UNIVERSIDADE D
COIMBRA

Inês Pereira Dias Marques

PROGRESS

Progression of Diabetic Retinopathy
Identification of Signs and Surrogate outcomes

Tese no âmbito do Programa de Doutoramento em Ciências da Saúde – ramo de Medicina, orientada pela Professora Doutora Maria da Conceição Lobo Fonseca e pelo Professor Doutor João Pereira Figueira e apresentada à Faculdade de Medicina da Universidade de Coimbra

Dezembro 2020

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This clinical study is registered in the public accessible database - Clinicaltrials.gov: NCT03010397-PROGRESS

The sponsor of the study was AIBILI – Association for Innovation and Biomedical Research on light and Image.

It was developed at the Clinical Trials Center (CEC) of AIBILI - Association for Innovation and Biomedical Research on Light and Image, with the permission and support of the President of the Administration Board, Professor Doutor José Cunha-Vaz.

The Study Protocol and the Patient Information/Informed Consent form were reviewed and approved by the Institutional Review Board and Ethics Committee of AIBILI, Comissão de Ética para a Saúde da AIBILI, and by the Portuguese Data Protection Authority before the study start.

The study was designed, implemented and reported in accordance with the ICH Harmonized Tripartite Guidelines for Good Clinical Practice, with applicable national regulations (Law Nr. 21/2014 changed by Law Nr. 73/2015), European Directive 2001/20/EC, United States Code of Federal Regulations Title 21 and Japanese Ministry of Health, Labor, and Welfare and with the ethical principles laid down in the Declaration of Helsinki.

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Este projeto teve início em 2013, enquanto me encontrava a trabalhar no Hospital de Sousa Martins, na Guarda, e fui desafiada pelo meu mentor, o Senhor Professor Doutor José Guilherme Cunha-Vaz, para iniciar a minha carreira de investigadora clínica na Universidade de Coimbra. Este foi um desafio que aceitei, naturalmente, com grande vontade e espírito de missão. Designar o Senhor Professor Cunha-Vaz de "mentor" é talvez um pouco redutor. O Professor Cunha-Vaz foi, de facto, o autor intelectual e o responsável máximo por todo o planeamento e orientação deste estudo, a quem devo tudo o que foi conseguido até aqui. Da minha parte, como boa "ovelha", contribuí com o meu conhecimento e dedicação para que o projeto decorresse com sucesso. Ao Senhor Professor Cunha-Vaz expresso a minha mais profunda gratidão, admiração, respeito e agradeço todas as palavras de entusiasmo e encorajamento. Espero não ter defraudado expectativas e manter esta linha orientadora pela minha vida: a investigação clínica como pilar basilar de melhores cuidados de saúde prestados na clínica.

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List of abbreviations

AIBILI	Association for Innovation and Biomedical Research on Light and Image
AUC	Area Under the Curve
BCVA	Best Corrected Visual Acuity
BRB	Blood-Retinal Barrier
CFP	Color Fundus Photography
CIME	Center Involved Macular Edema
CORC	Coimbra Ophthalmology Reading Centre
CRT	Central Retinal Thickness
CSF	Central subfield
CSME	Clinically Significant Macular Edema
DCP	Deep capillary plexus
DM	*Diabetes Mellitus*
DME	Diabetic Macular Edema
DR	Diabetic Retinopathy
DRCR.net	Diabetic Retinopathy Clinical Research Network
DRIL	Disorganization of the Retinal Inner Layers
DME	Diabetic Macular Edema
DRP	Deep retinal plexus
ERM	Epiretinal Membrane
ETDRS	Early Treatment Diabetic Retinopathy Study
EUROCONDOR	European Consortium for the Early Treatment of Diabetic Retinopathy
EZ	Ellipsoid Zone
FA	Fluorescein Angiography
FAZ	Foveal Avascular Zone
HbA1c	Glycosylated Haemoglobin
ICAM-1	Intercellular Adhesion Molecule 1
ILM	Inner Limiting Membrane
INL	Inner Nuclear Layer
IPL	Inner Plexiform Layer
IRMA	Intraretinal Microvascular Abnormalities
IRC	Intra-Retinal Cysts

IS	Inner Segments
MA	Microaneurysms
mfERG	Multifocal Electroretinography
NPDR	Non-Proliferative Diabetic Retinopathy
NSD	Neurosensorial Serous Detachment
LOR	Lower than normal Optical Reflectivity
OCT	Optical Coherence Tomography
OCTA	Optical Coherence Tomography Angiography
OCTL	Optical Coherence Tomography Leakage
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
OR	Odds Ratio
OS	Outer Segments
PD	Perfusion Density
PEDF	Pigment Epithelium Derived Factor
PDR	Proliferative Diabetic Retinopathy
ROC	Receiver Operating Characteristic
RPE	Retina Pigment Epithelium
SCME	Subclinical Macular Edema
SCP	Superficial capillary plexus
SD	Standard Deviation
SD-OCT	Spectral Domain Optical Coherence Tomography
SRF	Subretinal Fluid
SRP	Superficial retinal plexus
SS-OCT	Swept-Source Optical Coherence Tomography
T2D	Type 2 diabetes
TNF-α	Tumor Necrosis Factor Alfa
UWF-FA	Ultra-widefield Fluorescein Angiography
VA	Visual Acuity
VD	Vessel Density

Resumo

A retinopatia diabética (RD) é a complicação mais frequente da *diabetes mellitus* e a principal causa de cegueira legal na população ativa de países industrializados. A progressão de RD não ocorre ao mesmo ritmo em todos os doentes. Alguns nunca desenvolvem diminuição da visão, enquanto outros progridem rapidamente para o edema macular ou neovascularização, levando à perda da visão. Os fatores de risco já descritos são insuficientes para prever os doentes que irão progredir e desenvolver complicações. O principal objetivo na diabetes é prevenir o desenvolvimento de RD. Quando as lesões de RD se desenvolvem, a intervenção precoce deve ser tentada para preservar a visão. Para isso, é essencial compreender os mecanismos pelos quais a diabetes afeta a retina, melhorar os métodos de deteção precoce da doença e encontrar novas moléculas para o tratamento direcionado. A compreensão dos mecanismos que levam ao agravamento da doença é o principal objetivo desta tese.

Esta tese representa os resultados de um estudo clínico longitudinal observacional, o estudo PROGRESS (NCT03010397), que acompanhou 212 doentes com *diabetes mellitus* tipo 2 sem RD ou com RD ligeira, por um período de 5 anos, com visitas anuais.

O objetivo geral desta investigação foi caracterizar funcional e morfologicamente as fases iniciais da RD. Identificamos que existem diferentes vias da doença alteradas em diferentes olhos, representando isquemia, neurodegenerescência e edema. Quisemos ainda caracterizar, num estudo com um seguimento alargado, os fenótipos da RD já identificados e que podem ser usados como biomarcadores de progressão de doença. Além disso, compreender a extensão das anomalias neuroretinianas e caracterizar a progressão da neurodegenerescência em doentes com ou sem danos microvasculares detetáveis foi também um objetivo do estudo.

Iniciei, no CAPÍTULO 1, com uma introdução geral, onde abordo a epidemiologia e fisiopatologia da RD, os seus principais fatores de risco, diferentes sistemas de classificação e as principais vias de progressão da doença.

Nos capítulos 2, 3 e 4 apresento os resultados do estudo de acompanhamento de 5 anos, com uma descrição das características demográficas e sistémicas da população em estudo e da progressão ao longo de 5 anos de seguimento, para as complicações ameaçadoras da visão e a progressão da RD baseada no nível de ETDRS. O valor preditivo de fatores de risco sistémicos e oculares foi explorado

bem como biomarcadores de imagem identificados. Os doentes com fenótipo C apresentam níveis de HbA1c mais elevados e valores mais elevados de triglicéridos quando comparados com outros fenótipos. O fenótipo C foi identificado principalmente nos olhos com ETDRS nível 35, sugerindo que o nível 35 do ETDRS poderá ser o ponto de viragem na progressão de RD. Os diferentes fenótipos identificados nos diabéticos tipo 2 mostram um risco diferente para o desenvolvimento de complicações ao longo de 5 anos: CSME, CIME e PDR. O fenótipo C identifica os olhos com maior risco para o desenvolvimento de CSME ou PDR. Em contraste, o fenótipo A identifica os olhos com um risco muito baixo de desenvolvimento destas complicações. O turnover dos microaneurismas e o fenótipo C correlacionam-se bem com as alterações nos níveis de gravidade da RD segundo o ETDRS, independentemente dos valores de espessura central da retina, validando a sua utilização como um biomarcador da progressão de RD. O fenótipo A e B, que representa 70% de toda a coorte, apresentam um risco muito baixo de agravamento do nível ETDRS de 2 ou mais níveis (2%).

No CAPÍTULO 5 apresentamos uma análise transversal de uma coorte de doentes com RDNP, agrupados de acordo com a classificação ETDRS, nos níveis 10-20, 35 e 43-47. Foram identificadas três vias diferentes da doença: neurodegenerescência, isquemia e edema, com prevalências diferentes em diferentes doentes, indicando que o mecanismo predominante da doença da retina pode ser diferente em diferentes indivíduos. Parecem ocorrer independentemente uns dos outros. Apenas as métricas da densidade vascular, indicando isquemia, parecem estar associadas ao nível ETDRS.

Nos 2 capítulos seguintes, CAPÍTULO 6 e 7, apresento os resultados de um estudo de acompanhamento de 2 anos e de 3 anos, realizados num subgrupo de doentes, caracterizando a evolução das três vias de doença identificadas na RD. Em cada grupo ETDRS, os valores de oclusão capilar (densidade vascular reduzida), edema e neurodegenerescência variam amplamente, identificando diferentes níveis de lesão em diferentes olhos. Os valores relativos à densidade vascular foram os únicos que permitiram distinguir entre os grupos ETDRS, mesmo após ajustamento a múltiplos fatores. Durante o período de seguimento de 2 anos, os valores da densidade vascular diminuíram em todos os plexos da retina, particularmente no plexo capilar superficial, e esta diminuição foi superior nos olhos que apresentaram agravamento do nível de ETDRS em comparação com os olhos que mantiveram a severidade de RD, enquanto o edema e a neurodegenerescência permaneceram estáveis. No estudo de seguimento de 3 anos, tronou-se evidente uma diminuição da espessura da camada GCL+IPL (representando a

neurodegenerescência) durante o seguimento que, no entanto, não permite discriminar entre os olhos que agravam em comparação com os olhos que mantém o nível de severidade do ETDRS.

No último trabalho apresentado, no CAPÍTULO 8, avaliámos 105 olhos com o Swept-Source OCTA (SS-OCTA, PlexElite, Carl Zeiss Meditec), que permite explorar uma área maior da retina, utilizando o protocolo de 15x9 mm e 3x3 mm. Observamos que a oclusão capilar na média periferia caracteriza uma fase avançada de retinopatia, enquanto a oclusão capilar limitada à perifóvea sugere uma fase mais inicial da doença.

No último capítulo realizei uma breve discussão sobre os resultados obtidos. A informação apresentada tem um grande impacto na gestão de RD, contribuindo para uma monitorização e cuidados individualizados e abre novas perspetivas relativamente a novas terapias a serem usadas na fase inicial, antes de ocorrerem complicações clinicamente significativas.

Abstract

Diabetic retinopathy (DR) is the most frequent complication of diabetes mellitus and the leading cause of legal blindness in active populations of industrialized countries. Progression of DR does not occur at the same rate in all patients. Some never develop vision loss, whereas others rapidly progress to macular edema or neovascularization leading to vision loss. The already known risk factors are unable to predict the patients that will progress and develop complications. The main goal in diabetes is to prevent the development of DR. When DR lesions develop, early intervention should be attempt in order to preserve vision. It is essential to understand the mechanisms by which diabetes affects retina, improve the methods for early disease detection and find new molecules for targeted treatment. The understanding of the mechanisms that balance in different direction is the main objective of this thesis.

This thesis represents the results of an observational longitudinal clinical study, the PROGRESS study (NCT03010397), that followed up 212 type 2 *Diabetes mellitus* patients with no or mild DR, in a 5-year period, with annual visits.

The overall purpose of this research was to characterize both functionally and morphologically initial DR stages. We found that different DR pathways of disease may be identified in different eyes, representing ischemia, neurodegeneration and edema. We wanted to further characterize the already identified DR phenotypes that may be used as biomarkers of progression. Furthermore, understanding the extent of neuroretinal abnormalities and characterize the neurodegeneration progression in patients with or without detectable microvascular damage was a main goal of the study.

I have started, in CHAPTER 1, with a general introduction, where I go through the epidemiology and pathophysiology of DR, principal risk factors, different classification systems and the main pathways of disease progression.

In CHAPTER 2, 3 and 4 I present the results of the 5 year follow up study, with a description of the demographic and systemic characteristics of the study population and the 5 year progression to vision threatening complications (VTC) and ETDRS level progression. The predictive value of systemic and ocular risk factors were explored, and imaging biomarkers identified. Phenotype C patients present higher HbA1c levels and higher values of triglycerides when compared to other phenotypes. Phenotype C was identified mainly in eyes with ETDRS grade 35 suggesting that

ETDRS grade 35 may be the turning point in the progression of DR. Different retinopathy phenotypes in T2D show different five-year risk for development of VTC: CSME, CIME and PDR. Phenotype C identifies eyes at higher risk for development of vision-threatening complications (CSME or PDR). In contrast, phenotype A identifies eyes that are at a very low risk of development of vision–threatening complications.
Microaneurysm turnover and phenotype C correlate well with changes in ETDRS severity levels, independent of CRT values, validating its use as a simple to use biomarker of DR progression. Phenotype A and B, representing 70% of the entire cohort, have a very low risk for 2-or-more-step ETDRS worsening (2%).

In CHAPTER 5 we presented a cross-sectional analysis of a cohort of NPDR patients, grouped according to the ETDRS grading protocol, in levels 10-20, 35, and 43-47. Three different pathways of disease were identified: neurodegeneration, ischemia and edema, with different prevalence in different patients, indicating that the predominant mechanism of retinal disease may be different in different individuals. They appear to occur independently of each other. Only the metrics of vessel density, indicating ischemia, appear to be associated with the ETDRS level.

In the next 2 chapters, CHAPTER 6 and 7, I present the results of a 2-year and a 3-year follow up studies, performed in a subset of patients, characterizing the evolution of the three identified retinal pathways occurring in DR. In each ETDRS group, values of capillary dropout (reduced vessel density), edema and neurodegeneration covered a wide range, identifying different levels of damage in different eyes. Vessel density remained the only metrics significantly different between ETDRS groups, even after adjusting for multiple baseline factors. During the 2-year follow-up period the vessel density decreased in all retinal plexuses, particularly in the superficial capillary plexus, and was more important in eyes with worsening in ETDRS level comparing to eyes that maintained DR severity, whereas edema and neurodegeneration remained stable. In the 3-year follow up study it was evident a GCL+IPL thickness decreased (representing neurodegeneration) during the follow up, however, this decrease do not discriminate between eyes that will present worsening comparing to eyes that maintain the ETDRS severity level.

In the last paper presented, in CHAPTER 8, we evaluated 105 eyes with the innovative Swept-Source OCTA (SS-OCTA, PlexElite, Carl Zeiss Meditec), that allow to explore a bigger area of the retina, using 15x9 mm and 3x3 mm protocol. We observe that capillary closure in the midperiphery in a diabetic retina is indicative of an advanced stage of retinopathy, whereas capillary closure limited to the perifovea suggests a milder stage of the disease.

In the last chapter I performed a brief discussion of the obtained results.

This information has a crucial impact in DR management, contributing for an individualized monitoring and care and open new perspectives concerning new therapies to be used in the early phase, before clinically significant complications occur.

List of publications

1. **Retinopathy phenotypes in type 2 diabetes with different risks for macular edema and proliferative retinopathy.**

   Marques IP, Madeira MH, Messias AL, Santos T, Martinho AC-V, Figueira J, Cunha-Vaz J.

   J Clin Med. 2020. https://doi.org/10.3390/jcm9051433

2. **Different retinopathy phenotypes in type 2 diabetes predict retinopathy progression.**

   Marques IP, Madeira MH, Messias AL, Martinho AC-V, Santos T, Sousa DC, Figueira J, Cunha-Vaz J.

   Acta Diabetol. 2020. https://doi.org/10.1007/s00592-020-01602-9

3. **Ocular and systemic risk markers for development of Macular Edema and Proliferative Retinopathy in type 2 diabetes. A five-year longitudinal study.**

   Martinho AC-V., Marques IP, Messias AL, Ana L. Messias, Santos T, Madeira MH, Sousa DC, Lobo C, Cunha-Vaz J.

   Diabetes Care. 2020 Nov. https://doi.org/10.2337/dc20-1125

4. **Multimodal imaging of the initial stages of diabetic retinopathy: Different disease pathways in different patients.**

   Marques IP, Alves D, Santos T, Mendes L, Santos AR, Lobo C, Durbin M, Cunha-Vaz J.

   Diabetes. 2019 Mar. https://doi.org/10.2337/db18-1077

5. **Characterization of disease progression in the initial stages of retinopathy in type 2 diabetes: A 2-year longitudinal study.**

   Marques IP, Alves D, Santos T, Mendes L, Lobo C, Santos AR, Durbin M, Cunha-Vaz J.

   Investig Ophthalmol Vis Sci. 2020. https://doi.org/10.1167/iovs.61.3.20

6. **OCTA Metrics Predict Severity Progression of Diabetic Retinopathy—3-Year Longitudinal Study**

   Marques IP, Kubach S, Santos, T, Mendes L, Madeira, MH, Sisternes, L, Tavares D, Santos, AR, Lewis W, Lobo, C, Durbin M, Cunha-Vaz J.

   Submitted to Retina

7. **Swept source OCTA quantification of capillary closure predicts ETDRS severity staging of NPDR.**

   Santos T, Warren L, Santos AR, Marques IP, Kubach S, Mendes L, Sisternes, L, Madeira MH, Durbin M, Cunha-Vaz J.

   British Journal of Ophthalmology. 2020. https://doi.org/10.1136/bjophthalmol-2020-317890

Chapter 1

General Introduction

Diabetes mellitus is a chronic systemic disease characterized by persistence of hyperglycemia. It is complex and requires a multidisciplinary evaluation and intervention. Diabetes affects individuals of all ages and socio-economic groups.

Diabetic retinopathy (DR) is the most frequent complication of diabetes mellitus and the leading cause of legal blindness in active populations of industrialized countries. Progression of DR has been up to now classified according to the ETDRS classification, based on a multicentric study that evaluated the effect of laser photocoagulation on advanced stages of DR. Although appropriate for late stages of DR, it does not grade progression well in the initial stages of the disease. Initial stages of DR require urgent and detailed characterization in order to act in the evolution of DR while lesions are still reversible. Furthermore, progression of DR does not occur at the same rate in all patients. Some never develop vision loss, whereas others rapidly progress to macular edema or neovascularization leading to vision loss. The duration of diabetes mellitus and the metabolic control are major risk factors for DR progression, but they are insufficient to explain the great variability observed in patients. The understanding of the mechanisms that balance in different direction is of outmost importance in order to achieve the best management of patients. In diabetes, the main goal is to prevent the development of DR. When DR lesions develop, early intervention should be attempt in order to preserve vision. It is essential to understand the mechanisms by which diabetes affects the retina and vision and to improve the methods for early detection of the retinal disease. Identifying patients at risk of developing vision threatening complications and visual loss and understanding its causes and development is fundamental for its appropriate management and treatment.

Incidence and Prevalence

The World Health Organization divides *diabetes mellitus* into type 1 (insulin-dependent) and type 2 (non-insulin-dependent). The first type, typically at a younger age, is characterized by a deficit in insulin production, while the second type, of appearance in adulthood/elderly, results from insulin resistance.

Type 1 *diabetes mellitus*, more prevalent in northern hemisphere countries, usually has its onset before age 30 and represent near 5% of all individuals with diabetes. Today there is a real worldwide epidemic of diabetes, especially type 2 diabetes, which is related to lifestyle changes, obesity and increase in average life expectancy with consequent population aging. The latest estimates from the International Diabetes Federation (IDF) (1) indicate, in 2019, a prevalence of 463 million people worldwide living with diabetes and, by 2045, this prevalence is estimated to increase to 700 million, 10,9% of the world population (Figure 1).

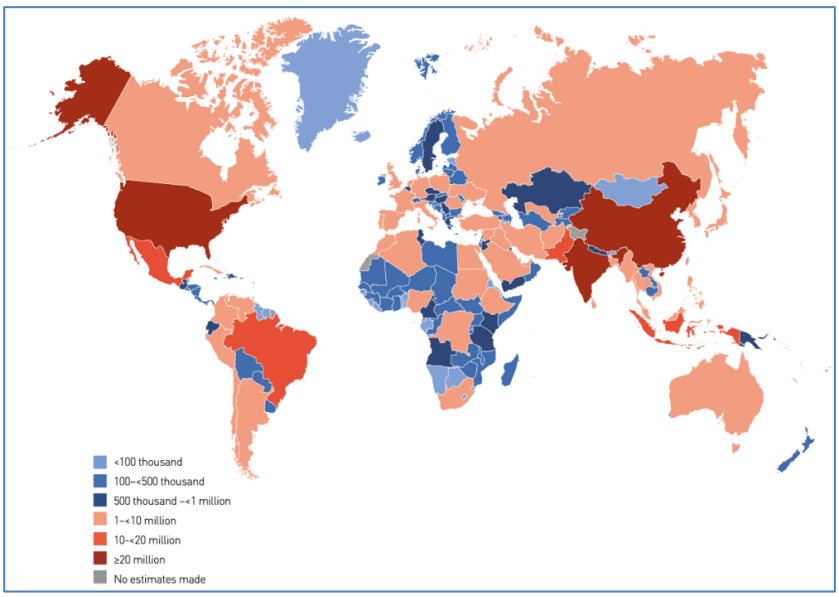

Figure 1: Estimated total number of adults (20–79 years) with diabetes in 2019 (1)

According to the Portuguese National Observatory, in 2009 diabetes affected 11.7% of the Portuguese population aged between 20 and 79 years, while in 2015 the prevalence raised to 13.3%, corresponding to more than 1 million Portuguese in this age group with the disease. In 2015 the estimated incidence of new DM cases were 591 to 699 per 100 000 people, corresponding to 61169 people (2).

Half of diabetic people are unaware of their condition, and so at risk of developing serious diabetes-related complications. Diabetes and its complications play a significant role in the causes of death, having led to 11.3% of deaths worldwide among people aged 20-79 years in 2019 (1). Early detection and appropriate treatment of those complications are essential to prevent premature death and disability.

In particular, diabetic retinopathy (DR) constitutes the leading cause of blindness in many countries, affecting working-age adults, and represent a major economic burden to the society. DR is the most common and most serious of the ocular complications of diabetes mellitus and affect nearly 30% of diabetic patients, with 4.4% having advanced disease, vision threatening retinopathy (3). The Wisconsin Epidemiologic Study of Diabetic Retinopathy refer that, in patients with type 2 diabetes mellitus (DM), 80% have evidence of any retinopathy after 20 years of disease, while in type 1 DM almost every patient present any retinopathy sign at this time (4)(5). The same study revealed an incidence of DR after 10 years of follow-up of 89, 79 and 67%, respectively, in insulin-treated patients whose diagnosis was established below 30 years old, in insulin-treated patients whose diagnosis was established at or after 30 years old, and in not insulin-treated diabetic patients. The 10 years incidence of proliferative DR was 30, 24 and 10% in the same groups (6).

The annual incidence of DR varies, however, according to different studies and regions, ranging from 2.4% in the SN-DREAMS-II (Sankara Nethralaya-Diabetic Retinopathy Epidemiology and Molecular Genetics Study II), India 2017 to 22.3% in the WESDR (Wisconsin Epidemiologic Study of Diabetic Retinopathy), USA 1989 (7). In fact, it is difficult to estimate the global incidence of DR, given the low number of studies reporting on its incidence, the different methods used in those studies as well as the different patient characteristics, type of diabetes and time of follow up, which makes it difficult to compare between countries.

