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CIÊNCIAS MÉDICAS

The Role of Vitreous and Vitreoretinal Interface in the Management of Diabetic Macular Edema

Bernardete Teixeira Pessoa

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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O trabalho descrito na presente tese foi desenvolvido no Serviço de Oftalmologia, no Laboratório de Química Clínica e no Laboratório de Citometria do Centro Hospitalar e Universitário do Porto.

“Já que muitos empreenderam compor uma narração dos factos que entre nós se consumaram, como no-los transmitiram os que desde o princípio foram testemunhas oculares e se tornaram servidores da Palavra, resolvi eu também, depois de tudo ter investigado cuidadosamente desde a origem, expor-tos por escrito e pela sua ordem...”

Lucas, 1, 1-4

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Aims of this thesis

The following were the main goals of this thesis

1. defining the role of humoral vitreous biomarkers and vitreoretinal interface (VRI) in the physiopathology and treatment of (diabetic retinopathy (DR) and diabetic macular edema (DME);
2. assess some vitreous changes found in diabetics that may have impact on DR and DME physiopathology and therapy;
3. integrate various imaging retinal and choroidal biomarkers in response to DME treatment, in vitrectomized (VIT) and non-vitrectomized (non-VIT) eyes;
4. final elaboration of operational algorithms for dealing with DR and DME, based on this research.

Summary

Introduction: DME is the primary cause of DR-related vision loss and the leading cause of vision loss among working-age adults in the developed world. Among the complex, multifactorial, and not fully understood pathogenesis of DME, the vitreous plays a relevant role, although not always sufficiently valued. In diabetes *mellitus* (DM), the vitreous acts as an unbalanced deposit of pathogenic/inflammatory, neuroprotector/anti-inflammatory molecules mostly produced by the retina, imbibing the retina itself. Their dynamic humoral and mechanical interactions were herein investigated from several perspectives with the purpose of highlighting the role of vitreous in DME management.

Purpose: The main goals of this thesis were: 1 - defining the role of humoral vitreous biomarkers and vitreoretinal interface (VRI) in the physiopathology and treatment of (diabetic retinopathy (DR) and diabetic macular edema (DME); 2 - assess some vitreous changes found in diabetics that may have impact on DR and DME physiopathology and therapy; 3 – integrate various imaging retinal and choroidal biomarkers in response to DME treatment, in vitrectomized (VIT) and non-vitrectomized (non-VIT) eyes; 4 – final elaboration of operational algorithms for dealing with DR and DME, based on this research.

Methods: This thesis is based on eleven research studies, with an additional sub-analysis conducted on the basis of one of them. For aim 1 patients with diabetes and without diabetes (group control) with tractional diabetic macular edema (tDME) and indication for vitrectomy were included in a prospective study for evaluation of functional and anatomical outcomes after 12 months of follow-up, based on serum and vitreous biomarkers (several cytokines, chemokines, vitamin A, erythropoietin and transthyretin). Two other studies, one retrospective (12 months of follow-up) and other retrospective-prospective (6 months-6 months) evaluated the correlation of VMI abnormalities or characteristics with anatomical/functional outcomes and its effect on DME evolution in response to therapy approach (vitrectomy and enzymatic vitreolyses, respectively).

For aim 2 a cross-sectional consecutive analysis, including 55 eyes with DME in at least one eye, excluding VIT eyes or tDMEs, was conducted to analyze vitreoretinal interface status at the posterior pole, using ultrasound (US) vs. 55x35º vs. 20 20º spectral domain - ocular coherence tomography (SD-OCT) and to stablish a comparison between the three exames.

For aim 3 a retrospective analises through functional and anatomical outcomes based on best corrected visual acuity (BCVA), central foveal thickness (CFT) and the OCT-ESASO biomarkers, of DME patients nonresponders to bevacizumab (BEV) who were switched to ranibizumab (RBZ) or aflibercept (AFL) was developed to use a non-inferior anti-VEGF for three other prospective investigations developed subsequently. In these prospective analyses, DME outcomes with RBZ therapy were evaluated, in VIT and non-VIT eyes, using

SD-OCT and OCTA potential prognostic and predictive biomarkers: retinal layer thicknesses, choroidal parameters (choroidal thickness – CT, choroidal vascular index – CVI, and choriocapillaris flow density – CCD), and OCT- ESASO biomarkers. Three other retrospective studies in patients treated with fluocinolone acetonide implant (Iluvien®, FAc) for chronic DME(cDME) were developed for the evaluation of predictive and prognostic biomarkers, comparing VIT and non-VIT, based on BCVA/CFT, ganglion cell layer thickness and OCT - ESASO biomarkers, respectively. Finally, the last research addressed a series of challenging chronic DME clinical cases needing additional therapy over FAc, a comprehensive analysis was undertaken through biomarkers, including the vitreous status, and outcomes.

Results and discussion: This analysis is based on the three main research objectives that have been proposed.

Assessing the vitreous humor biomarker's role in n DR and DME physiopathology and therapy, the vitreous levels of IL-6, IL-8, MIG and IP-10 revealed to have the greatest post-vitrectomy prognostic and DR severity prediction capabilities among the biomarkers tested. IL-8 and IP-10 raised vitreous levels could indicate DR worsening before further functional compromise, whereas IL-6, EPO, and MIG increased levels may indicate a more serious central macular lesion.

About VRI role, vitreomacular traction (VMT), T3, has been identified as a negative prognostic factor. The release of vitreomacular adhesion, VMA, <2.500 µm has been linked to better DME control and a reduction in the number of intravitreal anti-VEGF injections required.

Concerning the DM-induced changes in vitreous status that may have an impact in DR and DME physiopathology and therapy, VMA was shown to be extremely common (100%) in patients with DME (according to US and SD-OCT 55x35º video display mode), but was undervalued by standard SD-OCT 20x20º, which only detected 43.6 % of VMA instances, the majority of which with thickened posterior hyaloid (40 %). The occurrence of focal VMA was relatively low (18.2 %).

The integration of various imaging retinal and choroidal biomarkers in response to DME treatment, in VIT and non-VIT eyes led to 3 primary achievements.

Regarding to OCT-ESASO Biomarkers: 1 - the clinical applicability comparing drugs' effectiveness was highlighted by the results. RBZ and AFL showed superiority over BEV. No clear superiority between AFL and RBZ was observed. The presence of DRIL have emerged as a key biomarker to further investigate in larger studies; **2** - Fewer INL cysts, lower INL and baseline CFT have been linked to normalization of macular anatomy as a result of the disappearance of DRIL, OPLd, and ONLc, as well as a better response to RBZ, in VIT and non-VIT eyes (no differences between them). In addition, INL cysts association with higher glycated haemoglobin levels reinforces the relevance of systemic metabolic control in diabetic microvascular manifestations; **3** - Even though no differences in the type of response to the

FAc implant were found between VIT and non-VIT eyes, VIT eyes had a more benign evolution through disease stabilization and a trend toward fewer additional treatments. SRF at baseline, a higher CFT, and a higher number of HRD at month 12 were linked to a more chronic and severe DME, as well as the need for additional treatment in DME patients who did not respond to anti-VEGF or short-term corticosteroids. The presence of SRF in an anti-VEGF non-responder appears to be a key factor for an increased risk of a lesser response even with CCT, the need for additional therapy, and even a possible increased progression of the non-controlled inflammatory process, which explains the increased number of HRF and CFT at month 12 in lesser responder cases to FAc.

Results addressing retinal inner layers: 1 - even in milder DMEs, treated with RBZ, the negative effect of edema was evidenced particularly in the inner retinal layers, in VIT eyes, which may explain poor functional results despite VIT and non-VIT eyes have started with comparable vision. This could be due to a decrease in the lower reservoir of the inner retinal layers in VIT eyes; **2** - FAc appears to arrest DR neurodegeneration, exhibited by non treated DME and in the natural course of DR, for the 2-year period following therapy with FAc, in VIT and non-VIT eyes (with no dfferences between them).

Results for choroidal parameters (CVI, CCD e CT): They not seem to be affected by vitrectomy by itself. Overall and in VIT eyes, none of choroidal parameters were affected by RBZ treatment. That was explained as a sign of a relatively undertreatment in VIT eyes that may have influenced the overall results, as **CT decreased only in non-VIT**. DME does not appear to alter CVI in the short-term (6 months), neither in VIT nor in non-VIT eyes. CVI have not changed significantly with RBZ therapy. When the global choroidal vasculature is assessed using CVI, the anti-VEGF beneficial effect on small CC arteries (through **CCD increase in non-VIT eyes**) may be attenuated.

After 6 months of an effective PRN therapy with RBZ as seen in our non-VIT eyes, RBZ appears to improve choroidal microvascular perfusion.

Conclusion: The vitreous condition has been shown to play an essential role not only in DME pathophysiology but also in therapy, together with other anatomical and laboratory biomarkers.

Keywords: Diabetic retinopathy, macular edema, vitreous, vitreoretinal interface, biomarkers

Sumário

Introdução: O edema macular diabético (EMD) é a principal causa de perda de visão relacionada com a retinopatia diabética (RD), em adultos em idade ativa, nos países desenvolvidos. A sua patogénese é complexa, multifatorial e não totalmente conhecida, tendo o vítreo um papel reconhecidamente importante, embora nem sempre adequadamente valorizado. No contexto da diabetes *mellitus* (DM*)*, o vítreo atua como um depósito não balanceado de moléculas inflamatórias / patogénicas, neuroprotetoras / anti-inflamatórias, produzidas pela retina e onde ela mesma se encontra integrada. A interação dinâmica, mecânica e humoral, entre o vítreo e a retina foi aqui investigada sob diferentes perspetivas, com o objetivo de se destacar o papel do vítreo na abordagem do EMD.

Objetivos: Foram 4 os principais objetivos desta tese: 1 – esclarecer qual o papel dos biomarcadores humorais vítreos e da interface vitreo-retiniana (IVR) na fisiopatologia e tratamento da RD e do EMD; 2 - Avaliar algumas alterações vítreas presentes em diabéticos com potencial influencia na fisiopatologia e tratamento da RD e do EMD; 3 – Integrar diferentes biomarcadores imagiológicos da retina e da coróide, em olhos vitrectomizados (VIT) e não vitrectomizados, na resposta ao tratamento do EMD; 4 – Elaboração final de um algoritmo de atuação para a abordagem da RD e EMD, com base na investigação aqui desenvolvida.

Métodos: Esta tese foi baseada num total de onze estudos diferentes, com diferentes amostras de doentes, tendo sido feita uma sub-análise adicional com base num deles.

Para o objetivo 1 foram estudados doentes com diabetes e sem diabetes (grupo controlo) com edema macular diabético tracional (EMDt) e indicação para vitrectomia. Foram incluídos num estudo prospetivo para avaliação de resultados anatómicos e funcionais ao longo de 12 meses de follow-up, com base em biomarcadores vítreos e séricos (várias citoquinas, quimiocinas, Vitamina A, eritropoietina e transtirretina). Dois outros estudos, um retrospectivo (12 meses de follow-up) e outro retrospectivo-prospetivo (6 meses-6 meses) avaliaram a correlação entre as anormalidades / características da interface vítreo-macular e os resultados anatómico-funcionais, bem como o seu efeito na evolução de EMD na resposta ao tratamento instituído (vitrectomia e vitreólise enzimática, respetivamente).

Para o objetivo 2, através de um estudo transversal, consecutivo, incluindo 55 olhos com EMD em pelo menos um olho, excluindo olhos VIT ou com EMDt, foi feita a avaliação por ultrassonografia (US) vs. tomografia de coerência óptica de domínio espectral (SD-OCT) 55x35º vs. 20 20º, de modo a ser estabelecida uma comparação entre os três exames relativamente ao real estado de adesão vítrea ao polo posterior.

Para o objetivo 3 foi realizada uma análise retrospetiva com base em resultados anatómico-funcionais de melhor acuidade visual corrigida (MAVC), espessura do sub-campo central

(ESC) e biomarcadores de OCT ESASO, em doentes com EMD não respondedor a bevacizumab (BEV) que fizeram swich para ranibizumab (RBZ) e aflibercept (AFL). Este estudo foi desenvolvido para a escolha de um anti-VEGF de eficácia não inferior para a sua aplicação para o tratamento de EMD, nos estudos prospetivos desenvolvidos a seguir. Nestas análises prospetivas foi avaliado o efeito do tratamento do EMD com RBZ, em olhos VIT e não VIT, com base em biomarcadores, com potencial valor prognóstico e preditivo: espessura das camadas retinianas, parâmetros da coróide (espessura da coróide – CT, índice vascular coroideu – CVI e densidade de fluxo coriocapilar -CCD) e biomarcadores de OCT ESASO, respectivamente.

Foram feitos 3 outros estudos retrospetivos em doentes com edema macular crónico tratados com implante de fluocinolona acetonido (Iluvien®, FAc) onde foram avaliados biomarcadores prognósticos e preditivos, comparando olhos VIT e não-VIT, com base na MAVC/CFT, espessura da camada de células ganglionares e biomarcadores de OCT- ESASO, respetivamente. Finalmente, numa última investigação, através de uma series de casos clínicos de doentes com EMD crónico e com necessidade de tratamento adicional ao FAc, foi feita uma análise abrangente de vários biomarcadores, entre os quais o estado do vítreo, e o resultado obtido.

Resultados e discussão: Esta análise foi baseada nos três objetivos propostos para esta investigação.

Na determinação do papel dos biomarcadores humorais vítreos na fisiopatologia e tratamento da RD e EMD, os níveis vítreos de IL-6, IL-8, MIG e a IP-10 no vítreo mostraram ter o melhor valor prognóstico no pós-vitrectomia e capacidade de predição de gravidade da RD. O aumento dos níveis de IL-6, EPO e MIG poderão indiciar lesão macular mais grave pela relação com o comprometimento não só anatómico, mas também funcional. Os níveis vítreos de IL-8 e IP-10 correlacionaram-se apenas com um pior resultado anatómico, o que poderá indiciar um aumento destes biomarcadores numa fase mais precoce, antes do comprometimento funcional.

Relativamente ao papel da IVR, a tração vítreomacular, TVM, T3, foi identificada como um fator de prognóstico negativo. A liberação da adesão vítreo-macular (AVM) <2.500 µm foi associada a um melhor controlo do EMD, com necessidade de um menor número de injeções intravitreas de anti-VEGF após libertação da mesma.

Com relação às alterações do vítreo induzidas pela DM com possível influência na fisiopatologia e resultado terapêutico da RD e do EMD – foi demonstrada uma elevada prevalência da adesão vítrea ao polo posterior (100%) nos doentes com EMD (pelo US e SD-OCT 55x35º, no modo de vídeo), sub-valorizada pelo SD-OCT 20x20º standard que apenas conseguiu detetar VMA em 43,6% dos casos, na sua maioria com evidência de hialóide posterior espessada (40%). A AVM focal revelou ser pouco prevalente (18.2%).

A integração dos diferentes biomarcadores imagiológicos da retina e coróide, em olhos VIT e não VIT, na resposta ao tratamento do EMD, levou a 3 resultados principais.

Resultados relativamente aos biomarcadores de OCT- ESASO: 1- A aplicabilidade clínica na comparação entre fármacos foi evidenciada pelos resultados. RBZ e AFL mostraram superioridade sobre BEV com base na ESC (RZB e AFL) e MAVC (RZB), sem superioridade entre AFL e RBZ. A presença de DRIL emergiu como um biomarcador chave para investigação e análise mais robusta com base em estudos maiores; 2- Um menor número de quistos na INL, bem como uma menor espessura retiniana central e da INL foram associados a uma resposta precoce com RBZ, normalização da anatomia macular, com desaparecimento de DRIL, OPLd e ONLc, em olhos VIT e não VIT (sem diferenças). Também evidenciada a relevância do controle metabólico nas manifestações microvasculares diabéticas (maior HgA1c) e associação a um maior número de quistos na INL; 3- Apesar de não existirem diferenças no tipo de resposta ao FAc entre olhos VIT e não VIT, os olhos VIT tiveram uma evolução mais benigna com estabilização do EMD e tendência para uma menor necessidade de tratamentos adicionais. SRF na baseline, uma maior CFT e um maior número de HRF aos 12 meses correlacionaram-se com um EMD mais severo, com maior necessidade de terapia combinada, em doentes não respondedores a anti-VEGF e corticoides de curta duração. A presença de SRF em não respondedores a anti-VEGF parece ser um fator chave para o aumento de risco de menor resposta mesmo à corticoterapia, com necessidade de terapêutca adicional e ainda assim progressão do processo inflamatório com aumento do número de HRF e da CFT aos 12 meses, em piores respondedores a FAc,

Resultados decorrentes da avaliação das camadas internas da retina: 1- Mesmo em EMDs menos severos, tratados com RBZ, foi evidenciado o efeito negativo do edema, especialmente nas camadas internas da retina e em olhos VIT, explicação para um pior resultado funcional apesar de AV semelhante entre grupos na *baseline*. Provavelmente devido à diminuição do seu já menor reservatório nas camadas retinianas internas; 2- FAc parece parar o processo de neurodegeneração, que caracteriza o não tratamento do EMD e o curso natural da RD, ao longo dos 2 anos analisados, em olhos VIT e não VIT (sem diferenças).

Resulados com base nos parâmetros da coróide (CVI, CCD e CT): Não parecem ser afetados pela vitrectomia. Na população total e no grupo de VIT, nenhum dos parâmetros da coróide foi afetado pelo tratamento com RZB. Um provável sub-tratamento no grupo de olhos VIT que terá condicionado o resultado global, já que a CT diminuiu significativamente em olhos não VIT. O EMD não parece alterar o CVI no curto prazo (6 meses), nem em olhos VIT nem em não VIT. A CVI não teve alteração significativa com RBZ. O efeito benéfico do RBZ nos pequenos vasos através do aumento da CCD nos não VIT e não na vasculatura coroideia global (CVI não alterado) pode dever-se a um menor impacto da microvasculatura quando

avaliada em conjunto com vasculatura coroideia global. Após 6 meses de uma terapia PRN eficaz com RBZ (como observado através dos olhos não-VIT) RBZ parece para melhorar a perfusão da coriocapilar.
Conclusão: O estado do vítreo revelou ter um papel essencial na fisiopatologia e no tratamento do EMD, juntamente com outros biomarcadores anatómicos e laboratoriais.

Palavras Chave: Retinopatia diabética, edema macular, vitreo, interface vitreoretiniana, biomarcadores

Abbreviations

ACTH -Adrenocorticotropic hormone

ADA - American Diabetes Association

AFL – Aflibercept

AGEs - Advanced glycated end-products

AMD - Age related macular degeneration

Ang-1 – Angiopoietin-1

Ang-2 - Angiopoietin-2

Anti - VEGF - Anti-vascular endothelial growth factor

ARVO - Association for research in vision and ophthalmology

ATEs - Arterial thromboembolic events

b - baseline

BCVA – Best corrected visual acuity

BAB – Blood aqueous barrier

BDNF - Brain-derived neurotrophic factor

BEV – bevacizumab

BM – Bruch´s membrane

BRB – Blood retinal barrier

BRZ - brolucizumab

CC – Choriocapillaries

CCD - Choriocapillaris flow density

CCT – Corticosteroid

CFT- Central subfield foveal thickness (in the 1 mm central ETDRS subfield)

CH – Chemokines

CI - contraindication

CK – Cytokines

CKD - Chronic kidney disease

CMT - central macular thickness

CNTF - Ciliary neurotrophic factor

CNV - choroidal neovascularization

CS – Choroidal stroma

CSME - clinically significant macular edema

CT - Choroidal thickness

CTGF – Connective tissue growth factor

CV – Cardiovascular

CVI - Choroidal vascular index

DAG - Diacyl glycerol

DAN – Diabetic autonomic neuropathy
DCCT - Diabetes Control and Complications Trial
DEXii - Dexamethasone intravitreal implant (Ozurdex®)
DHAP - Dihydroxyacetone phosphate
DHPH Glyceraldehyde-3-phosphate dehydrogenase
DM - Diabetes mellitus
DME - Diabetic macular edema
DMO - Diabetic macular oedema
DR - Diabetic retinopathy
DRCR.net Diabetic retinopathy clinical research network
DRIL - Disruption of the inner retinal layers
DRSS - Diabetic Retinopathy Severity Scale
DVC – deep vascular complex
EASD - European Association for the Study of Diabetes
EC – Endothelial cell
ECM - Extracellular matrix
EDI - Enhanced deep imaging
EGF - epidermal growth factor
“E” layers – ELM and EZ
ELM - External limiting membrane
EMA - European Medicines Agency
eNOS - Endothelial nitric oxide synthetase
ER - Early responders
ERF 2 - Erythroid-2- related factor 2
ESASO - European School for Advanced Studies in Ophthalmology
ET-1 - Endothelin-1
ETDRS - Early treatment diabetic retinopathy study
EZ - Ellipsoid zone
d – Disruption
FAc - Fuocinolone acetonide implant (Iluvien®)
FAF – Fundus autofluorescence
FAR – Faricimab
FDA - US Food and Drug Administration
FDT - Frequency doubling technology
FGF - Fibroblast Growth Factor
FOV – Field of view
GAPDH - Glyceraldehyde-3- phosphate dehydrogenase

GCL - Ganglion cell layer

GP – general practitioner

GSSG – Gluthatione

GSH – reduced Gluthatione

HA – Hyaluronan

Hb A1c - Glycated hemoglobina A1c

HCs - Horizontal cells

HBP – High blood pressure

HE - hard exudates

HRF Hyperreflective foci

ICAM-1 - Intercellular adhesion molecule 1

ICP - Intermediate capillary plexus

iFAF – Increased fundus autofluorescence

Ig – Immunoglobulin

IGF-I - Insulin-like growth factor

IL – interleukin

IL-1Ra – Interleukin-1 receptor antagonist

ILM - Internal limiting membrane

IFN- γ – Interferon- γ

INL- Inner nuclear layer

INLc - Inner nuclear layer cysts

IOP – intraocular pressure

IP-10 - IFN-γ–induced protein-10, CXCL10

IPL- Inner plexiform layer

IRC – intraretinal cysts

IRF – intraretinal fluid

IRMAs – Intraretinal microvascular abnormalities

IS-OS - the inner and outer segments of the photoreceptors

IV – Intravitreal therapy

K-ras - Kirsten rat sarcoma virus

MA - microaneurysm

MAT - Microaneurysms turnover

MC - Müller cells

MCP-1 - monocyte chemoattractant protein-1, CCL2

mfERG - Multifocal electroretinography

MIG - monokine induced by interferon-γ, CXCL9

HA – Hyaluronan

HIF-1α - Hypoxia-inducible factor 1-alpha

n - Number

NAD+ - Nicotinamide adenine dinucleotide (oxidized form)

NADH - Nicotinamide adenine dinucleotide (reduced form)

NADP+ - Nicotinamide adenine dinucleotide phosphate (oxidized form)

NADPH - Nicotinamide adenine dinucleotide phosphate (reduced form)

NFE2L2 Nuclear factor erythroid-derived 2-like 2

NF-κB Nuclear factor kappa-B

NLRP3 - NOD-, LRR- and pyrin domain-containing protein 3

NRF2 – Nuclear factor erythroid 2-related factor 2

NO - Nitric oxide

Non-VIT – Non-vitrectomized

NPA – Non-perfused areas

NPDR - Non-proliferative diabetic retinopathy

NR - Non-responders

NV – Neovascularization

OCP – Ocriplasmin

OCT - Optical coherence tomography

OCTA - Optical coherence tomography angiography

OHT - Ocular hypertension

OLM - Outer limiting membrane

ONL - Outer nuclear layer

ONLc - Outer nuclear layer cysts

OPL - Outer plexiform layer

OR - Odds ratio

ORTs - Outer retinal tubulations

PAI-1 - plasminogen activator inhibitor-1

PDGF AA - Platelet-derived growth factor AA

PDGF AB/BB - Platelet-derived growth factor AB/BB

PDR - Proliferative diabetic retinopathy

P ERG - Pattern electroretinogram

PEDF - Pigment epithelium derived factor.

PIGF - Placental growth factor

PKC - Protein kinase C

PPV – pars plana vitrectomy

PRN - Pro re nata

PRP - Panretinal photocoagulation

PR - Photoreceptors

PVD - Posterior vitreous detachment

RAGE - Receptor for advanced glycation end products

RANTES - Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted

RBX - Ruboxistaurin

RBZ - Ranibizumab

RCT – randomized clinical trial

RGC - Retinal ganglion cells

RMG - Retinal Müller glial cells

RNFL - Retinal nerve fiber layer

RPE - Retinal pigment epithelium

RNP- Retinal nonperfusion

ROS - Reactive oxygen species

RRD- rhegmatogenous retinal detachment

SCP - Superficial capillary plexus

SD - Square deviation

SDF-1 - Stromal Cell-Derived Factor-1

SD-OCT - Spectral domain optical coherence tomography

SE - Standard error

SFCT - Subfoveal choroidal thickness

SLRP -Small leucine-rich repeat protein/proteoglycan

SND - Subfoveal neuroretinal detachment

SPD - Diabetes Portuguese Society

SRF – Subretinal fluid

SS-OCT - Swept source optical coherence tomography

SSPiM **-** suspended scattering particles in motion

sVAP-1 - Vascular adhesion protein - 1

SVC – Superficial vascular complex

SVN - superficial vascular network

TA - Triamcinolone acetonide

TE - treat and extend

T1D - Type 1 diabetes

T2D - Type 2 diabetes

TCA - Tricarboxylic acid

TGF-α - Transforming growth factor alfa

TGF-β - Transforming growth factor beta

Tie2 - tyrosine kinase with Ig (immunoglobulin) and EGF (epidermal growth factor) homology domains 2

TMV - total macular volume

TNF-α - Tumor necrosis factor alpha

TRP - targeted retinal photocoagulation

TSH - thyroid stimulating hormone

TXNIP - Thioredoxin-interacting protein

UDP- GlcNAc - UDP-N-acetylglucosamine

UKPDS - United Kingdom Prospective Diabetes Study

UWF- Ultrawidefield

VA – Visual acuity

sVAP-1 - Vascular adhesion protein - 1

VE-PTP - Vascular endothelial tyrosine phosphatase

VEGF - Vascular endothelial growth factor

VEGF-A - Vascular endothelial growth factor A

VEGFR1 - Vascular endothelial growth factor receptor 1

VEGFR2 - Vascular endothelial growth factor receptor 2

VCAM-1 Vascular cell adhesion molecule 1

VIT – Vitrectomized

VMA – Vitreomacular adhesion

VMI – Vitreomacular interface

VMT – Vitreomacular traction

VR - Vitreoretinal

VRI – Vitreoretinal interface

WF- Widefield

Wnt – glycoproteins signaling regulation, function and biological outputs

Y – Years

Publications

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2. Bernardete Pessoa, David Afonso Dias, Pedro Baptista, Constança Coelho, Melo Beirão, Angelina Meireles. Vitrectomy Outcomes in Eyes with Tractional Diabetic Macular Edema. Ophthalmic Research, 2018; 1–6. doi:10.1159/000489459.
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4. Bernardete Pessoa, João Coelho, Luísa Malheiro, Diana José, Saúl Pires, Constança Coelho, João Figueira, Angelina Meireles, João Melo-Beirão. Comparison of Ocular Ultrasound Versus SD-OCT for Imaging of the Posterior Vitreous Status in Patients With DME. Ophthalmic Surg Lasers Imaging Retina, 2020; 51:S50-S53. doi: 10.3928/23258160-20200401-07.
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8. Bernardete Pessoa, João Leite, André Ferreira, João Ramalhão, João Poças, João Coelho, Nuno Alves Correia, Constança Coelho, João Figueira, Angelina Meireles, João Melo Beirão. Optical coherence tomography biomarkers - Early prognostic factors in diabetic macular edema treatment with ranibizumab. Submitted.

9. Bernardete Pessoa, João Coelho, Nuno Correia, Natália Ferreira, Melo Beirão, Angelina Meireles. Vitrectomized versus Nonvitrectomized Eyes for the Treatment of Chronic Diabetic Macular Edema Fluocinolone Acetonide Intravitreal Implant 190 µg (ILUVIEN®). Ophthalmic Res 2018; 59:68–75. DOI: 10.1159/000484091.
10. Bernardete Pessoa, André Ferreira, João Leite, João Figueira, Angelina Meireles, João Melo Beirão. Optical Coherence Tomography Biomarkers: Vitreous Status Influence in Outcomes for Diabetic Macular Edema Therapy with 0.19-mg Fluocinolone Acetonide Implant. Ophthalmic Res, 2021. 64:639–647. DOI: 10.1159/000515306.
11. Bernardete Pessoa, Catarina Castro, André Ferreira, João Leite, João Heitor, Pedro Menéres, João Figueira, Angelina Meireles, João Melo Beirão. Changes in ganglion cell layer thickness after treatment with the 0.2 µg/day fluocinolone acetonide implant in vitrectomized and nonvitrectomized eyes with diabetic macular edema. Accepted for publication in Ophthalmic Research. DOI: 10.1159/000520411.
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Overview of the thesis

Despite the fact that this thesis is about diabetic macular edema (DME), it is impossible to isolate it from the context of diabetic retinopathy (DR) and its severity stage. They have a lot in common, not only in terms of risk factors, but also in terms of clinical decision-making. The relevance of the vitreous in DR and its two main causes of vision loss, DME and proliferative diabetic retinopathy (PDR), is undeniable, yet not always adequately recognized. The published literature on DME and DR is increasingly adding new, laboratory, and imaging biomarkers of evaluative stratification, prognostic, and predictive significance.

The vitreous condition and vitreoretinal interface (VMI) will be combined with fresh insights into their significance in the DME approach, among other biomarkers.

There are **six chapters** in the thesis.

Chapter 1 covers the background and fundamental principles of DME, such as the pathophysiology of DR and DME, as well as specific considerations about the retina as a neurovascular unit and its link to the vitreous state. A brief overview of the current state of the art in DR and DME staging, categorization, predictor / predictive imaging and laboratory biomarkers is followed by a discussion of DR and DME therapy options.

The foundation for this thesis, which is based on scientific investigations that have been published, accepted, or submitted for publication, are presented in **Chapters 2.1-2.12**. They were designed to fulfill **3 main purposes**: 1- to enrich the knowledge about **the role of humoral vitreous biomarkers and vítreo-retinal interface (VRI) in the physiopathology and treatment of RD and EMD; 2 – to assess some vitreous changes found in diabetics that may have impact on RD and EMD physiopathology; 3 – to integrate various imaging retinal and choroidal biomarkers in response to EMD treatment, in vitrectomized (VIT) and non-vitrectomized (non-VIT) eyes.**

Chapter 2.1 provides a one-year prospective study that includes laboratory vitreous and blood sample analysis at the time of vitrectomy for tractional DME, highlighting the vitreous' potential as a valuable source of knowledge concerning DR and DME evolution as well as new therapeutical approaches.

In **Chapters 2.2 and 2.3**, two published papers, one retrospective and the other combining a retrospective and prospective phase, aim to show the prognostic and predictive value of removing different sub-types of thickened visible VMI connections that can be easily defined by Spectral Domain Optical Coherence Tomography (SD-OCT).

In **Chapter 2.4**, a cross-sectional study is presented revealing our ignorance of the genuine vitreoretinal interation in patients with DME using standard macular 20x20º SD-OCT, pointing out its limits and inaccuracy in comparison to WF SD-OCT technology.

A retrospective study was published in **Chapter 2.5**, to validate ranibizumab as a non-inferior anti-VEGF choice for the design of one of the major studies considered for this thesis, a one-year prospective study evaluating the effect of IV ranibizumab (RBZ) in several SD-OCT and Optical Coherence Tomography Angiography (OCT-A) predictor and predictive biomarkers for DME treatment, in vitrectomized (VIT) and non-vitrectomized (non-VIT) eyes.

Several analises were then exposed in **Chapters 2.6, 2.7 and 2.8** atwart 3 papers (2 published and one submitted for publication).

Chapters 2.9, 2.10, and 2.11 explore the effects of a long-acting corticosteroid (Iluvien®) on visual acuity and DME regression in VIT and non-VIT eyes, as well as its impact on various OCT retinal biomarkers and the neurodegenerative process. **Chapter 2.12** represents a publication showing five clinical cases where is debated the vitreous status influence, among others biomarkers, in Iluvien® efficacy and the need of supplemental therapy.

Chapter 3 is a comprehensive discussion that includes all of the research conducted for this thesis as well as new therapies on the horizon through vitreous as a vehicle for longer lasting therapies. Finally, based on the dissertation's findings, this chapter concludes with four DME approach algorithms.

The conclusions and future perspectives emerging from the thesis' primary results and thoughts are exposed in **Chapter 4**.

Finally, **chapter 5** contains the list of references of this thesis and **chapter 6** the appendices.

1. Introduction

1.1 DME background - Updated basic concepts

1.1.1 Epidemiology

Diabetes mellitus (DM) is a chronic condition that occurs when there are raised levels of glucose (Fig. 1.1) in a person's blood because their body cannot produce any or enough of the hormone insulin, or cannot effectively use the insulin it produces. Diabetes can be classified into 4 general categories: 1 – type 1 diabetes; 2 – type 2 diabetes; 3 - specific types of diabetes due to other causes, e.g., diseases of the exocrine pancreas (such as pancreatitis and cystic fibrosis), drug- or chemical-induced diabetes (such as with glucocorticoid use, in the treatment of HIV/AIDS, or after organ transplantation) and monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes of the young); 4 - Gestational diabetes mellitus (diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation). The vast majority of cases of diabetes fall into the first 2 types of DM[1]. Type 1 is caused by an autoimmune reaction in which the body's immune system attacks the insulin-producing beta cells of the pancreas. In type 2, hyperglycaemia is the result, initially, of the inability of the body's cells to respond fully to insulin, a situation termed 'insulin resistance'. Type 2 DM accounts for around 90-95% and type 1 for 5-10% of all DM worldwide[2].

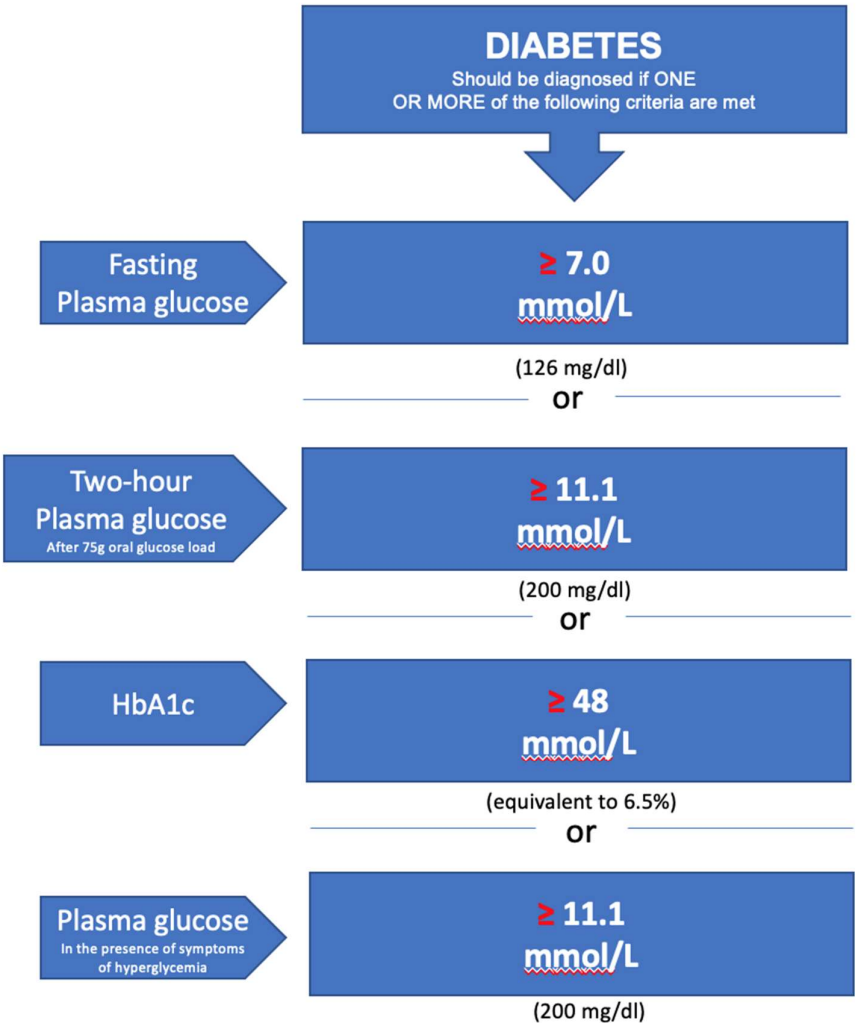


Figure 1.1: Modified diagnostic criteria for diabetes (adapted from IDF Diabetes Atlas, ninth edition, 2019)[2]

Type 1 diabetes, is known to have a strong genetic component, predominantly affecting individuals of European ancestry[3]. In contrast, Type 2 diabetes has racial variations thought to be related to socioeconomic factors and less likely genetic in origin[4].

DM affects mainly 'middle aged working people, usually between 40 and 59 years old, but owing to rising levels of obesity, physical inactivity and inappropriate diet type 2 DM is increasingly seen in children, teenagers and younger adults, with serious personal, social and economic implications[2, 5, 6].

In 2018 the DM prevalence in Portuguese population, estimated ages between 20-79 years (7.7 millions of people), was 13.6%, which means more than one million with DM[7].

Regarding glycaemic control target in DM, although Diabetes Control and Complications Trial (DCCT) and United Kingdom Prospective Diabetes Study (UKPDS) trials observed a favorable effect of an intensive glycemic control ($HbA_1C < 7$ %) reducing the risk of development and progression of diabetic retinopathy (DR), other trials have failed to show a clear benefit of improved glycaemic control for patients with existing DR, which is a believed to be a reflection of a gradual cumulative effect on DR[8-10]. This may represent the long-term benefit from earlier treatment through a mechanism called "metabolic memory"[11].

The precise mechanism for a metabolic memory linked to all diabetic microvascular lesions in addition to other macro-vascular complications although not fully understood, it seems that in the eye, the vitreous may play a major role in this long-term effect, as a potential reservoir of oxidative reactive species (ROS), non-enzymatic glycation of proteins, products of epigenetic changes and inflammation resulting from chronic Hyperglycemia, as later will be discussed.

It also extremely important to stress that according to DPS National Guidelines for the Treatment of Hyperglycaemia in Type 2 Diabetes – Update Proposal (adaptation of the Update 2015 of the Joint Position Statement of American Diabetes Association, ADA / European Association for the Study of Diabetes, EASD) the therapeutic goal should not be to reach strictly a HbA1c of 6.5% or 7% for every patient, as the risk for some patients may overcome the benefits of a perfect metabolic control, namely in older patients, longer DM duration, with cardiovascular pathology or other comorbidities with an increased risk for cardiovascular and hypoglycemia negative consequences, as well in patient-specific psychological, social, and economic conditions, including underlying capacities for self-management. (Fig. 1.2)[12].

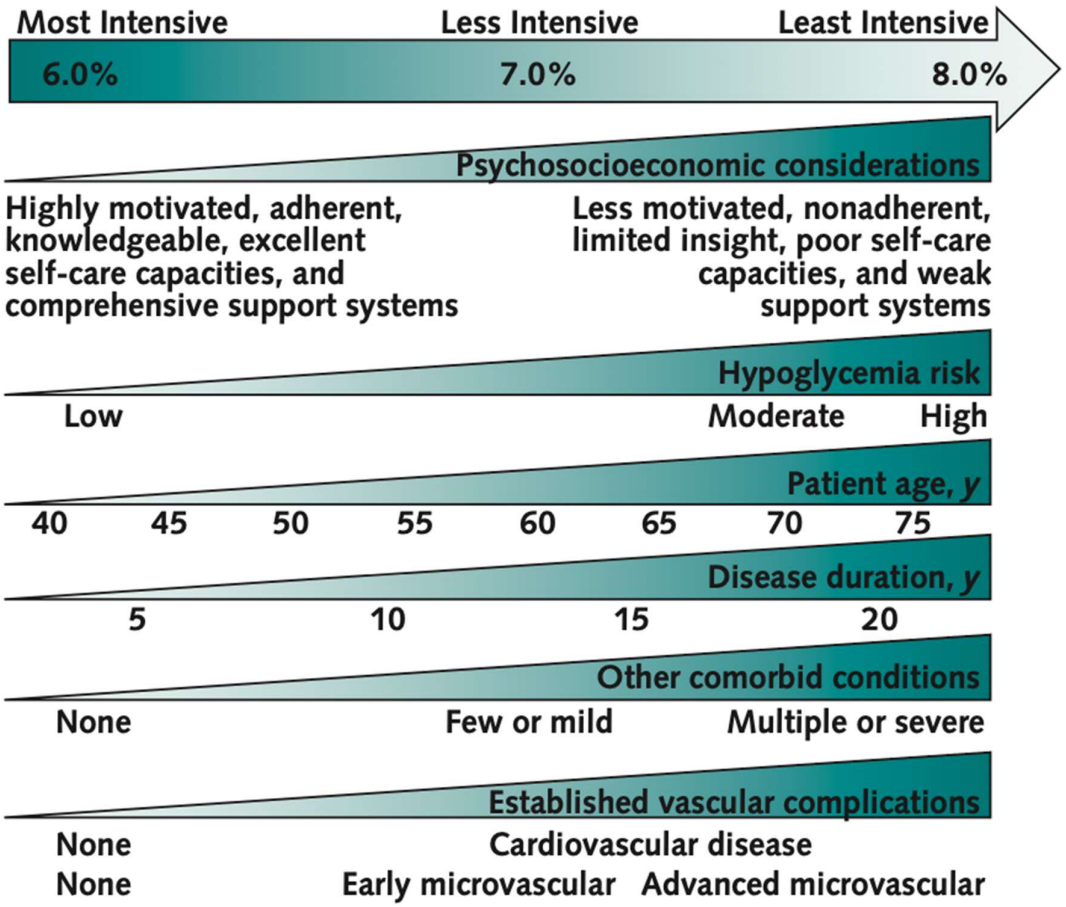


Figure 1.2: A framework for defining glycemic therapy targets in type 2 diabetic patients. Glycemic goals and treatment intensities are depicted in terms of increasing severity or magnitude of clinical factors, as well as with limitations set by the psycho socioeconomic context. Greater height of a triangle indicates increased clinical concern about the considered variable. The depicted targets assume stable outpatient treatment protocols and competent clinical judgment should always be used in these situations[13].

DR is in fact the most frequent complication of DM and is the leading cause of vision loss and preventable blindness among adults aged 20–74 years in the developed world[10, 14-18]. It is estimated that in 2019 DM affected 463 million people worldwide. Thereabout, one third of diabetics have signs of DR and of these one third (10% of all DM patients) have vision-threatening DR, as diabetic macular edema (DME) the main cause of vision loss in DR, which is present in 6-7% of diabetics, and proliferative diabetic retinopathy (PDR) (7.5%)[5, 6, 19].

In accordance with the latest report of the Vision Loss Expert Group of the Global Burden of Disease Study, the prevalence of visual impairment and blindness caused by DR increased substantially between 1990 and 2015[20]. Even though, a decrease in the incidence of blindness related to DR is also explained due to concerted public health efforts including improvement in metabolic control, screening modalities and advances in DR therapy[14, 21, 22].

Studies in the literature have reported that the prevalence of severe DR decreased from 1990 to 2010, while simultaneously the prevalence of diabetes increased, and this implies a reduction in the prevalence of severe DR per person with diabetes[23].

The prevalence of DR and DME in all types of diabetes increases with DM duration and age of the patient DR lesions usually only starts after 3-5 years of systemic disease. After 20 years of Hyperglycemia almost all type 1 DM and more than 60% of type 2 have some degree of DR.

Type 1 DM are prone to have more severe and frequent ocular complications resulting in PDR, the most severe DR form, in opposition to type 2 DM, where a higher percentage of older patients had, instead, visual loss due DME.

DME may be present in any DR severity degree and its prevalence is directly correlated with DR duration and poor metabolic control. DME development seems to be an earlier event in type 2 DM, with a higher incidence in the first 5 years, in opposition to type 1 DM. Furthermore, the need for insulin therapy patients is correlated with the higher rate of DME emergence among all types of DM[16, 24-26].

Based on large-scaled population-based studies and meta-analyses risk factors, duration of DM, HbA1C level/bad metabolic control, hypertension, dyslipidaemia, nephropathy, insulin treatment, type 1 vs. 2 of diabetes mellitus, younger age, shorter axial length (hyperopia) and pregnancy are considered risk factors for DR and DR progression[4, 27-32].

Risk factors for development and progression of DME have usually been assessed together with risk factors for DR. Only few epidemiological studies have been performed so far to specifically investigate risk factors for DME. A poor glycemia control, diabetic nephropathy, dyslipidemia, younger age and poor blood pressure control have been more robustly associated with DME[4, 16, 33-41]. Despite evidence from the cohort studies and meta-analysis of the case-control studies suggesting a strong relationship between lipid levels and DME, this was not confirmed by the meta-analysis that included only prospective randomized clinical trials (RCTs). Hence relationship between lipid levels and DME deserves further investigation[42].

In addition, other modifiable risk factors have been broadly described and associated with DME as anemia, sleep apnea, glitazone usage, and pregnancy[43-51].

Furthermore, type 2 DM with DME and PDR were more likely to have incident and fatal cardiovascular disease compared with those without DME or PDR[52].

Although there are several effective ocular treatments available for DME, a subset of patients does not respond to ocular treatment. Due to multifactorial and systemic nature of DME all the modifiable systemic risk factors referred above should be considered always in the management of DME, particularly in persistent DME cases.

The estimated 20 million patients worldwide having DME[53], the main cause for DR vision loss with particular incidence in active age population, makes this disease a major global health, social and economic problem, dictating an urgent implementation of most effective and precocious screening programs to chase the primary goal which is avoid vision loss and minimize the high burden related with the state of art for DME therapy.

It is so, essential to identify prognostic biomarkers as guides to the prediction risk for a specific result to prevent and avoid end stage conditions and also predictive biomarkers as parameters that can be used to predict the differential result of a therapy or of some special treatment[54].

1.1.2 The essential of Biochemistry in Diabetes

Hyperglycaemia is responsible for a negative lesion cycle, low-grade and chronical inflammation that seems to be mediated by the increased production of mitochondrial superoxide due to high levels of intracellular glucose and oversupply of metabolites from glycolysis into the tricarboxylic acid (TCA) cycle. This causes oversupply of electrons to the electron transport chain of mitochondria with subsequent superoxide overproduction and inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), diverting upstream metabolites from glycolysis into four pathological pathways, the polyol, hexosamine, protein kinase C (PKC) and advanced glycated end-products (AGEs) pathways. ROS and stress oxidation have its own additionally negative role activating cell death pathways such as apoptosis[55-57] (Fig. 1.3)

An important regulator of oxidative stress is nuclear factor erythroid-derived 2-like 2 (NFE2L2) also known as nuclear factor erythroid 2-related factor 2 (NRF2), with anti-inflammatory properties. Several studies suggest that enhancement of the NRF2 pathway is a potential protective strategy for the treatment of DR[58-64].

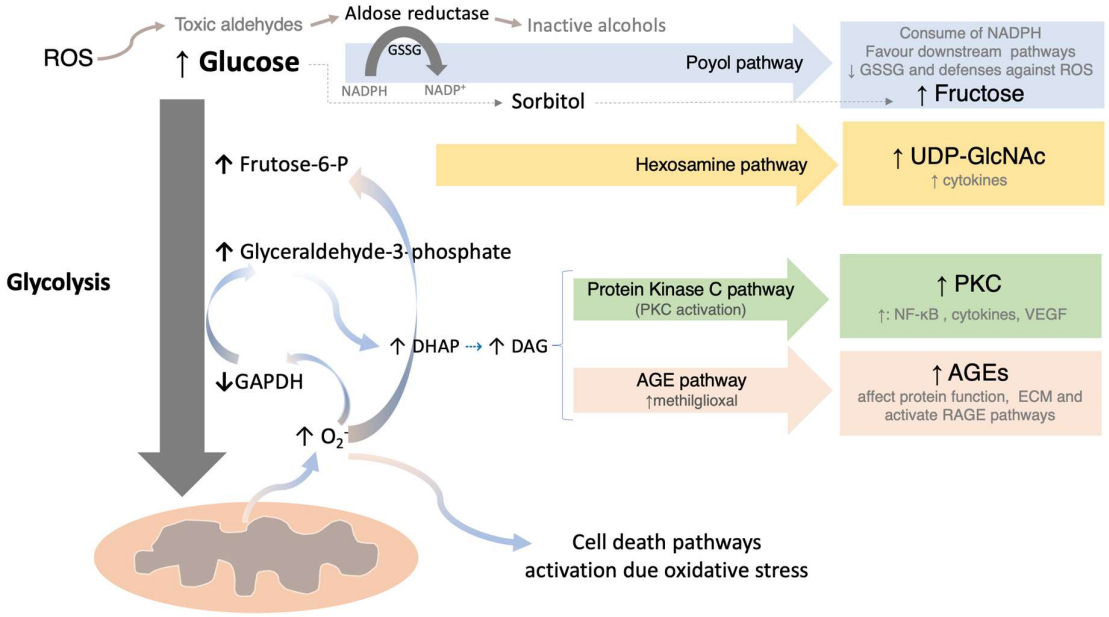


Figure 1.3: Hyperglycaemia-induced mitochondrial superoxide overproduction activates four pathological pathways, the polyol, hexosamine, PKC and AGEs pathways. Data summarized from[55-57]. AGE - advanced glycation end products, DAG - diacyl glycerol, GAPDH - glyceraldehyde phosphate dehydrogenase, DHAP - dihydroxyacetone phosphate, ECM - extracellular matrix, GAPDH - glyceraldehyde-3-phosphate dehydrogenase, GSSG - gluthatione; NADP+ - oxidized nicotinamide adenine dinucleotide phosphate, NADPH - reduced nicotinamide adenine dinucleotide phosphate, NF-κB - nuclear factor - kappa B, PKC - protein kinase C, RAGE - receptor for AGE, ROS - reactive oxygen species, UDP- GlcNAc - UDP-N-acetylglucosamine, VEGF - vascular endothelial growth factor.

The AGE pathway

Oxidized sugars as methylglyoxal (a precursor of AGEs) may attach to proteins forming cross-linked protein compounds irreversibly altered and collectively termed AGEs. This process occurs normally with aging but is accelerated in diabetes.

AGEs may act directly to induce cross-linking of long-lived structural proteins as collagen from extracellular matrix (ECM), changing the structure and function of the vessels promoting their stiffness and vitreous liquefaction, among others changes[65]. In fact, vitreous AGE levels correlate with diabetic retinopathy[66]. It may also interact with certain receptors, such as the receptor for AGE, RAGE (receptor for AGE), to induce intracellular signalling that leads to enhanced oxidative stress and elaboration of key proinflammatory and prosclerotic cytokines[67-69].

The polyol pathway

In this pathway, aldose reductase reduces aldehydes generated by ROS to inactive alcohols, and glucose to sorbitol, using NADPH (reduced nicotinamide adenine dinucleotide phosphate) as a co-factor. With a low affinity for glucose, aldose reductase only converts significant amounts of glucose into sorbitol only under a hyperglycemic background. The consuming of NADPH also decreases glutathione (GSSG) levels, which reduces the cell defenses against ROS. Increases in cytosolic NADH:NAD + (reduced nicotinamide adenine dinucleotide: oxidized nicotinamide adenine dinucleotide).

ratio can additionally result in downstream increases of AGE precursor methylglyoxal and diacyl glycerol (DAG) which activates PKC[56].

Beneficial effects of the angiotensin-converting enzyme inhibitor enalapril and the aldose reductase blocker losartan have been noted in terms of reducing the risk of progression of non-proliferative diabetic retinopathy (NPDR)[70, 71].

The hexosamine pathway

In Hexosamine pathway fructose-6-phosphate is converted into glucosamine-6-phosphate. Inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by superoxide is also a trigger for this pathway activation in diabetes. Enzymatic glycation of certain transcription factors with glucosamine alters the expression of relevant pathological cytokines such as transforming growth factor alfa (TGF-α), transforming growth factor - β1 (TGF-β1) and plasminogen activator inhibitor-1 (PAI-1)[56, 57, 72].

This pathway is also important role in Hyperglycemia induced and fat-induced insulin resistance[73, 74].

The PKC pathway

The inhibition of GAPDH by intracellular Hyperglycaemia and superoxide production also increases production of the metabolite diacyl glycerol (DAG), which is the specific physiologic activator of PKC primarily the β- and δ- isoforms.

Activation of RAGE and increased activity of the aldose reductase pathway may also activate PKC by increasing reactive oxygen species and inhibiting GAPDH.

When activated, PKC represents a key factor in the pathological processes of diabetes by affecting expression of endothelial nitric oxide synthetase (eNOS), endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), TGF-β, and PAI-1, and by activating nuclear factor - kappa B (NF-kB) and NAD(P)H oxidases (Fig. 1.4)[56, 75].

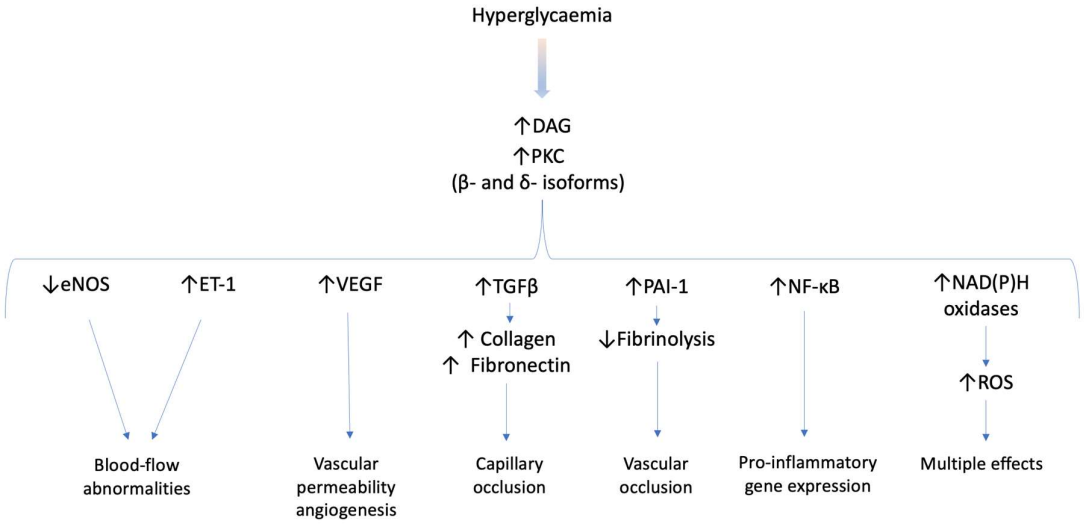


Figure 1.4: Consequences of hyperglycaemia-induced activation of protein kinase C (PKC). Hyperglycaemia increases diacylglycerol (DAG) content, which activates PKC, primarily the b- and d-isoforms. PKC activation has a variety of pathogenic implications by influencing expression of endothelial nitric oxide synthetase (eNOS), endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGF-β) and plasminogen activator inhibitor-1 (PAI-1), and by activating NF-kB and NAD(P)H oxidases and production of reactive oxygen species (ROS) – adapted from Brownlee M.[56]

The isoform-selective PKC β inhibitor ruboxistaurin (RBX) have been investigated in several clinical trials for the treatment of DR and DME, however there has been no clear therapeutic benefit to date[76-78].

1.1.3 Pathophysiology of Diabetic Retinopathy and Diabetic Macular Edema – The importance of the Retina as a neovascular unit

The retina is separated from the systemic circulation by the blood retinal barrier (BRB) and blood aqueous barrier (BAB) and receives its nutritional supply from the retinal and choroidal circulations and perhaps from the ciliary body by diffusion through the vitreous gel - vitreous could act as an ”emergent reservoir” for the retina[79]. The BRB is essential to maintaining the eye as an immune-privileged site and is essential for normal visual function. The BAB is formed by an epithelial barrier located in the nonpigmented layer of the ciliary epithelium and in the posterior iridial epithelium, and by the endothelium of the iridial vessels. The BRB consists of inner and outer components, the inner BRB being formed of tight junctions between

retinal capillary endothelial cells and the outer BRB of tight junctions between retinal pigment epithelial cells[80, 81].

The retina is a multi-layer high specialized tissue (Fig. 1.5 and 1.6) with alternating layers of neurons (outer and inner nuclear layers and ganglion cell layer) interposed with two plexiform layers, where neurons communicate at synapses between dendrites and between axons and dendrites.

It is composed by five major types of cells: the **neurons**, the **retinal pigment epithelium** (RPE), the **glial cells**, **microglia** and the **vascular endothelial cells / pericytes.** (Fig 1.5)

A sequence of 10 retinal layers from the innermost to the outermost layer can be individualized in: 1- the inner limiting membrane (ILM) - the basal membrane of the retinal Müller glial cells; 2- the nerve fiber layer (RNFL); 3- the ganglion cell layer (GCL); 4- the inner plexiform layer, (IPL) - synapses between ganglion cells and interneurons - bipolar, amacrine, and horizontal cells); 5- the inner nuclear layer (INL) - formed by nuclei of bipolar, amacrine, horizontal and Müller cells; 6- the outer plexiform layer (OPL) - synaptic connections between photoreceptors and interneurons; 7- the outer nuclear layer (ONL) - nuclei of cone and rod photoreceptors; 8- the external limiting membrane (ELM) - cellular connections between Müller cells and photoreceptor inner segments; 9- the inner and outer segments (IS-OS) of the photoreceptors (PR); 10- retinal pigment epithelium (RPE). Bruch's membrane (BM) represents in its innermost part the basal membrane of RPE and in its outermost part the basal membrane of the choriocapillaris (the innermost vascular layer of the choroid)[82, 83]. (fig. 1.6)

The **neurons** (photoreceptors, bipolar, horizontal, amacrine, and ganglion cells) are responsible for sensory functions, spatial resolution, color perception, and contrast discrimination[80].

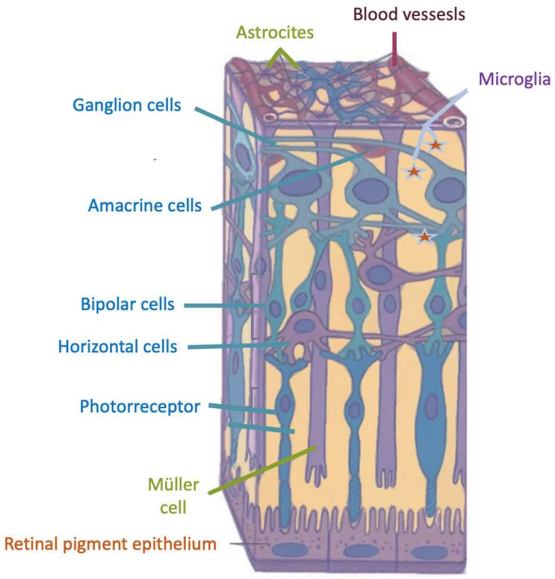


Figure 1.5: The lamellar retinal structure is depicted with Neurons, Glial cells, Microglial cells, Retinal pigment epithelium and Blood vessels. Adapted from Antonetti et al, 2006.[80]

The **glial cells** (Müller cells and astrocytes) - the interface between the neurons and the vasculature, are key regulators of neuronal metabolism and nutrition. Müller cells span the retina from the RPE to the ILM, a basement membrane formed by Müller cell end-feet that interfaces with the vitreous gel.

The **microglia** - resident macrophages which interact with neurons, glia, and endothelium. They become activated under stresses as infection, trauma, or retinal detachment, by release of proinflammatory cytokines and clearance of necrotic or apoptotic cells via phagocytosis, to resolve local injury[84-87].

RPE – phagocytes and removes toxic products, as shed photoreceptor outer segments and retinal lactic acid; reduces phototoxic damage to the retina absorbing light; participates with the PR in the cycling of the vitamin A isoforms retinol and retinal; allows oxygen diffusion from the choroidal circulation to the outer retina; secretes trophic factors such as pigment epithelium derived factor (PEDF). It is crucial in reconstitution and maintenance of the BRB[80, 88].

Vascular endothelial cells and pericytes - nutritional support and waste product removal of the inner retina.

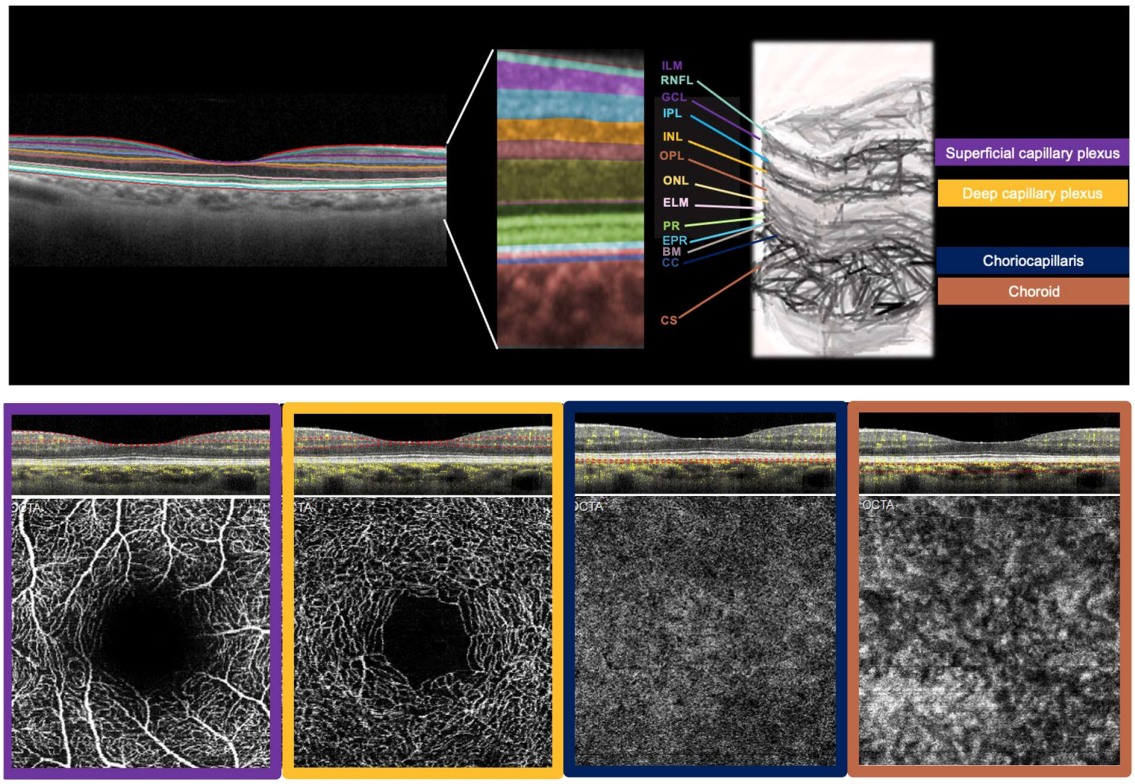


Figure 1.6: Representation of the multi-layer high specialized tissue of the retina and choroid through cross-sectional images in a near-histological level through spectral domain optical coherence tomography (SD-OCT) and OCT angiography (OCTA) (Spectralis HRA + OCT, version 1.10.2.0; Heidelberg Engineering, Germany). In the left side of the upper image: SD-OCT depiction of the retinal and choroidal layers from the innermost to the outermost layers : 1- the inner limiting membrane (ILM) 2- the nerve fiber layer (RNFL); 3- the ganglion cell layer (GCL); 4- the inner plexiform layer, (IPL); 5- the inner nuclear layer (INL); 6- the outer plexiform layer (OPL); 7- the outer nuclear layer (ONL); 8- the external limiting membrane (ELM); 9- the inner and outer segments of the photoreceptors (PR); 10- retinal pigment epithelium (RPE); 11- Bruch's membrane (BM); 11- choriocapillaris (CC); 12- choroid (CS – choroidal stroma). The bottom images are OCTA images from left to right, of the superficial vascular complex, deep vascular complex, choriocapillaris and choroidal vascular tissue.

The retina is vascularized by two independent vascular beds, the retinal and choroidal vasculatures. The larger retinal vessels, branches of the central retinal artery and vein, lay below the ILM, and are surrounded by astrocytes, pericytes and Müller cells. Between pre-capillary arterioles and post-capillary venules, the retinal capillary network is arranged in three plexus layers: the superficial (located in the nerve fiber and ganglion cell layers), the intermediate (at the inner border of the IPL) and the deep (at the inner and outer border of the INL)[89, 90].

Some authors have considered the retinal vasculature to consist of 2 layers: superficial and deep, with the latter including both layers flanking the INL (considered intermediate and deep plexuses by Hwang et al and Park et al)[91-93]. Bonnin et al[91] proposed a model that could explain the differences in flow resistance and perfusion between the two plexuses, the superficial capillary plexus (SCP) and the deep capillary plexus (DCP). (Fig. 1.7).

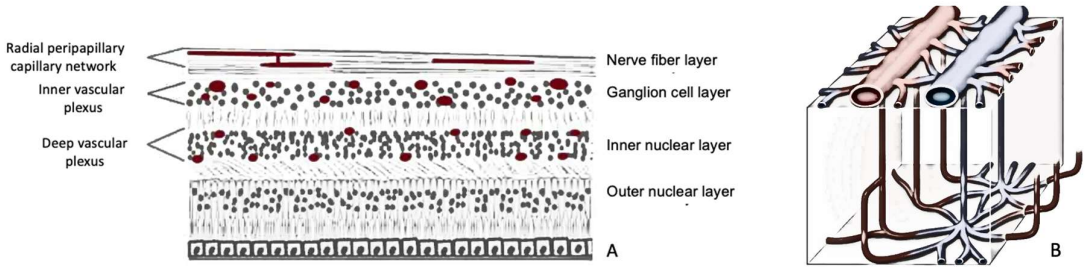


Figure 1.7: - Vascular layers in the retina. In panel A the vessels are represented in reddish color. The radial peripapillary capillaries course is parallel to the nerve fibers. Panel B is a representation of a simplified model proposed by Bonnin et al. for the relationship between the superficial vascular network, SPD (made up of long, horizontal arterioles and venules that originate from the superior and inferior arcades around the foveal avascular, with transverse capillaries connecting the arterioles and venules to form an interconnected plexus) and deep capillary plexus (DCP). The DCP, composed of polygonal units, in which the capillaries converged radially toward an epicenter, the capillary vortex, then drain into the superficial venules of SCP. Panels A and B were adapted from Spaide et al, 2015, and Bonnin et al, 2015, respectively[91, 93].

As an example, the most recent German SD-OCT, Spectralis HRA + OCT, version 1.10.2.0; Heidelberg Engineering, Heidelberg, includes in the segmentation for the superficial vascular complex (SVC) the nerve fiber layer vascular plexus in addition to the SVP, and for deep vascular complex (DVC) the intermediate capillary plexus and the DCP. (Fig 1.8)

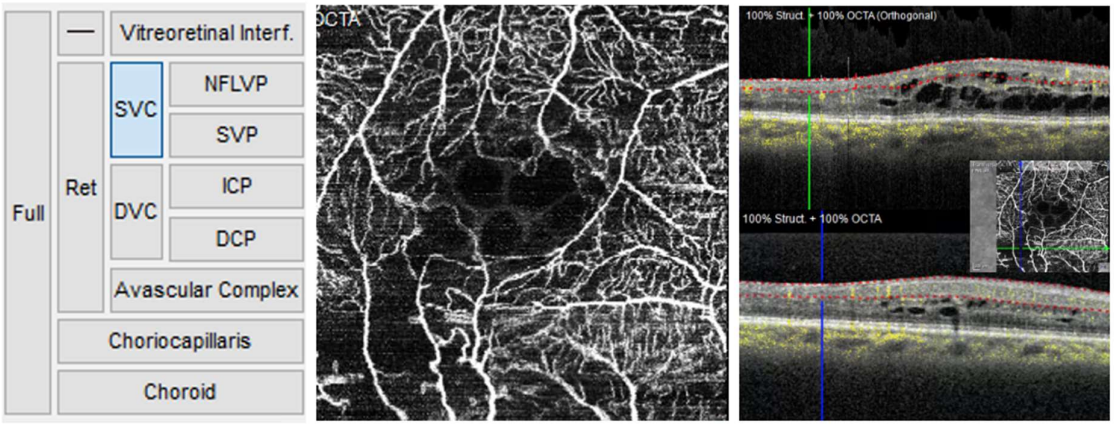


Figure 1.8: The display of segmentation through optical coherence tomography angiography (OCTA) mode (Spectralis HRA + OCT, version 1.10.2.0; Heidelberg Engineering, Germany). Left image: highlighted in blue, is the chosen segmentation, the superficial vascular complex (SVC), which includes the nerve fiber layer vascular plexus (NFLVP) and the superficial vascular plexus (SVP). In the middle is the OCTA slab which corresponds to the SVC. On the right are two different OCT B-scan angiograms identified by the selected cuts, blue and green lines, respectively, representing the structural OCT and functional imaging (OCT-A) in an overlapped mode.

Despite the fact that blood vessels are the only structures visible on clinical examination, they account for less than 5% of total retinal mass, while neurons, glial cells, and microglia account for over 95%[94].

The neurons, glia, and microglia are metabolically linked, neuroglial cells generate vision and blood vessels provide the nutrients.

The unique retinal structure to optimize light delivery to neuro retina system avoiding its absorption through relatively low density of blood vessels and few mitochondria (riches in heme compounds) determine the relatively low oxygen tension of the inner retina. Thus, the inner retina depends heavily on glycolysis, a less efficient generating energy process in comparison with oxidative phosphorylation, which predominates in the outer retina[95, 96].

The combination of high metabolic demand and minimal vascular supply limits the inner retina to adapt to the metabolic stress of diabetes[97]. By contrast, the outer retina receives its oxygen and nutrients by diffusion from the choroid through the RPE which is relatively spared from the early insults of diabetes[80].

Nevertheless, neural outer retinal rod photoreceptor dysfunction has been detected through dark adaptation tests in patients without DR[98, 99].

There are many other evidences suggesting that neural retina is affected by diabetes before vascular changes and implicating neurodegeneration as an early event in the pathogenesis of

DR. In fact, retinal ganglion cells (RGCs) seem to be the earliest cells affected having the highest rate of apoptosis[100, 101].

It has been demonstrated that there is a higher rate of thinning of the RNFL and GCL/IPL in patients with no to minimal NPDR compared with that observed in healthy eyes related with aging[102-106]. This is also corroborated by abnormalities detected in neuro retinal tests - electrophysiology tests (mfERG and P ERG); frequency doubling technology (FDT) perimetry (for inner retinal function); dark adaptation, loss of color discrimination and contrast sensitivity (for outer retinal function) - that may precede or predict vascular abnormalities[99, 107-112].

In the retina, glial, neural, and vascular cells are closely linked creating a 'neurovascular unit' closely associated by metabolic synergy and paracrine communication, bolstered by vitreous cavity which acts as a deposit of pathogenic/inflammatory, neuroprotector/anti-inflammatory molecules mostly produced by the retina, imbibing the retina itself (Fig. 1.9) This neurovascular unit, closely interacting with vitreous and vitreous-macular interface, exists not only in health but also in disease[94, 113].

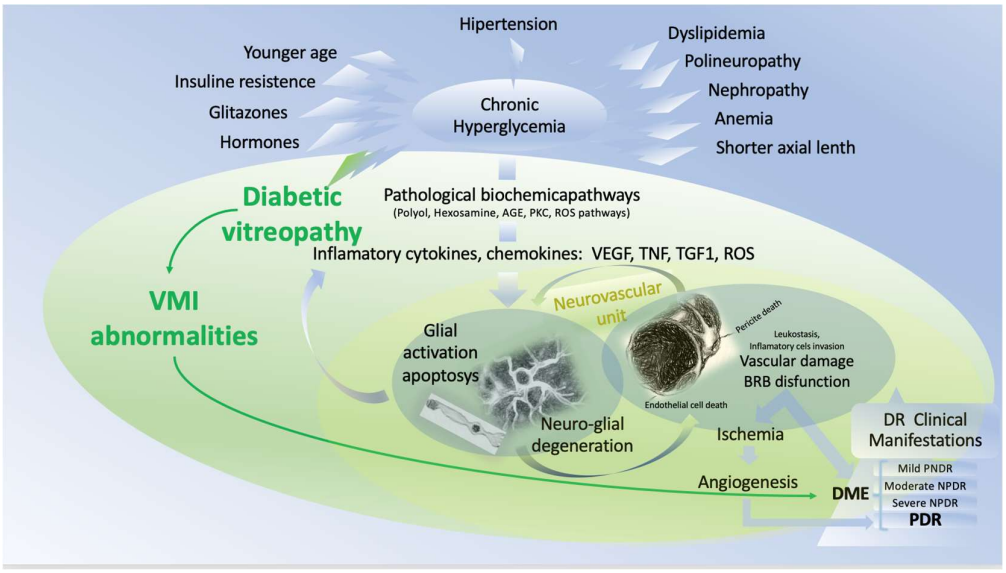


Figure 1.9: The activation of the pathological pathways induced by hyperglycaemia compromises the normal pro-blood retinal barrier stimuli from neural retina to blood-derivate elements to invade the retina and damage neural cells inducing a process of immune cell activation, inflammatory cell infiltration, cytokine and chemokine expression, and diverse cell type lesions, including neural and vascular cells. A feed-forward cycle of combined vascular and neural damage leads to diabetic retinopathy (DR) clinical manifestations and ultimately to impaired vision. In the retina, glial, neural, and vascular cells are closely linked creating a 'neurovascular unit' closely associated by metabolic synergy and paracrine communication, bolstered by the vitreous which acts as a deposit of pathogenic/inflammatory, neuroprotector/anti-inflammatory molecules mostly produced by the retina, imbibing the retina itself. Other systemic risk factors for DR manifestations are also represented in the upper part of the figure.

