyzing the impact of cyst size in the functional response (BCVA improvement after treatment), showing an association between large cysts and a poor BCVA response to anti-VEGF injections.

Previous works have suggested several classifications of DME based on morphologic patterns identified by OCT[6–8]. These classifications are important as they represent an attempt to predict the prognosis of DME with the aim of helping physicians guiding

treatment approaches. By our results, it appears that the larger the cystoid spaces more are the chances of a poor visual outcome.

As it was proposed in the literature, accumulation of fluid in the extracellular space may develop either by enhanced fluid efflux from blood vessels, or by disturbed fluid clearance from the retinal tissue into the blood, namely by the external BRB through EPR[9,10]. As the accumulation of fluid remains, cystoid spaces can increase their size along with a stretching of all retinal layers to form big coalescent spaces to accommodate larger amounts of fluid[2,9]. Stretching of neurosensorial retina leads to stretching of retinal cells involved in the visual transduction process, causing a decrease of the vision quality in these patients. On the other hand, presence of small cysts, especially in the inner retinal layers, has been associated with atrophic DME, a chronic and late stage of the disease[10], characterized by a thinning of the retina below minimal normal values, inner and outer retinal layers disruption and loss of the central depression. Therefore, cystoid spaces size and location are easily identified in the clinical practice nowadays with the spread of OCT technology and should be considered valuable biomarkers in the evaluation and treatment of DME patients.

3. **DISORGANIZATION OF THE RETINAL INNER LAYERS (DRIL) AND DISRUPTION OF THE ELLIPSOID ZONE (EZ) OF PHOTORECEPTORS LAYER ARE GOOD DISCRIMINATORS OF POOR AND GOOD RESPONDERS TO ANTI-VEGF TREATMENT, BEING RELIABLE PROGNOSTIC FACTORS FOR THERAPEUTIC DECISIONS.**

Presence of DRIL, caused by both diffuse or cystoid DME, and disruption of the EZ showed to have a crucial impact on visual function of patients with DME and the extension of these changes in the macular area, was able to predict the response to the therapy. As shown in **chapter 3**, presence of DRIL and EZ disruption at baseline represent a risk 7 to 10 times higher of having a poor BCVA response after treatment.

Different morphological parameters as presence and size of intraretinal cysts, hyperreflective foci, subretinal fluid and disruption of the outer retinal layers, have been extracted from OCT imaging of patients with DME, with a number of studies[2,11–13]

suggesting modest associations between them and functional vision. However, none of the published correlations have been strong enough to predict in a reliable way what would be the visual acuity after treatment and most of the reported studies were performed retrospectively in mixed treatment cohorts. Recently, DRIL was considered one of the most valid predictive biomarkers for treatment outcomes[14,15] being highly associated with current and future vision not only in DME but also in other retina conditions[16,17]. Our work adds value to these findings, as it was obtained in a prospective research protocol with *naïve* patients under the same treatment regimen. DRIL seems to be an important parameter to be included in future clinical trials to validate its role as a functional marker and its pathophysiological mechanism should be explored in future histological studies.

Apart from DRIL, our study reinforced the role of photoreceptors integrity on BCVA. EZ disruption was shown to be even more relevant predictor of visual function before and after treatment, being a useful structural marker to be evaluated. Automated methods to quantify this layer integrity on the OCT equipment's need to be developed so they can be used and applied in the clinical practice.

In a time when an adequate DME classification is still needed[8,18,19], with the objective of standardization and stratification of this condition to improve ophthalmic care of these patients and to reduce vision loss, these features should be taken in consideration not only for categorization purposes, but more importantly for development of treatment algorithms with the aim of a more personalized care to obtain the best structural and functional results.

4. **OCT - LEAKAGE WAS ABLE TO DISCRIMINATE, BEFORE TREATMENT, PATIENTS THAT WILL IMPROVE BCVA AFTER TREATMENT, WITH A 85% OF SENSITIVITY AND 80% OF SPECIFICITY, SHOWING BETTER PREDICTIVE VALUE THAN DRIL AND EZ DISRUPTION.**

Despite OCT ability to quantify retinal thickness, to evaluate retinal layers integrity and presence of fluid, it is important also to quantify the amount of retinal extracellular fluid. As described before[20,21], presence of fluid within retina layers was shown to correlate with

response to treatment, being therefore pertinent the development of such method, allowing identification and quantification of extracellular fluid by OCT technology.