In England, 20 686 patients with type 2 diabetes have been followed up for 17 years. From patients with no DR at baseline, only 4.0% of patients developed pre-proliferative retinopathy, 0.59% developed sight-threatening maculopathy and 0.68% PDR, after 5 years of follow up. After 10 years, the cumulative incidences were 16.4, 1.2 and 1.5%., respectively. From patients with non-proliferative retinopathy at baseline, after 1 and 10 years, 5.2% and 9.6% developed maculopathy and 6.1% and 11% developed PDR (8).

The RETINODIAB (Study Group for Diabetic Retinopathy Screening) program was performed in Portugal between July 2009 and December 2014 and provides the first Portuguese incidence DR

data in a large-scale population-based cohort of type 2 diabetes. The yearly incidence of any DR in patients without retinopathy at baseline was 4.6% in the first year, decreasing to 3.87% in the fifth year and the cumulative incidence at 5 years was 14.47%. The cumulative incidence of any referable retinopathy at 5 years was 1.37%. In patients with mild NPDR at baseline, the cumulative progression rate to higher grades of retinopathy was 4.59% after 5 years (9).

Approximately one in three people with diabetes have DR, and between them, one in three people have developed vision threatening complications, diabetic macular edema (DME) and proliferative diabetic retinopathy (PDR). Diabetic Macular Edema constitute the major cause of vision loss in type 2 diabetic patient while PDR is the most common vision-threatening lesion among patients with type 1 diabetes (10), representing a big problem for daily life activities such as driving, working and cooking.

It is a general consensus that progression of DR does not occur at the same rate in all patients. Some patients never develop vision loss whereas others rapidly progress to DME and neovascularization leading to vision loss. Since DME can be present at all DR severity stages and goes unnoticed by the patient until late, early screening and detection is crucial. Also crucial is the ability to identify eyes at risk of developing such vision threatening complications before it occurs. The understanding of the mechanisms underlying that phenotypic variability are of outmost importance. The hope that in the future we can practice a more personalized and assertive medicine helping to reduce the burden of the health system while enabling a more effective treatment of our patients is the goal of our research.

With this purpose in mind it is essential to understand the physiopathology of the retinal diabetic complications.

Pathophysiology

Much is still unknown about DR. Ashton, who has contributed so extensively to our knowledge of DR, remarked in 1974 that "we must continue to look for more fundamental scientific investigations and at the same time develop new ways of examining the diabetic retina in an effort to unravel the still unsolved mysteries of diabetic retinopathy" (11).

Diabetic Retinopathy was first described in the nineteenth century and, at that time, it was considered a disease of the retinal blood vessels. It is accepted that the first clinically observable signs of the disease are microaneurysms and "dot-and-blot" hemorrhages.

The chronopathology of DR is slow and can be separated in four overlapping clinical stages: retinal damage without any visible abnormalities in clinical examination, non-proliferative retinal microvascular changes (mild and moderate NPDR, with and without macular edema), more advanced pre-proliferative stages (severe NPDR) and proliferative DR (advanced stages).

It is a complex disease that has chronic **hyperglycemia** as its starting point. Abnormal glucose metabolism results from low levels of insulin and leads to numerous biochemical pathways that are responsible for some of the lesions characteristics of DR. Advanced Glycation Endproduct (AGE) formation is increased in diabetes. The interaction of these sugar-derived substances with their receptors (RAGE) evoke vascular inflammation, macrophage activation and prothrombotic endothelial activation, as well as has been linked to reduced survival signals and oxidative stress (increased production of reactive oxygen and nitrogen species) (12). Diabetes causes numerous pro-inflammatory changes in the retina, with impact in different inflammation-associated cytokine, including VEGF, IL-6, IL-1, TNFα and MCP-1. There is also a strong association between **dyslipidemia** and the development of DR (13). Lipid peroxidation was found to be significantly higher in DR patients, probably due to induced apoptosis, oxidative stress and inflammation (14). Changes on fatty acid status of the diabetic retina were associated with increased levels of inflammatory markers, IL-6, VEGF and ICAM-1 (15). The elevated levels of diacylglycerol resulting from hyperglycemia lead to the activation of protein kinase C, with altered blood dynamics and capillary hyperpermeability and consequent retinal edema. Furthermore, retinal **hypoxia** contributes to the VEGF overproduction, with its vaso-proliferative and vaso-permeability effects. Laser photocoagulation, a therapy used to treat neovascularization and inhibit progression of advanced retinopathy, acts by increasing oxygen availability in the central retina, proving the role of the oxygen in the progression of the retinopathy. Actually, low levels of light during sleeping,

in order to prevent complete darkness and dark adaptation (reducing oxygen consumption by rods), have been reported to improve visual acuity (16).

Before visible microvascular abnormalities, those metabolic pathways can lead to neurodegeneration, early microvascular impairment and neurovascular unit impairment. The neurovascular coupling allows that retina regulate blood flow according to metabolic demands and neural activity (17)(18). The accumulation of the extracellular glutamate, the principal excitatory neurotransmitter, results in neuronal death (excitotoxicity). An imbalance of retinal production of neuroprotective factors, such as pigment-derived growth factor (PEDF), somatostatin, interstitial retinol-binding protein and several neurotrophins, which are downregulated in the diabetic retina, might contribute to retinal neurodegeneration. On the other hand vascular endothelial growth factor (VEGF) as well as erythropoietin are upregulated in the early stages of neurodegenerative process. This increase in VEGF levels links neurodegeneration with early microvascular impairment (BRB breakdown). Erythropoietin is synthesized in the retina, mainly in the retinal pigment epithelium (RPE) cells, and is involved in the remodeling of damaged tissue, being a potent neuroprotective factor. Its overexpression may counteract the reduction in neuroprotective factors in the early phases. The overproduction of endothelin 1, retinal inflammation, overexpression of RAGE and RAS activation contributes to the crosstalk between endothelial cells and neuroretina and contribute to neurodegeneration (Figure 2).

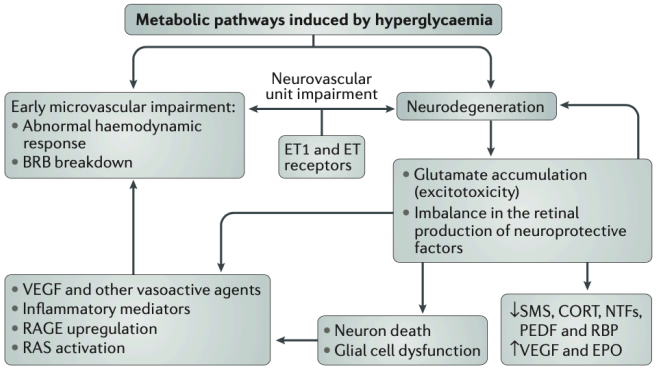

Figure 2: Potential mechanisms linking neurodegeneration and microangiopathy in diabetic retinopathy (Adapted from Wong et al (2016))

1. Histological lesions

The first morphological anomaly of DR is the thickening of the basal membrane of retinal capillaries, which conditions the loss of control of endothelial proliferation. The disappearance of intramural pericytes and the modification of endothelial cells lead to the loss of the self-regulation function of retinal capillaries. In 1961, Cogan and Ashton, described the presence of pericyte degeneration, microaneurysms formation, basement membrane thickening and arteriolar hyalinosis with consequent venular dilation and tortuosity in diabetic retina (19). More recently, in a rat model of DR, it has been demonstrated that the cellular population of the normal retinal vasculature seems to be quite stable. The replacement of senescent endothelium or pericytes may occur by mitosis of cells resident in the vessel or by recruitment of circulating relatively undifferentiated endothelial progenitor cells (EPC) or pluripotent cells (PSC). The release of these cells from the bone marrow is, however, depressed in diabetes (20). Dilation and increased tortuosity of capillaries and vascular tree assembly as well as the appearance of capillary microocclusions occur and can be visible in fluorescein angiography. With the progression of the disease, microaneurysms arise. These ectasic structures resulting from a reactive vasodilation of capillaries in response to hypoxia in an unperfused retinal territory may rupture, causing superficial (flame) or deep (blot and dot) retinal hemorrhages. On the other hand, they present a deficient blood-retinal barrier (BRB), hyperpermeable to the passage of fluid and proteinaceous material, clinically manifesting as retinal thickening and exudates appearance that may involve the macula, decreasing visual acuity. Vascular endothelial growth factor (VEGF) retinal levels have been shown to be increased early in the course of diabetes. This growth factor induces capillary hyperpermeability by opening endothelial tight-junctions and increases the adhesion of leukocytes to the endothelial wall of retinal capillaries (21).

2. Blood flow

At least three processes can contribute to retinal capillary occlusion and obliteration in diabetes: proinflammatory changes and leukostasis, increased microthrombosis and accelerated apoptosis of retinal capillaries (22). Endothelial adhesion of leukocytes to the vascular wall is responsible for the appearance of micro-territories of retinal hypoxia. This leukostasis seems to be initiated by the presence of AGE (advanced glycosylation end products) with the aid of the protein ICAM-1 (intercellular adhesion molecule). These products result from non-enzymatic glycation, more pronounced in diabetics, of which the most known product is glycated hemoglobin A_{1c} (HbA_{1c}). It has been found that blood flow is decreased in diabetic patients, which is in part explained by the occurrence of leukostasis (23).

With the progression of the disease, the capillaries on the arterial side of the retinal circulation show hyaline thickening of the arteriolar wall, increased cell loss and closure. Simultaneously, on the venous side of the circulation, there is venular dilation and tortuosity, initially reversible and latter permanent due to sclerosis (19). There is an increase in the number of microaneurysms, that evolutes with progressive thickening with eventually disintegration of their walls (24). As the areas of capillary closure enlarge, they are seen to be traversed by a few enlarged capillaries, which appear to act as arteriovenous shunts, receiving the blood diverted from the surrounding closed capillary net. These "shunt" enlarged vessels appear close to totally acellular and occluded capillaries and result whether from primary distension of one channel with consequent closure of alternate capillary pathways or, more probably, from primary occlusion of some capillaries with secondary distension of one or more channels. They present earlier in the central portion of the retina, where they are subject to high intravascular pressure, near capillaries arising from side branches of the main retinal artery (24).

These observations are summarized in Table 1.

Table 1: Evolution of Retinal vascular lesions in diabetes

Stage	Ophthalmoscopy	Pathology, small vessels	
		venous side	arterial side
0	–	?	
1 – initial	Rare aneurysm	Endothelial proliferation ++ Aneurysm ++	Endothelial degeneration +
2 – intermediate	Numerous aneurysms Hemorrhages Exudate	Endothelial proliferation ++ Aneurysm ++	Endothelial degeneration ++ Focal capillary closure
3 – advanced	Same lesions as in stage 2 Large hemorrhages Venous beading	Endothelial degeneration +++ Aneurysm +++ A-V shunts Large area capillary closure	
4 – final	Same lesions plus retinitis proliferans	Endothelial degeneration ++++ Aneurysm +++ Generalized capillary closure	

Modified from Cunha-Vaz [4].

Capillary occlusion and stasis of axoplasmic flow result in infarcts of the retinal nerve fiber layer (RNFL) clinically visible as cotton wool spots. Longitudinal studies in diabetic patients have shown that capillary closure is mostly irreversible but that a percentage of capillaries can be recanalized (25).

3. Neovascularization

The end stage in the natural history of DR is proliferative DR. When retinal ischemia occupies a sufficient area of retina, with severe hypoxia, vasoproliferative factors are released and cause the appearance of neovessels from veins adjacent to unperfused territories. Several growth factors have been implicated in this phenomenon such as fibroblast growth factors 1 and 2 (FGF 1 and 2), platelet derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1) and VEGF. The inhibition of anti-angiogenic factors, as PEDF, also contributes to neovascularization. Initially, small abnormal vascular formations, intraretinal microvascular abnormalities (IRMAs), appear, resulting from the proliferation of endothelial cells. With the evolution of the disease, the extracellular matrix is broken by proteases and neovessels from retinal venules penetrate the internal limiting membrane forming a capillary network between the inner layer of the retina and the posterior hyaloid. Neovessels are most often observed at the boundaries of the non-perfused retina, along the vascular arcades (neovascularization elsewhere, NVE) and in the optic nerve head (neovascularization of the disc, NVD). They are fragile and hyperpermeable vascular structures that disrupt to minimal vitreous traction, with consequent hemorrhage into the vitreous cavity and preretinal space. With the progressive maturation of the neovessels the degree of the associated fibrous tissue increases. In the late stages these fibrous materials can regress leaving only an avascular network adherent to the retina and posterior hyaloid. With the contraction of the vitreous the tractional forces on the retina can cause edema and tractional or rhegmatogenous retinal detachments.

Risk factors

We have assisted to an increase in the prevalence and incidence of diabetes mellitus and its complications. The increased life expectancy in the industrialized world as well as the sedentary lifestyle and the change in the eating habits with overweight have been pointed as reasons for this increase.

Previous studies indicate poor glycemic control, longer disease duration age, albuminuria, high systolic blood pressure , hypercholesterolemia, high body mass index and insulin use as risk factors of DR (26).

DR and DME share many common risk factors.

As modifiable risk factors, serum glucose and lipid levels as well as hypertension appear to be the most important and have been associated with the progression of diabetic retinopathy (DR). Disease duration, age and genetic predisposition are the main non-modifiable risk factors of DR progression.

1. Hyperglycemia

The quality of glycemic control is the best established risk factor for DR. The Wisconsin study demonstrated a relationship between the incidence and progression of DR with glycemic control at the beginning of the study as well as with HbA_{1c} throughout the study in both type 1 and 2 diabetics (6).The Diabetes Control and Complications Trial, DCCT, involving 1441 type 1 diabetic participants, demonstrated that the incidence of DR lowered by 76% in the group with HbA1c lower than 7.2% under insulin therapy or insulin pump use, as well as reduced the progression of established DR by 54% and the frequency of PDR by 47% (27). The United Kingdom Prospective Diabetes Study, UKPDS, also showed that a lowering of HbA_{1c} by 11% decrease the need of laser photocoagulation in one-third of patients with type 2 diabetes and demonstrated the unequivocally importance of a "tight" blood glucose control for reducing the incidence and progression of DR (28). The prevalence of macular edema increases with the severity of DR but can be present at any stage. Chronic hyperglycemia proved to be a strong and independent predictive factor for macular edema at 10 years of follow-up (27).

The actual recommendation is to maintain a glycemic control near normal values (HbA1c ≤ 7.0%) (29).

2. Dyslipidemia

Hyperlipidemia is also under discussion as a risk factor for development of DR. The randomized FIELD study (Fenofibrate Intervention and Event Lowering Diabetes) included 995 type 2 diabetic patients and found that treatment with fenofibrate significantly reduced progression retinopathy as well as the need of laser photocoagulation (30). This effect was independent from the blood lipid levels. The primary endpoint of this study, the reduction in cardiovascular events, was not achieved, and so a definite recommendation was not given. However, treating of hyperlipidemia is usually recommended.

3. Arterial Hypertension

A large number of studies have confirmed the effect of the elevated blood pressure on the development and progression of DR. Notable, the UKPD Study showed that, in hypertensive patients, a reduction in arterial blood pressure to a target value <150/85 mmHg lowered the rate of DR progression by 34% and the need of laser photocoagulation by 35%, being associated with 47% less loss of visual acuity (31). The EUCLID study (Eurodiab Controlled Trial of Lisinopril-Dependent Diabetes), included 530 insulin-dependent normotensive patients, demonstrated that patients under lisinopril therapy present a lower rate of DR progression, comparing to the placebo group (32). Other studies with normotensive diabetic patients, showed little or no effect in blood pressure reduction. The ACCORD-Eye Study (33)(34) showed that blood pressure reduction in type 2 diabetic patients had no effect on the development and progression of DR and the Diabetic Retinopathy Candesartan Trials (DIRECT), including only normotensive patients with both type 1 and 2 diabetes, failed to demonstrate a significant protective effect (35)(36). A recent systematic review of studies dealing with blood pressure reduction in type 1 and 2 diabetic patients, showed a little evidence for a beneficial effect of blood pressure control on DR progression (37). However, arterial hypertension seems to worsen DME and promotes retinal vascular proliferation. Furthermore, the diabetic nephropathy sometimes associated, can induce a major worsening of macular edema.

The UKPD study found no difference in DR progression in patients with type 2 DM taking atenolol and those taking captopril (31). Thus, actually there is no definitive medical recommendation concerning the recommended antihypertensive agent. The only recommendation is to maintain a tight control of arterial hypertension to values ≤ 130/80 mmHg.

4. Obesity

The effect of **obesity** on DR presents inconclusive and conflicting findings. In the EURODIAB Prospective Complications Study, involving patients with type 1 diabetes, larger waist to hip ratio was associated with incidence of DR after more than 7 years of follow-up (38). In contrast, many studies in type 2 diabetes found an inverse relationship between obesity and DR (10). Weight loss, exercise and good nutrition is a recommendation that should be maintained in order to lower diabetes prevalence and the development of its complications.

5. Duration of diabetes

It is recognized that the duration of diabetes is one of the most important risk factors for the development of DR.

In the Wisconsin Epidemiologic Study of Diabetic Retinopathy, 80% of patients with type 2 diabetes mellitus (DM) present, after 20 years of disease, evidence of any retinopathy, while in type 1 DM almost every patient present any retinopathy sign at this time (4)(5). The Wisconsin study revealed that the incidence of DR and PDR were higher in insulin-treated patients and increase with the disease duration. The prevalence of DR in persons who had diabetes for less than 5 years is 28.8%, while it increases to 77.8% in persons who had diabetes for 15 or more years. In the same way, PDR prevalence is 2.0% and 15.5%, respectively, in persons with less than 5 years and 15 or more years of disease duration (5) .

6. Ocular Risk Factors

Previous cataract surgery has been associated with progression of DR, with a doubling of DR progression rates 12 months after surgery (39). A study in 50 diabetic patients underwent uncomplicated phacoemulsification surgery concluded that the surgery did not cause acceleration of DR postoperatively and, despite macular oedema has been common after surgery, it may follow a benign course and probably represent the natural disease progression rather than a direct effect of surgery (40). Furthermore, a myopic refractive error appears to protect against DR (41)(42).

7. Genetic Predisposition

Genetic predisposition exists in diabetes, however robust genetic markers have not yet been detected. Strong familial clustering has been identified in different studies, both in type 1 and 2 diabetes (43). Hereditability estimates of 25% have been reported in patients with type 1 DM with PDR in the FIND-EYE sample of 2368 diabetic subjects (44).

The possible candidate genes contributing to the development of diabetic retinopathy are genes for Aldose Reductase (ALR), Nitric Oxide Synthase (NOS) genes, genes for Receptor for Advanced Glycation Endproducts (RAGE), genes coding for Angiotensin Converting Enzyme (ACE gene), Human Leucocyte Antigen (HLA) genes and genes for Vascular Endothelial Growth Factors (VEGF).

Candidate and genome-wide association studies have identified few genetic markers for DR in type 2 DM (45) (46). However, genes associated with DM, T2D and vascular diseases do not appear to be consistently associated with DR and should be investigated in additional DR cohorts (46). Polymorphisms involved in differences in RAGE gene regulation appear to influence the pathogenesis of diabetic vascular complications in type 2 diabetic patients with and without retinopathy (47). Polymorphisms identified in the regulatory region of the aldose reductase gene have been strongly associated with diabetic retinopathy in the Chinese population with type 2 DM (48). Specific VEGF polymorphisms, increasing VEGF production from high-expressing haplotypes, have been identified as positive predictive factors for the development of proliferative diabetic retinopathy (49).

Furthermore, specific gene variants in ICAM1, PPARGC1A and MTHFR have been linked to different NPDR phenotypes, and could potentially increase the knowledge about the mechanisms underlying the different phenotypes evolution (50).

Complex interaction between genes and environment influence the development of DR. Hyperglycemia, lipidemia and other biochemical perturbations of the diabetic state can potentially modify the DNA with consequent epigenetic changes. Diabetes increases oxidative stress in the retina and its capillary cells with interactions with other major metabolic abnormalities. As the duration of the disease progresses, mitochondrial DNA (mtDNA) is damaged and the DNA repair system may be compromised (51). This hypothesis is supported by the observation of persistence of beneficial effects due to “metabolic memory” in patients with type 1 and 2 DM that have been previously under intensive hypo-glycemic treatment (52)(53).

In clinical practice, these risk factors are often considered when determining the risk of DR progression. In some countries they are even used to determine the frequency of screening(54). However, despite being important in determining the initial DR development, these risk factors do not explain the great variability that characterizes the evolution and rate of progression of the retinopathy in different diabetic individuals (55)(56)(57). A proportion of variance remain unexplained. Many diabetic patients never develop sight-threatening retinal changes after many years of disease, maintaining good visual acuity. However, there are other patients that even after only a few years of diabetes show a retinopathy that progresses rapidly and may not even respond to available treatments.

Classification

Diabetic Retinopathy

Classifying and staging DR is important to establish degrees of severity of retinopathy and the consequent prognosis in view of different indications of surveillance and treatment. Since the first classification of DR appeared at the symposium of Airlie House in 1968, several classifications have been proposed. The classification adopted in the ETDRS (58) is based on the modified Airlie House classification used in the Diabetic Retinopathy Study (DRS), that included 1758 patients and studied the benefit of prompt photocoagulation versus no treatment in PDR (59). This is still the reference classification used nowadays (Table 2).

Table 2: Classification of Diabetic Retinopathy based on ETDRS level

	SEVERITY	LESIONS
10	No retinopathy	No DR lesions
14-15	Questionable DR	≥ 1 Haemorrhage but microaneurysms absent
20	Minimal NPDR	Microaneurysms (MA) only
35	Mild NPDR	Hard exudates (HE); mild retinal haemorrhages (H) or cotton wool spots
43	Moderate NPDR	Moderate H/MA in 4-5 fields or severe H/MA in 1 field or mild IRMA in 1-3 fields
47	Moderately severe NPDR	Both characteristics from level 43 or IRMA in 4-5 fields; severe H/MA in 2-3 fields or venous beading (VB) in 1 field
53	Severe NPDR	Two or more characteristics from level 47 or severe H/MA in 4-5 fields; moderate-severe IRMA in 1 field or VB in 2 fields
61	Mild PDR	NVE (neovessels elsewhere) < ½ DA (disc area) or NVE Isolated fibrosis
65	Moderate PDR	NVE ≥ 1/2 DA in 1 field and/or NVD (optic disc neovessels) < 1/4 to 1/3 DA
71	Severe PDR	NVD ≥ 1/4 to 1/3 DA (≥ standard photog 10A) NVE or NVD associated with VH (vitreous haemorrhage) or PRH (preretinal haemorrhage)
81-85	Advanced PDR	Non-visible FO, macula obscured by VH or PRH and or retina detached ate the centre of the macula

Fundus imaging of the posterior pole of the retina has been accepted as the reference standard to grade DR for decades and relies on a 20–50° field of view for each of the 7 fields. The standardized

7-field images show hemorrhages, MAs, exudates, cotton-wool spots, intra-retinal microvascular abnormalities and neovascularization, as well as retinal thickening, to be recognized, staged, and quantified in terms of size, number and location, and graduated by comparison to reference standards photographs. Nonproliferative diabetic retinopathy is subdivided into five stages according to the severity of 4 retinal signs: hemorrhages and microaneurysms, cotton wool spots, intraretinal microvascular abnormalities (IRMAs) and venous anomalies. Severe NPDR (formerly called pre-proliferative) corresponds to stage 53 and presents a five-year probability of developing neovascularization as high as 40%, depending on the associated risk factors (60). Proliferative diabetic retinopathy is subdivided into 4 states according to the severity of neovascularization.

In 2003 a consensus regarding clinical disease severity classification systems for diabetic retinopathy was proposed as the International Clinical Diabetic Retinopathy Severity Scale (61). This simplification of the ETDRS scale consists of five levels with increasing risks of retinopathy. The first level is "no apparent retinopathy," and the second level "mild NPDR" includes ETDRS stage 20 (microaneurysms only). The risk of significant progression over several years is very low in both groups. The third level, "moderate NPDR," includes eyes with ETDRS levels 35 to 47, and the risk of progression increases significantly by level 47. The fourth level, "severe NPDR" (ETDRS stages 53 and higher), have the worst prognosis concerning progression to PDR and is based on the "4:2:1 rule". The fifth level, "PDR" includes all eyes with definite neovascularization (Table 3).