The normal neural cells produce essentials factors for the maintenance of a local biochemical environment and a normal BRB, which protects the retina from circulating antibodies, inflammatory cells, and amino acids[80, 102].

Hyperglycaemia-induced mitochondrial superoxide overproduction and the activation of the pathological pathways compromises the normal pro-BRB stimuli from neural retina to blood-derivate elements to invade the retina and damage neural cells inducing a process of immune cell activation, inflammatory cell infiltration with macrophages and leucocytes, cytokine and chemokine expression, and diverse cell type lesions, including neural and vascular cells (as pericyte and endothelium cells). A feed-forward cycle of combined vascular and neural damage ultimately leads to impaired vision. Vascular damage is represented by capillary occlusion (retina ischemia), increased permeability (DME) and reactive neovascularization.[80, 102, 114, 115]. (fig.1.9)

At initial stages, the edema is predominantly intracellular, as a result of cytotoxic damage of the Müller cells and of other neuronal cells (**cytotoxic edema**, resulting from a neurodegeneration process) with no visible retinal vascular dilations. As the disease progresses, the breakdown of the BRB results in extracellular **vasogenic edema** - retinal thickening with visible retinal vascular abnormalities (microaneurysms, dilated capillaries).

There is another type of edema, also related with chronic hyperglicemia, diabetic vitreopathy and retinopathy - the tractional DME, when there is an OCT-detectable macular traction, including the epiretinal membrane, partial vitreous detachment with focal traction, and taut and thickened posterior hyaloid membrane. When there is an OCT-detectable traction associated with vasogenic or nonvasogenic edema, that is classified as mixed DME[83, 116, 117]. That explains why macular edema can occur independently of the severity of DR[83].

Furthermore, the background of DME as a multifactorial and systemic disease also can enhance the inflammatory, ischemic, pro-oedematous and angiogenic ocular state induced by Hyperglycaemia itself. There are other concomitant factors that can aggravate endothelial disfunction/lesion with subsequent ischemia, local inflammatory responses, cytokine and VEGF upregulation, such as increased hydrostatic vascular pressure induced by high-blood pressure; nephropathy through EPO production reduction and subsequent anaemia; dyslipidaemia; glitazones, placental or puberty hormones, as well as insuline resistence. Moreover, all haemodynamic factors that cause a decrease in vascular osmotic pressure (as proteinuria due to nephropathy), an increase in hydrostatic pressure (HBP), or a greater difference between the hydrostatic pressure in the blood vessels and the retina tissue (as vitreomacular traction, VMT), will make DME management more difficult[29, 30, 35-40, 47-51, 118-134]. (Fig 1.9)

The importance of the existence of other target organs of diabetes-induced damage beyond the retina (DR), such as kidney (diabetic nephropathy, DN), peripheral nerves (diabetic

polyneuropathy), heart (ischaemic heart disease, cardiomyopathy, congestive heart failure) and peripheral arteries (peripheral artery disease) further reflects the complexity of the physiopathology of this disease. An example is the diabetic autonomic neuropathy (DAN) and DN[135].

Systemic EPO (which role in DR will be further discussed in detail in chapter 3) is mainly produced by peritubular fibroblasts, epithelial distal tubular cells, collecting tubules and glomerular cells, and its release is thought to be modulated by the splanchnic innervation of the kidneys. Both DN and DAN (which can occur even early than DN) can cause the EPO deficiency observed in these patients, due to efferent sympathetic denervation of the kidney and/or direct damage to the EPO-producing fibroblasts.[136, 137].

In addition to DR, there are other effects of DM in eye structures correlated directly or indirectly with vascular and neurodegenerative lesions, such as ischemic optic neuropathy, cranial nerve palsies, recurrent corneal erosion syndrome, cataract, glaucoma and vitreopathy manifestations[138].

1.2 Vitreous: from health to diabetic vitreopathy

The vitreous is the largest structure within the eye occupying about 80% of the ocular volume[139]. It is a highly hydrated transparent extracellular matrix containing 98-99% of water[140]. Its gel structure is maintained by a 3-dimensional network of randomly spaced, nonbranching collagen fibrils, held apart by hyaluronan (HA) and other macromolecules[141].

Although the collagen concentration is low, approximately 300 µg/ml, type II collagen being the main component, it is the collagen fibrils that imparts gel-like properties to the vitreous[142, 143].

Type II collagen combines with types IX, and V/XI collagen to form fibrils. It is believed that collagen fibrils are spaced apart by hyaluronan via interaction with the chondroitin sulfate chains of type IX collagen[144]. (Fig. 1.10)

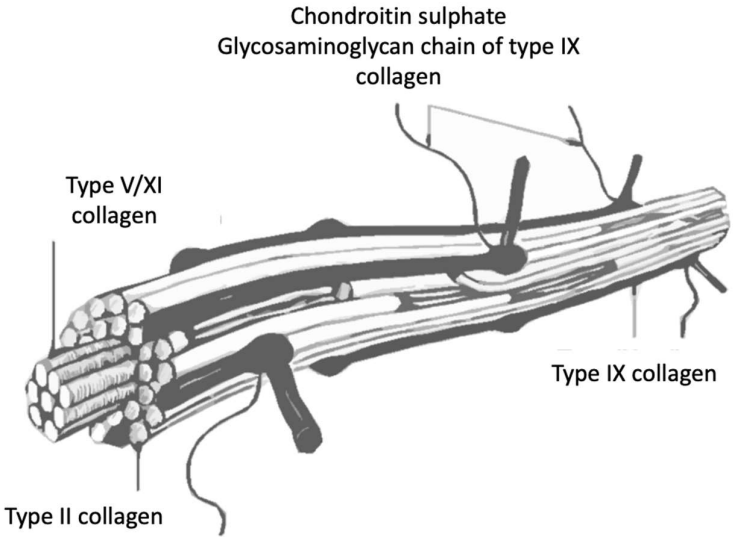


Fig 1.10 A schematic representation of the structure of collagen fibrils in vitreous. Adapted from Bishop, 1996.[140]

HA provides the viscoelastic properties to the vitreous[145-147]. It is mostly acellular, with the exception of the scant number of hyalocytes and phagocytic type cells, more concentrated in the vitreous base and adjacent to the posterior pole[145, 148-150].

The distribution of collagen within the eye is not uniform. Vitreous structure in adults is characterized by macroscopic fibers with an anteroposterior orientation[148].

The highest concentration is present at the vitreous base followed by the posterior vitreous cortex, and the vitreous core[142].

Collagen fibrils of the vitreous cortex are organized in a lamellar type structure, with stronger adhesions between the posterior vitreous cortex and ILM, mainly at the vitreous base and posterior pole, in a fascial mode, and also at the disc, fovea, and along retinal blood vessels, with a more focal type of adhesion[142, 151-153].

The network of collagen fibers provides mechanical strength to the vitreous, allowing it to sustain impacts, and transmit tractional forces to the retinal surface[140].

The dense matrix of collagen fibrils of posterior vitreous cortex are attached to the retina via tight attachment mediated by ECM proteins, mainly fibronectin and laminin[154-156].

The VMI represents a complex formed by ILM, the posterior vitreous cortex, and the aforementioned ECM that is thought to be responsible for vitreoretinal adhesion. The ILM of the retina, formed mainly by the basal lamina of Müller cells, is composed of collagen types I and IV, proteoglycans, fibronectin, and laminin[152, 157-160]. (Fig. 1.11)

The ILM continuous over the entire surface of the fundus, is thinnest at the vitreous base and over the disc and fovea[161-163].

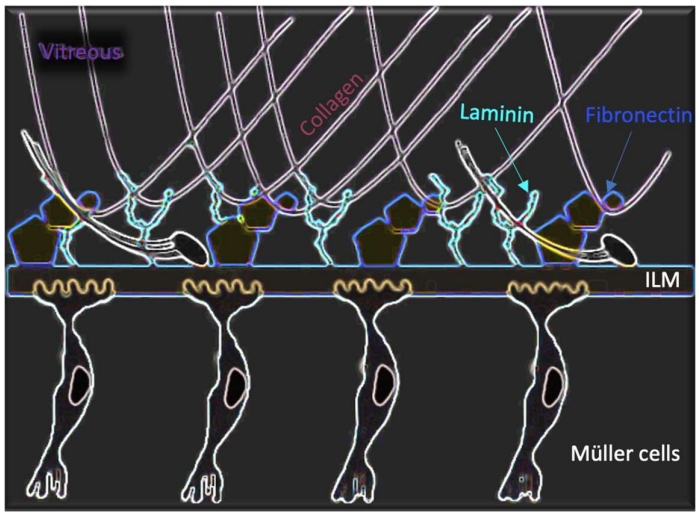


Figure 1.11: Representation of the vitreous-macular interface (VMI), formed by the inner limitant membrane (ILM), the posterior vitreous cortex, and the extracellular matrix. The ILM, formed mainly by the basal lamina of Müller cells, is composed of collagen types I and IV, proteoglycans, fibronectin, and laminin. Adapted from Sebag 2014[164].

Thanks to modern proteomic techniques many hundreds of vitreous proteins could be identified and quantified, including many of the signalling pathways implicated in DR[165-167]. Non-collagenous extracellular glycoproteins have important roles in many cell-surface interactions, such as adhesion, migration, phenotype differentiation and polarization, wound healing, tumour-cell invasion, and metastasis[168].

Human vitreous is normally anti-angiogenic as it contains proteins/glycoproteins that inhibit angiogenesis such opticin, thrombospondins and pigment epithelium-derived factor (PEDF). Vitreous collagen contributes to preretinal neovascularization in two ways: providing mechanical support for the growth of new blood vessels and stimulating angiogenesis through sustained signalling via the collagen binding integrins (including α1β and α2β1 integrins) expressed on endothelial cells (ECs) leading to an initial and critical change in cell shape, contractility, and polymerization and arrangement of cytoskeletal actin into stress fibers[146, 169-173].

Hyalocytes syntheses not only vitreous components such HA, collagen fibrils, fibronectin but also other molecules as VEGF, involved in DR pathological process (PDR and DME)[174].

Opticin

Opticin is a member of the ECM, SLRP (small leucine-rich repeat protein/proteoglycan) family and is the only member of this family of molecules that has been identified conclusively in vitreous[175, 176]. In the eye, opticin is secreted by the posterior non-pigmented ciliary epithelium during development, and high level expression is maintained in the adult eye, which is not common for other vitreous ECM molecules[177-179].

Opticin coats the collagen fibrils, weakens integrin-mediated endothelial cell adhesion to the collagen and inhibits the outside-to-inside integrin-mediated signalling that is required for angiogenesis[146, 180].

Thombospondins

Thombospondin-1 and trombospondin-2 are glycoproteins that act as potent endogenous inhibitors of angiogenesis. They inhibit angiogenesis through direct effects on endothelial cell migration, proliferation, survival, and apoptosis and by antagonizing the activity of VEGF. They exert their direct effects through CD36, CD47, and integrins[181, 182]. In diabetic vitreous, thrombospondin-1 levels have been reported to be lower, predisposing to PDR[183].

Pigment Epithelium-Derived Factor

It has multiple biological activities: neuroprotective and neurotrophic; it can regulate cell survival, differentiation and migration and has anti-angiogenic properties[184]. Overall levels of PEDF are not altered in diabetic vitreous. Even though, the shift to a higher molecular weight isoform was suggested to be the reason for a shift a pro-angiogenic shift[183].

1.2.1 The effect of vitreous in diffusion and fluid currents

Diffusion and fluid currents are slower in a highly versus less viscous mediums as happens in an eye with vitreous versus a vitrectomized (VIT) eye, where viscous vitreous gel is replaced by water. That applies to all diffusing molecules, both beneficial and potentially harmful nutrients, drugs, oxygen, growth factors, and other cytokines[185, 186].

1.2.2 The importance of vitreous in oxygen physiology and DR

The vitreous gel has antioxidants that act as free radical scavengers mitigating the nefarious effects of elevated oxygen levels. The ascorbic acid-oxygen reaction, where both oxygen and ascorbate are consumed is a classic example of that[187].

After vitrectomy, in vitreous cavity, oxygen diffuses from the anterior segment to the retina (when lensectomy is added), it is transported more effectively from well perfused to ischemic

areas of the retina[188, 189]. The oxygen gradients flatten out and more oxygen is transported to the posterior pole of the crystalline lens, which functions as an additional stimulus for cataract development[190, 191,188, 192-204].

Hence, hypoxia in the anterior segment in a VIT eye leads to oxygen diffusion away from the anterior segment towards the retina faster, especially if the lens is also absent, and that may contribute to iris neovascularization[188, 205].

In a low-viscosity fluid as a VIT eye, or an eye with posterior vitreous separation, the hypoxic areas of the retina receive extra amounts of oxygen from vitreous cavity, thus reducing growth factor production. In addition, the clearance of growth and inflammatory factors from the retina is also enhanced. This may help to explain why retinal neovascularization stops after vitrectomy, potentiating panretinal photocoagulation (PRP) effect[185, 206].

In addition, patients with DR have a decreased oxygen supply to the inner retina and the retina is the source of most of the oxygen in the vitreous body, it seems likely that diabetic patients would have decreased levels of oxygen in the vitreous body[207-210]. Which may explain their lower incident of surgery for post vitrectomy nuclear sclerotic cataract and a lower prevalence and incidence of nuclear sclerotic cataracts[211-213].

It is also important to highlight that in the diabetic eye approach there is still controversy, both clinically and experimentally, regarding the vitrectomy role in open-angle glaucoma development, in non-aphakic eyes[214-218].

Moreover, it is also possible to enhance artificially vitreous viscosity to deal with a neovascular glaucoma. Silicone oil does appear to act as a diffusion/convection barrier to oxygen and may alter the stimulus for anterior segment neovascularization[219]. Silicone, 1000-cp oil will not change the diffusion characteristics dramatically from vitreous gel, whereas 5000-cp oil will slow down diffusion compared with vitreous gel[185].

1.2.3 The role Vitreous and VMI abnormalities in DR and DME

Glucose is usually lower in the vitreous than the serum but has been found to be higher in diabetics, with increased levels of an excess of 11 mmol/L[220].

DM affects the extracellular matrices of connective tissue through the thickening of the basement membrane of the retinal vasculature as a result of an accumulation of collagen[221, 222].

Because the vitreous is principally an extracellular matrix in which collagen is an important component, the increased nonenzymatic glycation of vitreous collagen and its cross-linking by AGEs is believed to underlie the observed structural and functional alterations in human diabetic vitreous[65, 223-225].

Glycation of the HA protein core appears to contribute to depolymerization and dissociation of HA from collagen, resulting in liquefaction not just in aging but also in diabetes. [226, 227].

These biochemical abnormalities induce premature liquefaction, earlier anomalous PVD and vitreoschisis which is corroborated by their highly prevalence in diabetic patients[224, 228-232].
An anomalous PVD occurs when liquefaction is not concurrent with dehiscence at the vitreo-retinal interface leading to vitreo-macular traction or vitreo-papillary adhesion[231].
Vitreoschisis may be instead considered a form of anomalous PVD, corresponding to a split in the posterior vitreous cortex, leaving the external layer attached to the macula, while the remainder of the vitreous collapses forward[233].
These vitreous and VMI abnormalities lead to formation of pockets of liquefied vitreous. These pockets, particularly important when located on the pre-macular area, may promote the accumulation of cytokines, VEGF, advanced glycation end products and other inflammatory molecules, enhancing their negative effect upon the macula[113]. (Fig. 1.12)
The ECM responsible for the tight attachment of posterior vitreous cortex to the retina is abnormally thickened in diabetic patients. Retinal glia, RPE, and fibroblast-like cells were largely recognized as components in the intraocular fibrocellular tissue in human PVR are known to be capable synthesis of fibronectin and laminin in vitro[154-156].
Increased amounts of fibronectin, laminin, type I and type IV collagen, as well as fibrocellular tissue in the ECM of VMI of diabetics are due not only to local cell production (in retinal glia, RPE, and fibroblast-like cells), but also to increased breakdown of the BRB, which contributes to the accumulation of some of these components, with additional plasmatic source (as fibronectin and vibronectin)[159, 234].
Moreover, the infiltration of the posterior vitreous cortex with glial and inflammatory cells induces a taut thick posterior vitreous cortex which further become a barrier to retinal oxygenation that is tethered by neovascular tufts with increased vitreoretinal adhesion contributing further to VMT, abnormal PVD, vitreoschisis, complex preretinal membranes, scaffolds for retinal fibrovascular proliferation and tractional retinal detachment genesis . (Fig 1.12)

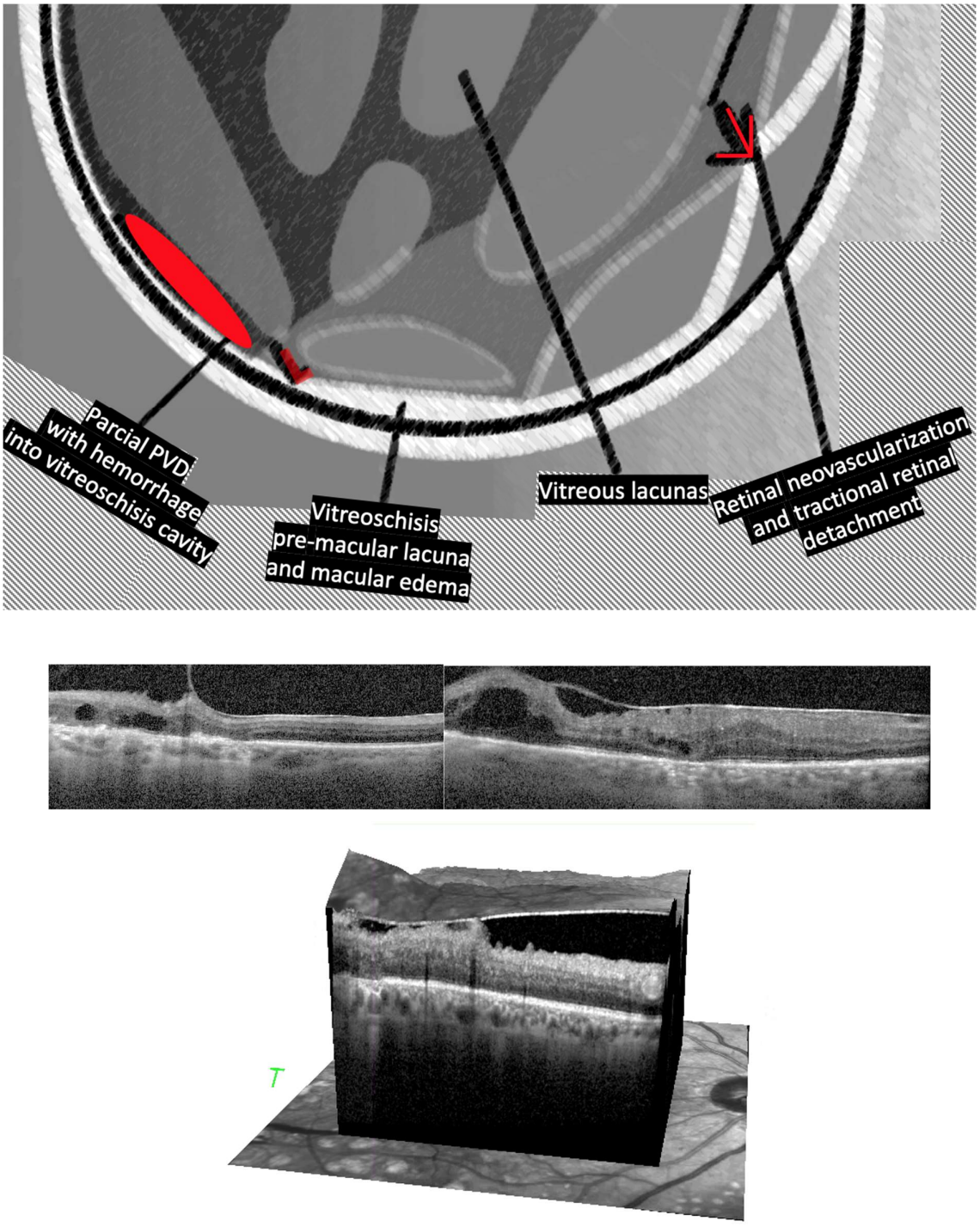


Figure 1.12: The upper image is the representation of infiltrated posterior vitreous cortex with glial and inflammatory cells, promoting the formation of taut thick posterior vitreous cortex, a scaffold for neovascular tufts contributing to vitreomacular traction (VMT), abnormal posterior vitreous detachment (PVD), vitreoschisis and complex preretinal membranes (PRM), which may facilitate a tractional retinal detachment genesis. The three bottom spectral domain optical coherence tomography images (SD-OCT from Spectralis HRA + OCT, version 1.10.2.0; Heidelberg Engineering, Germany) are a practical example from a patient, with PDR VMT and complex PRMs.

These proliferations in the macular area induce retinal traction and act as an additional contributor to the development of DME[113, 235].

The abnormal thickened VMI leads to an additional metabolic barrier between retina and vitreous. Overall, these mechanic factors related to traction forces cannot be eliminated with the effect of intravitreal (IV) anti-VEGF or corticosteroid therapy, thus being responsible for pharmacological resistance to those treatments in a subpopulation of DME patients[236, 237].

Supporting the evidence based on all these anatomopathogical diabetic vitreous and VMI retinal changes there are other several clinical reports supporting the relationship of the posterior cortical vitreous to the macula playing a role in the development of DME[142, 163, 238-241].

Additionally, some authors have highlighted that vitrectomy and the presence of posterior vitreous detachment (PVD) have a positive effect on the evolution of DME[163, 238-247].

Chronic diffuse macular edema might also be related to extrafoveal vitreoretinal traction[248, 249].

Schulze and colleagues suggested that VMA/VMT induces a cut-off from blood supply in the macula, leading to ischemia-induced vascular endothelial growth, thus VMA/VMT removal would improve capillary macular flow[250].

As reflected above, a simply attached cortical vitreous implies a gel-like vitreous with low intraocular oxygen, allowing VEGF levels and other inflammatory molecules implicated in DME to be high[185].

Even though, there is still many controversial regarging the real effect of VMA/VMT, type of VMA adhesion (broad or focal anterior-posterior or tangential), vitrectomy effect or type of vitrectomy approach, with or without ILM peeling when dealing with DME[251-255].

As previously discussed, the vitreous, via several mechanical and physiological mechanisms that lead to increased vascular permeability, has been implicated as a cause of DME.

Although vitrectomy has been consensually indicated and to be effective in the resolution of DME when an evident tractional cause is involved in its pathogenesis[53, 243, 246, 256-261].

That benefit is not that clear or consensual when a tractional component is not evident[245, 262-277].

The main controversies related with vitrectomy with ILM peeling in nontractional DME lay in its transient benefit (of near 6 months) because it may induce macular atrophy (CFT<220 µm and a worse BCVA) and due to its invasive nature, although with low rate of complications[262, 264, 265, 268, 273-275].

Iglicki et al, in a multicenter study including a reasonable number of non-tractional naïve DME cases (n=120), with at less than 12 months of DME duration and 24 months of follow-up after PPV with ILM peeling, observed a significant functional and anatomical improvement of DME 24 months after primary PPV, without the need for additional treatment. Neverthless, a significant number of macular atrophies were observed (35.8%, n=43 eyes) and there was no group control for comparison[264].

Regarding post-PPV macular atrophy, Romano et al, suggested, based in their study, that the risk of macular atrophy is higher when DME is associated with intraretinal cysts larger than 390 mm, and that vitrectomy with ILM peeling should be not be recommended in those cases[268].

The rational for ILM peeling in DME (most widely applied when a tractional DME exists) is to eliminate all traction, vitreous remnants, inflammatory factors, the scaffold for epiretinal membrane recurrence[278].

Several studies have reported reductions in GCL thickness following ILM peeling, which was associated with worse postoperative BCVA[279-282].

However, other studies have assessed postoperative changes in GCL thickness in the eyes with or without ILM peeling during pars plana vitrectomy[283, 284].

Overall, these mechanic factors related to traction forces cannot be eliminated with the effect of intravitreal (IV) anti-VEGF or corticosteroid therapy, thus being responsible for pharmacological resistance to those treatments in a subpopulation of DME patients[236, 237].

The force of vitreoretinal traction will be met by an equal and opposite force in the retina, and this tends to pull the tissue apart, resulting in a lowering of the tissue pressure in the retina. The lowered tissue pressure increases the difference between the hydrostatic pressure in the blood vessels and the tissue and contributes to edema formation according to Starling's law[185, 285].

Even-though the relevance of vitreous macular status is of unquestionable relevance in DME its real status is not always clear. The variability in the thickness of the walls of the vitreoschisis cavity may, in many cases, be below the level of resolution of the method used to define the vitreous structure[141].

1.3 Staging and Classification of DR and DME

The main purpose of DR and DME Staging, and Classification is to perform a feasible, practical and an accurate clinical diagnosis with prognostic and predictive value[54, 286].

Early treatment diabetic severity scale (ETDRS) - diabetic retinopathy severity scale (DRSS) is the current standard for assessing DR severity in RCTs through 7 estereoscopic color fundus photos to document vascular lesions. This standard classification system, known as the Airlie House Classification, was established by a group of experts in 1968 and was modified for use in the ETDRS grading system (fig.1.13) This classification system although accurate and reproducible requires labor analysis that has limited its clinical applicability[287-290].

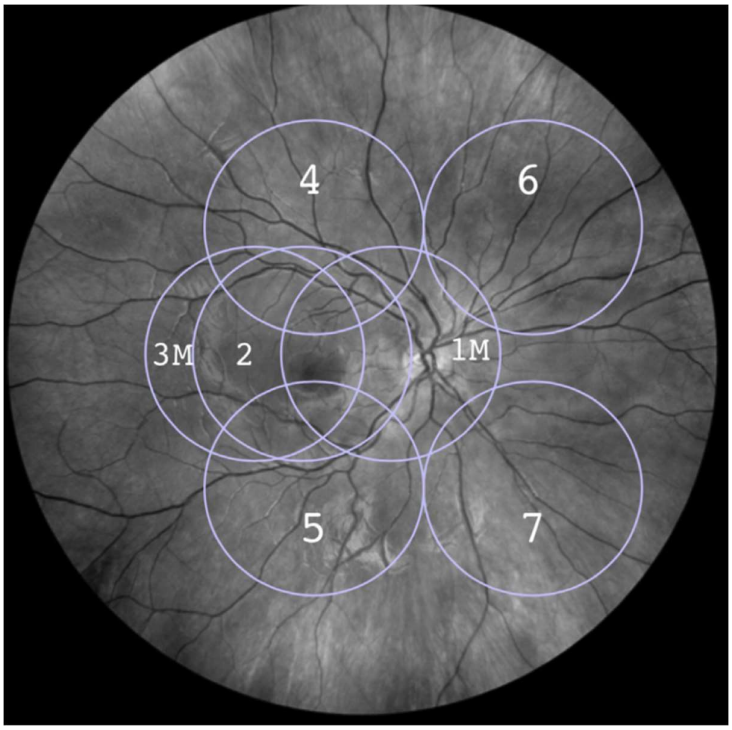


Figure 1.13 Representation of the seven standard fields of the modified Arlie House Classification of diabetic retinopathy. Field 1 is centered on the optic nerve, field 2 is centered on the macula and field 3 is temporal to the macula. Fields 4, 5, 6 and 7 capture the mid-periphery. Adapted from Goldberg & Fine, 1969.[287]

The scale progresses from DR absent (levels 10-12) through degrees of nonproliferative DR (NPDR) (levels 35-53) to proliferative DR (PDR; levels 60-85) and has demonstrated a predictor and predictive value[290, 291].

Its valuable predictor value is overt when we observe that cases with moderately severe to severe NPDR (DRSS levels 47/53) and DRSS level of 53 or more in comparison with DR level of 47 or less were at greatest risk of progression to PDR at month 24[291, 292].

This has clear implications in clinical management, because PDR is associated with an increased occurrence of DME and fibroproliferative events, such as retinal traction, vitreous haemorrhage and retinal detachment.

Furthermore, the risk of decline in visual function is higher in patients with DRSS level of 43 or more and vision-related functional burden is also significantly greater in patients with severe NPDR or worse ETDRS-DRSS has also a main predictive value for DR outcomes[293, 294].

In patients with DR with and without DME, anti-VEGF treatment resulted in DR improvements up to 47% of all patients, regardless of baseline DR level, experienced 2-step or more DR

improvement at 24 months with anti-VEGF treatment[295-299]. Although patients with moderately severe and severe NPDR (DRSS levels 47/53) experienced the greatest benefit of ranibizumab treatment, with nearly 80% of ranibizumab-treated patients achieving 2-step or more DR improvement at month 24 and month 36[292].

Even though, for daily bases, clinical practice DRSS based on the International Clinical Diabetic Retinopathy and Diabetic Macular Edema Severity scale seems to be more applicable. According to this classification based also in seven standards fields on color fundus photos the DR levels of severity is divided into 5 levels: No apparent retinopathy (nDR), mild NPDR (with microaneurysms only), moderate NPDR (more than just microaneurysms but less than severe NPDR), severe NPDR (any of the following: more than 20 diabetic retinopathy intraretinal haemorrhages in each of 4 quadrants; venous beading in 2 quadrants; prominent intraretinal microvascular abnormalities in 1 quadrant and no signs of PDR), and PDR. For DME the classification is categorized as “apparently present” or “apparently absent”. In addition, additional levels of DME are based on the distance of retinal thickening and/or lipid from the fovea: the existence of central involvement by edema or lipid is categorized as “severe DME” and when they are relatively distant from the macula is graded as “mild DME”[300]. However, there are other relevant pathological manifestations that are not fully visible on clinical evaluation, with an additional predictor and predictive role, as macular and peripheral micro perfusion, changes in macular microstructure, neurodegeneration, a quantitative measurement of macular thickness and last but not least, the knowledge of a precise evaluation of vitreous status.

1.4 Imaging in diabetic retinopathy beyond retinography

1.4.1 Ultrasonography

US and OCT methods rely on the reflection of waves on the structures to be imaged: mechanical waves in US and electromagnetic light waves in OCT[301, 302].

The main advantage of US in ophthalmology is its ability to scan through opaque light-conducting media, such as the normal iris, corneal opacities, dense cataract, or vitreous haemorrhage. US is the only imaging method that, thanks to is large focus depth, can capture the entire eye in a single cross-section. It may be performed dynamically in time, as the patients moves the eye and this may help distinguish the vitreous (totally loose and mobile) from a detached retina (loose but attached to the optic nerve) and from solid lesion such as haemorrhage, fibrosis or tumour[301].

1.4.2 Optical coherence tomography (OCT)

Although ocular US may be considered the gold standard exam to establish the vitreous status, spectral-domain OCT is the retinal imaging method most used worldwide. OCT is currently the backbone in diagnosis and staging of diabetic macular edema, where US has little to no applicability[233, 303].

From time-domain OCT to ultimate spectral-domain (SD), swept source (SS) widefield/ultrawidefield (WF/UWF) OCT and the advances of the visualization of the choroid by enhanced depth imaging OCT (EDI-OCT) technology advances, it becomes the most essential, accessible, non-invasive, and reproducible technology to image the fundus in diabetic patients. It provides high-resolution cross-sectional images of the neurosensory retina, a near-histological assessment of different retina layers and structure (fig.6) allowing in vivo precise measurements and an improved follow-up[303]. In opposition to the superficiality and subjective nature of fundoscopy and retinography, OCT allows an objective measurement of the mean thickness of each individual retinal layer in the nine individual ETDRS subfields (figure 1.14), and thus, an accurate detection for DME existence, type of edema (central - involving the central 1mm subfield zone - versus noncentral DME) beyond other potential relevant information based on several structural changes[53].

Two types of OCT instruments are currently in use: Spectral domain (SD-OCT) and Swept source (SS-OCT). The SD-OCT uses a broadband near-infrared super-luminescent diode as light source, centre wavelength of approximately 840 nm and a spectrometer as the detector. The SS-OCT uses tuneable swept laser as light source, centre wavelength of approximately 1050 nm and a single photodiode detector and penetrates tissue better allowing image acquisition in seconds due to the higher speed nature of the imaging, as well as improved choroidal and CC visualization[304-306].

1.4.2.1 OCT angiography (OCTA)

Another innovative branch in OCT technology is **OCT-Angiography** (OCT-A). **OCT-A** is based on the concept that in a static eye, the only moving structure in the eye fundus is blood, flowing in the vessels. OCT-A uses motion-contrast imaging to represent the erythrocyte movement in retinal blood vessels by comparing the decorrelation signal (differences in the backscattered OCT signal intensity or amplitude) between sequential OCT B-scans performed in the same area, in few seconds[307]. Both morphological (structural OCT) and functional (OCT-A), may be simultaneously shown in an overlapped mode (figure 1.14). A series of OCT section images (or B-scans) are acquired in order to create a cube of data. The acquisition of OCTA volume scans provides a three-dimensional cube of data that includes structural OCT and OCTA images. *En face* images generated from slabs are sections

of this 3D volumetric data (https://www.heidelbergengineering.com), as represented by OCT, slab correspondent to superficial vascular complex, SVC (fig 1.14).

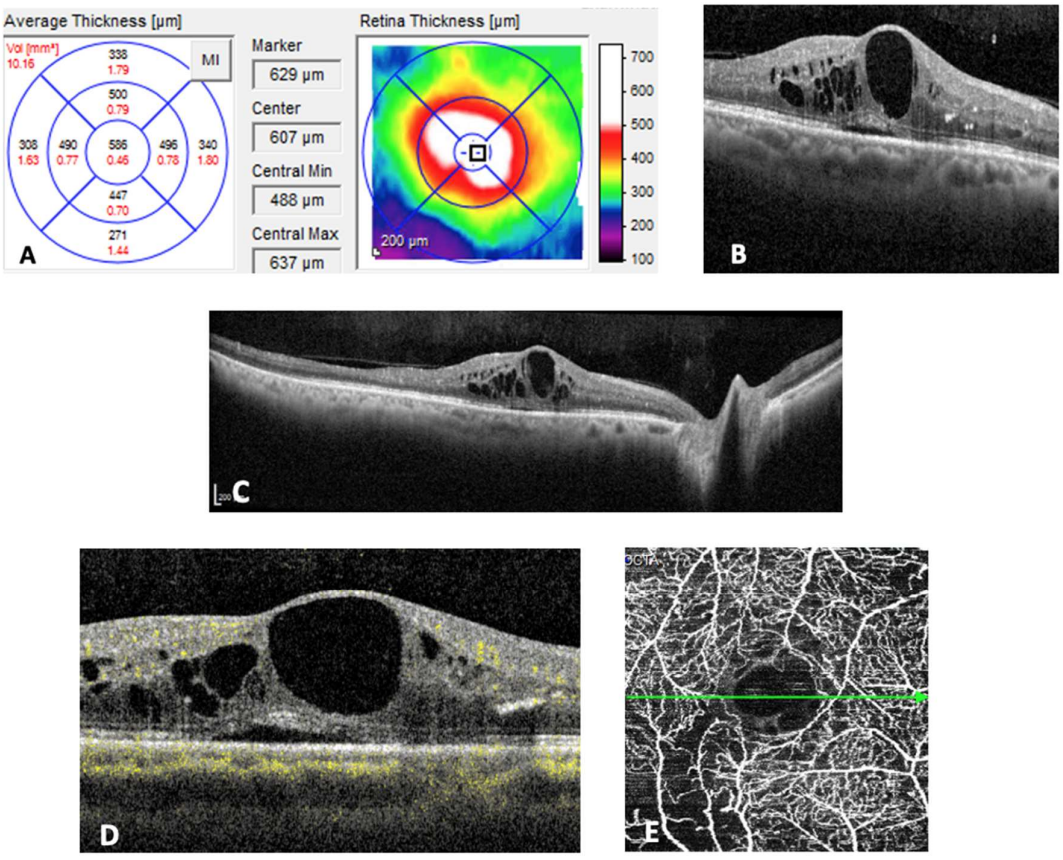


Figure 1.14: Spectral domain optical coherence tomography, SD-OCT, from Spectralis HRA + OCT, version 1.10.2.0; Heidelberg Engineering, Germany. Multimodal imaging display of a diabetic macular edema (DME) providing complementary information: A - sectorial thicknesses of the 9 ETDRS subfields and the topographic location of the DME through the retina thickness map; B – B-scan standard 20x20º SD- OCT image; C - widefield 55x35º showing more accurately the vitreomacular interface (VMI); D – B-scan morphological (structural OCT) and functional (OCT-A), simultaneously shown in an overlapped mode; E – OCTA slab correspondent to superficial vascular complex (SVC).

OCT-A allows to obtain qualitative and quantitative information from the retina and the choroidal vasculature. Even though it is highly dependent on an accurate segmentation and the absence of fake deep projection images from superficial vessels (projection artifacts)[308-314].

In DME, the use of OCT-A presents more limitations due to the high rates of segmentation errors Hypo-reflective intraretinal cyst on en face OCTA slab can also be misinterpreted as areas of ischemia[310, 313].

Qualitative analysis

It allows the evaluation of the shape of the *FAZ*; the presence of *retinal lesions* (microaneurysms, non-perfusion areas, cystoid spaces, collaterals, new-vessels, morphology of retinal capillaries), *CC structure analysis and the presence of choroidal lesions* (detection of choroidal neovascularization, its type and morphology)[308-312].

Quantitative analyses

Obtained through binarized images (or skeletonized image for vessel length measurement), with metrics for the evaluation of the *FAZ* (area, perimetry and circularity index) and the *retinal perfusion parameters* (perfusion and vessel density, vessel diameter index, perfused capillary density, vascular complexity parameters)[315-319].

FAZ: the area, the perimeter (mm) and circularity index (CI, (4π x area) / perimeter) which expresses the regularity of a shape, the more its value is closer to 1 (near a perfect circle)[320].

Perfusion density (PD): the total area of perfused vasculature per unit area[321].

Vessel density (VD) (mm^{-1}): the total length of perfused vasculature per unit area[322].

Vessel diameter index: the average vessel caliber (total vessel area /total vessel length)[323].

Perfused capillary density: the percentage of capillary area divided by the total analyzed area, once noncapillary blood vessel areas have been subtracted, on full vascular slab[316].

Fractal dimension (FD): reflects the degree of complexity of a biological structure, using the box-counting method on binarized image[317] (total sum of the single branches' length in the analyzed area, on skeletonized image)[315, 319].

CC perfusion: the term signal voids represent CC non-perfusion or flow signal strength is below the decorrelation threshold[324, 325].

The concept of *Intercapillary spacing* have also been explores – it seems to be more sensitive than VD and FAZ to detect early capillary dropouts or areas of non-perfusion[326].

OCTA enabled differentiation of NV from large vessel vasculitis, and distinguished areas of fibrovascular proliferation with regressed NV from active NV[327].

In OCTA neovascularization appears as disorganized vessels originating from retina into vitreous which can be located on disk or near to NPAs and intraretinal microvascular abnormalities (IRMAs) (fig 1.15)[328].

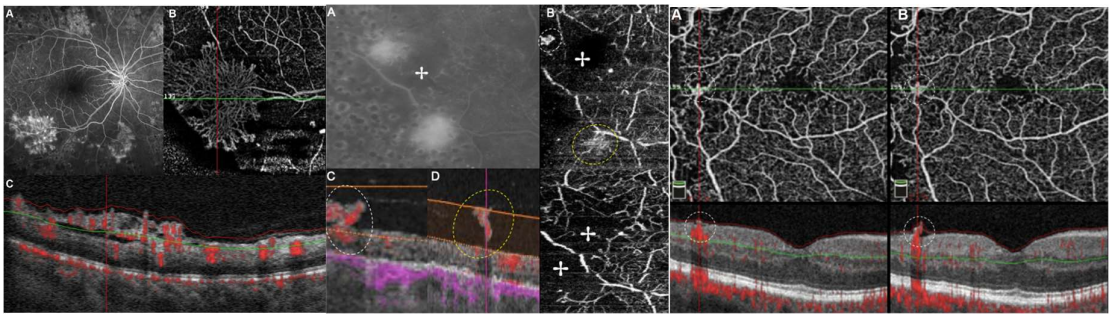


Figure 1.15: (from left to right) – Red square: A) FA image of an eye with PDR. Note NVE alongside the temporal inferior arcade with a sea fan configuration and leakage. (**B**) Corresponding OCTA image of the same complex, in which the filamentous irregular new vessels are well outlined and not obscured by fluorescein leakage. (**C**) OCT B-scan with flow overlay in red showing a flat complex with ILM breaching and flow signal, consistent with active disease. **Blue square**: Montage showing two NVEs. (**A**) Late FA image shows leakage associated with both NVEs. (**B**, top) In OCTA both NVEs appear as vascular loops with irregular new vessels, each one circled by a dashed ellipse. (**B**, bottom) Areas with absent flow signal adjacent to NVEs (⊹) are seen, corresponding to the capillary nonperfusion evidenced on FA. (**C** and **D**) B-scans of the NVEs show ILM breaching and flow signal, indicating disease activity, in agreement with the FA. Each NVE is circled by a colored dashed ellipse matching the en face image in (**B**, top). **Yellow square:** Case showing progression of NVE over time. Left images were obtained in an early disease stage. OCTA en face image (**A**, top) showing a small temporal NVE within the dashed circle. Corresponding OCT B-scan with flow overlay (**A**, bottom) shows a flat NVE with breaching of the ILM and flow signal (located within the dashed circle). Follow-up OCTA images revealed the same features in the en face OCTA, (**B**, top; NVE located within the dashed circle) while the corresponding OCT B-scan (**B**, bottom) revealed NVE progression (situated within the dashed circle), with ILM breaching and new protrusion. **Abbreviations:** FA, fluorescein angiography; ILM, internal limiting membrane; NVE, neovascularization elsewhere; OCT, optical coherence tomography; OCTA, optical coherence tomography angiography; PDR, proliferative diabetic retinopathy.[329]

IRMAs can be diagnosed as dilated retinal vessels without internal limiting membrane (ILM) or posterior hyaloid breach in opposition to neovascularization with expansion into vitreous with ILM breakdown[329].

Beyond the identification of DME and other OCT signs of diabetic retinopathy, B-scans that corresponded to en face angiograms provides additional information about whether NV is sub-hyaloid, growing along the posterior hyaloid, or associated with retinal traction[330, 331].

1.4.3 Fluorescein angiography

Although fluorescein angiography (FA) remains the gold standard for the analyses of the retinal ischemia, neovascularization, particularly of the retinal periphery, detailed features of new vessels and surrounding retinal microvasculature cannot be shown because of fluorescein leakage. In opposition to OCTA, FA is a time-consuming invasive procedure, unrepeatable on the same day or in the short term and can cause severe life-threatening, although extremely rare, complications.

Furthermore, does not differentiate whether leakage is from preretinal NV or from other sources and only one eye can be chosen as the early transit eye for characterization[327, 332].

When compared to FFA, OCT-A may be more sensitive in detecting microvascular changes in retinal diseases by enabling high resolution depth-resolved visualization of multiple layers, providing a more detailed view of the alterations of FAZ, disruption of the peri-foveal capillary net, and better visualization of the microaneurysms in the deep capillary plexus (DCP) even if FFA is able to detect more microaneurysms[313, 333-335].

Out as main disadvantages of OCTA are the need for visual acuity that allows to fixate on a target, whereas FA does not require fixation, and OCTA have less performance than FA in the presence of vitreous haemorrhage[327].

With current devices OCTA *en face* images may be obtained at varying sizes ranging from 2.0 x 2.0 mm to 12.0 x 12.0 mm or 15.0 x 9.0 mm without the use of montage algorithms[336, 337].

Although conventional 3 x 3-mm and 6 x 6-mm images produce high-resolution images allowing accurate assessment of the macular capillaries and vasculature, the scan quality decreases with larger fields of view (FOV)[337, 338]. Currently, 3×3-mm scans appear to have an equivalent or better resolution of blood vessels than available FFA and ICGA methods.

To assess the vasculature beyond the vascular arcades with OCTA, using currently available devices, montage methods are used[339]. Prototypes capturing up to 100 degrees FOV at once have been developed as well[340].

In the same field of view OCTA seems to be equivalent or even superior to FA in identifying non-perfused areas (NPA) and wide field swept-source OCT (SS OCT) could identify areas of NV as well as ultra-wide FA (UWFFA)[304, 313, 334, 341, 342].

However, an unmet major need for OCTA is a complete of standardization in nomenclature and methodology towards uniformization, a universal interpretation of data and compatible analyses. The multiplicity of perfusion metrics is a classic example of that. Even though some consensuses have been already achieved[343-345].

Hence, among the most important mandatory consensus is the definition of what is considered wide-field OCTA, based on the field of view (FOV), as the term “wide-field” OCTA is heterogeneously used in the literature[346-351].

Also, important to have in mind the correspondence between the degrees in FOV and area in mm ^{2}. Hence, 12 x 9 mm, 9 x 9 mm, 6 x 6 mm, and 4.5 x 4.5 mm—provides, respectively, 40° x 30°, 30° x 30°, 20° x 20°, and 15° x 15° fields of view, a composite of 2 images of 15 x 9 mm corresponds to 70º and 5 images of 12 x 12 mm corresponds to 90º [344, 352].

1.4.4 Widefield/Ultra-widefield imaging (fig 1.16-1.18)

The term widefield (WF) has been used inconsistently in the literature in describing retinal images from all methods (e.g., fundus photography, fundus autofluorescence, fluorescein angiography, ICGA, OCT, and OCTA)[336].

The International Widefield Imaging Study Group have recommended a consensus terminology for Widefield and Ultra-Widefield (table 1)

Table 1: Recommendations for definitions of Widefield and Ultra-Widefield Imaging from The International Widefield Imaging Study Group[336].

Region within the Retina	Field of View	Anatomic Location
Posterior pole	Approximately 50º	Retina just beyond disc and arcades
Midperiphery	Approximately 60º - 100º (widefield)	Retina up to posterior edge of vortex vein ampulla
Far periphery	Approximately 110º - 220º (ultra widefield)	Anterior edge of vortex vein ampulla and beyond to pars plan
Panretinal	Complete 360 º	Ora-to-ora view of the retina

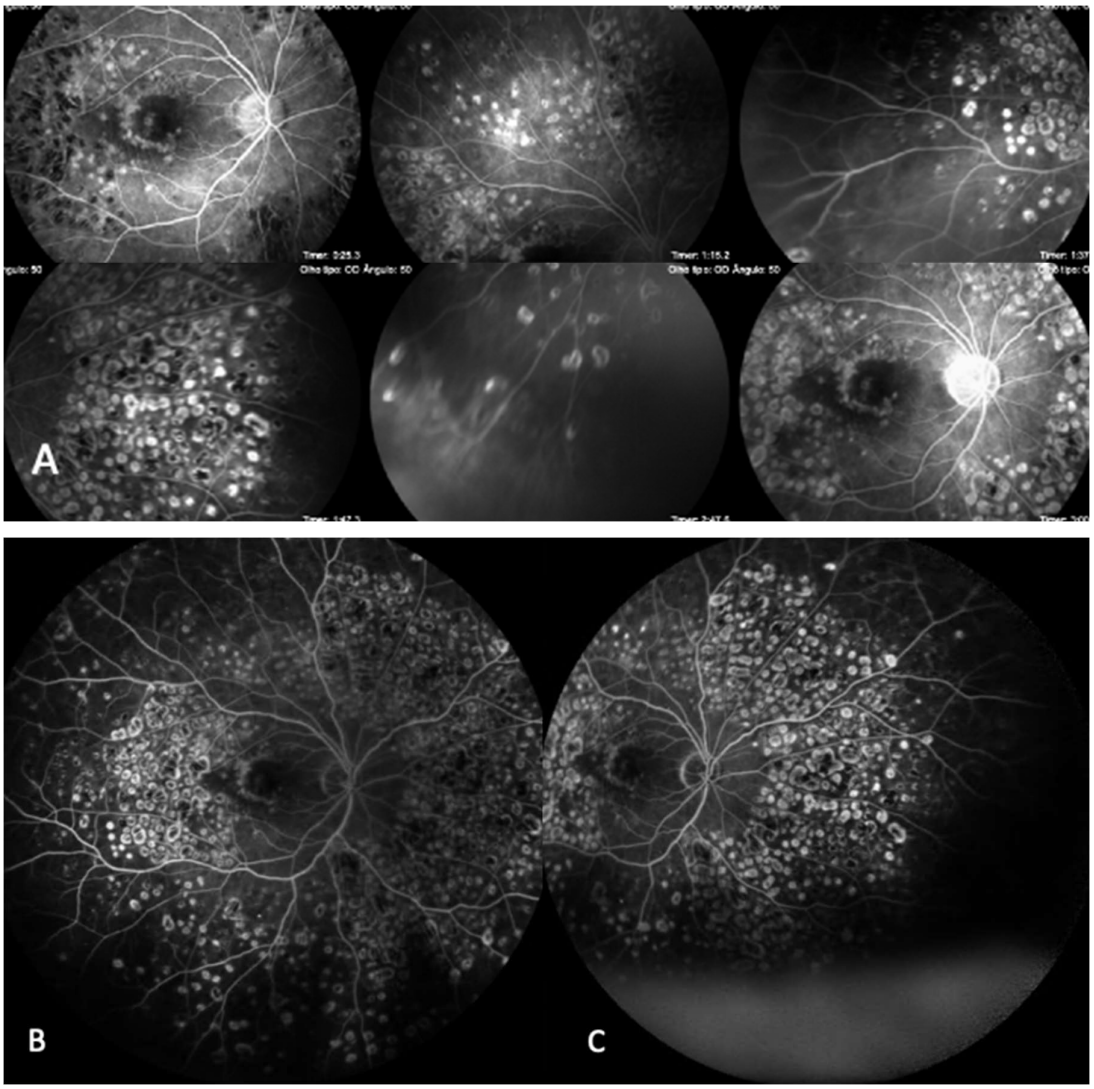


Figure 1.16: Standard (55º - image A) and widefield (WF) (102º - images B and C) fluorescein angiography (FA) exames from the same patient. WF offers a better perspective of peripheral ischemia. (Spectralis HRA + OCT, version 1.10.2.0; Heidelberg Engineering, Germany)

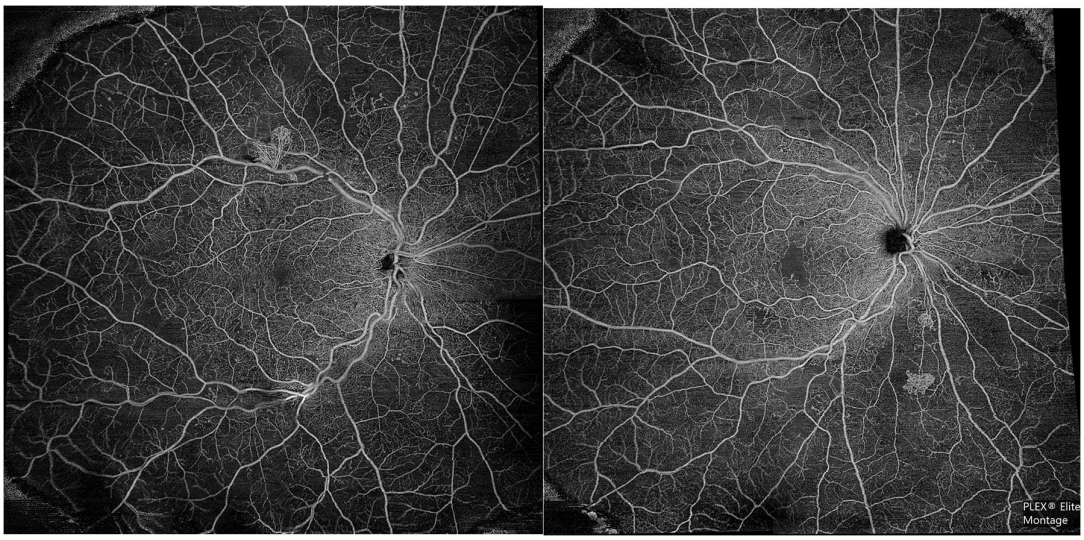


Figure 1.17: Plex Elite widefield optical coherence tomography angiography (OCTA) of 90º: montage of 5 images of 12 x 12 mm of rights: shows clearly a superior retinal neovascularization (left image) and an inferior neovascularization (right image). Courtesy of João Figueira.

Recently advances with technology ultrawide-field angiography has been shown to capt nearly 3 times more total retinal area compared with standard 7-field imaging (7SF), and to demonstrate retinal non-perfusion and neovascularisation in 10% of eyes that would have been missed by 7SF[353].

The advances in ultrawide-field (UWF) retinal imaging technique led to the discovery that the majority of nonperfusion areas (NPAs) in eyes with DR are located outside the posterior pole and retinal NPAs are significantly correlated with the DR severity scale (DRSS)[354, 355].

WF/UWF-OCT is an additional up-grade in OCT imaging, covering a larger retina area characterization, as the macula and optic disc area simultaneously, also allowing a more precise definition of vitreous-retinal status[254]. The needed of a high number of A-scans, time of acquisition, increase difficult collaboration and potential motion artifacts may limit its generalized application[340].

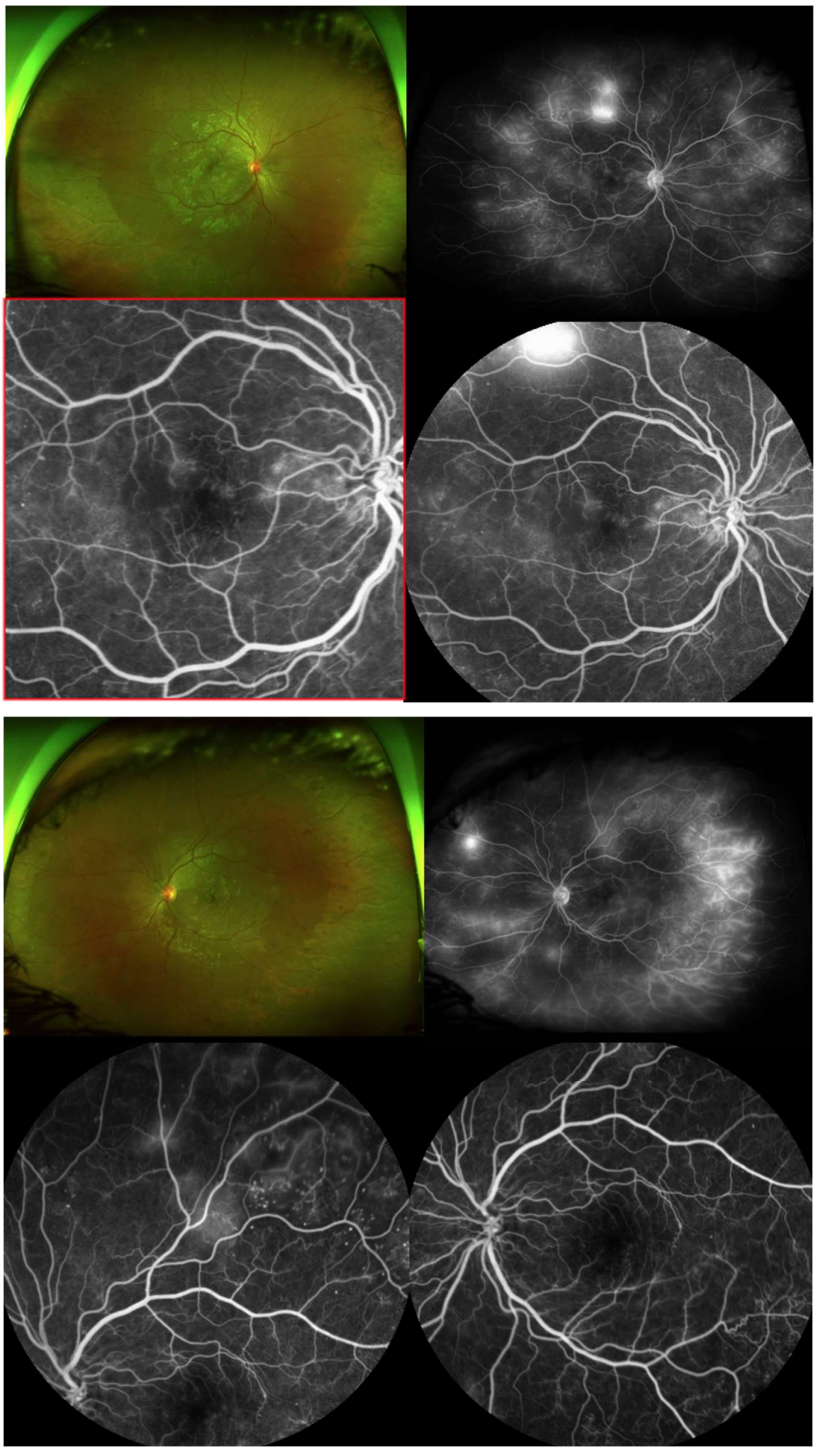


Figure 1.18: Images from the same patient with proliferative diabetic retinopathy (PDR). Right eye (upper four images) - shows the superiority capturing the periphery with ultra-widefield 200º of Optos Daytona ultra-wide-field retinography and fluorescein angiography, FA (the two upper images) over spectralis 102º FA and particularly over the standard 55º FA (left inferior image). Left eye (bottom four images) - shows the superiority capturing the periphery with ultra-widefield 200º of Optos Daytona ultra-wide-field retinography and FA (the two upper images) over spectralis 102º FA. Courtesy of João Figueira.

For SD-OCT B-Scans WF definition is not clearly defined, however, in comparison with a standard 20x20º (fig 1.14 B), the 55ºx35º SD-OCT (fig.1.14 C) image should be considered a widefield image, providing additional and relevant information, especially regarding vitreous-retinal interface.

According to a consensus on OCTA nomenclature involving members of The Retina Society, the European Society of Retina Specialists, and the Japanese Retina and Vitreous Society the Classification and Guidelines for Wide-field Imaging cannot be applied to OCTA in retinal vascular diseases, in line with the results of the standardization approach in uveitis OCTA nomenclature[336, 344, 345].

Hence there was a near consensus considering Field of view >70 degrees as a wide-field image for OCTA[336].

1.5 Update in biomarkers for diabetic retinopathy and diabetic macular edema – *Screening, Staging, Predictor and Predictive* role*

**See therapy options section to complement (section 1.6)*

The main rational for the identification of biomarkers is to anticipate and effectively treat lesions in a reversible state and to predict a promptly response to a specific therapy in order to obtain an early response and avoid the nefarious effect of a chronic persistent DME.

The group of Coimbra, using a multimodal approach, found that in the initial stages of DR, the eyes with DME from different patients included in the same Early Treatment Diabetic Retinopathy Study (ETDRS) grading category **displayed different prevalence of the main disease pathways, neurodegeneration, edema, and ischemia** (figure 1.19)[356, 357]. This observation supports the theory of different phenotypes of disease progression.

Hence, multimodal imaging is essential to effectively define the stage and severity of the disease, through a comprehensive understanding of the main disease pathway, for clinical decision-making.

1.5.1 Imaging biomarkers – Multimodal approach

1.5.1.1 Microperimetry

In clinical practice, VA is still considered the gold standard of vision testing. However, neither VA nor conventional visual field testing allows the detection of small scotomas or mild changes in retinal sensitivity, especially when retinal fixation is altered. Microperimetry make possible to overcome retinal fixation instability with a fundus image visible during the examination[358].

In DME is used to correlate macular sensitivity to retinal thickness, VA, and FAF images[359]. Many studies have shown an inverse correlation between macular sensitivity and retinal thickness[358, 360, 361].

1.5.1.2 Fundus Autofluorescence

Fundus Autofluorescence (FAF) is a rapid, non-invasive imaging technique, whose major source for autofluorescence is represented by lipofuscin of the retinal pigment epithelium (RPE)[362].

In DR, local ocular inflammation and oxidative stress lead to increased amount of lipofuscin and decreased amount of lutein and zeaxanthin in the macula. In addition, activation of microglia in diabetes could cause oxidation of proteins and lipids[363, 364]. Histologic studies have found lipofuscin to accumulate in microglia much more than in RPE[364].

Vujosevic et al 2011, hypothesize that the increased FAF (iFAF) areas observed in DME are caused by accumulation of oxidative product induced by activated microglia[358].

Another hypothesis is the mechanical effect of cystoid macular edema. Cysts, mostly located in the outer plexiform and inner nuclear layers, where there is a maximum accumulation of luteal pigment, may displace luteal pigment preventing the normal blockage of foveal FAF signal[365, 366].

Hence, three main different increased patterns of FAF may be distinguished (multicystic, single cyst, and combined single and multicystic iFAF) in patients with cystoid DME that correlated positively with FA and OCT findings[358, 367, 368].

Although areas of iFAF significantly decrease after both steroid and anti-VEGF therapies, when the iFAF area is bigger, steroids appear to improve retinal sensivity more, whereas anti-VEGF have a more restricted effect. After a 3 loading dose of ranibizumab VA and retinal sensitivity increase in a more limited manner in case of larger iFAF area at baseline in comparison with dexametasone[369].

1.5.1.3 OCT A

Clinically, microaneurysm is considered the earliest sign to reveal DR initiation[328]. Compared with healthy eyes without diabetes, **OCT angiography** reveals the presence of microaneurysms, in the diabetic eyes without clinical retinopathy demonstrating potential utilization as a ***screening tool***[312, 370-373].

Even IRMAs, capillary non-perfusion area, and neovascularization can be identified in OCTA before they are appreciated clinically, in fundus photography or on FA[330, 374-376].

According to the consensus on **OCTA** nomenclature group previously mentioned, OCTA have a **role as an alternative to *ETDRS grading***. The presence of **NV**, **area of nonperfusion** and **FAZ** should be implemented in the identification and ***Severity Staging of DR***. Thus, an area **>0.5 mm ^{2}** should be considered as a **"large area of decreased flow" on conventional OCTA** and **large flow decrease** should be defined as **> 30%** of the **total wide-field area**; and an OCTA ILM/vitreous slab should be considered preferably used to detect NVE/NVD (although a minority defended that any slab should be used for NV detection)[336].

The ***Prognostic Role of OCT A*** can be seen through several clues. In fact, **DCP** that seems to be the most affected retinal vascular network in DR. Microaneurysms are preferentially located in DCP and their increased number has a **positive correlation with an increased retinal volume**[377, 378]**.** This is in line with what have been described, **INL with a higher and most frequent increase in retinal thickness (RT)**[83]. (Figure 1.19)

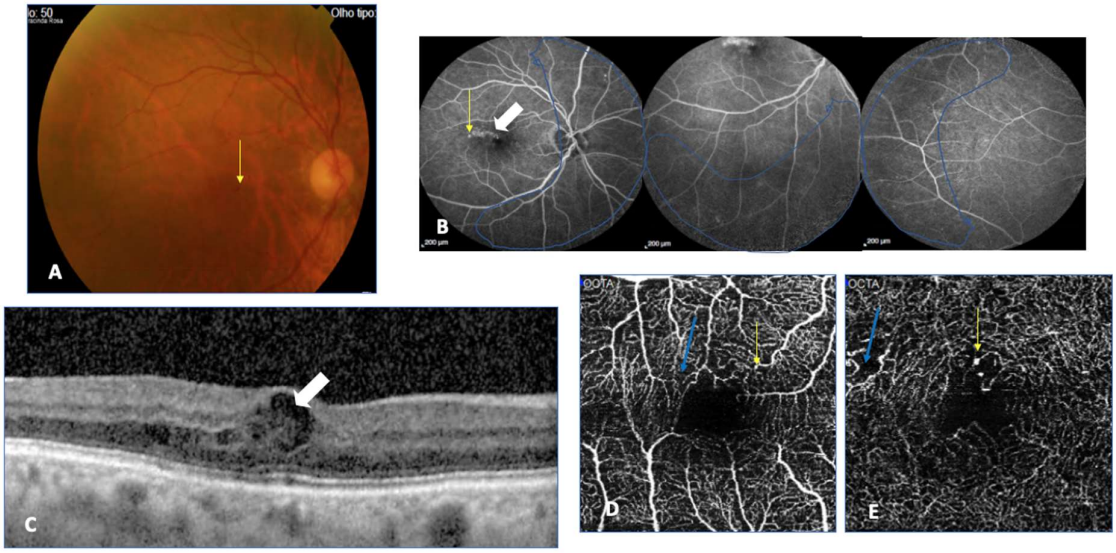


Figure 1.19: Multimodal imaging demonstrating features of non-proliferative diabetic retinopathy of the same patient, sequentially obtained at same time point (A) Color digital fundus photography (*Topcon IMAGEner 1-base*) exhibiting sparse microaneurysms (yellow arrow). (B), (C), (D),(E) are images from Heidelberg Spectralis® : (B) fluorescein angiogram obtained with the 55° lens, (C) structural OCT, (D) superficial capillary complex (SCC), (E) deep capillary complex (DCC) OCT-angiography (OCT-A) (version 1.10.2.0, Heidelberg Engineering, Heidelberg, Germany). (B) Mid-late phase fluorescein angiography demonstrating an hyperfluorescent area correspondent to leaking microaneurisms at the posterior pole – macular edema (white arrow) and peripheral ischemic areas (circle blue areas). (C) OCT passing through the macula, showing cystoid central macular edema, with a greater cyst with an hyperreflective content, in the inner nuclear layer, and smaller cysts and mild intra-retinal fluid in the outer nuclear layer. 3 x 3 mm OCTA of the SCC (D) and DCC (E) shows a relatively reduction in fine

capillaries, irregular perifoveal network with areas of dropout (blue arrows) and hyperreflective microaneurysms (yellow arrows).

Furthermore, **microaneurysms turnover (MAT) and capillary closure (areas of nonperfusion**) are biomarkers that are also indicators **of DR progression over a 5 years period**[41, 379].

Macular vessel density progressively deteriorates from healthy controls to people with diabetes with no diabetic retinopathy to NPDR to PDR, with closely correlating quantification values[321, 322, 373, 380, 381].

Giant intraretinal cysts (>200μm) on OCT and the **intraretinal reflective material** (suspended scattering particles in motion, **SSPiM**) observed on OCTA are correlated with macular ischemia and could be an indirect expression of **poor visual prognosis**[303, 382]. Extravasated lipid is believed to be involved in the formation of these SSPiM and subsequently hard exudates. SSPiM seems to be an extravascular signal on OCTA due to Brownian motion of particles within intraretinal fluid pockets, usually found at vascular–avascular junctions[382]. Hence, OCTA may offer an objective method for monitoring diabetic maculopathy as well DR severity.

OCT-A parameters considered as ***predictive Biomarkers*** (**biomarkers of DME treatment response**) are the characteristics of the FAZ in different retinal layers (size and circularity index), the characterization of microaneurysm - MA (visibility, internal reflectivity, number and location), the measurement of VD, NPAs and vessel tortuosity. [343].

At the level of **DCP**, internal hyperreflective MA (related to macular ischemia) and a great number of MAs, a lower VD and larger FAZ have been reported as biomarker of **poor response to anti-VEGF treatment**[374, 383-386]**.**

For eyes with DME, **parafoveal VD in the superficial layer at the baseline** was an **independent predictor for visual improvement after the loading ranibizumab** treatment[386].

Better BCVA after aflibercept treatment was significantly associated with **larger retinal vascular area** (percentage of the entire area occupied by large vessels and microvasculature), in the SCP and the DCP, at baseline, **and** with the **resolution of MAs and DME**[387].

The precise location of neovascularization (NV), above or below ILM, by OCTA has also implications as a predictive biomarker, as an important classification sub-type of NV[341, 388].

Compared with above ILM type, the below ILM type is more sensitive to PRP treatment. Hence, the enhance of retinal circulation by PRP treatment has little effects on the above ILM

type, attached to the posterior vitreous membrane and growing into the vitreous cavity, and anti-VEGF therapy is pointed out as a better option for those cases[389].

1.5.1.4 OCT

Retinal thickening

DME definition is an accumulation of intra and/or subretinal fluid in the macular area associated with retinal thickening on OCT[390].

A normal **CFT** and central macular volume (MV) in normal eyes is between 225–315 μm and 0,17–0,26 mm,respectively[342]. The normal CFT in patients with diabetes and minimal or no diabetic retinopathy are < 320 μm for males and 305 μm for females[391].

However, in the both groups analyzed near 100% of eyes, without edema, have bellow 300μm[83, 390].

The most worldwide accepted definition for chronic persistent DME, essential for the detection of a DME nonresponder to the first line anti-VEG therapy (see section 1.3.3) is the failure to achieve a CFT <300μm (based on the latest SD-OCT technology) and at least a 10% reduction from the 24-week visit on at least 2 consecutive study visits[342, 390, 392-395].

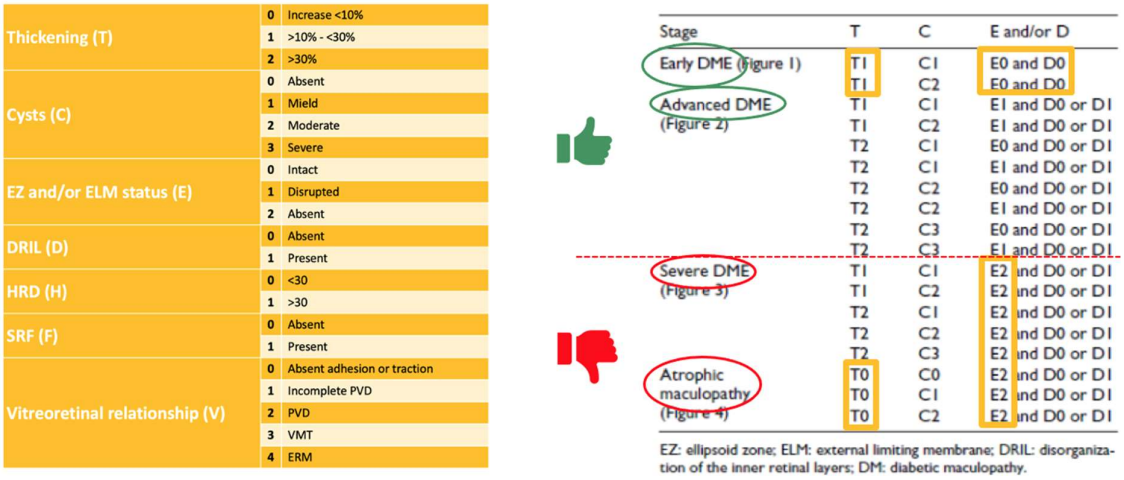
Even though CFT is not an isolated reliable biomarker to evaluate the prognostic in patients with DME, a **CFT >400 μm** is widely recognized as a **marker for a more severe DME/cpDME**[303, 390, 396-400].

According protocol T sub-analyses **persistent DME through 24 weeks – have greater baseline CFT** (median 415μm)[400].

An international expert panel on behalf of the European School for Advanced Studies in Ophthalmology (ESASO), and according to the literature, proposed for **grading of diabetic maculopathy**, **7 morphologic parameters on the SD-OCT**, under the acronym, **TCED/HFV** (T – grade of thickness; C – size of intra-retinal cysts, IRC; E – ellipsoid and external limitant membrane layers integrity; D – presence of DRIL – disorganization of retinal inner layers; H – presence and number of hyperreflective foci, HRF; F -presence of sub-retinal fluid; V – the vitreoretinal relationship[342]. **ESASO OCT biomarkers** are a mainstay for guidance to obtain predictors for disease control and predictive biomarkers indicators of response to therapy.

According to this classification and with the support of the literature a lower thickness (<400 μm), smaller IRC, intact E layers, absence of DRIL, lower number of HRF (<30) and absence of SRF indicates an early stage of diabetic maculopathy with better, early, responses to intravitreal therapy, as anti-VEGF. In opposition, the worsening of those biomarkers

represents a more severe and potentially cumulative inflammatory disease, as expected with the increase of RD severity[401]. That may benefit the most with CCT or combined therapy.



Thickening (T)	0	Increase <10%
	1	>10% - <30%
	2	>30%
Cysts (C)	0	Absent
	1	Mield
	2	Moderate
	3	Severe
EZ and/or ELM status (E)	0	Intact
	1	Disrupted
	2	Absent
DRIL (D)	0	Absent
	1	Present
HRD (H)	0	<30
	1	>30
SRF (F)	0	Absent
	1	Present
Vitreoretinal relationship (V)	0	Absent adhesion or traction
	1	Incomplete PVD
	2	PVD
	3	VMT
	4	ERM

Figure 1.20 – European School for Advanced Studies in Ophthalmology (ESASO) grading of diabetic maculopathy. Adapted from Panozzo et al, 2020.[342]

Hence, a higher CFT have been associated with a more chronic, severe DME and the need for combined treatment in DME non-responder to anti-VEGF or short term CCT[399, 402, 403]. Corroborating this data but in another perspective, a study from our group showed that 69% of good-earlier responders to ranibizumab had a baseline CFT<400 µm[404].