OCT-Leakage was recently developed by our team[22] and uses data from simple OCT B-scans obtained in the daily practice in a complete non-invasive way, removing the need of dye injection to map the presence of extracellular fluid[23,24]. Despite the advances in non-invasive technology to evaluate the retina vasculature, as OCT-Angiography (OCTA), important limitations still exist in routine examination, namely the capacity to measure fluid accumulation in the retinal tissue without the injection of a tracer, such as fluorescein.

We consider that this new method can be an important tool to understand the pathophysiology of DME or other retina diseases, allowing to obtain information on the fluid flow of retina. Our results **(chapter 4)** showed that the most important accumulation of fluid in DME seems to occur in the OPL and EZ. As the fluid comes from the internal layers and drains through the RPE, its accumulation reveals failure or, at least, a breakdown of the elimination mechanisms, confirming previous theories of a dysfunction of the RPE and/or choroidal vessels being involved in DME development[24,25]. This can be relevant for the development of new therapeutic targets to improve RPE and choroid performance.

Despite the exploratory character of this study, considering only early changes immediately after treatment (1-week after 1 ranibizumab injection), OCT-Leakage was able to show the efficacy of anti-VEGF drugs in drying extracellular fluid. It also revealed to be a good predictor of functional recovery after treatment, even better that DRIL and EZ, which means that accumulation of fluid is still one of the major factors for vision impairment in DME. As shown by previous works[20,25], if left untreated, persistent subretinal fluid may be harmful to photoreceptors and RPE. It is therefore part of our plan to apply this method in future studies with longer follow-ups.

5. **CHOROIDAL VESSELS SEEM TO REACT DIFFERENTLY TO ANTI-VEGF TREATMENT IN ASSOCIATION WITH DIFFERENT FUNCTIONAL RESPONSES. DILATION OF CHOROIDAL VESSELS, WITH CONSEQUENT INCREASE OF CHOROIDAL THICKNESS AND VOLUME, WAS OBSERVED IN PATIENTS THAT HAD A POOR FUNCTIONAL RESPONSE, WHILE GOOD RESPONDERS SHOWED AN ALMOST UNCHANGED CHOROID DURING TREATMENT COURSE.**

Choroid was another structure evaluated in the present thesis due to its potential role in DR and DME[26–29]. As it is highly vascularized, we hypothesized that it could be easily used to detect the effects of anti-VEGF drugs. Swept Source OCT was the most adequate technology to be used in our analysis. As described **in chapter 5**, it enables a better visualization of the choroid and permits automatic choroidal segmentations increasing data reliability. It also gives good quality *en*-face OCT images, which have been increasingly used in the evaluation of several pathologies[30,31] as they produce transverse images of retinal and choroidal layers at any specified depth providing a more extensive overview of pathological structures in a single image, making possible to delineate and precisely measure microstructural and morphological changes in a coronal view.

Using Swept-Source OCT technology, we were able to show that choroidal vessels density and volume (CVD and CVV) can be potential biomarkers to explore in DME patients. As it was showed also by recent works, including from our team[32–34], it is expected that choroidal vascular indexes and choroidal thickness are reduced in DME patients. Surprisingly, we found distinct behaviors in these parameters depending on their functional response to anti-VEGF treatment. It seems that, right after the loading dose, there is a dilation of choroidal vessels with an increase of its volume and thickness in patients that do not improve BCVA after treatment (poor responders), while there are no significant changes in the choroidal vessels of patients that improved their BCVA (good responders). We raised the possibility that VEGF expression in circulation could be unequal among DME patients or an inadequate blockade of VEGF could happen in a part of the subjects submitted to this treatment. Furthermore, these results also open the hypothesis of a different pathway of the disease, not predominantly related to vascular proliferation. It is known that pro-inflammatory agents play an important role in DME pathogenesis causing vasodilation and breakdown of the BRB[35,36]. If these patients are not responding to

anti-VEGF therapy, maybe they could benefit from switching therapies early on, namely, to anti-inflammatory agents. In both cases, CVD and CVV can be useful and reliable markers of treatment response that can be used to monitor results in order to adapt the better strategy as earlier as possible and avoid vision loss.

It is worth to explore these findings in different cohorts of patients submitted to different therapies. New image modalities, as Swept Source OCTA, are also promising methods to investigate the contribution of choroidal vessels changes to DME pathogenesis, as it enables retina and choroidal vasculature reconstruction with high resolution images. There is still the need to solve some technical limitations that can compromise possible investigations with this technology, namely the impact or artifacts projections, which cannot be ignored when analyzing such deep structures as choroid, and the lack of reliable metrics regarding vessels density and flow.