Table 3: Classification of Diabetic Retinopathy based the International Clinical Diabetic Retinopathy and Disease Severity Scales

PROPOSED DISEASE SEVERITY LEVEL	LESIONS
No retinopathy	No DR lesions
Mild NPDR	Microaneurysms only
Moderate NPDR	More than just MA but less than severe NPDR
Severe NPDR	Any of the following: intraretinal haemorrhages (≥20 in each of 4 quadrants), definite venous beading (in 2 quadrants) or intraretinal microvascular abnormalities (in 1 quadrant); no signs of proliferative DR
PDR	One or more of the following: neovascularization, vitreous or preretinal haemorrhages

In the UK, the ETDRS classification has been simplified in screening protocols and applied to one-field (Scotland) and two-field (England, Wales and Northern Ireland) fundus photography (62).

These simplified approaches were created to be used in a simple way by optometrists and non-ophthalmologists so that diabetic retinopathy features can be detected and referred for expert opinion to an ophthalmologist.

A uniform qualitative and quantitative analysis with a reproducible grading approach is needed to critically evaluate diabetic retinopathy. The ETDRS grading represents the morphological picture of DR progression and any new information on classification should be evaluated in the context of this well-established classification/grading.

However, this 7 field imaging methodology only image 30º of the total retinal area and does not visualize retinal peripheral lesions. Current non-proliferative retinopathy classifications are also limited in more severe cases, owing to their broad description of moderately severe and severe non-proliferative retinopathy, giving little information about the risk of developing sight threatening diabetic complications of individual patients.

Several observations in the past have indicated that the retinal periphery plays a key role in predicting the risk of diabetic retinopathy progression (63)(64).

Ultra-wide field (UWF) imaging devices capture up to 200º of the retina in a single image, representing approximately 82% of the retinal area, with a resolution of 14 μm, in only 0.25 seconds. Paolo Silva, Aiello and colleagues have identified what they referred to as predominantly peripheral lesions (PPL), DR lesions with a greater extent outside versus inside the standard ETDRS fields (65)(66). They found that eyes with PPL at baseline had a 3.2 fold increased risk of 2 step or greater DR progression and a 4.7 fold increased risk of progression to proliferative DR and so the peripheral retina should be taken into account in DR classification systems.

Valuable information on the progression of NPDR could be acquired in future clinical trials. Suggested supplementary measurements for NPDR lesion assessment have been proposed. Shape and size of lesions, as diferent MA shape may have different risks of leakage or oclusion; type of intraretinal microvascular abnormalities, a precursor to proliferative diabetic retinopathy; concomitant hypertensive retinopathy, with presence of cotton wool spots and flame-shaped haemorrhages; peripheral location of microaneurysms and intraretinal microvascular abnormalities, that may be more predictive of progression to sight-threatening diabetic complications and turnover of lesions (microaneurysms and cotton wool spots) which may predict the risk of progression as well as the treatment response (67).

Further research is required to determine new blood biomarkers, retinal imaging markers, and genetic determinants of diabetic retinopathy. These findings will enable us to improve our understanding of the pathogenesis, risk prediction, prevention and treatment of diabetic retinopathy, and finally improve the management of the retinopathy.

Diabetic Maculopathy

Diabetic macular edema is an important complication whose staging is independent of retinopathy staging.

The ETDRS introduced the term clinically significant macular edema (CSME) defined from the biomicroscopic examination. It was defined as retinal thickening involving or threatening the center of the macula (even if visual acuity was not reduced). Clinically significant macular edema was considered when, at least, one of the following characteristics were present: 1) any retinal thickening within 500 μm of the center of the macula or 2) hard exudates at or within 500 μm of the center of the macula, if associated with thickening of the adjacent retina (not residual hard exudates after the resolution of the thickening) or 3) a zone /zones of retinal thickening ≥ 1 disk area, any part of which is within 1 disc diameter (1500 μm) from the center of the macula (58). It includes both center involving (criterion 1) and non-center involving (criterion 2 and 3) macular edema.

With the emergency of the OCT machine, the possibility of quantifying the edema became available, offering new opportunities for a more objective follow up of the edema. Optical Coherence Tomography has improved the investigator's ability to follow macular changes, allowing the detection and localization of increased retinal thickness (RT) even before clinical detection by slit-lamp examination.

One concept that will be widely addressed in the thesis and that arose with the appearance of the OCT, is center-involving DME (CIME). It is defined on OCT as an increased retinal thickening, above a specified threshold. The Diabetic Retinopathy Clinical Research Network (DRCR.net) have conducted a study in 122 eyes with diabetes mellitus and no or minimal retinopathy, in the absence of macular thickening on clinical examination, in order to evaluate the macular thickness in the nine standard OCT subfields using Heidelberg Spectralis OCT and Stratus OCT scans (68). The mean central subfield (CSF) thickness was approximately 70 μm thicker when measured with Heidelberg Spectralis OCT as compared with Stratus OCT in this group of patients. They also

agreed that the retinal thickness measured in individuals with diabetes with no or mild NPDR was comparable to the thickness obtained in healthy individuals without diabetes (69)(70). Central subfield (CSF) thickness values ≥ 320 μm for males and 305 μm for females (~2 SD above the average for this normative cohort) were proposed as gender-specific thicknesses levels to have reasonable certainty that diabetic macular edema involving the central subfield is present, using Heidelberg Spectralis measurements. According to DRCR.net, values of 305 μm in men and 290 μm in women may be used as cutoff point for central subfield thickness, in Zeiss Cirrus OCT machine (Figure 3), to presume macular edema is present in diabetic eye disease clinical trials (71).

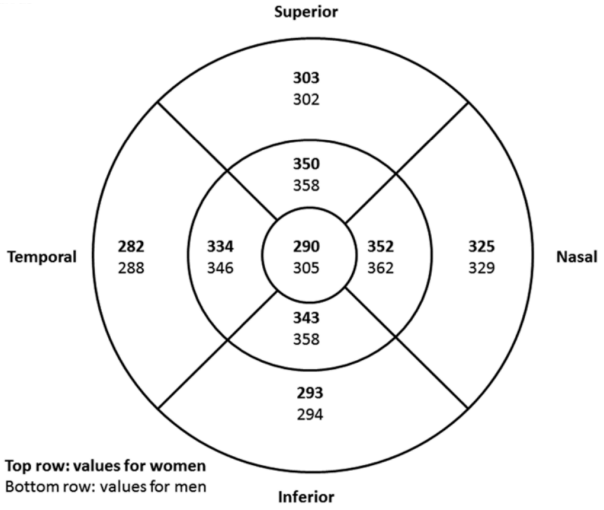

Figure 3: OCT threshold values to define CIME in Zeiss Cirrus OCT (71)

It is important to check the accuracy of the OCT measures, ensuring that the scan is centered and of good quality.

Non-center involving DME is defined clinically as definite retinal thickening due to DME within 3000 μm of the center of the macula but not involving the center. On SD-OCT it is defined as cystic spaces and/or retinal thickening in non-central macular subfields.

This definition of DME according to the location of the edema is important in order to better delineate the treatment strategy, particularly the use of focal laser photocoagulation in non-center involving DME, according to the ETDRS guidelines.

The term subclinical macular edema (SCME) has been proposed to describe the early stages of macular edema, even before the development of CIME. Subclinical macular edema was defined as: (1) no edema involving the center of the fovea as determined by slit-lamp biomicroscopy without reference to whether an indirect lens or contact lens viewing system was used and (2) a CPT measurement on Stratus OCT of ≥ 225 μm and ≤ 299 μm (the value judged to be recognized as clinically apparent thickening) (72). It may be a good biomarker for CIME development (73) (74)(75)(76).

Diabetic macular edema may also be classified based on specific morphological alterations of the retina and the presence of macular traction, either by epiretinal or vitreomacular membranes (77).

An OCT-based method, the OCT-Leakage software, have been recently introduced by our group and is capable of identifying and quantifying sites of alteration of the blood retinal barrier (BRB), in a non-invasive way, by mapping areas of lower-than-normal optical reflectivity (LOR ratios), reflecting changes in the retinal extracellular fluid (78). We found good correspondence between the location of increased areas of low optical reflectivity identified by OCT-Leakage and the main sites of leakage on fluorescein angiography (79). Furthermore, with OCT-Leakage the areas of abnormal fluid accumulation can be identified in specific retinal layers, clearly offering more information than previously obtained with fluorescein angiography. This imaging method can complement OCT and OCTA in characterization of DME.

Fluorescein Angiography (FA) also contributed to the characterization of diabetic macular edema. It has been traditionally used to locate the sources and extent of vascular leakage. Based on FA, the leakage of DME may be focal, with localized areas of leakage from microaneurysms or dilated capillaries, diffuse, with diffuse leakage involving the entire circumference of the fovea, or diffuse cystoid leakage, when there is mainly diffuse leakage but accumulation of the dye within cystic areas of the macula during the later phase of the angiogram. There is, however, a significant correlation between the features of fluorescein angiography and OCT in clinically significant diabetic macular edema (80), and thus there is no strong rationale for using standard FA in routine examination. The exam is not free of side effects and can cause adverse reactions, including nausea, vomiting, acute hypotension and even in rare cases anaphylactic shock (81).

Ischemic maculopathy is an important component of diabetic maculopathy, in which microvascular changes occur, with loss of capillaries in the macula. It is a vision-threatening disorder and it does not respond well to treatment and may be associated with a severe irreversible visual loss. Ischaemic maculopathy may occur in the presence of macular edema. Ischemic maculopathy is characterized by enlarged or irregular FAZ (foveal avascular zone), loss of

perfusion in the capillary network surrounding the FAZ, presence of FAZ abnormalities, capillary nonperfusion areas in the macula and the presence of microaneurysms at the border of FAZ (82)(83).

Fluorescein Angiography can be used to assess the outline characteristics of the foveal avascular zone (FAZ), as reported in the Early Treatment Diabetic Retinopathy Study group report 11 (84). This classification grades the foveal avascular zone according to the FAZ outline, the size of FAZ and the presence of capillary loss, in Grade 0 (with normal FAZ outline, size < 300 μm, no capillary loss) to Grade 4 (with capillary outline totally destroyed and severe capillary loss). Once again, FA in this context has been replaced by the non-invasive and more sensitive OCTA technology, that allows a more detailed morphological evaluation of central macular vascular changes than FA and perform quantitative metrics, without the need of an intravenous injection of fluorescein (85).

Recent studies using ultra-wide field fluorescein angiography (UWFFA) appear to offer added value, allowing a better evaluation of peripheral retinal lesions as areas of ischemia, which appear to be significantly correlated with occurrence of DME (86).

The recent developed wide-angle OCTA provide approximately 100º OCTA images of the fundus and may be more suitable in DR evaluation, visualizing the peripheral retina (87).

A study comparing the ability of a wide-angle swept source OCTA 12×12 mm fields of five visual fixations (PLEX Elite 9000®), with that of UWFFA (Optos® panoramic 200T) to detect non-perfusion areas or retinal neovascularization in eyes with DR conclude that the wide-angle OCTA present a similar sensitivity and specificity as UWFA in detection of such lesions (88).

These new imaging methods allow a bigger retinal area to be imaged and may, in the future, contribute with new classification systems of diabetic retinopathy and diabetic maculopathy.

Different components of diabetic retinal damage: 3 Main Disease Pathways

1. Edema

An abnormality of the BRB, demonstrated both by vitreous fluorometry and fluorescein angiography, is an early finding both in human and diabetes animal models (89)(90). The alteration of this barrier is well demonstrated by fluorescein leakage and leads to the development of retinal edema. It may occur at any ETDRS stage of disease progression.

Ultrastructural studies showed that, despite the accumulation of the extracellular fluid, the primary change is intracellular, with intracytoplasmic swelling of the Müller cells that later spills over into the extracellular compartment (91)(92). The transudation from the perifoveal blood vessels also contribute to the edema, as well as to the ischemia, with malfunction of the RPE and prejudice of the normal depuration function of this structure (93)(19). These histologic studies might, however, present critical limitations, as tissue preparations involved fixation of the specimens, using fixative chemicals and embedding material led to significant tissue shrinkage and dehydration, with consequent structural alterations in the intracellular spaces and relevant structural artefacts (94).

Accurate assessment of the retinal structure, central to disease understanding and diagnosis, is obtained with *in vivo* measurements by a variety of digital imaging technologies.

Fundus Fluorescein Angiography (FFA) has been used to identify the source and extent of capillary leakage. Standard FFA use digital cameras and captures an image 30 to 45 degrees in width. Protocols such as the seven standard fields have been utilized in order to obtain midperipheral angiographic information, but they are mainly used for clinical research purposes, due to the extra time needed to perform and grade the exam. The seven standard 30º fields images was developed for the Diabetic Retinopathy Study (59) and image approximately 75º of the posterior pole.
Ultra-wide field angiography is a panoramic digital technique that may capture up to 200º of the fundus at once, allowing to visualize virtually the entire fundus in each frame of the study. Recent studies have proven the importance of the identification of major peripheral pathology, including NV and peripheral capillary non-perfusion areas, that would be missed with traditional imaging

methods (95). Ultra-wide field fluorescein angiography may also reveal peripheral abnormalities of undetermined clinical significance, including peripheral capillary non-perfusion and late peripheral vascular leakage (PVL). Peripheral vascular leakage (PVL) was described as an angiographic phenomenon of late leakage from retinal veins and arteries, and frequently occurred in the setting of active retinopathy. In the study of Oliver and colleagues, peripheral vessel leakage was associated with both neovascularization and macular leakage and it is suggested as a possible indicator of retinal ischemia (95).

Optical coherence tomography (OCT) allows an objective assessment of macular thickness and vitreous-macular interface and is currently an essential tool for the assessment of diabetic macular edema. The presence of retinal edema is well demonstrated by increased retinal thickness and it is a frequent finding since the earliest retinopathy stages (75)(96).

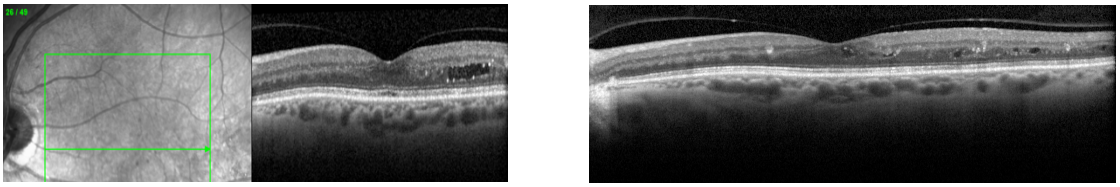

Figure 4: SD-OCT images with presence of intraretinal fluid at the level of the ONL

Spectral domain-OCT, with high resolution and reduced speckle noise, has enabled segmentation of the individual retinal layers. Several OCT instruments provide automated segmentation. Our research group have implemented and validated a graph based segmentation algorithm applied on Cirrus HD-OCT (Carl Zeiss Meditec, Dublin, CA, USA), that automatically identify 8 retinal interfaces, namely Vitreous to Inner Limiting Membrane (ILM), Retinal Nerve Fiber Layer (RNFL) to Ganglion Cell Layer (GCL), Inner Plexiform Layer (IPL) to Inner Nuclear Layer (INL), INL to Outer Plexiform Layer (OPL), OPL to Outer Nuclear Layer (ONL), ONL to Inner Segment (IS), Outer Segment (OS) to RPE and RPE to Choroid. The segmentation methodology is well suited for detecting individual retinal layer changes and has been used in our research work (97).

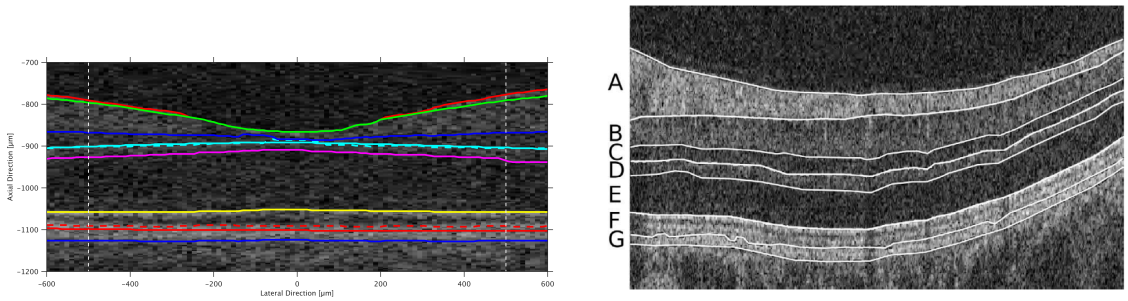

Figure 5: Image with labeled intralayer segmentation. Eight surfaces and corresponding intra-layers labeled from (A) to (G).
(A) Retinal nerve fiber layer (RNFL); (B) ganglion cell layer and inner plexiform layer (GCL-IPL); (C) inner nuclear layer (INL); (D) outer plexiform layer (OPL); (E) outer nuclear layer and photoreceptor inner segments (ONL-IS); (F) photoreceptor outer segments (OS); (G) retinal pigment epithelium and Bruch's membrane (RPE)

Bandello *et al* described, in a study using SD-OCT Cirrus and automated segmentation software, in 194 patients with mild NPDR, that the increase in retinal thickness in eyes with subclinical (i.e. retinal thickness (RT) 260-290 μm in women and 275-305 μm in men) and clinical DME (i.e. RT ≥ 290 μm in women and ≥ 305 μm in men) is predominantly located in the inner nuclear layer (INL) but extends to a lesser degree to the ganglion cell-inner plexiform layer (GCL-IPL) and outer plexiform layer (OPL), indicating that it is probably due to extracellular fluid accumulation originating from the deep retinal vascular capillary net and not from any specific neuronal or glial cell swelling (98). The investigators have proposed a composite grading of DME taking into account the location of thickening (central thickness involvement, involvement of the inner ring and/or of the outer ring), its extent (highest absolute mean RT in any subfield) and the presence of vitreous traction, and concluded that the increased thickness of the central subfield was the best predictor for the development of clinical macular edema, with 85.7% sensitivity and 71.9% specificity (99). Some authors, however, defend the intracellular nature of fluid accumulation, with a diabetes-induced hypertrophy of Müller cells, responsible for the INL thickening and consequent INL-OPL thickening on OCT verified in diabetic patients with mild to moderate NPDR with no macular edema (100). In these initial stages, there was no significant differences between ONL/ELM or photoreceptor/RPE layer thickness between diabetics and controls. It seems that the outer retina is not significantly influenced by diabetes in the early stages of the disease, while the inner retina is initially more affected (100).

The characterization of the magnitude of edematous changes is fundamental, particularly when realizing that macular edema is the most frequent sight threatening complication of DR. An increasing number of studies have reported the relevance of measuring the retinal thickness and individual layer thickness after treatment for DME as a surrogate marker (101)(102). Spectral domain-OCT based central subfield thickness (CSF) or cube average thickness (CAT) provide reliable objective measurements for screening and follow up of DME. There is an increase in thickness measures in SD-OCT with the increasing severity of retinopathy. So, these metrics are frequently used as surrogate markers for prognosticating disease severity.

The Diabetic Retinopathy Clinical Research Network (DRCR.net) (72) performed an observational 2-year study of diabetic eyes and found that approximately 38% of eyes identified has having SCME increased thickening of at least 50 μm and have a center point thickness (CPT) of at least 300 μm at an annual visit or needed treatment for DME by 2 years. Our group have also proved those findings, in a 2 year study of 348 mild NPDR diabetics, reporting that DME progression was 3.686 times higher in eyes with SCME at baseline (74). Also, a study of 205 patients followed for 2 years, demonstrated that patients with increased RT in the central subfield at baseline showed a 12-fold

risk of progression to CIME compared with patients without SCME. We can conclude that SCME is a good predictor of progression to CIME (76).

The OCT leakage software is able to identify sites of low optical reflectivity (LOR) depicted as 2-dimensional images of retinal areas by analyzing a simple representative value for each OCT A-scan (78). These LOR sites represents the existence of reflectivity values falling below a predefined threshold of optical reflectivity from A-scans obtained from a healthy control population. The white areas depicted in the LOR maps represent the location of the A-scans having reflectivity values below the predefined threshold and the black areas represent A-scans with reflectivity above that threshold (Figure 6). Extracellular fluid distribution of given areas of the retina can be measured by LOR ratios, representing the number of A-scans with optical reflectivity values below the threshold divided by the total number of A-scans within the considered area. To identify LOR sites in the different retinal layers, the segmentation algorithm described above is used (97). For the OCT leakage, the sections with LOR ratios above the mean plus 2 standard deviations from the healthy population, for the full retina and for layer-by-layer analysis, were classified as having increased retinal fluid.

In a study performed to compare the location and extent of abnormal fluid accumulation determined by OCT leakage with the location and extent of fluorescein leakage identified by FA, in eyes with NPDR (79), the changes in extracellular space, represented by the LOR area ratio, agreed well with the sites of leakage on the FA examinations. The OCT leakage software identified, in general, larger areas of abnormal fluid accumulation in the retina. In the eyes with no visible fluorescein leakage, evidence of abnormal fluid accumulation was present in 52.6% of the eyes, demonstrating the higher sensitivity of OCT leakage to detect abnormal fluid in the retina when compared with FA. OCT leakage was able to identify the location and extent of the abnormal fluid accumulation in each retinal layer.

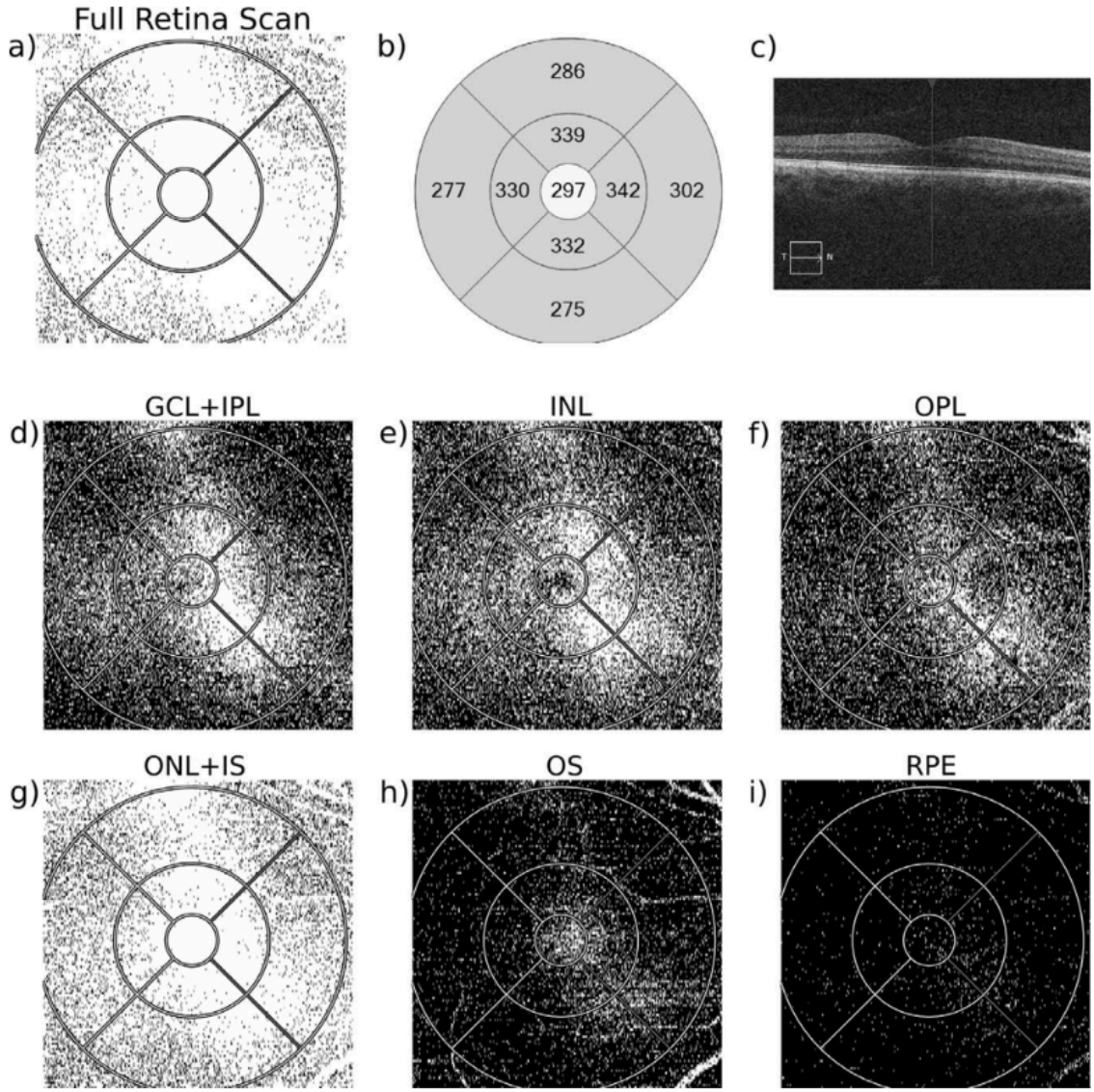

Figure 6: OCT-Leakage LOR maps for the right eye of a male diabetic patient with subclinical macular edema. (a) Full retina scan LOR map; (b) ETDRS grid map; (c) B-scan centered on the fovea; (d–i) LOR maps layer by layer, showing increased LOR ratios reflecting increases in the retinal extracellular space in the GPL-IPL, INL, OPL, and ONL-IS and extending to the OS layer.