Large cysts

Cysts have been classified as small (<100 µm), large (101–200 µm), or giant (>200 µm)[303, 405] and cystoid degeneration in DME has been defined as the largest cyst in the central fovea having a horizontal diameter >450 µm and vertical diameter >300 µm Both horizontal and vertical diameter of cyst increases with severity of macular ischemia[406]. Larges and giant cysts are correlated with a poor visual prognosis. Location of giant intraretinal cysts about the center and its lateral extension, ONLc, the degree of the anatomical damage caused to the inner and outer layers by the cystoid change and associated photoreceptor or RPE lesion are correlated with a poor visual prognosis[303, 405-407].

Fibrin and other inflammatory by-products that may fill these the cystoid spaces **and as hyperreflective material forms septa within the cysts, in more severe cases of BRB disruption with anticipated poor functional outcome**[382, 408]. These type of cysts, with inflamatory nature, seem **not to be directly affected by anti-VEGF in their natural course**[409].

When edema overcomes the stretching capability of the retina, bipolar axons are damaged with subsequent disruption of visual signal transmission. As a consequence of these morphological changes, the recovery of visual acuity does not parallel the resolution of edema[410]. This is why **retinal bridging** should be considered a sign for a **rapid intervention to avoid irreversible damage**[390, 411, 412]**.** No predictive nature has been associated to retinal bridging towards anti-VEGF or CCT.

Subretinal fluid

In addition to large ONL cysts, the SRF accumulation appears relatively late during the course of DME[405]. DME associated with SRF is a specific pattern of DME associated with higher concentration of inflammatory cytokines. SRF decrease after either anti-VEGF or CCT, although major SND decrease seems to occur with CCT[369, 413-416].

DRIL

DRIL is significantly associated with the severity of DR and associated with worse outcomes of visual acuity. Its presence is considered a bad prognostic biomarker for DME. Furthermore, it has been correlated with disruption of the outer retinal layers (ELM and EZ) and with central foveal thickning[385, 417-420].

ELM and EZ disruption

Is a hallmark of advanced maculopathy. An intact EZ is critical to achieve a good functional outcome in DME. A greater percentage of VA improvement after switch from anti-VEGF to CCT was associated with EZ and ELM disruption[313, 342, 390, 419, 421].

Hyperreflective foci

Hyperreflective foci (HRF) are identified in OCT images as small (<30µm), punctiform, with reflectivity similar to the nerve fiber layer and no back shadowing, thus not clinically detected. Although Bolz et al. have hypothesized that HRF may represent subclinical features of lipoprotein extravasation that act as precursors of hard exudates supported by their distribution across all retina layers, even though their unequal distribution, according to several studies[422-427]. The most defensible theory seems to be that they represent activated microglial cells[423, 426, 428-432].

HRF were mainly located in diabetics, even when clinical retinopathy is undetectable (which is against the hard exudates theory) in the inner retinal layers, from ILM to GCL layer, where precocious inflammation changes occur, at the level of retinal neuronal cells[423]. Then the release of inflammatory mediators (including VEGF) provokes the extension of the inflammatory process through the entire retina including RPE, with the spread of activated microglia[426]. Their number increase with the progression of DR.

This also is line with what is already known, the resting retinal microglia is physiologically located in the inner retinal layers[429].

There are studies that concluded that the number of HRFs did not influence either functional or anatomic outcomes. Instead in others, HRF number have been associated with a poorer visual outcome[433]. Although there are evidences that HRF number decrease with both anti-VEGF and CCT, a higher number of HRF is correlated with poor responders to anti-VEGFs. Additionally, and corrobrating the background of inflamatory nature of HRF, there is an increased number of HRF in CCT responders in comparison with poor responders to CCT[369, 413, 416, 419, 427, 434-436].

Nevertheless a **higher CFT, a higher number of HRD and the presence of SRF were associated with a more chronic and severe DME and the need for additional treatment** in the first 12 months after FAc implant, in DME non-responder to anti-VEGF or short term CCT[385, 399, 402, 403].

Anoher apparent contraditory finding was observed by *Zur et al*., where cases with submacular fluid, no HRF, and a continuous IS-OS layer responded better to DEX implant than those without these features[437].

At this point it is important to sentece **two fundamental key messages** when dealing with DME biomarkers, first, a DME in a more precocious state of evolution probably may respond better not only to anti-VEGF but also to CCT and second, they are orphans devoid of clear meaning when they are alone, even though they are essential pieces to face the complex puzzle of DME approach.

Other biomarkers correlated of advanced maculopathy and DR

Outer retinal tubulations (ORTs)

ORTs have been described in numerous different retinal diseases**,** including DME, are characteristically correlated with lower BCVA and worse prognosis resulting from severe disruption of subfoveal photoreceptor integrity. A persistent ORT was also pointed as a negative biomarker of outcome of DME[438-444].

OPL disruption

OPLd may be predictive of **Good Response to Steroids** and worse or non-response to anti-VEGF therapy[395, 434].

Hard exsudates

The breakdown of the BRB in DME is the responsible for an enhanced lipid and protein exudation clinically labeled as hard exudates (HE)[445, 446].

HEs are frequently associated with a high level of serum lipids, can contribute to visual loss when they are located in the foveal area

and are associated with the development of sightthreatening complications in DME such as subretinal fibrosis[126, 127, 447-453]. Various treatments including lipid-lowering agents photocoagulation, surgical excision intravitreal injections of steroids, or antiangiogenic agents can induce their regression. Ranibizumab have demonstrated a significant greater reduction of HE area compared with sham[126, 452, 454-458]. In the context of intravitreal anti-VEGF (ranibizumab), the presence of HE is not a prognostic indicator of poor visual outcomes.

Intravitreal steroids (triamcinolone, dexamethasone implants) significantly reduce Hes in DME patients on short-term follow-up, whereas intravitreal bevacizumab does not. **They may be more effective** in reducing HE in patients with DME compared with anti-VEGF agents (bevacizumab), especially when the exudates are subfoveal[303, 459].

Choroidal Biomarkers

The choroid provides the blood supply to the RPE and photoreceptor cells, playing a major role in the metabolic exchange to the foveal avascular zone[410].

Choroidal thickness

There are evidences that treatment with PRP, anti-VEGF and triamcinolone acetonide IV injections can affect the central choroidal thickness (CT). Removing the effect of therapy, even though there are conflit data regardind choroidal thickness central CT in treatment naïve DME, which have been reported to be thickened, unchanged or thinned[282, 460-469].

The retrospective and cross-sectional nature of most previous studies, the influence of different factors such as age, refractive error, different DR severity/DME/PRP status, as well as the possible effect of previous anti-VEGF therapy, circadian cycle or even other systemic vascular factors such as blood pressure, and vitreous status[282, 460, 463, 470-472].

Sub-foveal choroidal thickness (SFCT) had a positive significant correlation with the central macular thickness (CMT) and total macular volume (TMV). The amount of thickened choroid may be an indicator of undertreatment[461, 473, 474].

Additionally, a **greater SFCT** seems to be a **predictive factor** of better outcomes following **anti-VEGF treatment** due a possible more preserved choriocapillaris, less ischemic outer retina, and better preservation of photoreceptors[461].

Choroidal Hyperreflective Foci and Choroidal Vascularity Index (CVI)

HRFs are commonly interpreted as inflammatory cell aggregates, affecting both the retina and the choroidal vascular index, CVI, (ratio of choroidal luminal area to total choroidal area) is a novel tool, that represents the proportion of choroidal vasculature including both large choroidal vasculature and choriocapillaris[475-477]. And may be considered a biomarker of choroidal congestion[478, 479].

Both the presence of a high number of choroidal HRF and low CVI can be considered signs of increased chorioretinal inflammation[465, 480].

Choroidal HRF and CVI seem to be good predictive biomarkers for steroids, in line with Arrigo et al. study where good responders to fluocinolone acetonide (FAc) were characterized by significantly higher choroidal HRF and lower CVI than poor responders. In addition, poor responders required significantly higher additional anti-VEGF treatments but globally maintained stable CMT and BCVA values at the end of the follow-up, thus indicating FAc treatment as a feasible treatment also in DME eyes characterized by less pronounced inflammatory profiles[478].

Choroidal HRF are also associated with PDR and more severe DR, association with ELM disruption (d), EZd and a poor functional prognostic biomarker in DME[481].

The analysis of choroidal changes using CT may have low reproducibility and low reliability. Hence, CVI may be a more stable and objective quantitative marker for the assessment of choroidal vascularity[465].

CVI correlates with progressing DR although DME seems not to be correlated significantly with the CVI[465, 474, 482].

Vitreomacular Interface Abnormalities
(VMIA) (see also section 1.2.3)

Noticeable OCT changes of vitreoretinal (VTI) or vitreomacular (VMI) interface (ERM involving the macular center VMT, VMA) and even with eccentric ERM have poorer response to anti-VEGF[483]. VMIA are associated with worse presenting vision in patients with DME[484].

1.5.2 Laboratory biomarkers

Diabetes is considered a chronic low level inflammatory disease, where numerous biomarkers are involved in the process of inflammation[485].

Cytokines (CK) are a small group of precursory solvable proteins attached to cell membranes that transmit signals between neighboring cells and are responsible for many pathways of biological processes (cell proliferation, inflammation, immunity, migration, fibrosis, tissue repair, and angiogenesis). Some of them are pro-inflammatory (IL-1, IL-6, IL-17, TNF-α and TNF-β) whilst others have anti-inflammatory functions (IL-1Ra - Interleukin-1 receptor antagonist, IL-4, IL-10 and IL-13). Chemokines, CH (as IL-8, MIG - monokine induced by interferon-γ - CXCL9, IP-10 - interferon-γ –induced protein-10 - CXCL10, Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted - RANTES) are multifunctional mediators, responsible for the recruitment of leukocytes to sites of inflammation, and they also boost the process, enhance immune responses, and promote stem cell survival, development,

and homeostasis. They play a pivotal role in mediating angiogenesis and fibrosis as well[486-491].

IL-6, IL-8 and monocyte chemoattractant protein-1 - CCL2 (**MCP-1**) have been independently reported to be regulated by nuclear factor-kappa B (NF-kB). NF-kB is found in almost all cell types and is involved in cellular response to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, and bacterial or viral antigens, in addition to its central role in immune response.

MCP-1 recruits monocytes, memory T cells, and dendritic cells to sites of tissue injury and infection and its upregulation may stimulate the infiltration of inflammatory cells into eyes with vitreoretinal disorders.

On the other hand, VEGF is upregulated by hypoxia through the hypoxia-inducible factor 1 alpha **(**HIF-1a), which is another transcriptional factor that regulates hypoxia-responsive genes[486, 492].

IL-6 is a multifunctional cytokine **produced in ischemic or inflammatory conditions** by ischemic **retina** hypoxia-induced or cytokine-stimulated **vascular endothelial cells** and **vascular smooth muscle cells**, and **inflammatory cells (**T cells and macrophages). IL-6 stimulates the **proliferation of glial cells and fibroblasts** and promotes **wound healing**, induce **VEGF** expression and increase **directly** endothelial cell permeability. It also i**nduces acute phase reactions** and modulates immune processes[493-497].

IL-8 is a pro-inflammatory chemokine with angiogenic properties, produced by activated monocytes, macrophages, endothelial and glial cells in ischemic retinas. IL-8 promotes the migration of neutrophils, basophils and T lymphocytes to endothelial cells, and consequently results in tissue injury[486].

The interferon-inducible chemokines, such as MIG (CXCL9) and IP-10 (CXCL10), which have been shown to be produced by retinal cells, are potent inhibitors of angiogenesis[498-502]. In addition, IP-10 inhibits angiogenesis *in vivo* at least in part by antagonizing the functions of IL-8.

Stromal Cell-Derived Factor-1 (SDF-1). SDF-1 is the predominant chemokine which is upregulated in many damaged tissues as part of the response to injury and mobilizes stem/progenitor cells to promote repair. In addition, induces VEGF expression in cells that are both hematopoietic and endothelial in origin, thus increasing the angiogenesis. SDF-1 works in conjunction with VEGF to promote the recruitment of endothelial progenitor cells (EPCs) from remote locations, such the bone marrow to the ischemic retina.

SDF-1 concentration increases in the vitreous of patients with either DME or PDR, and this increase was correlated with disease severity.

Tumor Necrosis Factor-α (TNF-α) is primarily synthesized by macrophages and T cells and its expression it is also regulated by NF-kB[503]. The serum levels of TNF-α have been reported

to be higher in diabetics versus nondiabetics and to have a strong correlation with severity of DR. It was also found in the vitreous fluid of diabetic patients and in a higher vitreous/serum ratio[504-506]. Another group of important molecules in DR are the adhesion molecules implicated in Leukostasis. The irreversible adhesion of leukocytes to the endothelium plays a major role in capillary nonperfusion and retinal vascular leakage in DR, through the induction of apoptotic changes to endothelial cells[507-510].

The intercellular adhesion molecule ICAM-1, the Vascular cell adhesion molecule 1 (VCAM-1), Eselectin and vascular adhesion protein 1 (sVAP-1) have been found increased in the vitreous of patients with PDR[511-516].

I-CAM-1 correlates with the number of migrated neutrophils in the retina and choroid, facilitating leukocyte recruitment VCAM-1 and E-selectin can act on endothelial cells as angiogenic factors and have a direct correlation between VCAM-1 and VEGF levels[512].

sVAP-1 is involved in leukostasis / leukocyte entrapment process and in the production of metabolites involved in cellular oxidative stress and advanced glycation end-product formation, through its additional enzymatic function[514, 515].

Takeuchi et al. reported that some growth and inflammatory factors are identifiable and measurable in both aqueous and vitreous fluid samples, however, they are not necessarily linearly related. Hence, aqueous humour alone does not provide a complete understanding of the extent of inflammation at the site of the disease in the posterior of the eye[517].

MIG (CXCL9), IP-10 (CXCL10) and IL-17 significant higher levels in vitreous samples of diabetic eyes[498, 517, 518]. ´

IL-6, IL-8 and VEGF, are elevated in both the **vitreous and aqueous humor** of patients with DR. **In the aqueous and vitreous humour,** they are **correlated with the severity of diabetic retinopathy** especially in the neovascular glaucoma-PDR group[493, 519-521].

Vitreous MCP-1 may play a role in the development of the proliferative phase of PDR[522].

Vitreous increased levels of of MCP-1 (accessed at the second vitrectomy) have also been correlated with **post-vitrectomy DME** and prolonged inflammation, (especially tracional retinal detachment)[523]. In those eyes VEGF levels did not change significatively[524].

There is robust evidence supporting that **aqueous and vitreous humour IL-6, IL-8 and VEGF** not only contribute to **DR development**, but also correlate with more severe disease stages, especially in **PDR** and **DME**[493, 520, 521, 525, 526].

Vitreous and aqueous PlGF have been significantly correlated in patients with **PDR**[527, 528].

Elevated IL-6 and subsequent **up-regulation** of various growth factors and cytokines, especially **VEGF,** may aggravate pre-existing disease. Recently, it has been suggested to consider intraocular **IL-6 as a target for management of DR**[494, 520].

Compared with the nondiabetic controls, diabetic patients had significantly higher concentrations of IL-1β, **IL-6, IL-8, MCP-1, IP-10, and VEGF** and significantly lower

concentrations of IL-10 and IL-12 in the aqueous humor. The aqueous humor levels of IL-1β, IL-6, IL-8, MCP-1, and IP-10 are closely correlated with the severity of DR; however, the *VEGF concentration was not correlated with the severity of DR*[529]*.* This observation may due the small number of patients with severe PDR included in that study. In fact, **Song et al.** in a more balanced sample observed that **increased levels of cytokines** (**IL-6, IL-8,** IL-10, **VEGF,** TGF-β , VCAM-1, ICAM-1 and **MCP-1) in the aqueous** humour **correlate with the severity of diabetic retinopathy** especially in the neovascular glaucoma-PDR group[519].

Among the cytokines and proinflammatory factors implicated in the pathogenesis of DME, one of the most relevant and investigated is vascular endothelial growth factor (VEGF)[530, 531]. VEGF is a crucial mediator of physiological and pathological angiogenesis and one of the most potent molecules promoting the increase of vascular permeability in humans[236, 509, 532].

VEGF level in the aqueous humor is absolutely positively correlated with its level in the vitreous[512, 533-535]. VEGF concentration is **comparable** in vitreous and aqueous humor[536].

Even though, vitreous levels of vascular endothelial growth factor seems not to be influenced by its serum concentrations in diabetic retinopathy as plasma cytokines which are not always correlated with those in vitreous and aqueous[527, 537]. Hypoxia-inducible factor 1-alpha (HIF-1α) is indeed the critical event implicated in the HIF-mediated cellular response to low oxygen, as HIF-1α is highly induced by hypoxia One of the best-known target genes induced by HIF-1 is VEGF.

Changes in glucose levels are another major factor involved in VEGF expression. In this regard, both long-term high glucose concentration and acute glucose deprivation increases the formation of AGEs[538, 539].

HIF-1a activation by AGEs through the extracellular regulated kinase pathway could be also involved in VEGF expression mediated by AGEs[540].

Insulin, IGF-I, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF), as well as proinflammatory cytokines (i.e., interleukin [IL]-1 and IL-6), several hormones (i.e., TSH, ACTH, and gonadotropin), and oncogenes (i.e., K-ras, Wnt) could upregulate VEGF[541].

Acute intensive insulin therapy produced a transient worsening of diabetic BRB breakdown via an HIF-1a–mediated increase in retinal VEGF expression[542].

Predictive role

A sudden decrease in VEGF levels after IV bevacizumab treatment, induces a compensatory **increase in other inflammatory cytokines such as IL-6** (day 1), which is associated with fibrosis. TGF-b is a multifunctional growth factor, which has a role in inducing a number of other growth factors, including VEGF, and aggravating capillary basal lamina thickening in DR. The TGF-b family of cytokines plays an important role in mediating fibrosis and scar contraction.

It is also known that there is a significant linear correlation between IL-6 and IL-8 after IV bevacizumab (BEV)[52, 494, 543-547].

Levels of IL8 also increase one week following antiVEGF injection[548].

Vitreous and aqueous VEGF-A levels were suppressed in patients with recent anti-VEGF injections Vitreous VEGF-A levels in patients with recent anti-VEGF injection were similar to those in nondiabetic controls. In contrast, vitreous and aqueous IL-6, IL-8, TNF-α, and PlGF level are the same, with or whithout **anti-VEGF in a mean of 55.8 days (within the past 90 days) prior to sample collection**[527].

IL-8 level in vitreous samples was higher in DME patients with subretinal fluid than those without subretinal fluid, suggesting that inflammation is an important factor in the progression of DME leading to the subretinal fluid formation in diabetic patients[549].

According Sonoda et al.and Yenihayat et al. IL-6 and IL-8, respectively, have also previously been associated with the presence of SRF in DME and a more inflammation status. In the study by Sonoda et al, significant correlations were found between the concentrations of IL-6 and VEGF and between the concentrations of IL-6 and IL- 8. Even though, in both studies, in all types with DME, VEGF was significantly higher compared with eyes of nondiabetic patients indicating that VEGF is equally important for any morphologic changes in eyes with DME[415, 549].

In addition increased vitreous VEGF level are associated with the progression of PDR after primary PPV and vitreous VEGF level was positively associated with plasma VEGF level in although vitreous levels were higher than serum[550].

Beyond cytokines and chemokines there are other substances associated with neurovascular protective functions such as, erythropoietin (EPO), 25HO-vitamin D (VitD) and transthyretin (TTR) that have also been implicated in DR pathogeneses. Even though, their precise role and relevance in DR approach is still not clear[45, 551-559].

1.6 Therapeutic Strategies for the Management of DR and DME

The two main causes of severe vision loss in DR, DME and PDR, are the focus of therapy. In fact DR and DME not only shares risk factors, but also coexists in the decision therapy process[6].

As previously mentioned, the breakdown of the BRB and inflammatory processes, influx of macrophages and leucocytes are related to DR damage[115].

1.6.1 Screening as the best treatment

In the context of a disease which is generally asymptomatic in early stages, an optimized screening programm has not only a major positive impact from a clinical perspective by preventing vision loss but also in a global economic and social broaded point of view. The costs of a screening and a systematized therapy program for DR, and more specifically for DME, are estimated to be a 1/10 of those spent in an erratic treatment approach by the time of symptoms emergence[560-563].

In Portugal, according to data from the National Diabetes Observatory's annual report for DM, in the Portuguese population aged between 20 and 79 years (7.7 million individuals) estimated prevalence for DM was 13.6%, with an increased prevalence of 16,3% in the last 10 years, and less than 50% of diabetic patients have ever been evaluated by an ophthalmologist[7, 564, 565]. In a retrospective analyses of our group the first 5 years of implementation of Diabetic Screening Program, promoted by the *Administração Regional de Saúde (ARS) do Norte*, in Centro Hospital Universitário do Porto, *CHUPorto* (which included 613 patients referenced for DR management according a retinography exam) revealed that more than 80% of patients observed in first visit did not present regular follow-up by ophthalmology, 70% of them had moderate to severe non-proliferative DR or PDR (PDR in 7%), 31.9% had DME, 17.4% had bilateral DME, and 51% indication for therapy[566].

1.6.2 Systemic Diabetic Disease and Management

A basic and permanent therapeutical measure should be a careful control of the main potential modifiable systemic risk factors, such as hiperglicemia, high blood pressure and dislypidemia[566]. Lipid lowering therapy seems to reduce the severity of HE and subfoveal lipid migration in diabetic patients with DME and dyslipidemia[454, 567].

Even though there is strong evidence that tight control of glycemia (with a glycosylated hemoglobin [HbA1c] of 7% or less) reduced the risk of development and progression of DR in both type 1 and type 2 diabetes mellitus[8, 9, 33, 568-574].

The therapeutic goal unfortunately should not be to reach strictly a HbA1c of 6,5% to 7% for every patient, as the risk for some patients may overcome the benefits of a perfect metabolic control, as previously discussed (fig. 1.2)[12].

Even-though, a lower HgA1c was associated with the magnitude of vision improvement following anti-VEGF therapy and better prognosis OCT characteristics[404, 575].

Hence, besides the importance of educating the patients to a healthy lifestyle with good control of their disease and risk factors, an interdisciplinary support involving the ophthalmologist, primary care doctor, nurse, endocrinologist for an optimized and individualized control and monitoring is, as well, crucial.

1.6.3 Laser therapy for DR and DME

Laser photocoagulation has represented the standard of care for the treatment of DME for many years prior to the advent of the intravitreal injection approach.

The standard treatment for DME since the mid-1980s has been laser photocoagulation (Early Treatment Diabetic Retinopathy Study, ETDRS, guidelines), applied to microaneurysms and other areas within a thickened macula ("clinically significant macular edema", CSME, criteria defined in this study). Laser therapy showed to diminish the risk of moderate visual loss by approximately 50% (from 24 to 12%), and improve vision in approximately 30% of eyes with vision impairment, although approximately 15% of eyes have vision loss despite this treatment[576, 577].

The mechanism of action which is responsible for laser therapeutic effect are still poorly understood[578]. The effect of focal laser has been related to the occlusion of leaking vessels, especially MA (focal laser) and stimulation of the RPE alone, not destroying of the PRs, to induce repair of the inner blood retinal barrier particularly in macular grid laser strategy (updated to a modified macular grid, in latest years)[576, 579, 580]. Sublethally injured RPE cells induce an up- and downregulation of various factors [pigment epithelium-derived factor (PEDF), VEGF inhibitors, VEGF inducers, permeability factors, etc.][578]. In addition, the destruction of ischemic retina, the rational for peripheral laser, leads to improved oxygenation of neighboring retinal areas[256].

Apart from the favored therapeutic effect, laser treatment can lead to undesirable side effects such as: epiretinal fibrosis; secondary to peripheral panretinal photocoagulation, PRP - permanent peripheral visual field loss, decreased night vision and exacerbation of DME; due to macular laser – loss of central vision, central scotomas, decreased colour vision, decreased contrast sensitivity and more rarely macular choroidal neovascularization, CNV in the area of the laser scar[295, 581-586].

The interference of visual field loss in ability to drive light vehicles (the legislation requires at least 120° in the horizontal plane and have a minimum extension of 50° to the left and right, and 20° to the top and bottom, and the absence of defects within a radius of 20° from the central axis) and contrast sensivity have been observed in cases with very confluent laser pattern, vitreoretinal surgery and peripheral crioterapy, i.e. more severe DR cases. However PRP interference in driving licence was not observed in the majority of patients[586].

In an attempt to reduce the macular adverse effects, many retinal specialists now treat with burns that are lighter and less intense, with widely spaced burns, avoiding the foveal region than originally specified in the ETDRS (modified-ETDRS technique)[580].

More advanced technologies, such as subthreshold micropulse lasertherapy, have additionally increased the therapeutic benefits of laser avoiding RPE or PRs damage[359, 587, 588].

Even though intravitreal anti-VEGF injections have now replaced laser as standard treatment for central DME, macular laser photocoagulation may still be used when a complete response is not seen[256].

PRP Laser therapy reduces the risk of vision loss in patients with high-risk PDR and, in some cases, severe NPDR. Therefore, it is indicated for **PDR and NPDR cases at high risk of progression to PDR** or poor compliance with follow-up[19].

New retinal vessels, both at surface and those extending slightly into the vitreous, may disapear in six to eight weeks after therapy with PRP. However, they may also persist for long periods and in some cases leakage detected in AF can be atributed to vessel dilation leaking[589].

Peripheral laser in ischemic areas as a **complement to DME therapy** is still **controversial.** Based on the association of DME and peripheral ischemia, especially in recalcitrant DME cases, to decrease VEGF production, many authors have proposed PRP to the mid-peripheral and peripheral retina and several case series and small, brief studies have implied that targeted retinal photocoagulation (TRP) guided by WF FA can decrease treatment burden (i.e., decrease the need for ongoing anti-VEGF injections)[590-595].

Three-Year Randomized DAVE Trial tried to validate this hypoteses[596]. Two arms were included: arm 1 – 4 monthy RBZ (and then Pro re nata - *PRN*) plus targeted laser directed to peripheral NPA retina (accessed with WF FA, OPTOS 200Tx; Optos, Peripheral) plus a 1 – disc area margin (at week 1 +- month 6 +- month 18 +- month 25); arm 2 – only monotherapy with RBZ (same IV injection protocol as arm 1). **Only severe NPDR or early PDR** were included in the study. A trend to better BCVA and CFT was observed in monotherapy arm. Even though being a randomized and prospective study it has limitations and bias (such as small samples sizes and macular ischemia not be considered an exclusion criteria).

Furthermore, these cases **may** not correspond to recalcitrant DME which have been more positive correlated with peripheral ischemia and retinal nonperfusion covering wider areas[595, 597]

This kind of clinical variability may affect the results of the study, as the VEGF levels may be increased with DR severity, particularly in more severe PDR cases[519, 529].

In addition, the thermic effect of PRP on macula without complete regression of DME may also affect the results in the short term after PRP laser have applied[584].

1.6.4 Intra-vitreal therapy

1.6.4.1 Intra-vitreal therapy for DR

There are studies that have documented retina reperfusion with anti-VEGF and even with corticosteroids, even though there are discrepancies in the literature[19, 586-594, 596].

Anti-VEGF therapy's genuine effect on retinal reperfusion is a matter of contention, with both pro and con views (it may slow the progression of retinal nonperfusion, but there is no clear evidence on reperfusion or prevention of retinal capillary closure in patients with DME, and it may even worsen the perfusion state)[598-604].

Phenomenons of capillary reopening, recanalization or reperfusion have been also recognized and described since 1960s, as a physiological response to the disease process as well as reactive to lasertherapy[589, 605-607].

New insights in this area may be gained thanks to recent improvements with UWF technology angiography, the expanded vascular imaging through flow-based modalities, such as OCT angiography and adaptive optics scanning laser ophthalmoscopy[353, 607-609].

There are evidences that anti-VEGF therapy have the highest effect inducing the regression of diabetic retinopathy particularly in patients at high risk of progression to proliferative diabetic retinopathy (DRSS score 47-53)[292, 610]. It is also non-inferior or even superior to PRP promoting NV regression, with lower incidences of DME and visual field loss.[295, 296, 611]. Despite the fact that, according to protocol S, visual field loss progressed on average in both PRP and ranibizumab groups from years 2 to 5, and the gap between groups shrank, in the long run, [295, 611]. Hence, although more aggressive, PRP laser approach have a more stable and predictable effect in the long-term, allowing permanent remissions of PDR in the majorty of cases. This is especially important because adherence, compliance with the high IV therapy burden, frequency of visits, and high therapy costs are all key concerns for diabetes patients, many of whom have additional comorbidities and are of active age[612-614].

Although anti-VEF can effectively reduce the progression of DR and improve retinal perfusion, it may not be able to stop the never-ending RNP process associated with DR[602, 608].

A mixed approach anti-VEGF and PRP seems to be more reasonable, and have proven to be more effective and even superior to PRP[615].

European Medicines Agency (EMA)[616] and Food and Drug Administration (FDA)[617] have already approved ranibizumab for DR therapy, as has aflibercept for FDA[618].

Regarding the effect of corticosteroids (CCT), recently Toto el al, through WF OCTA scan of 15 × 9 mm, showed that DEXii reduce ischemic areas at month 1 with a slightly not significantly increase thereafter not reaching preinjection values, i.e., a strong effect 1 month after treatment and subsequent gradual loss of effectiveness[608]. This achievement was in line with a pilot study conducted by Querques et al., who showed not only a decrease in BRB

breakdown but also an improvement in ischemic index in all patients with DME treated with DEXii, measuring peripheral ischemia status with UWF FA[619].

In the same line, in subjects with DME, sustained intraocular delivery of FAc slows development of PDR and slows progression of diabetic retinopathy[620].

Discrepancies among studies regarding the effect of anti-VEGF in RNP may be due different criteria for grading and imaging method used for diagnosis, FA, UW FA or WF OCTA[621, 622].

For the eyes that do not show reperfusion, it can be hypothesized that these areas are either irreversibly infarcted or may require a higher or more frequent dose of antiVEGF therapy[622].

Given the burden and cost of anti-VEGF injections for patients, it is important to identify factors with our imaging modalities that can help guide and predict treatment response to anti-VEGF agents. In clinical practice, physicians will need to utilize a multimodal imaging approach to appropriately characterize the degree and progression of ischemia in DR and choose the most valid therapy.

1.6.4.2 Intra-vitreal therapy for DME

For DME anti-VEGF is worldwide accepted as the first-line therapy for most eyes[19, 53, 256].

Two anti-VEGF treatments have been approved by FDA and EMA, to treat DME: ranibizumab, RBZ (Lucentis®; Genentech, South San Francisco, CA)[616, 617] and aflibercept, AFL (Eylea®; Regeneron, Tarrytown, NY)[618].

Bevacizumab, BEV (Avastin®; Genentech, South San Francisco, CA), which is approved for the treatment of certain types of cancer, although off-label to treat DME, its lower cost, perceived effectiveness and relative safety, makes it a widely accepted therapy option particularly in the developing world[623, 624].

Additional information

- Unlike **RBZ**, which is composed of the Fab fragment of IgG, **BEV** contains the full-length human IgG framework, making the molecule three times larger (149 kiloDaltons, kDa) than **RBZ** (48 kDa)[1]. **AFL**, which was initially developed and approved for oncological use, is a fusion protein with the Fc portion, that binds to VEGF-A, VEGF-B, and PlGF[2].
- **AFL** like **BEV** contains an Fc portion. This makes it two times larger (115 kDa) than **RBZ**[3]. The Fc antibody domain contributes to immune activation[4].
- No major differences regarding systemic safety have been reported between **RBZ** and **AFL**[5].
- Regarding **BEV** there are studies reporting significant higher risks of hemorrhagic stroke and mortality by other causes, arterial thromboembolic events (ATEs) and intra-ocular inflammation[6-8].
- In terms of ocular side effects, Souied et al. observed in a sample of 432794 IV injections an increased risk of severe inflammatory reactions in **AFT** in comparison with **RBZ**[9].

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Anti-VEGF therapy can stop vision loss and even improve visual acuity[625].

Nevertheless, VEGF inhibition is not 100% effective in all patients. Despite six consecutive monthly injections of a VEGF inhibitor, 40%–60% of eyes had persistent DME[626].

This suggests that in some eyes, the main driver of DME is VEGF, whereas in other eyes, other cytokines predominate[627, 628].

According to sub-analyses from DRCR.net protocol I (a prospective, multicenter, randomized Phase III study with a large sample size of 335 eyes), a strong response (>=20 percent) after a loading dose of 3 RBZ IV injections showed significantly greater improvement in visual acuity over a 3-year follow-up period compared to a limited early anatomical response (<20% thickness reduction).

Within the cases without a strong response after the 3-loading dose injection, 31% respond after 1 year, 45% after 2 years and 52% after 3 years[629].

However, in order to acquire this late response, we would have to penalize all 50% of non-early respondents. Furthermore, the long-term (3-year) visual acuity outcome was significantly worse in eyes with chronic persistent edema through the entire 3-year follow-up period than in eyes with shorter lasting edema (mean BCVA improvement from baseline to 3 years: 7 vs. 13 letters)[394]. This was also consistent with the functional outcome of this group of patients, since the visual improvement acquired after 12 weeks of therapy was maintained over the course of the three-year follow-up period[630]. Similar results were obtained with the sub-analyses from DRCR.net protocol T, which included 363 patients comparing the 3 anti-VEGF drugs (RBZ, AFL and the off-label BEV).

Among eyes with persistent DME, eyes assigned to 1.25 mg BEV were more likely to have chronic persistent DME than eyes assigned to 0.5 mg AFL or 0.3 mg RBZ. Albeit RBZ 0,3 mg dose is approved for monthly therapy by FDA that was not the standard and approved dose for EMA and the PRN-like regimen adopted in protocol T[575, 616, 617, 624, 631].

If anti-VEGF therapy is contraindicated or fails to produce a satisfactory response (despite proper injection frequency and regular monitoring), then intravitreal (IV) CCT is indicated[632] and macular laser may be used as an adjunctive therapy[19, 53, 256].

Available CCT include dexamethasone intravitreal implant (DEXii), (Ozurdex®; Allergan Inc., Irvine, CA), an injectable fluocinolone polymer (FAc), (Iluvien®; Alimera Sciences, Alpharetta, GA), and triamcinolone acetonide (TA) (an off-label therapy)[628].

IVTA injections must be repeated every 2 to 4 months to maintain their effectiveness. As a result, sustained-release steroid formulations have been developed to extend treatment intervals and are recommended[633]. The sustained-release biodegradable 0.7-mg DEXii has a duration of action of 4 to 6 months[634]. Fac implant is nonbiodegradable implant containing 0.19 mg of FAc that delivers a continuous therapeutic dose of 0.2 µg/day of fluocinolone acetonide

for up to 3 years. There is less than a fivefold variation in mean aqueous levels over the 3-year life of the implant explained by zero-order kinetics of FAc, with a relatively flat profile of release[635].

1.6.4.3 Special considerations for DME

DEXii is preferred for patients not suitable for first-line VEGF (for example, those with recent cardiovascular or arterial thromboembolic events; during pregnancy or breast feeding) while FAc has been used for more chronic and resistant DME[636-638].

In those who are unavailable for frequent injections and clinic visits or with needle´s phobia Fac can be considered earlier. Further advantages of sustained intravitreal release of the FAc implant include lower rates of complications related to IV procedure (such as retinal detachment, endophthalmitis and lens iatrogenic injury), higher patient compliance and lower healthcare costs[639-641].

The potential risks of CCT include increased intraocular pressure (IOP), in nearly 1/3 of healthy patients (globally from 11–79%)[642], glaucoma development and cataract formation in phakic eyes. However, these side effects are usually manageable[632, 643].

Anti-VEGF is preferable in patients with in PDR or major ischemia (see sections 1.5.1.4 and 1.5.3), uncontrolled or severe glaucoma (double or triple combination therapy). Even though, DEXii and FAc are acceptable in patients with no glaucoma or glaucoma treated with monotherapy[644-646].

After DEXii patients should perform a safety visit after 6–8 weeks of implantation to evaluate the therapeutic response to corticosteroids and any potential increase in IOP, or earlier when existing other risks factors, as double or triple combination therapy[403, 647, 648].

With Fac IOP should be checked one-week post-injection to exclude any adverse effects related to the intravitreal injection, and then follow the patient every 3 months[649].

In VIT eyes the functional and anatomical efficacy seems to be achieved slower, with the need of a higher number of injections at least during the first 12 months of treatment[299, 650], probably due more rapid clearance of drugs than in non-VIT eyes[650-655].

In aphakic/without capsular or zonular integrity anti-VEGF or triamcinolone (when CCT is indicated) are the safest options[637].

Thus it is defensable to start with a 3 loading dose of anti-VEGF as first line therapy, unless contra-indicated or in the presence of a vitrectomized eye. Then, if there is not a strong response (>=20%) after the loading dose it should be considered an early switch, as discussed above.

Anti-VEGFs are normally used in *pro re nata* (PRN), fixed or a treat and extend (TE) regimens, after loading dose[637].

Patients with DME represent a heterogeneous group with varied responses to therapy that have led to individualized dosing regimens of anti-VEGF. The TE regimen seems to be the regimen more suitable for that purpose, in the real-world setting, to optimize the therapy approach, allowing the incremental extension of inter-treatment intervals based on disease stability and severity biomarkers. A VA loss due to disease recurrence triggers a return to a step-back interval until VA stability is re-established[656]. The extension intervals and pattern of extended intervals should be adapted to DR/maculopathy severity.

The TE regimen seems to ensure the maintenance of an individualized strategy, with the potential to reduce the burden of the visits in comparison to a pure PRN.

This is well demonstrated by Protocol T Extension Study results (with a PRN-like regimen), with loss of gained vision from year 2 to 5, regardless of the type of anti-VEGF used, due to insufficient follow-up and therapy. In the extension period of 3 years the median of follow-up visits and injections was 14 and 4, respectively. Although the number of injections was equivalent to DRCR.net protocol I (n=4), the number of visits was lower (n=20 in protocol I vs n=14 in protocol T)[394, 611].

Furthermore, there is the most extensive published evidence that an intensive therapy during the first year of therapy is crucial to maximize the possible gain of vision[292, 394].

A large-scale real live retrospective study (including data from a database, Vestrum Health Retina Database, obtained from a panel of more than 240 United States private-practice retina physicians) including 28 658 eyes, have demonstrated a correlation between gain of vision and intensive therapy during year 1. Near 50% of the patients received ≤6 injections during year 1. A mean number of 7 injections in year 1 was the cutoff observed to obtain a significant gain of vision (>5 letters)[657]. In protocol T, as an example, a mean of 9-10 injections was the needed according to the protocol.

As a result, correct treatment, rather than sparse, random, or reactive, as well as intensive therapy in the first year, are critical approaches for DME.

Tractional DME (tDME)

Patients with VRI abnormalities (ERM involving the macular center or VMT) which result in significant traction forces, may be recommended for vitrectomy, and even for eccentric ERM. Even for eccentric ERM, depending on the grade of its eccentricity and tangencial traction effect, vitrectomy may be considered. That can be accurately determined after a single IV injection[483].

When tDME and PDR coexist, anti-VEGF pre-vitrectomy is used to reduce intra- and post-operative bleeding, make epiretinal membrane dissection and removal easier, reduce surgical time and the risk of recurrent DME[658, 659].

Nonethless anti-VEGF may induce a compensatory increase of connective tissue growth factor (CTGF) and IL-6, fibrovascular contraction and development or agravation of tractional retinal detachment (tRD)[494, 660].

A reduced time between anti-VEGF and vitrectomy, a lower dose of anti-VEGF and PRP pre-vitrectomy decreases the risk or aggravation of tractional RD[660, 661].

According to Castillo Velazquez vitrectomy should be performed within 1 week, preferably between 3-5 days, after anti-VEGF.

As previously discussed in section 1.2.3, there is no consensus regarding the advantages of PPV when there is no evidence of traction[256].

Until the advent of Ocriplasmin, OCP (Jetrea; ThromboGenics, Leuven, Belgium) the only management options for symptomatic focal VMT and full-thickness macular hole (FTMH) were observation or pars plana vitrectomy surgery. EMA approved Ocriplasmin (Jetrea, Thrombogenics USA, Alcon/Novartis EU) in 2013 for the treatment of symptomatic VMT.

Ocriplasmin is a truncated recombinant form of human plasmin (a serine protease) which degrades fibronectin and laminin and promotes vitreous liquefaction. It has a time dependent, dose-dependent effect, and its biologic action in vitreous is anticipated to last 16-42 days, depending on serine protease inhibitor levels. (alpha(2)-antiplasmin, antithrombin III, alpha(1)-antitrypsin) in vitreous. In a liquefied vitreous its autolysis further decreases[662, 663].

The efficacy of OCP was demonstrated in two phase three clinical trials (the MIVI-TRUST study group)[664].

However, the relatively high cost of OCP, as well as the low and variable success rate in achieving VMT release of around 30–40%, even with a wider range of success rate from 0 to 71 percent, largely due to case mix, influenced the current low uptake of OCP as a treatment option, compared to vitrectomy (with a near 100% of success for VMT release)[664-666].

1.7 The vitreous as a vehicle for longer lasting therapies - New therapies on the Horizon

Only Phase 3 therapies trials with positive safety and efficiency results anticipating their potential approval and gene therapy, will be addressed in this final section.

1.7.1 Brolucizumab (BRZ) and Faricimab (FAR)

New anti-VEGF **brolucizumab** (Beovu®, Novartis, Basel, Switzerland) and the first bispecific antibody **Faricimab** molecule (Roche, Genentech) have their efficacy, durability, and safety findings, 52 weeks results, of phase 3 clinical trials in DME, (*KITE and KESTREL* for BRZ and

YOSEMITE and RHINE for FAR) presented lately at Euretina 2021 virtual annual meeting[667, 668].

These new therapies were developed with the purpose of further extension of treatment intervals.

Additional information

Brolucizumab

- Is a 26 kDa humanized single-chain antibody fragment that inhibits **all isoforms of VEGF-A**, including VEGF110, VEGF121, and VEGF165, by preventing interactions with VEGFR-1 and VEGFR-2[1].
- It **enables the delivery of a much higher molar dose in the same volume** as the current VEGF inhibitors in clinical use, potentially supporting an earlier initiation and a prolonged duration of treatment effect[1-4].

Faricimab

- Is a novel bispecific antibody that targets both angiopoietin-2 (Ang-2) and VEGF-A.
- **Anti–Ang-2** Fab enhances vascular stability, reduces inflammation and vascular leakage, the **Anti–VEGF-A** Fab inhibits vascular leakage and neovascularization and modified Fc intends to reduces systemic exposure and inflammatory potential[5].

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BRZ is the newest anti-VEGF drug already approved for the treatment of neovascular age related macular degeneration (nAMD) by the FDA on October 7, 2019, followed by the EMA approval on February 17, 2020. BRZ 6 mg in *KESTREL (NCT03481634) and KITE (NCT03481660) trials* revealed non inferiority to 2 mg aflibercept (5q4w followed by q8w fixed regimen) with BCVA mean change at Week 52, with fewer injections. Additionally, there was a higher proportion of patients with fluid resolution (IRF/SRF) on BRZ 6 mg at Week 52 and after 5 loading doses every 6 weeks (q6w) followed by q12w dosing. In Year 1 more than half of brolucizumab 6 mg patients were maintained on q12w treatment interval up to Week 52 immediately after the loading phase, with a well-tolerated safety profile. No negative impact on the brolucizumab-related incidence of intraocular inflammation (IOI) where added to previous reports. Comparing 6 mg BZB and the AFL arms no significant differences were observed regarding IOI (1.7 vs 1,7 % in *KITE* and 3.7% vs 0.5 and *KESTRE*l , respectively), retinal vascular occlusion (0.6 vs 0.6% in *KITE* and 0.5 vs 0% in *KESTREL*), retinal vasculitis (0 vs 0% in *KITE* and 0.5 vs 0% in *KESTREL)*, and ≥15 letter loss from baseline at Week 52 (1.1 vs 1.7% in *KITE* and 0 vs 0.5% in *KESTREL*)[667].

FAR is a novel bispecific antibody that targets both angiopoietin-2 (Ang-2) and VEGF-A. **Anti–Ang-2** Fab enhances vascular stability, reduces inflammation and vascular leakage, the **Anti–VEGF-A** Fab inhibits vascular leakage and neovascularization and modified Fc intends to reduces systemic exposure and inflammatory potential[668].

Additional information

- **Angiopoietin-1 (Ang-1)** and **angiopoietin-2 (Ang-2)** are peptide ligands for the tyrosine kinase with Ig (immunoglobulin) and EGF (epidermal growth factor) homology domains 2 (Tie2) receptor[1].
- **Ang-1 is a Tie2 receptor agonist** while **Ang-2 is a Tie2 receptor antagonist**, blocking Tie2 signaling. Tie2 signaling pathway is important to the maintenance of vascular health, promoting endothelial cell (EC) survival, maturation, and stability[2].
- There are **5 key components of the Tie2 pathway** relevant to ocular angiogenesis: the Tie1 and Tie2 receptors, angiopoietin-1 and -2, and the vascular endothelial tyrosine phosphatase (VE-PTP) receptor[3].
- **Ang-1** and **Ang-2** are Tie2 receptor ligands; both bind to Tie2 at the same site with similar affinity[690]. Although **Ang-2** is a Tie2 receptor antagonist in resting ECs, it may also serve as a partial agonist when ECs are in activated or stressed states such as in the setting of inflammation as may occur in diabetic patients[4,5].

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The 1-year results of the pivotal Phase 3 *YOSEMITE and RHINE* trials showed favourable results for 6 mg IV injections with FAR for DME treatment after a loading dose of 4 monthly injections (in the arm of a treat-and-extend concept - PTI). The visual gains achieved with every-16-week dosing (PTI arm) were non-inferior to those of AFL (Eylea, Regeneron Pharmaceuticals) dosed every 8 weeks (q8w) and the anatomic gains also favoured FAR compared with aflibercept. FAR also demonstrated a good safety profile with very low rates of inflammation. Comparing 6 mg FAR and the 2 mg AFL arms no IOI (2.24 vs 0.96 % in *YOSEMITE and* 0.63 vs 0.32% in *RHINE*, respectively), retinal vascular occlusion (0.32 vs 0.32% in *YOSEMITE* and 0.32 vs 0.32 % in *RHINE*) and retinal vasculitis (0.0 vs 0.0 % in *YOSEMITE* and 0.0 vs 0.0% in *RHINE)* significant differences were observed.

Of note is the CFT criteria for edema (for inclusion and retreatment) of 325 µm[668] which is, by definition, considered edema. This could lead to a possible bias in the results.

FDA requests for brolucizumab and faricimab have already been annouced for DME. Thereby, it is anticipated eminent approval of these new drugs in DME treatment armamentarium in 2022.

1.7.2 The Port Delivery System with ranibizumab (PDS)

PDS is an investigational drug delivery system that includes a surgically placed permanent refillable ocular implant that continuously delivers a customized formulation of RBZ into the vitreous. The implant reservoir can be repeatedly refilled through a self-sealing septum in the center of the implant flange, allowing for drug replenishment via clinic-based refill-exchange procedures[669].

In *Archway randomized phase 3 trial* the results for neovascular age macular degeneration (nAMD), using with a 100 mg/mL with fixed 24-week refill-exchanges (PDS Q24W) or IV RBZ,

0.5 mg, injections every 4 weeks, PDS Q24W was both noninferior and equivalent to monthly RBZ, with 98.4% not needing to receive supplemental RBZ treatment before the first refill[669].

In 22 October 2021 - Roche (SIX: RO, ROG; OTCQX: RHHBY) announced that the FDA has approved SusvimoTM (ranibizumab injection) 100 mg/mL for intravitreal use via ocular implant for the treatment of people with nAMD who have previously responded to at least two anti-vascular endothelial growth factor (VEGF) injections (https://www.roche.com/investors/updates/inv-update-2021-10-22b.htm).

Two phase 3 trials to evaluate PDS 100 mg/mL in DR/DME *Pavilion (NCT04503551)* will evaluate the prophylactic effects of PDS 100 mg/mL Q36W versus clinical observation in moderately severe-severe non-proliferative DR without DME (DRSS 47-53). *Pagoda (NCT04108156)* will evaluate the tolerability of PDS 100 mg/mL Q24W and its efficacy versus ITV RBZ 0.5 mg Q4W injections in DME[670].

Counteracting the positive effect of the reduced burden with PDS is the increased percentage of adverse events of special interest (AESIs), with PDS 19.0% (1.6% of endophthalmitis cases, 0.8% of retinal detachments, 5.2% of vitreous haemorrhages, 2.4% of conjunctival erosions, and 2.0% conjunctival retractions) vs 6% in RBZ group. 20.2% manifested also iritis and most ocular adverse events in PDS patients occurred within 1 month of implantation. Hence although manageable after appropriate therapy, these adverse events are the major problem to consider when choosing this therapy option.

1.7.3 Gene therapy

This chapter will also cover gene therapy, because of its novelty and high expectations around the world, as well as its potential futuristic long-term and multi-targeting possibilities in a multifactorial condition like DR.

Gene therapy studies for DR can be divided into two categories: those that target existing neovascularization and vascular hyperpermeability, and those with the purpose to protect blood vessels or neurons from damage. An exciting potential application of gene therapy for DR is to intervene in the early stages of the disease prior to the development of significant vascular and neuronal pathology, something that is not possible with current therapeutic options[671].

Since November 22, 2018, with voretigene neparvovec approval (Luxturna®)[672], the first ocular adeno-associated virus (AAV)-based gene therapy (delivering a functional copy of the RPE65 gene by subretinal injection to patients with Lebers congenital amaurosis), **gene therapy** is in the horizon as a real future option. It represents a unique drug delivery platform to **chronically express drugs as anti-VEGF proteins** for the treatment of DME[673].

Different delivery methods (nonviral and viral-based vectors - adeno-associated virus - AAV, adenovirus and lentivirus), injection sites (subretinal, intravitreal, suprachoroidal) and methodologies (gene replacement, silencing, editing) are currently being tested[674].

Synthetic vectors, known as nanoparticles, are low production cost compared to viruses and have large transport capacity, low immunogenicity and the absence of integration into the genome alleviating insertional mutagenesis. However, their delivery efficiency is much lower than viral vectors, with an increase in efficacy being related also to an increase in toxicity[671].

For now, the synthetic vectors are not as efficient as viral vectors[675]. Lentiviruses and adenoviruses can deliver genes exceeding the carrying capacity of AAVs. AAVs cannot replicate by itself, requiring coinfection with an adenovirus or a herpes simplex virus[675].

The superior safety and efficacy of intraocular AAV administration in humans have led to efforts in engineering AAV-based vector systems for delivering large genes (Dual AAV–mediated large gene delivery)[676, 677].

An adeno-associated virus vector (ADVM-022) encoding AFL, optimized for intravitreal, lesser invasive, therapy delivery and strong protein expression, revealed promising results in preclinical studies with non-human primates. A good safety profile and long-term continuous expression of ADVM-022-derived AFL, out to 30 months with negligible systemic exposure to aflibercept was the motor for the initiation of a Phase 2 trial in DME (INFINITY study)[673, 674, 678, 679].

Even though INFINITY trial raised a major safety concern. An eye of a patient who got the high dose manifested a severe unexpected severe adverse event (SUSAR) hypotony with other infinity patients treated with a single high dose also manifesting concerning adverse events, AEs, (rapid, clinically-relevant decreases in IOP refractory to steroids and requiring a different form of treatment). The AEs surfaced 16 to 36 weeks after treatment with the high dose. No similar events have been charted so far in Infinity subjects given the low dose, or in any wet AMD Optic patients who got the high or low dose.

In the last decade, a paradigm shift can be observed from gene supplementation strategies to gene surgery, where the host genome or the transcriptome is altered directly, either transiently or permanently[680]. Gene silencing using endogenously produced micro RNAs (miRNA) and introducing small interfering RNAs (siRNA) or short hairpin RNAs (shRNA) has become an essential technology in studying gene function and a possible approach in gene therapy[681].

Multiple **miRNA**-based therapies are another potential novel class of drugs therapy target[682]. Micro RNAs are small non-coding RNAs that have been introduced as key controllers of gene expression by binding to their target messenger RNAs. Their altered expression influences several pathological pathways in DR. Hyperglycemia upregulates miRNAs that bind sirtuin 1 (**SIRT1)** suppressing its expression.

The **Sirtuins SIRTs** are a group of 7 enzymes, SIRT1-7, with different distributions inside the eukaryotic cell. SIRT1 is the most studied member of the SIRTs family involved in the regulation of many patterns such as DNA repair, oxidative stress, angiogenesis, inflammation, and senescence. Most SIRTs exert numerous actions in neuronal cells. Indirect activators of the SIRTs (AntagomiR, Fenofibrate, Extendin-4, long non-coding-RNA "MEG3", Kallistatin, Resveratrol, Flavonoids, Ginsenoside Rb1, Coumarins, Glycyrrhizin, dihydrocloride "BGP-15", and Ergothioneine) represent potential therapeutic targets for treating DR, could be used to slow down or stop the degenerative process of the disease. Gene allowing a multi-target therapy approach may also play a role, directly or indirectly[683].

Thanks to combination of high polyunsaturated fatty acids (PUFA) content, high oxygen consumption, and a high level of metabolic activity, the retina is a site of high ROS, lipid hydroperoxide (LOOH), and lipid aldehyde production. The accumulation of **damaging levels of lipid aldehydes and advanced lipoxidation end products** (ALEs) in DR is a consequence of a shift in the balance between the rate of production and clearance. Two of the most promising approaches are the use of aldehyde scavenging molecules or gene therapeutic enhancement of clearance and detoxification pathways[684].

The role of **Gap Junctions** (GP) in cell death and neuromodulation have also been addressed in multiple ocular diseases, as DR. GPs seems to play a role in neuronal cell death and the possible routes of rescuing neuronal and glial cells in the retina succeeding illness or injury. Allowing permeability for macromolecules, able to cross the cellular barriers, they show duality in illness as a cause and as a therapeutic target. Henrein, is the rational for GP neuromodulation through strategies as **gene therapy,** pharmacological blockade, electrical and light stimulation[681].

Using an expressing vector encoding both the pigment epithelium-derived factor gene and a shRNA targeted to the placental growth factor (**dual-acting antiangiogenic non-viral gene therapy**) Araujo R et al. reached a reduced inflammation as well regression of neovascularization in a diabetic mouse retina[685].

In another perspective, the known potential negative effect of anti-VEGF therapy that may increase the degree of retinal fibrosis in PDR patients have been correlated to the up-regulation of **Follistatin-like protein 1** (FSTL1). This was discovered thanks to a dual-target model using a RNA sequencing (RNA-Seq) technology which allowed to determine the genes that were differentially expressed between the **dual-target treatment** (anti-VEGF + connective tissue growth factor, CTGFshRNA) and the anti-VEGF treatment alone. With the dual-target therapy FSTL1 was then down regulated[686].

Gene therapy advances using induced pluripotent stem cells (iPSCs)

Differentiating iPSCs into endothelial cells (iPSC-ECs) from full responders and nonresponders to anti-VEGF, observed that a higher expression of neuronal pentraxin 2 (NPTX2) also related to C-reactive protein (one of the inflammatory biomarkers involved in the pathogenesis of DR) higher expression of this protein in the endothelium of nonresponders could be driving edema through a VEGF parallel pathway, which could be an additional reason why these patients fail to respond to VEGF inhibition. Silencing NPTX2 in nonresponders diminished the effect of VEGF in the phosphorylation of VEGFR2[687, 688].

Gene therapy is **an endless source of opportunities to change the natural curse of DR and DME**. Nonotheless, difficulties for the clinical translation of gene therapy in DR are related with safety concerns, technical limitations, the long time it takes to develop, besides the complex and multifactorial pathogenesis of this disease[671].

Several aspects of transgene delivery are among the issues that need to be addressed. For example, over-expression of the transferred gene could result in excessive protein production to harmful levels[671].

2. Results

2.1 Vitreous biomarkers – new insights in diabetic retinopathy[689]

TITLE: Vitreous biomarkers – new insights in diabetic retinopathy

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KEY MESSAGES:

- Several proteins related with inflammation and neurovascular protection have been suggested to be potential biomarkers for diabetic retinopathy.
- Here, we observed that IL-6, IL-8, MIG and IP-10 from vitreous were the more promising biomarkers for diabetic retinopathy severity.

ABSTRACT

Purpose: Diabetic retinopathy (DR) is a microvascular inflammatory and neurodegenerative disease. The purpose of this study was to analyze the relationship between DR severity and the levels of potential biomarkers in the serum and/or vitreous. **Methods:** Prospective, consecutive, controlled, observational study performed between June 2018 and January 2020. Blood and vitreous samples were collected at start of vitrectomy in patients without diabetes and in patients with diabetes with epiretinal membrane, macular edema and indication for vitrectomy.. **Results:** Transthyretin (TTR) was the only blood biomarker with levels statistically higher in patients with diabetes ($p=0.037$). However, no correlation with DR severity was observed. Erythropoietin (EPO) was the only blood biomarker whose levels were associated with DR severity ($p=0.036$). In vitreous samples, levels of EPO ($p=0.011$), Interleukin (IL)-6 ($p<0.001$), IL-8 ($p<0.001$), IL-17 ($p=0.022$), Monokine induced by interferon-γ (MIG) ($p<0.001$), and Interferon gamma induced protein 10 (IP-10) ($p=0.005$) were significantly higher in patients with diabetes. Additionally, in vitreous, IL-6, IL-8, MIG and IPL-10 levels were also higher in more severe DR cases ($p<0.05$). **Conclusions:** Among the studied biomarkers, vitreous IL-6, IL-8, MIG and IP-10 were the ones whose levels have the strongest coherent relationship with DR severity prediction and, thus, have the best potential post vitrectomy prognostic value.

INTRODUCTION

Diabetes mellitus (DM) is a highly prevalent disease that affects multiple organs. In 2019, approximately 463 million people worldwide were affected by DM.[1] Among DM patients, 10% reach vision-threatening states, such as diabetic macular edema (DME) or proliferative diabetic retinopathy (PDR).[1, 2]

Diabetic retinopathy results from a low-grade inflammatory neuro-vascular degeneration due to chronic hyperglycemia.[3, 4] Pro-inflammatory cytokines and chemokines have been shown to be upregulated in the serum as well as in ocular samples of DM patients, being proposed that their levels correlate with retinopathy severity.[5]

The vitreous promotes the accumulation of cytokines, VEGF, advanced glycation end products and other inflammatory molecules, enhancing their negative effect upon the macula.[6] Increased inflammatory mediators in DR could trigger the production of protective agents to counter-regulate the harmful effects (of chronic inflammation) and induce neuroprotection.[7] Beyond cytokines and chemokines there are other substances associated with neurovascular protective functions such as, erythropoietin (EPO), 25HO-vitamin D (VitD) and transthyretin (TTR). VitD deficiency is associated with an increased risk of DR and lower serum levels of VitD were associated with more severe DR.[8-10] EPO has been shown to provide endogenous neuroprotective effects in various vitreoretinal ischemic diseases,[11] and previous studies have suggested its involvement in DR pathogenesis.[12, 13] Serum and vitreous levels of TTR have been associated with DR progression.[14] Moreover, microalbuminuria is among widely investigated biomarkers for DR risk progression.[15]

In this study, we performed a comprehensive analysis of the levels of some known mainstay pro-inflammatory, anti-inflammatory and potential neuroprotective mediators in blood and vitreous samples of patients with tractional Diabetic Macular Edema (DME) to clarify their pathogenic and prognostic role in DR.

METHODS

Study Design

This was a single center, prospective, observational study, conducted at the Department of Ophthalmology from Centro Hospitalar e Universitário do Porto (CHUPorto). Consecutive eyes with indication for pars plana vitrectomy (PPV), for macular edema and vitreous-macular interface pathology, were included. Patients were subsequently divided in two groups: group 1 – patients with diabetes; control group – patients without diabetes.

The severity of DR was graded according to the Diabetic Severity Scale Score (DRSS) on the basis of the International Clinical Diabetic Retinopathy and Diabetic Macular Edema Severity scale according to seven standard fields on color fundus photos and divided into five levels: No apparent retinopathy (nDR), mild, moderate and severe non-proliferative diabetic retinopathy (NPDR), and proliferative diabetic retinopathy (PDR).[16]

Patients were followed-up according to the standard of care and a final analysis of the results was conducted at 12 months of follow-up, which was the minimum follow-up period required for each patient. The recruitment period was from June 2018 to January 2020.

This study was conducted according to the tenets of the Declaration of Helsinki in its latest amendment (Brazil, 2013), and was approved by the Ethics Committee of CHUPorto. All patients signed an informed consent form.

Inclusion criteria

The inclusion criteria were: 1) presence of tractional macular edema [central subfield foveal thickness, CFT, accessed by Spectral Domain Optical Coherence Tomography (SD-OCT) of at least 300μm associated with the presence of an epiretinal membrane (ERM), and/or vitreomacular traction (VMT)]; 2) age ≥18 years; 3) type 1 or type 2 DM, for patients with diabetes; 4) best corrected visual acuity (BCVA) of 20 to 75 letters, using Early Treatment of Diabetic Retinopathy Study (ETDRS) letters chart; 5) ability and willingness to provide written

informed consent. If the inclusion criteria were fulfilled for both eyes, bilateral inclusion was allowed.

Exclusion criteria

The exclusion criteria included: 1) History of other retinal vascular diseases; 2) (LASER) photocoagulation or intravenous (IV) anti-VEGF injections in the studied eye or systemic anti-Vascular Endothelial Growth Factor (VEGF) or pro-anti-VEGF treatment, in the 4 months previously to study inclusion; 3) IV ocriplasmin injections, IV or peribulbar corticosteroid injections in the 6 months previously to study inclusion (in the studied eye); 4) previous vitrectomy (in the studied eye); 5) history of IV implant of fluocinolone acetonide (in the studied eye); 6) other major ocular surgery (such as cataract extraction, scleral buckle, or any other intraocular surgery) within 6 months prior to or anticipated within the next 6 months following vitrectomy (in the studied eye); 7) YAG capsulotomy performed within 6 months prior to vitrectomy; 8) vitreous haemorrhage or opacification; 9) rubeosis; 10) active ocular inflammation (or infection) in either eye; 11) uncontrolled glaucoma in either eye (intraocular pressure > 24 mmHg with treatment); 11) history of stroke in the previous 6 months; 12) uncontrolled arterial hypertension (systolic blood pressure >160 mmHg or diastolic blood pressure > 100 mmHg); and 13) ward of the state.

Patient Assessment

Demographic data, systolic and diastolic pressure, body mass index and information about concurrent pro-edematous medication (glitazones, prostaglandins) were collected at baseline and at every follow-up visit (between day 1 and 4, and at months 1, 3, 6, 9, and 12), for all patients. Additionally, an SD-OCT and an ophthalmological evaluation (symptoms, BCVA using ETDRS letters chart, intraocular pressure measurement, and anterior and posterior segment biomicroscopy) were also performed at these time-points. Fluorescein angiography was performed on all patients from group 1 at study entrance and whenever needed.

Blood samples were collected at baseline, within one week pre-vitrectomy, to assess glycemic control (HbA1c), and to determine the levels of hemoglobin, EPO, VitD and TTR. Moreover, for patients belonging to group 1, albuminuria was evaluated in a 24h urine collection. At the moment of vitrectomy, vitreous samples were collected for the measurement of cytokines, chemokines, EPO, VitD and TTR. All samples were carefully protected from light.

Procedures

A standard transconjuntival 23G 3-port pars plana vitrectomy (PPV) using the CONSTELLATION® Vision System (Alcon Laboratories, Inc., Fort Worth, TX, USA) was performed by a single senior surgeon, BP. The surgical technique consisted of vitrectomy with the removal of vitreous, delamination and dissection techniques to remove ERMs and internal limiting membrane peeling with cromodissection with DOUBLEDYNE® (Horus Pharma, Saint-Laurent du Var, France). Endolaser photocoagulation and cryotherapy were performed as necessary, such as in the presence of rhegmatogenous retinal lesions, PDR or significant peripheral ischemia. Endotamponade was performed with air or gas, as needed.

The need for post-vitrectomy ME was considered if there was an increase in CFT of 10% or more over baseline. Intravitreal therapy (IV) of ME (dexamethasone implant - ozurdex®) during follow-up was allowed if there was a persistent CFT >10% after 3 months of therapy with topical anti-inflammatory eye drops (nepafenac 3mg/ml qd), for both groups of patients.

Vitreous Sample Collection

Under a safe air eye infusion, undiluted vitreous samples (UVS) of at least 1 mL were collected at the start of every vitrectomy by aspiration with a 2 mL syringe attached to the aspiration line of the vitreous cutter. After injected into sterile tubes, UVS were immediately placed on ice and sent to the Cytometry Laboratory (for cytokines and chemokines) and to the Clinical Chemistry Laboratory (for EPO, vitD and TTR), where they were frozen at -80°C until analysis.

Cytokine and chemokine measurements

Three multiplex bead-based immunoassays (Cytometric Bead Arrays, CBA), from BD Biosciences, San Jose, CA, USA, were used to determine the levels of cytokines and chemokines in vitreous samples by flow cytometry: BD™ CBA Human Th1/Th2/Th17 Cytokine Kit (Catalog No. 560484), BD™ CBA Human Inflammatory Cytokines Kit (Catalog No. 551811), and BD™ CBA Human Chemokine Kit (Catalog No. 552990). The first allows for the quantification of interleukin (IL)-2, IL-4, IL-6, IL-10, IL-17A, Interferon-γ (IFN-γ), and Tumor Necrosis Factor (TNF); the second permits the quantification of IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF; the third allows for quantitatively measure of IL-8 (CXCL8), RANTES (Regulated upon Activation, Normal T cell Expressed, and Secreted, CCL5), MIG (monokine induced by interferon-γ, CXCL9), MCP-1 (monocyte chemoattractant protein-1, CCL2), and IP-10 (IFN-γ–induced protein-10, CXCL10).

The staining protocols were performed following the manufacturer instructions and the samples were acquired in a BD FACSCanto II flow cytometer, previously set up for the BD CBA Flex Set. For each cytokine, at least 300 beads per sample were acquired. The FCAP Array Software (BD Biosciences; Catalog No. 641488) was used for data analysis. The standard curve for each cytokine and chemokine covered a defined set of concentrations from 20 to 5000 pg/mL and from 10 to 2500 pg/mL, respectively. The minimum detection levels were: 7.2 pg/mL for IL-1β, 2.6 pg/mL for IL-2; 4.9 pg/mL for IL-4; 2.4 pg/mL for IL-6; 0.2 pg/mL for IL-8/CXCL8; 3.3 pg/mL for IL10; 1.9 pg/mL for IL-12p70; 18.9 pg/mL for IL17A; 3.7 pg/mL for IFN-γ; 3.7 pg/mL for TNF; 1.0 pg/mL for RANTES/CCL5; 2.5 pg/mL for MIG/CXCL9; 2.7 pg/mL for MCP-1/CCL2; and 2.8 pg/mL for IP-10/CXCL-10.

EPO, VitD and TTR measurements

UVS were centrifuged at 16 000 x g for 5 min at 4°C. The samples were immediately frozen at -80°C until assayed. For serum determination, 5 ml venous blood samples were collected

and centrifuged at 3000 x g for 15 min at 4°C to obtain serum and stored at -80°C until assayed. Vitreous and serum concentrations of EPO, VitD and TTR were measure by chemiluminescence in an automatic Xpi Immulite 2000 analyzer (Siemens Healthcare Diagnostics, Siemens AG, Germany).

SD-OCT acquisition

Central subfield foveal thickness and vitreous-macular interface evaluation was determined by spectral domain optical coherence tomography, SD-OCT (Spectralis HRA+OCT, version 1.10.2.0 (Heidelberg Engineering, Heidelberg, Germany).

Two highly trained technicians conducted SD-OCT scans. Macular thickness measurements were performed with automated segmentation, 25 scans and a 20x20º acquisition mode. The follow-up function and auto-rescan with active eye tracking were also utilized. CFT was obtained automatically from equipment readings.

Outcome measures

Primary endpoint

Differences in the levels of serum and vitreous biomarkers between the control group and group 1.

Secondary endpoints

1) Differences according to DR severity in group 1 concerning baseline characteristics, microalbuminuria, and serum (EPO, VitD and TTR) and vitreous (EPO, VitD, TTR, cytokines and chemokines) biomarkers; 2) Correlation between serum and vitreous biomarkers levels and BCVA and CFT at 12 months of follow-up in both groups; 3) Correlation between correspondent serum *vs* vitreous EPO, VitD and TTR levels and microproteinuria *vs* serum EPO, VitD and TTR; 4) Differences between groups regarding BCVA and CFT evolution during follow-up.

Safety

Cataract formation in phakic eyes and post-vitrectomy ME in both groups.

Statistical analysis

Data were analysed using non-parametric statistics. BCVA values of ETDRS letters were converted to LogMar before analyses. For statistical sub-analyses diabetic patients were grouped in 3 sub-groups according to DR severity: less severe DR (mDR, including nDR, mild NPDR and moderate NPDR), severe NPDR (sDR), and PDR. Between-group analyses of continuous variables were performed using the Mann-Whitney U test or the Kruskal-Wallis test corrected for multiplicity. Within-group analyses were performed using the Wilcoxon test or the Friedman test corrected for multiplicity. Nominal variables were analysed using the chi-square test and the Bonferroni correction was used when appropriate. Correlations between variables were tested using the Spearman rank correlation or the Kendall's τ-b, as appropriate. A $p<0.05$ (two-sided) was considered statistically significant.

RESULTS

Baseline demographics and clinical data by presence or absence of diabetes

From the 90 eyes enrolled in the study, a total of 87 (45 from group 1 and 42 from the control group) completed the entire follow-up and were included in the analysis. Most of the participants were males (59%, n = 36) with a higher proportion of females in the control group, although differences between groups did not reach statistical significance. The median age in group 1 and in the control group was 69 and 72 years, respectively, ranging from 67 to 74 years. In group 1, the mean DM duration was of 21.5 years, and 52.3% of the patients were on insulin therapy. Most of the DM patients had severe NPDR (42.2%) or PDR (24.4%), and only 20% had absence of DR lesions. As expected, group 1 had higher HbA1c levels compared to the control group. Also, group 1 had a higher prevalence of macular LASER and PRP laser compared to the control group – Table 1.

Baseline biomarkers by presence or absence of diabetes

Regarding blood biomarkers, the only statistical significantly difference was seen in the level of TTR, which was higher in group 1, compared to the control group (268.5 *vs* 260.1 mg/L, respectively, p=0.037). In vitreous samples, significant differences were found in EPO (p=0.011), IL-6 (p<0.001), IL-8 (p<0.001), IL-17 (p=0.022), MIG (p<0.001), and IP-10 (p=0.005), with group 1 having higher levels of these molecules. There were no other statistically significant differences – Table 2.

Baseline biomarkers by DR severity sub-groups

When the baseline biomarkers were analysed by DR severity sub-groups additional information was obtained. Regarding the blood biomarkers, the only statistically significant difference was obtained for EPO, with patients with PDR having lower serum EPO levels when compared with patients with sDR (p=0.036). For vitreous biomarkers, higher values of IL-6 (mDR vs PDR, p=0.009; mDR vs sDR, p=0.008), IL-8 (mDR vs PDR, p<0.001; mDR vs sDR, p=0.025), MIG (mDR vs PDR, p=0.005), and IPL-10 (mDR vs PDR, p=0.001; mDR vs sDR, p=0.025) were observed in the higher severity sub-groups. There were no other statistically significant differences – Table 3.

Correlation between serum and vitreous biomarkers levels, BCVA and CFT at 12 months of follow-up by presence or absence of diabetes

In group 1, and regarding 12 months BCVA, negative and moderate correlations were observed for vitreous IL-6 (-0.523, p=0.001), MIG (-0,474, p=0.003), and EPO (-0.442, p=0.016). As for CFT at 12 months, also negative and moderate correlations were observed for vitreous biomarkers IL-6 (-0.523, p=0.003), IL-8 (-0.540, p=0.001), MIG (-0.428, p=0.009), IP-10 (-0.516, p=0.001), and EPO (-0.473, p=0.01). No correlations were observed for the control group.

Correlation between serum and vitreous EPO, VitD and TTR levels

In group 1, serum TTR showed a moderate positive correlation with microproteinuria (+0.550, p=0.001). No correlations were observed between serum and vitreous levels of EPO, VitD and TTR levels in both groups.