Functional characterization of the DME response to anti-VEGF treatment

Visual acuity is the standard measurement of visual function both in daily practice and in clinical trials, but can be reductive and limited[37]. BCVA, as the number of letters a patient can identify in a chart, is a fovea-biased retinal function test. Therefore, it fails to evaluate functional improvement of the remaining macular and peripheral retina, which might have a huge impact in vision-related quality of life[38]. In fact, patients frequently report a mismatch between their visual performance and the objective measurements in the course of their ophthalmic evaluation[39,40]. Also, the entire process of testing BCVA yields information not only associated with a patient's ocular health but also with his neurological health in regard to intracranial visual pathways and processing[41].

A successful and reliable BCVA test requires a certain degree of mental capacity and compliance, which constitutes perhaps the biggest disadvantage of this method. Ideally, vision assessment should be objective, reproducible, and accurate, without depending on patient compliance. The use of differentiated functional methods, as microperimetry and mfERG, is useful to evaluate the impact of DME in the phototransduction process and in

the way patients perceive the world. These techniques together permit a full characterization of visual function in a larger area of the retina, decreasing BCVA limitations[42] and can be used as potential biomarkers of treatment response.

Therefore, these two methods were applied in our prospective and self-controlled study of naïve DME patients. Our results **(chapter 6)** revealed that:

1. **DME CAUSES A SIGNIFICANT IMPAIRMENT IN VISUAL FUNCTION, NOT ONLY IN VISUAL ACUITY BUT ALSO IN RETINAL ELECTROPHYSIOLOGICAL RESPONSE (mfERG) AND LUMINOUS SENSITIVITY (MICROPERIMETRY). WHEN COMPARED WITH HEALTHY SUBJECTS, DME PATIENTS SHOWED A SIGNIFICANTLY LOWER mfERG AMPLITUDE AND MICROPERIMETRY SENSITIVITY NOT ONLY IN THE FOVEA BUT ALSO IN PARAFOVEAL AND PERIFOVEAL AREAS.**

One possible explanation for the functional disturbance noted in DME is a dysfunction of Muller cells and bipolar cells that are located in the outer retinal layers due to the accumulation of fluid in the neurosensorial retina[10]. The presence of fluid, by itself, have a relevant impact on the function and survival of macular cells that are undergoing mechanical and toxic stress induced by edema. Macula is the retina area with higher densities of cone photoreceptors and their second- and third-order neurons which causes a high level of metabolic activity, accompanied by high oxygen and glucose demands[43]. This can explain the vulnerability of the macula during impaired oxygen supply due to vascular diseases and/or inflammatory conditions[43] and the occurrence of local neuronal cell death and gliosis in case of fluid accumulation. Additionally, the consequent increase of retina thickness causes displacement and stretching of the different retinal neuronal components compromising the connectivity between photoreceptors and ganglion cells with impact in visual function[9].

Again, BCVA may be a gross and delayed assessment to evaluate retinal cells disfunction, so the application of other methods capable of an early detection, mapping and monitoring of retina outer and inner dysfunction are of useful application in DME. Both mfERG and microperimetry have been reported as capable of detecting diabetic retinopathy functional damage earlier than other functional and structural methods[44]. The

EUROCONDOR study was able to demonstrate a correlation between functional changes in mfERG and OCT findings in the early stages of DR[45] highlighting a possible neurodegenerative process in these patients. Also, changes in microperimetry have shown to predict the outcome of DME after treatment, as it documents individual areas where function is altered[46]. Both mfERG and microperimetry enabled a topographic mapping of retinal disfunction allowing a precise localization of visual defects and quantification of their depth. Therefore, these methods may together act as useful functional biomarkers in early and late stages of the disease.

2. MICROPERIMETY SENSITIVITY AND mfERG AMPLITUDE SHOWED A CLEAR IMPROVEMENT IN VISUAL FUNCTION DURING THE PERIOD OF MONTHLY ANTI-VEGF INJECTIONS, ESPECIALLY IN PATIENTS WHO HAD A MAJOR IMPROVEMENT OF BCVA LETTERS (GOOD RESPONDERS).

These results show that microperimetry and mfERG are able to identify functional improvements resulting from anti-VEGF therapy and were significantly correlated with BCVA, reinforcing the usefulness of these methods as functional discriminators of different levels of response to therapy, with potential clinical utility.

Despite response to treatment in the present work was defined according to the improvement in number of letters after the loading dose of ranibizumab, mfERG and especially microperimetry sensitivity, were able to show clear differences between good responders (increase ≥ 10 letters), responders (increase between ≥ 5 and < 10 letters) and poor responders (decrease or increase < 5 letters) after monthly treatment.