A recent study from our group aiming to characterize the type of retinal edema in the initial stages of type 2 diabetic retinopathy was performed (103). One-hundred and forty two eyes from 142 patients (28% women) aged 52-88 years were analyzed with Cirrus HD-OCT 5000 with AngioPlex® OCT Angiography (Carl Zeiss Meditec, Dublin, CA, USA), with layer by layer analysis of the retina using the segmentation software implemented by AIBILI (104)(97). The OCT-Leakage algorithm was applied, capable of performing automated analysis of changes in the retinal extracellular space by identifying sites of LOR (78). Using OCT-Leakage it was possible to identify in the full retina and in its different layers, the location and extent of the sites of extracellular edema in the retina. Macular edema, either SCME or CIME, was present in 43% of eyes in group 10-20, in 41% of eyes in group 35, and in 38% of eyes in group 43-47. The inner nuclear INL was the layer

showing higher and most frequent increases in retinal thickness (79%). The edema was predominantly intracellular in group 10-20 (65%) and extracellular in groups 35 (77%) and 43-47 (69%). The prevalence of SCME and CIME was independent of the severity of retinopathy. The authors concluded that retinal edema is mainly located in the INL and appears to be mostly extracellular, except in the earliest stages of diabetic retinal disease, where intracellular edema predominates.

With the progression of disease macular edema, the presence of cystoid spaces in the INL and OPL, cystoid macular edema (CME), is one of the first morphologic alterations visible on OCT. Sponge-like retinal swelling at the fovea occurs in the OPL, sometimes in association with pathologic findings in the vitreomacular interface. Vascular hyperpermeability in DME may increase the osmotic and oncotic pressure of the intraocular fluids and result in extracellular fluid pooling between the outer segments and retinal RPE, serous retinal detachment (SRD). Chronic macular edema may also contribute to retinal neuronal damage, even if little apparent ischemia. Subretinal fluid, particularly, causes detachment of the photoreceptor layer from the underlying RPE, breaking the normal interactions between those cells. The eyes with SRD have poor prognosis after treatment (105).

Studies comparing FA imaging with OCT alterations demonstrate that cystoid spaces in the INL have a honeycomb pattern of fluorescein pooling, whereas petalloid-shaped pooling corresponds to cystoid spaces in the OPL near the fovea (106). These cystoid spaces at the foveal center on OCT images are associated with an enlarged foveal avascular zone (FAZ) and with higher numbers of microaneurysms (MA) in the perifoveal capillary network, suggesting that both ischemia and leakage form MA contribute to the maintenance of cystoid spaces in DME (107).

According to some studies, retinal thickness is only modestly correlated with visual acuity (VA) in DME and even paradoxical VA changes after treatment were reported by DRCRnet, and so retinal thickness values cannot reliably substitute VA as a surrogate for visual acuity (108). Interestingly, the thickness in the inner retinal layers was correlated positively with visual acuity gain, whereas the outer retinal thickness was associated negatively (102). This suggests that thinning of the outer retinal layers is related to photoreceptor degeneration (or atrophy), contributing to visual disturbance and may support the paradoxical VA changes reported by the DRCRnet.

Visual acuity represents only one, possibly the most relevant, aspect of macular function. Microperimetry depicts a macular sensitivity mapping and is able to quantify macular sensitivity (and fixation) in an exact point of the retina, adding detailed information about the pattern of

macular function alteration. In a study of Vujosevic and collegues, microperimetry was successfully used in the diagnosis and follow-up of diabetic macular edema, with a decrease in macular sensitivity observed when diabetic macular edema developed and correlated with the severity of the edema (109). Microperimetry may be a valuable tool in predicting the outcome of diabetic macular edema, and may supplement the predictive value of OCT and visual acuity.

Disorganization of retinal inner layers (DRIL), as well as disruption of the external limiting membrane (ELM) and ellipsoid zone (EZ) on SD-OCT, have been investigated as important biomarkers for predicting visual outcomes in eyes with diabetic macular edema and its response to treatment. Disorganization of the retinal inner layers affecting > 50% of the central 1 mm foveal area was associated with worse VA in eyes with CIME, and early changes in DRIL predicts future changes in VA, identifying eye with a higher likelihood of improvement or decline in VA (110). Photoreceptors markers, as ELM and junction between the inner and the outer segments (IS/OS), also known as ellipsoid zone (EZ), and RPE have also been studied as useful hallmarks for use in evaluation of foveal photoreceptor layer integrity being closely associated with final VA in DME. They appear to be correlated with levels of serum markers, as nitric oxide (NO) and lipid peroxide (LPO), vascular endothelial growth factor (VEGF) and intercellular adhesion molecule-1 (ICAM-1), urea and creatinine (111)(112)(113), as well as with severity and progression of DR. Therefore, ELM and EZ disruption may be used as surrogate biomarkers in progression of disease. The ELM represents the intercellular junction between the Müller cells and photoreceptors and can be disrupted by extension of the cystoid spaces in adjacent retina or a tear in the outer retinal layers. External limiting membrane disruption is associated with visual impairment in DME and poor response to treatment (114). The transverse length of the disrupted or absent EZ has been correlated with visual impairment (115). The photoreceptors outer segment length appear to be more directly related to visual function than macular thickness measurements and may be a useful physiologic outcome measure (116). Phadikar and colleagues have shown that ELM disruption occurred early than EZ disruption, and the disruption of the later occurred secondarily (117). The reason for that is the fact that ELM, like RPE, has tight junctions and act like a third outer blood retinal barrier, contributing to maintain the retinal homeostasis. The disruption of this barrier contributes to fluid accumulation.

Hyperreflective foci (HF) in retina, appearing as dot-like lesions in SD-OCT, have been studied as biomarker of retinal inflammation, retinopathy progression and response to treatment (118)(119). These HF appear to be present since the early stages of diabetic retina even with no visual impairment, and are in higher number in diabetic patients with a poorer glycemic control or with hypertension (120). A study from Frizziero and colleagues identified hyperreflective intraretinal

foci in the inner and outer retina in diabetic eyes without DR or with mild NPDR (121). It has been speculated that, in DME, they represent lipid-laden macrophages and precursors of hard exudates (122) whereas, in other diseases, they are considered to be degenerated photoreceptors and RPE hyperplasia (123). The presence of hyperreflective foci in the outer retina of patients with DME was closely associated with a disruption of the ELM and EZ on SD-OCT images and with a decrease in VA (124).

2. Microvascular changes with ischemia

The baseline severity of DR is a good predictor of clinical outcome and is used to determine the rescreening strategy (125). Traditionally, DR severity is classified using fundus color photographs, with different grading systems, in which the 7 fields fundus color photograph ETDRS classification, based on the modified Airlie House classification, is, so far, the most acceptable for research purposes.

However, subtle changes in microvascular hemodynamics may be the earliest changes occurring in preclinical diabetic retinopathy, which are not visible in retinal fundus images.

Ludovico *et al*, in 2003, studied the alterations of the retinal capillary blood flow in a group of type 2 diabetic patients with preclinical diabetic retinopathy, using a laser Doppler flowmeter, and found that there was a significant increase in the retinal blood flow in those patients in comparison to healthy control eyes (126). However, the individual response was highly variable between subjects and this increase was only present in half of the eyes. They conclude that the changes in the retinal capillary blood flow were an early alteration in diabetic retina but do not occur to the same degree or at the same time in every retina. It remains to be understood which eyes are at risk of progression of the retinopathy. Would it be the eyes with increased capillary blood flow, apparently creating conditions for more rapid and progressive damage of the capillary walls, or the eyes that do not show this autoregulatory response, possibly due to a failure in this compensating mechanism?

The research of new retinal biomarkers of disease progression will permit an individualized rescreening strategy, enabling earlier intervention and stopping the progression of the retinopathy before vision loss occurs.

Microaneurysms and dot hemorrhages as well as presence of exudates (signs of vascular hyperpermeability) and capillary closure are observed in the initial stages of DR.

The presence of local retinal factors, such as the number of microaneurysm (MA count), MA formation and disappearance rates, as well as the presence of some degree of DR in the other eye, have been implicated in DR progression and in the development of vision threatening complications (127)(128)(129)(130).

Microaneurysm turnover (MAT) is a composite of MA formation and disappearance rate. Microaneurysm formation rate represents microvascular disease activity while MA disappearance rate is considered to be sign of capillary closure and progressive vascular damage. Microaneurysm values have been shown to have a wide range in eyes in the same ETDRS retinopathy level (129), confirming previous observations by Sharp *et al* (131) indicating that MAT values may represent different microvascular disease activity in different eyes. In a prospective follow up study of 10 years, using a computer-assisted method (MA-Traker) capable of mapping the specific location of each microaneurysm and co-register it in the same location in multiple visits, it was found that a MAT of at least 2 MA/year (computed in the first 2 years of the study) was present in 70.8% of eyes that developed CSME, whereas it was found in only 8.3% of eyes that did not develop CSME, making MAT a good indicator of DR progression (132)(128). The method has a sensitivity of 70.6% and a specificity of 91.7% of detecting CSME development in a 10 year period. Cunha-Vaz and colleagues developed an automated software program, the Retmarker DR (Critical Health, Coimbra, Portugal), that is able to automatically detect "red-dot-like" vascular lesions occurring in eye fundus digital images, comparing successive visits to the reference image, based on co-registration and localization of changes. A group from Munich, in the CALDIRET study, have analyzed 160 diabetic patients treated with calcium dobesilate with Retmarker software, during a period of 5 years, and confirms our results, with the higher MA formation rate being associated to the development of CSME (133). Another 2 year prospective study with 410 patients also shows that MA turnover has an high predictiveness for CSME, higher that the remaining parameters, and is a good indicator of retinopathy worsening (129).

Our group has proposed the existence of three different **phenotypes** of mild nonproliferative DR (NPDR) (134). Phenotypes were defined based on MA turnover value (Retmarker) and on macular thickness measures on central subfield (CST), on spectral domain OCT (SD-OCT). The increased thickness in central subfield and in the inner and outer rings were based on the reference values for subclinical macular edema for Cirrus HD-OCT (73)(135). Phenotype A is characterized by a low MA turnover (< 6) and a normal retinal thickness (CST < 260 μm in women and < 275 μm in men); phenotype B, also with a low MA turnover but with thicker retina (CST ≥ 260 μm in women and ≥ 275 μm), the "leaky" phenotype, and phenotype C, characterized by an high MA turnover (≥ 6), with or without increased retinal thickness, the "ischemic" phenotype. Those phenotypes can be identified at as early as 6 months of follow up. This theory was confirmed by the results of a pooled analysis of four different longitudinal observational studies of mild NPDR in type 2 diabetes patients (136)(137). Eight hundred and eighty two patients with NPDR were included, ETDRS grades 20-35, and enabled the collection of 103 progression events. Using only noninvasive procedures, easy to repeat in the clinical practice, the study showed that

characterization of mild NPDR phenotypes has a prognostic value. The chance of developing macular edema within 2 years is 7-25 times higher if the patients have increased CST (phenotype B) and 14-62 times higher if the patients have increased CST measurements and MA turnover ≥ 6 in a period of 6 months (phenotype C), when comparing with phenotype A patients. Phenotype A represent approximately 50% of the mild NPDR patient population and shows a negative predictive value of 97% for the development of macular edema. A recent study performed at AIBILI, Portugal and LV Prasad Institute, India, followed 205 in T2D for 2 years and found that MAT, calculated at 6 months of follow up, could predict with a high degree of confidence the eyes that do not progress for a period of at least 2 years (129). Furthermore, the study confirms the prognostic value of phenotype classification, with phenotype A eyes showing a very low risk of developing CIME, in contrast to phenotype B and C, and phenotype C presenting the higher risk of DR worsening based on ETDRS scale. Of notice is the fact that this worsening was mainly seen in the indian population, probably due to higher HbA1c levels or to a genetic predisposition (138).

The **size of microaneurysms** may also be important, with smaller MA presenting higher risk of rupture and leakage comparing to larger MA (139).

The role of **cotton wool spots**, representing nerve fiber layer infarcts, and **hard exudates**, composed of extracellular lipids, as predictors of DR progression to sight threatening complications, has not yet been established (140)(141)(142).

Histologic descriptions of retinal vessels have identified the presence of one, two, three or four vascular plexuses depending on the eccentricity, corresponding to different metabolic needs and different cell population in the different retinal layers. In the macular region, the four proposed existing plexuses are the radial peripapillary capillary network, the superficial vascular plexus, the intermediate capillary plexus and the deep capillary plexus (143). A study by Snodderly *et al* in macaque retinas, suggested that the retinal capillary circulation consist on 4 layers, with 2 planes of superficial capillaries nourishing the RNFL and GCL and 2 planes of deep capillaries in the inner nuclear layer (144). Two studies in human donor eyes, using confocal scanning laser microscopy for capillary imaging, have also identified the 4 capillary networks in the following retinal layers: RNFL, GCL and superficial portion of IPL, deep portion of IPL and superficial portion of INL, and deep portion of INL. Laminar configurations were presented in RNFL and deep INL networks and the remaining networks demonstrated a three-dimensional irregular pattern, at least in the perifoveal area (145)(146). However, histology studies have major limitations, in the sense that post-mortem tissue fixation and sectioning artifacts may alter the human vascular anatomy interpretation.

Fluorescein Angiography enables the visualization of retinal vasculature in physiologic conditions and has been the gold standard for evaluating vascular alterations that occur in DR (84). However, FA is invasive, involves risks and can cause nausea or allergic reactions (147).
Previous pioneering studies comparing FA and anatomic drawings of retinal vessels from primate retina demonstrated that only 43% of capillaries with diameter from 4.1 μm to 4.5 μm were visible on FA and that the capillaries visibility decreased linearly with the increasing depth in retina (148). Furthermore, it has been suggested that OCTA examination allows better discrimination of the central subfield and parafoveal macular microvasculature than FA (149).

OCT angiography (OCTA) allows in vivo imaging of the retinal capillary bed without contrast dye, identifying the motion of vascular flow. The basic principle of OCTA involves determining the change in backscattering between consecutive B-scans and differences are attributed exclusively to the flow of the erythrocytes through the retinal blood vessels. Different OCTA devices make use of different algorithms to quantify vascular flow: OCT-based optical microangiography (OMAG), split-spectrum decorrelation angiography (SSADA), OCT angiography ratio analysis (OCTARA), speckle variance, phase variance and correlation mapping (150). In did, direct comparisons between those different OCTA algorithms are limited. The image contrast of the OMAG algorithm is a complex OCT signal based, using both amplitude and phase in the OCT signals to show the blood flow within the tissue (full spectrum). It is the algorithm of the ZEISS Angioplex system, that includes a 3x3 mm and a 6x6 mm scanning pattern, and generates high resolution maps of the Superficial Capillary Plexus (SCP), Deep Capillary Plexus (DCP) and Full Retina (FR). A recent research device, the swept-source OCT ZEISS PlexElite 9000, provides a 3x3 mm and a 15x9 mm protocol, that added 3 extra concentric rings to the standard 9 ETDRS areas with 31, 42 and 52º of field of view. Vascular density metrics for the SCP, DCP and FR are calculated using a density quantification algorithm available on the Advanced Retina Imaging (ARI) portal that uses multi-layer segmentation. The SSADA algorithm uses multiple spectrums from a single B-scan, and is based on the decorrelation of OCT signal amplitude due to flow. It is the algorithm utilized in the Optovue AngioVue imaging system, that includes a 2x2 mm, 3x3 mm, 6x6 mm and 8x8 mm scanning pattern and provides numerical vessel density (VD) measurements in the whole area (central 3 mm), foveal (central 1 mm) and parafoveal (1-3 mm) areas (150).

Changes in amplitude can be detected by speckle (intensity) decorrelation, when detected changes are in the intensity on OCT structural images, or phase variance, when detected changes are in the phase of a light wave. If the changes are above a given threshold, flow is considered to be present.

Optical coherence tomography angiography examination allows a volumetric rendering of blood vessels and vessel density metrics that may be easily compared in follow up studies offering an objective method for monitoring disease progression and response to treatment (151). Quantitative analysis of blood flow or circulation requires conversion to 2D metrics, which obscure the topological information in the original 3D vasculature networks. It requires accurate segmentation of slabs of interest and may mask underlying capillaries due to projection artifacts (152). The segmentation slab designed to isolate the retinal vascular plexus (superficial and deep) in different OCTA devices may not correctly correlate to relevant anatomic layers, and consequently, study bias can occur when using manufacturer-recommended OCTA settings (150).

The commercially available OCTA devices generate information for superficial and deep plexuses, using automated segmentation of anatomic reference planes such as the inner limiting membrane (ILM) and Bruch membrane. Stratification of the blood supply follows 4 *en face* zones: superficial plexus (in ganglion cell layer), deep plexus (network between the outer bounder of the IPL and the midpoint of the OPL with total thickness about 55 microns) , outer retina (photoreceptors, with no vessels) and choriocapillaris (30 microns below RPE) (150). Some authors have described the identification of a middle capillary plexus (MCP), as a unique vascular network qualitatively and functionally distinct from the superficial (SCP) and deep capillary plexus (DCP), using customized manual segmentation analysis in OCTA (153).

Optical coherence tomography angiography is helpful in understanding the normal variation in retinal vascular anatomy as well as interpret retinal images with pathology. The studies with OCTA have demonstrated that OCTA assessment of capillary density and morphology are very similar to histology-based studies, while providing new information (152).

The mean capillary density in the superficial and deep capillary plexus decreases with age and foveal avascular zone (FAZ) area increases with age (154). Measurements of retinal capillary density ranging from 30-60% have been reported with different OCTA devices (155). The normal FAZ is described as a well-defined round or oval area of absent vessel signals, with no gaps or interruptions in its border, and with its longest diameter oriented in either vertical or horizontal axis. However, the FAZ characteristics, as the size, depth and shape show a wide variability between healthy subjects (156). Furthermore, the FAZ diameter is not identical in the SCP and DCP. Studies with OCTA in healthy subjects demonstrated FAZ values ranging from 0.25 to 0.30 mm^2 in SRL and up to 0.495 mm^2 in DCP (157)(157). The area of FAZ is significantly influenced by the central retinal thickness, gender, spherical equivalent, axial length and choroidal thickness (158). Two methods of quantifying the irregularity of the FAZ are more independent of the axial

length: the circularity index and axis ratio of the FAZ. The circularity index is defined as the ratio of the perimeter of the FAZ to the perimeter of a circle with equal area. A perfectly circular FAZ area has a circularity index of 1, with an increase with the deviation from the circular shape. The axis ratio is the ration between the major and minor axis of an ellipse generated by custom software. A perfectly circular FAZ as an axis ratio of 1, increasing with elongated FAZ. FAZ area has been associated with visual function and VA in some studies (159)(160).

Choriocapillaris (CC) alterations are also believed to contribute to the pathogenesis of DR, with non-perfusion areas in CC detected in patients with DR (161). In patients without DME CC non-perfusion area correlated with central thickness as well as with logMAR VA. The extent of non-perfusion area corresponded to ellipsoid zone (EZ) distortion. Alterations in the CC layer, with greater areas of flow void, are present in diabetic macular ischemia (DMI).

A variety of studies have demonstrated that changes in quantitative OCTA microvascular metrics were present since the preclinical stages of diabetes, before the manifestations of clinically apparent retinopathy and have investigated their association with systemic risk factors (152)(162)(163).

Retinal and choriocapillaris microvascular changes have also shown to be correlated with the severity of DR.

Diabetic macular ischemia is a key component of DR, and the accurate quantification of foveal avascular zone (FAZ) can be a potential biomarker in management of the disease. It has been shown that OCTA allows better discrimination of the central foveal and parafoveal macular microvasculature than FA, especially for FAZ disruption and capillary dropout (149). The avascular area of FAZ is significantly greater in eyes with DR compared with controls. Reduced in parafoveal (12.6% decrease) and perifoveal (10.4% decrease) vessel density was evident in one study in diabetic retinopathy compared to controls (164). However, other studies report that FAZ area is not a predictor of NPDR severity (165) and that significant enlargement of FAZ can occur only in more advanced stages of DR, in transition to PDR (166). A study from Frizziero and colleagues (121) aiming to evaluate the earliest retinal morphological and functional changes in diabetic eyes without or with early signs of diabetic retinopathy comparing to healthy controls using OCTA Spectralis HRA+ OCT2 platform, found no significant differences in FAZ area in neither capillary plexus between both DM groups and controls. All OCTA vessels parameters were significantly reduced in all diabetic groups in the 3 capillary plexuses (superficial, intermediate and deep) versus controls. In this study major vascular changes in the SCP were present in diabetic eyes with no clinical microvascular signs of DR. Vessel density measured by OCTA provides a

quantitative metric of capillary closure that correlates with severity of DR and may allow staging, diagnosis, and monitoring that do not require subjective evaluation of fundus images (167).

Nesper *et al* have evaluated, in 137 eyes, superficial and deep OCTA parameters as FAZ, VD, percent area of nonperfusion (PAN) and adjusted flow index (AFI), and found that all OCTA parameters showed a significant linear correlation with DR severity except for AFI in the SCP. The increased AFI in the SCP of eyes with no DR represent an higher flow in the SCP and is presumed to be an early marker of diabetic microvascular changes, before clinical signs of DR (168). In a study from Cao and colleagues, in 71 type 2 DM patients and 67 healthy controls, parafoveal and average vessel density metrics were decreased in SCP, DCP and choriocapillaris (CC) in eyes in the preclinical stage of DR (MAs found in OCTA but no detected anomalies in fundus examination) (162). The authors described microvascular alterations in OCTA, as MAs, FAZ enlargement and irregularity and macular capillary dropout as more reliable indicators of preclinical DR than the reduction in VD alone. In another study, FAZ area measures were found to be statistically significant higher in diabetic (mean 0.348 mm^2; 0.1085-0.671 mm^2) in comparison to control eyes (mean 0.288 mm^2; 0.07-0.434 mm^2) and FAZ remodeling and capillary nonperfusion were more prevalent in diabetic eyes (169). In a study of Krawitz *et al* the average circularity index was 1.32 in control subjects, 1.32 in the no DR group, 1.57 in the NPDR group and 1.78 in the PDR group (170). The authors found statistically significant trends of higher circularity index and axis ratio in more severe stages of DR.

Li and colleagues, in a recent study that included 259 in a wide range of DR, from no DR to PDR, analyzed the microvascular and neural parameters related to the severity of DR by using OCTA images (171). They could identify sensitive OCTA parameters related to the severity of DR: the FAZ perimeter, circularity index (AI) and FD-300 are more influenced by DR progression that the FAZ area both in 3.0 and 6.0 mm scans; the VD in DCP in macular fovea and parafovea and the VD in SCP in temporal perifovea are differently affected by progression of DR. The authors postulate that 3.0 mm scan may be more appropriate for early DR evaluation while 6.0 would be preferable for advanced DR.