Evolution of BCVA and CFT by presence or absence of diabetes and by DR severity sub-groups

BCVA and CFT evolution during follow-up and differences between groups by presence or absence of diabetes and by DR severity sub-groups are depicted in Figure 1A and 1B and Figure 2A and 2B, respectively. Regarding BCVA, eyes from group 1 have a significantly worse BCVA at baseline when compared with eyes from the control group (p=0.009). In both groups, the improvement in BCVA was significant from baseline to 6 months (p<0.001) and then stabilized (p=1.000) – Figure 1A. The same evolution was observed in all severity groups of diabetics, with a trend for worse BCVA in the more severe stages, although not always significant – Figure 1B.

As for CFT, there were no differences at baseline between groups (p=0.105). The most significant decrease occurred in both groups in the first 6 months, with stabilization in the control group, and a continued decrease in group 1 from 6 to 12 months (p=0.016). At month 6 and 12 a significantly lower CFT was observed in group 1 when compared with the one obtained for the control group (p=0.026 and p<0.001, respectively) – Figure 2A. The decrease in CFT was only significant from baseline to month 6 in PDR cases (p=0.005) – Figure 2B.

Safety

Post-vitrectomy DME was observed in 25.6% (n=11) of group 1, and 4.8% (n=2) of the control group. Postoperative IV therapy was needed only in the first group (6 out of 11 ME cases; 54.5%).

Cataract formation was observed in 30.8% (n=4) and 50% (n=12) of phakic eyes in group 1 and in the control group, respectively. All patients, except two (one in group 1 and one in the control group), performed cataract surgery before the final follow-up visit. Only one eye from the control group developed DME post-cataract surgery.

In 3 eyes (2 in the control group and 1 in group 1) gas tamponade with SF6 was applied due to intraoperative retinal detachment, without no further complications during the follow-up. No other complications occurred during follow-up.

DISCUSSION

Multiple vitreous-resident proteins, such as cytokines and chemokines, produced in retina cells, not only contribute to the pathogenic mechanisms of DR but also seem to be a reflection of DR severity.[5, 17, 18]

In accordance with the literature, we observed significant higher levels of IL-6, IL-8, MIG (CXCL9), IP-10 (CXCL10), EPO and IL-17 in vitreous samples of eyes from patients with diabetes.[19-24] Also, in more severe grades, significantly higher values of IL-8, IL-6, MIG and IP-10, were observed. This difference was particularly significant in the transition from the stages mDR to PDR.

These observations are in accordance with the literature as there is robust evidence supporting that IL-6 and IL-8 not only contribute to DR development, but also correlate with more severe disease stages, especially in PDR and DME.[20, 25-27] Similarly, the interferon-inducible chemokines, such as MIG (CXCL9) and IP-10 (CXCL10), are potent inhibitors of angiogenesis, which are thought to be associated with different stages of DR severity [28, 29]. In addition, IP-10 inhibits angiogenesis *in vivo* at least in part by antagonizing the functions of IL-8 [30, 31].

Regarding IL-17, although there is also evidence supporting that the levels of IL-17 increase with DR severity up to end-stage PDR,[18] we did not observe significant differences in IL-17 levels between severity stages. The lack of significant differences observed for IL-17 might be due to our grouping of the severity stages of DR, as if it increases continuously, grouping of the initial stages can increase the levels of IL-17 and decrease the difference with end-stage IL-17.

The role of EPO on DR progression is controversial [24] as EPO may be considered a pathogenic factor in PDR, and simultaneously a protective factor in early DR.[32-35] In this work, patients with diabetes had a higher level of EPO in the vitreous than patients without diabetes. This observation might be a reflection of the proposed role of EPO as a neuroprotector of retinal cells, which involves the increased production and binding of EPO to its receptor as a compensatory response to tissue hypoxia and hyperglycemia.[36, 37] Additionally, the increase in permeability of the blood retinal barrier caused by DR could also contribute to this difference [22] but PDR cases with vitreous hemorrhage were excluded from this study. When comparing the levels of EPO for different DR severity stages, a significant lower concentration of serum EPO was observed for PDR when comparing with sDR. These results are in line with the results from Tian et al, where it is suggested that lower levels of EPO might be related with excessive apoptosis and oxidative stress.[38] Moreover, given that systemic EPO release is thought to be modulated by the splanchnic innervation of the kidneys, the diversity of end stage degree for diabetic nephropathy and autonomic neuropathy can explain the divergent results observed between studies.[36, 37, 39-42] No difference between vitreous EPO levels regarding disease severity were observed. Although it would be expected to have higher levels of vitreous EPO due to its neuroprotector role, EPO levels might not increase under significant ischemia or in case of retinal pigment epithelium lesion, as the induced by pan-retinal photocoagulation. Additionally, this result might seem contradictory to the ones obtained in other studies, but the

comparison of vitreous EPO levels between eyes with PDR and eyes without diabetes, without having a control group with less severe DR, may result in the misleading conclusion that EPO levels are highest in the proliferative end stages of DR.[11, 43, 44]

TTR was the only blood biomarker significantly increased in patients with diabetes, accordingly to what has been previously reported in literature.[45-49] TTR is thought to regulate the key genes for DR neovascularization, including Tie2, VEGFR1, VEGFR2, Angpt1, and Angpt2, repressing neovascularization in DR. There are reports associating serum and vitreous TTR levels with DR progression,[50] but we did not observe an association between serum or vitreous TTR levels and DR severity. Nevertheless, levels of serum TTR in patients with diabetes had a moderately positive correlation with microproteinuria, which is also considered a reliable marker of retinopathy and of increased risk for PDR.[51]

VitD has been associated with anti-inflammatory, immunomodulatory and neurovascular protective roles. Additionally, relevance in DR pathophysiology and correlation between lower levels of VitD and DR progression have been previously reported.[52, 53] However, we did not observe any difference between groups nor any correlation within diabetic sub-groups, both in serum and vitreous. Thus, in our study neither vitreous VitD nor serum VitD seem to be appropriate biomarkers or to have a clear relationship with DR.

Increased vitreous levels of EPO, IL-6 and MIG are associated with a negative anatomical and functional prognosis as reflected by the moderately negative correlation between these levels and both BCVA and CFT at the end of follow-up. A similar negative and moderate correlation of IL-8 and IP-10 with the final CFT but not with the final BCVA suggests that their increase may be indicative of DR worsening, before functional compromise. Thus, increased levels of vitreous IL-6, EPO and MIG may be indicative of a more severe central macular lesion.

The differences in BCVA and CFT between group 1 and the control group, as well as between the groups with different DR severity are in accordance with the expected benefit of vitrectomy

in tractional DME.[54-60] In addition, the worse BCVA and CFT observed for patients with diabetes are also expected, due to the effect of chronic hyperglycaemia and DME in the neuroretina.[4, 61, 62]

Since all but two patients (one in each group) were subjected to cataract surgery before the final follow-up visit, this procedure should not affect the prognostic value of the studied biomarkers. Moreover, only one patient from the control group developed ME post-cataract surgery. As previously described by Smiddy et al [63], we observed that the rate of post-vitrectomy cataract development seemed to be lower in group 1, in which patients were relatively younger and the vitrectomized eyes are under a rich glucose environment. Additionally, a higher incidence of post-vitrectomy ME occurred in DME patients[64-66] comparatively to patients without diabetes.[67] Hence, as expected, we observed a more benign ME evolution for patients without diabetes, as post-vitrectomy ME had a spontaneous resolution, whereas in patients with diabetes, postoperative IV therapy was needed in more than half of the eyes (54.5%).

The prospective and controlled design of this study, the exclusion of PDR cases with vitreous hemorrhage and having the same surgeon performing all vitrectomy procedures, minimize bias and strengthen the results. However, the relatively small sample used, especially when considering the stratification of patients with diabetes into severity sub-groups, can be a limitation of this study.

In conclusion, our results reinforce the knowledge that DR is a complex multifactorial disease where anti-inflammatory/antiangiogenic cytokines counterbalance proinflammatory/ angiogenic cytokines. These molecules have a challenging dynamic and can have different roles in different stages of the disease, as seems to be the case with EPO. Among the studied biomarkers, vitreous IL-6, IL-8, MIG and IP-10 are the ones with best post-vitrectomy prognostic and DR severity prediction potential. In addition, higher levels of vitreous IL-6,

EPO and MIG can indicate a more severe central macular lesion. Therefore, early monitoring of the levels of these molecules can help prevent, stop or control end-stage disease and, consequently, vision loss. Moreover, this work reinforces the importance of developing alternative, multitargeted and tailored therapies.

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DECLARATIONS

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Conflicts of Interest/Competing Interests

The authors declare no conflicts of interest.

Ethics approval

This study was conducted according to the tenets of the Declaration of Helsinki in its latest amendment (Brazil, 2013), and was approved by the Research Coordination Office and the Ethics Committee of the CHUPorto, and authorized by the Administration Board of the Hospital [Study number: 2017.093 (084-DEFI/082-CES)].

Consent to participate

All patients signed an informed consent form.

Consent for publication

All listed authors have provided consent for publication of this article.

Availability of data and material

The raw data on which this manuscript is based is available from the authors upon reasonable request.

Code availability

Not applicable

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FIGURE LEGENDS

Figure 1 – Best corrected visual acuity (BCVA) evolution from baseline to 6 and 12 months follow-up and differences between groups by presence or absence of diabetes **(A)** and by diabetic retinopathy severity sub-groups **(B)**. Values represent mean; error bars are 95%CI.
mDR=no diabetic retinopathy + mild non-proliferative diabetic retinopathy + moderate non-proliferative diabetic retinopathy; sDR=severe non-proliferative diabetic retinopathy; PDR=proliferative diabetic retinopathy. *p<0.01 versus baseline, p-values from Friedman's test adjusted for multiplicity; **p<0.05 between groups, p-values from Mann-Whiney U test **(A)** or from Kuskal-Wallis test adjusted for multiplicity **(B)**.

Figure 2 – Central foveal thickness (CFT) evolution from baseline to 6 and 12 months follow-up and differences between groups by presence or absence of diabetes **(A)** and by diabetic retinopathy severity sub-groups **(B)**. Values represent mean; error bars are 95%CI.
mDR=no diabetic retinopathy + mild non-proliferative diabetic retinopathy + moderate non-proliferative diabetic retinopathy; sDR=severe non-proliferative diabetic retinopathy; PDR=proliferative diabetic retinopathy. *p<0.01 versus baseline, p-values from Friedman's test adjusted for multiplicity; **p<0.05 between groups, p-values from Mann-Whiney U test **(A)** or from Kuskal-Wallis test adjusted for multiplicity **(B)**.

Table 1 – Baseline characteristics by presence or absence of diabetes

Parameter	Group 1 (n=45)	Control group (n=42)	p-value
Age, mean [95%CI]	69.07 [66.99–71.14]	71.86 [69.68–74.03]	0.068
HBP, n (%)	40 (88.9)	19 (45.2)	**<0.001**
Dyslipidemia, n (%)	27 (62.8)	13 (31.0)	**0.014**
Gender female, n (%)	14 (31.1)	22 (52.4)	0.052
Phakic, n (%)	13 (28.9)	24 (57.1)	**0.026**
DM duration (years), mean [95%CI]	21.54 [17.52–25.55]	NA	NA
Insulin therapy, n (%)	23 (52.3)	NA	NA
Macular LASER, n (%)	16 (35.6)	0 (0.0)	**<0.001**
PRP LASER, n (%)	26 (57.8)	0 (0.0)	**<0.001**
Naïve IV, n (%)	33 (73.3)	42 (100.0)	**<0.001**
Diabetes without DR lesions, n (%)	9 (20.0)	NA	NA
Mild NPDR, n (%)	2 (4.4)	NA	NA
Moderate NPDR, n (%)	4 (8.9)	NA	NA
Severe NPDR, n (%)	19 (42.2)	NA	NA
PDR, n (%)	11 (24.4)	NA	NA
HbA1c, mean % [95%CI]	7.71 [7.27–8.14]	5.38 [4.72–6.03]	**<0.001**
Hemoglobin, mean [95%CI]	13.03 [12.49–13.57]	13.63 [13.10–14.16]	0.107
Microproteinuria (mg/g), mean [95%CI]	733.94 [208.17–1,259.71]	NA	NA

Group 1=patients with diabetes; Control group= patients without diabetes; 95%CI=95% Confidence Interval; HBP=high blood pressure; DM=Diabetes mellitus; macular LASER=Prior focal-grid photocoagulation treatment in the study eye; PRP LASER=Prior panretinal photocoagulation treatment in the study eye; naïve IV=no prior anti-VEGF or corticosteroid intravitreal treatment in study eye; DR=diabetic retinopathy; NPDR=non proliferative diabetic retinopathy; PDR=proliferative diabetic retinopathy; NA=not applicable.

Table 2 – Baseline biomarkers by presence or absence of diabetes

Parameter	Group 1 (n=45)	Control group (n=42)	p-value
Serum Epo, U/L	26.80 [10.17–43.43]	11.69 [9.33–14.05]	0.412
Serum VitD, nmol/L	37.65 [31.53–43.78]	43.62 [36.74–50.50]	0.349
Serum TTR, mg/L	268.46 [246.52–290.41]	260.05 [243.66–276.44]	**0.037**
Vitreous Epo, U/L	84.81 [54.25–115.36]	45.52 [25.98–65.05]	**0.011**
Vitreous VitD, nmol/L	91.91 [72.47–111.36]	97.68 [79.42–115.93]	0.262
Vitreous TTR, mg/L	34.47 [6.19–62.76]	34.93 [6.32–63.55]	0.653
Vitreous IL6, pg/mL	32.05 [20.06–44.04]	6.03 [3.97–8.09]	**<0.001**
Vitreous IL8, pg/mL	31.57 [19.95–43.20]	5.71 [2.96–8.46]	**<0.001**
Vitreous IL17, pg/mL	0.27 [0.06–0.48]	0.02 [0.00–0.07]	**0.022**
Vitreous MIG, pg/mL	58.46 [36.32–80.59]	18.19 [10.80–25.58]	**<0.001**
Vitreous IP-10, pg/mL	87.70 [59.38–116.02]	42.66 [24.24–61.08]	**0.005**

Group 1=patients with diabetes; Control group=patients without diabetes; Epo=erythropoietin; VitD=25 *hydroxy vitamin D; TTR*=transthyretin; IL6=interleukin 6; IL8=interleukin 8; IL17=interleukin 17; MIG=monokine induced by interferon-γ; IP-10=IFN-γ–induced protein-10. All values presented as mean [95% Confidence Interval].

Table 3 – Baseline biomarkers by DR severity sub-groups

Parameter	mDR (n=15)	sDR (n=19)	PDR (n=11)	p-value
Serum Epo, U/L	21.57 [6.88–36.25]	41.10 [0.96–81.24]*	10.55 [3.62–17.47]*	***0.036**
Serum VitD, nmol/L	42.72 [27.13–58.31]	31.22 [25.05–37.40]	41.27 [30.77–51.77]	0.287
Serum TTR, mg/L	239.86 [182.42–297.30]	295.06 [272.10–318.02]	266.36 [249.06–283.67]	0.160
Vitreous Epo, U/L	55.19 [15.52–94.86]	85.85 [51.83–119.88]	152.34 [0.00–355.20]	0.113
Vitreous VitD, nmol/L	105.24 [60.10–150.38]	88.88 [69.27–108.50]	70.24 [0.00–155.95]	0.431
Vitreous TTR, mg/L	69.04 [0.00–154.23]	13.25 [6.85–19.66]	29.59 [0.00–104.22]	0.586
Vitreous IL6, pg/mL	10.44 [4.04–16.84]*/**	44.51 [21.98–67.04]*	44.54 [16.58–72.51]**	***0.008; **0.009**
Vitreous IL8, pg/mL	10.42 [4.98–15.86]*/**	31.31 [20.26–42.37]*	77.61 [21.72–133.51]**	***0.025; **<0.001**
Vitreous IL17, pg/mL	0.27 [0.00–0.65]	0.36 [0.00–0.74]	0.03 [0.00–0.10]	0.845
Vitreous MIG, pg/mL	29.94 [9.90–49.98]*	64.33 [28.09–100.57]	103.64 [19.65–187.64]*	***0.005**
Vitreous IP-10, pg/mL	35.94 [19.80–52.08]*/**	93.04 [57.17–128.91]*	184.11 [62.03–306.20]**	***0.025; **0.001**

mDR=no apparent diabetic retinopathy+mild non-proliferative diabetic retinopathy+moderate non-proliferative diabetic retinopathy; sDR=severe non-proliferative diabetic retinopathy; PDR=proliferative diabetic retinopathy; Epo=erythropoietin; VitD=25 *hydroxy vitamin D; TTR*=transthyretin; IL6=interleukin 6; IL8=interleukin 8; IL17=interleukin 17; MIG=monokine induced by interferon-γ; IP-10=IFN-γ–induced protein-10. All values presented as mean [95% Confidence Interval]. All p-values from Kruskal-Wallis corrected for multiplicity.

Figure 1

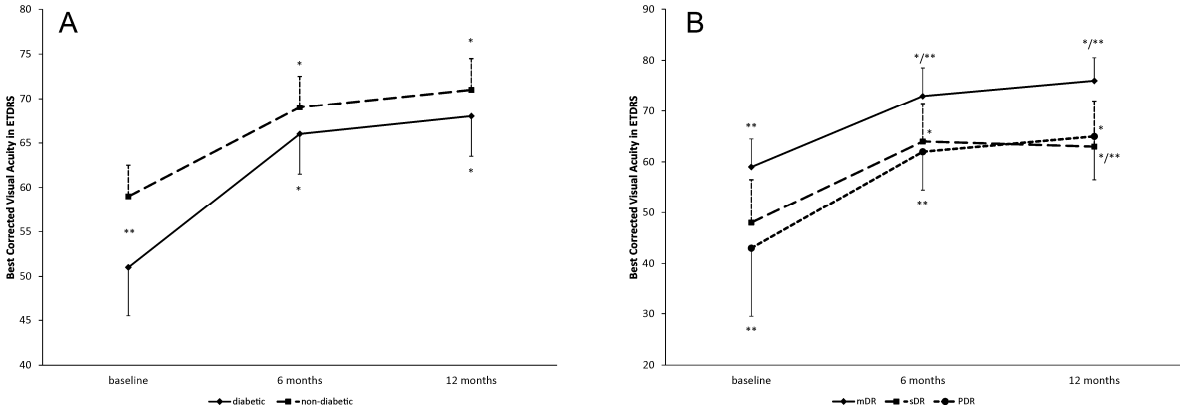
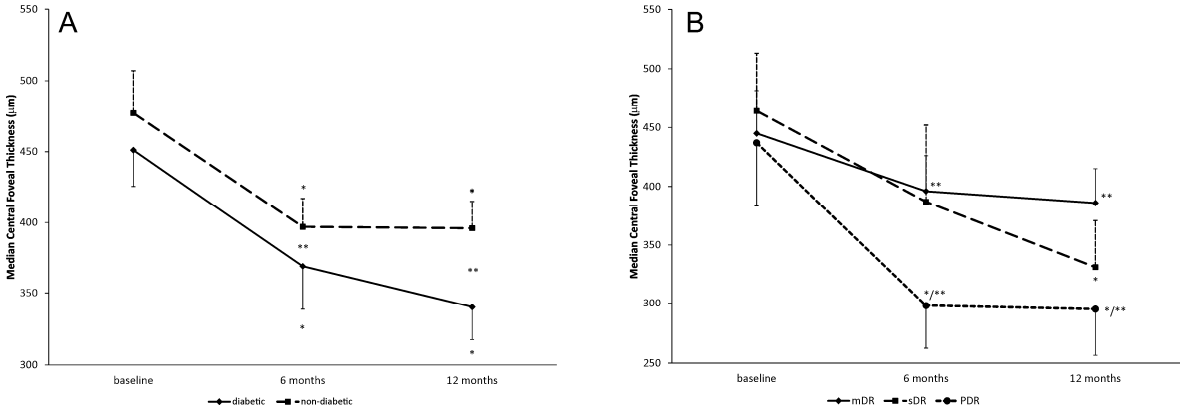


Figure 2



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Vitrectomy Outcomes in Eyes with Tractional Diabetic Macular Edema

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Keywords
Vitrectomy · Tractional diabetic macular edema · Internal limiting membrane · Epiretinal membrane · Best corrected visual acuity · Central foveal thickness

Abstract
Purpose: To evaluate pars plana vitrectomy (PPV) outcomes in cases with tractional diabetic macular edema (tDME). ***Methods:*** We conducted a single-center retrospective study with a follow-up of 12 months. Forty-six eyes with tDME of 38 patients submitted to PPV between 2013 and 2015 were assessed. A standard PPV was performed and surgical outcomes were registered at the 3-, 6-, and 12-month follow-up. ***Results:*** The baseline median best corrected visual acuity (BCVA) in ETDRS (Early Treatment Diabetic Retinopathy Study) letters and the median central foveal thickness (CFT) were 43.0 letters and 491.0 µm, respectively. At the 12-month follow-up, a median decrease in CFT of 232.7 µm was observed. A CFT <300 µm was achieved in 65.2% of the cases (52.2% needing no further treatment); a BCVA improvement by ≥10 letters was achieved in 60.0%, but there was a decrease of ≥10 letters in 13.0% of the cases. DME recurrence was observed in 10.9% of the cases, with a median time of development after vitrectomy of 6 months. As a major postoperative complication, a macular hole was observed in 1 patient (2.1%). ***Conclusions:*** In our series, PPV for tDME induced an improvement in retinal thickening and visual outcome in more than 50% of the cases, with low recurrence rates and a low number of postoperative complications.

Introduction

Diabetic macular edema (DME) is a disorder with an increasing prevalence worldwide [1] and is the leading cause of visual impairment in patients with diabetes mellitus [2]. The vitreous, via several mechanical and physiological mechanisms that lead to increased vascular permeability, has been implicated as a cause of DME [1]. Vitrectomy has been proven to be effective in the resolution of DME when a tractional cause is involved in its pathogenesis [2, 3]. In fact, the observation of DME reduction after release of mechanical traction on the macula, either by spontaneous posterior vitreous detachment or by vitrectomy, lends support to this line of reasoning [4]. Furthermore, the removal of accumulated cytokines, VEGF,

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and advanced glycation end products resulting from diabetic disease from the vitreous cavity – as well as evidence that vitrectomy improves retinal oxygenation, taken together with evidence that increased oxygenation can reduce DME – suggests an additional physiological advantage potentially conferred by vitrectomy [5, 6]. Some additional procedures may be conducted during vitrectomy, and internal limiting membrane (ILM) peeling appears to be effective in reducing DME and improving visual acuity in the long term [7–12], although the authors suggesting this acknowledge that the ILM peeling approach still remains controversial [13, 14].

While vitrectomy has been performed as a treatment for DME, information on the precise benefits and risks has been limited by the lack of substantial prospective data [15]. The aim of this study was to evaluate the effectiveness and safety of pars plana vitrectomy (PPV) for the treatment of tractional DME (tDME).

Subjects and Methods

Study Population

This was a single-center retrospective study, performed at Centro Hospitalar Universitário do Porto, Porto, Portugal, between 2013 and 2015, which included a study population of 46 eyes of 38 patients submitted to PPV as treatment for tDME with presence of vitreomacular traction (VMT) and/or epiretinal membrane (ERM).

Inclusion Criteria. The inclusion criteria were: age ≥18 years; type 1 or 2 diabetes; tDME as the indication for vitrectomy; central foveal thickness (CFT) ≥300 μm as measured by spectral-domain optical coherence tomography (SD-OCT); and best corrected visual acuity (BCVA) <75 letters according to the ETDRS (Early Treatment Diabetic Retinopathy Study) letter chart.

Exclusion Criteria. Excluded were patients with: a history of macular photocoagulation or peripheral scatter photocoagulation, intravitreal (IV) anti-VEGF or corticosteroid (CCT) injection, or other treatment for DME within 4 months before PPV; a history of IV implantation of fluocinolone acetonide; a history of other retinal vascular diseases; or active ocular inflammation or infection.

Procedures

All patients underwent comprehensive ophthalmic history-taking and examination including BCVA measurement, anterior segment examination, and fundus examination with SD-OCT image acquisition (Spectralis HRA+OCT; Heidelberg Engineering, Heidelberg, Germany). The data were analyzed preoperatively and after surgery at the 3-, 6-, and 12-month follow-up.

A CFT <300 μm was defined as DME resolution. Significant functional improvement was considered when a gain of ≥10 letters was achieved during follow-up.

Standard PPV was performed according to the investigator's usual routine. General steps during the intervention included: 3 pars plana sclerotomies; core vitrectomy with induction of posterior vitreous detachment, if attached, and removal of the peripheral vitreous; staining using trypan blue ophthalmic solution 0.15% (MembraneBlue; DORC, Zuidland, The Netherlands); and peeling of the ERM and ILM. In some cases, additional maneuvers such as endolaser, cryotherapy, and intraocular CCT/anti-VEGF were also used. Treatment of DME during follow-up – by macular, peripheral laser and/or CCT and/or IV anti-VEGF injections – was at the investigator's discretion.

The main outcome measures were BCVA and CFT changes and safety findings at the 3-, 6-, and 12-month follow-up. The effect of IV injections (at the end of the PPV procedure), age, and type of traction (VMT, ERM, or both) in the main outcome measures (BCVA and CFT) were also analyzed. The age at baseline was compared between the traction type groups. The rate of DME recurrence was evaluated at the 12-month follow-up.

Statistical Analysis

After testing for the normality of all variables using the Shapiro-Wilk test, nonparametric statistical methods were used. Changes in BCVA and CFT in the overall sample over time were evaluated with a Friedman or Wilcoxon test for paired samples, as appropriate. The Kruskal-Wallis or Mann-Whitney U test was used for comparisons between groups, as appropriate. The χ^2 test was used for comparison of proportions. Correlations between variables were evaluated with the Spearman coefficient. Bonferroni correction was applied whenever necessary. Values are presented as median (range, interquartile range) unless otherwise specified. Data analysis was performed using SPSS 23th edition (IBM, USA). Tests were considered significant at the $\alpha = 0.05$ significance level (two-sided).

Results

Baseline Characteristics

Between 2013 and 2015, 46 eyes of 38 patients who met the primary cohort criteria underwent PPV. The baseline characteristics were as follows: the median age was 71.0 years (58.5–82.3, 10.0); the median HbA_{1c} was 7.8% (6.0–13.0, 2.5); the median diabetes duration was 17 years (8–28, 8); and 60.5% were male patients.

Combined cataract surgery at the moment of vitrectomy was performed on 10 eyes (21.7%); 7 (15.2%) underwent cataract extraction during follow-up, and 11 (23.9%) remained phakic. During surgery, additional maneuvers included ILM peeling in 100.0%, ERM peeling in 82.6%, and an IV injection in 28.2% of the cases at the end of the procedure (bevacizumab in 30.8%, ranibizumab in 15.4%, and triamcinolone in 54.8%).

Functional Results

The median BCVA at baseline and at the 3-, 6-, and 12-month follow-up was 43 letters (3–74, 48), 51 letters (4–80, 48), 54 letters (5–85, 38), and 66 letters (5–85, 45), respectively (Fig. 1). There was a statistically significant improvement in median BCVA in all follow-up periods

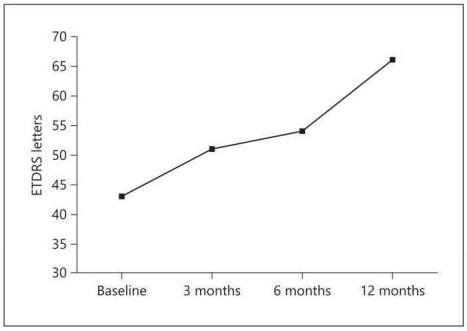

Fig. 1. Best corrected visual acuity (BCVA) evolution during follow-up. The increase in BCVA was most significant during the first 6 months after surgery ($p = 0.003$ between baseline and 3 months of follow-up; $p = 0.005$ between 3 and 6 months; and $p = 0.07$ between 6 and 12 months).

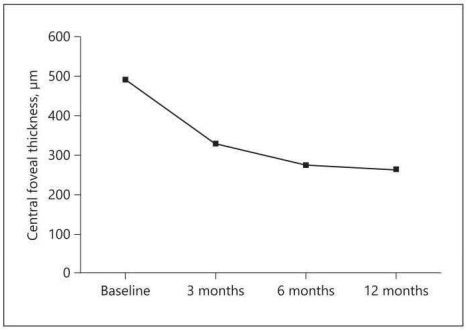

Fig. 2. Central foveal thickness (CFT) evolution during follow-up. The decrease in CFT was significant during the first 3 months after surgery ($p < 0.001$ between baseline and 3 months of follow-up; $p = 0.056$ between 3 and 6 months; and $p = 0.178$ between 6 and 12 months).

compared with the baseline value ($p < 0.001$). The increase in BCVA was most significant ($p < 0.001$) during the first 6 months after surgery. The BCVA improvement achieved at month 6 was maintained until the end of follow-up, with no statistically significant difference between months 6 and 12.

A BCVA improvement by ≥10 letters was achieved in 42.2, 53.3, and 60.0% of the cases at 3, 6, and 12 months, respectively. The median BCVA change from baseline was +8, +11, and +23 letters at 3, 6, and 12 months, respectively.

The differences in age, baseline BCVA, baseline CFT, and CFT at 12 months between those who improved ≥10 letters and those who did not were not significant ($p > 0.05$). A worsening of ≥10 letters was seen in 13.0% of the cases at the 12-month follow-up. This worsening was observed in phakic patients with evidence of cataract. Of the eyes that experienced an improvement by ≥10 letters at the 12-month follow-up ($n = 27$), 40.7% were also submitted to phacoemulsification (in 25.9% of the cases during the surgical procedure and in 14.8% of the cases during the follow-up period).

A significant increase in BCVA was weakly and inversely correlated (Spearman correlation; $r = -0.316$) with final CFT at 12 months ($p = 0.039$).

Anatomical Results

The median CFT at baseline and at the 3-, 6-, and 12-month follow-up was 491 µm (302–819, 202), 329 µm (107–727, 137), 276 µm (112–804, 120), and 264 µm (120–752, 163), respectively. The median CFT change from baseline was –161, –189, and –215 µm at 3, 6, and 12 months, respectively. The decrease in CFT was most significant during the first 3 months after surgery ($p < 0.001$). This decrease was not statistically significant between 3 and 6 months or between 6 and 12 months of follow-up (Fig. 2), although all time points remained significant compared to baseline.

Table 1. Cases of recurrence/no improvement of DME

PDR	ERM	Anti-VEGF/ TIV periop.	BCVA before PPV, letters	CFT before PPV, µm	DME recurrence, months
No	No	No	5	581	12
No	Yes	Ranibizumab	39	573	4
No	Yes	TIV	31	724	6
No	Yes	No	61	303	3
No	Yes	No	36	351	11

Cases of recurrence/no improvement of DME. DME, diabetic macular edema; PDR, proliferative diabetic retinopathy; ERM, epiretinal membrane; anti-VEGF/TIV periop., perioperative intravitreal anti-VEGF/triamcinolone injections; BCVA, best corrected visual acuity; CFT, central foveal thickness; PPV, pars plana vitrectomy.

Table 2. Comparison of the subgroup with IV injection (at the end of the PPV procedure) versus that with no IV injection

Median	Injection	No injection	*p*
BCVA, letters			
Baseline	39	47	0.386
3 months	65	45	0.010
6 months	61	52	0.483
12 months	66	64	0.310
CFT, μm			
Baseline	482	500	0.779
3 months	294	330	0.674
6 months	310	264	0.252
12 months	288	248	0.693

There were statistically significant differences ($p < 0.05$) within both subgroups between baseline and the 12-month follow-up for BCVA and CFT. IV, intravitreal; PPV, pars plana vitrectomy; BCVA, best corrected visual acuity; CFT, central foveal thickness.

Table 3. Comparison between the VMT, ERM, and ERM+VMT subgroups

Median	VMT	ERM	ERM+VMT	*p*
Age, years	71	71	70	1.000
BCVA, letters				
Baseline	5	48	49	0.028
3 months	29	56	59	0.168
6 months	28	62	64	0.012
12 months	30	68	72	0.012
CFT, μm				
Baseline	609	471	492	0.007
3 months	391	294	313	0.308
6 months	219	281	271	0.131
12 months	149	273	295	0.009

There were statistically significant differences ($p < 0.05$) within the subgroups between baseline and the 12-month follow-up for BCVA and CFT. VMT, vitreomacular traction; ERM, epiretinal membrane; BCVA, best corrected visual acuity; CFT, central foveal thickness.

A final CFT <300 μm at the 12-month follow-up was achieved in 65.2% ($n = 30$) of the cases (52.2% [$n = 24$] needing no further treatment). A median CFT decrease of 216 μm was observed; no DME improvement occurred in 8.7% ($n = 4$) of the cases, and recurrence was observed in 10.9% ($n = 5$) of the cases during follow-up (of those, 60.0% [$n = 3$] achieved a CFT <300 μm at the 12-month follow-up with additional intravitreal treatment). The median time to recurrence was 6 months (from 3 to 12 months) (Table 1).

Between-Group Comparisons

The results of the analysis of the evolution of BCVA and CFT from baseline until the end of follow-up, comparing the subgroup of patients who had undergone IV injections at the end of the PPV procedure with the subgroup of patients who had not, are shown in Table 2. At month 3 of follow-up, BCVA was better in the injection subgroup ($p = 0.01$). There were no statistically significant differences between these subgroups beyond 3 months of follow-up ($p = 0.483$ at 6 months and $p = 0.310$ at 12 months).

Regarding the type of tDME, VMT was present in 17.4%, an ERM in 78.3%, and an ERM associated with a VMT component in 4.3% of the cases. Table 3 shows the comparison between these subgroups. There was no statistically significant difference in age between the three traction type subgroups. A statistically significant difference ($p < 0.05$) was observed regarding baseline CFT and BCVA, which was higher and lower, respectively, in the VMT group. BCVA beyond month 3 was significantly worse ($p = 0.012$) in the VMT group. An improvement of >10 letters between baseline and the end of follow-up was observed in 50.0, 58.3, and 100.0% of the cases in the VMT, ERM, and ERM plus VMT subgroups, respectively. Moreover, the VMT subgroup showed a lower median CFT at month 12 than the other groups ($p = 0.009$).

Discussion

As described previously in the literature, PPV was effective in significantly reducing tDME [1, 3, 12, 15]. After vitrectomy, retinal thickening was significantly reduced to <300 μm in 65.2% of the eyes. It is also possible to state that these results are mainly achieved during the first 3 months after surgery. In our study, ILM peeling was performed in all cases, because it has been proposed that ILM peeling in DME is a way to eliminate all traction, vitreous remnants, and inflammatory factors [16].

Regarding functional outcomes, we achieved a significant improvement in visual acuity in approximately 60.0% of the cases with this approach. The most significant part of the improvement occurred during the first 6 months of follow-up. A BCVA worsening of >10 letters was observed in 13.0% of the patients and was probably related to cataract progression during follow-up. However, unlike what has been described in some of the literature with respect to predictive factors for visual outcome after vitrectomy for tractional syndromes [17], our study did not find any positive correlation of age, baseline

BCVA, and baseline CFT with significant functional improvement in tDME ($p = 0.196$, $p = 0.702$, and $p = 0.594$, respectively).

Analyzing the influence of the type of tDME, the greatest BCVA improvements were seen in the VMT subgroup (anterior-posterior traction [18]), but the highest final BCVAs were observed in the subgroups with presence of ERM (tangential traction [18]). As described in other studies, we also achieved a significant BCVA improvement at the end of follow-up in around half of the patients with VMT [19].

Moreover, the lowest final CFT was observed in the VMT subgroup (with a median of 149 μm), revealing a greater level of macular atrophy in comparison with the other subgroups. This may be related to a higher level of anterior-posterior traction on the macula, inducing a higher degree of macular edema and more irreversible damage to the structure of the retinal layers, which subsequently led to a worse functional outcome.

Regarding recurrent macular edema, the literature suggests that it tends to occur late during follow-up (after 24 months) in approximately 11% of cases [12]. In our study, it occurred as soon as 3 months after surgery. However, our lower rate of recurrence (10.9%) may be related to our relatively short follow-up period.

We also performed an analysis concerning the influence of IV injections at the end of the PPV procedure (either an anti-VEGF or a CCT) on anatomical and functional outcomes. A total of 28.3% of the cases were submitted to IV injections during surgery; the analysis of the functional and anatomical results found no significant differences between those who did and those who did not receive an IV injection beyond month 3 of follow-up. Our study thus suggests that a perioperative IV injection does not change the long-term outcome. The surgical complication rates were low and similar to what has been reported for this procedure [12].

An important limitation of this study is its retrospective nature. Some important data that may influence outcomes and the rate of recurrence could not be precisely assessed, such as DME duration, glycemic control, blood pressure, and serum lipid levels [12]. In addition, any changes in macular perfusion (which can be observed after vitrectomy) were also not documented in this retrospective study [12].

Conclusions

The type of traction in DME – anterior-posterior (VMT) and/or tangential (ERM) – should be considered a relevant prognostic factor for a vitrectomy indication. Our study pointed out VMT as a negative prognostic factor after vitrectomy, leading to worse functional and anatomical outcomes. Perioperative IV injections of anti-VEGF and corticosteroids do not seem to change the long-term evolution of DME after vitrectomy and therefore are not indicated. This study demonstrates that PPV with ILM peeling for tDME provides early and significant functional and anatomical results that are sustained over a period of 12 months, with a low recurrence rate. These data provide estimates of surgical outcomes and support vitrectomy as an indication for DME in eyes with at least moderate vision loss and vitreomacular anterior-posterior and/or tangential traction.

Statement of Ethics

The study was conducted in agreement with the Declaration of Helsinki in its latest amendment (Brazil, 2013). All patients signed an informed consent form and the study was approved by the Ethics Committee of Centro Hospitalar Universitário do Porto.

Disclosure Statement

This research was not funded by any institution, public or private. None of the authors have any conflicts of interest.

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2.3 Enzymatic vitreolysis for the treatment of tractional diabetic macular edema[691]

Therapeutic Advances in Ophthalmology Original Research

Enzymatic vitreolysis for the treatment of tractional diabetic macular edema

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Abstract

Background: A new approach to address focal vitreomacular adhesion in patients with diabetic macular edema may control and stabilize diabetic macular edema with fewer anti-vascular endothelial growth factor injections.

Objectives: The aim of this study was to demonstrate that diabetic macular edema can be improved by inducing the release of a vitreomacular adhesion, with less than 2500 μm, with enzymatic vitreolysis.

Methods: From a retrospective analysis of clinical records from patients with diabetic retinopathy, patients with diabetic macular edema and vitreomacular adhesion <2500 μm were selected for a single-arm prospective study. The primary endpoint was to control diabetic macular edema with fewer anti-vascular endothelial growth factor injections after an observed vitreomacular adhesion release. A statistical subanalysis was performed for the following two groups: the group with vitreomacular adhesion release (group 1) and the group without vitreomacular adhesion release (group 2).

Results: A total of 23 eyes from 19 patients were included. A reduction of the median number of injections was achieved in group 1 ($p = 0.006$). Adverse events were mild and transitory.

Conclusion: Release of vitreomacular adhesion <2500 μm through enzymatic vitreolysis contributed to the control and stabilization of diabetic macular edema with fewer anti-vascular endothelial growth factor injections, reducing the burden and the risks related to these invasive and frequently chronic treatments.

Keywords: anti-vascular endothelial growth factor injections, diabetic macular edema, ocriplasmin, vitreolysis, vitreomacular traction

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Introduction

The pathogenesis of diabetic macular edema (DME) is multifactorial and not fully understood.[1–3] Many reports correlate the posterior cortical vitreous interaction with the macula with DME development.[3–7] Furthermore, some authors have highlighted that the presence of posterior vitreous detachment (PVD) has a positive effect on the evolution of DME.[1,4–8]

Schulze and colleagues[9] suggested that vitreomacular adhesion/traction (VMA) induces a cut-off from blood supply in the macula, leading to ischemia-induced vascular endothelial growth factor (VEGF) release. Moreover, VMA is regarded as a risk factor for proliferative-type complications such as proliferative vitreoretinopathy and epiretinal gliosis.[10] These macular proliferations may induce additional edema which further impairs vision.[11] Taken together, these tractional mechanic factors may antagonize the effect of anti-VEGF or corticosteroid intravitreal (IV) injections and may be the lead causing factors for pharmacological resistance in patients with macular edema.[12]

Until recently, the only treatment option available for VMA was vitrectomy. Given its risks, the

standard of care has generally been conservative until visual symptoms from VMA have deteriorated sufficiently to justify surgical intervention. Furthermore, post-vitrectomy DME is considered more difficult to treat with IV therapy, as those eyes eliminate drugs more quickly than nonvitrectomized eyes, usually needing a higher number of injections during the first 12 months of treatment.[13–16]

Ocriplasmin has been reported to be efficient in improving different types of macular edema through VMA release, total PVD, and increase in oxygen concentration in the vitreous cavity.[4,7,17–19] In this study, we report the results of ocriplasmin treatment in patients with DME and a VMA with less than 2500 μm, with the purpose to induce a VMA release and better DME control.

Materials and methods

Study design

From a retrospective analysis of 1484 clinical records from patients with diabetic retinopathy, 23 eyes from 19 patients with DME and VMA with less than 2500 μm were selected for a prospective single center study, conducted at the Department of Ophthalmology, Hospital de Santo António—Centro Hospitalar do Porto (HSA-CHP), Portugal, between July 2016 and July 2017. This study was divided into two phases: a retrospective pre-vitreolysis phase, in which patients received anti-VEGF injections for DME in a *pro re nata* (PRN) regimen and were followed-up for 26 weeks, and a second prospective phase, after administering the ocriplasmin injection, in which patients were also treated with anti-VEGF, if needed, and followed-up for 24 weeks, with a 1 month possible interval between ocriplasmin injection and the next anti-VEGF IV, if needed.

Inclusion and exclusion criteria

Patients aged ⩾18 years with type 1 or 2 diabetes and DME were eligible if their best-corrected visual acuity (BCVA) was between 20 and 80 Early Treatment Diabetic Retinopathy Study (ETDRS) letters and met the following inclusion criteria: (1) DME with central subfield foveal thickness (CSFT) of at least 300 μm, with a VMA less than 2500 μm in length within the 6-mm central retinal field using spectral domain optical coherence tomography (SD-OCT, Spectralis HRA + OCT, version 1.10.2.0; Heidelberg Engineering, Heidelberg, Germany) and (2) ability to provide written informed consent. Patients were excluded if they had the following: (1) evidence of epiretinal membrane (ERM) on optical coherence tomography (OCT); (2) history of other retinal vascular diseases; (3) previous vitrectomy; (4) undergone intraocular surgery in the previous 6 months, namely, phacoemulsification, retinal photocoagulation, or YAG LASER capsulotomy; (5) history of IV corticosteroid therapy; (6) suffered vitreous hemorrhage or other opacifications which can conceal fundus visualization and OCT measurements; (7) proliferative diabetic retinopathy (PDR); (8) active ocular inflammation or infection in either eye; (9) uncontrolled glaucoma in either eye [intraocular pressure (IOP) > 24 mmHg with treatment]; (10) history of stroke in the previous 6 months; (11) uncontrolled arterial hypertension (systolic blood pressure > 160 mmHg or diastolic blood pressure > 100 mmHg); or (12) be a ward of the state.

The study was conducted according to the tenets of the Declaration of Helsinki in its latest amendment (Brazil, 2013) and was approved by the ethics committee of HSA-CHP [2017.093 (084-DEFI/082-CES)]. All patients signed an informed consent form.

Patient assessment

Baseline demographics and glycemic control (HbA1c) data as well as history of previous ocular treatments were collected. Each patient had a complete ophthalmological evaluation, along with a papillary and macular OCT to assess the status of posterior vitreous cortex, VMA, DME, and PVD, conducted at baseline, on the day of the ocriplasmin injection, on day 4 after the ocriplasmin injection and then monthly until the end of follow-up. Maintenance of vitreous adhesion on the papillary area was considered a non-PVD achievement. In cases of doubt of the vitreous adhesion, an ocular ultrasonography (Eye Cubed™, Ellex, version 2.5.0.1) was performed with a 10-MHz sealed B-Scan probe. PVD was considered complete when the posterior vitreous cortex was well defined and completely separated from the retina situated posterior to the equator. Fluorescein angiography was performed on all patients at baseline.

During the follow-up period the following two phases were considered: 26 weeks before and 24 weeks after ocriplasmin treatment.

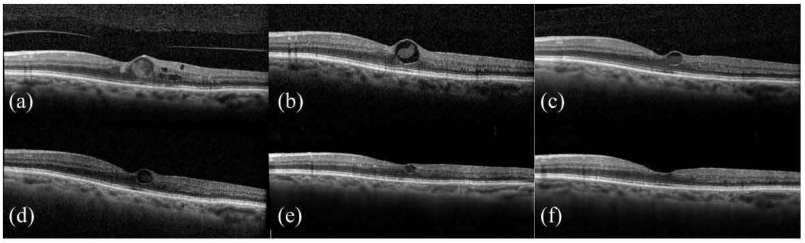

Figure 1. Spectral-domain optical coherence tomography macular images of one case with a central focal edema, a VMA and a CSFT between 300 and 350 μm. The images are from a 71-year old female patient, phakic, with a DME and a focal VMA with at least 16 months of duration, treated only with LASER therapy and no intravitreal injections. (a) immediately pre-ocriplasmin—OCT image with a CSFT of 350 μm and a BCVA of 7/10; (b–e) day 4, month 1, 3, and 6 post ocriplasmin, respectively, with a progressively normalization of the macular anatomy. At month 6 of follow-up (e and f) the patient had a BCVA of 20/20, 85 ETDRS letters, and a CSFT of 295 μm. The release of VMA was documented at the day 4 after ocriplasmin.

SD-OCT acquisition

A highly trained technician conducted SD-OCT scans. The acquisition infrared (IR) 30° + OCT 20° was applied. The circle scan OCT mode, for vitreous-optic disk adhesion analyses, and dense line scan OCT mode, for macula and posterior pole, were used. The follow-up function and auto-rescan with active eye tracking were also utilized. SD-OCT images were classified according to presence and length of VMA, CSFT, detachment of neurosensory retina, and integrity of the ellipsoid zone. CSFT was obtained automatically from equipment readings. The longer VMA length was measured using the macular scan, by two experienced medical retinal specialists, B.P. and J.N.M.B., and the mean value of these measurements considered. The presence of VMA was also evaluated by the same doctors and incongruent cases assessed by a third senior medical retinal specialist, A.M.

Treatment protocol

All patients were monitored with SD-OCT scan. Patients with DME and CSFT > 350 received anti-VEGF treatment. If CSFT was above 350 μm, anti-VEGF treatment was performed in a PRN regimen, with at least a 1 month interval (Figure 1). The choice of anti-VEGF treatment drug (ranibizumab or aflibercept), when required, was dependent on the previously administered anti-VEGF. The same anti-VEGF option was maintained during the follow-up period. LASER or steroid treatments were not allowed during the follow-up period. The same anti-VEGF protocol treatment was applied for the retrospective phase, when DME and VMA with less than 2500 μm coexisted.

Patients with persistent VMA in the pre-ocriplasmin phase were treated with an IV injection of 125 μg/0.1 ml of ocriplasmin (Jetrea; ThromboGenics, Inc., Iselin, New Jersey, US; Alcon/Novartis Farma, Porto Salvo, Portugal) on week 26, 2 weeks after administering the IV anti-VEGF if that had been the case. Anti-VEGF injections were counted from 26 weeks previously to ocriplasmin because the last anti-VEGF injection was performed, if needed, 2 weeks before ocriplasmin. The 24-week period after ocriplasmin is sufficient to include the same maximum potential number of anti-VEGF injections (six). All injections were performed in the operating room following standard protocol: injection through *pars plana* (3.5 or 4.0 mm from the limbus for pseudophakic and phakic patients, respectively), under sterile conditions.

Study endpoints

The primary endpoint was to determine the number of anti-VEGF injections to control DME after the ocriplasmin injection, comparing eyes that exhibited VMA release with eyes with persistent VMA. Secondary endpoints included the following: (1) the percentage of eyes with spontaneous nonsurgical resolution of VMA during follow-up, (2) BCVA and CSFT changes after ocriplasmin injection, (3) achievement of PVD, and (4) safety. An increase of ⩾5 letters was considered to be a clinical significant increase.

Table 1. Baseline characteristics of the study population.

Parameter	Study population	Group 1	Group 2	*p* value
Age, years	68.0 (59.0–75.00; 8.1)	70.7 (58.9–75.0; 8.4)	66.9 (60.6–72.6; 6.7)	0.089
Age > 65 years, eyes *n* (%)	18 (75.0)	11 (78.6)	7 (77.9)	0.964
Males; eyes *n* (%)	15 (65.2)	9 (64.3)	6 (66.7)	0.906
High blood pressure; eyes *n* (%)	19 (82.6)	12 (85.7)	7 (77.8)	0.624
Phakic; eyes *n* (%)	14 (60.9)	9 (64.3)	5 (55.6)	0.675
Laser therapy (peripheral and macular)	23 (100)	14 (100)	9 (100)	1.000
Macular LASER	21 (91.3)	12 (85.7)	9 (100)	0.668
Peripheral LASER	22 (95.7)	13 (92.9)	9 (100)	0.820
HbA1c (%)	7.4 (6.5–10.0; 1.5)	7.0 (6.5–10.0; 0.7)	8.1 (6.7–9.1; 1.8)	**0.032**
Duration of DME (months)	22.0 (5.7–68.9; 13.6)	19.5 (5.7–68.9; 19.4)	28.3 (12.3–59.0; 19.7)	0.369
Duration of VMA (months)	5.0 (1.0–16.0; 6.0)	5.5 (1.0–15.0; 7.8)	3.8 (1.8–16.0; 5.5)	0.643
VMA length (μm)	425 (128–2115; 528)	417 (128–2115; 428)	582 (324–1820; 1002)	0.680
Eyes with focal VMA (<1500 μm), *n*	20	7	13	0.538
Baseline BCVA (letters)	65.0 (40.0–80.0; 10.0)	67.5 (60.0–80.0; 9.0)	65.0 (40.0–83.0; 15.0)	0.926
CSFT (μm)	326.0 (199.0–416.0; 67.0)	322.5 (199.0–416.0; 43.0)	328.0 (206.0–400.0; 105.0)	0.829

BCVA, best-corrected visual acuity; CSFT, central subfield foveal thickness; DME, diabetic macular edema; IQR, interquartile range; VMA, vitreomacular adhesion.
Group 1—with VMA release; group 2—without VMA release. Values are presented as median (range, IQR). Baseline defined as the day of ocriplasmin injection (pre-injection). BCVA in ETDRS letters; CSFT at ocriplasmin injection day. *p* value pertains to the comparison between groups. All significant values are represented in bold.

Statistical analysis

After testing for the normality of all variables using the Shapiro–Wilk test, nonparametric statistics methods were used. Changes in BCVA, CSFT, VMA length, number of IV injections, and glycemic control in the overall sample over time were evaluated with a Wilcoxon test for paired samples. A statistical subanalysis was performed for two groups, group 1 with VMA release and group 2 without VMA release. The Mann–Whitney test was used for comparisons between patients with and without VMA release. The χ^2 test was used for comparison of proportions. Values are presented as median (range, interquartile range) unless otherwise specified. Data analyses were performed using SPSS 23th edition (IBM Corporation, Armonk, New York, US). Tests were considered significant at $\alpha < 0.05$ significance level (two-sided).

Results

Demographic and baseline data

No cases initially enrolled in the study were excluded during the study period. Before inclusion in the prospective study 18 eyes (78.3%) had received IV anti-VEGF. The remaining demographic and baseline data are summarized in Table 1.

Primary endpoint

From a total of 23 eyes, 17 received a median of 3 injections (1–6, 2) in the pre-ocriplasmin phase and a median of 2 injections (0–6, 3.5) post-ocriplasmin ($p = 0.005$). The remaining six eyes did not receive anti-VEGF treatment because CSFT was successfully maintained below 350 μm, during the entire follow-up period. The median

Table 2. Median number of anti-VEGF injections, pre- and post-ocriplasmin, between and within groups.

	All patients	Group 1	Group 2	*p* value
Number of intravitreal anti-VEGF in the pre-ocriplasmin phase	3.0 (1.0–6.0; 2.0)	3.0 (1.0–6.0; 1.5)	4.0 (1.0–6.0; 3.5)	0.376
Number of intravitreal anti-VEGF in the post-ocriplasmin phase	2.0 (0.0–6.0; 3.5)	1.0 (0.0–5.0; 2.5)	4.0 (1.0–6.0; 2.75)	**0.022**
p value	0.005	**0.006**	0.564	

IQR, interquartile range; IV, intravitreal; VEGF, vascular endothelial growth factor; VMA, vitreomacular adhesion.
Group 1—with VMA release; group 2—without VMA release. Values are presented as median (range, IQR). For these analyses, only the 17 eyes that needed IV injections of anti-VEGF in pre-ocriplasmin phase were considered. *p* value on the right column refers to comparison between groups 1 and 2 in pre- and post-ocriplasmin phase, respectively. Statistically significant values are represented in bold.

number of anti-VEGF injections, pre- and post-ocriplasmin, between and within groups is summarized in Table 2. The number of anti-VEGF injections was significantly higher before VMA release, with a median of three injections (1–6, 1.5), than after VMA release, with a median of one injection (0–5; 2.5), $p = 0.006$.

Secondary endpoints

The overall VMA resolution rate was 60.9% ($n = 14$). VMA resolution was achieved in 50% of the eyes between the first and fourth day following the ocriplasmin injection, in 21.4% between day 4 and the first month, and in 28.6% after the first month of follow-up. VMA resolution was observed in one of the three broad adhesion VMA cases (≥1500 μm; Figure 2).[20]

In the six eyes with no indication for treatment with anti-VEGF previous to the ocriplasmin injection, five exhibited VMA release with resolution of the focal macular edema (Figure 1 exemplifies one of those cases), and the eye without VMA release maintained DME with less than 350 μm during the entire follow-up period.

In both groups, BCVA was clinically and statistically higher at the end of follow-up, having increased in group 1 ($p = 0.012$) from a median of 67.5 (60.0–80.0; 9.0) to 76.0 ETDRS letters (60.0–85.0; 14.0) and in group 2 ($p = 0.038$) from a median of 65.0 (40.0–83.0; 15.0) to 76.0 ETDRS letters (53.0–85.0; 17.0; Figure 3).

In the last visit, 8.7% of the eyes lost 5–10 ETDRS letters with no statistical differences between groups (7.1% in group 1 and 11.1% in group 2, $p > 0.05$). None of the eyes lost more than 10 ETDRS letters. With regard to CSFT, there were no differences between groups at any time point ($p > 0.05$; Figure 4). The number and percentage of patients who needed injections at each time point (1, 2, 3, 4, 5, and 6 months, respectively) was: group 1—4 (28.6%), 2 (14.3%), 1 (7.1%), 3 (21.4%), 2 (14.3%), and 3 (21.4%); group 2—7 (77.8%), 6 (66.7%), 7 (77.8%), 5 (55.6%), 4 (44.4%), and 1 (11.1%).

Complete PVD was achieved in 50% of the eyes in group 1, with no statistically significant differences in the number of IV injections between patients with and without complete PVD.

With respect to glycemic control, HbA1c was not statistically significant different within groups during the follow-up period (median of 7.0% at baseline and at end of follow-up in group 1, $p = 0.310$, and 8.1% at baseline and 7.8% at the end of follow-up in group 2, $p = 0.684$). Comparing group 1 with group 2, HbA1c was statistically significant inferior in group 1 both at baseline ($p = 0.032$) and at the end of follow-up ($p = 0.009$).

There were no statistically significant differences between groups regarding the remaining baseline characteristics ($p > 0.05$).

Safety

The adverse events reported (mild visual acuity decrease in 21.7% of the patients, pain or discomfort in 26.1%, floaters in 26.1%, and photopsias in 8.7%) were transitory, well tolerated, and none was present or reported beyond the first follow-up visit. No changes in the ellipsoid layer or the occurrence of a neurosensory retinal detachment[17] were observed. No suspected unexpected serious adverse reactions (SUSARS) were

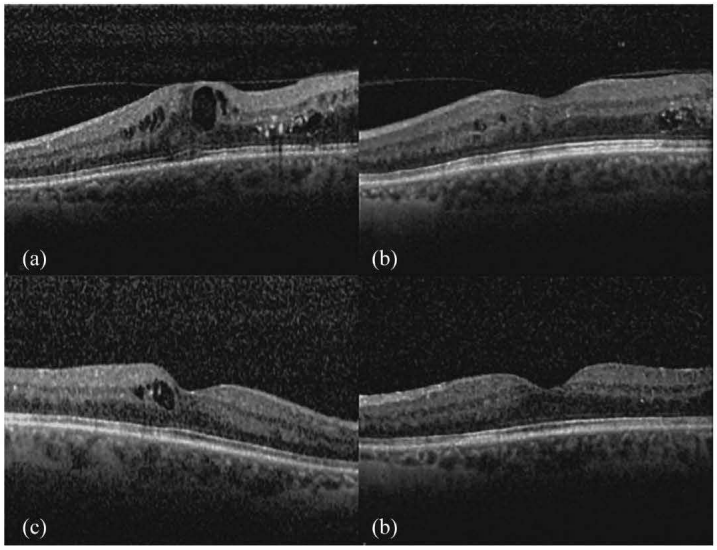

Figure 2. Spectral-domain optical coherence tomography images of a 71-year old phakic male patient with DME and a broad VMA adhesion. (a) DME status after LASER therapy and out of the window effect of multiple anti-VEGF intravitreal injections (CSFT of 559 μm). (b) Pre-ocriplasmin injection condition, 15 days after an anti-VEGF injection (CSFT of 382 μm). (c) VMA resolution 1 day after ocriplasmin injection. (d) Six months later the patient had a BCVA of 20/20, 85 ETDRS letters, and a CSFT of 310 μm. There was no need for further additional treatment during the follow-up period, with a stable macular anatomy since the first month post ocriplasmin.

observed regarding drugs and procedures applied in this study.

Discussion

DME, even when VMA is present, may recede with different therapeutic measures (metabolic control, LASER therapy, and anti-VEGF or corticosteroid IV injections). However, it is believed that the response to these measures might be less efficient and last less time when a VMA is present.

The Food and Drug Administration (FDA) approved ocriplasmin in 2012 and the European Medicines Agency (EMA) in 2013 for the treatment of symptomatic VMA. As inflammatory, sometimes ischemic and fibrovascular proliferation stimuli underlie diabetic retinopathy disease, these eyes are particularly at risk of experiencing cellular migration, taut posterior hyaloid, ERMs' formation, and vitreomacular traction, which can also be promoted by the laser retinal treatments;[11,20,21] it would be expected that the effect of ocriplasmin, in this particular patient subgroup, would be less efficient. However, in this study, VMA resolution was achieved in a relatively high percentage of patients (60.9%), compared with other series which reported rates from 26.5% to 64% mainly in nondiabetic patients.[10,22] Positively skewing our results may be the exclusion of patients with ERMs, known to be a negative prognostic factor for the efficacy of ocriplasmin.[23] Nonetheless, our sample has some possible negative prognostic factors, such as the percentage of previous LASER therapy (100%) and broad adhesions (13%).[20,23]

Reflecting on other possible determining factors, there were no statistically significant differences in baseline characteristics (phakic status, age, gender, type of DR, history of hypertension,

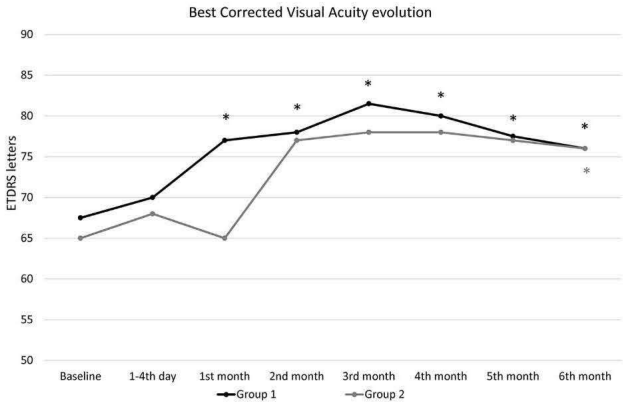

Figure 3. Best-corrected visual acuity (BCVA) evolution in ETDRS letters after ocriplasmin injection. Group 1—with VMA release; group 2—without VMA release. Values are presented as median. There were no differences between groups at any time point ($p > 0.05$).
*$p < 0.05$ within groups compared with baseline.

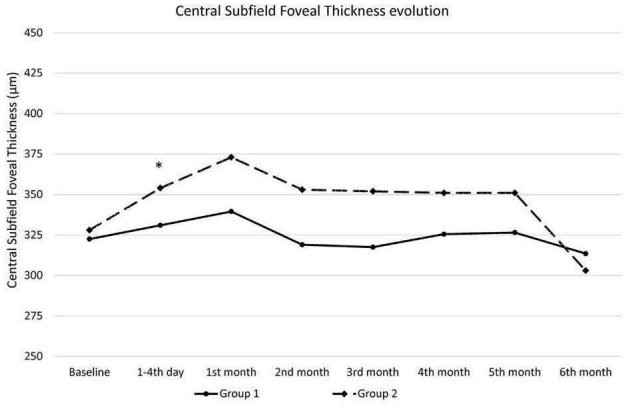

Figure 4. Central subfield foveal thickness (CSFT) evolution after ocriplasmin injection. Group 1—with VMA release; group 2—without VMA release. Values are presented as median. There were no differences between groups at any time point ($p > 0.05$).
*$p < 0.05$ within groups compared with baseline.

duration of DME, duration and length of VMA, BCVA, and CSFT) between the two groups analyzed (with and without VMA release). HbA1c levels were statistically significantly lower in group 1 compared with group 2, both at baseline and at the end of follow-up.[21,22] However, HbA1c levels do not correspond robustly to anti-VEGF treatment effects in the eye, and neither the absolute benefit nor the prognosis is associated with HbA1c levels.[21] Future studies are required to prove that HbA1c level is not relevant for the VMA release process.

The VMA resolution occurred in the majority of the patients until the first month after ocriplasmin, which is in line with outcomes described in other series.[10,22]

At the end of follow-up, an increase in BCVA, overall and in each assessed group, was noted. However, BCVA values showed a more rapid increase in group 1 compared with group 2. In group 1 that achievement occurred since the first month after ocriplasmin injection, whereas a similar increase was only observed in group 2 at the end of the follow-up period. This difference may be explained by the stability induced by the elimination of the traction effect on DME evolution, in group 1, with less fluctuations in macular thickness, due to the edema. Previous trials have shown that the number of anti-VEGF injections decrease with time.[24] In our series that decrease was documented only in group 1. Patients in group 2 maintained the same median number of injections in post-ocriplasmin phase because DME was not controlled without them, and the anti-VEGF treatment maintained the edema under control. This may explain the median CSFT value, without variation during the post-ocriplasmin phase. In group 1, the same stability in CSFT was achieved but with a lower number of IV injections needed, and in some cases without the need of any IV injections.

The increase in BCVA, both in group 1 and in group 2 compared with baseline (in eyes already being under treatment before), may be explained by a more tight monthly follow-up after the inclusion in this prospective study as pre-defined in the study protocol. The authors acknowledge that in real-life, PRN IV anti-VEGF regimens may lead to a DME insufficient treatment approach with subsequent suboptimal functional and anatomical results. Also, the known positive effect of ocriplasmin on increasing oxygen concentration in the vitreous cavity may have played a role.[4]

Regarding safety issues, side effects of ocriplasmin were transitory and well tolerated. Mild visual acuity of less than six letters decrease, pain or discomfort, floaters, and photopsias were not referred beyond the first follow-up visit (performed at the fourth day post-ocriplasmin). Although there are evidence suggesting that DME patients with VMA have a higher potential to improve visual acuity,[24–26] these are based on study methods in which a non-VMA adhesion was assumed based only on OCT images and not confirmed with eye ultrasonography. OCT is not completely accurate to diagnose vitreous attachment or PVD, particularly when the adhesion cannot be seen, especially in diabetic eyes in which vitreoschisis is highly prevalent.[27] There is still a lack of studies comparing both methods on the evaluation of vitreoretinal interface. In the Read 3 study,[24] there is also a contradiction: the best functional and anatomical outcomes were verified in PVD cases (obtained spontaneously in four out of the low number of five eyes with focal VMA), the paradigm of a complete non-VMA status. In fact, a broad *versus* a focal type of adhesion are different types of adhesions and cannot be assumed as having the same influence in DME evolution.

The lack of a standardized anti-VEGF drug may be considered a weakness of this study. However, the type of anti-VEGF used has not been reported as a relevant factor for VMA occurrence. The same anti-VEGF option was maintained during both the retrospective and prospective phases.

In addition, although the majority of studies use less than 350 μm of thickness as the threshold for treatment, it is our opinion that when a focal VMA exists (Figure 1) an extra mechanical factor influences the distribution of intra-retinal macular edema, near the focal adhesion region, sometimes with an almost dry adjacent peri-foveal area.

It may also be questionable why vision improvement was relatively higher after inclusion in the prospective period, in both groups (8.5 letters in group 1 and 11 letters in group 2). This result may suggest that patients were being under-treated before entering the trial. In our opinion that fact can add even more value to the results of this study: with a higher previous number of injections a more significant reduction in the number of injections would have been expected in group 1.

The results of this study favor the positive effect in DME obtained by the release of a focal VMA even without a PVD achievement. The VMA release seems to be more important than the PVD occurrence itself for DME evolution. Nevertheless, that inference cannot be claimed as relevant in this study because of the small absolute number of eyes with PVD in the group with VMA release.

To our knowledge, this is the first study reporting the use of ocriplasmin specifically in a DME

group of patients. On the subanalyses of the two phase 3 clinical trials, the basis for the approval of ocriplasmin for vitreomacular traction and macular holes, ocriplasmin was referred to be used in diabetic retinopathy in 6.9% of the 652 patients included (45 patients), with no specific considerations regarding DME control or diabetic retinopathy status.[22,23]

Furthermore, although the authors acknowledge that a focal VMA release may be anticipated earlier as a spontaneous occurrence, especially in eyes that underwent IV injections,[25] in this study the majority of the eyes showed a VMA release within the first month after ocriplasmin injection, increasing the probability of this event as a result of ocriplasmin treatment and not a haphazard development.

This study has some limitations, such as the absence of a control group, the small cohort included and also the relatively short duration of follow-up, with the additional difficulty inherent to the multifactorial nature of DME. Future studies with a larger number of cases are warranted to confirm the benefits attained in our study population.

Conclusion

The main result of this study was that if the VMA disappears after ocriplasmin injection the anti-VEGF injection burden could be reduced. However, if inflammation is the main cause of sustained DME rather than VMA, VMA release may be less effective in improving DME, and this approach can be unsuccessful in some patients. Indeed, in one case there was still a need for five anti-VEGF injections for 6 months. Therefore, a VMA release may not be the solution for all DME cases. However, when the gold standard therapy fails or if the anti-VEGF burden is too high, this treatment strategy should be considered. The results of this study emphasize the importance of VMA as an anatomic biomarker, when there is a need to decide which is the best treatment approach in DME.

Author contributions

All authors contributed equally to this paper regarding the design of the study, data collection, and statistical analysis. B.P. wrote the first draft of the paper which was critically reviewed and discussed by all authors prior to submission. All authors have seen and approved the submitted version of the paper.

Conflict of interest statement

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

The study protocol has been approved by the research institute's committee on human research.

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Informed consent

Subjects have given their written informed consent.

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2.4 Comparison of Ocular Ultrasound Versus SD-OCT for Imaging of the Posterior Vitreous Status in Patients With DME[254]

Comparison of Ocular Ultrasound Versus SD-OCT for Imaging of the Posterior Vitreous Status in Patients With DME

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BACKGROUND AND OBJECTIVES: To assess the percentage of vitreous adherence to the posterior pole in patients with diabetic macular edema (DME) with ocular ultrasonography (US) and establish a comparison with spectral-domain optical coherence tomography (SD-OCT).

PATIENTS AND METHODS: Cross-sectional consecutive analysis of patients followed in a diabetic retinopathy consultation. Vitrectomized eyes and patients with epiretinal membranes were excluded. A comparison between macular SD-OCT 20 × 20°, SD-OCT 55 × 35°, and ocular US for the vitreous status was performed. A subanalysis of the percentage of eyes with thickened posterior hyaloid and focal vitreous macular adhesion (VMA) was determined with SD-OCT 20 × 20° and SD-OCT 55 × 35°.

RESULTS: From 78 eyes of 39 patients, 55 eyes were included. All patients had type 2 diabetes mellitus with a median duration of 20 years (range: 3 to 40 years); 60% were phakic, and 61.8% were male. Previous treatments included intravitreal injections in 54.5% eyes, macular laser in 67.3%, and panretinal photocoagulation in 56.4%. All eyes had a non-posterior vitreous detachment (PVD) status on US. The 55 × 35° SD-OCT detected a non-PVD status in 96.4% (100% in video display mode) and a VMA in 87.3%. The 20 × 20° SD-OCT only detected a VMA in 43.6% of cases, with a thickened posterior hyaloid in 40% and a focal VMA in 18.2%.

CONCLUSIONS: In the authors' DME patients, vitreous adherence to the posterior pole was highly prevalent, with a total agreement between US and SD-OCT 55 × 35° video display mode. SD-OCT 20 × 20° is not an accurate method to diagnose VMA compared to SD-OCT 55 × 35°.

[*Ophthalmic Surg Lasers Imaging Retina.* 2020;51:S50-S53.]

INTRODUCTION

Vitreoschisis, anomalous or incomplete posterior vitreous detachment (PVD), is believed to be highly prevalent in diabetic patients and to play a role in the pathogenesis of diabetic macular edema (DME).[1-3] Although ocular ultrasonography (US) may be considered the gold standard exam to establish the vitreous status, spectral-domain optical coherence tomography OCT (SD-OCT) is the retina imaging method most used worldwide.[4]

Over the years, different technologies with different threshold resolution capabilities to detect the multilamellar vitreous thickness may be responsible for the differences reported concerning prevalence results.[5]

The vitreous cortex may be erroneously assumed to be detached from the underneath macula when it is not visible in a current 20 × 20° SD-OCT acquisition.

In this study the perception of vitreous adherence in the 20 × 20° central macular and 55 × 35° areas, including macula and optic disc, were evaluated with SD-OCT technology, establishing a comparison with ocular US.

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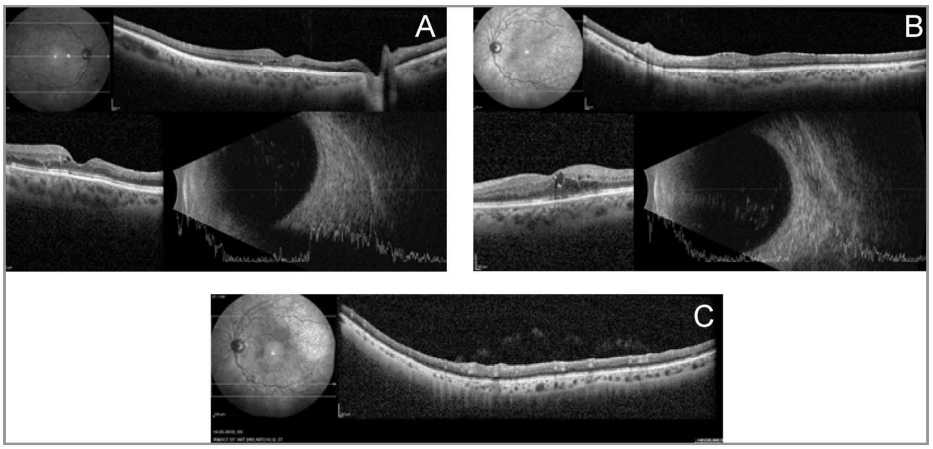

Figure 1. Two cases with a doubtful non-posterior vitreous detachment (PVD) status in spectral-domain optical coherence tomography (SD-OCT), in spite of evidence of non-PVD on ultrasonography (A and B, respectively). (C) SD-OCT in video display mode from the same eye of B where a vitreomacular adhesion posterior to the arcades can also be seen.

PATIENTS AND METHODS

Study Design

This was a cross-sectional analysis of patients followed in a Diabetic Retinopathy consultation of one vitreoretinal surgeon (BP) at Centro Hospitalar Universitário do Porto (CHUPorto), Portugal. Patients were recruited from September 2018 to November 2018.

The study was conducted according to the tenets of the Declaration of Helsinki in its latest amendment (Brazil, 2013) and was approved by the Ethics Committee of CHUPorto. All patients signed an informed consent.

Participants, Inclusion and Exclusion Criteria

Inclusion criteria were patients with type 1 or type 2 diabetes mellitus; those older than 18 years; and those with center-involved DME, defined as central subfield thickness (CST) of more than 300 μm on SD-OCT. Non-DME eyes could be included if there was DME in the fellow eye.

Exclusion criteria included additional ocular diseases or conditions that could significantly affect vitreous-macular status, as well as a correct fixation or ocular medium transparency that could compromise an accurate acquisition of the exams under analyses, such as previous vitrectomy, presence of epiretinal membrane (ERM), age-related macular degeneration, retinal vascular occlusion, central corneal opacity, amblyopia, advanced glaucoma, optic neuropathy, history of ocular trauma, or surgery other than uncomplicated cataract surgery. Both eyes of a single patient could be included only if both met all of the inclusion criteria and did not meet any of the exclusion criteria.