If, by definition, a biomarker is “a characteristic that is objectively measured and evaluated as an indicator of pharmacological responses to a therapeutic intervention” that, apart from other values, may forecast the response to different treatment modalities, facilitating individualized care[47], we can securely say that these two methods can be used for that purpose. Nevertheless, further studies with higher number of patients using microperimetry and mfERG as response to treatment criteria should be conducted to consolidate these findings.

3. When monthly treatment with anti-VEGF is changed to PRN, a decrease in mfERG and microperimetry features was observed, despite maintenance of BCVA.

Several clinical trials conducted worldwide have demonstrated the safety and efficacy of ranibizumab for the treatment of DME. Different doses[48], regimens[49–51] and/or combinations with other therapeutic approach's as laser photocoagulation[52], have been studied in order to identify the most cost-effectiveness treatment with improved DME prognosis and reducing the incidence of other severe complications of DR that threaten vision. The literature shows that the best functional results are obtained with monthly injections (e.g. RISE and RIDE[48]) even though there are evidences that other treatment regimens decrease patients' burden and health costs, as PRN[51], treat-and-extend[49] or even just observation if BCVA is still preserved, as it was suggested recently [53]. However, all those strategies based their re-treatment decision on BCVA number of letters.

Our study confirms that changing from monthly injections to other regimens is associated with visual function loss not shown by BCVA number of letters, and emphasizes that BCVA and OCT CRT, as used in clinical practice, may be insufficient measurements in a re-treatment decision since patients quality of vision may be significant impaired if only those criteria are evaluated. Further studies using microperimetry and mfERG to evaluate visual function are needed, as they may be of added value to OCT and BCVA to determine the timing for PRN or treat-and-extend regimens.

4. Microperimetry is a validated method to access visual function in DME patients before, during and after anti-VEGF treatment, with immediate applicability in clinical practice.

Despite the demonstrated value of both functional methods, mfERG can be a longstanding and complex examination, with wide variability, expensive equipment and specifically-build normative databases[54]. That said, microperimetry seems to be an adequate and useful test to add to clinical practice. It is simple, reproductible[55], and with the new generation technology as MAIA[56], less time consuming to perform. It generates a report with immediate and reliable results as sensitivity maps, fixation stability information and macular integrity indexes that can be compared along time[57]. These two last parameters

can constitute additional information regarding patient's visual performance, which is important for daily activities as reading or driving. Further validation and standardization of strategies for DME application is needed.

5. **DESPITE THE NEW FUNCTIONAL INFORMATION THIS THESIS ADDS TO THE CURRENT KNOWLEDGE ABOUT DME VISION IMPAIRMENT, FUTURE STUDIES WITH ADDITIONAL FUNCTIONAL METHODS ARE STILL NEEDED IN ORDER TO BETTER CHARACTERIZE VISUAL FUNCTION BUT ALSO VISUAL PERFORMANCE ON THESE PATIENTS, TO UNDERSTAND THE IMPACT OF DME ON VISION DISABILITY AND HEALTH-RELATED QUALITY OF LIFE.**

Visual acuity, electrophysiology and retina sensitivity provide only a partial representation of a patient's overall visual function. Additional functional measurements as contrast sensitivity, chromatic vision, fixation stability, or presence of metamorphopsia[58] are other parameters that should also be explored in patients with DME as they may provide a better characterization of the impact of visual performance on day-to-day functioning and quality of life. Studies have shown that a reduction in contrast sensitivity is associated with a self-reported increase of difficulty on everyday tasks and with mobility and balance decrements, independently of visual acuity loss[40,59], whereas instability in fixation is associated with reduced reading speed and reading ability[60], which also influences a patient's ability to work or perform everyday tasks. If we think in DME as a maculopathy of working-age people, these disabilities can conduct to a loss of productivity or even loss of jobs increasing even more the burden and costs associated with this disease.

Additional tests to quantify other aspects of visual performance like reading speed, stereoacuity (the ability to discriminate depths[61]), or glare (indicating how patients handle light scatter), are also required in routine tasks like driving, reading, shopping or cooking and should be explored in future studies. Lack of standardization and a lack of access to the correct equipment are some of the drawbacks to implement these measurements in the clinical practice, so studies are needed to address these issues. It is also important to include these and other functional tests in future clinical trials of therapeutic agents. Visual function and performance can help to prove the safety and effectiveness of new drugs as well as be used in retreatment algorithms as they may help to identify patients who might benefit from early interventions/retreatments or switch of therapies.

Implications for clinical practice

1. Degree of decrease of CRT is still a valuable biomarker for functional recovery, since it is reliable and easy to be evaluated with generally available OCT equipment.