Vujosevic and colleagues had recently analyzed the microvascular changes present in a group of diabetic patients with and without retinopathy using swept source OCT (Plex Elite 9000 and DRI OCT-A Triton Plus) (172). They concluded that circularity index (CI) of the FAZ in SCP and DCP is an early parameter that shows a decreasing trend from controls to no-DR and DR groups, meaning that FAZ regularity was gradually lost with damage induced by diabetic disease. VD

(vessel density) and PD (perfusion density) were also decreased in DR group comparing to controls. The differences were confirmed for both instruments.

Different authors have found that the decrease in the perfusion index was correlated with the severity of the retinopathy and was more pronounced in the DCP, preceding the changes in the SCP (173)(174)(165). In a study of 101 eyes with NPDR, using OCTA RTVue-XR 100, VD in CC, SCP and DCP, as well as duration of disease, HbA1c and systemic hypertension were found to be associated with NPDR ETDRS level. However, after adjusting for relevant clinical features, the only and most robust parameter found to be statistically associated with ETDRS level was the parafoveal VD (in the annular zone 1-3 mm around the foveal center) in the DCP (165). Agemy *et al* found that the average capillary perfusion density values for normal subjects in 3x3 mm^2 scan were 0.1849, 0.2462 and 0.3106 for the superficial and deep retinal capillaries and choriocapillaries, respectively (175). For NPDR patients these values reduced to 0.158, 0.2011 and 0.2787 and in PDR subjects further reduced to 0.1508, 0.1874 and 0.2822, respectively. The vessel density percentages quantified using OCT angiography AngioAnalytics demonstrated the same trend, with significantly lower values in nearly all layers of DR groups compared to controls, lower in the most severe NPDR grades and further decreased in PDR group. The decrease in vessel perfusion was more consistent in the deep plexus. Another study of 102 eyes, using the OCTA RTVue Avanti and a built in automated software to generate perfusion indices (VD and FI) at superficial and deep plexus, outer retina and choriocapillaris, also agreed that as DR progresses in severity the decrease in the perfusion index was more pronounced in DCP, preceding changes in SCP (176). Furthermore, vessel density in DCP appear to have the strongest association with functional deficit (177). This finding is in line with histologic studies that describes that vascular abnormalities are more pronounced in the deep capillary plexus (178), where there is a watershed zone of oxygen supply and a higher sensibility to ischemic injury, and the hypothesis that DCP contribute more to the metabolic demands of photoreceptor metabolism in eyes with diabetic macular ischemia (179).

In a prospective study of Durbin *et al*, including 50 diabetic eyes with a wide range of DR versus 50 healthy eyes, the mean vessel density measured in the superficial retinal layer (SRL) was the metric with the highest area under the receiver operating characteristic curve, comparing to perfusion density in the same layer and the DRL metrics, indicating that it is the best method at differentiating healthy eyes from eyes with DR and should be preferred at least as a diagnostic tool. They also found negative correlation of SRL measurements and DR severity. Foveal avascular zone measurements showed a wide range in the healthy eyes, and were not useful distinguishing eyes with DR from the healthy population (167). The authors pointed out that measurements in the DRL

are affected by decorrelation tails artifacts, and after their remotion the measurements made in this layer improved.

The discrepancies in OCT metrics results may be attributable to differences in segmentation and layer definitions among studies.

Optical coherence tomography angiography can visualize the origin of microaneurysms within the superficial and deep capillary plexus, the areas of retinal non-perfusion and provides quantitative information on new vessels, enabling closer observation of each layer of retinal capillaries (180). There was a significantly higher number of MAs detected in the DCP compared to the SCP, suggesting their primary origin at that level (181). Optical coherence tomography angiography also enables a qualitative classification of MAs in different shapes, round dots, round with dark center and fusiform, and shows looping vessels adjacent to areas of impaired capillary perfusion (clinically defined as IRMA), cotton-wool spots and some forms of intraretinal fluid (182). Microaneurysms in the deep plexus might contribute more to DME than MA in the superficial plexus (183). A study comparing MA detection between SD-OCT and OCTA concluded that hyporeflective MAs were less likely to be visualized on OCTA, probably due to a flow rate below the threshold, and that 8% of hyporeflective vs 66% of hyperreflective MAs developed extracellular fluid after 1 year of follow up (184). It is believed that a blood flow of less than 0.3 mm per second cannot be detected by SSADA algorithm (185).

Neovascularization, hallmark of PDR, can also be detected by OCTA. With the segmentation of the flow signal at the inner boundary of the superficial plexus (ILM) and projecting the signal in the cross-sectional orientation, it was evident that these lesions were retinal new vessels protruding into the vitreous. This flow signal of the elevated new vessels appear as shadows rather than flow signals because they are outside the depth range of OCTA (164). Different patterns of NV have been described in relation with previous treatment: exuberant vascular proliferation, more frequent in treatment naïve patients and early leakage in FA, and "pruned vascular loops" of filamentous vessels, with faint leakage on FA (186). Singh *et al* demonstrated that OCTA can differentiate between optic disc venous collaterals and new vessels of the disc in DR (187).

Optical coherence tomography angiography has also proven to have value in DR prognosis and risk evaluation.

In a prospective study of Sun and colleagues, evaluating 205 eyes using a swept source Triton OCT, OCTA-derived biomarkers were identified as predictors of DR progression and DME development over 2 years of follow-up (188). A larger foveal avascular zone (FAZ) area, lower VD and lower

fractal dimension (FD) on DCP were associated with a higher risk of DR progression, whereas eyes with lower VD on SCP were more likely to develop DME. Such associations are independent of established risk factors including age, duration of diabetes, HbA_{1c}, blood pressure and severity of DR at baseline.

It is generally accepted that in DR the posterior pole is the region initially affected, in contrast to the peripheral involvement that characterizes retinopathies from blood disorders, like sickle cell disease, macroglobulinemia and multiple myeloma (189).

However, recent retinal imaging technology developments bring some new clues concerning retinal periphery.

The ETDRS 7-standard fields photography reveals approximately 30% of retinal surface, whereas the new ultra-wide field system (Optos P200MA, Optos plc, Scotland, UK) visualizes extensive area of retinal periphery, up to 200º, near 82% of the retinal area, covering areas of retina traditionally not visualized by the ETDRS 7 fields protocol. Ultra-wide field fundus imaging has shown peripheral lesions that were so far undetected. Silva *et al*, using ultra-wide field imaging, showed that approximately one-third of H/Ma, IRMA, and NVE were located predominantly outside the ETDRS defined 7-standard fields, suggesting a more severe assessment of DR in 10% of cases (190). The evidence of predominantly peripheral lesions (PPL: MA and hemorrhages, but also IRMA) had a greater that fourfold increased risk of DR progression to PDR (65). Predominantly peripheral lesions were more frequently identified in eyes with less severe DR (66).

Early **wide-angle angiography** systems raised the hypothesis whether capillary non-perfusion in diabetic retinopathy occurred more commonly in the mid-periphery than in the posterior pole (63). According to the authors, the examination with a super-wide (130°) fluorescein angiographic montage technique demonstrated that the midperipheral retina was more prone to undergo capillary nonperfusion than the posterior retina. A study by Shimizu and colleagues demonstrated that midperipheral fundus area, outer to the radial peripapillary capillaries, was prone to undergo capillary nonperfusion and that that area was more extensive in eyes with retina and optic disc neovascularization (191). In a study of 130º wide-field FA for non-proliferative retinopathy, capillary non-perfusion tended to enlarge from its site of origin, with peripheral location associated with more rapid progression of the non-perfusion (64).

Diabetic Retinopathy has been considered a microvascular complication of diabetes, clinically identified by changes produced either due to progressive cell degeneration and vasoregression or due to abnormalities of the blood retinal barrier, limiting the diagnostic and therapeutic focus to

the vascular system. However, it is now accepted that diabetic retinal disease may at the same stage involve the neuronal as well as the retinal vascular compartments (192). Recent studies carried out by our group confirmed that retinal neurodegeneration may precede the microvasculopathy of DR and may occur in the absence of any signal of microvascular damage (193).

3. Neuroretinal changes

Neuronal integrity is essential for vision. In the early stages of diabetes, a number of patients presented deficits that they are commonly unaware of in daily life.

Retinal function depends on the synergy of several neuronal subtypes, including photoreceptors, horizontal and bipolar cells, amacrine and ganglion cells, and their supporting glia (astrocytes and Müller cells) as well as the inner vascular (endothelial cells and pericytes) and outer blood-retinal (RPE and choroidal vessels) barriers, responsible for nourish the retina and control the transport of ions and water in the retinal tissue.

Functional changes secondary to neural retinal dysfunction, such as loss of chromatic discrimination, contrast sensitivity and dark adaptation can be detected on electrophysiological studies even before microvascular lesions can be detected in ophthalmologic examination (194)(195)(196)(197).

The extent of involvement of the cellular elements of the neural parenchyma, neurons and glia in the onset of DR is still object of debate. It is recognized that retinal neurodegeneration is an early event in the pathogenesis of DR, and that it is already present even before any microcirculatory abnormalities can be detected in ophthalmoscopic examination. It is unknown which of the two primordial pathological elements (apoptosis or glial activation) is the primary event of neural deficits.

Neuroretinal degeneration can initiate and activate several metabolic pathways that will participate in the microangiopathic process as well as in the disruption of the blood-retinal barrier (BRB) (198)(199). The outer retina is compromised, with an elevated rate of apoptosis observed in the photoreceptors (outer nuclear layer) and in the RPE (200). Photoreceptor cells contribute most to DM-induced retinal oxidative stress and are major sites of superoxide generation and production of inflammatory proteins (201). At the inner retina, the retinal ganglion cells present the highest rate of apoptosis. These cells are responsible for integrating electrochemical information received from bipolar and amacrine cells and transmit it to the brain. Apart from the inner and outer retina, also the neurovascular unit is prone to damage in diabetic retinopathy. Neurovascular unit covers the functional coupling and interdependency between neurons, glial cells, and retinal vascular elements (endothelial cells and pericytes) and is essential to maintain the normal functioning of the inner BRB (Figure 7). The glia, including the macroglia and the microglia, seems to be an early target of the vascular permeability and its behavior may contribute decisively to the onset and development of neuropathy in the diabetic retina (202). Retinal macroglial cells (astrocytes and

Müller cells) are intercalated between vasculature and have an important role in the uptake of glucose from the circulation, its metabolism and transfer of energy to neurons. Similar to macroglia, microglial cells representing the resident tissue macrophages, are highly dynamic and capable of assuming different morphologies and functions in response to changes in their local chemical and cellular environment. Microglial activation is rapid and often precedes that of macroglia (203). The acquisition of gliotic features enables both macro and microglia to participate in the initiation of an immune response. Local vascular leakage and reactivity of all three glial cell types, manifesting as hyperplasia of Müller cells, a decrease in cell density of astrocytes, and activation of microglial cells, are the earliest structural changes observed in diabetic retina. Müller cells are strongly involved in neurodegeneration via glutamate signaling. Microglia could directly influence Müller cells, which increased the inflammatory response across the retinal layers increasing further the mobilization of migratory microglia (204).

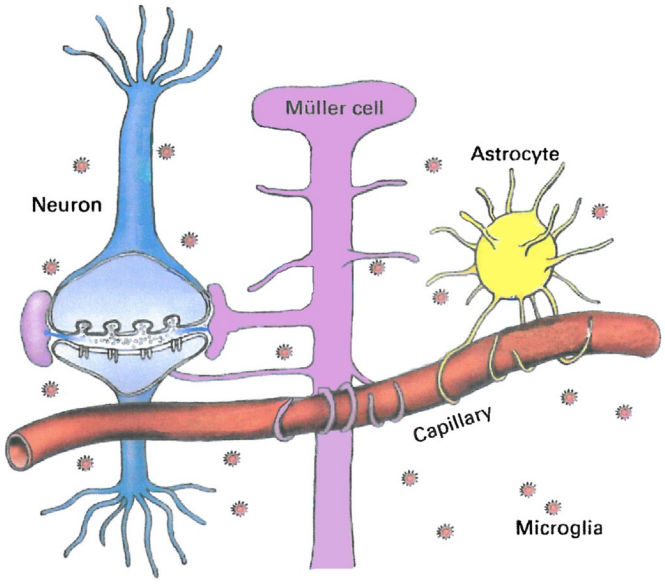

Figure 7: Retinal neurovascular unit (Adapted from Antonetti et al (2006)).

The neurovascular unit allows the integration of metabolic needs and vascular tone by integrated multiple molecular signals maintaining normal visual function in a variety of physiologic conditions (205). Deficient neurovascular coupling is an early sign of retinal disequilibrium caused by diabetes.

Multifocal ERG (mfERG) enables the assessment of retinal neuroglial function. Harrison and colleagues have studied 78 eyes for several years with mfERG and found that focal areas of electrical depression and increased latency (implicit time, IT) can predicted the development of retinal vascular lesions in specific retinal zones (194). Another study from Lecleire-Collet *et al*, found that alterations in the amplitude and IT pattern of ERGs in patients with diabetes correlate with deficits in flicker light–induced vasodilation (206). These alterations may be related to the altered function of Müller, ganglion, amacrine and bipolar cells. Furthermore, the development of recurring retinopathy over a 3-year period was shown to be predicted by using a multivariate model based on mfERG implicit time with a sensitivity and specificity of 88% and 98% (195).

A large clinical trial (EUROCONDOR Study) was performed in diabetic patients with no DR or DR ETDRS level 20-35, and found that 58% of diabetic patients with no visible microvascular lesions and 66% of diabetic patients with ETDRS level 20–35 showed multifocal mfERG abnormalities at baseline compatible with neurodysfunction (delayed IT and lower P1 amplitude) (207). The authors reported an association between SD-OCT thinning and mfERG abnormalities in 67% of the eyes with no DR and in 83% of the eyes with ETDRS level 20-35.

A recent study from Frizziero and colleagues investigated the correlation between the new morphological and vascular findings by means of OCT and OCTA and the functional parameters given by the mfERG in patients with diabetes with no or mild DR comparing to healthy controls (121). Concerning mfERG parameters, a greater number of altered hexagons were present in no DR versus mild DR group. No correlations between morphologic and functional parameters were found. As we could appreciate in EUROCONDOR study, here again, it is suggested that vascular damage, neurodegeneration and retinal inflammation contribute independently to the pathogenesis of diabetic retinal disease.

The ERG is a highly sensitive index of retinal function but difficult to apply in routine clinical practice due to the time length of the test.

The recent **Optical Coherence Tomography (OCT)** technology enables microstructural analysis of the retina to become part of routine clinical practice. It allows the precise measurement of different retinal layers, using automated methods of retinal segmentation, and high resolution images in only a few minutes of exam.

Different studies have reported reduced retinal thickness, particularly thinning in the ganglion cells layer (GCL) and RNFL in diabetic patients with no or minimal capillary changes when compared with healthy controls, especially around the macula (208).

This parameters may differ with variation of the HbA1c levels or duration of DM (209). Kim *et al* have studied various clinical features in association with retinal neurodegeneration in type 2 diabetes and found that hypertension, diabetic retinopathy, statin medication, smoking status, estimated glomerular filtration rate, peripheral nerve conduction and autonomic nerve function appear to be significantly correlated with GCL-IPL thickness, whereas HbA1c and diabetes duration did not (210). The authors suggested that several genetic and environmental factors may be protective for the development of diabetic complications.

In a study of 76 eyes from 62 type 2 diabetic subjects versus controls, using the Cirrus HD-OCT, early thinning on the inner retina was identified even before visible vascular signs of DR (211). The GCL-IPL layer was found to be the most affected by thinning, mainly in the nasal area (papillomacular region), but no significant differences were found between different DR levels. The RNFL thickness was not significantly reduced in these cohort, except for the minimum RNFL thickness that was decreased in each of the DR stages. The authors point that the neuronal damage in diabetes primarily affects the RGC nuclei and dendrites and secondarily the RGC axons and this fact explain such results.

Another study was performed on 124 subjects, 74 diabetics and 50 healthy controls, using a SD-OCT with an automatic algorithm to detect 5 different retinal layers: ILM-RNFL, GCL-IPL, INL-OPL, ONL-ELM and IS/OS-RPE (100). The authors report a decrease in RNFL thickness in the macula of diabetic eyes even without any clinical sign of retinopathy explained by progressive ganglion cells and astrocytes loss induced by diabetes. No differences were found in the decreased RNFL thickness between diabetics with no DR and with NPDR, probably due to the fact that all of the patients had only mild to moderate NPDR and a good metabolic control. Although there was a trend of decreasing GCL-IPL thickness in diabetics without DR versus controls, the differences were not statistically significant. The INL and OPL showed an increased thickness in NPDR patients versus controls. This increased thickness was already demonstrated in a study of Bandello *et al* (135). The authors suggested the Müller cells activation with consequent hypertrophy in the early DR stages as a reason for that. Finally, the outer retina (ONL-ELM and IS/OS-RPE) was not significantly different between diabetics and controls, appearing that this layer is not significantly affected by diabetes at least at earlier stages. Another study in type 1 diabetic patients with no or early DR, using the Spectralis segmentation software, with segmentation of the 9 distinct retinal layers, has also highlighted the neurosensory retinal impairment in the initial DR stages demonstrating an increased thickness in INL as well as decreased in GCL thickness in diabetics versus controls, when data adjusted for inner retinal thickness measurements. No differences were found in RNFL thickness, in the outer retinal layers or in the choroidal thickness in these early stages.

Srinivasan *et al* reported an association between GCL-IPL and RNFL thickness with the severity of diabetic periphery neuropathy (212). Frizziero and colleagues used the spectralis OCT with automated segmentation in patients with diabetes with no or mild DR and in healthy controls (121). They found that RNFL, GCL, IPL and ONL were thinner in both diabetic group, however not in a statistically significant manner, whereas the ORL thickness was higher in this group. Li and colleagues, in the previously referred study of 259 diabetic patients, with no DR to PDR, analyzed also the neural parameters related to the severity of DR by using OCTA images (171). Measurements of GCL macular and RNFL peripapillary thicknesses decreased in early DR stages but increased with the progression of DR.

Sohn *et al* reported, in a 4-year follow up study in patients with DM and no or minimal DR, a significant progressive thinning of the RNFL (0.25 μm/y) and of the GCL-IPL (0.29 μm/y) on OCT (versus 0.133 μm/y and 0.149 μm/y, respectively, in normal subjects), independent of glycated hemoglobin, age, sex or presence and progression of DR (213). In the animal mouse model these changes in the ganglion cell density were noticed before any changes in the pericyte density, preceding microvascular manifestations of DR.

Kim *et al* (214) performed a longitudinal study of 87 diabetic patients with no or minimal DR and 40 healthy controls that were followed up for at least 4 years. A two-step progression of DR was found in 44.8% of the eyes and 6.9% developed PDR. They found a decreasing rate in GCL-IPL thickness of 0.38 μm/year versus the 0.18 μm/y observed in controls. These change was related to the baseline GCL-IPL thickness. Reduced cardiac autonomic and peripheral nerve function were also associated with a two-step or greater progression of DR. The authors conclude that these parameters can predict long term progression of DR in patients with type 2 diabetes.

A 3-year follow up prospective study evaluates the reduction rates of GCL-IPL in diabetic patients with or with no DR , using the Cirrus HD-OCT and the ganglion cell analysis algorithm of the device (215). The GCL-IPL and RNFL thicknesses were lower in DR groups at all time points, and lower in NPDR comparing to no DR group. The estimated reduction rates of the average GC-IPL thickness in the no-DR (−0.627 μm/year) and NPDR (−0.987 μm/year) groups were 2.26-fold and 3.56-fold faster, than in the control group, respectively (−0.277 μm/year). Age, duration of diabetes, and baseline average GC-IPL thickness were associated with longitudinal changes in average GC-IPL thickness .

Another longitudinal 2-year study, using OCT angiography in 40 eyes with no or mild NPDR, reported a strong positive correlation between loss of GCL-IPL and VD from baseline to 24

months while with no correlation found with FAZ parameters (216). Thinner baseline GCL-IPL and greater loss of layer during the follow up were significantly associated with changes in VD. The authors conclude that in the early stages of DR progressive structural retinal neurodegeneration and parafoveal microvascular change seem to be highly linked and that advanced GCL-IPL thinning might precede microvascular impairment in early DR.

A recent study performed by van de Kreeke and colleagues in 80 eyes of type 1 DM patients with no or minimal DR, wanted to clarify the association between diabetic retinal neurodegeneration and DR development/presence within 4 different quadrants of the retina (217). RNFL and GCL both showed a significant thinning whereas IPL showed a slight but significant thickening over time. For GCL and IPL, more pronounced changes were associated with the presence or development of DR in those eyes/quadrants. The study has shown, for the first time, that spatially, regional structural retinal neurodegeneration is associated with regional DR, confirming earlier studies that regional functional retinal neurodegeneration is associated with regional DR.

The actual classification systems of DR are clearly insufficient, in the sense that they are not possible to use in daily clinical practice. The implement of additional lesion measurements into current classification systems may help in characterizing the DR progression profiles.

A better characterization of the earliest changes induced in the diabetic retina, whether neuroretinal degeneration or microvascular occlusion, is an important goal to delineate a better strategy of prevention and therapeutical approach.

The use of more sensitive markers of progression that allow clinicians to predict diabetic progression is desirable. Measurements of retinal neurovascular structure and function can help in a better characterization of the phenotypic presentations of diabetic patients.

In order to solve some of these issues, we designed a prospective longitudinal study of 5 years of follow up, in diabetic patients with no or mild NPDR.

Main Objectives

1- To characterize the 5-year progression of NPDR to vision threatening complications (VTC) using non-invasive retinal imaging biomarkers.

2- To evaluate risk factors and biomarkers in the development of DR complications and DR progression. To identify markers of risk progression to VTC.

3- To characterize the initial stages of diabetic retinal disease related to neurodegeneration, in patients with or without detectable microvascular damage.

4- To assess the correlation between the presence and 5-year progression of neuretinal changes and the microvascular alterations, in relation to the progression of retinopathy severity.

5- To identify and validate diabetic retinopathy phenotypes as potential biomarkers of disease progression in a 5-year follow up period.

The next chapters will present our findings.

In the chapter 2 to 4, I present the results of our research concerning the identification of 3 different phenotypes with different risks of developing vision threatening complication and DR progression over the 5 years of follow up, and explore possible risk factors and imaging biomarkers of progression. In the fifth chapter, I present the results of a cross-sectional study that evaluated the prevalence of different disease pathways (ischemia, neurodegeneration and edema) in the initial stages of diabetic retinopathy. In chapter 6 and 7 I will characterize the 2-year and 3-year follow up changes occurring in the 3 identified disease pathways, neurodegeneration, edema, and capillary dropout, in non-proliferative diabetic retinopathy and their relation with development of complications and DR progression. In chapter 8, I present a study using the swept source OCTA (SS-OCTA, PlexElite, Carl Zeiss Meditec), that allow to explore a bigger area of the retina, using 15x9 mm and 3x3 mm protocol. We discuss the value of a single or composite set of OCTA parameters evaluated as NPDR staging predictors. Finally, in the last chapter, I will make a brief discussion and summary of the overall results and present the future perspectives.

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Chapter 2

Retinopathy Phenotypes in Type 2 Diabetes with Different Risks for Macular Edema and Proliferative Retinopathy

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Research Question

Diabetic Retinopathy is a multifactorial disease. Different factors or different pathways may predominate in different subjects.

Our group has proposed, in previous studies, the existence of three different phenotypes of mild nonproliferative DR (NPDR), based on MA turnover values in a field 2 fundus photograph evaluated by Retmarker DR and on CRT on spectral domain OCT (SD-OCT). These phenotypes appear to represent different risks for development of vision-threatening complications in studies with 2 and 3 year of follow up.

Are ocular markers and phenotype classification good biomarkers for development of vision-threatening complications in longer follow up studies?

In this first paper we could confirm the predictive value of this phenotypic classification in 5 years of follow up. Phenotype C, which comprises near 30% of the included subjects, was the phenotype with the higher rate of complications, including 79% of the eyes that developed clinically significant macular edema (CSME) and all the cases that developed PDR. Phenotype C presents, in a multivariate analysis, an OR of 17.4 for CSME development, when compared to the other phenotypes.