Patient Assessment

Demographics and relevant clinical and treatment information, including complications, were collected directly from the patient on a routine ophthalmology visit. Additional clinical important data were completed by consulting the patient clinical record.

A comparison between a high-resolution (HR) macular SD-OCT 20 × 20º and SD-OCT 55 × 35º (Spectralis HRA+OCT, version 1.10.2.0; Heidelberg Engineering, Heidelberg, Germany) with ocular US (Eye Cubed, version 2.5.0.1; Ellex, Adelaide, Australia) (10 MHz sealed B-scan probe) was performed for the prediction ability of a vitreous adhesion to the posterior pole and/or PVD.

In ocular US, the vitreous status was determined using a high gain (90 dB), real-time, through-the-lid contact technique with gel. Both vertical and horizontal views were used and the mobility of the posterior vitreous was examined during saccadic eye movements. A PVD status was considered when posterior vitreous cortex was well defined and completely separated from the retina situated posterior to the equator.

For vitreous macular adhesion (VMA) detection, a comparison between SD-OCT HR 20 × 20° and SD-OCT 55x35° was performed and was considered to be present if there was a vitreous reflectivity in continuous contact with the retina inner surface in the central macular 5.8 mm^2.

As a subanalysis, the percentage of the cases with thickened posterior hyaloid and focal (< 1,500 µm) VMA was also determined with SD-OCT 20 × 20° and SD-OCT 55x35°.

SD-OCT scans were acquired by a trained technician and US by the same physician (BP). All the exams were analysed by two experienced retina specialists, BP and MB, and incongruent cases assessed by a third senior retina specialist (AM).

Statistical Analysis

Nonparametric statistical methods were used. Results are presented as n, %, or median (range; interquartile range).

RESULTS

From a total population of 78 eyes of 39 patients, 23 eyes were excluded (22 vitrectomized). All patients had type 2 diabetes with a median duration of 20 years (range: 3 to 40 years; 13); 60% were phakic and 61.8% were male. Previous treatments with intravitreal injections were performed in 54.5% eyes, macular LASER in 67.3% and panretinal photocoagulation (PRP) in 56.4%. All eyes (n = 55) had a non-PVD status on US. The 55 x 35° SD-OCT detected a non-PVD status in 96.4% (n = 53) and a VMA in 87.3% (n = 48). In two cases (Figures 1A and 1B) there was doubtful non-PVD status. When a 55x35° SD-OCT using a video display imaging mode was performed, the non-PVD status was evident (Figure 1C). The 20 × 20° SD-OCT only detected a VMA in 43.6% of cases (n = 24), with a thickened posterior hyaloid in 40% (n = 22) and a focal VMA in 18.2% (n = 10). A bilateral DME was present in 75% of patients (n = 41).

DISCUSSION

Systemic conditions such as diabetes mellitus induce biochemical and structural modifications in the vitreous that may contribute to an anomalous PVD and vitreoschisis (a split in the posterior vitreous cortex).[1,2,6,7] There are many reports supporting the relationship of the posterior cortical vitreous to the macula playing an important role in the development of DME.[8-10] Moreover, vitreous adhesion on the retinal surface is regarded as a risk factor for proliferative complications such as proliferative vitreoretinopathy (PDR) and epiretinal gliosis.[11] Frequently, these proliferations in the macular area induce an additional edema, which further decreases visual acuity.[12,13] Chronic diffuse macular edema might also be related to extrafoveal vitreoretinal traction.[14,15]

Although when dealing with DME and diabetic retinopathy vitreous-macular adhesion is of unquestionable importance, the relevance of the real status of the vitreous is not fully known.

The variability in the thickness of the walls of the vitreoschisis cavity may, in many cases, be below the level of resolution of the method used to define the vitreous structure. In 1996, Schwartz et al. identified a higher incidence of posterior vitreoschisis detected during vitrectomy (81%) than by US (17%).[5] The technological evolution over time may also be a factor responsible for increasing the resolution to detect the real vitreous status.

Currently, OCT technology is the most widely used to analyze the macula and the vitreous-macular interface. However, in diabetic eyes where vitreoschisis prevalence is expected to be high, OCT may not be the most accurate method to distinguish a vitreoschisis and/or a totally adherent vitreous from a DPV. Instead, US may be considered the gold standard to provide a more accurate diagnosis of vitreous status.[4]

Among macular OCT acquisition modes, 20 × 20° SD-OCT is the most commonly used in clinical practice for macula evaluation. However, its value to establish the diagnosis of a real VMA status has not been fully studied. Therefore, the 55x35° SD-OCT acquisition mode, covering a larger retina area, the macula and optic disc area, could allow more precision in the vitreous-retinal characterization.

In our sample of 55 eyes from patients with DME in at least one eye (bilateral in 75%), more than 50% had macular LASER PRP and intravitreal injections treatments. We observed vitreous adherence to the posterior pole in 100% of the eyes, with a total agreement between US and 55x35° SD-OCT video display mode methods. VMA presence was also identified in a high percentage of eyes with 55x35° SD-OCT technology (87.3%).

The 20 × 20° SD-OCT only detected a VMA in about one-half of the cases (43.6%) in comparison with 55x35° SD-OCT. The presence of a thickened posterior hyaloid was identified in a percentage similar to the whole VMA cases identified by the 20 × 20° SD-OCT. Therefore, 20 × 20° SD-OCT may be considered a good method only for detecting thickened posterior vitreous-macular adhesions. It remains to be demonstrated if these are the only clinically relevant vitreous-macular adhesions to be considered in DME approach.

The number of cases with focal VMA was low (18.2%), as expected. However, it was higher than the observed in the VMA analysis performed in the

124 eyes of the READ study subanalysis (4%).[17] Our superior percentage may be explained by the referral nature of our practice.

Our study, through US and widefield SD-OCT equivalent results, corroborated the expected high prevalence of some degree of vitreous adherence to the posterior pole in DME patients. These results highlight the need of further investigation to establish the real importance of vitreous in DME pathogenesis, as SD-OCT 20° x 20°, the most common macular evaluation method used in clinical practice, did not reveal the required level of accuracy for VMA detection.

In conclusion, and although the real vitreous status is not yet fully understood, even during vitrectomy, our results show that US and 55x35° SD-OCT video display mode methods detected vitreous adherence to the posterior pole in 100% of the eyes, with a total agreement between both methods. Moreover, the 20 × 20° SD-OCT is not an accurate method to diagnose VMA compared to SD-OCT 55x35°. Given this is the first reported comparison between these methods, future studies with different cohorts and a larger number of cases are warranted to confirm our results.

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2.5 Intravitreal Ranibizumab or Aflibercept After Bevacizumab in Diabetic Macular Edema: Exploratory Retrospective Analysis[692]

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ORIGINAL RESEARCH

Intravitreal Ranibizumab or Aflibercept After Bevacizumab in Diabetic Macular Edema: Exploratory Retrospective Analysis

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Clinical Ophthalmology

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Aim: To evaluate the efficacy of switching from bevacizumab to ranibizumab or aflibercept in eyes with diabetic macular edema (DME) unresponsive to bevacizumab.

Methods: Single-center retrospective comparative study of patients with DME unresponsive to intravitreal bevacizumab that was switched to ranibizumab or aflibercept. Best-corrected visual acuity (BCVA) and central foveal thickness (CFT) were analysed prior to and 4 months after the switch. Ocular coherence tomography (OCT) biomarkers were also analysed.

Results: Fifty-six eyes from 40 patients were included in the study, 33 eyes switched to ranibizumab and 23 to aflibercept. A significant median CFT decrease was observed in both groups ($p<0.001$), with no between-group differences. BCVA gain was only significant in the ranibizumab group ($p<0.001$). None of the pre-baseline or baseline parameters were associated with the response to ranibizumab or aflibercept.

Conclusion: In persistent DME unresponsive to bevacizumab, both anatomical and functional improvements were observed with ranibizumab whereas aflibercept only showed an anatomical improvement. Clinicaltrials.gov NCT04018833.

Keywords: aflibercept, bevacizumab, diabetic macular edema, ranibizumab, refractory

Introduction

Diabetic retinopathy (DR) is the leading cause of vision loss among working-age adults in the developed world,[1] and diabetic macular edema (DME) is the main responsible for the vision loss related to DR.

The International Council of Ophthalmology and EURETINA Guidelines recommend anti-vascular endothelial growth factors (anti-VEGF) agents, ranibizumab[2,3] and aflibercept,[4] as well as off-label bevacizumab,[5] as first-line therapy for treating central DME.[6,7] The lower cost of bevacizumab, perceived effectiveness and relative safety, makes it a widely accepted therapy option particularly in the developing world.[8] Protocol T is the only head-to-head study in DME with the three anti-VEGF agents.[9] However, ranibizumab was used at 60% (0.3mg) of the dose approved by the European Medicines Agency (EMA), which is 0.5mg.[2] Although in the first 3 years of the RIDE and RISE (A Study of Ranibizumab Injection in Subjects With Clinically Significant Macular Edema (ME) With Center Involvement Secondary to Diabetes Mellitus, NCT00473382 and NCT0047330, respectively) trials no statistically significant difference in functional or anatomical outcomes has been detected between the 0.3mg and the 0.5mg doses, their

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comparison was not performed under a pro re nata (PRN) regimen. In fact, in an extended dose regimen strategy, those differences could have emerged.[9] In more severe or refractory cases, this is the rational for switching from a less effective off-label anti-VEGF to ranibizumab or aflibercept.

There are numerous data showing the efficacy of ranibizumab or aflibercept for the treatment of patients with DME refractory to bevacizumab, as well as with an anti-VEGF switch approach in cases where tachyphylaxis or tolerance to a previously effective anti-VEGF may occur.[10–12] Much less evidence exists on the real efficacy differences between aflibercept versus ranibizumab treatment after an initial bevacizumab regimen.[13]

Retinal edema is responsible for retinal microstructural changes, retinal atrophy of photoreceptors and ganglion cell lesions.[14] It may also be considered consensual that the best improvements in visual acuity are achieved when retinal edema is resolved. In a background of a chronic and progressive disease, DME should be faced as a condition to control as efficiently and promptly as possible. Therefore, it is crucial to identify early non-responders to an anti-VEGF considering also an early therapeutical switch with the purpose of achieving the best anatomical and functional outcomes. The key to an early identification of a nonresponder lies on the identification of prognostic biomarkers. According to the literature, the presence of OCT biomarkers such as subretinal fluid (SRF), ellipsoid layer disruption (ELd), external limiting membrane disruption (ELMd), hyperreflective foci (HRF), cysts in the outer nuclear layer (ONLc) and their size, hard exudates (HE) and disorganization of the retinal inner layers (DRIL) are correlated with a more severe, chronic DME and poor retina function but not with an expected negative anti-VEGF response.[15–19] Only the presence of outer plexiform layer disruption (OPLd), epiretinal membrane (ERM) with retina wrinkling and the loss of deep capillary plexus in OCT angiography have shown to be predictive of a poor response to anti-VEGF.[15–19]

A vitreomacular detachment from the macular area (VMA) with some possible anterior-posterior traction (considered in this study when at least one-third of VMA was present) may also play a role in DME evolution, particularly in treatment response. The tangential as well as anterior-posterior traction, through a synergic effect, may contribute to a higher hydrostatic pressure towards the retina tissue.[20–22]

The purpose of this study was to evaluate the safety and efficacy of switching to ranibizumab or aflibercept in cases of DME refractory to off-label bevacizumab treatment, and to identify any parameters associated with positive or negative prognosis following this switch.

Subjects, Materials and Methods

Study Design

This was a single-center retrospective comparative study of consecutive DME cases unresponsive or incompletly responsive to intravitreal (IV) bevacizumab (1.25mg/0.05mL) that were switched to IV ranibizumab (0.5mg/0.05mL) or aflibercept (2.0mg/0.05mL).

The study was conducted according to the tenets of the Declaration of Helsinki in its latest amendment (Brazil, 2013) as was approved by the Ethics Committee of HSA-CHUP. All patients signed an informed consent prior to entering the study. This study is registered on clinicaltrials.gov under the number NCT04018833.

Setting and Participants

The study was performed at Hospital de Santo António-Centro Hospitalar e Universitário do Porto (HSA-CHUP), Portugal.

The clinical records of 188 eyes from 128 patients with DME under IV treatment between January 2012 and October 2015 were reviewed. A total of 147 eyes were identified as having started IV treatment for DME with bevacizumab.

Inclusion criteria were patients with type 1 or type 2 diabetes mellitus, older than 18 years, with center-involved DME, defined as central foveal thickness (CFT) of more than 300μm on spectral-domain OCT (SD-OCT). All patients included were considered nonresponsive to bevacizumab, defined as having persistent intraretinal and/or subretinal fluid on OCT, ie, CFT>300μm and ≤10% CFT decrease from the last two consecutive bevacizumab IV, after a minimum of 3 monthly injections, with CFT assessed during the fourth week after the last bevacizumab injection before switching, regardless of visual acuity (VA).

Exclusion criteria included additional ocular diseases that could significantly affect the VA: a significant vitreoretinal interface anomaly on SD-OCT that might contribute to macular edema, such as an ERM with inner retinal distortion including proliferative diabetic retinopathy (PDR) with tractional retinal detachment or vitreous haemorrhage; age-related macular degeneration; retinal vascular occlusion; central corneal opacity; amblyopia; advanced glaucoma;

optic neuropathy; vitreous opacity; history of ocular trauma or surgery other than uncomplicated cataract extraction; cataract surgery within 6 months before bevacizumab switch; and inability or unwillingness to provide informed consent. Previous vitrectomy was not considered an exclusion criteria if DME occurred after a minimum period of 6 months post-vitrectomy. Both eyes of a single patient could be included only if both met all of the inclusion criteria, none of the exclusion criteria, and if the patient was switched to the same anti-VEGF at the time.

Methods of Assessment

Demographic characteristics, type of diabetes, presence or absence of diabetic retinopathy, HbA1c, CFT, BCVA in Early Treatment Diabetic Retinopathy Study (ETDRS) letters, and comorbidities were collected from the clinical records before starting bevacizumab injections (pre-baseline).

Baseline was considered as the time point immediately before the switch. At this time, collected variables were previous laser therapy, number of previous bevacizumab IV injections, CFT and BCVA. Moreover, we have considered as OCT biomarkers the presence of 10 different morphological parameters (Figure 1A and B), six in 1 mm central foveal area and four within the 20x20º scan area. In the 1 mm central foveal area: 1) SRF presence; 2) OPLd; 3) DRIL; 4) ELd; 5) ELMd; 6) existence of more than 10 HRF. Within the 20x20º scan area: 7) cysts in the outer nuclear layer more expressive than in the inner nuclear layer (ONLc>INLc); 8) HE evidence; 9) ERM without inner retinal distortion (ERMn); and 10) vitreomacular adhesion with at least one-third of VMA.

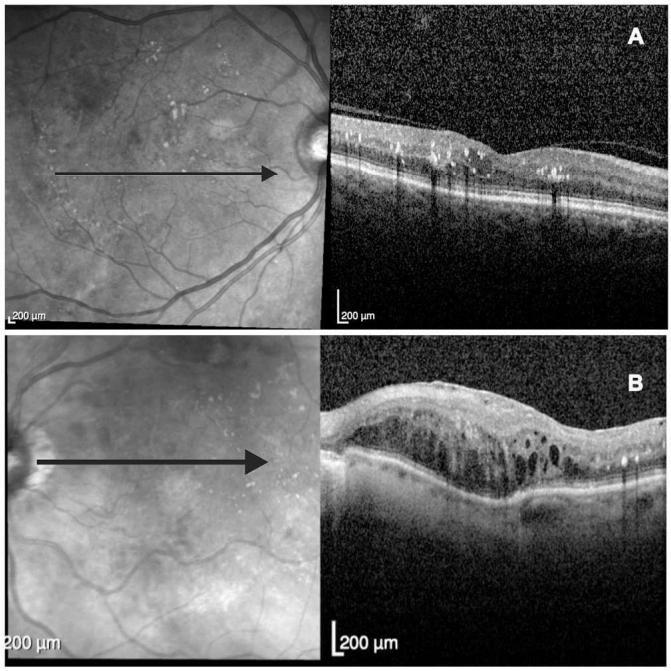

Figure 1 (**A**) Example of a central foveal image of an OCT 20x20° scan area (5.8 mm) acquisition where the existence of hard exudates (HE); hyperreflective foci (HRF) with small size (<30µm), with reflectivity similar to the nerve fiber layer and no back-shadowing; hard exudates (HE) with back-shadowing; and a vitreomacular adhesion with at least 1/3 of vitreomacular detachment from the macular area (VMA) can be observed. (**B**) Example of a central foveal image of an OCT 20x20° scan area acquisition with the presence of an epiretinal membrane without inner retinal distortion (ERMn); outer plexiform layer disruption (OPLd); disorganization of the retinal inner layers (DRIL); ellipsoid layer disruption (ELd); external limiting membrane disruption (ELMd); HE; and HRF.

A single 180º SD-OCT line scan (5.8mm length) centered onto the fovea was analyzed for HRF evaluation. A manual count of HRF, defined as small (<30µm), punctiform, with reflectivity similar to the nerve fiber layer and no back-shadowing, was performed in the central 1 mm in length.

After the switch, all patients received three monthly consecutive doses of ranibizumab or aflibercept and were observed during the fourth week after the last injection, when CFT and BCVA were assessed.

OCT scans were obtained by an SD-OCT (macular dense line scan mode HR 20x20º Spectralis HRA+OCT; Heidelberg Engineering, Heidelberg, Germany). The OCT data evaluation was performed by two experienced medical retina specialists, BP and MB, and the mean value of these measurements was considered.

The CFT was automatically measured by the software in the central 1 mm. A CFT<300µm was defined as DME resolution. After switching, during the fourth week after the last injection, a clinically significant functional improvement was considered when a gain of ≥5 EDTRS letters was achieved and a clinically significant anatomical improvement was considered if a reduction in CFT≥10% was achieved.

Quantitative Variables

Quantitative variables reported on this paper are HbA1c, CFT, and BCVA in ETDRS letters.

Statistical Methods

Non-parametric statistical methods were used. Values are presented as median (range, interquartile range) or n (%) unless otherwise specified. Within-group analyses were performed with the Friedman or Wilcoxon tests for paired samples, depending on the number of time points analysed. The McNemar test was used for discrete variables. Between-group analyses (ranibizumab versus aflibercept) at each time point were performed with the Mann–Whitney test. The x^2 test was used for comparison of proportions. Whenever necessary, p-values were adjusted for multiple comparisons. Data analyses were performed using SPSSv23 (IBM., USA). Tests were considered significant at $\alpha<0.05$ significance level (two-sided).

Results

Participants

A total of 56 eyes from 40 patients were included in the study.

Demographics and Clinical Baseline Characteristics

Demographic, pre-baseline and baseline clinical data from the total population sample and patients that switched to ranibizumab or aflibercept are shown in Table 1. There were no between-group statistically significant differences in demographic characteristics, pre-baseline or baseline parameters, except for DRIL presence in baseline OCT parameters – Table 2. There were more eyes without DRIL in the ranibizumab group than in the aflibercept group (63.6% vs 26.1%, p=0.007).

Between- and Within-Group Anatomical Differences Post-Switch

Median CFT decrease was significantly different in both switch groups after 4 months both compared to pre-bevacizumab and to the moment of the switch (p<0.001), with no between-group differences – Figure 2.

Between- and Within-Group Functional Differences Post-Switch

BCVA gain was only statistically significant in the post-switch ranibizumab group after 4 months, both compared to pre-bevacizumab and the moment of the switch (p<0.001) – Figure 3. Moreover, more patients increased more than 15 letters in the ranibizumab group compared with the aflibercept group (18.2% vs 0%, respectively, p=0.037) after 4 months of the switch.

Discussion

This single-center retrospective comparative study describes the efficacy of switching from bevacizumab to ranibizumab or aflibercept in eyes with DME unresponsive to bevacizumab. Of the 40 patients included in the study 56 eyes were analyzed.

The only difference between groups, among baseline characteristics, was the presence of more eyes without DRIL in the ranibizumab group which may explain the BCVA gain differences between groups, with only a statistically significant improvement in the ranibizumab group. Following the same pattern, more eyes had an increase of more than 15 letters in the ranibizumab group compared with the aflibercept group. Similar outcomes have been already described by Ashraf et al[13] who reported that despite a significant CFT decrease after the switch to ranibizumab or aflibercept in eyes with DME refractory to bevacizumab, a significant improvement in

Table 1 Demographics, Pre-Baseline and Baseline Clinical Characteristics for the Whole Population and by Switch Group (Ranibizumab or Aflibercept)

Parameters	Whole Population Sample (n=56)	Switch to Ranibizumab (n=33)	Switch to Aflibercept (n=23)	p-value*
Age in years, median (range, IQR)	67.0 (47.0–85.0, 11.0)	65.5 (47.0–85, 11.0)	69.0 (52.0–81.0, 14.0)	0.648
HT, n (%)	28 (77.8)	13 (65.0)	15 (93.8)	0.053
Dyslipidemia, n (%)	24 (66.7)	11 (55.0)	13 (81.2)	0.157
BMI, median (range, IQR)	27.8 (20.6–40.2, 5.1)	27.3 (20.6–35.0, 7.6)	27.9 (24.0–40.2, 10.0)	0.300
Type 2 DM, n (%)	39 (69.6)	24 (72.7)	15 (65.2)	0.400
DM duration in months, median (range, IQR)	16.0 (0.0–32.0, 10.0)	15.5 (0.0–31.0, 16.0)	18.0 (5.0–32.0, 8.0)	0.705
DME duration in months, median (range, IQR)	10.3 (0.0–48.0, 12.0)	8.5 (0.0–36.0, 9.0)	12.9 (1.0–48.0, 22.0)	0.842
PDR, n (%)	2 (3.6)	1 (3.0)	1 (4.3)	1.000
HbA1c in %, median (range, IQR)	7.9 (6.0–9.0, 3.0)	7.8 (6.0–8.8, 0.8)	7.9 (6.7–9.0, 2.3)	0.606
Phakic, n (%)	48 (85.7)	30 (90.9)	18 (78.3)	0.252
Pre-switch macular laser, n (%)	12 (21.8)	7 (21.9)	5 (21.7)	1.000
Pre-switch PRP laser, n (%)	14 (25.9)	11 (35.5)	3 (13.0)	0.115
Number of pre-switch beva injections, median (range, IQR)	3.0 (3.0–14.0, 3.0)	3.0 (3.0–14.0, 2.0)	4.0 (3.0–11.0, 3.0)	0.231
Pre-beva CFT in µm, median (range, IQR)	473.0 (320.0–808.0, 119.0)	483.0 (320.0–808.0, 78.0)	459.0 (332.0–644.0, 136.0)	0.191
Pre-switch CFT in µm, median (range, IQR)	468.5 (312.0–707.0, 131.0)	449.0 (312.0–707.0, 117.0)	473.0 (331.0–603.0, 153.0)	0.868
Pre-beva BCVA, median (range, IQR)	60.0 (3.0–80.0, 25.0)	60.0 (3.0–80.0, 20.0)	60.0 (10.0–80.0, 25.0)	0.712
Pre-switch BCVA, median (range, IQR)	65.0 (3.0–85.0, 25.0)	65.0 (3.0–85.0, 23.0)	61.0 (10.0–80.0, 27.0)	0.893
Time in months from last beva to first post-switch injection, median (range, IQR)	3.1 (1.0–9.0, 3.0)	3.0 (1.0–9.0, 3.0)	3.3 (1.0–8.0, 2.0)	0.434

Note: *p-value between ranibizumab and aflibercept groups.
Abbreviations: IQR, interquartile range; DM, diabetes mellitus; HT, hypertension; BMI, body mass index; DME, diabetic macular edema; PDR, proliferative diabetic retinopathy; HbA1c, glycated hemoglobin; PRP, photocoagulation; beva, bevacizumab; CFT, central foveal thickness; BCVA, best-corrected visual acuity in ETDRS letters.

Table 2 OCT Parameters for the Whole Population and by Switch Group (Ranibizumab or Aflibercept)

OCT Parameters	Whole Population Sample (n=56)	Switch to Ranibizumab (n=33)	Switch to Aflibercept (n=23)	p-value*
SRF, n (%)	6 (10.7)	4 (12.1)	2 (8.7)	1.000
OPLd, n (%)	49 (87.5)	27 (81.8)	22 (95.7)	0.220
DRIL, n (%)	29 (51.8)	12 (36.4)	17 (73.9)	0.007
ELd, n (%)	27 (48.2)	13 (39.4)	14 (60.9)	0.174
ELMd, n (%)	24 (42.9)	11 (33.3)	13 (56.5)	0.105
ONL>INL, n (%)	29 (51.8)	17 (51.5)	12 (52.2)	1.000
HE, n (%)	36 (64.3)	19 (57.6)	17 (73.9)	0.264
>10 HRD, n (%)	53 (94.6)	30 (90.9)	23 (100.0)	0.261
ERMn, n (%)	17 (30.4)	11 (33.3)	6 (26.0)	0.734
VMA, n (%)	9 (16.1)	3 (9.1)	6 (26.1)	0.139

Note: *p-value between ranibizumab and aflibercept groups.
Abbreviations: OCT, optical coherence tomography; SRF, subretinal fluid; OPLd, outer plexiform layer disruption; DRIL, disorganization of the retinal inner layers; ELd, ellipsoid layer disruption; ELMd. external limiting membrane disruption: ONL>INL. cysts in the outer nuclear layer more expressive than in the inner nuclear layer: HE. hard exudates; >10 HRD, more than 10 hyperreflective dots; ERMn, epiretinal membrane without inner retinal distortion; VMA, vitreomacular adhesion with at least 1/3 of vitreomacular detachment from the macular area.

BCVA was observed only with ranibizumab but not with aflibercept. As it is already well established, there is an association between DRIL, increasing severity of diabetic retinopathy, and a poor VA outcome with treatment.[23,24] In our cohort, the higher prevalence of baseline DRIL in the aflibercept group did not parallel a lower baseline visual acuity in comparison with the ranibizumab group. Until now, the influence of OCT biomarkers, such as DRIL, on functional outcomes with anti-VEGF therapy, has not been addressed in hallmark clinical trials such as protocol T.[9]

Protocol T, the only head-to-head study comparing the effect of the three anti-VEGFs in DME, corroborates these

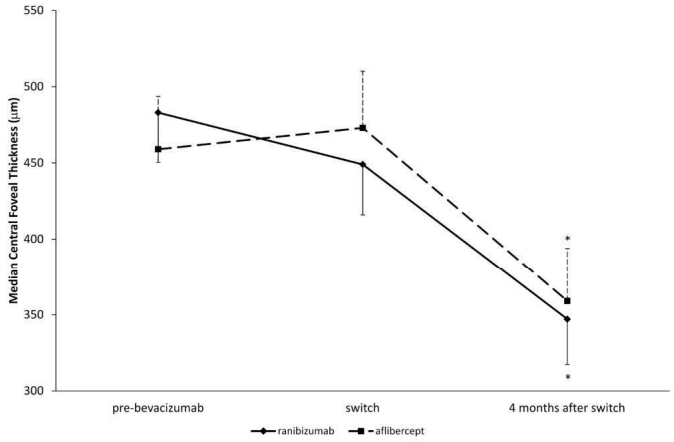

Figure 2 Median central foveal thickness change between- and within- switch groups (ranibizumab or aflibercept) pre-bevacizumab, at the time of switch and 4 months after switch. *p<0.001 compared both to pre-bevacizumab and to switch (within-group).

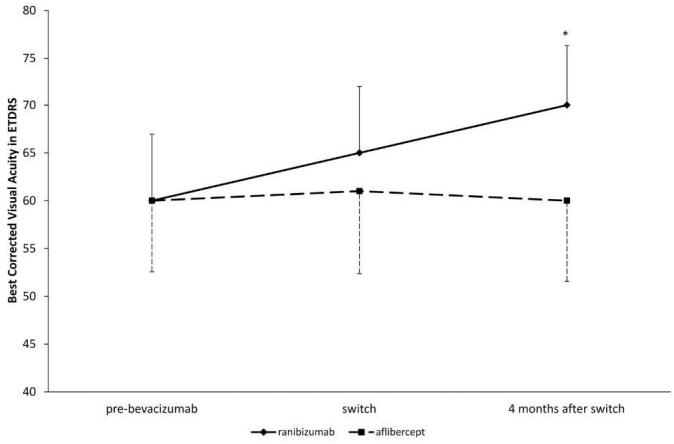

Figure 3 Best-corrected visual acuity change between- and within- switch groups (ranibizumab or aflibercept) pre-bevacizumab, at the time of switch and 4 months after switch. *p<0.001 compared compared both to pre-bevacizumab and to switch (within-group).

findings, although with a 0.3mg ranibizumab dose (which has not been tested against the 0.5mg dose under a PRN regimen). In the United States ranibizumab, 0.3mg is only approved for DME treatment in a monthly regimen and not in a PRN regimen.[3] In fact, the Resolve Phase II study ended with a target average treatment dose of 0.47mg.[25]

Interestingly, and even using a possible suboptimal ranibizumab dose whilst using the recommended aflibercept dose, the 2 years results of protocol T have shown that there were no statistically significant differences between ranibizumab and aflibercept, regarding CFT decrease and BCVA improvement. On the contrary,

bevacizumab was less efficient in comparison with ranibizumab and aflibercept in achieving a BCVA gain in patients with less than 69 letters at baseline and in reducing macular edema (even in patients with a good baseline BCVA, >69 letters).[9] Our results reinforce the conclusion of protocol T in as much as a sub-optimal response to bevacizumab was verified in patients with pre-bevacizumab low vision, <69 letters, and our study population had a mean BCVA lower than 69 letters in both pre-bevacizumab and pre-switch time points.

Favoring our study design with early switch criteria is the post-hoc analysis of the DRCR.net Protocol I.[26] According to this study, and for patients with a sub-optimal visual response after the first 3 IV anti-VEGF injections, it may be appropriate to consider adjustments to the treatment regimen and an early switch to achieve the best functional outcomes. Eyes with sub-optimal early BCVA response (after 12 weeks with monthly IV ranibizumab treatment) had poorer long-term visual outcomes than eyes with a pronounced early response.[26]

Following this line of reasoning, and although the average number of bevacizumab injections prior to switching (3–4) may be considered insufficient, it is possible that if patients had continued with bevacizumab treatment they might eventually improve. However, and in order to minimize this possibility, only patients with ≤10% CFT decrease from the last two consecutive bevacizumab IV were considered for switching in our cohort. The same rationale was used for the post-switch follow-up period.

In addition, the majority of studies accept structural criteria on OCT rather than functional outcomes as relevant for refractoriness. Although OCT may not be considered the best method to be used, it is still the most widely, reproducible and accessible method in real-world clinical practice. Moreover, a functional negative outcome is induced by previous structural retinal lesions such as DME.

The present study had limitations, such as its retrospective non-randomized nature, the small number of eyes, the absence of a control arm, the relatively short duration of follow-up, and the additional difficulty inherent to the multifactorial nature of DME.

This study also has strengths: our results were achieved using the same baseline anatomical and visual selection inclusion criteria for both groups, and therefore there is no selection bias; it reinforces that other possible, yet unidentified, anatomical macular biomarkers may influence the functional outcomes in response to different treatments. In our cohort, DRIL has emerged as an important biomarker to explore, but also other possible prognostic factors should be pursued, eg, macula vascularization profile through future, more accurate, and fully developed non-invasive imaging OCT technology such as OCT angiography (OCTA).

Conclusion

In persistent DME unresponsive or with incomplete response to bevacizumab, a significant anatomical and functional improvement was observed with ranibizumab therapy. In the aflibercept group, the anatomical response was not followed by an improvement on BCVA after 4 months of switch. Novel biomarkers, other than visual acuity and CFT outcomes, should be pursued, to clarify these achievements and the real efficacy of each individual therapy.

Data Sharing Statement

All data used to support the findings of this study are at the Ophthalmology Department, Hospital de Santo António, Centro Hospitalar Universitário do Porto, Portugal, and available from the corresponding author upon request. Any data intended for sharing is deidentified.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have no conflicts of interest to declare for this work.

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2.6 Vitrectomized versus non-vitrectomized eyes in diabetic macular edema response to ranibizumab – retinal layers thickness as prognostic biomarkers[404]

TITLE: Vitrectomized versus non-vitrectomized eyes in diabetic macular edema response to ranibizumab – retinal layers thickness as prognostic biomarkers

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ABSTRACT

Background: To evaluate the role of the vitreous in the management of diabetic macular edema with ranibizumab intravitreal injections in a pro re nata regimen. **Methods:** Prospective study of 50 consecutive eyes with diabetic macular edema treated with ranibizumab and 12 months of follow-up. Primary endpoint: to assess differences between non-vitrectomized and vitrectomized eyes in the number injections needed to control the edema. Secondary endpoints: comparison of groups regarding best corrected visual acuity, central foveal thickness and thickness of seven retinal layers. **Results:** 46 eyes from 38 patients, 10 vitrectomized and 36 non-vitrectomized, completed the follow-up. At month 12, the two groups achieved an equivalent anatomical outcome and needed a similar number of ranibizumab intravitreal injections. In vitrectomized eyes final visual acuity was worse when baseline retinal nerve fiber layers in the central foveal subfield were thicker, showing a strong correlation ($r=-0.942$, $p<0.001$). A similar, albeit moderate correlation was observed in non-vitrectomized eyes ($r=-0.504$, $p=0.002$). A decrease of retinal nerve fiber layers inner ring thickness was correlated with a better final visual acuity only in vitrectomized eyes ($r=0.734$, $p=0.016$). **Conclusions:** The effect of diabetic macular edema seems to be worse in vitrectomized eyes, with a thinner inner retina reservoir. Clinicaltrials.govNCT04387604.

KEYWORDS: diabetic macular edema; ranibizumab; vitrectomy; retinal layers thickness; prognostic biomarkers

INTRODUCTION

Diabetic retinopathy (DR) is one of the most important diabetes mellitus microvascular complications and diabetic macular edema (DME) is the main responsible for the vision loss related to DR (1). The International Council of Ophthalmology and EURETINA Guidelines recommend anti-vascular endothelial growth factors (anti-VEGF) agents as first-line therapy for treating central DME (2). Among the approved anti-VEGF products, ranibizumab (RBZ) is the one with more safety and efficacy data in the long term (3-8).

DME pathogenesis is multifactorial and the posterior cortical vitreous seems to play a major role in its development, via several mechanical and physiological mechanisms that lead to increased vascular permeability (1,9-12). Vitrectomy has been proven to be effective in the resolution of DME, through the removal of growth factors and cytokines in a background of an ischemic retina and inflammatory response, particularly when a tractional cause is involved in its pathogenesis (13,14). Although DME recurrence post-vitrectomy has been reported in a low percentage of cases (10.9%) (15), this type of edemas is considered more difficult to treat particularly with intravitreal (IV) anti-VEGF agents since those eyes have a more rapid clearance of drugs than non-vitrectomized eyes (16-19). There is a lack of long term, prospective, comparative studies addressing vitrectomized and non-vitrectomized eyes with DME and otherwise comparable characteristics. There is some evidence that RBZ is also an effective treatment for DME in vitrectomized eyes although the functional and anatomical efficacy seems to be achieved slower, with the need of a higher number of injections at least during the first 12 months of treatment (1,20,21).

Neurodegeneration is an early event in DR, documented through the thinning of the inner retina due to ganglion cell apoptosis, reflected also in retinal nerve fiber layers (RNFL), the

axons derived from ganglion cells (22,23). It has already been described that inner and outer temporal ganglion cells complex layer (GCL) thickness is decreased in vitrectomized eyes (24).

The total retinal thickness in DME seen on OCT may represent edematous or degenerative changes. The effect and negative impact of DME in inner retinal layers and of the latter on functional outcomes has been objectively demonstrated, with the achievement of a positive correlation of inner retinal layers thickness reduction, particularly in the nasal quadrant, and visual gain, both with RBZ and triamcinolone applied to DME treatment (25). As opposed to non-vitrectomized eyes, the influence of DME in eyes without vitreous is much less explored.

The purpose of this study was to deepen the knowledge of the real effect of the vitreous status in the management of DME with RBZ IV injections, a first line treatment approach for DME. A comprehensive analyses of the different retinal layers thickness before and after treatment, and their influence on functional and anatomical outcomes, was assessed.

RESULTS

Demographic and clinical baseline data

From the 50 eyes enrolled in the study, 46 eyes of 38 patients, 10 vitrectomized and 36 non-vitrectomized, completed the entire follow-up. There were no differences between any of the analysed demographic or clinical parameters at baseline – Table 1.

At baseline, group 2 had thinner inner retinal layers, particularly the GCL layer, with a mean thickness of 35.5µm (26-43, 95%CI 31.2-39.7) compared to 41.3µm (32-51, 95%CI

39.8-42.8) in group 1 (p=0.011). A more expressive thinner GCL in group 2, compared with group 1, was observed in inner and outer temporal ETDRS subfields and also in inner ring ETDRS subfields (p<0.001 and p=0.002, respectively) – Table 2.

A percentage of 30.4% of the patients was treatment *naïve* (33% and 20%, p=0.699, in group 2 and group 1, respectively). The causes for vitrectomy were PDR in 7 cases, ERM in one case (in these 8 cases ILM was peeled without indocyanine green staining), one vitreous hemorrhage with no PDR and one retinal detachment.

Number of RBZ IV injections needed to control DME

The mean number of RBZ IV injections needed to control DME was 7.86 (95%CI 5.39–10.33) in vitrectomized eyes and 7.72 (95%CI 6.71–8.74) in non-vitrectomized eyes (p=0.815). Although the number of RBZ IV was similar in both groups, there was an overall association between DME resolution and the number of RBZ IV injections, favoring a lower number of injections (p=0.002). Overall BCVA at month 12 showed a positive moderate correlation with the number of RBZ IV injections (r=0.562, p<0.001).

BCVA and CFT evolution at baseline and 12 months follow-up

There were no differences between groups regarding BCVA and CFT evolution from baseline to the end of follow-up (Figures 2 and 3, respectively). Nevertheless, at month 12, there were more eyes with <70 ETDRS letters in group 2 compared to group 1 (50% vs 13.9%, p=0.027).

Type of responders

There were no differences between groups 1 and 2 regarding the type of responder, good-earlier responders versus partial/non-responders (p=1.000). Overall 33% of patients were good-earlier responders and 69% of good-earlier responders had a baseline CFT<400μm.

When analyzing good-earlier responders versus partial/non-responders, good responders were significantly associated with a lower INL thickness at baseline and at the end of follow-up, especially in the inner ring of ETDRS subfield ($p=0.002$ and $p=0.004$, respectively). There were no differences between responder groups regarding rescue LASER approach ($p=0.225$).

Analysis of the thickness of the seven retinal layers

A statistically significant difference between groups was observed in differences from 12 months follow-up to baseline only for INL and, to a lesser extent, for GCL layers – Table 3.

Correlations between parameters

In vitrectomized eyes final visual acuities were strongly correlated with baseline RNFL and GCL, in the central foveal subfield ($r=0.942$ and $r=0.871$, respectively, $p<0.001$). Also, a positive correlation, although moderate, was observed in non-vitrectomized eyes only for RNFL in the central foveal subfield ($r=0.504$, $p=0.002$). A decrease of RNFL inner ring thickness was negatively correlated with the final visual acuity in vitrectomized eyes ($r=-0.734$, $p=0.016$), with no correlation in non-vitrectomized eyes.

Safety outcomes

Ocular adverse events included a retinal detachment fifteen days after a third IV injection and an iatrogenic cataract at the eighth month of follow-up, both leading to drop-out of the study. No other serious ocular events, such as endophthalmitis, were registered. The two remaining causes of drop-out of the study were due to non-ocular serious adverse events: one acute myocardial infarction (AMI) and one death due to a stroke at the fifth (after four ranibizumab injections) and the fourth month (after three ranibizumab injections) of follow-up, respectively.

DISCUSSION

This prospective long term study tried to comprehensively analyze vitrectomized and non-vitrectomized eyes in their vitreoretinal anatomical features beyond the simple vitreous' existence, in the process of DME treatment with a first line treatment approach with RBZ IV injections.

There is some controversy regarding the real effect of the vitreous status and intravitreal anti-VEGF therapy in DME. The majority of studies was performed in animals and showed more rapid clearance rates of bevacizumab, RBZ and triamcinolone acetonide placed inside the vitreous cavity of vitrectomized eyes (16-18), although others did not (19). In humans this evidence is even more scarce and there are no studies addressing anti-VEGF drugs (20). Another variable to consider is the vitreous concentrations of VEGF that are increased and correlated with the severity of macular edema in diabetic patients (21). Vitrectomy itself may have other positive effects that may offset an eventual inferior half-life of IV agents, through the removal of cytokines, VEGF and advanced glycation end products from the vitreous, facilitating the fluid circulation inside the vitreous cavity, along with the increased macular capillary flow, retinal oxygenation and, not less important, promoting the vitreous macular traction release (13,26).

According to our study, and in line with this rationale, the number of RBZ IV injections needed to control DME was similar in groups 1 and 2. Another interesting observation was the overall association between DME resolution, a better visual outcome and a lower number of RBZ IV injections, the paradigm of good-earlier responders. These sub-types of patients were not different in groups 1 and 2.

Regarding treatment efficacy between groups this was confirmed in anatomical and

functional outcomes, as no differences existed between groups regarding BCVA and CFT evolution during the entire follow-up period.

In vitrectomized eyes, the trend to inferior outcomes and different results, according to different studies, concerning the number of IV anti-VEGF injections, with higher number at least in the first 6 months and first year of treatment (20,21), and the type of anatomical and functional response (less expressive (20,21), variable (27), ineffective (28,29) or achieved slower (20,21)) can be related with several factors: 1) the different anti-VEGF used (8,30); 2) the retrospective nature of the studies (20,27,28); 3) the poor design of studies with different or short term (20,28,29,31) follow-ups; and 4) the different baseline/demographic characteristics (21). All of these factors may contribute to the different reported results, in addition to the particular background of a vitrectomized eye.

Regarding the baseline characteristics of our cohort the two groups were very similar. The only significant difference was the expected decrease in inner retinal layers (32), particularly in the GCL thickness of inner-outer temporal and superior sectors and in inner ring ETDRS subfields in vitrectomized eyes, with ILM peeling performed in 80% of the eyes in our study. A possible explanation for these results could be the preference for ILM removal in temporal superior sectors due both to ease of access and to preserve the nasal part, where the papillomacular bundle is located, leaving the remaining sectors to be removed clockwise with a minimum touch (32). Although ILM peeling is indicated to prevent re-proliferation of ERM (33), one retrospective study found that ILM removal was correlated with worse visual outcomes (34). Furthermore, retina from diabetic patients is particularly susceptible, as neurodegeneration is also an early event in DR, reflected on the thinning of the inner retina due to ganglion cell apoptosis with GCL and RNFL thickness

decrease, even before microvascular lesions are evident (20,23,35). The observation that the inner layer thickness further decreased in vitrectomized eyes stresses the need of a special care during the treatment of DME in these eyes.

This was in line with our results. Better visual outcomes were correlated with a lower baseline RNFL, GCL, IPL and INL thickness in central and inner ring ETDRS areas, in both vitrectomized and non-vitrectomized eyes. Moreover, a higher decrease in RNFL inner ring during the 12 months of follow-up was also correlated with a better final vision, in both groups. However, correlations were by far stronger in vitrectomized eyes. These data favors a higher fragility of those eyes when DME does not regress, highlighting the particular nefarious effect of edema in vitrectomized eyes, particularly in the inner ring RNFL, where the axons responsible for central VA are located.

Even though these effects on retinal layer thickness have already been described for non-vitrectomized eyes with DME treated with RBZ or triamcinolone (25), no prior studies have addressed that effect in vitrectomized eyes. Our study reinforces the need of an optimized DME therapy, especially in non-responder cases and particularly in vitrectomized eyes with a more limited neuroretina reservoir. Although both groups have started with similar BCVA, in vitrectomized eyes the functional outcome was lower, with poor vision in a higher percentage of cases at the end of follow-up, in comparison with non-vitrectomized eyes (50% vs 13.9%, p=0.027).

Another important result was the significant association between good-earlier responders and a lower INL thickness at baseline and at the end of follow-up, especially in the inner ring of ETDRS subfield (p=0.002 and p=0.004, respectively). This may be an important biomarker to predict earlier responders with less treatment burden and a more benign

evolution, and may also contribute to the clarification of RD pathophysiology. As it has been described, focal vessel dilations and microaneurisms are among the preclinical vascular changes in DR at the level of the deep capillary layer (DCL) which is located at the INL (36). According to previous histopathology and OCT studies, microaneurysms are preferentially located at the DCL (37,38), and a positive correlation has been described between an increased retinal volume and the number of microaneurysms (37).

The onset of edema in the INL can be considered a consequence of the natural evolution of DCL DR vascular lesions and a sign, when isolated and low grade, of a non-chronic DME, with higher probability to behave as a good-earlier responder to anti-VEGF therapy. Hence, the invasion of adjacent layers can be considered a sign of progression, a higher level of severity and chronicity, particularly when DCL and OPL disruption is evident, characteristics that have been described as predictors of poor response to anti-VEGF and more advanced DR stages, with intra-retinal cysts invading also the ONL, further compromising visual function (39). From 12 months follow-up to baseline a difference between groups was observed only for INL and, in a lesser extent, for GCL, favoring group 2. The easy access of ranibizumab to the superficial and deep capillary plexus, located in GCL and INL, respectively, through the thinner inner layers which features vitrectomized eyes (32), may facilitate the stabilization of capillary plexus of those eyes in comparison with non vitrectomized eyes.

CFT may be considered a predictor of early remission of DME under PRN RZB IV injections, as already described (40). In our study we have also observed a positive correlation between baseline CFT and CFT evolution during the follow-up period, for both

vitrectomized and non-vitrectomized eyes. According to our results, 69% of good-earlier responders had a baseline CFT<400 μm.

In conclusion, vitrectomized and non-vitrectomized eyes achieved an equivalent anatomical outcome and needed a similar number of RBZ IV injections in a PRN regimen. Lower baseline CFT and lower INL thickness seem to be associated to a good-earlier response to RBZ IV therapy. Moreover, the expected compromised inner retina layers thickness in vitrectomized eyes showed to be relevant to their lower functional outcome. Therefore, an optimal treatment choice in order to resolve DME, should be considered promptly to avoid irreversible damages, particularly in those eyes. The severity of the edema compromising the inner retinal layers, in central and inner ring ETDRS areas and its correlation with poor functional outcomes highlight the negative effect of DME to retinal function, especially in those locations.

The results of this study have practical implications when planning schedules for anti-VEGF IV injections, such as loading dose strategy and overall treatment decisions regarding DME approach. Even though the low number of vitrectomized eyes included in this study is a reflection of the relatively infrequent post-vitrectomy DME occurrence (15), future studies with larger samples should be carried out to further investigate and confirm our results.

METHODS

Study Design

This was a two-center, prospective, observational study, conducted at the Departments of Ophthalmology from Centro Hospitalar e Universitário do Porto (CHUP) and Hospital

Santa Maria Maior de Barcelos, Portugal. Fifty consecutive eyes with DME were considered for treatment with RBZ IV injections following a PRN regimen, of which 46 completed the 12 month follow-up. Patients were included in two groups according to the vitreous status: group 1 – non-vitrectomized eyes (n=36); group 2 – vitrectomized eyes (n=10). Patients were followed-up according to the standard of care and a final analysis of the results was conducted at 12 months of follow-up, which was the minimum follow-up period required for each patient. The recruitment period was from January 2018 to January 2019.

This study was conducted according to the tenets of the Declaration of Helsinki in its latest amendment (Brazil, 2013) and was approved by the ethics committee of CHUP [2017.093 (084-DEFI/082-CES)]. All patients signed an informed consent form. This study is registered at www.clinicaltrials.gov (NCT04387604, date of registration 14/05/2020).

Inclusion criteria: 1) ≥18 years with either type 1 or type 2 diabetes mellitus; 2) central subfield foveal thickness (CFT) >300μm, measured using spectral domain optical coherence tomography (SD-OCT, Spectralis HRA+OCT, version 1.10.2.0, Heidelberg Engineering, Heidelberg, Germany); 3) best corrected visual acuity (BCVA) of 20 to 80 letters, using Early Treatment of Diabetic Retinopathy Study (ETDRS) letters chart; 4) minimum period of 6 months post-vitrectomy for inclusion in group 2; 5) ability and willingness to provide written informed consent. 6) if the inclusion critria were fullfilled for both eyes bilateral inclusion was allowed.

Exclusion criteria: 1) Epiretinal membrane (ERM) existence in the study eye; 2) persistent posterior hyaloid adherence after vitrectomy for group 2; 3) previous vitrectomy for group

1; 4) history of other retinal vascular diseases in the study eye; 5) LASER photocoagulation or anti-VEGF IV or systemic anti-VEGF or pro-anti-VEGF treatment and cataract surgery in the 6 months prior to study inclusion; 6) IV or peribulbar corticosteroid injections in the 6 months prior to study inclusion; 7) history of IV of implant of fluocinolone acetonide in the study eye; 8) vitreous hemorrhage or opacification in the study eye; 9) proliferative diabetic retinopathy (PDR) in the study eye; 10) active ocular inflammation or infection in either eye; 11) aphakia in the study eye; 12) other causes for macular edema, e.g., after cataract surgery in the study eye; 13) other causes of visual loss in the study eye; 14) pro-edematous medication (such as systemic glitazones or prostaglandins) or other pathologies that might influence the course of macular edema in the study eye; 15) uncontrolled glaucoma in either eye (intraocular pressure >24mmHg with treatment); 16) history of stroke in the previous 6 months; 17) uncontrolled arterial hypertension (systolic blood pressure >160mmHg or diastolic blood pressure >100mmHg).

Treatment

All patients were treated with RBZ IV injections (0.5mg/0.05ml) following a PRN regimen. In the PRN regimen adopted, the rationale was to follow the patient every 4 weeks and treat every 4 weeks until a maximum BCVA (85 letters) without edema or stability were achieved. Stability was considered when CFT was <300mm or when a change in BCVA of <5 letters or CFT<10% in two consecutive visits within the 24 week period, considered a critical period of therapy, were observed.

When required, adjunct treatment with macular LASER (rescue LASER) was also admitted at or after 24 weeks, in case of persistent DME.

Treatment Schedule

Repeat injections every 4-weeks if eye "improved" or "worsened" (defined as ≥5 letter change from last injection, or ≥10% CFT increase on OCT from last injection), or if CFT>300μm at any time point. BCVA worsening was only considered a treatment criterion if it was due to DME and not with other ocular cause.

Defer injections if either BCVA of 85 letters and OCT CFT was "normal" (CFT≤300μm and non-existent intra- or sub-retinal fluid); or OCT CFT was "normal" (CFT≤300μm) and stable BCVA (defined as <5 letters change from last injection) after two consecutive injections during the first 24 weeks, or after one injection if OCT and VA criteria were equal or better than those obtained in a previous stability period.

Patient Assessment

At baseline, demographic and clinical data, including, serum levels of hemoglobin and glycated hemoglobin (HbA1C), microalbuminuria, body mass index and systolic and diastolic blood pressure were recorded. Each patient performed an SD-OCT, and underwent a complete ophthalmological evaluation (symptoms, BCVA, intraocular pressure measurement, anterior and posterior segment biomicroscopy) at baseline and every month until the end of follow-up. Fluorescein angiography was performed on all patients at study entrance and at the end of follow-up.

SD-OCT acquisition, data collection and data assessment

Two highly trained technicians conducted SD-OCT scans. Macular thickness measurements were performed with automated segmentation, 25 scans and a 20x20° acquisition mode. The follow-up function and auto-rescan with active eye tracking were also utilized. CFT was obtained automatically from equipment readings.

The thickness of seven retinal layers – retinal nerve fibre layer (RNFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL) and outer retinal layer (ORL) – between the external limiting membrane and the bruch membrane - were also measured. Automatic segmentation errors were corrected manually when necessary.

The mean thickness of each individual retinal layer was analysed in: 1) the nine individual ETDRS subfields; 2) the inner ring ETDRS subfields; 3) and the outer ring ETDRS subfields. For RNFL and GCL the mean layer thicknesses of the inner and outer nasal, temporal, superior and inferior ETDRS subfields were also calculated. Figure 1 shows a representative image of the retinal layers identified by automatic segmentation in SD-OCT scan and a schematic representation of the nine individual ETDRS subfields, this latter adapted from Won et al (32).

Outcome measures:

Primary outcome

To assess the differences between groups in the number of RBZ IV injections needed to control DME.

Secondary outcomes

Secondary endpoints included: 1) comparison of groups 1.1) BCVA and CFT at baseline and after 12 months of follow-up; 1.2) differences in type of responders; 1.3) analysis of the thickness of seven retinal layers; 1.4) correlation between retinal layers thickness and BCVA; 2) safety.

Functional and anatomical outcomes criteria

A significant functional improvement was defined as a gain $\geq$5 ETDRS letters. A DME

resolution was defined as CFT ≤300μm. BCVA ≥70 letters was defined as a good visual acuity and BCVA <70 as a poor visual acuity. Type of responder was classified as: 1) good-earlier responder – when beyond the 24th week of follow-up (maximum 7 injections) there was a complete anatomical response (CFT≤300 μm) with an increase in BCVA ≥5 letters; 2) non-responder – 13 injections and final CFT >400μm or ≤10% of CFT reduction and BCVA gain <5 letters; 3) partial responder – between good-earlier responder and non-responder criteria. According to our definition, a late responder was considered a partial responder.

Statistical analysis

Data were analyzed using non-parametric statistics. BCVA values in ETDRS were converted to LogMar before analyses. Between-group analyses of continuous variables were performed using the Mann-Whitney U test. Within-group analyses were performed using the Wilcoxon test. Nominal variables were analyzed using the chi-square test. Correlations between variables were tested using the Spearman rank correlation or the Kendall's τ-b, as appropriate. A $p<0.05$ (two-sided) was considered statistically significant.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author Contribution Statement

We declare that all authors have made substantial contributions, have approved the submitted version, and have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

Competing Interests Statement

The authors declare no competing interests, either financial or non-financial.

FIGURE LEGENDS

Figure 1 - retinal layers were identified by automatic segmentation in SD-OCT – retinal nerve fibre layer (RNFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL) and outer retinal layer (ORL) – between the external limiting membrane and the bruch membrane (top panel). The mean thickness of each individual retinal layer was analysed in: 1) the nine individual ETDRS subfields (A); the fovea (or central circle with a diameter of 1mm) (B); the inner ring ETDRS subfields (C); the outer ring ETDRS subfields (D); and globally (I). For RNFL and GCL the mean layer thicknesses of the outer and inner temporal (E), nasal (F), superior (G) and inferior (H) (bottom panel). Bottom panel adapted from Won et al[26].

Figure 2 – BCVA evolution from baseline to the end of follow-up. Mean±95%CI. p=ns at each time point using the Mann-Whitney U test.

Figure 3 – CFT evolution from baseline to the end of follow-up. Mean±95%CI. p=ns at each time point using the Mann-Whitney U test.

TABLES

Table 1 – Baseline Characteristics

Parameter	Group 1 [n=36]	Group 2 [n=10]	p-value
Age, mean [95% CI]	66.45 [62.87 – 70.02]	67.22 [60.34 – 74.11]	0.973
Gender, female	55.6%	41.4%	0.703
DM duration (years), mean [95% CI]	17.00 [13.90 – 20.10]	22.56 [16.26 – 28.85]	0.068
DME duration (months), mean [95% CI]	28.08 [19.62 – 36.55]	30.60 [6.39 – 54.81]	0.803
Type 2 DM	100%	100%	1.000
Macular LASER	80%	63.9%	0.460
PRP LASER	80%	58.3%	0.282
Naive IV	20%	33.3%	0.699
Number previous IV treatments, mean [95% CI]	5.31 [3.10 – 7.53]	2.50 [(-0.84) – 5.84]	0.179
HbA1c, mean [95% CI]	7.45 [7.12 – 7.78]	7.59 [6.76 – 8.42]	0.867
Hemoglobin, mean [95% CI]	12.98 [12.29 – 13.66]	12.91 [11.49 – 14.33]	0.946
Microalbuminuria, mean [95% CI]	299.14 [20.24 – 578.03]	117.74 [(-35.8) – 271.27]	0.647
BMI, mean [95% CI]	28.35 [26.96 – 29.74]	28.17 [24.99 – 31.35]	1.000
SBP, mean [95% CI]	137.6 [132.5 – 142.7]	138.5 [127.1 – 149.9]	0.697
DBP, mean [95% CI]	75.9 [73.2 78.7]	74.3 [65.6 – 83.0]	0.600

Group 1 = non vitrectomized; Group 2 = vitrectomized; 95%CI= 95% Confidence interval; DM = Diabetes mellitus; DME = diabetic macular edema; macular LASER = Prior focal-grid photocoagulation treatment in the study; PRP LASER = Prior panretinal photocoagulation treatment in the study eye; IV = intravitreal, naïve IV = no prior anti-VEGF intravitreal treatment in study eye; BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure.

Table 2 – Baseline thickness of retinal layers with significant difference between groups

Parameter	Group 1 [n=36]	Group 2 [n=10]	p-value
GCL int baseline, mean [95% CI]	49.8 [48.2 – 51.4]	41.8 [35.9 – 47.6]	**0.002**
IPL ext baseline, mean [95% CI]	31.2 [29.9 – 32.5]	28.3 [25.4 – 31.2]	**0.047**
GCL global baseline, mean [95% CI]	41.3 [39.8 – 42.8]	35.5 [31.2 – 39.7]	**0.011**
GCL temp baseline, mean [95% CI]	41.1 [38.8 – 43.3]	32.4 [27.4 – 37.4]	**< 0.001**
GCL sup baseline, mean [95% CI]	43.0 [41.4 – 44.7]	37.0 [31.5 – 42.5]	**0.010**

Group 1 = non vitrectomized; Group 2 = vitrectomized; GCL = Ganglion cell layer; IPL = inner plexiform layer; int = inner ring ETDRS subfields; ext = outer ring ETDRS subfields; global = the nine ETDRS subfields; temp = inner and outer temporal ETDRS subfields; sup = inner and outer superior ETDRS subfields. 95%CI = 95% Confidence interval.
Only statistically significant differences are reported.

Table 3 – Differences from 12 months follow-up to baseline of the different ETDRS subfields

Parameter	Group 1 [n=36]	Group 2 [n=10]	p-value
Dif INL ext, mean [95% CI]	-0.5 [(-1.5) – 1.4]	-1.9 [(-3.2) – (-0.6)]	**0.031**
Dif GCL M, mean [95% CI]	-0.1 [(-1.9) – 1.7]	-2.5 [(-3.8) – (-1.2)]	**0.033**
Dif INL M, mean [95% CI]	-1.4 [(-2.6) – (-0.2)]	-4.4 [(-5.7) – (-3.2)]	**0.007**

Group 1 = non vitrectomized; Group 2 = vitrectomized; GCL = Ganglion cell layer; INL = inner nuclear layer; ext = outer ring ETDRS subfields; M = the nine ETDRS subfields; 95%CI = 95% Confidence interval. Only statistically significant differences are reported.

FIGURES

Figure 1

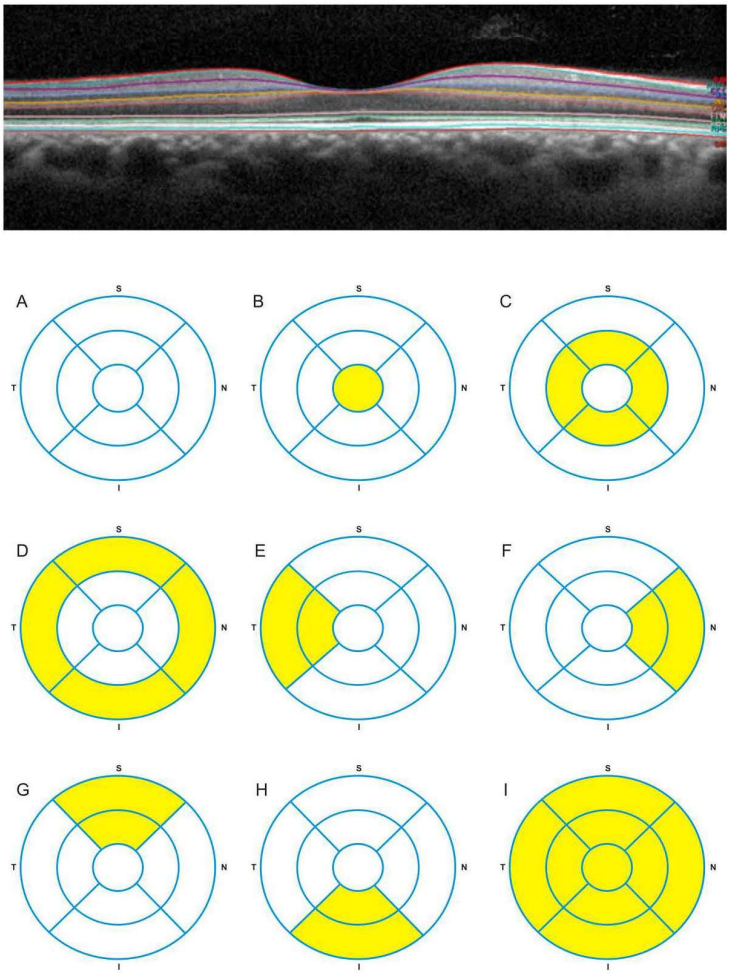


Figure 2

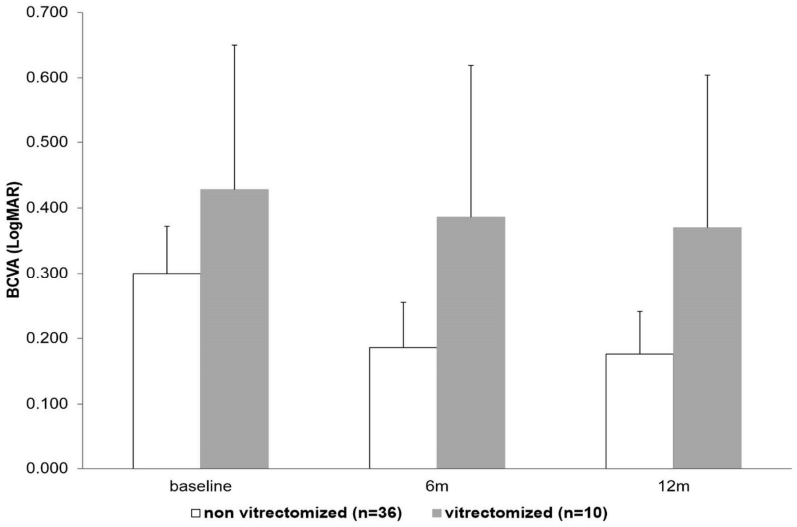
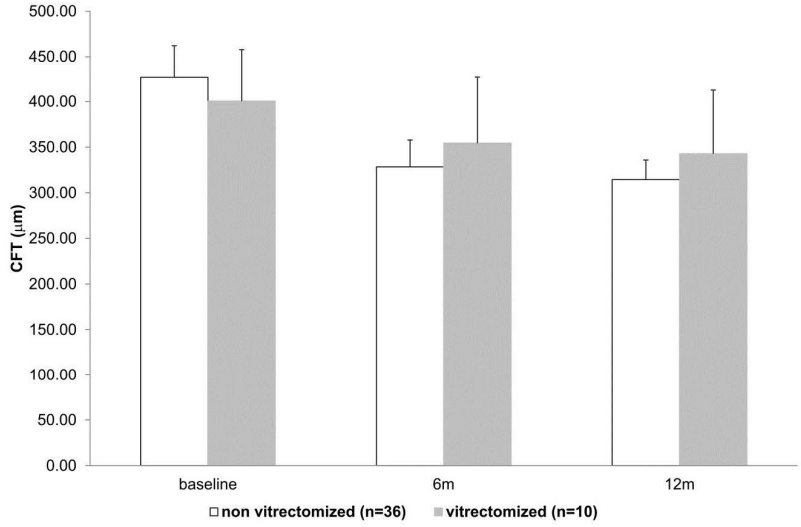

0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
BCVA (LogMAR)
baseline
6m
12m
non vitrectomized (n=36)
vitrectomized (n=10)

Figure 3


500.00
450.00
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
CFT (µm)
baseline
6m
12m
non vitrectomized (n=36)
vitrectomized (n=10)

2.7 Choroidal blood flow after intravitreal ranibizumab in vitrectomized and non-vitrectomized eyes with diabetic macular edema[282]

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ORIGINAL RESEARCH

Choroidal Blood Flow After Intravitreal Ranibizumab in Vitrectomized and Non-Vitrectomized Eyes with Diabetic Macular Edema

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Aim: Diabetic retinopathy staging system and progression predictors are soon to be considered insufficient for ophthalmologic practice. Given the growing evidence of the role of choroidal dysfunction, our purpose was to assess choroidal vascular changes with intravitreal ranibizumab (RBZ) treatment in diabetic macular edema (DME).

Methods: This was a prospective longitudinal cohort study. The study included DME eyes, grouped in vitrectomized (group 1) and non-vitrectomized (group 2) eyes, submitted to RBZ in a pro re nata regimen, with 24 weeks of follow-up. Main outcome measures such as central subfield foveal thickness (CFT), choroidal thickness (CT), and choroidal vascular index (CVI) were obtained from structural OCT, and choriocapillaris flow density (CCD) was obtained from OCT angiography and analyzed before and after treatment.

Results: Thirty-one patients were included, 10 eyes in group 1 and 24 eyes in group 2. The mean number of injections was 5.18 (range 2–6). Globally, there was an improvement in BCVA (+4.3 ETDRS letters, p=0.004) and CFT (−84.6 µm, p<0.001) with no changes in CT, CVI, or CCD (p>0.05). When considering only group 2, there was a significant decrease in CT (p=0.033) and a significant increase in CCD (p=0.010) 6 months after treatment, with no differences in CVI (p=0.111). Baseline CVI was correlated with visual acuity at week 24 both globally (r=0.406, p=0.029) and in group 2 (r=0.604, p=0.004).

Conclusion: In non-vitrectomized eyes, choriocapillaris blood flow improves with RBZ. Baseline CVI may correlate with visual function after RBZ. ClinicalTrials.gov NCT04387604.

Keywords: diabetic macular edema, ranibizumab, vitrectomy, choroidal vascular index, choriocapillaris flow

Introduction

Diabetic retinopathy is the leading cause of preventable blindness among working-age individuals in most developed countries, and diabetic macular edema (DME) is the main cause of visual impairment in diabetic patients. DME pathogenesis is complex, multifactorial, and not completely understood. Several mechanisms such as oxidative damage, microvascular hypoperfusion, and up-regulation of vascular endothelial growth factor (VEGF) are implicated in increased fluid leakage into retinal tissue.[1,2] Clinical and experimental findings suggest that not only the retinal capillaries but also choroidal vasculopathy in diabetes play a role in the pathogenesis of diabetic retinopathy.[3–5] Obstruction of the choriocapillaris, vascular degeneration, choroidal aneurysms, and choroidal neovascularization have been reported

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in histopathologic studies of diabetic eyes.[3] Central choroid thinning in type 2 diabetic eyes and choroidal blood flow reduction in eyes with DME have already been described.[5,6]

There is some controversy regarding the real impact of anti-VEGF therapy in choroidal and retinal vessels. VEGF has an undoubtful role in normal vascular physiology through neurovascular trophic effects, namely on endothelial survival and in the allowance of some fenestration of vascular tissue in adults.[7] In the choroid, VEGF receptors have been identified both in the choriocapillaris and in large blood vessels.[8,9] Additionally, it has been shown that the choroid is highly dependent on VEGF.[9]

Although there is evidence that sustains that anti-VEGF therapy slows or even reverses retinal non-perfusion in diabetic retinopathy,[10] Falavarjani et al demonstrated that there are no differences in the retinal blood flow before and after a single intravitreal injection (IV) of anti-VEGF for macular edema.[11] In choroidal tissue, Okamoto et al observed that a single anti-VEGF IV reduces choroidal vascular index (CVI) and choroidal blood flow in DME eyes not previously submitted to panretinal photocoagulation (PRP).[12] The long-term effect of anti-VEGF intravitreal therapy in choroidal vasculature is much less explored.

Our purpose was to analyze choroidal vascular changes with intravitreal ranibizumab (RBZ) treatment in a pro re nata (PRN) regimen in DME during 24 weeks of follow-up.

Materials and Methods

Study Design

This was a single-center, prospective, observational study, conducted at the Department of Ophthalmology of Centro Hospitalar e Universitário do Porto (CHUP), Portugal. This study was conducted according to the tenets of the Declaration of Helsinki in its latest amendment (Brazil, 2013) and was approved by the ethics committee of CHUP [2017.093 (084-DEFI/082-CES)]. All patients signed an informed consent form. This study is registered at www.clinicaltrials.gov (NCT04387604).

Study Population

The recruitment period was from January 2018 to January 2019. Patients were included in two groups according to the vitreous status: group 1 – vitrectomized eyes and group 2 – non-vitrectomized eyes. Inclusion criteria were as follows: 1) ≥18 years with either type 1 or type 2 diabetes mellitus; 2) central subfield foveal thickness (CFT) >300μm and a minimum signal strength for optical coherence tomography angiography of 7 out of 10, measured using spectral domain optical coherence tomography (SD-OCT); 3) best-corrected visual acuity (BCVA) of 20 to 80 letters, using Early Treatment of Diabetic Retinopathy Study (ETDRS) letters chart; 4) minimum period of 6 months post-vitrectomy for inclusion in group 1; 5) ability and willingness to provide written informed consent. If the inclusion criteria were fulfilled for both eyes, bilateral inclusion was allowed. Exclusion criteria were as follows: 1) epiretinal membrane (ERM) in the study eye; 2) persistent posterior hyaloid adherence after vitrectomy for group 1; 3) history of other retinal vascular diseases in the study eye; 4) scatter and macular LASER photocoagulation, IV anti-VEGF, systemic anti-VEGF, pro-anti-VEGF treatment or cataract surgery in the 6 months prior to study inclusion; 5) IV or peribulbar corticosteroid injections in the 6 months prior to study inclusion; 6) previous IV implant of fluocinolone acetonide in the study eye; 7) vitreous hemorrhage or opacification in the study eye; 8) active proliferative diabetic retinopathy (PDR) in the study eye; 9) active ocular inflammation or infection in either eye; 10) aphakia in the study eye; 11) other causes for macular edema, eg, after cataract surgery in the study eye; 12) other causes of visual loss in the study eye; 13) pro-edematous medication (such as systemic glitazones or topical prostaglandins) or other pathologies that might influence the course of macular edema in the study eye; 14) uncontrolled glaucoma in either eye (intraocular pressure >24mmHg with treatment); 15) pathologic myopia (spherical equivalent of ≥–8 diopters, or axial length of ≥25mm[19]); 16) history of stroke in the previous 6 months; 17) uncontrolled arterial hypertension (systolic blood pressure >160mmHg or diastolic blood pressure >100mmHg).

Study Protocol

Patients were followed according to the standard of care, with visits every 4 weeks. All patients were treated with RBZ IV injections (0.5mg/0.05mL) following a PRN regimen. No adjunct therapies such as LASER were admitted during the 24 weeks of follow-up.

Treatment Schedule

Injections were repeated every 4 weeks if eye "improved" or "worsened" (defined as ≥5 letter change from the last injection, or ≥10% CFT increase on OCT from the last

injection), or if CFT>300μm at any time point. Injections were deferred if either BCVA of 85 letters and OCT CFT was "normal" (CFT≤300μm and non-existent intra- or subretinal fluid); or OCT CFT was "normal" (CFT≤300μm) and stable BCVA (defined as <5 letters change from the last injection) after two consecutive injections during the first 24 weeks.

Patient Assessment

At baseline, demographic and clinical data, including serum levels of hemoglobin and glycated hemoglobin (HbA1C), microproteinuria, body mass index (BMI), and systolic and diastolic blood pressure (BP), were recorded. Each patient underwent a complete ophthalmological evaluation (ocular symptoms, BCVA, intraocular pressure measurement, anterior and posterior segment biomicroscopy), OCT and OCT-angiography (OCT-A) exams at baseline and every 4 weeks until the end of follow-up. BMI and BP were assessed at every visit. Fluorescein angiography was performed on all patients at baseline and at the end of follow-up. A final visit was conducted at 24 weeks of follow-up, which was the minimum follow-up period required for each patient.