2. Cysts size and location matter. Large and coalescent cysts are usually signs of severe DME or chronic stages of the disease with poorer prognosis.

3. DRIL and EZ disruption should be analyzed before and during treatment as they can be valid predictors of functional outcomes to treatment. These features have a useful role when considering retreatment decisions.

4. Choroidal vessels could play a role on DME pathophysiology and seem to present distinct behaviors according to the response to anti-VEGF treatment. In the era of new imaging modalities as OCTA, that permit vessels density and length quantification, choroidal vasculature should be analyzed during the course of the treatment with potential interest in switching treatment regimens and agents.

5. Functional parameters, as retina sensitivity, identify functional impairment in DME, earlier than BCVA. It should be a tool to consider when monitoring this disease and considering retreatment algorithms.

Implications for future research

1. Development of automatic methods for identification and quantification of retina layers disruption to improve these biomarkers applicability in clinical practice.

2. Investigation of retinal and choroidal vascular indexes variations between treatment agents and treatment response groups, with new imaging methods, as Swept Source OCTA or Ultra-Wide Field Imaging.

3. Validation of OCT-Leakage software in longer studies and different ocular diseases.

4. Development of a new classification of DME considering all validated biomarkers assessed by OCT and novel diagnostic tools (CRT, location and size of cystoid spaces, presence of hard exudates and/or hyperreflective foci, disruption of internal and external retinal layers, integrity of the vitreo-retinal interface, etc.). A clear and consensual classification will give a global overview on the macular structure and function, providing useful information about patient prognosis and treatment decisions.

5. Validation and standardization of additional functional tests to assess endpoints, others than BCVA, that can be used in DME clinical studies to evaluate the impact of the disease in visual function and vision-related quality of life.

6. Exploration of new functional outcomes, like fixation stability, reading speed, stereoacuity, color vision, scotopic sensitivity, etc., as surrogate markers of efficacy for current and new therapeutic agents. Supportive surrogate markers are needed to better assess the overall benefit of a treatment, achieved in individual patients.

General conclusions

Retinal structural features are important not only for prognosis but also for the selection of the best treatment strategy. It is therefore, of utmost importance to develop automatic approaches to quantify these features in daily clinical practice.

The main conclusions regarding retinal structural features with potential relevance for the DME treatment response are:

1. Despite CRT being considered an imperfect biomarker of DME response, it seems that a certain degree of decrease after anti-VEGF loading dose (-20%) is able to predict patients' functional response.

2. Of all OCT structural parameters analyzed, disruption of the photoreceptors complex (EZ layer), revealed to be the most robust predictor of treatment response.

3. Using the non-invasive OCT imaging technology, together with OCT-Leakage, it is possible to extract qualitative and quantitative information about extracellular fluid accumulation in the retina. Accumulation of fluid in the external retinal layers (OPL and EZ) showed to be directly correlated with functional treatment response.

4. Choroidal vasculature seems to be involved in DME and showed distinct changes over the course of anti-VEGF treatment that correlates with different treatment responses. These findings should be explored in future larger studies with potential interest in assessing the effects of different treatment agents.

Retina function is also an important factor to be monitored in DME patients. BCVA evaluation seems not to be enough to characterize the functional state of DME patients' vision.

The main conclusions of the functional analysis of the DME treatment response are:

1. Microperimetry and mfERG were able to show a reduction of retinal function in DME patients, not only in the fovea but also in the parafoveal and perifoveal areas, revealing that functional impairment is not only limited to fovea, assessed by BCVA measurements, but extends to the entire macula area, compromising other functions as contrast sensitivity, visual fields and reading performance.

2. Microperimetry and mfERG parameters showed to detect earlier changes in visual function, when compared with BCVA. During a monthly anti-VEGF treatment regimen, microperimetry and mfERG were able to detect an improvement of visual function along with BCVA increase. However, when treatment regimen changed to PRN, a decrease on microperimetry luminous sensitivity and implicit time of mfERG was detected without an impairment of BCVA.

3. Despite the difficulties in applying these two functional methods in daily clinical practice, microperimetry underwent a considerable evolution on its applicability. It is nowadays an easy and fast approach with good reproducibility and reliability indexes that adds valuable information about the functional state of DME patients before and during the course of treatment.

DME is a sight threatening disease presenting different degrees of damage and progression. Therefore, personalized care should be increasingly applied. For that, a combination of different structural and functional aspects of each DME patient should be evaluated and algorithms of treatment need to be developed to help physicians on their decisions.

We look forward to continuing answering these and other research questions, in order to provide a better understanding of DR and DME pathophysiology and progression so we can contribute to overcome and manage this disease's complications and sight impairment.

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