Abstract

Our group reported that three diabetic retinopathy (DR) phenotypes: A, characterized by low microaneurysm turnover (MAT < 6) and normal central retinal thickness (CRT); B, low MAT (<6) and increased CRT, and C, high MAT (≥6), present different risks for development of macular edema (DME) and proliferative diabetic retinopathy (PDR). To test these findings, 212 persons with type 2 diabetes (T2D) and mild nonproliferative retinopathy (NPDR), one eye per person, were followed for five years with annual visits. Of these, 172 completed the follow-up or developed an outcome: PDR or DME (considering both clinically significant macular edema (CSME) and center involved macular edema (CIME)). Twenty-seven eyes (16%) developed either CSME (14), CIME (10), or PDR (4), with one eye developing both CSME and PDR. Phenotype A showed no association with development of vision-threatening complications. Seven eyes with phenotype B and three with phenotype C developed CIME. Phenotype C showed higher risk for CSME development, with 17.41 odds ratio (p = 0.010), compared with phenotypes A + B. All eyes that developed PDR were classified as phenotype C. Levels of HbA_{1c} and triglycerides were increased in phenotype C ($p < 0.001$ and $p = 0.018$, respectively). In conclusion, phenotype C identifies eyes at higher risk for development of CSME and PDR, whereas phenotype A identifies eyes at very low risk for vision-threatening complications.

Keywords: diabetes; retinopathy; macular edema; proliferative retinopathy

Introduction

Diabetic retinopathy (DR) is one of the major complications of diabetes and leading cause of vision loss and blindness in the world, particularly among working-age adults in the United States [1]. This serious ophthalmic condition creates significant disabilities that threatens independence and impact on life quality [2]. The two major vision-threatening complications, diabetic macular edema (DME) and proliferative diabetic retinopathy (PDR) develop only in a limited number of individuals [3].

It is generally accepted that diabetes duration and level of metabolic control play a role. Still, these risk factors *per se* cannot explain the variability observed in DR evolution in diabetic individuals [4]. Whereas several individuals with diabetes do not develop vision-threatening retinal changes, maintaining good visual acuity after many years of diagnosis, others show progression of DR even after only a few years of diabetes, leading to vision loss.

Our group has reported on two-year and three-year follow up studies of people with type 2 diabetes (T2D) and mild nonproliferative DR (NPDR) and found marked individual variations in the progression of DR and development of complications [5–9]. Namely, using noninvasive methods, we have identified three different phenotypes of NPDR, based on microaneurysm turnover (MAT) and central retinal thickness (CRT), that appear to be related with different risks for the development of clinically significant macular edema. Briefly, Phenotype A is characterized by low MAT (<6) and normal CRT; Phenotype B by low MAT (<6) and increased CRT; Phenotype C by higher MAT (≥6) with or without increased CRT.

This new study is a prospective five-year study of a large cohort of people with T2D and with mild NPDR examining disease progression to vision-threatening complications, using only non-invasive examination methodologies that are easily used in clinical practice, digital color fundus photography (CFP), and optical coherence tomography (OCT), to test if different DR phenotypes show different risks for development of vision-threatening complications.

Methods

A prospective longitudinal observational cohort study (ClinicalTrials.gov identifier: NCT03010397) was designed to follow people with T2D with mild NPDR (one eye per person), graded 20 or 35 on Early Treatment Diabetic Retinopathy Study (ETDRS classification) grading scale [10]. This study is an extension of three previous studies (NCT0114599 [9], NCT01607190 [7], and EudraCT2012-001200-38 [11]). The patients in the study here reported were included according to specified inclusion and exclusion criteria and they were followed for a period of five years or until the time of development of PDR or DME, considering clinically significant macular edema (CSME) and center involved macular edema (CIME). The tenets of the Declaration of Helsinki were followed, and the Institutional Ethical Review Board approved the study. Each participant signed a written informed consent, agreeing to participate in the study, after all procedures were explained.

A total of 212 people with T2D were included, men (68.4%) and women (31.4%) with diagnosed adult-onset of T2D, aged 42 to 82 years, with an average duration diabetes of 14.1 ± 7.4 years and a mean range of hemoglobin A_{1c} (HbA_{1c}) levels of 7.47 ± 1.27 (Table 1).

The study exclusion criteria comprised the presence of age-related macular degeneration, glaucoma, vitreomacular disease, high ametropia (spherical equivalent greater than -6 and +2 D), any previous laser treatment or intravitreal injections, or any patient comorbidity likely to affect the eye and not related with diabetes or cardiovascular disease. Excluded were also people with T2D with uncontrolled systemic hypertension (values outside normal range: systolic 70–210 mmHg and diastolic 50-120 mmHg), HbA_{1c} levels above 10%, during the first 6 months of the study, and a history of ischemic heart disease. Eyes with baseline central thickening identifying CIME, defined as a retinal thickness (RT) ≥ 290 μm in women and ≥ 305 μm in men [12], were also excluded.

At baseline visit (V0), demographics such as age, duration of diabetes, co-morbidities, and concomitant medication were collected for each participant. Physical assessment with biometric measures (body weight and height) and blood pressure evaluation were performed by an experienced nurse, as well as blood tests for determination of HbA_{1c} and lipid profile.

Visual acuity (best corrected visual acuity, BCVA) was measured for each eye using the ETDRS protocol and Precision Vision charts at 4 m [13].

The DR severity level was determined by two independent graders within the context of an experienced reading center and was based on the seven-field protocol following the ETDRS classification, performed by the Coimbra Ophthalmology Reading Center (CORC) [10]. One eye per person with T2D was selected at baseline as the study eye based on the inclusion/exclusion

criteria. When both eyes fulfilled the criteria, the eye showing the more advanced ETDRS grading in any given individual was chosen to be the study eye.

Study follow up visits were performed at 6 months (V1), 12 months (V2), 24 months (V3), 36 months (V4), 48 months (V5), and 60 months (V6) or last visit before treatment (in the eyes that developed either CSME or PDR). At all study visits, participants underwent a complete eye examination, which included BCVA, slit-lamp examination, intraocular pressure measurement, and OCT.

During the period of the study and outside of the study visits, participants were followed in our institution in accordance with usual clinical practice.

The outcomes in the study here presented were CIME, CSME, and PDR. Center involved macular edema is defined as CRT ≥ 290 μm in women and ≥ 305 μm in men (Zeiss Cirrus SD-OCT), according to pre-defined OCT values [12]; CSME was identified on clinical examination as defined by the Early Treatment Diabetic Retinopathy Study group as retinal thickening within 500 μm of the center of the fovea or presence of hard exudates (with thickening of the adjacent retina) within 500 μm of the center of the fovea, or thickening of at least 1 disc area located less than 1 disc diameter from the center of the fovea [14]. Finally, PDR was identified by the presence of abnormal new vessels in the retina.

Laboratory analyses included HbA_{1C} concentration, glucose, creatinine, red blood cell count, white blood cell count, platelet amount and packed cell volume. Plasma concentrations of lipid fractionation identifying total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides, were measured to assess metabolic control.

The calculation of the sample size was based on previous studies [6–9], where the existence of three distinct phenotypes of DR progression in T2D was proposed. Their incidence on the DR population was 50% for phenotype A, 30% for phenotype B and 20% for phenotype C. Individuals with phenotypes B and C were shown to be at higher risk of developing CSME needing treatment, with 11% of phenotype B and 30% of phenotype C developing CSME in a two-year interval. In order to have at least five eyes from phenotype B and 10 eyes from phenotype C developing CSME needing treatment, a total of 200 eyes were considered to be needed for the study.

Baseline characteristics of the study population are presented in Table 1.

Table 1: Demographic and clinical characteristics

Characteristics	Included patients *N* = 212	Study population *N* = 172	p-value
Demographics			
Males / Females, *N* (%)	145 (68.4) / 67 (31.4)	117 (68.0) / 55 (39.0)	0.865
Age, mean ± SD, y	63.0 ± 7.3	62.7 ± 7.2	0.187
Diabetes duration, mean ± SD, y	14.1 ± 7.4	14.2 ± 7.4	0.481
Systemic characteristics			
BMI, mean ± SD, kg/m^2	30.1 ± 5.8	30.10 ± 5.87	0.554
Systolic BP, mean ± SD, mm Hg	137.9 ± 15.8	138.1 ± 15.9	0.874
Diastolic BP, mean ± SD, mm Hg	72.2 ± 8.6	71.9 ± 9.0	0.431
HbA1c, mean ± SD, %	7.47 ± 1.27	7.5 ± 1.3	0.182
Total cholesterol, mean ± SD, mg/Dl	182.79 ± 38.40	184.02 ± 38.56	0.191
HDL cholesterol, mean ± SD, mg/dL	47.29 ± 11.65	47.45 ± 11.08	0.517
LDL cholesterol, mean ± SD, mg/dL	121.16 ± 31.69	122.24 ± 32.78	0.210
Triglycerides, mean ± SD, mg/dL	169.29 ± 116.23	166.38 ± 93.48	0.647
ETDRS level			
20, *N* (%)	58 (27.4)	48 (27.9)	0.359
35, *N* (%)	154 (72.6)	124 (72.1)	
Phenotypes, *N* (%)			
Phenotype A	84 (39.6)	66 (38.4)	
Phenotype B	60 (28.3)	50 (29.1)	0.980
Phenotype C	68 (32.1)	56 (32.6)	

HDL: high-density lipoprotein; LDL: low-density lipoprotein; ETDRS: Early Treatment Diabetic Retinopathy Study.

Color Fundus Photography

Color fundus photography (CFP) was performed according to the ETDRS protocol. The seven-fields photograph were obtained at 30/35º, using a Topcon TRC 50DX camera (Topcon Medical Systems, Tokyo, Japan), with a resolution of 3596x2448 pixels for ETDRS DR classification at CORC, according to the ETDRS grading scale [10].

Additionally, 45/50º 2-field images were obtained and subjected to automated microaneurysm (MA) analyses using the RetmarkerDR (Retmarker SA, Coimbra, Portugal). This automated computer-aided diagnostic system is a software that allows earmarking MA and red dot-like vascular lesions in the macula (all referred to as MA); it includes an algorithm co-registration that facilitates the comparison within the same retinal location between different visits for the same eye [15,16], as previously described [6]. Briefly, the algorithm computes for each eye the number of MAs in each visit, and the number of MAs that appear and/or disappear from one visit to the other, allowing a calculation of the number of MAs appearing and/or disappearing per time interval (i.e.,

the MA formation rate and the MA disappearance rate, respectively). The microaneurysm turnover (MAT) is computed as the sum of the MA formation and disappearance rates.

Optical Coherence Tomography

OCT was performed using the Cirrus Zeiss 5000 AngioPlex (Carl Zeiss Meditec, Dublin, CA, United States of America (USA)).

The Macular Cube 512 × 128 acquisition protocol, consisting of 128 B-scans with 512 A-scans each, was used to assess the subjects' average central retinal thickness (CRT). Retinal layers segmentation for layer thickness calculation was performed on the structural OCT or OCTA (in the last visit) using the segmentation software implemented by AIBILI [17]. In the first step of our implementation, the algorithm segments large voxel intensity variations, i.e., vitreous to Retinal Nerve Fiber Layer (RNFL), Inner Segment (IS) to Outer Segment (OS), and Retinal Pigment Epithelium (RPE) to choroid interfaces. The two outer most interfaces are then used as boundaries to find the remaining inner retinal layer interfaces. All surface interfaces are then smoothed by cubic splines. This method is able to segment the OCT volume into seven retinal layers, namely, RNFL, Ganglion Cell Layer and Inner Plexiform Layer (GCL + IPL), Inner Nuclear Layer (INL), Outer Plexiform Layer (OPL), Outer Nuclear Layer and Inner Segment (ONL + IS), OS, and RPE. Automated analysis results were reviewed by a masked grader.

Eyes with CIME were identified in agreement with the reference values established by the DRCR.net for Cirrus SD-OCT (retinal thickness greater than or equal to 290 μm in women and 305 μm in men [12]). Retinal Nerve Fiber Layer (RNFL) and/or GCL + IPL thickness decreases were considered to identify neurodegeneration [18], whereas full retina thickness increases were considered to identify edema [9], comparing to a healthy control population [17].

Data was also collected for OCT analysis of retinal segmentations in the inner and outer-rings, OCT-leakage [19], and OCT-Angiography [20]. OCT-A was performed only in the last two annual visits and the data collected could not be analyzed in correlation with the outcomes CSME, CIME or PDR.

Characterization of DR Phenotypes

The three different DR phenotypes for NPDR, previously described by our group [3,6], were identified according to the following rules: Phenotype A: MAT < 6 and normal RT values (CRT <

220 μm, i.e., normal mean ±1 SD); Phenotype B: MAT < 6 and increased RT values (CRT ≥ 220 μm); Phenotype C: MAT ≥ 6, with or without increased CRT.

Statistical Analysis

Data on each eye/patient is represented as means and corresponding standard deviations for continuous variables or absolute and relative frequencies for categorical and ordinal variables. Accordingly, a comparison of baseline characteristics of the cohorts was performed using Mann-Whitney test (due to violation of assumption of normality) or the Chi-square test with Monte-Carlo correction.

Univariate logistic regression models were used to determine the odds ratio of developing the outcomes, CSME and CIME, associated with each demographic (age, diabetes duration, gender, BMI), systemic (HbA_{1C}, total cholesterol, HDL, LDL, triglycerides, systolic blood pressure and diastolic blood pressure) and ocular (MAT, MA formation rate, MA disappearance rate, CRT and GCL + IPL parameters) variables. A multivariate analysis, considering the set of systemic and non-collinear ocular parameters with p-values ≤ 0.10, was performed to determine the adjusted odds ratio for the development of outcomes for people with T2D categorized with phenotypes B and C. A multivariate logistic regression with block entry of the systemic and non-collinear ocular parameters/variables that presented p-values ≤ 0.10 in the univariate analysis was performed to model the adjusted odds ratio for the development of outcomes for people with T2D categorized with phenotypes B and C.

All statistical analyses were performed with IBM® SPSS® Statistics version 24.0 (IBM Corp ©, New York, USA), and p-values < 0.05 were considered statistically significant.

Results

From the 212 eyes included in the study, 172 completed the five-year follow-up or achieved one of the outcomes: CSME, CIME, or PDR (Figure 1). Forty participants dropped out of the study (nine died, 10 were lost to follow-up, and 21 chose to withdraw from the study.

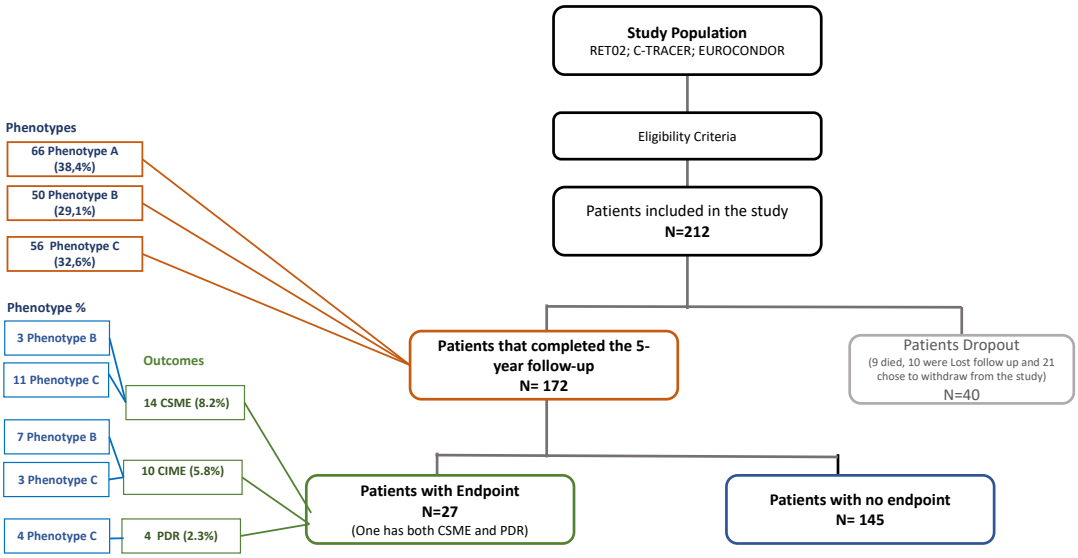

Figure 1: Composition of the patients included in the study over the study period: CONSORT flowchart.

CIME: center-involved macular edema; CSME: clinically significant macular edema; PDR: proliferative retinopathy.

The eyes included in the study had only mild DR, with 58 (27%) graded as ETDRS level 20 and 154 (73%) graded as ETDRS level 35. No statistically significant differences were found at baseline between the 172 eyes/patients that reached the study outcome or that performed the last visit of the study (five-year visit) and the 40 eyes from persons with T2D that dropped out of the study (Table 1).

Characterization of the eyes according to the different phenotypes previously described, was performed at the six-month visit. There were 66 (38%) eyes categorized as phenotype A, 50 (29%) eyes identified as phenotype B and 56 (33%) eyes as phenotype C within the people with T2D that completed the study, indicating a similar distribution to baseline (p = 0.980) and no attrition bias due to loss of follow-up (Table 1).

The demographic, systemic and ocular characteristics of each phenotype at baseline (except MAT, which was defined at six-month visit) are described in Table 2. No significant differences in age,

duration of diabetes, sex, blood pressure levels, body mass index and blood lipid levels, with the exception of triglycerides, could be found between the three different phenotypes at baseline. There was a statistically significant difference in HbA_{1c} between groups ($p < 0.0001$), attributable to higher levels of HbA_{1c} in Phenotype C when compared to phenotypes A ($p = 0.023$) and B ($p < 0.0001$). Baseline values of triglycerides were also not similar across phenotypes ($p = 0.018$), which was attributable to higher values associated with Phenotype C when compared to phenotype B ($p = 0.014$).

Table 2: Characteristics of each phenotype at baseline (n=172)

	Phenotype A *N* = 66	**Phenotype B** *N* = 50	**Phenotype C** *N* = 56	***p*-value***
Demographics				
Age, y	62.83 ± 7.4	64.6 ± 6.3	61.0 ± 7.4	0.060
Duration of diabetes, y	13.4 ± 7.4	15.2 ± 8.7	14.2 ± 6.0	0.393
Males/Females, frequency (%)	44/22 (66.7/33.3)	36/14 (72.0/28.0)	37/19 (66.1/33.9)	0.808
Left eye/Right eye, frequency (%)	29/37 (43.9/56.1)	28/22 (56.0/44.0)	23/33 (41.1/58.9)	0.270
Systemic characteristics				
HbA1C, %	7.4 ± 1.1[a]	7.1 ± 1.2[b]	8.1 ± 1.3[a, b]	**<0.001***
Total cholesterol, mg/dL	186.2 ± 33.3	183.6 ± 36.7	181.8 ± 46.7	0.750
HDL, mg/dL	46.5 ± 9.9	49.9 ± 12.1	46.3 ± 11.3	0.172
LDL, mg/dL	124.9 ± 32.0	121.0 ± 30.4	120.2 ± 36.0	0.533
Triglycerides, mg/dL	162.6 ± 88.0	146.9 ± 88.3[b]	188.5 ± 101.3[b]	**0.018***
Systolic blood pressure, mmHg	136.3 ± 14.2	136.8 ± 17.2	141.3 ± 16.3	0.345
Diastolic blood pressure, mmHg	70.9 ± 7.9	71.5 ± 9.9	73.6 ± 9.1	0.248
BMI, kg/m^2	30.9 ± 6.1	28.6 ± 5.4	30.5 ± 5.9	0.079
Ocular characteristics				
BCVA, letters	85.4 ± 3.7	85.7 ± 4.2	85.7 ± 4.0	0.846
MA turnover, no. per 6 M	1.9 ± 1.8[a]	2.1 ± 1.8[b]	17.5 ± 17.7[a, b]	**<0.001***
MA formation rate, no. per 6 M	0.7 ± 1.1[a]	0.7 ± 1.0[b]	8.0 ± 7.4[a, b]	**<0.001***
MA disappearance rate, no. per 6 M	1.1 ± 1.6[a]	1.4 ± 1.4[b]	9.5 ± 9.3[a, b]	**<0.001***
Central subfield RT, µm	252.0 ± 18.2[a, c]	285.6 ± 9.3[b, c]	267.3 ± 20.2[a, b]	**<0.001***
GCL+IPL CSF thickness, µm	35.1 ± 8.1[a,c]	44.3 ± 8.7[b, c]	39.4 ± 9.5 [a, b]	**<0.001***
GCL + IPL InRing thickness, µm	89.8 ± 8.2	91.3 ± 12.7	91.5 ± 9.1	0.192
ETDRS 10-20/35, frequency (%)	23/43 (34.8/65.2)[a]	23/27 (46.0/54.0)[b]	2/56 (3.6/96.4)[a, b]	**<0.001***

* and Bold values represent statistically significant alterations, with $p < 0.05$.
Similar superscript letters denote groups that differ at 0.05 level: [a] = Phenotype A vs C, [b] = Phenotype B vs C and [c] = Phenotype A vs B.

HDL: high-density lipoprotein; LDL: low-density lipoprotein; BCVA: best corrected visual acuity; MA: microaneurysm RT: retinal thickness; GCL: Ganglion Cell Layer; IPL: Inner Plexiform Layer; CSF: Central Subfield; ETDRS: Early Treatment Diabetic Retinopathy Study.

As a consequence of the definition criteria of phenotypes, MAT values were significantly increased in phenotype C ($p < 0.0001$). Accordingly, central subfield retinal thickness was increased in phenotype B when compared to both phenotypes A and C ($p < 0.0001$). Phenotype C also presented significantly higher retinal thickness than phenotype A. No significant alterations were observed in the GCL + IPL thickness in the inner ring between different phenotypes.

Phenotype C was identified mainly in eyes graded with ETDRS level 35 (97%), when compared with the other phenotypes, showing its association with the more advanced stage of the retinal disease included in the study.

Over the five-year follow-up period, of the eyes with phenotype A there was two-step ETDRS grade improvement in 9% (6/66 eyes) and two-step worsening in only 3% (2/66). Phenotype B showed two-step ETDRS grade improvement in 10% (5/50) with no eyes showing two-step ETDRS grade worsening. In eyes with phenotype C, only one eye showed two-step ETDRS grade improvement, whereas 23% (13/56 eyes) showed two-step ETDRS grade worsening. Twenty-seven eyes developed vision-threatening complications during the period of five-year follow-up: CSME in 14, CIME in 10 and PDR in four, with one eye/patient developing both CSME and PDR. The distribution of these outcomes in the different phenotypes is depicted on Table 3.

Table 3: Distribution of outcomes across phenotypes

	Outcome N (%)					
Phenotype	**No outcome**	**CIME**	**CSME**	**PDR**	**Total**	**p-value**
A	66 (100%)	0 (0%)	0 (0%)	0 (0%)	66	**<0.001***
B	40 (80%)	7 (14%)	3 (6%)	0 (0%)	50	
C	39 (69.6%)	3 (5.4%)	11# (19.6%)	4# (7.1%)	56+	
Total	145 (84.3%)	10 (5.8%)	14 (8.1%)	4 (2.3%)	172	

* and Bold values represent statistically significant alterations, with $p < 0.05$.
# One patient developed both CSME and PDR and is considered in the two outcomes

Clinically significant macular edema developed in three eyes (21%) categorized as phenotype B, and in 11 eyes (79%) categorized as phenotype C, corresponding to a crude odds ratio of 9.97 (2.64–37.62) for CSME development in phenotype C. The univariate logistic regression analysis also determined younger age, higher HbA_{1c}, and higher LDL cholesterol as systemic significant risk factors for the development of CSME. All ocular parameters are identified as significant in the determination of the risk of development of CSME (Table 4). The multivariate logistic regression

analysis, considering all those variables potentially influencing the risk associated of development of CSME ($p \leq 0.10$ in univariate analysis) determined an adjusted OR for phenotype C of 17.41 (1.98–153.00), $p = 0.010$. Only age, BMI and GCL + IPL CSF changes further contributed to increase the overall prediction of cases. The model presented an accuracy in classification of 95.4%, with 99.3% specificity. Despite the sensitivity of 57.1%, the model positively predicted 88.9% of the cases.