OCT Acquisition and Imaging Protocol

At every visit, two highly trained technicians conducted a fovea-centered 20ºx20º SD-OCT scan (Spectralis® OCT, version 1.10.2.0, Heidelberg Engineering, Heidelberg, Germany) to automatically assess central foveal thickness (CFT) in the central 1-millimeter circle, using the proprietary Heidelberg Eye Explorer® software. Choroidal imaging was performed using structural OCT and OCT-angiography (OCT-A). CVI was calculated in a single 3 mm-cropped image exported from a structural high-resolution foveal-centered B-scan. In ImageJ®, the choroid limits were manually drawn by two experienced examiners (DJ, SP) that were masked to patient clinical data at the time of acquisition. Segmentations were repeated by a senior specialist for all cases where differences between the two masked observers were equal or superior to 20%. The senior specialist used the same method described above and was also masked to previous measurements. The outer border of the hyperreflective line representing the retinal pigment epithelium was considered the inner choroidal limit. The outer choroidal limit was defined as the inner border of the hyperreflective sclera. The presence of a visible suprachoroidal space (SCS), defined as a continuous homogenous hyporeflective layer between the choroid and the sclera, was recorded and, in such cases, SCS was not considered part of the choroid. As previously described by other groups,[39] the image was binarized using the Niblack method, and the total choroidal area (CA), hyperreflective stromal area, and hyporeflective vascular luminal areas (LA) were automatically calculated. CVI was calculated as LA/CA. CA was converted to average choroidal thickness (CT). Choriocapillaris flow density (CCD) was calculated from a 10º×10º OCT-A C-scan, segmented automatically in the choriocapillaris layer using the OCT proprietary software. The image was binarized in ImageJ® using Phansalkar´s method[40] and CCD was defined as the ratio between vascular area and total area. Auto-rescan with active eye tracking was applied for OCT acquisitions. The minimum signal strength of OCT-A images was 7 of 10. The signal strength index was registered and compared between visits, given that it may directly influence the outcomes. Automatic segmentations were confirmed by the investigators (BP, JH). OCT was performed at the same time of the day, between 8 a.m. and 10 a.m, to avoid circadian fluctuations.[18]

Outcome Measures

Primary Outcome

To analyze choroidal vascularity changes, through CVI, CCD, and CT, 6 months after PRN treatment with RBZ.

Secondary Outcomes

Secondary endpoints were as follows: 1) BCVA and CFT 6 months after treatment; 2) prognostic value of baseline choroidal measurements for functional (BCVA) and anatomical (CFT) response at 12 weeks (early response) or 24 weeks of follow-up; 3) correlation between choroidal vascularity parameters at baseline and after 6 months; 4) effect of baseline and demographic characteristics in choroidal vascularity parameters at baseline and after treatment; 5) BP and BMI association with choroidal vascularity parameters, at baseline and at the end of follow-up.

Functional and Anatomical Response Criteria

Functional and anatomical improvements were defined as a gain of ≥5 ETDRS and a CFT decrease of ≥50μm, respectively. DME resolution was defined as a CFT ≤300μm.

Statistical Analysis

Data were analyzed using non-parametric statistics. Between-group analyses of continuous variables were performed using the Mann–Whitney *U*-test. Within-group analyses were performed using the Wilcoxon test. Nominal variables were analyzed using the χ^2 test. Correlations between variables were tested using the Spearman rank correlation or Kendall's τ-b, as appropriate. A $p<0.05$ (two-sided) was considered statistically significant.

Results

Fifty consecutive eyes with DME were considered for treatment with RBZ IV injections, of which 34 eyes (10 vitrectomized and 24 non-vitrectomized) from 31 patients fulfilled the inclusion criteria and completed the 24-week follow-up period.

Demographic and Clinical Baseline Data

Comparison between groups regarding baseline demographic and clinical characteristics are summarized in Table 1. The only difference observed between groups was DM duration, which was longer in group 1 (p=0.020). Causes for vitrectomy were PDR in seven cases and ERM in one case. In these eight cases the internal limiting membrane was peeled after membrane blue staining. One vitreous hemorrhage with no PDR and one macula on retinal detachment were registered.

Associations with Choroidal Vascularity at Baseline

Regarding the choroidal vascularity parameters, CVI, CCD, and CT, there were no baseline differences between groups (p>0.05). There was no association between baseline CVI, CCD, and CT and previous treatment with intravitreal injections, macular laser, or pan-retinal photocoagulation (p>0.05). At baseline, age and BP were not associated with baseline CT, CVI, or CCD.

Number of Injections in the 24-Week Period

The mean number of injections during the study period was 5.18±1.17 (minimum 2 and maximum 6), with no significant differences between groups (5.70±0.48 in group 1 and 4.96±1.30 in group 2, p=0.080). Treatment burden was moderately correlated with years on insulin

Table 1 Baseline Characteristics

Parameter	Group 1 (n=10)	Group 2 (n=24)	p-value
Age, mean [95% CI]	66.5 [60.2–72.8]	68.4 [65.1–71.7]	0.467
Gender female, n (%)	5 (50.0)	14 (58.3)	0.718
DM duration (years), mean [95% CI]	22.1 [16.5–27.7]	15.7 [12.4–19.0]	**0.020**
DME duration (months), mean [95% CI]	34.0 [7.8–60.2]	23.0 [13.7–32.4]	0.619
Type 2 DM, n (%)	10 (100.0)	24 (100.0)	1.000
Macular LASER, n (%)	8 (80.0)	15 (62.5)	0.437
PRP LASER, n (%)	8 (80.0)	13 (54.2)	0.251
Naive IV, n (%)	2 (20.0)	6 (25.0)	1.000
HbA1c, mean [95% CI]	7.6 [6.8–8.3]	7.3 [6.9–7.7]	0.589
Hemoglobin, mean [95% CI]	13.1 [11.8–14.4]	13.1 [12.3–13.9]	0.985
Microproteinuria, mean [95% CI]	112.7 [19.8–245.3]	181.1 [51.7–310.6]	0.908
BMI, mean [95% CI]	28.0 [25.1–30.9]	27.8 [26.3–29.3]	0.724
SBP, mean [95% CI]	138.5 [127.1–149.9]	138.4 [133.1–143.7]	0.832
DBP, mean [95% CI]	74.3 [65.6–83.0]	77.1 [73.6–80.6]	0.406
BCVA (letters), mean [95% CI]	63.3 [52.5–74.7]	72.3 [52.5–74.7]	0.196
CFT (μm), mean [95% CI]	400.5 [343.1–457.9]	433.7 [390.7–476.7]	0.381
CVI, mean [95% CI]	0.633 [0.603–0.662]	0.641[0.611–0.671]	0.324
CCD (%), mean [95% CI]	0.392 [0.337–0.448]	0.373 [0.319–0.428]	0.764
CT, mean [95% CI]	233.7 [172.1–295.3]	256.1 [232.1–280.1]	0.349

Note: Significant P-value in bold.

Abbreviations: Group 1, vitrectomized eyes; Group 2, non-vitrectomized eyes; 95% CI, 95% confidence interval; DM, diabetes mellitus; DME, diabetic macular edema; macular LASER, prior focal-grid photocoagulation treatment in the study eye; PRP LASER, Prior panretinal photocoagulation treatment in the study eye; IV, intravitreal; naïve IV, no prior anti-VEGF intravitreal treatment in study eye; CCT, corticosteroid; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CVI, choroidal vascularity index; CCD, choriocapillaris flow density, CT, choroidal thickness.

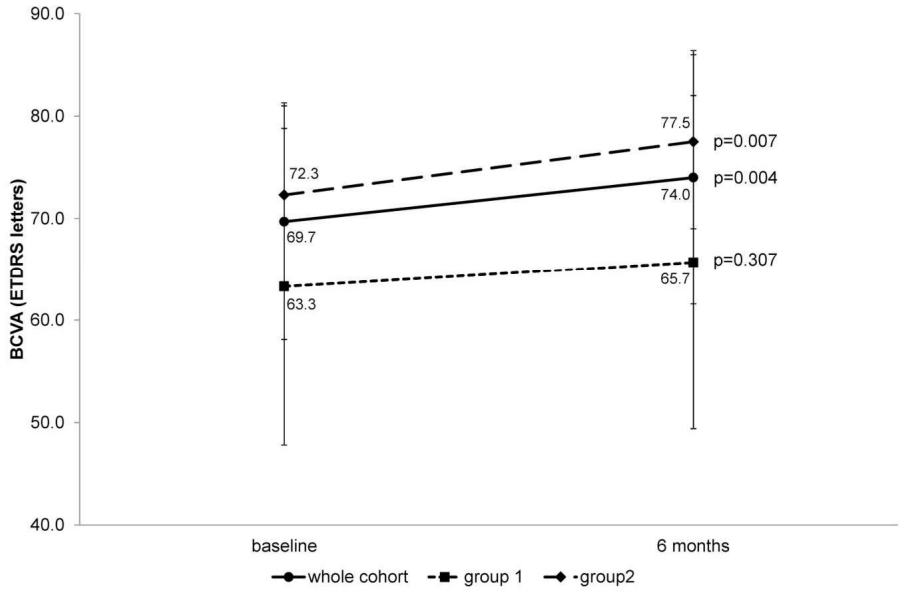

Figure 1 Change in BCVA from baseline to 6 months of follow-up.

therapy (0.462, p=0.023) and DME duration (0.484, p=0.017).

BCVA and CFT Changes Over Time

There was an improvement in BCVA both in the whole cohort and in group 2 (+4.3 ETDRS letters, p=0.004 and +5.2 ETDRS letters, p=0.007, respectively), with no differences in group 1 (Figure 1). A significant decrease in CFT was observed in the whole cohort and in both groups (Figure 2).

Choroidal Vascularity Changes Over Time

Globally, the choroidal vascularity parameters CVI, CCD and CT, did not change with ranibizumab treatment (p>0.05). Considering only group 2, there was a significant decrease in CT (p=0.033) and a significant increase in CCD (p=0.010) 6 months after treatment, with no differences in CVI (Table 2).

Correlations with Choroidal Vascularity at Month 6

At month 6, CVI was moderately negatively correlated with body mass index (BMI) –r=0.660, p=0.001. The duration of treatment with insulin and previous treatments (macular LASER, PRP LASER, or anti-VEGF IV treatments) were not associated with choroidal vascularity parameters at week 24. Blood pressure did not show any influence in choroidal variables at week 24.

Prognostic Value of Baseline Choroidal Vascularity for Anatomical or Functional Response

Despite no correlation with baseline BCVA, baseline CVI was positively correlated with final BCVA globally (r=0.406, p=0.029) and in group 2 (r=0.604, p=0.004). No other baseline choroidal parameters were correlated with final BCVA or final CFT. At 6 months, the same correlation was maintained between CVI and BCVA (r=0.450, p=0.041).

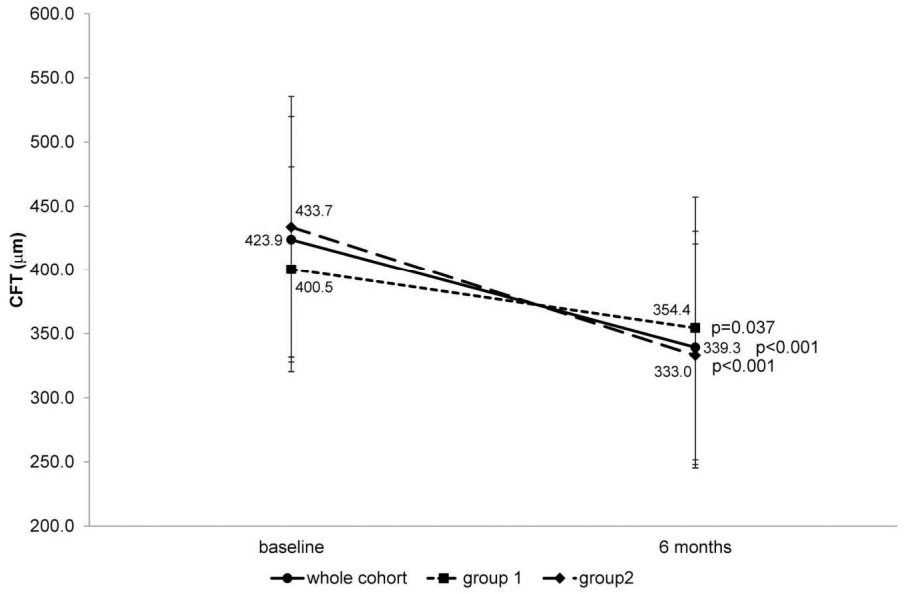

Figure 2 Change in CFT from baseline to 6 months of follow-up.

Discussion

There is growing evidence that choroidal vascularization, blood flow, and thickness are affected in diabetes and also influenced by intravitreal corticosteroid (CCT) and anti-VEGF treatments. Nonetheless, their exact influence is not completely clarified.[12–14] There are contradictory results regarding choroidal thickness in eyes with diabetic retinopathy, with or without DME, with different reports suggesting choroidal thickening, thinning, or no change.[4,15–17] The retrospective and cross-sectional nature of most previous studies, the influence of different factors such as age,[18] refractive error,[19] different DR severity/DME/PRP status,[16,17] as well as the possible effect of previous anti-VEGF therapy,[17] circadian cycle or even

Table 2 Changes in CVI, CCD, and CT, Globally and by Group

	Group 1 [n=10]	Group 2 (n=24)	Total (n=34)	
CT, µm	233.7 ± 73.7 272.6 ± 87.1 +38.9, p=0.109	256.1 ± 52.7 245.5 ± 55.9 −10.6, **p=0.042**	250.0 ± 58.7 254.2 ± 67.3 +4.2, p=0.508	Baseline 6-months Change, *p*-value
CVI, %	63.3 ± 3.5 63.5 ± 6.1 +0.2, p=0.945	64.1 ± 6.5 66.2 ± 5.6 +2.1, p=0.111	63.9 ± 5.8 65.3 ± 5.8 +1.4, p=0.169	Baseline 6-months Change, *p*-value
CCD, %	39.3 ± 7.2 39.3 ± 11.3 0.0, p=0.109	37.3 ± 11.6 41.2 ± 10.9 **+3.9, p=0.042**	37.9 ± 10.4 40.6 ± 10.9 +3.7, p=0.100	Baseline 6-months Change, *p*-value

Notes: Significant P-values in bold. Values are shown as mean±standard deviation. P-values from Wilcoxon tests.
Abbreviations: group 1, vitrectomized eyes; group 2, non-vitrectomized eyes; BCVA, best-corrected visual acuity; CFT, central foveal thickness; CT, choroidal thickness; CVI, choroidal vascularity index; CCD, choriocapillaris flow density.

other systemic vascular factors such as blood pressure[20] may be among the reasons for these disparities.

Our study aimed to clarify the effect of intravitreal RBZ therapy in a PRN regimen for DME in choroidal vascular tissue, comparing vitrectomized with non-vitrectomized eyes, during 24 weeks of follow-up, an acceptable period to reach anatomical and functional stability or to establish the type of response to anti-VEGF.[21]

Regarding baseline demographics, the only difference between groups was DM duration. This was to be expected since vitreopathy with vitreoretinal interface pathological changes, and a more severe and refractory DR, are consequences of the cumulative effect of hyperglycemia, which increases the probability of the need for vitrectomy.[22] There are evidences in the literature that in cases with ERM and indication for vitrectomy, CT and CVI are increased, with gradual reduction after surgery.[23,24] In one study evaluating and comparing CVI with a control group, the decrease occurred until the thickness matched that of the normal fellow eye. The secondary inflammation resulting from mechanical traction was the explanation for the increased choroidal thickness by way of increased vascularization of the choroid.[24] In line with this last study, our observation that baseline choroidal parameters were not significantly different between groups may reflect the fact that vitrectomy (with ILM peeling, performed in the majority of patients) by itself does not induce detectable choroidal changes in diabetic eyes.

In order to decrease the bias due to other important factors that could influence choroidal flow or thickness, all measurements were performed at the same time of the day, high myopic eyes were excluded, and the remaining baseline characteristics were included in the analysis. Age, BMI, BCVA, CFT, and previous treatments, such as LASER and anti-VEGF treatments, were not different between groups. The absence of correlation of any baseline characteristics with CT, CVI or CCD, reinforces the uniformity among patients included in our study.

The main clinical consequence of anti-VEGF, the first-line therapy for DME,[25] is to induce DME regression that leads to the expected functional improvement. This was observed in our study for both groups, although with a statistical significance only for group 2, particularly regarding BCVA. We may speculate that the lower functional outcome in group 1 may be due to a faster clearance of drugs in vitrectomized eyes, leading to a final higher CFT, which may have an impact on the final BCVA,[26] on the ILM peeled feature, in 80% of these eyes, or on the longer DM duration. These last two factors are correlated with retina neurodegeneration, manifested by lower inner retina layers' thickness, which may negatively influence the functional outcome.[27–29] The need for vitrectomy in a context of PDR (present in 80% of the eyes) may also be a sign of a more severe DR, in both neuronal and vascular degenerative components related to DR. Our results are also in line with the evidence that RBZ is an effective treatment for DME in vitrectomized eyes, although the functional and anatomical efficacy seems to be achieved slower, with the need for a higher number of injections at least during the first 12 months of treatment.[30] Nevertheless, it has to be taken into account that our population is a cohort of diabetic patients that are well known for their clinical heterogeneity.

Analyzing the choroidal vascularity parameters, we found a decrease in CT and a significant increase in CCD only in group 2, even though choroidal parameters at baseline were similar in both groups. According to the literature, anti-VEGF treatment reduces choroidal thickness, probably due to the reduction of the choroidal vasculature permeability.[14,15] On the other hand, we hypothesize that the reduced permeability of the large choroidal vessels reduces the interstitial fluid pressure and may favor blood flow in the choriocapillaris, as confirmed by the increase in CCD. Although there is robust evidence towards Diabetic Retinopathy Severity Scale (DRSS) score improvement and neovascularization regression in PDR cases, objective reperfusion in ischemic areas with anti-VEGF therapy, based on fluorescein angiography and OCT-A, yields more conflicting results.[31] Sub-analyses of the Phase 3 RISE and RIDE trials concluded that monthly injections of RBZ can slow, but not completely prevent, retinal capillary closure in patients with DME.[10] In other recent studies, choriocapillaris and retinal capillary perfusion density remained unchanged after 3 or 12 months of treatment with anti-VEGF.[32–34] Pongsachareonnont et al observed that all anti-VEGF medications reduce not only the number of microaneurysms but also the foveal avascular zone (FAZ) in the superficial and deep capillary plexus after one injection, with a positive correlation with visual improvement.[35]

Our results suggest that non-vitrectomized eyes may have a different response to anti-VEGF therapy in choroidal vascular anatomical readjustment, through the reduction of choroidal vasculature permeability, in addition to the renewal of the blood-retinal barriers.[25] In the existing literature regarding choroidal thickness and retinal

vascular density, vitrectomy is not an exclusion or grouping criteria. If that distinction had been done, then different conclusions could have emerged regarding macular perfusion across a great number of studies.

The absence of similar results for vitrectomized eyes, although it can be influenced by the small number of eyes included, may be due to 1) the less expressive effect of anti-VEGF in reducing not only retinal but also choroidal vasculature permeability, which is in line with a less dry macula observed in vitrectomized eyes and 2) the knowledge, already discussed above, that functional and anatomical efficacy also occur in vitrectomized eyes, although more slowly and depending on a high treatment burden, at least during the first year.[30] A longer follow-up would help to corroborate these hypotheses. Otherwise, the potential higher neurovascular disease severity or intense PRP, usually added intraoperatively in the vitrectomized eyes included in this study, may also be possible explanations.

A novelty highlighted by our analyses was the observation that baseline CVI seems to play a role as a potential functional predictor of anti-VEGF response in DME, overall and in non-vitrectomized eyes.

In our study, and contradicting the results obtained by Rayess et al[36] after three monthly injections of anti-VEGF, CT and CCD did not show an additional prognostic value regarding anatomical or functional response after 12 and 24 weeks of treatment. A possible explanation for otherwise early outcomes by these authors may lay on their different inclusion criteria: only naïve eyes, without previous treatments related to diabetic retinopathy before the first IV anti-VEGF injection. An expected higher baseline sub-foveal choroidal thickness in those eyes may represent a subgroup with more preserved choriocapillaris and thus greater potential for significant improvements following anti-VEGF therapy. In our cohort, the majority of patients were not naïve. Particularly, PRP LASER therapy is associated with a significant reduction of the subfoveal choroidal thickness and subfoveal choroidal blood flow.[17] A similar effect of anti-VEGF therapy on CT has already been reported, although the data is scarce in the long term, such as a treatment pause of 6 months, considered for inclusion in our study.[12–14]

Another expected result from our findings was the correlation between the number of injections needed to control DME with DME duration and insulin therapy, anticipating a more severe vascular damage and a higher treatment burden to stabilize the diabetic neurovascular disease.

The main limitation of this study is the reduced sample size, particularly in group 1. The small number of vitrectomized eyes included in this study is a reflection of the relatively infrequent post-vitrectomy DME occurrence.[37,38] The prospective evaluation of our cohort, the relatively uniform demographic characteristics (besides the expected differences associated with the need for vitrectomy), and our attempt to control for potential confounders are the strong points of our methodology.

Although we chose 24 weeks of follow-up as an acceptable and robust period to reach anatomical and functional stability, a longer prospective observation period might be needed to confirm our results. In addition, the known artifacts associated with OCT-A images and their analysis might lead to misinterpretations.

Conclusions

In conclusion, and in our population sample, CCD increased and CT decreased in non-vitrectomized eyes after 6 months of PRN treatment with IV RBZ. Moreover, CVI demonstrated to be a potential predictor for functional response to RZB therapy in DME. Choroid vascular status may help to understand different functional prognosis observed with anti-VEGF treatment, that classical retinal biomarkers cannot otherwise explain.

Future studies with larger samples should be carried out to further investigate and confirm our results with regards to the role of vitreous status in response to choroidal vascular tissue to anti-VEGF therapy.

Abbreviations

BCVA, best-corrected visual acuity; BMI, body mass index; BP, blood pressure; CA, choroidal area; CCD, choriocapillaris flow density; CFT, central subfield foveal thickness; CT, choroidal thickness; CVI choroidal vascular index; DM, diabetes mellitus; DME, diabetic macular edema; DR, diabetic retinopathy; DRSS, diabetic retinopathy severity scale; ERM, epiretinal membrane; ETDRS, early treatment of diabetic retinopathy study; FAZ, foveal avascular zone; HbA1C, glycated hemoglobin; ILM, internal limiting membrane; IV, intravitreal injection; LA, luminal areas; OCT, optical coherence tomography; PDR, proliferative diabetic retinopathy; PRN, pro re nata; PRP, panretinal photocoagulation; RBZ, ranibizumab; SCS, suprachoroidal space; SD-OCT, spectral domain optical coherence tomography; VEGF, vascular endothelial growth factor.

Data Sharing Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Informed Consent

This study was conducted according to the tenets of the Declaration of Helsinki in its latest amendment (Brazil, 2013) and was approved by the ethics committee of CHUP [2017.093 (084-DEFI/082-CES)]. All patients signed an informed consent form. This study is registered at www.clinicaltrials.gov (NCT04387604).

Author Contributions

We declare that all authors have made a significant contribution to the work reported, whether in the conception, study design, execution, acquisition of data, or analysis and interpretation; have drafted or written, or critically reviewed the article; have agreed on the journal to which the article was submitted; reviewed and agreed on all versions of the article before submission, during revision, the final version accepted for publication, and any significant changes introduced at the proofing stage; and agree to take responsibility and be accountable for the contents of the article.

Disclosure

The authors declare no competing interests, either financial or non-financial.

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2.8 Optical coherence tomography biomarkers - Early prognostic factors in diabetic macular edema treatment with ranibizumab[693]

Manuscript Category: Original Article – Clinical Science

TITLE: Optical coherence tomography biomarkers - Early prognostic factors in diabetic macular edema treatment with ranibizumab

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Conflicts of Interest

None of the authors has any conflicts of interest regarding this work.

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ABSTRACT

Background: Diabetic macular edema (DME) is the main cause of vision loss in patients that suffer from diabetic retinopathy. In this work, we aim to evaluate the prognostic role of biomarkers in DME, detected by Optical Coherence Tomography (OCT) in patients treated with ranibizumab. **Methods:** Prospective study of 46 consecutive eyes with DME under ranibizumab intravitreal therapy and 12 months of follow-up. The primary endpoint was to assess the association between the baseline characteristics of OCT biomarkers and the type of responder (good-earlier responder or partial/non-responder). **Results:** Good responders had at baseline and at the end of follow-up a lower number of inner nuclear layer cysts (26.5% vs 73.5%, p=0.035) and at end of follow-up a lower percentage of disorganization of retinal inner layers, disruption of outer plexiform layer and outer nuclear layer cysts than partial/non responders (12.0% vs 88.0%, p=0.001; 8.7% vs 91.3%, p<0.001; 17.4% vs 82.6%, p=0.013, respectively). At month 12 an association between inner nuclear layer cysts and higher glycated haemoglobin (p=0.028) was observed. **Conclusion:** This study highlights the prognostic value of SD-OCT parameters, such as fewer INLc, which was associated with a better therapeutic response. A normalization of the macular anatomy with ranibizumab is more likely to happen in early complete responders. The association between INLc and higher glycated haemoglobin levels reinforces the relevance of systemic metabolic control in diabetic microvascular manifestations. Clinicaltrials.gov NCT04387604.

Keywords: diabetic macular edema; diabetic retinopathy; vitrectomy; ranibizumab; biomarkers

1. Introduction

It is estimated that in 2019 diabetes *mellitus* affected 463 million people worldwide. Approximately one third of these 463 million have signs of diabetic retinopathy (DR) and of these, near one third have vision-threatening DR, including diabetic macular edema (DME), the main cause of vision loss in DR. On a global scale, diabetes affects mainly 'middle aged' people, who are between 40 and 59 years old with serious economic and social implications.[1, 2]

Anti-vascular endothelial growth factors (anti-VEGF) agents are recommended as first-line therapy for treating central DME.

Approximately 50% of patients with DME lose two or more lines of visual acuity within 2 years if left untreated.[3] DME patients have a higher probability of treatment delay when compared to patients with age macular degeneration and retinal vein occlusion, due to the disruption of normal retinal microstructure induced by the edema and to the neurovascular lesion induced by chronic hyperglycemia.[4] Moreover, diabetic patients have, in general, lower treatment compliance.[5-7] This highlights the relevance of pursuing optimized strategies for DME treatment, anticipating and delivering a successful therapy as soon as possible, or considering an early therapeutical switch. Additionally, timely treatment will lead to better visual outcomes as eyes with suboptimal early best-corrected visual acuity (BCVA) response have poorer long-term visual outcomes than eyes with pronounced early response[8, 9].

For this purpose, spectral domain optical coherence tomography (SD-OCT) seems to be a powerful, non-invasive, repeatable and widely used method to obtain qualitative and quantitative information on potential DME biomarkers other than central subfoveal thickness (CST). Examples of these potential biomarkers are subretinal fluid (SRF), ellipsoid layer disruption (ELd), external limiting membrane disruption (ELMd),

hyperreflective foci (HRF), presence and size of outer nuclear layer cysts (ONLc), hard exudates (HE) and disorganization of the retinal inner layers (DRIL) which have been described in the literature and are correlated with a more severe, chronic DME and with poor retina function. Nevertheless, they are not associated with a negative anti-VEGF response. Only the presence of outer plexiform layer disruption (OPLd), epiretinal membrane (ERM) accompanied by retina wrinkling and the loss of deep capillary plexus in OCT angiography have shown to be predictive of a poor response to anti-VEGF [10-16].Vitreous status seems to influence both anti-VEGF and corticosteroid response as well as evolution of OCT biomarkers through DME therapy [10-18].

The relevance of these SD-OCT biomarkers and vitreous status in diabetic maculopathy have been reinforced by an international panel of retina experts which proposed their inclusion in an OCT-based grading of diabetic maculopathy. This is particularly important to predict response to potential highly frequent treatments with antivitreal (IV) injections anti-VEGF to provide the patients with the most effective therapy and realistic expectations regarding prognosis and therapy burden in the context of a chronic illness.

The main goal of this study was to increase the knowledge of the prognostic role of biomarkers detected by OCT in patients treated with ranibizumab for DME. The patients were followed prospectively during 12 months in a *pro re nata* regimen and divided according to the vitreous status into the vitrectomized or non-vitrectomized group.

2. Methods

Methods concerning the population sample and treatment protocol have been described previously in detail[19]. Briefly, the study was conducted at the Department of Ophthalmology from Centro Hospitalar Universitário do Porto (CHUP) in Portugal and involved 46 consecutive eyes from 38 DME patients under treatment with *pro re nata*

(PRN) ranibizumab (RBZ) IV injections (0.5mg/0.05ml), followed prospectively for 12 months. Patients were included in two groups according to the vitreous status: group 1 – non-vitrectomized eyes and group 2 – vitrectomized eyes.

The study was conducted according to the tenets of the Declaration of Helsinki in its latest amendment (Brazil, 2013) and was approved by the ethics committee of CHUP [2017.093 (084-DEFI/082-CES)]. All patients signed an informed consent form. This study is registered at www.clinicaltrials.gov (NCT04387604).

This analysis comprises demographic and clinical data before study inclusion, at baseline and at 12 months. These data includes BCVA assessment using Early Treatment of Diabetic Retinopathy Study (ETDRS) letters chart, central foveal thickness (CFT) and other SD-OCT (Heidelberg Spectralis, Heidelberg, Germany) parameters described below.

SD-OCT parameters

CFT was obtained automatically from equipment readings. The presence of 9 different morphological parameters was determined within the 1-millimeter central foveal area. These included: 1) the presence of SRF; 2) the number of hyperreflective dots (HRD); 3) DRIL; 4) OPLd; 5) ELMd; 6) the disruption of ellipsoid zone (EZ); 7) ONLc; 8) the presence of cysts in the inner nuclear layer (INL); and, 9) number of cysts in ONL versus those in the INL. The methodology for SD-OCT measurements have been previously described [17].

OCT scans were obtained by an SD-OCT (macular dense line scan mode HR 20x20° Spectralis HRA+OCT; Heidelberg Engineering, Heidelberg, Germany). All measurements were determined by two highly trained medical doctors (A.F. and J.L.) with disagreements solved by a third senior medical retinal specialist (B.P.).

Outcome measures

Primary endpoint

To assess the association between baseline characteristics of OCT biomarkers and type of responder, which were classified as 1) good-earlier responder – when after the 24th week of follow-up (maximum 7 injections) there was a complete anatomical response (central foveal thickness, CFT, ≤ 300 μm) with an increase in best corrected visual acuity, BCVA, ≥5 letters; 2) non-responder – 13 injections and final CFT >400μm or ≤ 10% of CFT reduction and BCVA gain <5 letters; 3) partial responder – between good and non-responder criteria.

Secondary endpoints

As secondary endpoints, demographic data that included age, gender, diagnosis of diabetes *mellitus* type 1 or 2, DME duration, haemoglobin A1c (HgA1c), dyslipidaemia, previous IV injections and LASER treatments were gathered. Moreover, we assessed 1) differences in OCT biomarkers between groups 1 and 2; 2) association between baseline characteristics and OCT biomarkers; 3) percentage of different type of responders; 4) percentage of patients who needed 3 injections or less.

Statistical analysis

Data were analysed using non-parametric statistics. BCVA values ETDRS letters were converted to LogM before analyses. Between-group analyses of continuous variables were performed using the Mann-Whitney U test. Within-group analyses were performed using the Wilcoxon test. Nominal variables were analysed using the chi-square test. Correlations between variables were tested using the Spearman rank correlation or the Kendall's τ-b, as appropriate. A $p<0.05$ (two-sided) was considered statistically significant.

3. Results

A total of 46 eyes (10 vitrectomized and 36 non-vitrectomized) from 38 patients completed the follow-up. There were no differences between any of the analysed demographic or clinical parameters at baseline between groups. Baseline characteristics have been detailed previously[19]. Mean age was 66.8±8.1 years.

Comparing the results for good-earlier responders vs partial/non-responders we observed that at baseline, the presence of INL cysts was lower in good-earlier responders (26.5% vs 73.5%, p=0.035; Figure 1).

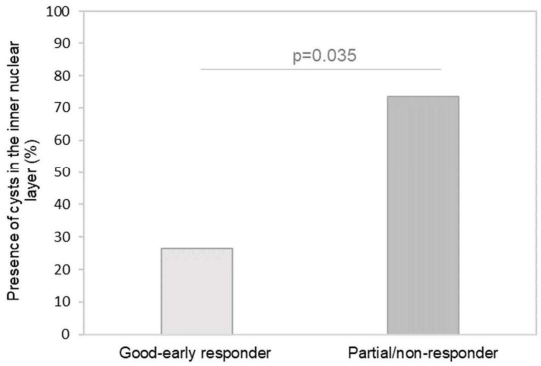

Figure 1. Percentage of inner nuclear cysts in the group of good-early responders and in the group of partial/non-responders, at baseline.

Good-early responders represented 33% of the whole treated sample and 69% of those had a baseline CFT<400µm as previously described[19]. At the end of follow-up, good responders maintained a lower percentage of INLc (20% vs 80%, p=0.027; Figure 2) and had a lower percentage of DRIL, OPLd and ONLc (12.0% vs 88.0%, p=0.001; 8.7% vs

91.3%, p<0.001; 17.4% vs 82.6%, p=0.013, respectively; Figure 2). An association between INLc and a higher glycated haemoglobin (p=0.028) was also observed at 12 months of follow-up.

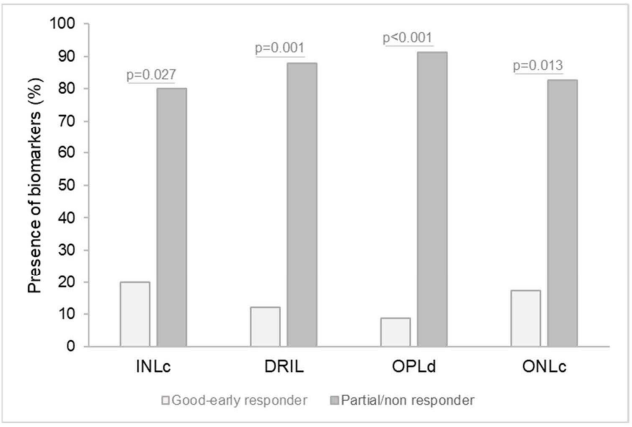

Figure 2. Percentage of different biomarkers present in the good-early responders and partial/non responders groups at the end of follow-up. DRIL: disorganization of the retinal inner layers, INL: inner nuclear layer cysts, ONLc: outer nuclear layer cysts and OPLd: outer plexiform layer disruption.

4. Discussion

The major OCT biomarker with prognostic impact for a good-early response with ranibizumab in DME was the presence of a lower number of INL cysts. We consider this decrease as a condition associated with the reestablishment of the macular anatomical configuration after 24 weeks of therapy without the need of further injections in the first year of follow-up. This result is in line with our previous observation that a lower INL

thickness seemed to be associated with the existence of fewer INL cysts, indicating a good-earlier response with less treatment burden and a more benign evolution[19]. If pathophysiologically intraretinal cystoid fluid first appears in the INL, at the level of the Deep Capillary Plexus (DCP), where other preclinical DR vascular changes also occur, we should be aware of the first signs of DR decompensation and precocious appearance of macular edema, as patients displaying these alterations may need to be given priority for IV therapy. [20, 21] Accordingly, our results showed that normalization of the macular anatomy due to the disappearance of DRIL, OPLd and ONLc upon treatment with ranibizumab was more frequent in the good-earlier complete responders. Thus, treating these patients precociously leads not only to lower therapy burden but also reversibility of OCT DR macular lesions and the avoidance of irreversible damage and additional treatments with functional compromise.

The association between the existence of INLc and higher glycated haemoglobin levels highlights the relevance of systemic metabolic control in diabetic microvascular manifestations, in DCP, that seems to be the most affected retinal vascular network in DR. Microaneurysms are preferentially located in DCP and their increased number has a positive correlation with an increased retinal volume.[22, 23] Indeed, chronic hyperglycaemia can influence all neurovascular pathological processes across the human body as it involves low-grade inflammation, immune cell activation and extracellular glutamate accumulation, phenomena responsible for neuronal degeneration of the retina and vascular lesions, through disruption of the blood–retinal barrier and impairment of several retinal cell types, as well as neural, glia, immune and vascular cells[4].

Isolated, several OCT biomarkers such as loss of integrity of EL and ELM, the existence of submacular fluid, HRD DRIL and large ONLc may negatively influence retinal function, but they do not indicate anti-VEGF therapy inefficiency.[24] Nonetheless, if the

patient presents different biomarkers simultaneously , they gain an additional prognostic value. An example of this hypothesis is the result reached by the DRCR.net protocol V in patients with DME[25].

In this study, the patients included had well-controlled diabetes (mean HgA1c of 7.6%), earlier stages of DR and mild DME (with a mean CFT at baseline of 311 μm). Hence, the results of Protocol V may not be applicable to patients with less controlled diabetes, advanced stages of DR or more severe DME (CFT >400 μm). [19, 26-28] Protocol V suggests observation without treatment of central DME with a good baseline VA (20/25 or better).[25]. Nonetheless, a recent study from Busch et al showed that there is an increased risk for VA loss if DRIL, HRF and EZ disruption are present at baseline. In those cases, earlier treatment with anti-VEGF agents may potentially decrease the risk of VA loss at 12 months [14].

Another example is the observationof Zur et al [29] that the presence of submacular fluid, absence of HRD and continuous inner/outer segment (IS-OS) line are indicators of a better results with Ozurdex® IV implant. This may be seen as a contradiction to the study by Vujosevic et al., as they observed that DME with a higher number of HRD seemed to show better morphologic and functional results if, at least initially, treated with steroids instead of anti-VEGF. Additionally, the presence of subretinal fluid (SF) also was related with better morphologic results when treated with steroids instead of anti-VEGF [30]. In more favourable DME cases, such as the ones where there is absence of HRD and continuous IS-OS, it is reasonable to expect that all current IV therapies, such as anti-VEGF and corticosteroid therapy, and even long term Fluocinolone Acetonide Implant (FAc), may be more efficient[31]. Following the same line of reasoning, chronic and more severe DME might need to be approached with combined therapy, as showed in a study from our group [17], where higher levels of CFT, a higher number of HRD and the presence

of SF where associated with chronic and more severe DME and with the need for additional treatment in DME non-responder to anti-VEGF or short term corticosteroid therapy[17, 32, 33]. The invasion of layers adjacent to the INL, can be considered a sign of progression and higher level of severity and chronicity, indicating the need for a large number of injections, or in more severe cases, non-response to isolated anti-VEGF therapy [17, 20].

In our analysis, the absence of differences between vitrectomized and non-vitrectomized eyes regarding good-earlier versus partial/non-responders may be explained by the similar baseline characteristics in both groups and by the inclusion criteria for this study, as a treatment pause of at least 6 months was mandatory (due to previously absent, stabilized, or undiagnosed DME). As opposed to other study from our group, which also compared vitrectomized and non-vitrectomized eyes treated with FAc implant, but that included eyes with recurrent or non-responsive DME, the sample of the present study is not expected to be considered mainly non-responsive to anti-VEGF therapy [17].

The results of the present study have practical implications when planning schedules for anti-VEGF IV injections, such as loading dose strategy and overall treatment decisions regarding DME approach, being a proposed treatment scheme based on OCT biomarkers. Our study provides relevant insights on how to adapt and plan DME treatment given the OCT biomarkers present. Moreover, prospective evaluation of the cohort of patients conducted is one of the strengths of this study. However, this study also has some limitations. The main limitation of this study is the reduced sample size, particularly in the vitrectomized group. The small number of vitrectomized eyes included in this study is a reflection of the relatively uncommon post-vitrectomy DME occurrence [34, 35]. The number of patients did not allow us to perform multivariable analysis therefore our conclusions might be misled by confounders. When two eyes of the same patient fulfilled

the inclusion criteria, both were used but due to the small sample no advanced statistical analysis were performed to adjust for the potential correlation between those eyes. Further studies with larger samples should be carried out to further investigate and confirm our results.

5. Conclusion

This study highlights the prognostic value of some SD-OCT parameters, such as fewer INLc, which were associated with a better therapeutic response. According to our results, normalization of the macular anatomy through the disappearance of DRIL, OPLd and ONLc due to treatment with ranibizumab is most likely to happen in early complete responders. The relevance of potential prognostic biomarkers in diabetic patients, known by their characteristic heterogeneity, is only valuable when integrated in the whole clinical context. Alone they are devoid of a clear meaning and their importance can be misvalued.

Authors' contribution:

- study design: BP, JF, AM and JMB
- data acquisition: BP, JL, AF
- data analysis: BP, CC, JL, AF
- critical interpretation of data: BP, CC, JL, JC, NC
- manuscript drafting BP and JL
- manuscript critical review: all
- final approval of manuscript: all

Ethics: The study was conducted according to the tenets of the Declaration of Helsinki in its latest amendment (Brazil, 2013) and was approved by the Ethics Committee of CHUP (2017.093 (084-DEFI/082-CES)).

Informed consent: All patients signed an informed consent form.

Conflict of interest: The authors have no conflicts of interest to declare.

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2.9 Fluocinolone Acetonide Intravitreal Implant 190 µg (ILUVIEN®) in Vitrectomized versus Nonvitrectomized Eyes for the Treatment of Chronic Diabetic Macular Edema[694]

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Fluocinolone Acetonide Intravitreal Implant 190 µg (ILUVIEN®) in Vitrectomized versus Nonvitrectomized Eyes for the Treatment of Chronic Diabetic Macular Edema

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Keywords

Diabetic macular edema · Pars plana vitrectomy · Intravitreal implant · Best corrected visual acuity · Central foveal thickness · Corticosteroid

Abstract

Purpose: To compare the functional and anatomical outcomes after a 0.2 µg/day fluocinolone acetonide (FAc) implant between vitrectomized and nonvitrectomized eyes with chronic diabetic macular edema (DME). ***Methods:*** This is a retrospective, comparative analysis of 43 eyes with chronic DME. All eyes were treated with a single 0.2 µg/day FAc implant and followed up for a mean period of 8.5 months (median, 6.0 months; range, 1–21 months). The patients with a 0.2 µg/day FAc implant were divided into 2 groups: 24 eyes which had undergone pars plana vitrectomy prior to 0.2 µg/day FAc (group 1) and 19 eyes which had not been vitrectomized (group 2). Outcome measures included mean changes in best corrected visual acuity (BCVA) in Early Treatment Diabetic Retinopathy Study letters, central subfield foveal thickness (CSFT), and intraocular pressure (IOP), and were measured prior to administration of the 0.2 µg FAc implant, in the first week, at month 1, and quarterly thereafter. ***Results:*** Following the 0.2 µg/day FAc implant, the mean change in BCVA at the last observation point, from baseline, was +16.9 ± 3.39 (mean ± SE) letters ($p \leq 0.001$) in group 1 and +8.2 ± 4.62 letters ($p = 0.092$) in group 2. From baseline, a gain of ≥15 letters was achieved in 37.5 and 36.8% of the eyes in group 1 and group 2, respectively. Additionally, an improvement in vision ≥20/40 in 29.2% of group 1 and 15.8% of group 2 was observed. The mean change in CSFT was –217.7 ± 40.8 µm and –155.6 ± 43.4 µm in group 1 and group 2, respectively. The mean change in IOP was +1.6 ± 0.7 mm Hg in group 1 and +0.8 ± 1.3 mm Hg in group 2, relative to baseline. At the last observation point, there were no significant differences between groups 1 and 2 ($p > 0.05$) in terms of their changes in BCVA, CSFT, and IOP. ***Conclusion:*** The results from the real-life practice study demonstrate that the 0.2 µg/day FAc implant is effective and well tolerated in vitrectomized and nonvitrectomized eyes of patients with chronic DME. Our results support the use of a 0.2 µg/day FAc implant to obtain long-term functional and anatomical improvements (mean, 8.5 months; median, 6.0 months) in vitrectomized and nonvitrectomized eyes.

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Introduction

Pars plana vitrectomy (PPV) has been suggested as a potential treatment option for diabetic macular edema (DME) due to the characteristics of the vitreous in diabetic patients [1]. When the vitreous gel is removed and replaced by balanced saline solution, oxygen transport to ischemic retinal areas is improved, as is clearance of vascular endothelial growth factor (VEGF) and other cytokines, thus reducing the edema and neovascularization [2]. Many studies have also addressed the advantages of PPV in DME patients with associated vitreoretinal traction, since it can also play an important role in the generation and/or maintenance of DME [3–5]. However, the chronic and multifactorial pathogenesis of DME usually requires continuous pharmacological treatments.

In vitrectomized eyes, drugs are believed to be more rapidly distributed throughout the eye than in an eye with an intact vitreous gel. There is evidence that the clearance of anti-VEGF agents and corticosteroids (i.e., triamcinolone) increases after vitrectomy, reduces the drug exposure, and impacts the treatment's success [3–9].

The continuous release of fluocinolone acetonide (FAc; 0.2 μg/day) from a sustained drug delivery system could potentially enhance the effect of vitrectomy in patients with DME. The 0.2 μg/day FAc intravitreal implant (ILUVIEN®, Alimera Sciences Inc., Alpharetta, GA, USA) is a nonbioerodible microimplantable cylindrical tube (3.5 × 0.37 mm) made from polyimide and loaded with 190 μg of FAc. It is approved for the treatment of vision impairment associated with chronic DME, considered insufficiently responsive to available therapies [10]. The implant releases 0.2 μg/day FAc for up to 36 months and offers an alternative therapeutic strategy, providing sustained delivery of the corticosteroid to maximize its anti-inflammatory, angiostatic, and antipermeability effects [11–13].

The purpose of this study is to compare the outcomes (functional, anatomical, and safety) in nonvitrectomized and vitrectomized eyes with chronic DME following the administration of the 0.2 μg/day FAc implant.

Materials and Methods

Study Design

This was a noninterventional, retrospective, comparative analysis of 43 eyes with chronic DME, defined as having at least 1 year of documented DME and having received at least 1 prior DME therapy. All subjects gave their informed consent, and the study protocol complies with the institute's committee on human research. All eyes were treated with a single FAc implant and treated per standard practices, and informed consent was obtained from all subjects prior to the injection of the 0.2 μg/day FAc implant. The treated patients were divided into 2 groups: 24 eyes which had undergone PPV prior to 0.2 μg/day FAc (group 1) and 19 eyes which had not been vitrectomized (group 2). Demographic data are summarized in Table 1.

The main outcome measure was the mean change in best corrected visual acuity (BCVA) reported as an Early Treatment Diabetic Retinopathy Study (ETDRS) letter score. Secondary outcome measures included: (a) the change in central subfield foveal thickness (CSFT) using spectral-domain optical coherence tomography (Spectralis® Tracking Laser Tomography, Heidelberg, Germany); (b) the change in BCVA between phakic and pseudophakic eyes; (c) the percentage of patients achieving ≥20/40 vision; (d) the percentage of patients with an improvement in BCVA ≥15 ETDRS letters; and (e) the changes in intraocular pressure (IOP) using the Goldmann applanation tonometry method.

Patients were examined prior to administration of the 0.2 μg FAc implant (baseline) and then during the first week and month after injection and quarterly thereafter. All changes were calculated by subtracting the baseline values from the last observed value. For all parameters, within-group comparisons were conducted using a paired Student *t* test, and an unpaired Student *t* test was used to compare between groups. For IOP, a correlation between IOP at last observation and vitreous status was performed using the Fisher Z transformation. Statistical differences were defined as a p value ≤0.05. All data are reported as means ± standard error unless stated otherwise.

Results

Following intravitreal injection of the 0.2 μg/day FAc implant there was a mean follow-up period of 8.5 ± 1.6 months (median, 6.0; range, 1–21). Twenty-four eyes were enrolled in group 1, and 19 eyes were enrolled in group 2.

Demographics, Baseline Characteristics, and Prior DME Therapies

Demographic data and baseline values for groups 1 and 2 are reported in Table 1, and prior DME therapies are reported in Table 2.

Effectiveness Outcomes

There was no difference in baseline BCVA values between groups 1 and 2 (Table 1). Following administration of the 0.2 μg/day FAc implant there was a mean increase in BCVA of +16.9 ± 3.39 letters ($p < 0.001$) in group 1 and +8.2 ± 4.62 letters ($p = 0.092$) in group 2, but the comparison between the groups revealed no statistical difference ($p = 0.130$) (Fig. 1).

Because there was a statistical difference in the pseudophakic:phakic ratio between groups, and the mean

Table 1. Demographics and baseline characteristics of the study patients and eyes

Parameters	Group 1, vitrectomized (n = 24)	Group 2, nonvitrectomized (n = 19)	p value
Pseudophakic/phakic, n	23/1 eyes	12/7 eyes	0.014
DME durations, years	2.5	3.5	0.223
Mean number of previous anti-VEGF injections	2.5±0.5	4.8±1.6	0.131
Mean number of prior steroid injections	2.7±0.3	1.5±0.3	0.008
Previous IOP-lowering medication	9 eyes	6 eyes	0.755
Mean time between vitrectomy and FAc implant injection, months	24.9±3.7	NA	NA
Mean BCVA, ETDRS letters	40.5±4.1	42.1±3.3	0.764
Mean CSFT, μm	543.9±38.2	523.6±45.7	0.733
Mean IOP, mm Hg	14.9±0.5	15.3±0.7	0.681
Mean follow-up, months	7.6±1.4	9.3±1.81	0.450

For p values, between-group comparisons were performed using an unpaired Student t test. NA, not applicable.

Table 2. Prior treatments in both the vitrectomized and nonvitrectomized groups

Prior treatments	Eyes with prior treatments, n (%)		p value
	group 1, vitrectomized (n = 24)	group 2, nonvitrectomized (n = 19)	
Focal laser	5 (20.8)	6 (31.6)	0.495
Panretinal photocoagulation	24 (100)	15 (78.9)	0.031
Intravitreal bevacizumab injection	18 (75)	14 (73.6)	1.000
Intravitreal ranibizumab injection	1 (4.2)	4 (21.1)	0.153
Intravitreal aflibercept injection	5 (20.8)	5 (26.3)	0.728
Intravitreal triamcinolone acetonide injection	23 (95.8)	13 (68.4)	0.033
Intravitreal dexamethasone implant injection	5 (20.8)	6 (31.6)	0.495

For p values, between-group comparisons were performed using an unpaired Student t test.

Table 3. Visual acuity changes at the last observation in vitrectomized and nonvitrectomized eyes

Change in vision	Group 1, vitrectomized (n = 24)	Group 2, nonvitrectomized (n = 19)	p value
Mean change in vision, letters	16.9 (3.94)	8.2 (4.62)	0.130
Stable/improved	23 (95.8)	15 (78.9)	0.153
≥15-letter gain	9 (37.5)	7 (36.8)	1.000
≥10-letter gain	17 (70.8)	9 (47.4)	0.209
≥5-letter gain	19 (79.2)	11 (57.9)	0.185
20/40 vision or better	7 (29.2)	3 (15.8)	0.470
>0-letter loss	1 (4.2)	5 (26.3)	0.189
>15-letter loss	0 (0.0)	2 (10.5)	0.470

Mean change in vision expressed with standard error in parentheses, all other results as numbers with percentages in parentheses. Stable/improved defined by any gain or any loss less than 5 letters from baseline. For p values, between-group comparisons were performed using an unpaired Student t test.

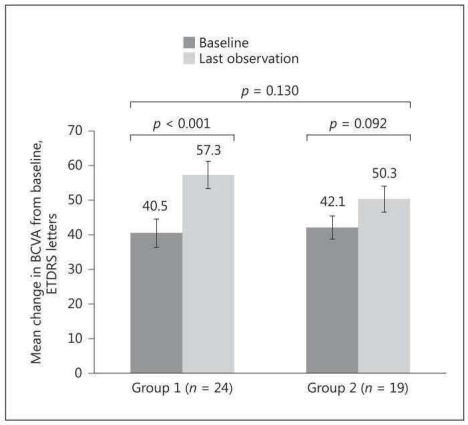

Fig. 1. Mean changes in BCVA in vitrectomized (group 1) and nonvitrectomized eyes (group 2). Between-group comparisons were performed using an unpaired Student *t* test.

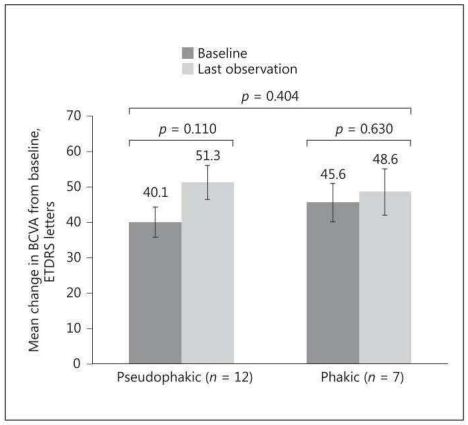

Fig. 2. Mean changes in BCVA in nonvitrectomized eyes (group 2) based on lens status. Between-group comparisons were performed using an unpaired Student *t* test.

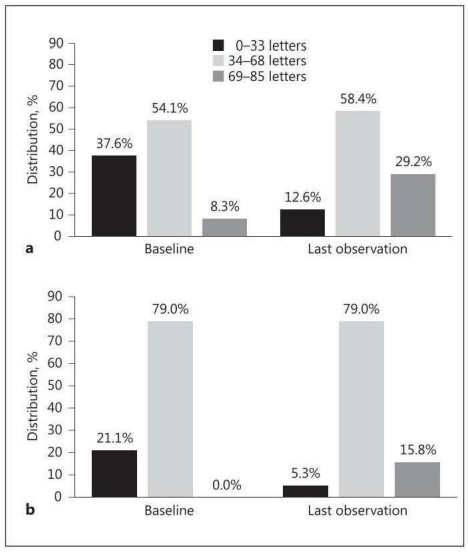

Fig. 3. Mean distribution of BCVA in vitrectomized (**a**, group 1, $n = 24$) and nonvitrectomized (**b**, group 2, $n = 19$) eyes.

change in BCVA in group 2 was found not to be statistically significant, a subanalysis carried out on group 2 (nonvitrectomized) showed that lens status had no effect on the mean change in BCVA ($p = 0.404$) (Fig. 2).

A gain of ≥15 letters, from baseline to the last observation, was achieved in 37.5 and 36.8% in group 1 and in group 2, respectively (Table 3).

BCVA at baseline and at the last observation point showed the majority of patients having a BCVA between 34 and 68 letters in both groups. After the 0.2 μg/day FAc implant there was a distribution change, with more patients achieving gains in vision that placed them in the good vision group (i.e., a BCVA between 69 and 85 letters or achieving ≥20/40 vision). Indeed, in vitrectomized eyes there was an increase from 8.3% at baseline to 29.2% at the last observation, and in the nonvitrectomized eyes there was an increase from 0 to 15.8% (Table 3; Fig. 3a, b).

After administering the 0.2 μg/day FAc implant, the mean change in CSFT, from baseline, was: group 1, –217.7 ± 40.8 μm ($p < 0.001$); group 2, –155.6 ± 43.4 μm ($p = 0.002$) (Fig. 4). No statistical difference was found between the groups ($p = 0.306$).

Safety Outcomes

IOP distribution and IOP-lowering medication at baseline and last observation are represented in Table 4.

Table 4. IOP and IOP-lowering medication at the last observation in vitrectomized (group 1) and nonvitrectomized (group 2) eyes

IOP parameter	Group 1, vitrectomized (n = 24)	Group 2, nonvitrectomized (n = 19)	p value
Baseline, %			
IOP <21 mm Hg	100	100	
IOP >21 mm Hg	0.00	0.00	
Not receiving IOP-lowering medication	62.50	68.40	0.755
Receiving IOP-lowering medication	37.50	31.60	
Last observation, %			
IOP <21 mm Hg	95.80	89.50	0.575
IOP >21 mm Hg	4.20	10.50	
IOP change >10 mm Hg	0.00	5.30	
Not receiving IOP-lowering medication	70.80	47.70	0.209
Receiving IOP-lowering medication	29.20	52.60	

Table 5. IOP changes over time in vitrectomized (group 1) and nonvitrectomized (group 2) eyes

	Baseline	2–4 weeks	3 months	6 months	9 months	12 months	15 months	18 months	21 months	Last observation
Group 1										
Mean IOP, mm Hg	14.9 (24)	16.9 (24)	16.5 (18)	14.7 (13)	16.1 (9)	16.1 (8)	16.0 (7)	15.5 (2)	16.0 (1)	16.5 (24)
Mean IOP change from baseline, mm Hg	0.0	2.0	1.4	–0.1	1.1	1.1	1.4	1.0	3.0	1.6
Group 2										
Mean IOP, mm Hg	15.3 (19)	17.1 (18)	18.2 (13)	17.5 (11)	19.6 (8)	21.9 (9)	19.4 (8)	16.4 (5)	15.0 (2)	16.1 (19)
Mean IOP change from baseline, mm Hg	0.0	1.7	1.8	1.1	2.6	5.0	1.9	–0.8	–3.0	0.8
p value (group 2 vs. group 1)	not reported	0.845	0.828	0.483	0.53	0.104	0.868	0.694	0.614	0.544

Figures in parentheses are numbers of eyes.

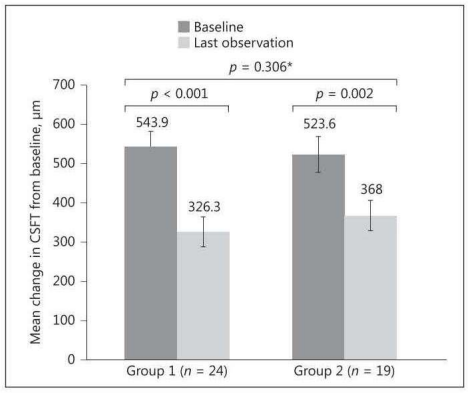

Fig. 4. Mean changes in CSFT in vitrectomized (group 1) and nonvitrectomized (group 2) eyes. Between-group comparisons were performed using an unpaired Student t test.

The mean change, at the last observation, in IOP was +1.6 ± 0.7 mm Hg (p = 0.023) and +0.8 ± 1.3 mm Hg (p = 0.547) from baseline values of 14.9 ± 0.52 and 15.3 ± 0.67 mm Hg (Table 5) in group 1 and group 2, respectively. There were no statistically significant differences in the mean changes of IOP between the groups at the last observation point (p = 0.544) or any time point up to 21 months (Table 5). No significant correlation between vitrectomy status and IOP was found (p = 0.431).

Prior to ILUVIEN, there were 6 (31.6%) nonvitrectomized and 9 (37.5%) vitrectomized eyes being treated with IOP medication. Over the subsequent 24 months, there were 7 (36.8%) nonvitrectomized and 4 (16.75%) vitrectomized eyes that required medication to manage new cases of raised IOP. Moreover, only 1 patient in the nonvitrectomized group presented an IOP >30 mm Hg at month 3. This patient had been treated with 4 different IOP-lowering eyedrops previously to FAc implantation. The IOP was successfully managed by cyclophotocoagulation guided with transillumination.

In total there were 8 patients with a phakic lens – 1 in the vitrectomized group and 7 in the nonvitrectomized group. Cataract extractions were conducted in 4 eyes – 1 phakic, vitrectomized eye (phacoemulsification performed at month 6) and 3 phakic, nonvitrectomized eyes (phacoemulsification performed at month 6 in 2 eyes and at month 3 in 1 eye) after administration without worsening or recurrence of DME. No other treatment-related adverse events were reported.

Additional DME Therapies

Additional therapies were carried out after the FAc implant had been administered – in group 1, 2 eyes (8.3%) were administered ranibizumab or laser, and in group 2, 5 eyes (26.3%) received ranibizumab or aflibercept.

Discussion

This real-life study compared the efficacy and safety outcomes of a 0.2 μg/day FAc implant in vitrectomized (group 1) and nonvitrectomized (group 2) eyes with chronic DME. Our findings show that despite the vitreous status, patients receiving a single 0.2 μg/day FAc implant had continuous and sustained exposure to treatment and had both functional and anatomical improvements over the study period. BCVA improved by +16.9 and +8.2 letters, whereas CSFT was reduced by –217.7 and –155.6 μm in group 1 and group 2, respectively. Although improvements in BCVA and CSFT seem to have been greater in group 1 there were no statistically significant differences between groups during the follow-up period. Additionally, an improvement in vision ≥20/40 of 29.2 and 15.8% was obtained in the vitrectomized group and nonvitrectomized group, respectively. To our knowledge, this is the first real-life study that shows that the 0.2 μg/day FAc implant (a) stabilizes/improves visual acuity in over 95% of vitrectomized eyes studied, (b) provided functional gains in visual acuity (i.e., ≥20/40) in previously vitrectomized eyes, and (c) reveals outcomes in vitrectomized eyes similar to those reported in the FAME studies where previously vitrectomized eyes were excluded [11–13].

The current results confirm the current knowledge concerning the use of a single 0.2 μg/day FAc implant in vitrectomized eyes. Meireles et al. [14] reported the results from 26 eyes that had undergone prior vitrectomy and had a mean follow-up period of 8.5 months (median, 6.0 months). Results showed improvements in visual acuity (+11.7 letters from baseline; range, –19 to +40; $p < 0.0004$) and CSFT (–233.5 μm from baseline; range, –678 to 274 μm), but, unlike the current study, there was no comparison versus nonvitrectomized eyes [10]. This was, however, performed by El-Ghrably et al. [15]. Subgroup analysis of 12 vitrectomized and 10 nonvitrectomized eyes achieving a 12-month follow-up showed the effectiveness of a single 0.2 μg/day FAc implant in both groups [15]. The current study provides new insights into vitrectomized eyes by assessing for 2 reasons – the first is by assessing the treatment history prior to the 0.2 μg/day FAc implant in vitrectomized and nonvitrectomized eyes (Tables 1, 2) as well as determining functional improvements in visual acuity (i.e., the stabilization/improvement in vision as well as the achievement of driving vision).

In this study all patients receiving the FAc implant had had an insufficient response to prior DME therapies, and the analysis of groups 1 and 2 revealed some subtle differences indicative of disease state and its progression. Indeed, statistical comparisons showed more eyes in the vitrectomized group had received prior panretinal photocoagulation (100% [vitrectomized] vs. 78.9% [nonvitrectomized]; $p = 0.031$) which may suggest a worsening in the overall diabetic retinopathy status.

Moreover, the increased percentage of eyes receiving intravitreal injections of triamcinolone acetonide, the most used steroid ($p = 0.033$; 95.8 vs. 68.4%, respectively) in the vitrectomized group may also be indicative of disease progression to a more proinflammatory state. From these findings we hypothesize that patients with previous vitrectomy to treat DME may already be at a chronic stage of the disease [16] and, therefore, should be treated earlier with an FAc implant complying with the approved indication or once an insufficient response to triamcinolone or dexamethasone has been established.

The advantages of PPV seem clear in DME patients with associated vitreoretinal traction, but the procedure remains controversial in nontractional cases, although several theories in favor of vitrectomy have been proposed: the removal of pathological vitreous and subclinical traction at the macula; the elimination of inflammatory mediators that could increase vessel permeability; the improvement in oxygen concentration in the vitreous cavity; and retinal vessel changes with normalization of the macular blood flow and decreased leakage [7].

Nevertheless, it is discussed that PPV could lead to more rapid drug diffusion and clearance from the vitreous cavity in vitrectomized eyes, therefore limiting the exposure of intravitreal therapies and reducing the treatment success [7] of therapies such as anti-VEGFs [8, 17–19] and maybe even dexamethasone implants [20–26], although the authors acknowledge that the treatment reg-

imen and duration for a dexamethasone implant in vitrectomized eyes still remain controversial [27].

Additionally, 8.3% of the eyes in group 1 and 26.3% in group 2 needed additional therapies for DME. Presently there is little or no guidance from the published literature on the use of additional therapies or when the decision to add them should be made [28]; a combination therapy approach in DME is being increasingly discussed, since there are multiple mechanisms which become more important in the chronic stages of DME. Some authors have suggested that the combination of anti-VEGF therapies with steroids would provide a more optimal pharmacotherapy in DME [29].

As expected and described in other studies [14, 15, 30–32], there was an increase in IOP during the mean follow-up period. The mean change in IOP was +1.6 mm Hg ($p = 0.023$) and +0.8 mm Hg ($p = 0.547$) from baseline in group 1 and group 2, respectively. Though group 1 presented a statistically significant mean change in IOP, no correlation was found between IOP and the vitreous status ($p = 0.430$). The increase in IOP was easily manageable with IOP-lowering medication in all eyes, except 1 eye from the nonvitrectomized group, which had to undergo cyclophotocoagulation. Nevertheless, IOP increases did not affect long-term efficacy outcomes, and IOP remained below 21 mm Hg in the vast majority of patients (95.8% in group 1 and 89.5% in group 2).

Cataract formation was developed in 1 phakic eye in group 1 and 3 phakic eyes in group 2 with subsequent cataract extraction during the follow-up period and without apparent impairment of functional outcomes, similarly to what was observed in the FAME studies [11].

Our analysis has some limitations that are related in part to the retrospective nature of the data and the small cohort. Another limitation is the different follow-up period of analysis between patients and the lack of general information concerning the underlying metabolic status.

Conclusions

The present study is the first real-life clinical comparison of the effectiveness and safety of the 0.2 μg/day FAc implant in vitrectomized and nonvitrectomized eyes with insufficient response to available therapies in Portugal, and, irrespective of vitrectomy, similar outcomes were achieved in chronic DME patients. Our results support that a 0.2 μg/day FAc implant is being used earlier in vitrectomized eyes as shown indirectly through the increased injection of steroids relative to anti-VEGFs (Table 1) and a possible indicator of an increased proinflammatory state as vitrectomy will have been conducted in eyes with long-standing macular edema. Patients will continue to be monitored to assess longer-term real-world benefits as the 0.2 μg/day FAc implant provided therapy that lasted for up to 3 years in its pivotal randomized controlled trials.

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Disclosure Statement

The authors have no conflict of interests to declare.

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2.10 Optical Coherence Tomography Biomarkers: Vitreous Status Influence in Outcomes for Diabetic Macular Edema Therapy with 0.19-mg Fluocinolone Acetonide Implant[399]

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Optical Coherence Tomography Biomarkers: Vitreous Status Influence in Outcomes for Diabetic Macular Edema Therapy with 0.19-mg Fluocinolone Acetonide Implant

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Keywords
Diabetic macular edema · Diabetic retinopathy · Vitrectomy · Fluocinolone acetonide · Biomarkers

Abstract
Background: The 0.19-mg fluocinolone acetonide (FAc) implant (ILUVIEN®; Alimera Sciences Ltd., Hampshire, UK) was approved for the treatment of vision impairment associated with chronic and refractory diabetic macular edema (DME). ***Objectives:*** To quantitatively assess functional and structural features in nonvitrectomized and vitrectomized DME patients after being treated with an FAc implant. ***Methods:*** Retrospective review of patients with DME receiving a single intravitreal injection of the FAc implant. The study was designed to analyze the presence of quantitative structural OCT biomarkers at baseline and 12 months after FAc therapy according to vitreous status. ***Results:*** A total of 41 eyes from 30 patients were included in this study. At 12 months after injection, vitrectomized patients had a lower central foveal thickness (p = 0.017) and fewer hyperreflective dots (p = 0.028) compared with nonvitrectomized. Thirty (73%) patients presented a significant functional improvement with 17 (42%) increasing at least 15 ETDRS letters. Overall, 22 (54%) eyes had a complete resolution of DME at the 12-month visit. Patients who needed additional therapy had a higher prevalence of subretinal fluid (42 vs. 3%, p = 0.005) at baseline. ***Conclusions:*** This study supports the effectiveness of the FAc implant and reports significant changes at 12 months after FAc injection.

Introduction

Diabetes was estimated to affect a total of 463 million people in 2019 [1]. It is expected that more than half a billion people will be living with diabetes in 2030 and will grow to around 700 million (10.9%) by 2045 [1].

Macular edema is the main cause of vision loss in diabetic retinopathy [2]. A course of at least 3 intravitreal injections of antivascular endothelial growth factor (VEGF) is recommended as the first-line therapy for diabetic mac-

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ular edema (DME) [3]. Insufficient response to anti-VEGF deems the resource to corticosteroid intravitreal injections, although in some cases they can be the first option if patients are unsuitable for anti-VEGF therapy [3].

The 0.19-mg fluocinolone acetonide (FAc) implant (ILUVIEN®; Alimera Sciences Ltd., Hampshire, UK) was approved for the treatment of vision impairment associated with chronic DME considered insufficiently responsive to available therapies such as anti-VEGF and short-term corticosteroid intravitreal injections. Its approval was based on data from FAME studies [4] that presented visual benefits over a 3-year period of follow-up in nonvitrectomized patients. Real-world studies have shown that the FAc implant is effective in both nonvitrectomized and vitrectomized eyes [5–10].

The main goal of this study was to quantitatively assess functional and structural features in nonvitrectomized and vitrectomized DME patients after being treated with an FAc implant. That purpose was enhanced by a comprehensive analysis of quantitative structural optical coherence tomography (OCT) biomarkers which have been correlated with severity and chronicity of DME, retina function, and influence in DME treatment response, which particularly deepen the knowledge of their potential role in predictive response to FAc therapy [11–17].

Methods

A retrospective review was conducted involving consecutive patients with DME who received a single intravitreal injection of the FAc implant at the Centro Hospitalar Universitário do Porto (CHUP), Portugal. The study was designed to analyze the presence of quantitative structural OCT biomarkers at baseline and 12 months after FAc therapy according to vitreous status: vitrectomized eyes (group 1) and nonvitrectomized eyes (group 2). The secondary objectives were to analyze differences in treatment response and the need for additional therapy as well as to correlate those biomarkers with response to therapy. The study was conducted according to the tenets of the Declaration of Helsinki in its latest amendment (Brazil, 2013) and was approved by the Ethics Committee of CHUP (2017.093 [084-DEFI/082-CES]). All patients signed an informed consent form.

Study Population and Data

Inclusion Criteria

Inclusion criteria were (1) type 1 or type 2 diabetes mellitus; (2) older than 18 years; (3) center-involved DME (central subfield foveal thickness [CFT] of >300 μm in the presence of intraretinal fluid), refractory to anti-VEGF and short-term steroid agents; (4) first treatment with FAc implant; and (5) both eyes (if indicated) could be included in this study. Refractory DME was defined as persistent intraretinal and/or subretinal fluid (SRF) on Spectral Domain-Optical Coherence Tomography (SD-OCT), after at least 3 anti-VEGF injections that were given at monthly intervals or 6 months after 1 short-term steroid injection, regardless of visual acuity.

Exclusion Criteria

Exclusion criteria were (1) other concomitant ocular diseases that cause macular edema or compromise visual acuity (as significant vitreoretinal interface abnormality on SD-OCT that might contribute to macular edema, such as ERM with inner retinal distortion, age-related macular degeneration, retinal vascular occlusion, central corneal opacity, amblyopia, advanced glaucoma, and optic neuropathy), except for the presence of cataract; (2) history of ocular trauma; (3) any intraocular surgery except uncomplicated cataract extraction and/or vitrectomy (in group 1) in <3 and 6 months before FAc, respectively; (4) previous treatment with intraocular corticosteroids within the 6 months before treatment with the FAc implant; and (5) inability or unwillingness to provide informed consent.

Procedures

Patient charts were reviewed for demographic data and previous treatments for DME. Based on the knowledge that steady-state aqueous FAc levels are achieved around 6 months after implantation and then maintained throughout to month 36 [18], we chose to assess patients 12 months after the injection of the FAc implant. At baseline and 12 months after FAc implant visits, assessment of BCVA using Early Treatment of Diabetic Retinopathy Study (ETDRS) letters chart, applanation tonometry, slit-lamp examination, dilated fundus examination, and SD-OCT (Heidelberg Spectralis, Heidelberg, Germany) was performed.

SD-OCT Parameters

CFT was calculated automatically by using the instrument. The presence of 9 different morphological parameters was determined using SD-OCT and measured with the 1-mm central foveal area. These included (1) the presence of SRF; (2) the number of hyperreflective dots (HRD); (3) the disorganization of retinal inner layers; (4) the disruption of outer plexiform layer (OPL); (5) the disruption of external limiting membrane (ELM); (6) the disruption of ellipsoid zone (EZ); (7) the presence of cysts in the outer nuclear layer (ONL); (8) the presence of cysts in the inner nuclear layer (INL); and (9) the number of cysts in ONL versus those in the INL.

Disruption of OPL, ELM, and EZ was defined as an evident discontinuity in those layers. HRD were defined as small (<30 microns) and punctiform, with reflectivity similar to the nerve fiber layer and no backshadowing. The presence of the 9 SD-OCT parameters was assessed in the central 1 mm of 5 scans: the foveal one and the 2 above and the 2 below it.

OCT scans were obtained by using an SD-OCT (macular dense line scan mode HR 20 × 20° Spectralis HRA + OCT; Heidelberg Engineering, Heidelberg, Germany). All measurements were determined by 2 highly trained medical doctors (A.F. and J.L.) with disagreements solved by a third senior medical retinal specialist (B.P.).

Functional and Anatomical Outcomes Criteria

A significant functional improvement was considered a gain of at least 5 ETDRS letters [19]. DME resolution was defined by CFT ≤300 μm. The type of response was classified as (1) a good responder when at 12 months after FAc implant, there was DME resolution with a significant functional improvement without ad-

Table 1. Baseline clinical and demographic characteristics of population

	Overall (n = 41)	Vitrectomized (n = 23)	Nonvitrectomized (n = 18)	p value
Age, mean ± SD, years	69.02±8.23	67.35±7.31	71.17±9.04	0.14
Female, n (%)	15 (37)	8 (35)	7 (39)	0.79
Right eye, n (%)	22 (54)	14 (61)	8 (44)	0.35
Phakic, n (%)	9 (22)	1 (4)	8 (44)	0.005
BCVA, ETDRS letters, mean ± SD	42.54±18.41	44.70±19.69	39.78±16.76	0.40
IOP, mean ± SD, mm Hg	15.63±2.98	15.57±3.31	15.72±2.59	0.87
IOP-lowering medication				
Patients using, n (%)	18 (44)	12 (52)	6 (33)	0.23
Median (range) of drops	0 (0–4)	1 (0–4)	0 (0–3)	0.30
Glaucoma surgery, n (%)	1 (2)	0 (0)	1 (6)	0.44
DME duration, mean ± SD, years	3.33±1.25	3.52±1.44	3.08±0.94	0.27
PRP, n (%)	38 (93)	23 (100)	15 (83)	0.042
Macular laser therapy, n (%)	11 (27)	6 (26)	5 (28)	0.90
Cryotherapy, n (%)	2 (5)	1 (4)	1 (6)	1.00
Anti-VEGF IV, median (range)	3 (0–25)	3 (0–11)	3.5 (0–25)	0.73
Steroid IV, median (range)	2 (0–11)	3 (1–11)	1.5 (0–4)	0.014

BCVA, best-corrected visual acuity; DME, diabetic macular edema; IOP, intraocular pressure; IV, intravitreal; PRP, panretinal photocoagulation; VEGF, vascular endothelial growth factor.

ditional treatments; (2) nonresponder, when there was a CFT >400 μm or ≤10% of CFT reduction and BCVA decrease (or BCVA gain <5 letters); (3) moderate responder – between good and nonresponder criteria; and (4) nongood responders include nonresponders and moderate responders. Macular atrophy was considered as CFT <230 μm [20].

Statistical Analysis

All statistical analyses were performed using Stata software (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX, USA: StataCorp LP). Shapiro-Wilk, Kolmogorov-Smirnov test, and normal probability plots were used to confirm the normal distribution of the data. Parametric or nonparametric tests were used for continuous variable comparison between defined groups, according to the normality of data. χ^2 or Fisher's exact tests were performed for categorical variable comparison. Statistical significance was defined as p value <0.05.

Results

A total of 41 eyes from 30 patients were included in this study. Table 1 summarizes baseline clinical and demographic characteristics for the full population with statistical comparison between groups. Table 2 summarizes clinical and SD-OCT biomarkers before and after FAc implant. At 12 months after injection, BCVA was significantly higher compared with baseline (p = 0.005) with a mean increase of 12.3 ± 18.6 ETDRS letters. CFT was significantly lower at 12 months (p < 0.001) with a mean reduction of 198.2 ± 211.3 μm. The prevalence of SRF (p = 0.026) and the number of HRD (p < 0.001) and cysts in INL (p < 0.001) and ONL (p < 0.001) were significantly lower after the FAc implant. Despite nonsignificant, there was a trend for other anatomical improvements, namely, a lower prevalence of OPL, ELM, and EZ disruption.

No cases underwent cataract surgery or vitrectomy in the first 12 months of follow-up. Macular atrophy was observed in 5 cases, 3 (13%) in group 1 and 2 (11%) in group 2.

Between-Group Demographic and Response to FAc Implant Differences

In group 1, more patients were pseudophakic (p = 0.005), more patients had been submitted to PRP (p = 0.042), and the number of previous steroid intravitreal injections was higher (p = 0.014) (Table 1). No other significant differences were found (p > 0.05) at baseline. At 12 months after the implant, lower IOP (p = 0.033), lower CFT (p = 0.017), and fewer HRD (p = 0.028) were observed in group 1 compared with group 2. There was a trend towards a lower need of additional treatment in group 1 (OR 0.26; 95%: 0.06–1.09, p = 0.066). No differences were found regarding the response to treatment (35 vs. 28% of good responders, p = 0.74, in groups 1 and 2, respectively).

Table 2. Clinical variables and SD-OCT biomarkers before and 12 months after FAc implant

	Baseline (n = 41)	p value	Vitrectomized (n = 23)	Nonvitrectomized (n = 18)	p value
BCVA, mean ± SD					
Baseline	42.54±18.41	0.005	44.70±19.69	39.78±16.76	0.40
12 months	54.85±19.84		58.39±18.30	50.33±21.32	0.20
IOP, mean ± SD, mm Hg					
Baseline	15.63±2.98	0.13	15.57±3.31	15.72±2.59	0.87
12 months	16.98±4.83		15.56±3.81	18.78±5.48	0.033
IOP-lowering medication patients using, n (%)					
Baseline	18 (44)	0.12	12 (52)	6 (33)	0.23
12 months	0 (0–4)		13 (57)	12 (67)	0.54
Median (range) of drops					
Baseline	25 (61)	0.16	1 (0–4)	0 (0–3)	0.30
12 months	1 (0–4)		1 (0–4)	1 (0–4)	0.68
CFT, mean ± SD, µm					
Baseline	535.46±192.46	<0.001	525.48±183.01	548.22±208.58	0.71
12 months	337.37±152.00		288.26±69.85	400.11±201.53	0.017
SRF, n (%)					
Baseline	6 (15)	0.026	2 (9)	4 (22)	0.38
12 months	0 (0)		0 (0)	0 (0)	–
HRD, mean ± SD					
Baseline	24.64±16.15	<0.001	22.73±15.87	27.12±16.66	0.41
12 months	13.00±13.16		9.04±7.07	18.05±17.16	0.028
DRIL, n (%)					
Baseline	40 (98)	0.17	23 (100)	15 (94)	0.25
12 months	37 (90)		21 (91)	16 (89)	0.99
OPL disruption, n (%)					
Baseline	41 (100)	0.078	23 (100)	18 (100)	–
12 months	38 (93)		21 (91)	17 (94)	0.99
ELM disruption, n (%)					
Baseline	36 (88)	0.095	12 (23)	14 (25)	0.82
12 months	30 (73)		17 (74)	13 (72)	0.99
EZ disruption, n (%)					
Baseline	36 (88)	0.095	20 (87)	16 (89)	0.85
12 months	30 (73)		16 (70)	14 (78)	0.73
INL cysts, n (%)					
Baseline	37 (90)	<0.001	22 (96)	15 (83)	0.19
12 months	18 (44)		8 (35)	10 (56)	0.22
ONL cysts, n (%)					
Baseline	38 (93)	<0.001	22 (96)	16 (89)	0.41
12 months	15 (37)		6 (26)	9 (50)	0.19

FAc, fluocinolone acetonide; BCVA, best-corrected visual acuity; CFT, central foveal thickness; SRL, subretinal fluid; DRIL, disorganization of retinal inner layers; ELM, external limiting membrane; EZ, ellipsoid zone; HRD, hyperreflective dots; INL, inner nuclear layer; IOP, intraocular pressure; ONL, outer nuclear layer; OPL, outer plexiform layer.

Response to FAc Implant

At 12 months after FAc implant, 30 (73%) patients presented a significant functional improvement with 17 (42%) having an increase of at least 15 ETDRS letters. Moreover, 22 (54%) patients had a DME resolution while 9 (22%) had a CFT >400 µm. Lower baseline BCVA was associated with an increase of at least 15 ETDRS letters (34.1 ± 11.6 vs. 48.5 ± 20.1, p = 0.012) after injection of the FAc implant. Baseline SRF was also associated with an increase of at least 15 ETDRS letters in BCVA (29 vs. 4%, p = 0.036) and with CFT above 400 µm (44 vs. 6%, p = 0.004) at 12 months after implant.

Overall, 13 (32%) eyes were judged to be good responders and 3 (7%) bad responders; therefore, 61% of

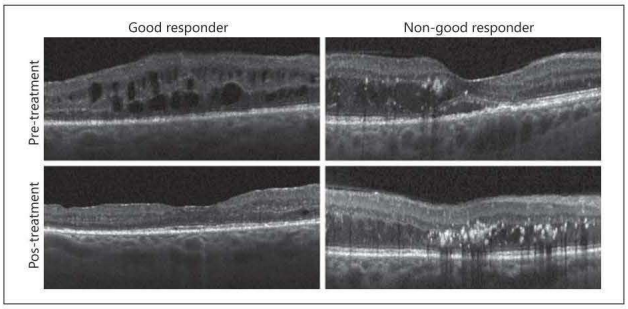

Fig. 1. SD-OCT of good and nongood responders at baseline (before treatment) and 12 months after FAc implant (after treatment). FAc, fluocinolone acetonide.

eyes had a moderate response. Figure 1 illustrates the SD-OCT scans of representative cases of good and nongood responders at baseline and 12 months after FAc implant. Regarding the 5 cases without functional improvement despite a DME resolution (which explains different percentages of functional improvement vs. DME resolution, 41 and 54%, respectively), they were all pseudophakic, with a better BCVA before FAc (mean + SD of 57.20 ± 16.24 vs. 34.23 ± 13.80 ETDRS letters, $p = 0.005$), and there was a trend for them to be younger (mean ± SD 63.40 ± 6.84 vs. 70.82 ± 8.29 years, $p = 0.08$), to have a higher DME duration (mean ± SD 4.6 ± 1.85 ± 3.35 ± 1.21 years, $p = 0.087$), and to have been submitted to a higher number of anti-VEGF intravitreal injections before FAc (median [range] 5 [1–8] vs. 1 [0–9], $p = 0.06$) compared with good responders.

Table 3 summarizes baseline characteristics and SD-OCT parameters by response to treatment. At baseline, when compared with the nongood responders, the eyes with good response had a tendency towards lower BCVA ($p = 0.052$) and lower prevalence of SRF ($p = 0.084$) and had received fewer prior injections of anti-VEGF ($p = 0.008$; Table 3). At 12 months after the implant, in comparison with nongood responders, the eyes with good response had lower CFT ($p = 0.012$) and fewer HRD ($p = 0.042$).

Need for Additional Therapy

Overall, 12 (29%) eyes needed additional therapy in the first 12 months after implant and all were submitted to further anti-VEGF injections. At baseline, patients that required supplemental therapy had a higher CFT (646.25 ± 185.45 vs. 489.62 ± 178.79 μm, $p = 0.016$) and a higher prevalence of SRF (42 vs. 3%, $p = 0.005$). At 12 months, those patients presented a higher number of HRD (20.00 ± 18.98 vs. 10.10 ± 8.73, $p = 0.026$). Further comparisons at baseline and at 12 months concerning demographic, clinical, and SD-OCT parameters were made, and no other significant differences were observed.

Discussion

This study supports the effectiveness of the FAc implant in the treatment of persistent or recurrent DME and reports significant changes in several SD-OCT parameters (CFT, SRF, HRD, INL, and ONL cysts) and BCVA 12 months after FAc injection. Comparisons between groups revealed that after 12 months, vitrectomized eyes had lower IOP, lower CFT, fewer HRD, and a trend towards a lower need for additional treatment. However, there was no difference in the response to the FAc implant between groups according to the criteria hereby defined. Data also suggest that biomarkers at baseline (i.e., higher CFT and higher prevalence of SRF) and at 12 months (i.e., higher number of HRD) may be important indicators as to whether additional therapy will be needed. This study emphasizes the important role of FAc in the treatment of DME that persists or recurs despite treatment and its positive effect on visual outcomes as well as SD-OCT parameters and are generally consistent with the published literature.

The main goal of this study was to quantitatively assess functional and structural features in nonvitrectomized and vitrectomized DME patients after being treated with an FAc implant, which is important as a history of vitrectomy was an exclusion criterion in the FAME/FAMOUS studies [4, 18, 21], and to the best of our knowledge, this is the first study to assess the importance of SD-OCT biomarkers in these eyes following therapy with the FAc im-

Table 3. Clinical and SD-OCT parameters according to type of response

	Good response (n = 14)	Nongood response (n = 28)	p value
Age, mean ± SD, years	71.85±7.87	67.71±8.20	0.14
Female, n (%)	4 (31)	11 (39)	0.73
Right eye, n (%)	9 (69)	13 (46)	0.17
Phakic, n (%)	2 (15)	7 (25)	0.69
Glaucoma surgery, n (%)	0 (0)	1 (4)	0.99
Previous vitrectomy, n (%)	8 (62)	15 (54)	0.71
DME duration, mean ± SD, years	3.42±1.22	3.28±1.29	0.75
PRP, n (%)	12 (92)	26 (93)	0.99
Macular laser, n (%)	3 (23)	8 (29)	0.99
Cryotherapy, n (%)	1 (8)	1 (4)	0.54
Anti-VEGF IV, median (range)	1 (0–9)	4.5 (0–25)	0.008
Steroid IV, median (range)	3 (1–7)	2 (0–11)	0.57
BCVA, mean ± SD			
Baseline	34.38±13.47	46.32±19.35	0.052
12 months	55.92±15.81	54.35±21.71	0.82
IOP, mean ± SD, mm Hg			
Baseline	14.77±2.55	16.03±3.12	0.21
12 months	17.77±4.13	16.61±5.16	0.48
IOP-lowering medication patients using, n (%)			
Baseline	7 (54)	11 (39)	0.50
12 months	8 (62)	17 (61)	0.99
Median (range) of drops			
Baseline	1 (0–4)	0 (0–3)	0.32
12 months	1 (0–4)	1 (0–4)	0.80
CFT, mean ± SD, μm			
Baseline	526.46±222.73	539.64±181.02	0.84
12 months	251.54±23.14	377.21±169.69	0.012
SRF, n (%)			
Baseline	0 (0)	6 (21)	0.084
12 months	0 (0)	0 (0)	–
HRD, mean ± SD			
Baseline	24.33±22.32	24.78±13.06	0.94
12 months	6.92±7.69	15.82±14.29	0.042
DRIL, n (%)			
Baseline	13 (100)	27 (96)	0.99
12 months	11 (85)	26 (93)	0.58
OPL disruption, n (%)			
Baseline	13 (100)	28 (100)	–
12 months	12 (92)	26 (93)	0.99
ELM disruption, n (%)			
Baseline	13 (100)	23 (82)	0.16
12 months	12 (92)	18 (64)	0.061
EZ disruption, n (%)			
Baseline	12 (92)	24 (86)	0.99
12 months	11 (85)	19 (68)	0.45
INL cysts, n (%)			
Baseline	11 (85)	26 (93)	0.58
12 months	5 (38)	13 (46)	0.74
ONL cysts, n (%)			
Baseline	12 (92)	26 (93)	0.99
12 months	4 (31)	11 (39)	0.73

Table 3 (Footnote)

BCVA, best-corrected visual acuity; CFT, central foveal thickness; DME, diabetic macular edema; SRL, subretinal fluid; DRIL, disorganization of retinal inner layers; ELM, external limiting membrane; EZ, ellipsoid zone; HRD, hyperreflective dots; INL, inner nuclear layer; IOP, intraocular pressure; IV, intravitreal; ONL, outer nuclear layer; OPL, outer plexiform layer; PRP, panretinal photocoagulation; VEGF, vascular endothelial growth factor.

plant. Baseline characteristics revealed that the vitrectomized group had a higher prevalence of pseudophakia and PRP treatment as well as a higher number of steroid intravitreal injections at baseline, with no other significant differences with regard to vitreous status. This may be explained by a higher severity of DME in vitrectomized eyes, as DME duration was similar in both groups.

Despite the lower CFT, lower IOP, and number of HRD in the vitrectomized group at month 12, no differences were found in the response to the FAc implant between vitrectomized and nonvitrectomized patient eyes. Other reports [6, 8, 10] have demonstrated the effectiveness of the FAc implant in vitrectomized and/or nonvitrectomized patient eyes. The current study, however, expands our understanding of SD-OCT biomarkers. This is important as they are considered relevant to functional outcomes and responses to DME therapy [11–17]. Here, we showed that vitrectomized eyes had fewer HRD compared with nonvitrectomized eyes at month 12. This was also observed in the subgroup analysis where good responders had lower HRD than nongood responders. According to Framme et al. [12], the number of HRD correlates with the severity of DME. It is also suggested to be a predictive factor for therapeutic outcomes and a hallmark of inflammatory retina tissue response [14].

The role of pars plana vitrectomy (PPV) in tractional DME is undoubtedly helpful while in nontractional DME, it remains controversial. Some theories advocate that PPV would eliminate some inflammatory mediators that contribute to vessel permeability and that it would normalize macular blood supply with decreased leakage [22]. In addition to the reduced vitreous viscosity due to vitreous removal, which increases the diffusion of molecules through the eye, promoting higher premacular oxygen concentrations and lower intraretinal VEGF concentrations, endo-PRP, usually performed during vitrectomy, may enhance its positive effect in DME control [23]. However, PPV may also lead to quicker drug diffusion and clearance, reducing the exposure to the treatment [22]. Our achievements may support the anti-inflammatory role of PPV enforcing the view that this procedure may act as an additional treatment for DME irrespective of tractional status. In fact, vitrectomized patients presented a tendency towards a lower need for additional treatment.

The importance of these findings may be relevant in the remaining course of the disease, as a more benign DME evolution through disease stabilization and less additional treatments, particularly in vitrectomized eyes [24, 25]. The lower CFT in vitrectomized eyes after 12 months of FAc treatment is also in line with the association of vitrectomy with macula atrophy, particularly when internal limiting membrane peeling is performed [20, 25, 26]. In this study, all vitrectomized eyes had undergone internal limiting membrane peeling [23]. Nonetheless, macular atrophy was observed in a low and similar number of cases in both groups. Regarding lower IOP in vitrectomized eyes at the end of follow-up seems not to be so clinically relevant as mean IOP and the median number of IOP-lowering medications were similar in both groups.

There was a difference in the number of prior steroid injections at baseline with the vitrectomized group having a higher number of treatments. However, this does not seem to explain the discrepancies at 12 months as both triamcinolone and dexamethasone implants have a short duration of action.

The current study also assessed SD-OCT parameters at baseline and, in-line with the literature, a higher CFT [27, 28], a higher number of HRD, and the presence of SRF [29] were associated with a more chronic and severe DME and the need for additional treatment in the first 12 months after FAc implant. This last finding was already expected as SRF is a predictor for anatomical resolution with anti-VEGF and steroid therapy, and its presence is associated with additional therapy burden to achieve DME regression [15, 17, 19, 24–26, 28]. This last fact justifies the trend toward a lack of good response among patients with SRF at baseline as our criteria included the absence of additional treatment. The need for additional therapy in eyes with a higher number of HRD also supports the hypothesis that HRD reflects increased disease

state and inflammatory activity [14]. Contrary to our study, Arrigo et al. [30] reported an association between higher CFT at baseline and good response to the FAc implant at month 12. This may be explained by their criteria where a good responder was based on the CFT outcome (improvement of at least 30%). Based on the published literature [27, 31], however, there is good argumentation that a higher mean CFT (535 vs. 476 μm) precludes a more chronic and refractory DME and the anticipation of a poor anatomical and functional outcome with a higher expectancy for additional therapies. The predictive value of prior PRP, as reported by Cicinelli et al. [29], could not be reproduced in our study and is probably explained by the low number of eyes without PRP ($n = 3$).

Limitations of the present study include its retrospective design and all its implications. The number of patients did not allow us to perform multivariable analysis; therefore, our conclusions might be misled by confounders. When 2 eyes of the same patient fulfilled the inclusion criteria, both were used but due to the small sample, no advanced statistical analyses were performed to adjust for the potential correlation between those eyes. Our population is a cohort of diabetic patients that are well known for their clinical heterogeneity. Further studies are needed to clarify all the implications of those baseline changes in the prognosis of these patients.

Conclusion

This study supports the effectiveness of the FAc implant in the treatment of persistent or recurrent DME and reports significant changes in several SD-OCT parameters at 12 months after FAc injection, besides the improvement of visual acuity, particularly in vitrectomized eyes, with lower CFT and number of HRD at 12 months after treatment and a trend to less additional therapy. SRF was the other important prognostic biomarker, with a clear connotation with the need of additional therapy. The importance of these findings may be relevant in the remaining course of the disease, as a more benign DME evolution through disease stabilization and less additional treatments, particularly in vitrectomized eyes. Moreover, the results hereby presented shed light on the relevance of biomarkers in treatment decisions and prognosis. Future studies with larger samples and longer follow-up should be carried out to further investigate and confirm our results.

Statement of Ethics

The study was conducted according to the tenets of the Declaration of Helsinki in its latest amendment (Brazil, 2013) and was approved by the Ethics Committee of CHUP (2017.093 [084-DEFI/082-CES]). All patients signed an informed consent form.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

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Author Contributions

Study design: B.P., J.F., A.M., and J.M.B. Data acquisition: B.P., A.F., and J.L. Data analysis: A.F. Critical interpretation of data: B.P. and A.F. Manuscript drafting: B.P. and A.F. Manuscript critical review: all. Final approval of the manuscript: all.

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2.11 Changes in ganglion cell layer thickness after treatment with the 0.2 µg/day fluocinolone acetonide implant in vitrectomized and nonvitrectomized eyes with diabetic macular edema[695]

Title: Changes in ganglion cell layer thickness after treatment with the 0.2 µg/day fluocinolone acetonide implant in vitrectomized and nonvitrectomized eyes with diabetic macular edema

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Running head: Ganglion cell layer thickness following the 0.2 µg/day fluocinolone acetonide implant

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Abstract:

Introduction: To compare changes in ganglion cell layer (GCL) between vitrectomized and nonvitrectomized eyes with diabetic macular edema (DME) over a 2-year period following treatment with 0.2 µg/day fluocinolone acetonide (FAc) implant.

Methods: Eighteen vitrectomized (group 1) and 8 nonvitrectomized (group 2) eyes were included in this cohort study. Changes in central macula GCL thickness were measured using the Spectralis spectral domain optical coherence tomography (SD-OCT) at baseline and 6, 12 and 24 months of follow-up. Other parameters analyzed included best-corrected visual acuity (BCVA), central foveal thickness (CFT) and intraocular pressure (IOP).

Results: Treatment with the FAc implant led to small reductions in mean global GCL thickness versus baseline and contrasts with the control group that was stable or slightly increased versus baseline. FAc therapy also led to improvements in mean BCVA and CFT that were observed at Month 6 and maintained to Month 24. For vitrectomised and non-vitrectomised eyes, no differences were observed between mean global GCL, BCVA and CFT values during follow-up. Linear correlations revealed that in all groups mean BCVA at Month 24 positively correlated with mean GCL thickness at baseline and at Month 24. IOP remained stable throughout the 24 months.

Conclusion: There was no evident retinal neurodegeneration in the 2-year period following treatment with FAc in both groups. GCL thickness may be a useful biomarker for assessing safety and effectiveness in patients with DME.

Introduction

Diabetic retinopathy (DR) is one of the most important microvascular complication of diabetes mellitus and is the leading cause of vision loss among working-age adults in the developed world. Diabetic macular edema (DME) is the main cause of the vision loss related to DR [1–3].

The concept of diabetic retinopathy as a microvascular disease has evolved in recent years, and it is now considered a more complex entity in which neurodegeneration plays a significant role [4]. Retinal neurodegeneration is an early event in the pathogenesis of diabetic retinopathy [4]. In the retina, glial, neural, and vascular cells are closely linked creating a 'neurovascular unit' to maintain the homeostasis necessary for physiological neuro-retinal function[5]. This neurovascular unit exists in health but also in disease. In addition, it has been demonstrated that there is a higher rate of thinning of the retinal nerve fiber layer (NFL) and ganglion cell/inner plexiform layer (GCL/IPL) in patients with no to minimal

nonproliferative diabetic retinopathy (NPDR) [5] compared with that observed in healthy eyes related with aging [6,7].

In diabetes, chronic hyperglycemia induces a process of low-grade inflammation, immune cell activation and extracellular glutamate accumulation, which is responsible for retina neuronal degeneration and vascular lesions through disruption of the blood–retinal barrier (BRB) and diverse cell type impairment, including neural cells [4]. Among them, retinal ganglion cells (RGCs) are the earliest cells affected and have the highest rate of apoptosis [8]. These key features of diabetic retinopathy stress the importance of glucocorticoids in the treatment armamentarium of DME through their anti-inflammatory response, direct effects on tight junction proteins, anti-angiogenic and neuroprotective actions [9].

The multifactorial pathogenesis of DME may require different and complementary therapeutic approaches, such as metabolic control, laser therapy, anti-vascular endothelial growth factor (anti-VEGF) or corticosteroid intravitreal injections as well as vitrectomy [10,11]. The most widely therapy approach for DME usually involves repeated pharmacological treatments, and, in vitrectomized eyes, some intravitreal (IV) treatments, such as anti-VEGF and triamcinolone, had an increased vitreous clearance and a higher number of anti-VEGF injections is expected during the first 12 months of treatment [12–14].

ILUVIEN (0.2 μg/day fluocinolone acetonide [FAc]) intravitreal implant is a long-acting steroid indicated for treatment of vision impairment associated with chronic DME considered insufficiently responsive to available therapies (Alimera Sciences Ltd, Hampshire, UK).

In accordance to previous studies of our group and other real-life studies, FAc implant have demonstrated to be effective in both nonvitrectomized and vitrectomized eyes [15–19]. Furthermore, there is also evidence that IV FAc may decelerate diabetic retinal neurodegeneration, through a decrease in the rate of inner retinal thinning in patients with persistent DME [20]. As opposed to nonvitrectomized eyes, the influence of DME in retinal degeneration process in eyes without vitreous and submitted to internal limiting membrane (ILM) peeling is much less explored. Reductions in GCL thickness following ILM peeling have already been reported in several studies [21,22]. and a worse postoperative visual outcome have also been documented [21].

This study sought to compare the changes of ganglion cell layer (GCL) in DME patients between vitrectomized eyes with ILM peeling and nonvitrectomized eyes following treatment with the 0.2 μg/day FAc implant, aiming to better understand of the vitreous status influence in the neurodegeneration process and DME behaviour, particularly after treatment with FAc implant.

Materials and methods

Study Population

This study was designed as a retrospective, single-center observational cohort study conducted at Centro Hospitalar Universitário do Porto (CHUPorto), Porto, Portugal. Patient records from April 2015 to September 2016 were reviewed for cases with DME treated with a single 0.2 µg/day FAc implant and followed up for a minimum period of 24 months. The effect of FAc was assessed in two groups: vitrectomized – group 1, and nonvitrectomized eyes – group 2.

Ethics

The study was conducted according to the tenets of the Declaration of Helsinki in its latest amendment (Brazil, 2013) and was approved by the ethics committee of CHUPorto (2017.093 [084-DEFI/082-CES]). All patients signed a written informed consent form.

Inclusion criteria

Patients with type 1 or type 2 diabetes mellitus, >18 years, and with center-involved DME, defined as central foveal thickness (CFT) of more than 300 µm on spectral domain-optical coherence tomography (SD-OCT) were eligible for the study. To be included in this analysis, patients in groups 1 and 2 had to have DME that was refractory to anti-VEGF and short-term steroid agents, defined as having persistent intraretinal and/or subretinal fluid on OCT (i.e. CFT >300 µm or ≤20% CFT decrease measured 1 month after at least 3 anti-VEGF injections that were given at monthly intervals or 6 months after 1 short-term steroid injection, regardless of visual acuity (VA)). For post vitrectomy cases, eyes were considered for inclusion provided the vitrectomy had been performed at least 6 months (range, 0.5 to 6.5 years) prior to intravitreal injection of the FAc implant.

Patients were excluded if they had additional ocular diseases that could significantly affect the VA, history of ocular trauma or surgery other than uncomplicated cataract surgery within the 3 months prior to FA therapy.

A control group of 12 fellow eyes (disease-free, nonvitrectomized eyes from non-diabetic patients; a third group separate from groups 1 and 2), of eyes submitted to previous vitrectomy for idiopathic epiretinal membrane (ERM), were included for comparison. For inclusion in the control group, the fellow eye (i.e. normal) was followed by one vitreoretinal surgeon, BP. These eyes were consecutively selected at 24-month follow-up period after vitrectomy in the contra-lateral eye, between December 2020 and February 2021.

Procedures

Baseline patient demographic data, including previous treatments, were recorded. All patients, including control eyes, had a complete ophthalmological evaluation, including intraocular pressure

(IOP) measurement (Goldmann Applanation Tonometry, Haag Streit GmbH, Wedel, Germany) along with a macular SD-OCT at baseline and at each follow-up visit (baseline, Month 1, Month 3 and then quarterly). For this analysis, the variation of GCL thickness (globally and by segmental quadrants: temporal, superior, nasal and inferior), best-corrected visual acuity (BCVA; measured with an Early Treatment Diabetic Retinopathy Study [ETDRS] letter chart), CFT, and safety outcomes [intraocular pressure (IOP), cataract development and IOP-lowering therapy needed] were assessed at baseline (the day of FAc implant injection), and then at 6, 12 and 24 months.

Optical coherence tomography

OCT scans were obtained by an SD-OCT (dense line scan mode HR 20x20º Spectralis HRA+OCT; Heidelberg Engineering, Heidelberg, Germany). A 3 mm area of the macular region centred on the fovea was examined with automated segmentation. For standardization, all examinations were performed by one well-trained technician to counteract a potential annotation bias. During the follow-up examinations, built-in automatic recognition system enabled scanning of the exact same location. SD-OCT scans were evaluated for the presence of intraretinal fluid, sub retinal fluid (SRF), epiretinal membrane (ERM), GCL thickness and CFT. CFT and GCL thickness were analyzed using the retinal thickness map analysis protocol with nine Early Treatment Diabetic Retinopathy Study (ETDRS) subfields.

The CFT was automatically measured by the software in the central 1 mm. The GCL thickness was measured globally (average) and by segmental quadrants: temporal, superior, nasal, and inferior, in the 3 mm region of the central macula.

CFT and GCL thickness variation were evaluated in comparison with baseline OCT scans as well as OCT scans from the previous timepoint.

Correlations between GCL thickness and BCVA in the overall population and per groups were also analysed regarding: a) VA at 24 months versus the difference between the maximum and minimum value recorded between months 6 and 24; b) mean GCL thickness at baseline versus BCVA at 24 months; c) mean BCVA at 24 months versus mean GCL thickness at 24 months.

For all vitrectomized eyes a standard pars plana vitrectomy with internal limiting membrane (ILM) peeling was performed using trypan blue ophthalmic solution 0.15% (doubledyne; Horus Pharma, Francheville, France).

Statistical analysis

The demographics and clinical characteristics of this study cohort were evaluated using traditional descriptive methods. A two sample Mann-Whitney test was used for comparisons between groups. A Fisher's exact two-tailed test was used to assess categorical differences. Linear correlations were performed using a Spearman rank correlation. Multiple-group comparisons were performed using a

Kruskall-Wallis test. A statistical difference was taken as a p-value <0.05. Statistical analysis was performed using a mix of MS Excel (Microsoft, Redmond, Washington, USA), SPC for Excel (BPI Consulting, LLC, Katy, Texas, USA) and GraphPad Prism (GraphPad Software, San Diego, California, USA). Values are reported as mean±standard deviation (SD) unless otherwise stated.

Results

Patient demographics

Overall, between April 2015 and September 2016, 26 eyes met the primary cohort criteria. The baseline characteristics of these 26 eyes are summarized in Table 1. This Table also presents outcomes by their groupings (i.e. group 1 or 2, respectively).

Best-corrected visual acuity, central foveal thickness

At baseline (**Table 1**), mean BCVA was 44.4 ± 18.9 letters in group 1 and 38.6 ± 18.9 in group 2 (p>0.05). A mean increase in BCVA was observed for both groups following injection of the FAc implant in the first 6 months (+16.1 letters in group 1 and +14.4 in group 2), and this was maintained to Month 24 (**Figure 1A**). There were no significant differences (p>0.05) between the two groups in absolute BCVA values at any of the timepoints measured.

In the whole population, by Month 6 mean BCVA increased by 15.6 ETDRS letters (median change of 13 letters; p=0.0193 versus baseline) and remained increased (15.2 ETDRS letters) to Month 24 (a median change of 13 letters; p=0.0038 versus baseline). Mean CFT decreased by 191.6 µm (a median change of 164 µm; p=0.000001 versus baseline) and was stable (-190.8 µm) to Month 24 (a median change of 170 µm; p=0.000003 versus baseline).

Mean CFT decreased from baseline in both groups 1 and 2 within the first 6 months (−212.8 ± 144.5 µm [p=0.00001)] and −140.0 ± 120.8 µm [p=0.0177], respectively), and mean CFT then remained stable in both groups with a significance difference from baseline being maintained to Month 24 (i.e. −198.6 ± 203.3 µm [p=0.0001] and −171.9 ± 117.7 µm [p=0.0092], respectively). Overall, there was no significant difference in absolute CFT between the groups at Months 6, 12 and 24 (p>0.05). Also of note was a general tendency for a lower mean CFT in group 1 compared with group 2 at Months 6 (281.2 ± 64.0 µm vs. 321.0 ± 82.5 µm; p=0.7032) and 12 (268.8 ± 40.7 µm vs. 359.4 ± 132.7 µm; p=0.3912), which was not evident at Month 24 (303.1 ± 99.5 µm vs. 289.1 ± 97.7 µm; p=0.3912), respectively) (**Figure 1B**).

Grouping the data based on lens status revealed no overall differences (p>0.05) between mean BCVA and CFT values at any timepoint for eyes with a phakic or pseudophakic lens. There was one small difference between mean CFT values at Month 6 (323.8 ± 60.2 µm vs. 282.4 ± 72.1 µm [phakic vs. pseudophakic]; p=0.3912),

GCL thickness

In the overall population treated with FAc implant, there was a decrease in mean global GCL thickness from 47.2 ± 9.2 µm at baseline to 43.0 ± 9.2 µm at Month 6 (i.e. -4.8 µm; p=0.1183 absolute values versus baseline values) and then remained stable at Months 12 (40.7 ± 9.9 µm; p=0.0344 vs. baseline) and 24 (42.6 ± 10.2 µm; p=0.1242 vs. baseline) (**Figure 2A(i)**). In the control group of patients, i.e. eyes without diabetes and included as a reference in the current study (group 3), GCL thickness remained relatively stable at baseline (49.5 ± 5.9 µm), Months 6 (49.8 ± 6.5 µm), 12 (50.6 ± 5.6 µm) and 24 (50.0 ± 5.4 µm) (**Figure 2A(i)**) with statistical changes observed between Month 6 and baseline (p=0.0359). Comparison between the overall population and the control group revealed no statistical differences at baseline (i.e. 47.2 ± 9.2 µm vs. 49.5 ± 5.9 µm [p=0.4878]) and Month 6 (43.0 ± 9.2 µm vs. 49.8 ± 6.5 µm [p=0.2271], but there were statistical differences at Months 12 (40.7 ± 9.9 µm vs. 50.6 ± 5.6 µm [p=0.0069]) and 24 (42.6 ± 10.2 µm vs. 50.0 ± 5.4 µm [p=0.0437], respectively) (**Figure 2A(i)**).

Similar changes were obtained in group 1 (-5.0 ± 4.8 µm at Month 6 [p=0.0875 for absolute values versus baseline values], −6.8 ± 8.7 µm at Month 12 [p=0.0279] and −5.1 ± 4.6 µm at Month 24 [p=0.1169]) and group 2 (-4.0 ± 5.2 µm at Month 6, −4.4 ± 6.5 µm at Month 12 and −6.5 ± 10.1 µm at Month 24 [p>0.05 for all absolute values versus baseline values]). Comparison between groups 1 and 2 at the follow-up timepoints revealed no statistical differences (p>0.05; **Figure 2A(ii)**).

In group 1, the smallest mean change from baseline was observed in the nasal segment at each timepoint (i.e. −3.7 µm at Month 6 [p=0.2482 absolute values vs baseline values], −5.6 at Month 12 [p=0.1737] and −3.2 at Month 24 [p=0.3554] and the largest mean change, again at each timepoint, was observed in the superior segment (−5.8 µm at Month 6 [p=0.1173], −8.5 µm at Month 12 [p=0.0227] and −6.3 µm at Month 24 [p=0.1813] (**Figure 2B–E**).

In group 2, the smallest mean changes from baseline were seen in nasal (0.0 µm at Month 6 [p=0.9491 absolute values vs baseline values], −0.8 µm at Month 12 [p=0.4822] and +1.8 µm at Month 24 [p=0.9491]). In the superior segmental region, there was a smaller variation at Month 12 in comparison to the other time points (−4.2 µm at Month 6 [p=0.5653 absolute values vs baseline values], −0.3 µm at Month 12 [p=0.6093] and −4.2 µm at Month 24 [p=0.7015]) (**Figure 2B–E**).

Figure 3 plots global GCL thickness for control eyes, all eyes, PPV and non PPV eyes.

Correlation between GCL thickness and BCVA in global treated population

For all eyes treated with the FAc implant, BCVA at 24 months was not correlated with the difference between the maximum and minimum value recorded between Months 6 and 24 (Rho=-0.22, p>0.05) (**Figure 4A**). However, a positive correlation was observed between BCVA at Month 24 and the mean GCL thickness at Month 24 (Rho=0.49, p=0.017) (**Figure 4B**) and mean GCL thickness at baseline and VA at 24 months (Rho=0.44, p=0.036) (**Figure 4C**).

Correlation between GCL thickness and BCVA in group 1 and group 2

BCVA at 24 months was not correlated (Rho=-0.09, p>0.05) with difference between the maximum and minimum GCL value recorded between Months 6 and 24 in vitrectomized eyes (**Figure 5A**); however, there was a strongly negative correlation that was not statistically significant (Rho=-0.70, p=0.105) observed in nonvitrectomized eyes (**Figure 5B**).

BCVA at 24 months was correlated mean GCL thickness at 24 months in vitrectomized eyes (Rho=0.64, p=0.006; **Figure 5C**) and nonvitrectomized eyes (Rho=0.37, p=0.003) (**Figure 5D**). Mean GCL thickness at baseline reach borderline significance (Rho=0.47, p=0.051) and statistical significance (Rho=0.54, p=0.003) when correlated with BCVA at 24 months in vitrectomized and nonvitrectomized eyes, respectively. (**Figures 5E and F**).

Safety

No statistically significant differences were observed between groups at any timepoints in terms of the absolute values or changes in mean IOP (p>0.05; **Figure 1C**). Mean IOP values remaining below 21 mmHg throughout. Compared with 50% at baseline, 61.5% of patients required IOP-lowering medication (p>0.05, Fisher's Exact test) during follow-up period. IOP-lowering surgery was performed on one eye in the vitrectomized group during this period. All phakic eyes (n=6) underwent cataract surgery between Month 9 and Month 12.

Supplemental therapies

Regarding additional therapy in the 24 months post-FAc implantation, 26.9% (n=7) required IV therapy and 19.2% (n=5) required laser therapy.

Discussion

Overall, there was no statistically significant difference in absolute CFT between vitrectomised and non-vitrectomised eyes groups between Months 6 and 24.

There were overall decreases in global GCL that were observed for all treated patients from around Month 6, before stabilizing and this compares with a stabilisation/slight increase in control eyes (without diabetes), which is in line with what was expected. Bonnin *et al.* [23] showed that GCL thickness in eyes treated for DME, following DME resolution, is reduced in comparison with diabetic eyes without DME, although their central macular thickness is within a normal range.

These results suggest that the FAc implant contributes to stabilization of GCL. While the effect of the FAc implant can start to appear within the first month, this usually peaks at 3–6 months and then stabilizes up to 36 months. This may explain the small decrease in GCL thickness within the first 6 months. Even though, the possible stabilization on GCL thickness by FAc after month 24 or until month 36 needs still to be addressed in future studies.

The total retinal thickening in DME seen on OCT may represent edematous or degenerative changes and that is expected to occur also for the GCL layer. We observed a significant positive mild correlation between baseline global GCL thickness and BCVA at Month 24 for all eyes. This result may mean that GCL against a background DME may be a predictive biomarker, although this needs further research. Under an effective DME therapy towards a dry macula, as demonstrated by a significant DME reduction, globally and separately in both groups following FAc therapy during the 2 years period analyzed in our study, a positive correlation between GCL thickness and VA observed at that timepoint can be expected to occur.

The effect and negative impact of DME in the inner retinal layers on functional outcome has been objectively demonstrated by Prager *et al.* [24] found a positive correlation between a reduction of inner retinal layers thickness, particularly in the nasal quadrant, and a visual gain, both with ranibizumab and triamcinolone applied to DME treatment [24].

Our study intended to analyze the effect of the FAc implant on GCL layer thickness preservation which has an eminent importance in visual function, as demonstrated with ranibizumab or triamcinolone [24].

What we believe is that the common effect among different therapeutical options is the achievement of an efficient edema reduction, which is continuous and longstanding (i.e. predictable) with the FAc implant. To our best knowledge there are no studies reporting the effect of the FAc implant on GCL thickness.

The observation of a BCVA at 24 months with no correlation with difference between the maximum and minimum GCL thickness value recorded between months 6 and 24 in vitrectomized and non-vitrectomised eyes, even though a negative correlation was observed with no statistical significance in non-vitrectomized, may indicate that the presence of vitreous play a role in the response pattern to the FAc implant, with a more constant, predictable and stable effect observed in vitrectomized eyes, which is also in line with another previous publication of our group [25]. In addition, may also reflect the negative effect of edema in the GCL layer and its negative correlation with VA, as demonstrated by Bonnin *et al.* besides the value of FAc in the management of DME and achieving the goal of retina neuroprotection in vitrectomized and non-vitrectomized eyes, particularly in vitrectomized eyes. [23,25]

The above finding reinforces the importance of a stable, effective therapy for DME and particularly in vitrectomized eyes that have a characteristic lower GCL thickness reservoir (see Figure 3). This is something that is related to the ILM peeling procedure during vitrectomy which, in our study, had been performed in all vitrectomized eyes included. Several studies have reported reductions in GCL thickness following ILM peeling [21,22], which was associated with worse postoperative BCVA [21].

However, other studies [26,27] have assessed postoperative changes in GCL thickness in the eyes with or without ILM peeling during pars plana vitrectomy. Hence in vitrectomized eyes with or without ILM peeled, the same effort towards an effective DME therapy should actively be pursued.

Regarding the differences between groups in the amount of GCL reduction by quadrants (superior, inferior, nasal or temporal) it is interesting to verify that FAc therapy induced the greatest change in the superior quadrant of vitrectomized eyes and the least affected was the nasal quadrant, in both sub-groups. A possible explanation may be both the easier access to superior quadrant for the ILM peeling approach and the intention to preserve, as much as possible, the nasal part where the papillomacular bundle is located. This leaves the remaining sectors to be removed clockwise with a minimal interruption [28]. The traumatic pinches in the superior quadrant may be a cause of some inflammatory retina response with a secondary reactive edema and a more pronounced response to intravitreal corticosteroid therapy in the most traumatized tissue. Furthermore, the smaller reduction in the nasal quadrant, also seen in nonvitrectomized eyes, may be a sign of a more pronounced BRB affected location in DME patients.

These results suggest that there was no evident neurodegeneration in the two-year period following treatment with the 0.2 µg / day FAc implant in both vitrectomized and nonvitrectomized eyes. Even though, the possible stabilization on GCL thickness by FAc after month 24 or until month 36 needs still to be addressed in future studies.

The sustained long-standing action of this intraocular delivery implant reduces the burden on the patient and allows the achievement of a sustained drying of the macula and improvement in vision. In addition, post hoc analysis of the FAME A and B trials also found that more subjects who received FAc experienced 2-or-more or 3-or-more step improvements in DR severity compared with subjects who received sham [29].

The current study has a number of limitations including its retrospective design, the small number of patients included in the primary groups analysed and no treated control arm, which would be useful in comparing the effect of different intravitreal therapies. Hence further research and trials are required at the current time to complement this research.

Conclusion

Overall, these results suggest that GCL thickness may be a useful biomarker for assessing the effectiveness of the FAc implant therapy in patients with DME.

Statement of ethics

The study was conducted according to the tenets of the Declaration of Helsinki in its latest amendment (Brazil, 2013) and was approved by the ethics committee of CHUPorto (2017.093 [084-DEFI/082-CES]). All patients signed a written informed consent form.

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Conflict of interest

The authors have no conflicts of interest to declare.

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Author contributions

BP was responsible for the study conception and design. Data collection was performed by BP and CC. All authors contributed to data analysis and interpretation. The first draft of the manuscript was written by BP and all authors reviewed on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

Data available from corresponding author upon request.

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Table 1. Baseline demographics.

Parameter	Full population	Vitrectomized Group 1	Nonvitrectomized Group 2	p value
Patient eyes, n	26[a]	18	8	N/A
Mean age, years ± SD (median)	67.9 ± 7.2 (68)	66.4 ± 7.0 (67)	71.3 ± 6.8 (71)	p=0.1012
Mean DME duration, years ± SD (median)	3.5 ± 1.3 (3)	3.6 ± 1.4 (3)	3.2 ± 1.0 (3)	p=0.6567
Mean GCL thickness, µm ± SD[b] (median)	47.2 ± 9.2 (50)	46.3 ± 9.1 (46)	49.4 ± 9.9 (51)	p=0.3640
Mean BCVA, letters ± SD[c] (median)	42.6 ± 18.7 (40)	44.4 ± 18.9 (40)	38.6 ± 18.9 (38)	p=0.5229
Mean CFT, µm ± SD[c] (median)	517.4 ± 185.7 (477)	516.8 ± 179.0 (477)	518.9 ± 212.9 (508)	p=0.8895
Mean IOP, mmHg ± SD (median)	15.0 ± 2.7 (15)	14.9 ± 2.4 (15)	15.3 ± 3.4 (16)	p=0.6974
Time between vitrectomy and FAc implant, years ± SD[d]	7.2 ± 1.5 (22 months)	7.2 ± 1.5 (22 months)	N/A	N/A
Pseudohakic eye, n (%)	20 (77)	16 (89)	4 (50)	p=0.0510[e]
Laser therapy (PRP and macular laser), n eyes (%)	26 (100)	18 (100)	8 (100)	p=1.000[e]
PRP laser, n eye (%)	26 (100)	18 (100)	8 (100)	p=1.000[e]
Macular laser, n eyes (%)	9 (35)	6 (33)	3 (38)	p=1.000[e]
Anti-VEGF IV injections, n eyes (%)	20 (77)	14 (78)	6 (75)	p=1.000[e]
Mean anti-VEGF IV injections, ± SD (median)	3.2 ± 3.6 (3)	2.8 ± 2.8 (2)	4.1 ± 5.1 (3)	p=0.6567
CCT IV injections, n eyes (%)	25 (96.2)	18 (100)	7 (87.5)	p=0.3077[e]
Mean CCT IV injections, ± SD (median)	2.5 ± 1.6 (2)	2.9 ± 1.6 (3)	1.6 ± 0.9 (2)	p=0.0668

BCVA, best-corrected visual acuity; CFT, central foveal thickness; DME, diabetic macular edema; PRP, panretinal photocoagulation; IV, intravitreal; VEGF, vascular endothelial growth factor; CCT, corticosteroids. Values are presented as mean ± standard deviation unless stated otherwise. Baseline defined as the day of FAc implant injection (pre-injection). BCVA in ETDRS letters. p values were derived using a Mann-Whitney test for two samples and pertains to the comparison between groups (a) 4 eyes out of 30 were excluded due to inconsistent segmentation; (b) at Month 2; (c) at Month 0; (d) all vitrectomized eyes had undergone internal limiting membrane (ILM) peeling; (e) these p values were derived from a Fischer's exact test.

FIGURE LEGENDS

Fig. 1. Mean changes from baseline in BCVA (A), CFT (B) and IOP (C) for vitrectomized and nonvitrectomized eyes.

A.

Note: p>0.05 for the comparison of non-PPV and PPV values at each timepoint.

B.

Note: p>0.05 for the comparison of non-PPV and PPV values at each timepoint.

C.

Note: p>0.05 for the comparison of non-PPV and PPV values at each timepoint.

Fig. 2. Mean changes from baseline in GCL thickness: global for all treated vs. controls and vitrectomzed vs. nonvitrectomized eyes (A) and segmental regions for vitrectomzed vs. nonvitrectomized eyes (B–E).

A. Global GCL thickness

(i) All treated (open circles) vs. controls (closed circles)

Comparison between the overall population and the control group revealed significance differences in the changes at Months 6 (p=0.0082) 12 (p=0.0016) and Month 24 (p=0.0010).

(ii) Victrectomized vs. nonvitrectomized

Note: p>0.05 for the comparison of non-PPV and PPV values at each timepoint.

B. Nasal

Note: p>0.05 for the comparison of non-PPV and PPV values at each timepoint.

C. Temporal

Note: p>0.05 for the comparison of non-PPV and PPV values at each timepoint.

D. Superior

Note: p>0.05 for the comparison of non-PPV and PPV values at each timepoint.

E. Inferior

Note: p>0.05 for the comparison of non-PPV and PPV values at each timepoint.

Fig. 3. Plots showing mean global GCL thickness in all eyes, vitrectomized (PPV), nonvitrectomized (non-PPV) and control eyes.

Notes: The comparison between groups using a Kruskall-Wallis test revealed a difference between groups at Month 12 (Chi square = 10.06, p=0.018, df=3). A two sample Mann-Whitney test revealed differences between control and all eyes groups (p=0.007), and between control and PPV eyes (p=0.002).

Fig. 4. Correlations between GCL thickness and BCVA in the overall population.

A. VA at 24 months versus the difference between the maximum and minimum value recorded between months 6 and 24.

B. Mean BCVA at 24 months vs. mean GCL thickness at 24 months.

C. Mean GCL thickness at baseline vs. BCVA at 24 months.

Fig. 5. Correlation of GCL thickness with VA for Group 1 (A, C, E) and Group 2 (B, D, F) eyes.

A. Group 1: Mean ETDRS VA at 24 months versus the difference between the maximum and minimum value recorded between months 6 and 24.

Notes: ETDRS VA at 24 months versus the difference between the maximum and minimum value recorded between months 6 and 24.

B. Group 2: Mean ETDRS VA at 24 months versus the difference between the maximum and minimum value recorded between months 6 and 24.

Notes: ETDRS VA at 24 months versus the difference between the maximum and minimum value recorded between months 6 and 24.

C. Group 1: Mean BCVA at 24 months versus mean GCL thickness at 24 months

D. Group 2: Mean BCVA at 24 months versus mean GCL thickness at 24 months

E. Group 1: Mean GCL thickness at baseline versus BCVA at 24 months

F. Group 2: Mean GCL thickness at baseline versus BCVA at 24 months

Figure 1

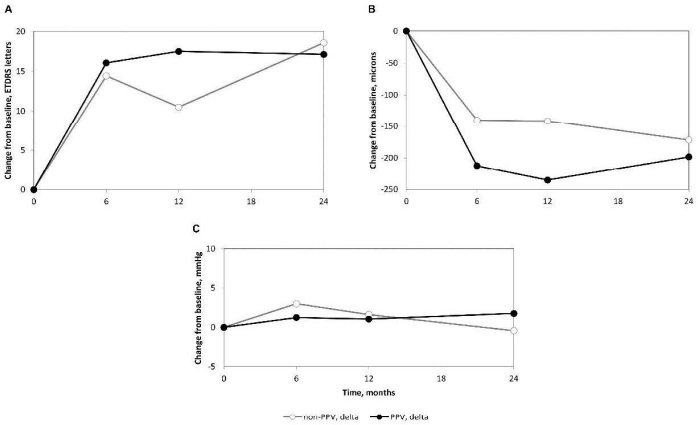

A
Change from baseline, ETDRS letters
20
15
10
5
0
0
6
12
18
24
B
Change from baseline, microns
0
-50
-100
-150
-200
-250
0
6
12
18
24
C
Change from baseline, mm/g
10
5
0
-5
0
6
12
18
24
Time, months
non-PPV, delta
PPV, delta

Figure 2

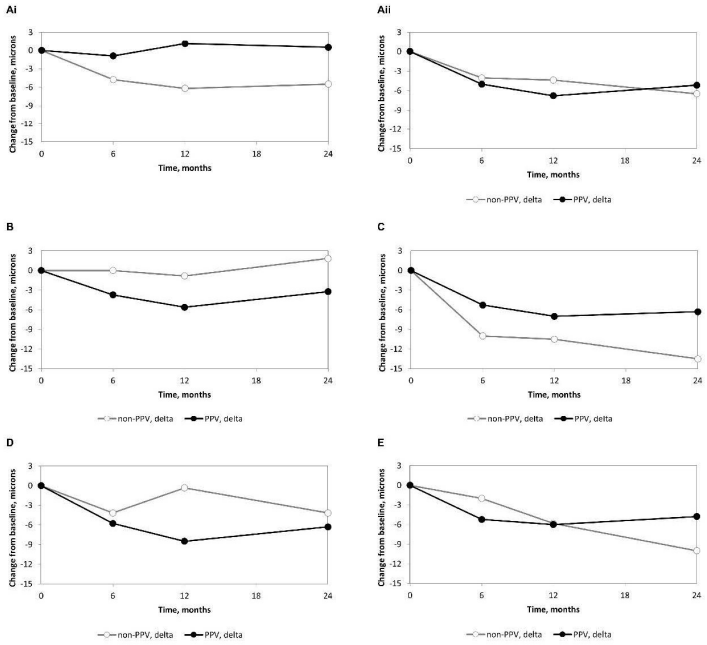

Ai
Aii
B
C
D
E
Change from baseline, microns
3
0
-3
-6
-9
-12
-15
0
6
12
18
24
Time, months
non-PPV, delta
PPV, delta

Figure 3

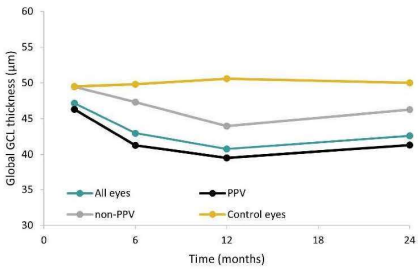

60
55
50
45
40
35
30
Global GCL thickness (µm)
All eyes
PPV
non-PPV
Control eyes
0
6
12
18
24
Time (months)

Figure 4

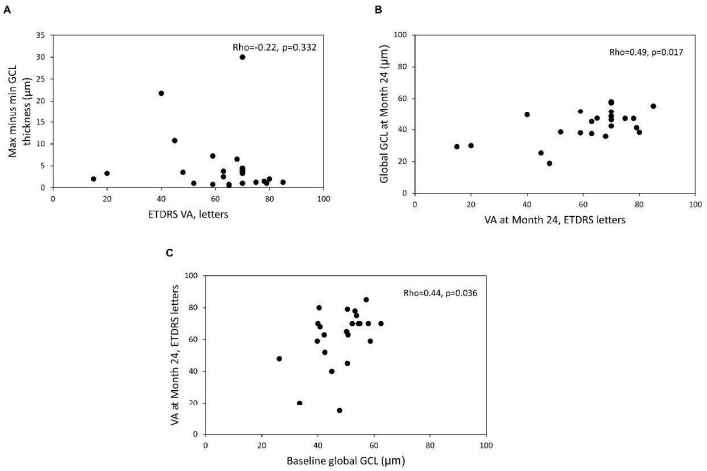
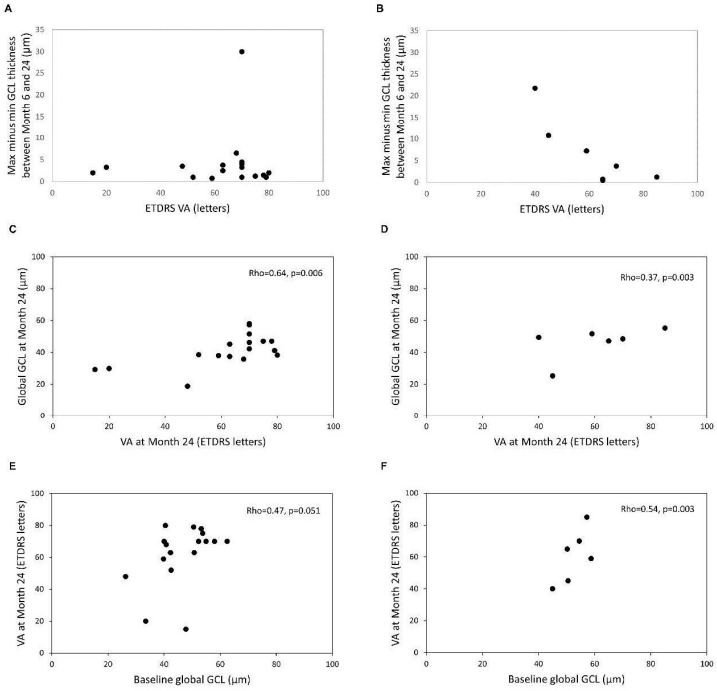

A
Rho=-0.22, p=0.332
Max minus min GCL thickness (µm)
ETDRS VA, letters
B
Rho=0.49, p=0.017
Global GCL at Month 24 (µm)
VA at Month 24, ETDRS letters
C
Rho=0.44, p=0.036
VA at Month 24, ETDRS letters
Baseline global GCL (µm)

Figure 5


A
Max minus min GCL thickness between Month 6 and 24 (µm)
ETDRS VA (letters)
B
Max minus min GCL thickness between Month 6 and 24 (µm)
ETDRS VA (letters)
C
Global GCL at Month 24 (µm)
Rho=0.64, p=0.006
VA at Month 24 (ETDRS letters)
D
Global GCL at Month 24 (µm)
Rho=0.37, p=0.003
VA at Month 24 (ETDRS letters)
E
VA at Month 24 (ETDRS letters)
Rho=0.47, p=0.051
Baseline global GCL (µm)
F
VA at Month 24 (ETDRS letters)
Rho=0.54, p=0.003
Baseline global GCL (µm)

2.12 Challenging Clinical Cases – A Walk Through Supplemental Therapy with Intravitreal Ranibizumab Therapy Following Treatment of Diabetic Macular Edema with the 0.19 mg Fluocinolone Acetonide Implant (ILUVIEN®)[696]

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CASE SERIES

Challenging Clinical Cases – A Walk Through Supplemental Therapy with Intravitreal Ranibizumab Therapy Following Treatment of Diabetic Macular Edema with the 0.19 mg Fluocinolone Acetonide Implant (ILUVIEN®)

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Purpose: There are limited published data regarding the use of supplemental intravitreal therapies in patients with diabetic macular edema (DME) following treatment with the 0.19 mg fluocinolone acetonide (FAc; ILUVIEN®) intravitreal implant. The aim of this report was to analyze five challenging eyes that required supplemental therapies after treatment with the FAc implant.

Methods: This is a retrospective case series conducted at the Centro Hospitalar Universitário do Porto in Porto, Portugal, between 2015 and 2019. It aimed to assess the patient background, treatment history and patient outcomes in challenging clinical cases in which intravitreal injections (IVI) of ranibizumab had been given pro re nata following treatment with the FAc implant (with a minimum follow-up of 33 months). Parameters measured included best-corrected visual acuity in early treatment diabetic retinopathy scale, central macular thickness and intraocular pressure.

Patients: Five eyes (three patients) diagnosed with persistent or recurrent DME and suitable for treatment with the FAc implant according to its licensed indication in Europe.

Results: In the first 2 patients, one bilateral, DME was refractory to IVI of short-acting corticosteroids and anti-VEGF. Following FAc therapy, there was a favorable evolution and a clear regression of diabetic retinopathy (DR) severity. Supplemental treatments were adopted, but a reduced number of treatments were needed beyond three years in these cases. The third case had bilateral DME. One eye had been vitrectomized and FAc therapy led to resolution of DME within 6 months. In the contralateral eye, the control of DME was dependent on anti-VEGF supplemental treatments until a pars plana vitrectomy was performed.

Conclusion: The multifactorial nature of DME means there is a need for an individualized treatment approach to the management of DME. It also explains why some patients need a combined or a more aggressive approach to therapy in order to achieve successful outcomes for the patient.

Keywords: anti-VEGF, diabetic macular edema, fluocinolone acetonide implant, ranibizumab, supplemental therapy

Plain Language Summary

This case series describes three cases from patients with diabetic macular edema (DME) that persisted or recurred despite treatment and were subsequently treated with a 0.19 mg fluocinolone acetonide implant (ILUVIEN®). Patient eyes still proved challenging to manage and required supplemental intravitreal therapies to improve overall outcomes for these patients.

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Introduction

In real-life practice, there are multiple factors contributing to suboptimal outcomes in patients with DME following anti-VEGF therapy (eg, intravitreal injections of bevacizumab [off-label], ranibizumab and aflibercept). These include the multifactorial nature of the underlying disease[1,2] as well as practical elements. For example the inability of physicians to administer therapies according to treatment strategies employed in randomized controlled trials due to the excessive treatment burden (ie, monthly injections in some cases) on ophthalmologists and patients[3] and missed clinical follow-up visits as a result of numerous other hospital appointments resulting in delayed or inappropriate treatment.[4]

The nature of intravitreal corticosteroids means they have angiostatic, anti-permeability and anti-inflammatory effects. The longer duration of effect of corticosteroids means that patients require fewer treatment injections and hospital appointments, and so they present a good treatment option in the management of diabetic macular edema (DME).[4]

Intravitreal corticosteroids are commonly used as second-line treatments for patients with DME.[5,6] Short-acting intravitreal injections (IVI) of corticosteroids included triamcinolone acetonide (IVTA; used off-label to treat DME) and the dexamethasone implant (OZURDEX®), both of which have a duration of action ≤6 months. In contrast, the fluocinolone acetonide (FAc) intravitreal implant (ILUVIEN®) is much longer-acting with a single implant lasting for up to 36 months when injected into the vitreous cavity.

The multifactorial nature of DME may be one explanation as to why some patients may require a combined treatment strategy to achieve the most efficient treatment outcomes,[2,7] especially in the more challenging DME cases where supplemental therapies are required. Indeed, outcomes achieved in real-world practice show that supplemental therapy is required in around 30% of cases following the administration of the FAc implant with a mean time to additional therapy of 356.1±274.8 days.[7] From a clinical perspective, the challenge is to identify patients requiring supplemental therapy.

The aim of this study was to analyze and understand the outcomes from five challenging cases where the FAc implant was administered in combination with IVI of ranibizumab.

Methods

This is a retrospective case series study performed at Centro Hospitalar Universitário do Porto (CHUP), Porto, Portugal, between 2015 and 2019.

At this center, 94 DME eyes (a total of 102 injections) have been treated with the FAc implant. Forty-six eyes of these eyes have 3 years of follow-up. In these, 25 eyes (54.3%) required supplemental intravitreal therapies and in 6 eyes (13.0%) supplementary treatment did not have any additional benefit.

Background disease, treatment history and patient outcomes were captured from five DME eyes (3 patients) with a minimum follow-up of 33 months following injection of the FAc implant. Patients were treated according to the European license and had DME that persisted or recurred despite treatment. Following treatment with the FAc implant, all patients had received IVI of ranibizumab (0.5 mg/0.05mL) as a supplemental therapy.

Data collection included patient demographics, diabetic retinopathy (DR) status and prior DME therapies.

At 1-week post-injection and then at least, every 3 months thereafter, each patient had a complete ophthalmological evaluation. This included an evaluation of best-corrected visual acuity (BCVA), assessed using the early treatment diabetic retinopathy (ETDRS) letter score; central macular thickness (CMT), assessed with spectral domain optical coherence tomography (SD-OCT Topcon 3D-OCT 1000 [used up to May 2015] or a Spectralis HRA + OCT, version 1.10.2.0; Heidelberg Engineering, Heidelberg Germany [used from June 2015]); and, intraocular pressure (IOP). Fluorescein angiography was performed on all patients at baseline and at subsequent time points as and when required.

The study was conducted according to the tenets of the Declaration of Helsinki in its latest amendment (Brazil, 2013) and was approved by the ethics committee of CHUP [2017.093 (084-DEFI/082-CES)]. All patients signed a written informed consent has been provided by the patients to have the case details and any accompanying images published.

Results

Clinical Case 1

A 68-year-old female with type 2 diabetes and diagnosed with center-involved DME in her right eye (RE) in 2012 and left eye (LE) in 2011 (please see Table 1). Comorbidities included hypertension and dyslipidemia. Both eyes were

Table 1 Case 1: Interventions, Treatments and Outcomes

	Year	Figure (Image)	Description of Interventions, Treatments and Outcomes
Case 1, right eye	2012	N/A	Center-involved DME diagnosed
	2012–2015	N/A	Treatments included: macular laser; peripheral laser; 3 injections of IVTA; 4 injections of bevacizumab IOP drops: Timolol (1 week after IVTA)
	2015	Figure 1 (fundus and OCT)	FAc implant injected (June 26, 2015; month 0) Visual acuity: 50 ETDRS letters; CMT: 621 μm
	2015	Figure 2 (OCT)	Treatment: one ranibizumab injection given (in 2015)
	2016	Figure 2 (OCT)	Intervention: cataract removal Treatments: three ranibizumab injections given (one was combined with the cataract surgery) (in 2016)
	2017	Figure 2 (OCT)	Treatment: no ranibizumab injections given (in 2017)
	2018	Figure 2 (OCT)	Treatments: four ranibizumab injections given (both were deemed unnecessary) (in 2018)
	2019	Figure 2 (OCT)	Treatment: one ranibizumab injection given (deemed unnecessary) (in 2019)
	2019	Figure 3 (fundus)	September 2019 (month 51) Visual acuity: 85 ETDRS letters; CMT: 298 μm
Case 1, left eye	2011	N/A	Center-involved DME diagnosed
	2011–2014	N/A	Treatments included: macular laser; peripheral laser; 5 injections of IVTA; 1 injection of bevacizumab IOP-drops: Timolol (1 week after IVTA)
	2014	N/A	Interventions (November 2014): Pars plana vitrectomy with internal limiting membrane peeling combined with phacoemulsification Treatment: 1 injection of IVTA
	2015	Figure 1 (fundus, OCT)	FAc implant injected (July 23, 2015; month 0) Visual acuity: 77 ETDRS letters; CMT: 590 μm
	2015	Figure 2 (OCT)	Treatment: one ranibizumab injection given (in 2015)
	2016	Figure 2 (OCT)	Treatments: two ranibizumab injections given (in 2016)
	2017	Figure 2 (OCT)	Treatments: six ranibizumab injections given (in 2017)
	2018	Figure 2 (OCT)	Treatments: two ranibizumab injections given (both were deemed unnecessary) (in 2018)
	2019	Figure 2 (OCT)	Treatment (January 2019): one ranibizumab injection given (deemed unnecessary)
	2019	Figure 3 (fundus)	September 2019 (month 50) Visual acuity: 77 ETDRS letters; CMT: 265 μm

treated with macular and peripheral laser and unresponsive to prior IVI therapies (LE, 5 IVTA and 1 bevacizumab; RE, 3 IVTA and 4 bevacizumab). In November 2014, the LE underwent a pars plana vitrectomy (PPV) with internal limiting membrane (ILM) peeling combined with phacoemulsification. At this time intravitreal injection of IVTA was administered with no regression of the DME. In mid-2015, changing therapy from IVTA to a FAc implant was considered based on this being a different corticosteroid and that it releases a sustained low dose of fluocinolone acetonide for up to 3 years.[8] FAc implants were injected into both RE and LE.

Figure 1 shows the baseline pre-FAc SD-OCT from the RE and LE.

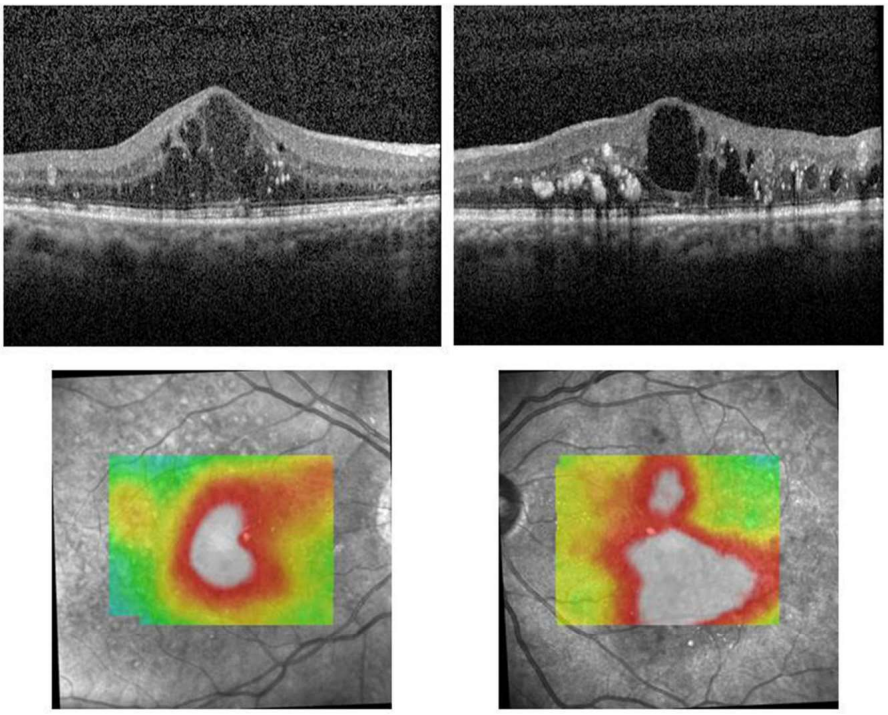

Figure 1 Clinical case 1: baseline pre-FAc therapy SD-OCT from the RE (right panels) and the LE (left panels). The most relevant optical coherence tomography (OCT) characteristics included the presence of outer photoreceptor segment disruption, significant amount of hard exudates (HE), hyper-reflective foci (HF), disruption in outer plexiform layer (dOPL), presence of confluent cysts in outer nuclear layer (ONLc) and in inner nuclear layer (INLc), ONLc in comparison with INLc were particularly more evident in the RE.

Supplemental Treatments Following FAc Therapy in the RE (51 Months of Follow-Up)

At 4, 6, 7 and 8 months post-injection of FAc, IVI of ranibizumab (combined with cataract surgery at month 8) were performed due to persistent DME. No other therapies were required from month 8 to 39 (Figure 2). At month 39, four IVI of ranibizumab were given to manage the recurrence of mild focal DME and no further injections were then required from month 48.

Supplemental Therapies Following FAc Therapy in the LE, Vitrectomized (50 Months of Follow-Up)

The LE was managed in a similar fashion to the RE with the first IVI of ranibizumab given at month 4 (Figure 2). A total of 3 IVI of ranibizumab were required each year, with the last 3 monthly IVI of ranibizumab been given at month 39, with no further treatments up to month 50.

After FAc implant, through more than 48 months of follow-up, 9 and 12 IVI of ranibizumab IVI were considered necessary to control refractory DME in RE and in LE,

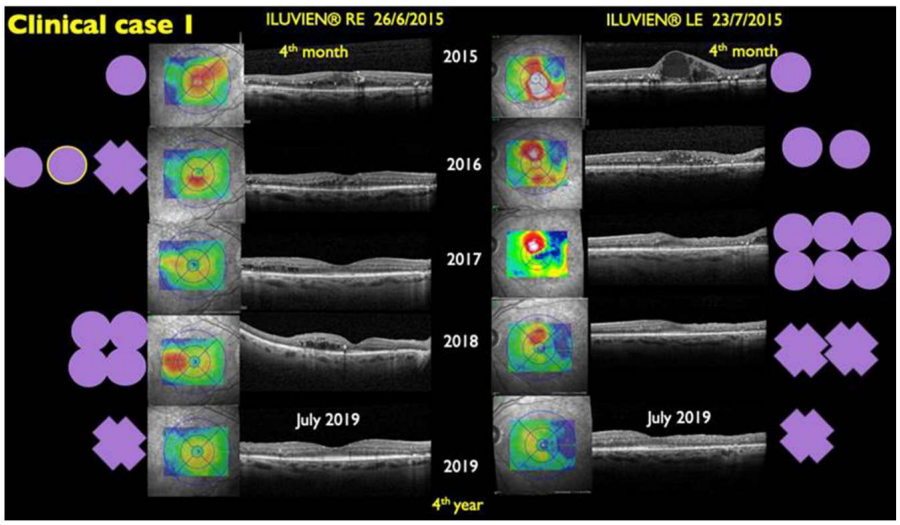

Figure 2 Clinical case 1: SD-OCT images from the RE (left panels) and the LE (right panels) showing the DME evolution from month 4 after the FAc implant was injected and up to the end of the follow-up period (51 and 50 months, respectively). Circles and crosses correspond to ranibizumab injections. The injections represented by the crosses may be considered unnecessary, as there is no significant edema. The circle surrounded by a yellow line was the ranibizumab injection performed at the time of the cataract surgery, in the RE.

respectively (Figure 2), with an improved DR status at the end of follow-up (see Figure 3). There were no visible hard exudates (HE) and an almost normal macular anatomy was observed, although some temporal macular atrophy with external photoreceptor disruption was noticed in the LE.