Center involved macular edema developed in seven eyes (70%) categorized as phenotype B and in three eyes identified as phenotype C, indicating an OR of 6.13 (1.51–24.85) for the first. The multivariate adjustment of the OR for the development of CIME was unable to detect a statistically significant impact of phenotype B ($p = 0.223$).

Eyes with phenotype A did not develop macular edema during the five-year period of follow-up. Finally, PDR developed only in eyes categorized as phenotype C.

Table 4: Univariate and multivariate analyses of OR for CSME and CIME on a logistic regression

	Univariate Model				Multivariate Model			
	CSME		CIME		CSME †		CIME †	
	OR (95% CI)	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*
Demographic characteristics								
Age	0.87 (0.79–0.95)	**0.001***	1.01 (0.92–1.11)	0.835	0.85 (0.74–0.99)	**0.033***		
Diabetes duration	0.96 (0.88–1.04)	0.305	0.96 (0.87–1.05)	0.385				
Gender (female)	1.72 (0.56–5.26)	0.340	0.57 (0.12–2.81)	0.493				
BMI	0.90 (0.81–1.00)	0.051	0.90 (0.79–1.03)	0.124	0.78 (0.64–0.96)	**0.017***		
Systemic characteristics								
HbA_{1c}	1.58 (1.02–2.43)	**0.040***	0.70 (0.39–1.24)	0.221	0.73 (0.33–1.63)	0.447		
Total cholesterol	1.01 (1.00–1.03)	0.087	0.99 (0.97–1.00)	0.106				
HDL	0.94 (0.88–1.00)	0.059	1.01 (0.06–1.07)	0.647	0.90 (0.79–1.02)	0.097		
LDL	1.02 (1.00–1.03)	**0.043***	0.98 (0.96–1.00)	0.102	1.03 (1.00–1.06)	0.061	0.99 (0.97–1.02)	0.601
Triglycerides	1.00 (1.00–1.01)	0.088	0.99 (0.99–1.01)	0.395	1.00 (0.99–1.01)	0.645		
Systolic blood pressure	0.99 (0.95–1.03)	0.552	0.96 (0.92–1.00)	0.054			0.9 4 (0.90–0.99)	**0.018***
Diastolic blood pressure	0.97 (0.91–1.04)	0.454	0.9 5 (0.88–1.03)	0.237				
Phenotype								
Phenotype B	0.72 (0.19–2.70)	0.622	6.13 (1.51–24.85)	**0.011***			2.82 (0.53–14.99)	0.223
Phenotype C	9.97 (2.64–37.62)	**0.001***	1.17 (0.29–4.73)	0.831	17.41 (1.98–153.00)	**0.010***		

Ocular characteristics								
MA turnover	1.08 (1.03–1.13)	**0.001***	0.96 (0.85–1.08)	0.507				
MA formation rate	1.16 (1.06–1.28)	**0.002***	0.70 (0.42–1.17)	0.173				
MA disappearance rate	1.13 (1.05–1.22)	**0.001***	1.00 (0.87–1.15)	0.961				
Retinal thickness at baseline	1.06 (1.03–1.11)	**0.001***	1.19 (1.09–1.31)	**<0.001***				
GCL + IPL CSF thickness	1.14 (1.05–1.22)	**0.001***	1.21 (1.09–1.35)	**<0.001***	1.20 (1.05–1.36)	**0.005***	1.24 (1.08–1.42)	**0.002***
GCL + IPL InRing thickness	1.12 (1.04–1.20)	**0.004***	1.05 (0.96–1.13)	0.283	0.98 (0.86–1.12)	0.762		
Ratio RT/GCL (CSF)	0.54 (0.32–0.90)	**0.019***	0.55 (0.30–1.00)	0.050				

* and Bold values represent statistically significant alterations, with $p < 0.05$.
†Multivariate models included variables with $p \leq 0.1$ in univariate analysis. Variables MA turnover, MA formation rate, MA disappearance rate and Retinal thickness not included due to the inherent inclusion in the definition of phenotypes. Total cholesterol and Ratio RT/CGL (CSF) not included due to multicollinearity issues with the variables that compose it.

Analysis of the time to event of the development of outcomes, CIME, CSME and PDR, along the five-year period, is shown in Figure 2A. There was, generally, a progressive decline in the number of cases presenting CIME, suggesting that their occurrence is not correlated with the duration of the disease but rather an individual response associated with retinal thickness baseline values. The predominance of complications in phenotype C is well demonstrated in Figure 2B.

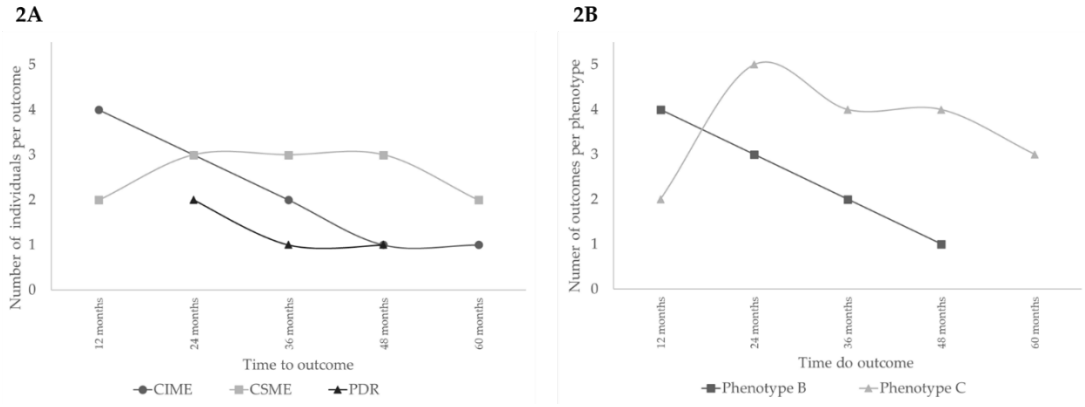

Figure 2: Analysis of the time to event of the development of outcomes. **A**: Number of people with type 2 diabetes (T2D) developing different outcomes; **B**: Number of people with T2D developing outcomes per phenotype.

Discussion

This five-year longitudinal study of a relatively large cohort of people with T2D and mild DR (ETDRS gradings 20 and 35) confirms and extends previous studies by our group [6–9] showing that there are different DR phenotypes with different risks for the development of vision-threatening complications, CSME, CIME and PDR.

Using only non-invasive procedures, easy to use repeatedly in clinical practice, the study shows that characterization of different NPDR phenotypes indicates that the chance of developing CSME and PDR within a period of five years is much higher if people with T2D have an increased MAT (≥ 6 at six months using the RetmarkerDR), which identifies phenotype C. In fact, our data suggests that Phenotype C is associated with an increased likelihood of development of CSME, whereas phenotype B is mainly associated with likelihood of development of CIME. These findings suggest that different factors underly the development of CIME and CSME. The association of increased values of MAT with CSME points to the potential relevance of capillary closure and ischemia in the process of CSME development. Therefore, CIME may not be necessarily a predictive factor for CSME [21]. It is also an argument against relying entirely on OCT metrics to identify DME [4]. Of major relevance for clinical management of diabetic retinal disease is the observation that eyes with phenotype A, which is characterized by low MAT and no increase in retinal thickness, representing approximately 40% of the mild NPDR population enrolled in the study, did not develop any vision-threatening complication, CSME, CIME or PDR, during the five-year period of follow-up. This observation is highly noteworthy, confirming our previous observations [6]. It indicates that a large proportion of eyes presenting already DR will progress very slowly and are not likely to develop vision-threatening complications in a period of five years. Phenotype A is also the phenotype associated with increased thinning of the GCL + IPL in the central subfield. This observation contrasts with the absence of changes in the GCL + IPL in the inner ring, outside the central subfield. It is also important to register that the relative proportion of the different phenotypes remains similar in the different studies [6–9]. People with T2D with initial stages of NPDR present phenotype A in 40% to 50%, whereas the remaining 50% are distributed between phenotypes B and C depending on other factors such as ethnicity [22].

Phenotype C was identified mainly in eyes with ETDRS grade 35 suggesting that ETDRS grade 35 may be the turning point in the progression of DR. Eyes with ETDRS grade 35 apparently reach a status of microvascular damage that creates the conditions for either stabilization or progression demonstrated by identification of Phenotype C. In this study, approximately 44% of the eyes graded as ETDRS 35 could be classified as phenotype C. Noteworthy, the eyes with phenotypes A

and B remained mostly stable through the five-year follow-up, with only two eyes with phenotype A (3%) and none with phenotype B presenting two-step ETDRS grade worsening, whereas phenotype C showed two-step ETDRS worsening in 13 eyes (23%).

A limitation of this study is the focus on the initial stages of DR, allowing conclusions to be made only on the development of vision threatening complications of people with T2D with ETDRS levels 20 and 35. Furthermore, the population studied is relatively well-controlled, chosen using exclusion criteria such as excessive HbA_{1C} levels (>10%) and uncontrolled blood pressure. However, the use of these criteria guaranteed a relatively homogenous population. Another possible limitation is the relatively small number of people with T2D that completed the five-year period of follow-up. However, the five years duration of the study is of major value and offers new insights into the progression of retinal diabetic disease.

The findings here reported have clear implications on clinical trial design to test new therapies to stop progression of DR. The development of an effective drug must take into account the need to demonstrate efficacy in the early and reversible stages of diabetic retinal disease by demonstrating its effect on surrogate endpoints which can be followed for relatively short period of time. The five-year period of follow-up of our study offers information that is crucial for such designs. This study also shows that noninvasive methodologies can be used to identify the eyes that are at risk of progression and develop vision-threatening complications. According to these observations, only eyes with phenotype C should be considered for inclusion in a clinical study expected to run for less than five years, particularly if the agent to be tested is directed at the prevention of vision-threatening complications of diabetic retinal disease.

Finally, the observations here reported offer promising perspectives for personalized management of DR. After diagnosis of NPDR and still in the initial stages of retinal disease, three different phenotypes can be identified through fundus photography, including MAT evaluation using the RetmarkerDR and OCT. These examinations are easy to perform and can be repeated easily without major inconvenience to the patient. This study confirms their value for improved management strategies of NPDR and timely diagnosis of vision-threatening complications of diabetes.

The retinopathy phenotypes identified in people with T2D show different risks for vision-threatening complications. Phenotype A is a slow progression phenotype that may not even need to be followed at short intervals, appearing that longer than one-year intervals are acceptable. On the other hand, people with T2D presenting phenotype C should receive the most attention.

Conclusions

Different retinopathy phenotypes in T2D show different five-year risks for development of CSME, CIME and PDR. Phenotype C identifies eyes at higher risk for development of vision-threatening complications (CSME or PDR). It is also the only phenotype associated with PDR. Phenotype B show higher risk for development of CIME. In contrast, phenotype A identifies eyes that are at a very low risk of development of vision-threatening complications.

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Chapter 3

Different retinopathy phenotypes in type 2 diabetes predict retinopathy progression

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Research Question

In the first study here reported we were able to confirm the predictive value of the NPDR phenotypic classification in a 5-year period of follow up. Vision threatening complications, such as CSME and PDR, were far more common in phenotype C eyes.

The development of such complications are the most acceptable primary endpoints in DR clinical trials. However , their development require a long period of follow up and a higher number of patients to include. Therefore, change on ETDRS severity level has been accepted as primary endpoint.

Are ocular markers and phenotype classification good biomarkers of DR severity progression? And are we able to predict DR severity progression based on other objective biomarkers?

In the second paper we wanted to address the question of whether more objective ocular characteristics could be used as biomarkers of DR severity score progression.

We could confirm that 23% of eyes characterized as phenotype C present a 2-step worsening in ETDRS level during the 5 years of follow up, while only 3% of eyes in phenotype A and B categories did so. Furthermore, MAT values were directly correlated with the worsening of the ETDRS score. Vessel Density metrics have also shown to be strongly correlated with ETDRS step changes, with lower vessel density presented in patients presenting DR ETDRS severity worsening.

Abstract

Purpose: To characterize the progression in retinopathy severity of different phenotypes of mild nonproliferative diabetic retinopathy (NPDR) in patients with type 2 diabetes.

Design and Methods: Patients with type 2 diabetes and mild NPDR (ETDRS 20 or 35) were followed in a five-year longitudinal study. Examinations, including color fundus photography (CFP) and optical coherence tomography (OCT and OCTA), were performed at baseline, 6-months and then annually. Phenotype classification was performed based on microaneurysm turnover (MAT, on CFP) and central retinal thickness (CRT, on OCT). Phenotype A is characterized by low MAT (< 6) and normal CRT; Phenotype B by low MAT (< 6) and increased CRT; and Phenotype C by higher MAT (≥ 6) with or without increased CRT. ETDRS grading of seven-fields CFP was performed at the initial and last visits.

Results: Analysis of ETDRS grade step changes showed significant differences in diabetic retinopathy (DR) progression between the different phenotypes ($p<0.001$). Of the 63 participants with phenotype A only 2 eyes (3%) presented 2-or-more-step worsening. None of the 53 participants characterized as phenotype B developed 2-step worsening, whereas 13 eyes (23.2%) characterized as phenotype C had 2-or-more-steps worsening. Phenotype C presents the higher risk for 2-or-more step worsening (OR: 15.94 95%CI: 3.45-73.71; $p<0.001$) and higher sensitivity, correctly identifying 86.7% of cases at risk (AUC: 0.84 95%CI: 0.72-0.96; $p<0.001$).

Diabetic retinopathy severity progression was associated with HbA1c ($p=0.019$), LDL levels ($p=0.043$), and ocular factors such as MAT ($p=0.010$), MA formation rate ($p=0.014$) and MA disappearance rate ($p=0.005$). Capillary closure at 5 years of follow up, identified by lower vessel density (VD) on OCTA, was also associated with diabetic DR severity progression ($p= 0.035$).

Conclusions: Different DR phenotypes in type 2 diabetes show different risks of retinopathy progression. Phenotype C is associated with increased HbA1c values and presents a higher risk of a 2-or-more-step worsening of the ETDRS severity score.

Keywords: type 2 diabetes, retinopathy, microaneurysm, retinal thickness, biomarkers, phenotypes

Introduction

Diabetic retinopathy (DR) is one of the leading causes of blindness worldwide (1) and the prevalence of vision-threatening DR is expected to double in the next decade (2). Considering that more than 90% of cases of vision loss can be prevented (3), accurate staging and classification of DR are paramount to guide treatment decision and determine prognosis. The Early Treatment of Diabetic Retinopathy Study (ETDRS) severity score is the gold-standard for DR staging (4). However, it is labor-intensive and has limited applicability outside of the research setting.

Our group has reported on two-year and three-year follow-up studies of people with type 2 diabetes (T2D) and mild nonproliferative diabetic retinopathy (NPDR) and found marked individual variations in the progression of DR and development of vision-threatening complications. Using non-invasive imaging methodologies (color fundus photography,CFP and optical coherence tomography, OCT), we have identified three different phenotypes of NPDR. These are based on microaneurysm turnover (MAT) assessed at 6 months of follow up and central retinal thickness (CRT) on OCT, being associated with different risks for development of vision-threatening complications (5).

Briefly, Phenotype A is characterized by low MAT (< 6) and normal CRT; Phenotype B by low MAT (< 6) and increased CRT and Phenotype C by higher MAT (≥ 6) with or without increased CRT (Figure 1).

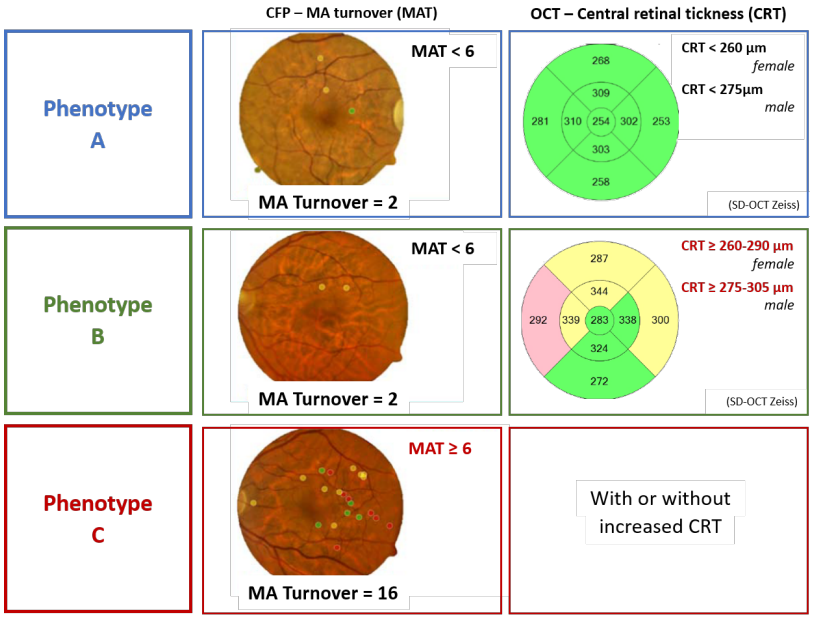


Figure 1: Representative cases for the three phenotypes of DR progression. Color fundus image: MA earmarked using the software RetmarkerDR at 6 months visit (V1): red dots are new MA, yellow dots are MA that disappeared from baseline to V1, and green dots are MA that were present in both visits. Central macular thickness maps obtained with the SD-OCT: Central Retinal Thickness (Reference values from Zeiss SD-OCT).

A recently published study revealed that these different retinopathy phenotypes in T2D show different five-year risk for development of center involved macular edema (CIME), clinically significant macular edema (CSME) and/or proliferative diabetic retinopathy (PDR). Phenotype C eyes are at higher risk for development of vision-threatening complications (CSME or PDR). It is also the only phenotype associated with PDR. In contrast, phenotype A identifies eyes that are at very low risk of development of vision-threatening complications (6).

This five-year longitudinal study of a large cohort of people with type 2 diabetes and mild NPDR aims to identify risk markers for DR severity progression using only non-invasive examination methodologies - digital CFP, OCT and OCT-Angiography (OCTA).

Methods

A prospective longitudinal cohort study was designed to follow eyes with minimal or mild NPDR (Early Treatment Diabetic Retinopathy Study (ETDRS) grades 20-35) for a 5-year period or until the time of development of diabetic macular edema (DME) or proliferative retinopathy (PDR) [6]. The tenets of the Declaration of Helsinki were followed, and the approval was obtained from the local Institutional Ethical Review Board. Each participant signed a written informed consent, agreeing to participate in the study, after all procedures were explained.

A total of 212 patients with diagnosed adult-onset T2D and ETDRS 20-35 retinopathy were included, with a maximum glycated hemoglobin A1c (HbA1c) value of 10%.

Exclusion criteria included any previous laser treatment or intravitreal injections, presence of age-related macular degeneration, glaucoma, vitreomacular disease and high ametropia (spherical equivalent greater than -6 and +2 DPT), or any other systemic disease that could affect the eye, with special attention for uncontrolled systemic hypertension and history of ischemic heart disease. Eyes with baseline central retinal thickening identifying CIME (defined as a central subfield retinal thickness (CRT) ≥ 290 μm in women and ≥ 305 μm in men, by the Diabetic Retinopathy Clinical Research Network [7]), were excluded.

At baseline visit (V0) demographics, such as age, duration of diabetes and concomitant medications were collected for each participant. Physical assessment with biometric measures (body weight and height) and blood pressure evaluation were performed by an experienced nurse, as well as blood analysis with determination of HbA1c and lipid profile. The remaining study visits were performed at 6 months (V1), 12 months (V2), 24 months (V3), 36 months (V4), 48 months (V5) and 60 months (V6) or last visit before treatment. At all study visits patients underwent a complete eye examination, which included best-correct visual acuity (BCVA, measured for each eye using the ETDRS protocol and Precision Vision charts at 4 m), slit-lamp examination, intraocular pressure (IOP) measurement, digital seven-field CFP and OCT. OCTA was performed only at last visit.

The study eye was selected at baseline based on the inclusion/exclusion criteria. When both eyes fulfilled the criteria, the eye showing the more advanced ETDRS grading in any given patient was chosen to be the study eye.

The patients did not receive any treatment during the follow-up period, as defined in the protocol of the study. Treatments were only applied when an outcome was developed, and therefore the patient left the study

Color Fundus Photography and RetmarkerDR - Microaneurysm quantification

CFP was performed according to the ETDRS protocol. The seven-fields photographs were obtained at 30/35º, using a Topcon TRC 50DX camera (Topcon Medical Systems, Tokyo, Japan). The DR severity level was determined by two independent graders within the context of an experienced reading center (Coimbra Ophthalmology Reading Center - CORC, Coimbra, Portugal) and was based on the seven-field protocol according to the modified Airlie House classification of diabetic retinopathy used by the Early Treatment Diabetic Retinopathy Study (4). According to the ETDRS final scale (ETDRS report number 12) a severity scale was used by identifying step changes. The DR severity scale used was: step 1, level 10, no retinopathy; step 2, level 20; step 3, level 35; step 4, level 43; step 5, level 47; step 6, level 53; step 7, levels 61 to 71.

Additionally, 45/50º field-2 images were obtained and subjected to automated microaneurysm (MA) analyses using the RetmarkerDR (Retmarker SA, Coimbra, Portugal). This automated computer-aided diagnostic system consists of software earmarking MA and red-dot-like vascular lesions in the macula (all referred to as MAs); it includes a co-registration algorithm that allows comparison within the same retina location between different visits for the same eye, as previously described (7)(8). Briefly, the algorithm computes for each eye the number of MAs in each visit, the number of MAs that appear and/or disappear from one visit to the other, allowing calculation of the number of MAs appearing and/or disappearing per time interval (i.e., the MA formation rate and the MA disappearance rate, respectively). The MAT is calculated as the sum of the MA formation and MA disappearance rates, determined at 6 month visit.

Characterization of retinopathy phenotypes

The three different DR phenotypes for NPDR, previously described by our group (5,9), were identified in the study eyes at the 6 month visits, based on the six-month MAT and CRT, and according to the following rules: Phenotype A: MAT < 6 and normal CRT values (central subfield RT < 260μm for females and < 275 μm in males , i.e., normal mean ±1 SD); Phenotype B: MAT < 6 and increased CRT values (CRT ≥ 260 μm for females and ≥ 275 μm in males); Phenotype C: MAT ≥ 6, with or without increased CRT. Central retinal thickness reference values presented are the reference for Zeiss Cirrus SD-OCT (10,11).

Optical Coherence Tomography

OCT was performed using the Cirrus Zeiss 5000 AngioPlex (Carl Zeiss Meditec, Dublin, CA).

The Macular Cube 512x128 acquisition protocol, consisting of 128 B-scans with 512 A-scans each, was used to assess the subjects' CRT, collected from the standard Cirrus examination reports. Segmentation of retinal layers to assess the average thickness value of the ganglion cell layer plus

inner plexiform layer (GCL+IPL) in both central subfield (CSF) and Inner Ring (InRing) were performed using the segmentation software implemented by AIBILI (6,12). Automated analysis results were reviewed by a masked grader.

Eyes with CIME were identified following the reference values established by the DRCR.net for Cirrus SD-OCT (13). GCL+IPL thickness decreases were considered as a surrogate marker of neurodegeneration (14) whereas full CRT increases were considered to identify edema (15), comparing to a healthy control population (12,16).

OCT-Angiography

OCTA was performed in all patients in the last study visit. OCTA data were collected by the CIRRUS™ HD-OCT 5000 with AngioPlex® OCT Angiography (Carl Zeiss Meditec, Dublin, CA, USA) device using Angiography 3x3 mm acquisition protocol, which consists of a set of 245 clusters of B-scans repeated 4 times, where each B-scan consists of 245 A-scans over a 3x3x2 mm^3 cube in the central macula.

To calculate vessel density (VD) a thresholding algorithm was applied to the superficial and deep retinal capillary layers (SRL and DRL) *en face* images to create a binary slab that assigns to each pixel a 1 (perfused) or 0 (background), from which a skeletonized slab was created, representing vessels with a trace of 1 pixel in width as previously described (17). We define VD (skeletal density) as the total length of perfused vasculature per unit area in a region of measurement. It is calculated by taking the mean of the skeletonized slab within a region of interest and scaling the result by the distance between pixels (in this case, 245 pixels per 3 mm).