At baseline, CMT was 621 µm in the RE and 590 µm in the LE and improved to 298 and 265 µm at year 4 post-FAc therapy. BCVA also improved in RE (increasing from 50 letters at baseline to 85 letters at last observation) and LE (increased from 70 to 77 letters). Both eyes received timolol 1-week post-IVTA, due to an elevation in IOP above 21 mmHg, and following treatment remained at ~18 mmHg, which was maintained throughout the follow-up period. Glycemic control also remained stable with glycated haemoglobin (HbA1c) remaining between 7.9% and 8% throughout.

Clinical Case 2

A 60-year-old female with type 2 diabetes and DME described for the first time in the RE in 2011 against a background of severe non-proliferative DR (Figure 4; Table 2). Macular LASER and pan-retinal photocoagulation (PRP) were performed in both eyes in 2011 and in 2012. Comorbidities included hypertension and dyslipidemia. In 2013, after referral to our department, several IV therapies were performed to the RE (4 bevacizumab, 1 IVTA, 3 bevacizumab, 7 ranibizumab, 2 aflibercept and 1 dexamethasone implant, by this order). DME was unresponsive to all these IV therapies. Prior to FAc therapy, administered in June 2016, IVTA evoked changes in IOP were being managed with timolol, brinzolamide and brimonidine. Subsequent therapy with the dexamethasone implant led to a further rise in IOP within one week and surgery (cyclophotocoagulation guided with transillumination) was performed. At this point, IOP was ~16 mmHg and managed without medication. One week after the administration of the FAc, timolol and brimonidine eye drops were required to manage IOP (at ~16 mmHg).

Supplemental Treatments Following FAc Therapy (39 Months of Follow-Up)

Cataract surgery was performed at day 40 with no worsening of DME. Due to persistence of DME, IVI of ranibizumab was added at months 4, 7, 9 and monthly thereafter

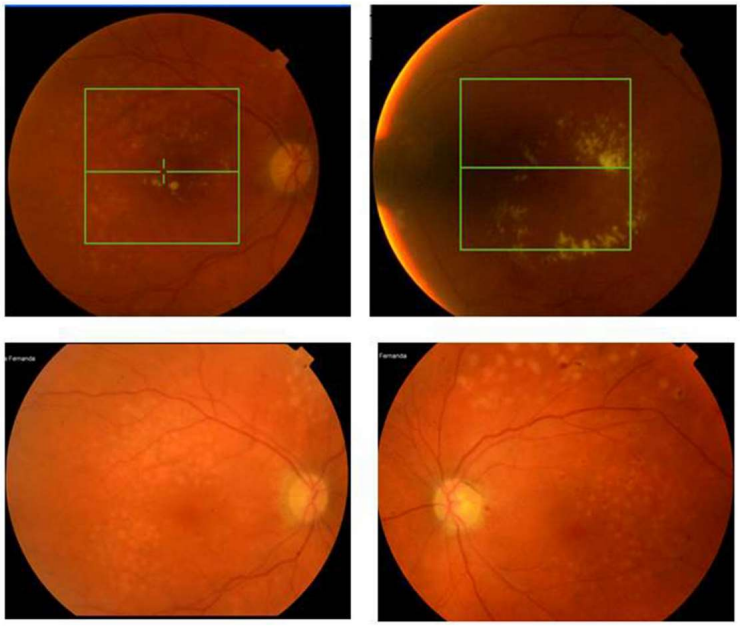

Figure 3 Clinical case 1: Retinography from the RE (top left panel = baseline and pre-FAc implant; bottom left panel = at the end of the follow-up period) and the LE (top right panel = at baseline; bottom right panel = the end of the follow-up period). The patient had moderate non-proliferative diabetic retinopathy (DR) according ETDRS Diabetic Retinopathy Severity Scale (DRSS). There was an improvement in DR severity at the end of follow-up (months 51 and 50, respectively) with the last ranibizumab treatment given at month 48 in the RE and month 42 in the LE.

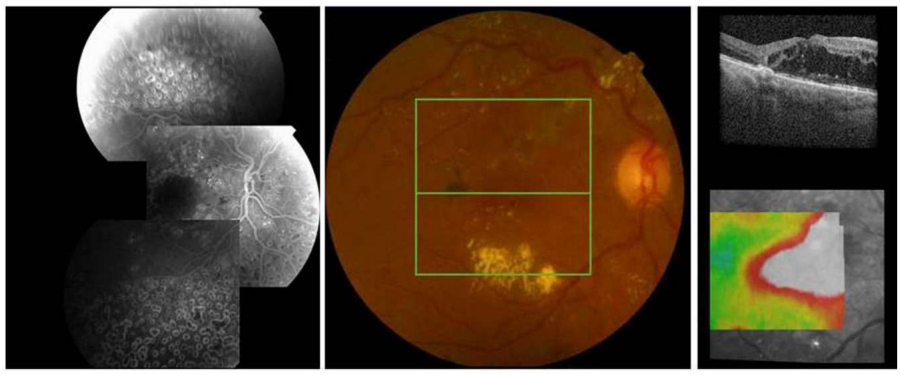

Figure 4 Clinical case 2: A severe non-proliferative DR case with DME that was unresponsive to anti-VEGF and corticosteroids. Left, middle and right panels show fluorescein angiography, retinography and SD-OCT images, respectively, at baseline, pre-injection of the FAc implant. The SD-OCT shows external photoreceptor disruption, a significant amount of HE, HF, dOPL, presence of confluent and expressive ONLc and not so relevant INLc (Figure 4, right panel).

Table 2 Case 2: Interventions, Treatments and Outcomes

	Year	Figure (Image)	Description of Interventions, Treatments and Outcomes
Case 2, right eye	2011	Figure 4 (fluorescein angiography, fundus, OCT)	Center-involved DME diagnosed against a background of severe non-proliferative DR
	2011	N/A	Macular LASER and pan-retinal photocoagulation was performed bilaterally
	2012	N/A	Macular LASER and pan-retinal photocoagulation was performed in bilaterally
	2013	N/A	Treatments: 4 injections of bevacizumab, 1 IVTA, 3 bevacizumab, 7 ranibizumab, 2 aflibercept, 1 dexamethasone implant (in this order) IOP-drops: Timolol, brinzolamide, brimonidine (post-IVTA) IOP-surgery: Cyclophotocoagulation (within 1-week of the dexamethasone implant injection)
	2016	N/A	Visual acuity: 40 ETDRS letters; CMT: 557 μm FAc implant injected (June, 2016; month 0) IOP-drops: Timolol, brimonidine (one week post-FAc implant injection)
	2016	N/A	Intervention (July 2016): Cataract surgery was performed at day 40
	2016	Figure 5 (OCT)	Treatment (October 2016): 1 ranibizumab injection
	2017	Figure 5 (OCT)	Treatments (March 2017): 8 ranibizumab injections (started)
	2017-2018	Figure 5 (OCT)	Treatments (November 2017): 5 ranibizumab injections (started)
	2019	Figure 6 (fundus, OCT)	September 2019 (39 months) -DR status: improved Visual acuity: 72 ETDRS letters; CMT: 251 μm

up to month 20 with two more injections given at months 23 and 24 (Figure 5). A total of 6 and 10 IVI of ranibizumab were added in year 1 and year 2, respectively. From month 24, the macula was dry and no supplemental therapies were required. The reduction in edema was accompanied by an improvement in DR status with no HE and a nearly normal central macular anatomy (Figure 6).

CMT and BCVA were 557 μm and 40 letters at baseline, respectively, and improved to 251 μm and 72 letters by month 39 post-FAc injection. At baseline the patient had poor glycemic control (HbA1c=8.5%) and progressively improved to 7.7% at the end of follow-up period.

Clinical Case 3

Fifty-six-year-old male, type 2 diabetic, diagnosed with bilateral DME in July 2014. The RE had severe non-proliferative DR and the LE had proliferative DR (please see Table 3). Both eyes were pseudophakic. Comorbidities included hypertension, dyslipidemia, stroke (in 2012) and acute myocardial infarction (2005). Both eyes were treated with macular (in 2014) and PRP (March 2015). The RE underwent PPV with ILM peeling in September 2014 due to an epiretinal membrane (ERM). RE had been treated with 1 IVTA in 2014 and with 2 IVTA in 2015 and LE with 1 IVTA in 2014 and another in 2015 with incomplete responses observed. Figure 7 shows the RE and LE status observed pre-FAc.

Supplemental Treatments Following FAc Therapy in RE (33 Months of Follow-Up)

The FAc implant was given in December 2016 and DME was controlled without additional therapy, although some mild recurrences were observed. At month 33, the macula was dry with a CMT of 256 μm, decreasing from a baseline of 763 μm. BCVA increased from 40 to 77 letters and IOP was stable (~12 mmHg) with no medication required (Figure 8).

Supplemental Therapies Following FAc Therapy in LE (33 Months of Follow-Up)

FAc therapy was started in January 2017 and supplemental IVI of ranibizumab every 1.5 months were needed to control DME between May 2017 and December 2017. A fluorescein angiography in September 2017 showed no evidence of new vessels or ischemia and up to April 2018 no supplemental IVI of ranibizumab were required. Severe recurrence of DME led to further supplemental IVI of ranibizumab every 1.5 months, which were ineffective. In May 2019, a PPV was conducted with concomitant

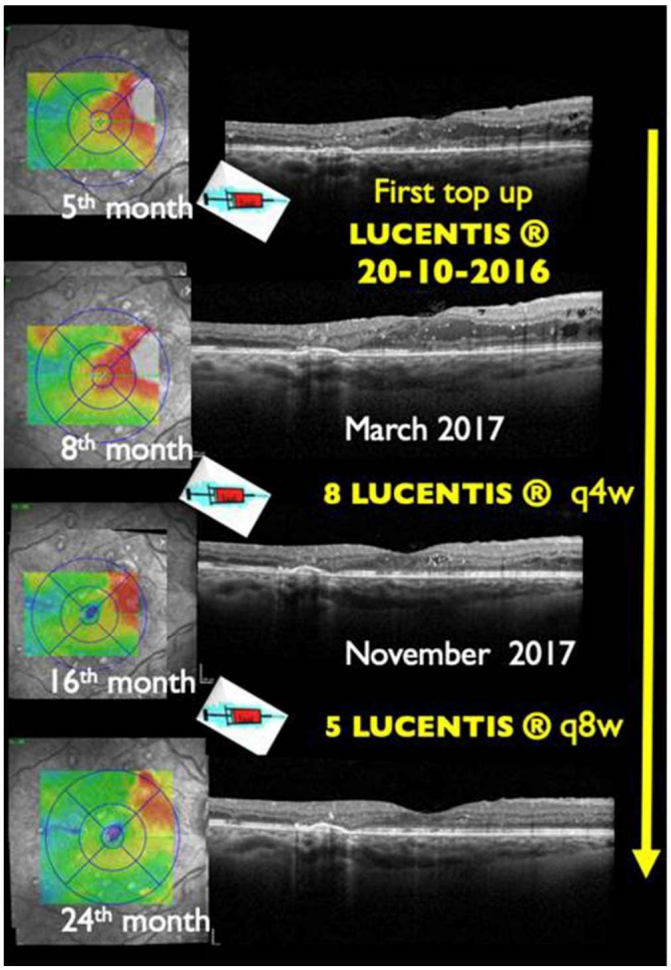

Figure 5 Clinical case 2: SD-OCT images showing the evolution while top-up treatment approach was undergone. A total of 14 IVI of ranibizumab were added between months 5 and 24.

confluence of PRP (see Figure 8, top panel). By month 33 (ie, 3 months after PPV) the macula was dry (CMT decreased to 251 µm from 631 µm at baseline), BCVA improved (to 73 letters from 50 letters) and IOP remained stable (~10 mmHg) without medication. During this period, glycemic control was quite varied and at the end of the follow-up period HbA1c had decreased to 6.1% from 11.6% at baseline.

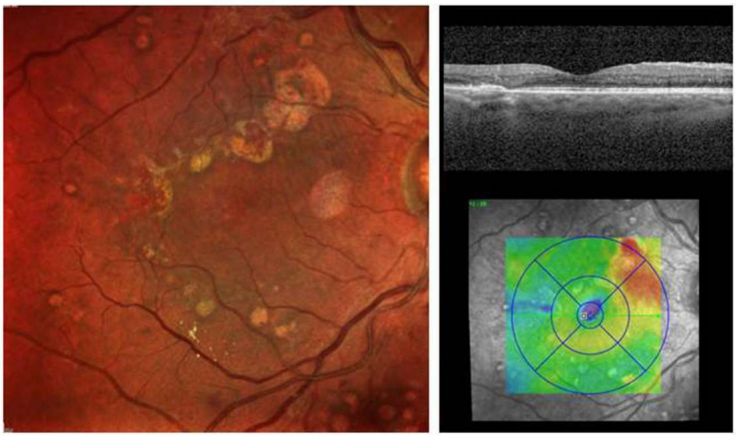

Figure 6 Clinical case 2: Multi-Color fundus image shown at the end of follow-up (month 39) and shows an improvement in DR status (left panel). The SD-OCT image (right panel) shows the DME control without any further treatments beyond month 24. DME was still under control up to 39 months (3 months beyond the duration of release) after the FAc implant had been given.

Discussion

The FAc implant is licensed for the treatment of DME that is insufficiently responsive to available therapies. The current review of challenging DME case series shows that DME can be effectively managed over the longer-term with the FAc implant when combined with

Table 3 Case 3: Interventions, Treatments and Outcomes

	Year	Figure (Image)	Description of Interventions, Treatments and Outcomes
Case 3, right eye	2014	N/A	Pseudophakic lens, center-involved DME diagnosed; severe non-proliferative DR
	2014	N/A	Treatments: Macular LASER, 1 injection of IVTA
	2015	N/A	Interventions: PPV with ILM peeling due to epiretinal membrane Treatments: Pan-retinal photocoagulation, 2 injections of IVTA
	2016	Figure 7 (fundus, fluorescein angiography, OCT)	Visual acuity: 40 ETDRS letters; CMT: 763 μm FAc implant injected (December 5, 2016; month 0)
	2017–2019	Figure 8 (fluorescein angiography, OCT)	Treatments: No supplemental therapies required
	2019	Figure 8 (fluorescein angiography, OCT)	September 2019 (month 33) Visual acuity: 77 ETDRS letters; CMT: 256 μm
Case 3, left eye	2014	N/A	Pseudophakic lens, center-involved DME diagnosed; proliferative DR
	2014	N/A	Treatments: Macular LASER, 1 injection of IVTA
	2015	N/A	Treatments: Pan-retinal photocoagulation, 1 injection of IVTA
	2017	Figure 7 (fundus, fluorescein angiography, OCT)	Visual acuity: 50 ETDRS letters; CMT: 631 μm FAc implant injected (January 5, 2017; month 0) Treatments (January 27, 2017): Pan-retinal photocoagulation, 5 ranibizumab injections (in 2017)
	2018	Figure 8 (fluorescein angiography, OCT)	Treatments: 5 ranibizumab injections (in 2018)
	2019	Figure 8 (fluorescein angiography, OCT)	Treatments: 2 ranibizumab injections (in 2019) Intervention (May 16, 2019): PPV
	2019	Figure 8 (fluorescein angiography, OCT)	September 2019 (month 33) Visual acuity: 73 ETDRS letters; CMT: 251 μm

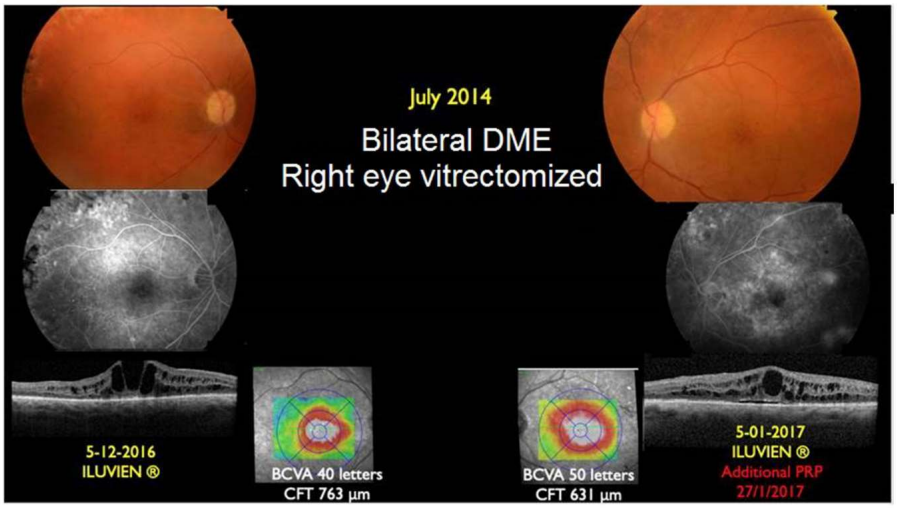

Figure 7 Clinical case 3: Images shown of the RE (left panel) and LE (right panel). Retinography (top panel of images), fluorescein angiography (middle panel of images) and SD-OCT (bottom panel of images). The most relevant OCT characteristics included the presence of outer photoreceptor segment disruption, dOPL, presence of central confluent ONLc and INLc, with a relatively equivalent distribution of cysts between both nuclear layers in both eyes. The case shows recurrent DME as the patient case was responsive to IVI of triamcinolone and PRP. Patient lived a long way from the hospital, had PDR in the LE (i.e. the non-vitrectomized eye).

supplemental treatments. A better knowledge of the patient's clinical background (patient characteristics and demographics, as well as the clinical condition of the patient) and results regarding supplemental treatments after the administration of the FAc implant is important to the treating physician as it provides insights into the timing of these therapies and the decision process involved. These are all important considerations that are not well documented in the literature but essential for making informed decisions about supplemental therapy.

Analyzing our first two clinical cases, both with more than 36 months of follow-up, it may seem controversial to have given additional therapies, particularly in case 1. Nevertheless, these cases were refractory to short-acting IVI of corticosteroid and anti-VEGF. With the treatment strategy adopted, we obtained a clear regression of DR severity, with a complete disappearance of the HE, a dry macula and treatment interval free from supplemental therapies lasting 3 (RE) and 11 (LE) months in case 1, and 15 months in case 2. This is particularly relevant in case 1, with four years of follow-up post-FAc implantation. In the LE of case 1 it can be also inferred that PPV with ILM peeling may be the explanation for the temporal macular atrophy and the reduced gain in visual acuity.[9] It is unlikely that differences between LE and RE are explained by changes in the clearance of FAc from the vitreous of the eye as past studies report similar outcomes in vitrectomised and non-vitrectomised eyes.[4,10]

In the third clinical bilateral case treated with the FAc implant, the different DR and vitreous status may be the explanation for the different response to treatment. In the RE, after the FAc implant, DME was effectively controlled without any additional treatment as opposed to LE with DME that was controlled with additional IVI of ranibizumab. At baseline, the RE was vitrectomized with PRP and a non-PDR status whereas the LE was not vitrectomized and received incomplete PRP and had PDR. After FAc implantation, despite supplemental PRP and the disappearance of the retina neovascularization according to the fluorescein angiography repeated 9 months post-injection of the FAc implant, the response of LE to treatment was not so favorable comparable with the RE. Nevertheless, after vitrectomy and confluence of PRP, at 33 months after the FAc implant was administered, a complete resolution of DME was achieved after 3 months. Probably VEGF

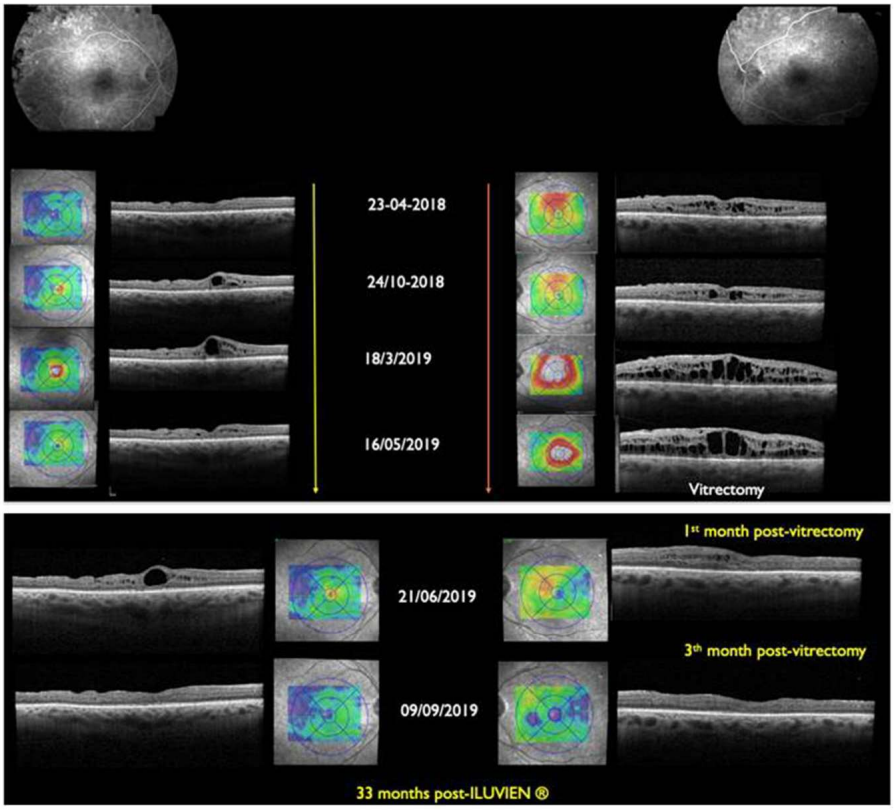

Figure 8 Clinical case 3: top panel: images of the RE (on the left side) and LE (on the right side) fluorescein angiography, on the top, and SD-OCT images, below. Both angiographies revealed a non-PDR DR status. In the vitrectomized eye (RE), although with some mild recurrences, the DME was controlled without additional therapy. In the LE, a total of 12 IVI of ranibizumab were administrated (5 in 2017, 5 in 2018, and 2 in 2019), with a complete response only within the first 5 anti-VEGF injections. A more evident epi-retinal membrane was noticed during the follow-up. Bottom panel: SD-OCT images from the RE (in the left side) and from the LE (in the right side) following vitrectomy. DME in the RE was still maintained under control and the LE followed the same pattern of evolution 3 months after vitrectomy.

levels were in fact increased in LE. That would explain the response to top-up ranibizumab and the absence of neovascularization at the time of the angiography (window effect during anti-VEGF treatment). Vitrectomy could also represent a way for the removal of angiogenic, inflammatory and tractional factors, and possibly with PRP confluence contributing to enhanced DME control.

In the third patient, the RE is an example, which illustrates that DME relapses during the 36 months after the FAc implant and shows it could be controlled without any additional treatment. A mild DME recurrence appeared in the second year after administration of the FAc implant and resolved in a period of 5 to 6 months.

Conclusion

This analysis intends to stress the importance of an individualized approach for DME, which is a multifactorial disease that is not fully understood, where many local and systemic factors (demonstrated by similar behavior in bilateral cases) may influence the course, presentation and the response to treatment. The challenging cases presented here show that DME can still be effectively

managed over the long term with the FAc implant combined with supplemental treatments as and when required.

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Disclosure

None of the authors has any conflicting interests to disclose for this work. B. Pessoa had full access to all the data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis.

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3. Global Discussion

The present work aimed to highlight the major role of vitreous as an essential piece to face the multifactorial, the multiple layers, complex and systemic nature behind DR physiopathology which transforms DME, the main cause of vision loss in DR, in a most challenging disease.

The two main causes of severe vision loss in DR are DME and PDR. In fact DME approach is inseparable of the DR severity as it can be present at any stage, although with higher prevalence in more severe DR forms[83, 293]. This implies that, the approach is expeted to be different in accordance to severity stage, including macular and peripheral ischemia[293].

As a first step of this journey, we have used the easy access of vitreous cavity at the time of vitrectomy to treat tractional DME in diabetic and non-diabetic patients as a way to collect vitreous, and information about its correlation with DR and DR severity with the intention to discover new biochemical biomarkers, as well, potential targets for future therapies[689].

As mentioned, growth and inflammatory factors identifiable and measurable in both aqueous and vitreous fluid samples are not necessarily linearly related[517].

Thusly, our approach of vitreous can also be considered more accurately representative of pathophysiological events in the retina in comparison with aqueous humour, although its sampling is considered too invasive to be used in regular clinical practice, except for vitrectomy applicants[697].

In addition, we attempted to overcome some of the limitations of many studies in this field, such as the low number of sample sizes, the limited number of growth and inflammatory factors investigated and inclusion grouping criteria. The majority of the studies compare PDR vs other several types of non-pure inflammatory diseases, such as retinal detachments, vitreous haemorrhages sub-luxated lens, macular holes among other examples, assuming that these pathologies may me comparable as a group control[519, 522-524, 528, 549, 698].

As an example, according Yoshimura et al, IL-6, IL-8 e MCP-1 are commonly upregulated in many vitreoretinal diseases DME, PDR, BRVO, CRVO, and RRD[486]. High levels of MCP-1 have also been reported in the vitreous of PVR (a major complication of RD surgery)[522, 699].

We cannot simply assume that the measurements obtained are higher or lower in comparison with other lesser severe DRSS stages. Another possible bias is related to the fact that, usually, vitreous haemorrhage is not an exclusion criterion in PDR cases and the compromise of the BRB may induce leakage of serum/plasma proteins into the vitreous, diluting the amount of cytokines[167, 517, 519, 698, 700].

Furthermore, an elevated intravitreal level of a particular protein is not necessarily a result of intraocular production and might simply reflect a nonspecific increase in protein levels due to serum diffusion[506]

In our study[689], notwithstanding the limitations induced by inherent BRB disruption in this kind of patients, we excluded vitreous haemorrhage presence, and choose a control group with the same main pathology (macular pucker) except for diabetes variable, gathering a balance accepted number for both groups and all the surgical procedures were performed by the same surgeon, with no other therapy, at least, performed in the 4 months previously to study inclusion. We are conscious that the exclusion of vitreous haemorrhage brought itself another limitation which is the possible exclusion of mores severe cases inside PDR sub-group. *Funatsu et al*. divided PDR patients into subgroups based on disease progression or regression after vitrectomy, and found that the vitreous levels of VEGF and IL 6 were significantly higher in the eyes of patients in the progression group compared with regression group[701].

Apart from VEGF already extensively study, we orientated this investigation around 17 potential inflammatory mediators that have been implicated in progressive retinal endothelial dysfunction, neurodegeneration, or in the counteracting side of neurovascular protection.

We found significantly greater amounts of IL-6, IL-8, MIG (CXCL9), IP-10 (CXCL10), EPO, and IL-17 in vitreous samples from diabetic eyes, which was consistent with the literature[498, 506, 525, 556].

Vitreous IL-6, IL-8, MIG, and IP-10 levels exhibit the greatest coherent association with DR severity prediction and thus have the best potential post vitrectomy prognostic usefulness of the biomarkers investigated. There is robust evidence supporting that IL-6 and IL-8 not only contribute to DR development, but also correlate with more severe disease stages, especially in PDR and DME[493, 521, 525, 526]. In contrast to IL-8 and IP-10, which were associated with a greater CFT but not with poor visual performance, IL-6, EPO, and MIG appear to be linked to a more severe central macular lesion with worsening visual function. Additionally, our results[689] favor the association between the levels of the potent inhibitors of angiogenesis produced by retinal cells, MIG (CXCL9) and IP-10 (CXCL10), with the increase of DR severity, as a mechanism to counteract the angiogenic effect of VEGF and other proinflammatory cytokines[498, 506, 689].

Although several studies previously reported increased levels of TNF-alpha, and MCP-1 (also observed in proliferative vitreoretinopathy) in DR patients, particularly in PDR eyes, they were not found elevated in our study. In accordance with our research[689], in PDR eyes, Loporchio et al did not reported TNF-alpha or MCP-1 elevated levels as well as ***Rusnak et al*** regarding MCP-1 levels[486, 523, 699, 702-706]

It is important to highlight that *Rusnak et al* compared, in their study, three groups based on the severity of PDR (PDR without the need for repeated surgical intervention; repeated vitreous bleeding; and refractory neovascular glaucoma) with a non-diabetic control group, as

well as aqueous humour collected samples. Only levels of IL-6,TGFβ-1, and VEGF were correlated with the severity of PDR (EGF- epidermal growth factor, IL-6, VEGF, TNF-α, IL-8, IP-10, MCP-1, PDGF AA, TGFβ-1, fractalkine, PDGF AB/BB, IL10, IFN- γ , FGF-2, CNTF-CNTF - ciliary neurotrophic factor, BDNF- Brain-derived neurotrophic factor , and RANTES)[707].

Intravitreal increased levels of MCP-1 (with concomitant increase of IL-6 and significant decrease in IL-8) have been observed after a successful vitrectomy for PDR with postoperative DME[523, 699, 706].

In a subsequent analyses of the few post-vitrectomy DME cases from our study population (n=11 in diabetic and n=2 in non-diabetics) no statistically significant differences were observed regarding any of the biomarkers analyzed ($p>0.05$)[689].

The different prevalence of the main disease pathways, neurodegeneration, edema, ischemia, and fibrovascular response in diabetic patients, as well as the balance of counteracting antiangiogenic, neurovascular protector substances, grade of lesion in the producing cells of these biomarkers, BRB disruption and its effect on their dilution, may all play a role in the differences in results between studies[356, 523, 706].

This is the bases for the controversy around EPO, its levels in the tissues of diabetic patients, namely in the retina, and its influence on the progression of DR (aggravation or protection)[556].

Our achievements and bibliographic framework helped to clarify its role in DR, as a pathogenic factor in PDR and more severe stages, and simultaneously a protective factor in early DR[689, 708-711]. EPO is a hematopoietic cytokine that promotes proerythroblast survival and maturation and is recognized as a member of the cytokine type 1 superfamily, which provides direct protection against hypoxia by its anti-apoptotic, anti-inflammatory and anti-oxidative properties, having additionally angiogenic capacity that allows the oxygen supply to ischemic tissues[712-714].

Several studies have found that EPO protects retinal ganglion cells, photoreceptor cells, and retinal pigment epithelial cells from apoptosis[715-724].

Although mainly produced in the kidneys, and near 10% in the liver, *Hernandez et al*. suggested that EPO is produced locally in the retina. Muller cells and retinal pigment epithelium (RPE) were identified by Fu et al. and Garcia-Ramírez et al., respectively, as the cells responsible for EPO production in the eye[45, 725, 726].

It acts by binding to transmembrane erythropoietin receptors (EPOR), which are primarily found on hematopoietic cells, but can also be found on the endothelial, myocardial, neural cells, and as well on the cells of liver, uterus, and retina[556, 726].

An increase in the number of erythropoietin receptors on retina cells in DR has been confirmed, which seems to be a compensatory response to tissue hypoxia and hyperglycemia during DM. Under these conditions, the increased production and binding of EPO to erythropoietin receptors (EPOR) is a mechanism of survival of retinal nerve cells[727]. EPO exert additionally negative proinflammatory and proangiogenic actions activating angiotensin I/II expression and VEGF pathway[711].

Although PDR is expected to generate increased vitreous levels of EPO as a compensatory angiogenic response to DR ischemia, EPO levels may not increase in the presence of a substantial ischemic retina or severe RPE lesion (as in widespread PRP).

This is the explanation for the absence of a significant in EPO vitreous levels with progression of DR, in our and other investigations[689, 728].

Due to a lack of a control group with less severe DR for comparison, the seemingly contradictory conclusions of other research where vitreous EPO levels are increased in PDR stage when compared to non-diabetic eyes may lead to the misleading conclusion that EPO levels are highest in the proliferative end stages of DR.[717, 729, 730].

The diversity of end stage degree for diabetic nephropathy and autonomic neuropathy[135-137] explains different results among studies, our achievement of a significant lower level of serum EPO in PDR stage in comparison with sDR stage[689], the absence of significant differences between diabetic and non-diabetic patients as reached by Semeraro et al, or even significantly elevated serum concentration of EPO in the advanced stages of DR as PDR, according to Davidović et al[556, 726, 727, 731].

The only blood biomarker significantly increased in diabetics compared with non-diabetics was TTR[689]. This is a protein mainly synthesized in liver (90 %) and choroidal plexus, being also produced in pancreas, placentae and in the eye (RPE, ciliary epithelium, iris epithelium, corneal endothelium and lens epithelium)[558, 732-736].

TTR appears to regulate the key genes for DR neovascularization, including Tie2, VEGFR1, VEGFR2, Angpt1, and Angpt2, and seems to repress neovascularization response in DR. In vitro there are evidences that TTR prevents DR progression[544, 558, 736]. According to Miao ZHUANG et al, the concentration of serum TTR is increased in early DR patients and steadily decreases with the advancement of DR[737].

Although there are reports associating serum and vitreous TTR levels with DR progression that achievement was not significant in our research[557, 689].

Nevertheless, in diabetic group serum TTR has a moderate positive correlation with microproteinuria, which elevated levels are also point as a reliable marker of retinopathy and increased risk for PDR, even though, our results have not accomplish that correlation[689, 738].

VitD is another molecule that has been associated with anti-inflammatory, immunomodulatory and neurovascular protective function, with relevance in DR pathophysiology and possible

association of lower serum levels of VitD with an increased risk of DR or more severe DR[552-555, 739-744].

Conversely, vitreous levels of Vit D are much less explored. There is investigative evidence of a local retinal positive effect of vit D against DR by inhibiting high-glucose-induced activation of the ROS/TXNIP/NLRP3 inflammasome pathway after intramuscular injections in rats[741]. According to research in enucleated rabbit eyes, Vit D was suggested to be synthesized in corneal epithelium following UV-B exposure, with aqueous and vitreous levels most closely matching plasma levels[745].

There was no connection between serum Vit D levels and DR or DR progression in our study. In the same way, no correlations were observed between serum and vitreous levels in neither in DM nor in non-DM patients, for Vit D, TTR and EPO[689].

To our best knowledge this is the first publication comparing Vit D vitreous and serum levels in diabetic and nondiabetic patients[689].

Thus, in our study neither vitreous VitD nor serum VitD seem to be appropriate biomarkers or to have a clear relationship with DR[689].

Unlike the continuously replenished fluid-like aqueous humor in the anterior chamber of the eye, the gel-like vitreous humor is more stagnant[186, 247, 1677–1684]. Although the vitreous lacks its own vasculature, surrounding blood vessels from retina and ciliary body nourish the vitreous with hyaluronic acid, prealbumin, transferrin, glycoproteins, and, in low quantities, hundreds of other proteins[746, 747]. (A sluggish turnover of hundreds vitreous components, local production of inflammatory mediators, ischemic stimulus, lesioned target cells, besides BRB disruption, all together may enclose the explanation for higher levels of these three proteins in comparison to serum.

With a year of follow-up, we were able to add some innovation to our prospective investigation by attributing extra prognostic and predictive knowledge to specific vitreous content molecules**.** Increased vitreous levels of EPO, as well IL-6 and MIG levels revealed to have a negative prognostic anatomical and functional value through CFT and BCVA outcomes. The observation of a similar negative moderate correlations of IL-8 and IP-10 and the final CFT but not with the final BCVA may preclude that their increase may precede a more severe DR disease, before functional compromise. Thus IL-6, EPO and MIG vitreous increased levels may anticipate a more severe central macular lesion[689]

According to our results[689], and in line with the literature, one year after VIT a higher incidence of post-VIT ME occurred in DME patients (24.4%) comparatively to patients without diabetes (4.8%)[690, 748-750]. Nonetheless a relatively low percentage of cases required treatment: 10.9% to 13.3%[689] in DM patients versus 0% in non-DM patients[689, 690].

In a different perspective is the vitreous mechanical interaction and influence on retina structure and function.

Different sub-types of thick visible vitreous macular interface, which can be easily identified with SD-OCT, may also have a relevant prognostic and predictor role in DME management.

According an OCT-based classification proposed by *Panozzo G. et al.* there are different morphology subtypes of epiretinal membranes (a well-defined and continuous hyper-reflecting line over the inner retinal surface with at least one point of adhesion to the retina) that may exert an effect of tangencial or anterior-posterior traction on the retina, with increased grades of severity[751].

The results of herein exposed research[690] based on this classification and grouping traction types in 3 groups: VMT (anterior-posterior traction, T3), ERM (tangencial traction, T1 or T2) and VMT plus ERM (mixed tangencial and anterior-posterior traction), lead to the identification of **VMT (T3) as a negative prognostic factor**. The greatest BCVA improvements were seen in the VMT subgroup, but the highest final BCVAs were observed in the subgroups with the presence of ERM[690].

With regard to anatomical and functional outcomes, globally, vitrectomy for tractional DME induces a CFT decrease more significantly until the 3rd to 6thmonth and BCVA reactively thereafter, increases in the first 6 months after regression of DME[690].

Afterwards there is a trend for BCVA stabilization with a slightly continuous decrease in CFT in PDR patients[689], This may reflect a progressive and acelarated neurodegeneration process, with inner retinal layers thickness decrease[404] particularly in more advanced DR stages, with further implications in future treatments and outcomes[102-104, 282, 404, 695].

Not all vitreo macular adhesions are equal, even when no clear traction is objectivated.

Hence, as pathway for our investigation, we used OCP IV injection with the purpose to obtain the release of focal VMA without the need for vitrectomy, more invasive, with inherent anesthesia and procedure risks[752-759].

Since 2017, our group started to perform OCP injections using the guided method of injection, under microscope visualization and proximally to VMA , with the purpose to increase the rate of the VMA release[663, 760-762].

What our research with OCP in DME linked with near focal VMA (< 2500) revealed was that the absence of that sort of VMA appears to lower anti-VEGF IV injection burden, resulting in more efficiently and long-lasting DME managemen[248,71].

Even so, we believe that in more chronic cases where inflammatory and angiogenic factors are more preponderant in the pathogenesis of the DME, VMA release can be unsuccessful.

We obtained a relatively high percentage of focal VMA resolution of 60.9%, with a guided injection method and in a specific subgroup of diabetic patients (as the majority of the series included mainly nondiabetics)[690, 763].

The exclusion of patients with ERMs, known to be a negative prognostic factor for the efficacy of ocriplasmin is an expectable contributor for that higher rate of efficacy[764].

The positive effect in DME may be obtained by the release of a focal VMA even without a PVD achievement. Even-though some authors have highlighted that the presence of posterior vitreous detachment (PVD) has a positive effect on the evolution of DME[202, 238, 241, 243, 245].
Based on evidence that vitreoschisis, or abnormal or incomplete posterior vitreous detachment (PVD), is common in diabetic patients and plays a role in the pathogenesis of diabetic macular edema (DME), and that standard SD-OCT, 20x20º, is not completely accurate in diagnosing the true status of VMI, particularly when the adhesion cannot be seen, [231, 232, 254, 765, 766], our research yielded fresh insights into the genuine vitreous retinal interaction, which may conceal key clues for an optimal DME treatment[141, 233, 254, 766].
Vitreous adhesion to the posterior pole is in fact extremely common in individuals with DME and a focal VMA occurs in only a small percentage of cases.[252, 254] Furthermore, when compared to SD-OCT 55x35º, which was 100 percent in accordance with US, SD-OCT 20x20º revealed to be inaccurate for the diagnosis of VMA status (detecting less than 50% of VMAs). As a result, SD-OCT 55x35º should be considered a required exam for DME patients, at the very least as a baseline approach, or in the event of aggravation during follow-up. (Fig. 3.1)

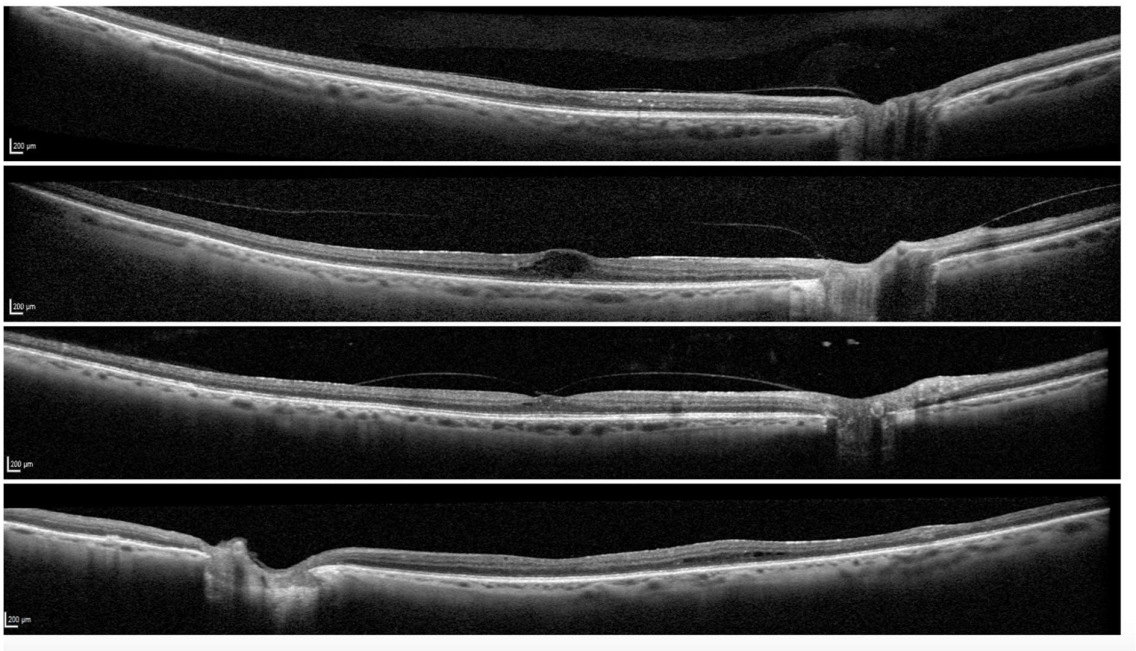

Figure 3.1: Different posterior vitreous status on 55ºx35º SD-OCT, that may be undetectable or barely undetectable in SD-OCT 20x20º: from top to bottom, complete posterior vitreous attachment; anomalous PVD with papillary adhesion; anomalous PVD with foveal adhesion and complete posterior vitreous detachment.

In order to validate RBZ as a non-inferior anti-VEGF choice for the design of one of the major studies of this thesis, the one year prospective study where RBZ effect, as anti-VEGF first line therapy in DME[53], was evaluated through several OCT and OCT-A predictor and predictive biomarkers in Vit and non-Vit eyes, we set out to analyze the outcomes of IV RBZ or AFL after BEV in DME unresponsive to BEV[19, 256, 282, 404, 692, 693].

The purpose was to avoid a possible additional bias using a different drug even being from the same class.

Among the approved anti-VEGF on-label products, RBZ is the one with more safety and efficacy data in the long term[393, 626, 767-774]. The possible relevant bias of the potential non-ideal dose used for ranibizumab in protocol T is extensively debated in our publication[692] and that could be the reason for relatively inferior results during the first year favoring AFL, including the cumulative effect along the 2 years as the area under curve (AUC) for BCVA[626].

Even though the 2 years result of protocol T have shown that there were no statistically significant differences between ranibizumab and aflibercept, regarding CFT decrease and BCVA improvement.

The consensual worldwide guidance statement indicating that only patients with ≤ 69 effectively benefit from on label drugs, based on protocol T results, is a matter of concern. BEV was less efficient in comparison with RBZ and AFL in achieving a BCVA gain in patients with less than 69 letters at baseline and in reducing macular edema (even in patients with a good baseline BCVA, >69 letters)[575, 626]. Among eyes with persistent DME, eyes assigned to BEV were more likely to have chronic persistent DME than eyes assigned to AFL or RBZ[575]. Furthermore, as observed in the sub-analyses of DRCR.net protocol I the long-term (3-years) VA outcome is significantly worse in eyes with chronic persistent edema than in eyes with shorter lasting edema (mean BCVA improvement from baseline to 3 years: 7 vs. 13 letters).

First comes the anatomical lesion and then the functional impact. DME has a cumulative negative effect, which explains why there is no direct association between CFT and BCVA[303, 394].

Therefore, a higher CFT even without correspondence in a BCVA decrease certainly should not be the goal of a proper therapy. BCVA is the result of reversible or irreversible (as “E” layes disruption) anatomical lesions during the DME evolution.

Additionally, CFT is not prognostic or predictive of final visual acuity[303]. As an example, according to *Pelosini L et al*. the cross-sectional area of retinal tissue between the plexiform layers in CME is a better predictor of visual acuity than macular thickness (80% versus 14%)[390].

This as many other biomarkers we choose for the outcome analyses across the several studies discussed hereafter intends to be a major foundation for this thesis, including the

mainstay ESASO biomarkers and vitreous status[282, 399, 404, 692-695]. They may affect the outcomes of the studies based on simple CFT and BCVA, as following will be described[692].

The seven ESASO biomarkers (SRF, DRIL, ELd, ELMd; HRF; IRC; VMI), OPLd and HE were evaluated at baseline before the moment of switch in non-responders to BEV to either RZB or AFL. Both CFT and BCVA improved with RBZ and only CFT improved with AFL. CFT improved equivalently with RBZ and AFL.

Besides the significant higher percentage of DRIL in AFL group, no other demographic or baseline characteristics were significantly different between groups. Even though the higher prevalence of baseline DRIL in the AFL group did not parallel a lower baseline VA acuity in comparison with the RBZ group (baseline VA was similar in both groups).

With a higher number of patients, a regression analyses would then be possible a to stablish a valid relationship between baseline DRIL and eventual other factors, as VMI (ERM without inner retinal distortion and VMA - at least 1/3 of vitreomacular detachment from the macular area), with final outcomes. DRIL has emerged as an important biomarker to explore in studies with larger numbers, as clinical trials.

Hence RZB and AFL revealed superiority in comparison to BEV and no superiority was observed between AFL and RBZ.

An important goal of this thesis was to increase the understanding of the clinical applicability of OCT and OCTA biomarkers in daily practice, beyond the limited BCVA and CFT.

Among these biomarkers there is more scant data about their prognostic and predictive role in vitrectomized eyes with regard to IV therapy with anti-VEGF and CCT.

Hereafter the key findings of our research in this topic will be described.

With the purpose to investigate the behavior of OCT and OCTA choroidal parameters (CT, CCD and CVI) **in the context of** DME cases treated with IV RBZ, in a PRN regimen, the outcomes in vitrectomized versus non-vitrectomized eyes have been compared in prospectively during 6 months of follow-up[282].

As previously described **our results also sustain that those choroidal parameters are not significantly affected by vitrectomy itself, at least 6 months after vitrectomy, the minimum accepted period for inclusion** criteria (with ILM peeling, performed in the majority of the patients)[775, 776]. That explains the absence of significant differences between groups regarding those choroidal biomarkers. The absence of no other baseline differences between groups also strengthens our achievements[282].

According to the literature SFCT had a positive significant correlation with the CFT and total MV and the amount of thickened choroid may be an indicator of undertreatment, a lower CVI can be considered a sign of increased chorioretinal inflammation and progression of DR, although DME seems not to be linearly correlated with the CVI[410, 461, 465, 473, 474, 479, 480, 482].

Again, our achievements corroborate these hypotheses bringing new insights into the disparity of outcomes regarding the effect of anti-VEGF therapy on the choroid, such the effect of vitrectomy[282].

First, it is relevant to observe that the mean CFT in relatively low in both Vit and non-Vit, near the cut-off of 400 µm (in opposition, as an example, to the over 500 µm CFT observed in studies[695], where a more severe DME/cpDME was expected for a FAc indication) recognized as the upper limit reference for a less severe DME, as previously extensively debated and also in line with our results[399, 404, 693, 694]. Second, although the reduction in CFT was significant in both groups, the group of Vit eyes started with a relatively lower mean CFT (400.5 µm) and still, the decrease to 354.4 µm (i.e. less than 50 µm) was more discrete after 6 months of therapy, in opposition to the near 100 µm of non-Vit eyes[282]. That explains the disparities in findings between BCVA, CT, and CCD, with no significant improvement in VIT eyes compared to non-VIT eyes following 6 months of PRN RBZ therapy (maximum 6 injections at the end of follow-up)[282].

We hypothesize that a reduction in choroidal vasculature permeability through an effective anti-VEGF therapy may reduce the interstitial fluid pressure, favoring blood flow in the small vessels of CC, reflected in an increase of CCD, reduction in CFT and increase in BCVA[777].

DME in the short term (6 months) seems not affect CVI, neither in Vit nor in non-Vit. Furthermore, CVI did not changed with RBZ therapy independently of the thickness outcomes[282].

We speculate that the positive effect on minor CC vessels (through CCD) may become diluted when global choroidal vasculature is evaluated through CVI. Even so, baseline CVI was positively correlated with BCVA at baseline and at 6 months only in non-Vit, independently of the existence of DME. Lower inner retina thickness due to neurodegenerative alterations, exacerbated by vitrectomy and correlated with a worse postoperative visual outcome[101, 404, 695, 778], may lead to a lesser influence of choroidal health in the overall retina tissue and its functional performance. The lesser energy expended by the reduced pool of inner neuro-cells in the high metabolic demand background of the macula may be an explanation [97, 778]. Thinner inner retina may have a more significant role in functional outcome, at least, in these less severe types of DME in VIT[404, 695].

In a separate study, we attempted to determine the prognostic and predictive significance of the thickness of seven retinal layers in VIT vs non-VIT patients treated with RBZ on a PRN basis with a 12-month follow-up[404].

The mean thickness of each individual retinal layer was analyzed in the nine individual ETDRS subfields. The only b difference between groups was the, expected, decrease in inner retinal layers, particularly the GCL thickness of inner-outer temporal and superior sectors and in inner ring ETDRS[404, 779]. The ILM peeling technique, which is commonly started in temporal superior

sectors due to ease of access, with the purpose of conserving the papillomacular bundle and allowing the remaining sectors to be peeled clockwise with minimal touch, was the explanation of this result[779]. In this research, in both VIT and non-VIT, better visual outcomes were correlated with a lower baseline RNFL, GCL, IPL and INL thickness in central and inner ring ETDRS areas[404]. Globally, VIT and non-VIT obtained an equivalent anatomical outcome, had similar percentage of good-earlier responders (33% overall) and number of RBZ IV injections. This may sound as a contradiction to the choroidal paper described earlier (with poor outcomes in VIT after a maximum of 6 injection). Nonetheless, the retinal layers thickness study used as criteria for a good-early responder the achievement of a dry macula and a maximum BCVA after a maximum of 7 injections until month 6, inclusively, and no need of further injections till month 12. Hence, 7 injections may be the cutoff for VIT to reach similar outcomes as non VIT, among non-chronic DME subtypes. However, this cumulative longer time to achieve response plus the negative effect of a lesser efficient therapy in partial/non-responder group are the arguments proposed for a lower functional outcome with poor vision (<70 ETDRS letters) in a higher percentage of cases at the end of follow-up with RBZ in VIT[404]. The BCVA rises gradually, but at a slower rate.

That is traduced by the strong correlation only verified in VIT eyes (only moderate in non-VIT) of better visual outcomes and higher decrease in RNFL at month 12 in the inner ring (where the axons responsible for central VA are located). The nefarious effect of the edema is then very clear, particular in the inner retinal layers and in Vit, which are in disadvantage due their characteristic lower inner retinal layers reservoir, as evince also by the analyzes showed in another study where DME in Vit and non-Vit was treated with FAc and followed during 24 months[695]. An interesting observation about GCL in this other study, comparing Vit vs non-Vit diabetic patients treated with FAc with a healthy control group, was that at baseline (b) GCL from non-VIT with DME was equivalent to normal eyes and clearly decreased in VIT. Following FAc, GCL thickness decreased significantly until month 6 and then stabilized until the end of the follow-up period (figure 3 from chapter 2.11)[695]. This evolution was also paralleled by CFT decrease and BCVA increase[695]. FAc, during its therapeutic effect seems to stop DR neurodegeneration[695], observed in the natural course of DR and enhanced by DME[404], in the 2-year period following treatment with FAc in VIT and non-VIT[695].

Another finding to be highlighted from this last study were the differences between VIT and non-VIT in the amount of GCL reduction by quadrants after FAc. The greatest reduction was in the superior quadrant of VIT and the least affected was the nasal quadrant, in both VIT and non-VIT[695, 779]. As a speculative explanation, the traumatic pinches in the upper quadrant were thought to generate an extra inflammatory retina response with secondary reactive edema and a more reactive reaction to intravitreal corticosteroid therapy in most traumatized tissue.

The smaller reduction in the nasal quadrant, also seen in nonVIT eyes, could be a sign of a more pronounced BRB affected location in DME patients[695].

Finalizing with the last group of OCT ESASO biomarkers plus OPLd. Their prognostic and predictive role was accessed in two different works with RBZ and FAc applied for the treatment of DME, through a prospective and a retrospective study, respectively, along 12 months of follow-up, comparing as well VIT and non VIT[399, 693]. Patients with VMI abnormalities that might contribute to macular edema, such as ERM with inner retinal distortion were excluded.

Our real-world experience comparing short-term CCT (ozurdex®) with FAc (Iluvien®) outcomes for the treatment of DME in VIT, provided the foundation for using FAc to access these biomarker analyses. An adequate response from DEXii was initially observed (BCVA gains at months 1 and 3, and a CFT decrease at month 1), but this response did not last until the sixth month, when both BCVA and CFT returned to close baseline values. The subset of eyes DEXii-treated that responded inadequately to DEX implantation when followed over 6 months responded well to FAc implantation, with sustained improvements in BCVA and CFT over 12 months post FAc implantation[780].

Our group have also accessed the long-term effectiveness of FAc throughout other challenging inflammatory diseases as Irving-Gass Syndrome, Non-ischemic Central Retinal Vein Occlusion and in Familial Amyloid Retinal Angiopathy Macular Edema ATTR V30M Portuguese[691, 781, 782].

The study where RBZ that was used to access OCT biomarkers[693] was a sub-analysis from the retinal thickness layers study previously described[404]**,** including the same group of patients and the same criteria for the definition of type of response.

This study allowed us to identify **early prognostic factors** in DME treatment with RBZ, associated with the reestablishment of the macular anatomical configuration after 24 weeks of therapy without the need for further injections within the first year of follow-up (good-early responder). A **fewer INL cysts**, the normalization of the macular anatomy due to the disappearance of DRIL, OPLd and ONLc were associated with a good-earlier response.

This was in line with the results of retinal thickness layers study[404]: **lower baseline CFT** (69% of good-earlier responders had a baseline CFT<400µm) and **lower INL thickness** seem also be associated to a good-earlier response to RBZ IV therapy.

With this regard no differences were observed between VIT and non-VIT. Even though, as previous discussed, BCVA improvement may be compromised with a delayed optimal therapy in VIT eyes[404].

Overall, treating these patients with early prognostic factors precociously leads not only to lower therapy burden but also reversibility of OCT DR macular lesions, the avoidance of additional treatments, irreversible damage and potential functional compromise.

Instead, and in opposition with RZB study (including lesser severe DME cases), with FAc, there were no differences at **baseline and at 12 months** concerning BCVA, its evolution was equally comparable in VIT and non-VIT over follow-up, even facing a **refractory and chronic DME**. Furthermore, when OCT biomarkers were studied, DME treated with FAc in VIT eyes evolved to lower CFT and number of HRD at month 12, having a more benign evolution through disease stability, and a trend to less subsequent treatments than non-VIT eyes. Despite this, there were no variations in the types of responses to the FAc implant between Vit and non-Vit patients[399].

After the FAc implant, at month 12, the prevalence of SRF, the number of HRD, INLc, and ONLc were significantly lower and, additionally, a trend for a lower prevalence of OPL, ELM, and EZ disruption was also observed[399].

A **key message from this paper** was that the presence of SRF in a non-responder to anti-VEGF seems to be a key factor for an increased risk of a lesser response even with CCT, further need of additional therapy and even a possible increased progression of non-controlled inflamatory process, which expains the increase number of HRF and CFT at month 12 in lesser responder cases to FAc, with current anti-VEGFs[399].

Suplemental therapy added to FAc (with a low burst height and near-zero release kinetics), with short term CCT with high burst heights, such as DEX implant; new therapies, as brolucizumab (with high molar concentration of anti-VEGF), the removal of vitreous or additional PRP should be considered[647, 783-786]. **This strategy is based on the notion that VEGF and other CK and CC levels rise in more severe DR patients, such as PDR and refractory DME**[493, 519-521, 525, 526, 707].

A walk through supplemental therapy with RBZ in challenging clinical cases treated with FAa was the focus of a series of clinical cases publicated with the purpose to show the multifactorial background of DME, which requires a careful, tailormade and comprehensive approach to be well succeed[696]. The clinical applicability of OCT biomarkers, retinal perfusion and vitreous status were altogether integrated and analysed.

From the simple need of topup with RBZ with complete regression of the DME, clear improvement of maculopathy based on OCT ESASO biomarkers, to self solving recurrent DMEs and one last case with a singular denouement. This final clinical case was the representation of what was interpretated as being a progressive severe DR stopping response to top-up RBZ after an initial response, although with non-visible neovascularization in FA. Vitrectomy with ILM peeling and reinforcement of PRP resolved that refractory DME case[696].

Herein the vitreous has demonstrated to be, not only a window to new insights for the acquaintance of DME and DR but also, many times, an essential piece to complete the complex dynamic puzzle of their approach.

The worldwide accepted first-line anti -VEGF therapy for DME has a major hitch, the burden related with frequent intravitreal dosing requirements and consequent lack of compliance that may result in inadequately treated disease, may lead to vison loss[657].

The vitreous has a major role in the disease and further in therapeutic solutions. It depictures a deposit in a privileged location, a vehicle for the delivery of new longer lasting and unmet targets therapies. This is the focus of many currently ongoing trials.

Based on the work developed across this dissertation are herein presented 4 algorithms for DME approach. BZB, already approved by FDA and EMA for nAMD, was also integrated in treatment protocol due its phase 3 clinical trial results, not inferior, or even superior, regarding safety, when compared with nAMD, antecipating its acceptance more granted in the short term also for DME.

The vitreous, OCT bimarkers, widefield AF, OCTA imaging and their comprehensive integration in other relevant clinical data from the DR context were herein depicted as essential pieces on the complex puzzle of DME clinical therapeutical decision. This guidelines for the approach of DR for DME were proposed, validated and adopted by the Ophthalmology Department from Centro Hospitalar e Universitário do Porto (CHUPorto), from 21st September 2021.

4. Conclusions and Future Perspectives

Based on our findings, we conclude that vitreous state plays a significant role in DME management.

Our three main research goals led to novel and previously unreported findings on RD and EMD physiopathology and potential target therapies based on humoral vitreous biomarkers and the VRI - **aim 1**; our unconscious regarding the real vitreous status may undervalue the vitreous macular interaction role in DME pathophysiology - **aim 2**; the relevance of integrating imaging retinal and choroidal biomarkers in response to EMD treatment, in VIT and non-VIT eyes - **aim 3**.

Therefore, early treatment approaches should be offered for more functionally negative prognosis VMI anomalies such as T3 VMT and in more reversible DMEs, by identifying good early responders with lower INL thickness and number of cysts, both in VIT and non-VIT.

When facing the burden of IV injections or non-response to IV therapy, focal VMA release should be considered.

WF- OCT has emerged as a valuable and feasible tool for determining the true state of the vitreous.

VIT eyes are effectively different and more prone to DR damage due to their advanced inner retina neurodegenerative stage and reduced sensitivity to standard anti-VEGF IV therapy. Although post-vitrectomy DME occurs in a small percentage of cases (10.9 percent to 13.3 percent in the first year after surgery, according to our research), it must be taken into account when making therapeutic options. To avoid irreversible damage, the best treatment option for resolving DME should be chosen promptly, especially in these eyes.

SRF was the biomarker that emerged in a non-responders to anti-VEGF, being associated to an increased risk of a lesser response even to CCT therapies available, indicating the need of combined therapy and an increased risk for a non-controlled inflamatory process, particularly in non-VIT.

Our analysis of the choroidal tissue using standard SD-OCT and OCTA imaging methods added further to the debate over anti-VEGF therapy's effect on the choroidal vasculature.

It appears to improve choroidal microvascular perfusion when it is effective, as demonstrated in our non-VIT compared to VIT eyes, and we can predict that retinal perfusion may improve as well.

Corticosteroids appear to be the preferred treatment option for DME in VIT eyes, according to our findings. Despite the fact that the effects of DR vasculopathy and their repair may demand further interventions, we believe that CCT's anti-inflammatory characteristics can independently halt the DR neurodegenerative process.

To gain a better understanding of the vitreous macular interaction role in DME pathophysiology, future studies applying increased valuable WF- OCT technology, with a larger number of patients, a better characterization of the different types of VMA, valuing its location regarding central macula, the area under posterior hyaloid, and their correlation to DME response to therapy are required.

Furthermore, our findings show that the vitreous cavity is a unique source of therapeutic targets, through the inhibition of pathogenic molecules like IL6 and IL8 or reinforcement of antiangiogenic mediators like IP-10.

Longer-acting medicines, such as novel anti-VEGF agents like brolucizumab and faricimab, as well as gene therapy, can be addressed using the vitreous drug delivery platform. In a complex condition like DR, gene therapy offers potential long-term and multi-targeting options. Gene therapy can modify the host genome or transcriptome directly, either transiently or permanently, ideally in the early stages of DR before functional compromise.

Dual acting technology for pathogenic molecules via gene silencing (using endogenously produced miRNA or injecting external siRNA or shRNA) and simultaneous introduction of expressing vectors encoding for therapeutic molecules such as IP-10 or PEDF is a near term horizon.

As a final message, OCT, OCTA and laboratory biomarkers for DME are orphan devoid of clear meaning when they are alone but essential pieces to face the complex puzzle of DME approach and **the vitreous and VMI are among those essential pieces**. Their clinical applicability was shown to be not only realistic but also essential in larger scale, randomized controlled studies to validate outcomes, deepen knowledge about prognostic and predictive biomarkers, and predict good early responders vs chronic / refractory DME, an optimized, individualized, tailored therapy approach.

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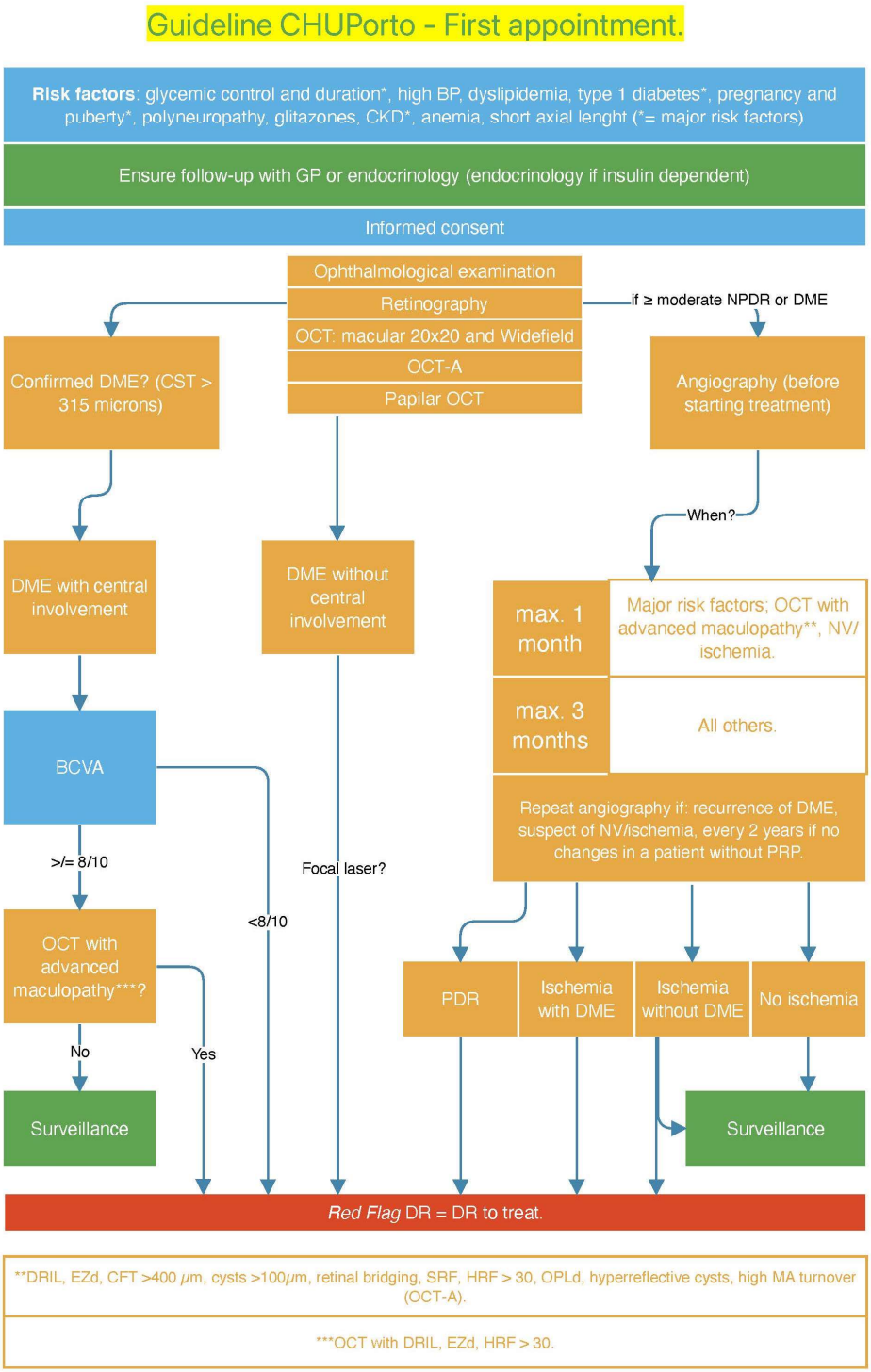
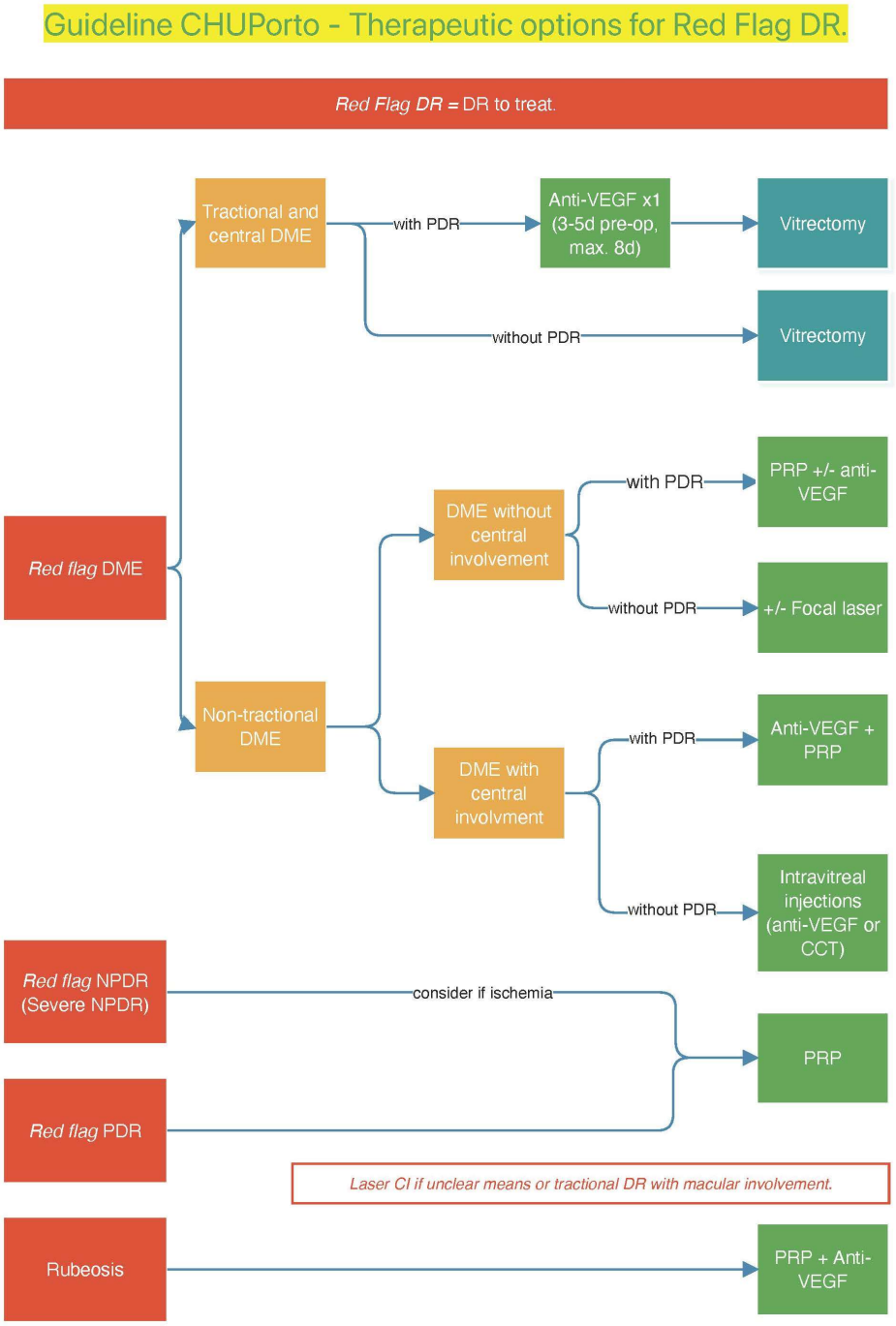
6. Appendices

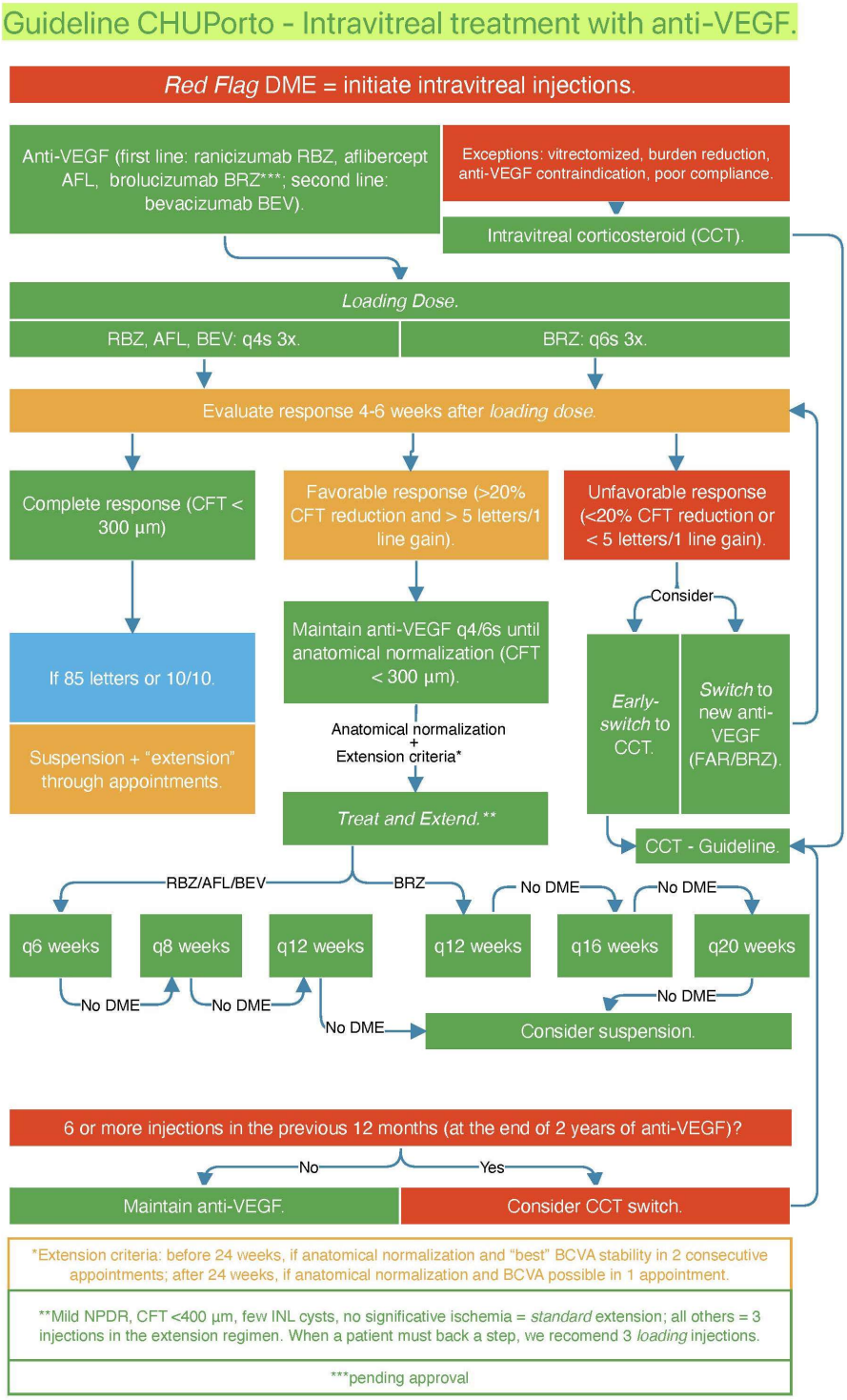
Appendix 1 – Guideline CHUPorto – First appointment



Appendix 2 – Guideline CHUPorto – Therapeutic options for Red Flag DR



Appendix 3 – Guideline CHUPorto – Intravitreal treatment with anti-VEGF



Appendix 4 – Guideline CHUPorto – Intravitreal CCT