For quality check, all OCTA acquisitions were reviewed by a masked grader. Only eyes that had OCTA examinations with signal strength greater or equal to 7, minimal motion artifacts and no evidence of defocus or blur were included in the analysis.

Outcomes and Statistical Analysis

The collected data on each eye/patient is presented as means and corresponding standard deviations for continuous variables or absolute and relative frequencies for categorical and ordinal variables. ETDRS step changes were determined as the difference between levels of ETDRS at baseline and at the 5 years follow-up and classified as improvement or worsening according to the reduction or increase of the retinopathy grade. Classification included "2-step improvement," "1-step improvement," "maintenance," "1-step worsening" and "2-or-more-step worsening." Due to the clinically less relevant 1-step change, patients with 1-step improvement and 1-step worsening were gathered under the category of maintenance—Maintenance (± 1 step deviation).

Comparison of baseline characteristics of patients with different ETDRS changes over the 5 years of follow-up was performed using the Kruskal–Wallis test (due to violation of assumption of normality) and all-pairwise post hoc comparisons with Bonferroni correction or the Chi-square test with Monte-Carlo correction.

A multinomial logistic regression was run to assess the likelihood of 2-step improvement or 2-or-more steps worsening associated with each demographic, systemic and ocular marker evaluated at the baseline or 6 months appointment—MAT, MA formation rate, MA disappearance rate, CRT, GCL + IPL CSF thickness, GCL + IPL InRing thickness, capillary closure metrics and phenotype. Results were presented as odds ratios (OR) and corresponding 95% confidence intervals. The significant predictors ($p<0.05$) were considered for multivariate analysis, and the obtained predicted probabilities were tested for the discriminatory performance using receiver operating characteristic (ROC) curves.

All analyses were performed with SPSS (IBM SPSS Statistics version 24; IBM Corp ©, New York, USA), and a p value <0.05 was considered statistically significant.

Results

Of the 212 eyes/patients included in the study, 172 completed the 5-year follow-up or achieved one of the endpoints, CSME, CIME or PDR. Forty patients dropped out of the study (nine died, twenty-one withdrew from the study and ten were lost to follow-up). The eyes included in the study had mild NPDR, 58 (27%) graded as ETDRS level 20 and 154 (73%) graded as ETDRS level 35. As previously described, no statistically significant differences were found for demographic, clinical and ocular characteristics at baseline between the 172 patients that reached the study endpoint or performed the last visit of the study and the eyes/patients that dropped out from the study [6].

The distribution of the ETDRS change over the 5-year period for the 172 eyes that completed the study is detailed in Table 1. Twelve eyes (7%) presented 2-step improvement and 15 (9%) had 2-or-more-step worsening of their retinopathy severity. The vast majority maintained the ETDRS classification (n=77) or presented 1-step variation: 1-step improvement (n=19) and 1-step worsening (n=49).

Among the demographic and clinical characteristics, statistically significant differences across the categories of ETDRS change were only found for HbA1c (p=0.025), for which was possible to register a continuous increase in the mean and median values from 2-step improvement to 2-or-more-step worsening. Pairwise comparisons indicated that the difference was set between those with 2-step improvement and 2-or-more-step worsening (p=0.024) (Table 1).

Table 1: Baseline characteristics of the population with different ETDRS changes over the 5-year period (n=172)

Characteristics	All patients included	2-step improvement	Maintenance	2-or-more-step worsening	*p*
Demographics					
Males/females, *n* (%)	117 (68.0)/55 (39.0)	9/3 (75/25)	98/47 (67.6/32.4)	10/5 (66.7/33.3)	0.891
Age, mean ± SD, y	62.7 ± 7.2	61.3 ± 7.1 (60.5)	62.9 ± 7.1 (63.0)	62.1 ± 8.4 (59.0)	0.683
Diabetes duration, mean ± SD, y	14.2 ± 7.4	14.1 ± 9.5 (12.50)	14.2 ± 7.4 (14.0)	14.3 ± 6.2 (15.0)	0.993
Clinical characteristics, mean ± SD (median)					
BMI, kg/m^2	30.1 ± 5.9 (29.7)	31.9 ± 8.1 (30.6)	29.8 ± 5.6 (29.3)	31.4 ± 5.9 (31.2)	0.478
HbA_{1c}, %	7.5 ± 1.3 (7.4)	6.9 ± 0.7 (6.5)	7.5 ± 1.3 (7.4)	8.3 ± 1.4 (8.0)	**0.025***
Total cholesterol, mg/dL	184.0 ± 38.6 (181.5)	194.4 ± 20.9 (193.0)	185.0 ± 38.8 (181.0)	167.1 ± 46.2 (160.0)	0.198
HDL cholesterol, mg/dL	47.4 ± 11.1 (47.0)	47.9 ± 12.5 (45.0)	47.4 ± 10.8 (46.5)	47.9 ± 12.9 (48.0)	0.893
LDL cholesterol, mg/dL	122.2 ± 32.8 (118.5)	127.6 ± 19.8 (130.0)	123.6 ± 32.9 (119.0)	105.4 ± 35.8 (109.0)	0.130
Triglycerides, mg/dL	166.4 ± 93.5 (142.0)	175.3 ± 78.5 (176.0)	164.6 ± 89.7 (144.5)	176.2 ± 135.9 (125.0)	0.692
Systolic BP, mmHg	138.1 ± 15.9 (139.0)	129.8 ± 15.7 (131.5)	138.4 ± 16.1 (140.0)	141.9 ± 11.7 (143.0)	0.206
Diastolic BP, mmHg	71.9 ± 9.0 (72.0)	70.1 ± 8.5 (68.0)	72.0 ± 9.1 (72.0)	73.1 ± 8.3 (72.0)	0.697

Ocular characteristics, mean ± SD (median)					
BCVA, letters	85.6 ± 3.9 (85.09	86.6 ± 3.2 (87.0)	85.5 ± 3.9 (85.0)	85.4 ± 4.7 (85.0)	0.484
MA turnover, no. per 6 months	7.0 ± 12.5 (3.7)	1.6 ± 2.5 (0.0)	6.2 ± 11.5 (3.6)	19.7 ± 18.3 (15.6)	**<0.001***
MA formation rate, no. per 6 months	3.1 ± 6.4 (1.9)	0.3 ± 1.1 (0.0)	2.7 ± 5.8 (1.9)	8.9 ± 10.9 (7.8)	**< 0.001***
MA disappearance rate, no. per 6 months	3.9 ± 6.7 (2.0)	1.3 ± 1.7 (0.0)	3.5 ± 6.3 (2.0)	10.8 ± 8.8 (8.3)	**< 0.001***
CRT, µm	266.8 ± 21.7 (267.0)	268.6 ± 18.4 (267.5)	267.3 ± 22.0 (268.0)	260.4 ± 20.6 (256.0)	0.314
GCL + IPL CSF thickness, µm	39.1 ± 9.5 (38.6)	40.4 ± 6.2 (40.2)	39.4 ± 9.9 (39.1)	35.5 ± 6.6 (33.9)	0.118
GCL + IPL InRing thickness, µm	90.8 ± 10.0 (91.2)	95.2 ± 6.4 (93.1)	90.7 ± 9.9 (91.2)	88.1 ± 12.0 (84.2)	0.106
Baseline ETDRS level, n(%)					
20	48 (27.9)	0 (0.0)	45 (31.0)	3 (20.0)	0.056
35	124 (72.1)	12 (100.0)	100 (69.0)	12 (80.0)	
Phenotypes, n(%)					
Phenotype A	66 (38.4)	6 (9.1)	58 (87.9)	2 (3.0)	**< 0.001***
Phenotype B	50 (29.1)	5 (10.0)	45 (90.0)	0 (0.0)	
Phenotype C	56 (32.6)	1 (1.8)	42 (75.0)	13 (23.2)	

BMI body mass index, *HbA*$_{1c}$ hemoglobin A_{1C}, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *BP* blood pressure, *BCVA* best corrected visual acuity, *MA* microaneurysm, *CRT* retinal thickness, *GCL* ganglion cell layer, *IPL* inner plexiform layer, *CSF* central subfield, *InRing* inner ring, *ETDRS* early treatment diabetic retinopathy study

*And bold values represent statistically significant alterations, with $p < 0.05$

MAT, MA formation rate and MA disappearance rate presented statistically significant differences across ETDRS change categories (p <0.001 for the three variables), with an increase in the median values from 2-step improvement to 2-or-more-step worsening. Pairwise comparisons indicated that the eyes with 2-or-more-step worsening presented statistically higher MAT than any other category (2-or-more-step improvement: p <0.001; maintenance: p =0.036).

Vessel density obtained from the OCTA at the last visit of the 5-year follow-up showed a decrease in all ETDRS levels. This decrease indicates a significant correlation with the DR severity progression. A consistent decrease in the median values of VD, obtained from the OCTA at 5-year of follow-up, was found when comparing the two-step worsening category (median: 16.8; interquartile range [IQR]: 2.6–2.7) with the improvement category (median: 18.9; IQR: 1.9–2.7) (Fig. 2).

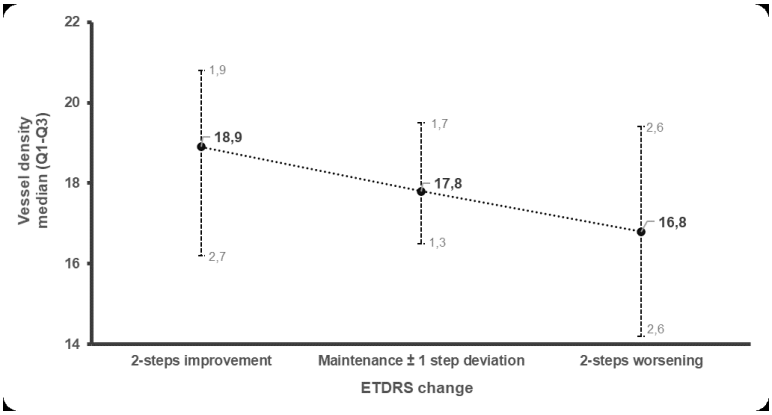


Figure 2: Correlation between vessel density at last visit with ETDRS step change over time. Vessel density was assessed at last visit of the study and is presented as Median and quartile 1 and 3 range. ETDRS step change was accessed by the difference between ETDRS grades in the baseline and last visit, considering step improvement, maintenance or worsening and represents diabetic retinopathy progression.

At the 6-month visit, the eyes were categorized as phenotype A, B or C, as detailed in the methods section. The distribution of ETDRS changes across phenotypes is detailed in Table 1. During the 5-year period of follow up, only two eyes (3%) of phenotype A presented 2-or-more-step worsening. In phenotype B, there were no cases of 2-or-more-step worsening. Finally, in phenotype C a total of 13 eyes (23%) presented 2-or-more-step worsening in the ETDRS score. These results indicate a non-independent distribution of ETDRS changes across the three phenotypes (p <0.001) with higher prevalence of 2-or-more-step worsening associated with phenotype C.

The univariate multinomial regression corroborated those patients that have 2-or-more-step worsening present particular features which are not observed in the patients falling into the other categories. While it was possible to identify higher HbA1c levels (OR = 1.66, 95% CI: 1.09–2.54), lower LDL cholesterol (OR = 0.98, 95% CI: 0.96–1.00) and elevated MAT (OR = 1.05, 95% CI: 1.01–1.09), MA formation rate (OR = 1.08, 95% CI: 1.02–1.15) and MA disappearance rate (OR = 1.10, 95% CI: 1.03–1.18) as significant univariate risk predictors of 2-or-more-step worsening when compared to maintenance (± 1 step deviation), no factor was identified as predictor of 2-step improvement (Table 2).

Table 2: Univariate multinominal regression of demographic, systemic and ocular predictors of ETDRS progression over the 5-year period, referencing to maintenance (± 1 step deviation)

	2-step improvement		2-or-more-step worsening	
	OR (95% CI)	p	OR (95% CI)	p
Demographic features				
Age	0.97 (0.89–1.05)	0.440	0.98 (0.92–1.06)	0.577
Diabetes duration	0.97 (0.92–1.08)	0.968	1.00 (0.93–1.08)	0.936
Gender (male)	1.44 (0.37–5.56)	0.598	0.96 (0.31–2.96)	0.942
BMI	1.06 (0.96–1.17)	0.239	1.05 (0.96–1.14)	0.325
Systemic features				
HbA_{1c}	0.65 (0.38–1.12)	0.117	1.66 (1.09–2.54)	**0.019***
Total cholesterol	1.01 (0.99–1.02)	0.442	0.99 (0.97–1.00)	0.088
HDL	1.00 (0.95–1.06)	0.874	1.01 (0.96–1.05)	0.849
LDL	1.00 (0.99–1.02)	0.692	0.98 (0.96–1.00)	**0.042***
Triglycerides	1.00 (1.00–1.01)	0.712	1.00 (1.00–1.01)	0.646
Systolic blood pressure	0.96 (0.93–1.00)	0.076	1.01 (0.98–1.05)	0.412
Diastolic blood pressure	0.98 (0.91–1.05)	0.481	1.01 (0.96–1.07)	0.634
Phenotypes				
Phenotype A	1.50 (0.46–4.88)	0.500	0.23 (0.05–1.06)	0.060
Phenotype B	1.59 (0.48–5.27)	0.451	Not possible to compute[a]	
Phenotype C	0.22 (0.03–1.78)	0.157	15.94 (3.45–73.71)	**<0.001***
Ocular markers				
MA turnover	0.76 (0.58–1.00)	0.049	1.05 (1.01–1.09)	**0.010***
MA formation rate	0.52 (0.26–1.02)	0.058	1.08 (1.02–1.15)	**0.014***
MA disappearance rate	0.78 (0.57–1.06)	0.116	1.10 (1.03–1.18)	**0.005***
CRT	1.00 (0.98–1.03)	0.837	0.99 (0.96–1.01)	0.245
GCL + IPL CSF thickness	1.01 (0.95–1.08)	0.720	0.96 (0.90–1.01)	0.126
GCL + IPL InRing thickness	1.06 (0.99–1.14)	0.103	0.98 (0.93–1.02)	0.345
Ratio GCL + IPL/RT (CSF)	0.84 (0.56–1.27)	0.409	1.09 (0.84–1.42)	0.530

BMI body mass index, *HbA_{1c}* hemoglobin A_{1C}, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *MA* microaneurysm, *CRT* retinal thickness, *GCL* Ganglion cell layer, *IPL* inner plexiform layer, *CSF* central subfield, *InRing* inner ring, *RT* retinal thickness

[a]Zero cases of phenotype B presented two steps worsening in the ETDRS classification over the 5 years

*And bold values represent statistically significant alterations, with $p < 0.05$

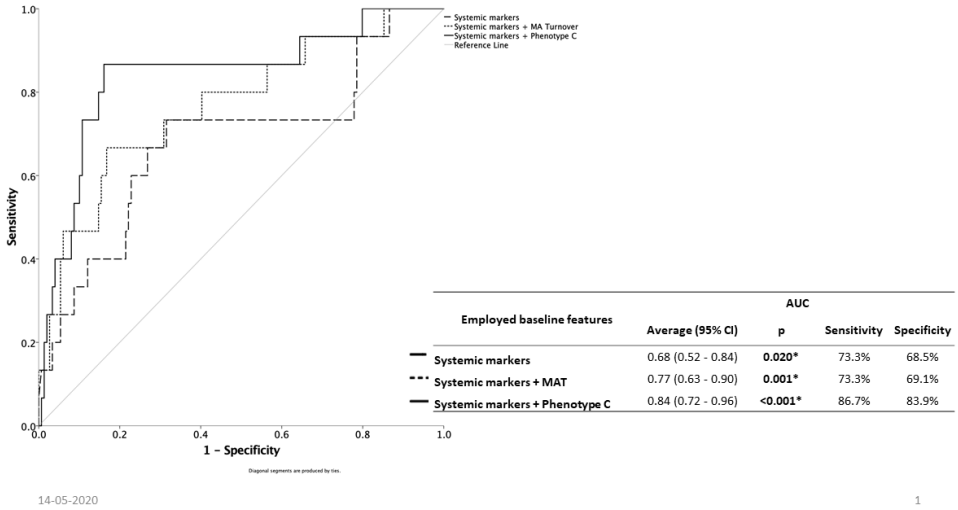
Phenotype C was associated with a significantly 16-fold higher risk of 2-or-more-step worsening (OR = 15.94, 95% CI: 3.45–73.71) while no phenotype was associated with an increased risk of improvement ETDRS score during follow-up.

Two multivariate analyses were performed (Table 3) using important metabolic systemic factors, HbA1c and LDL cholesterol. In one case, the analysis included MAT (adjusted OR = 1.04, 95% CI: 1.00–1.08), whereas in the other case the analysis included phenotype C (adjusted OR = 12.23, 95% CI: 2.53–59.18). Though, both models presented good discriminatory capacity of determining 2-or-more-step progression with area under the curve (AUC) > 75%, the model considering MAT could not increase the sensitivity of the systemic markers alone model. Contrarily, phenotype C, presented higher sensitivity, correctly identifying 87% of cases at risk of 2-or-more-step progression, and good specificity, correctly determining 84% of the cases not at risk (Figure 3).

Table 3: Multivariate regression analysis of 2 or more steps worsening risk

Table 3: Multivariate regression analysis of 2-or-more-step worsening risk							
		OR (95% CI)	**p**			**OR (95% CI)**	**p**
Systemic features	HbA_{1c}	1.83 (1.09 - 3.07)	**0.021***	**Systemic features**	HbA_{1c}	1.66 (0.98 - 2.81)	0.057
	LDL	0.97 (0.95 - 0.99)	**0.010***		LDL	0.98 (0.96 - 1.00)	**0.021***
Ocular markers	MA Turnover	1.04 (1.00 - 1.08)	**0.033***	**Phenotypes**	C	12.23 (2.53 - 59.18)	**0.002***

* and bold values represent statistically significant alterations, with p <0.05.
HbA1c: hemoglobin A1C; LDL: low-density lipoprotein; MA: microaneurysm.



Employed baseline features	AUC Average (95% CI)	p	Sensitivity	Specificity
Systemic markers	0.68 (0.52 - 0.84)	**0.020***	73.3%	68.5%
Systemic markers + MAT	0.77 (0.63 - 0.90)	**0.001***	73.3%	69.1%
Systemic markers + Phenotype C	0.84 (0.72 - 0.96)	**<0.001***	86.7%	83.9%

Figure 3: ROC curve for Systemic markers, Systemic markers + MAT and Systemic markers + Phenotype C on the prediction of 2-or-more ETDRS grade step worsening. Includes indication of AUC (95% confidence interval), sensitivity and specificity.

Discussion

This 5-year prospective longitudinal study of patients with type 2 diabetes and mild NPDR (ETDRS 20 and 35 at baseline) shows that phenotype C, is a good predictor of retinopathy worsening as demonstrated by 2-or-more-step increase in ETDRS score. Of particular interest is the observation that MAT determined over a period of only 6 months, predict, with a high degree of confidence, the eyes that do not progress for a period of at least 5 years. This finding potentially may have impact in clinical trial design allowing programmed recruitment and in the clinical management of the initial stages of diabetic retinal disease.

We know from previous epidemiological studies that the incidence of clinically significant endpoints (e.g. PDR or CSME), is very low in patients with mild NPDR. For that reason, clinical trials based on these primary endpoints require a long period of follow-up and a large number of patients to include. Thus, other clinically meaningful measures have been proposed as primary endpoints, such as 2 or 3-step progression on the ETDRS DR severity scale [18] or change in MA counts [19]. Although of some complexity, the ETDRS DR severity scale has become the "de facto" gold standard for grading diabetic retinopathy and any evaluation of progression in severity of diabetic retinopathy should refer to it. Indeed, recent studies have shown the relevance of retinopathy severity evaluation based on ETDRS grading as a clinically important outcome. In eyes treated with anti-VEGF agents [20] or with corticosteroids [21], greater degrees of improvement in ETDRS score correlate with greater magnitudes of functional and anatomic improvement. In fact, 2-step worsening in ETDRS grades has been accepted as clinically relevant and allowing to use smaller number of patients or shorten the duration of the trial [22]. However, ETDRS classification has some limitations to be used in routine screening of DR patients in clinic, as it is difficult to perform and time consuming.

The present study shows that automated analysis of MAT correlates well with changes in ETDRS severity levels validating its use as a simple to use biomarker of DR progression. Automated analysis techniques offer advantages of repeatability and consistency associated with ease of use. It is also relevant that MAT calculated by the RetmarkerDR is much less time consuming than ETDRS grading and only needs a CFP to be performed.

Increased values of MAT and phenotype C, independent of CRT values, appear to identify the eyes that will be progressing and developing vision-threatening complications such as CSME and PDR, which are not expected to improve without intervention. Our observations suggest that the eyes/ patients that can be identified by these methods are the ones that need most close follow-up.

Phenotype C was identified mainly in eyes with baseline ETDRS level 35 (97%) suggesting that ETDRS level 35 may be the turning point in the progression of diabetic retinopathy. Eyes with ETDRS level 35 apparently reach a status of microvascular damage that creates the conditions for either stabilization or progression demonstrated by identification of Phenotype C. In this study approximately 44% of the eyes graded as ETDRS level 35 at baseline were classified as phenotype C. Of the eyes classified as phenotype C 23% experienced a 2-or-more steps ETDRS grade worsening of their retinopathy severity during the 5-year period of follow- up; whereas for patients classified as phenotype A or B only 3% presented a 2-or-more steps grade worsening.

We have also observed a correlation between capillary closure, identified by decreased VD, and retinopathy severity progression, indicating that this OCTA metric is a potential early marker of DR severity progression. An automated non-invasive examination such as OCTA offers a promising option to identify retinopathy progression [16].

When considering systemic variables HbA1c stands out as being associated with retinopathy severity progression. It is the systemic variable that shows a clearer association with retinopathy progression.

Our study identified a large group of eyes/patients, phenotypes A and B, which combined represent 70% of the entire cohort and which are at a very low risk for 2-or-more-step ETDRS worsening (2%). This observation is particularly relevant for appropriate planning of eye care for the large numbers of patients with type 2 diabetes and mild retinopathy.

Limitations of this study include the focus on the initial stages of DR, allowing conclusions to be made only on the progression of ETDRS grades 20–35. The fact that there was no correction of the VD according to signal strength differences between 7 and 10 is another limitation of the study, as it has been suggested that differences in VD can be found according to the signal strength, and quantification algorithms for OCTA should ideally remove the signal strength bias [23]. However, the 5-year prospective follow-up is a major strength as it offers new insights into the progression of retinal diabetic disease, particularly when it may still be reversible and amenable to treatment. Fundus photography, including MAT evaluation using the RetmarkerDR, OCT and OCTA are easy to perform and can be repeated without major inconvenience to the patient or clinics' flow. This study confirms the potential of these variables for the evaluation of DR severity progression, opening new avenues for improved management strategies of NPDR and timely identification of eyes at risk for retinopathy progression.

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Chapter 4

Ocular and systemic risk markers for development of Macular Edema and Proliferative Retinopathy in type 2 diabetes. A five-year longitudinal study.

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Research Question

In the previous studies here reported we were able to confirm the predictive value of the NPDR phenotypic classification in a 5-year period of follow up. Vision threatening complications, such as CSME and PDR, as well as DR severity progression based on ETDRS classification, were far more common in phenotype C eyes.

The definition of phenotypes is based on MAT and CRT at 6 months visit.

Are ocular biomarkers, including microaneurysms activity and retinal thickness measurements, but also neurodegenerative markers based on OCT analysis, sufficiently powerful to predict development of DR complications and severity progression? Does them increase the predictive value of systemic parameters?

In the third paper presented in the thesis, we could confirm that the systemic markers, namely age, HbA1c, LDL and BMI showed a risk association with CSME, but no association was proven with PDR. After adjustment for the systemic characteristics, ocular factors such as MAT, CRT, GCL-IPL thickness in central subfield and GCL-IPL thickness in the inner ring were good predictors of the development of CSME, with an AUC of 0.87 (0.75 – 0.98), with 85.7% sensitivity and 83.4% specificity.

The eye markers remain determinant in the risk of development of STC.

Observation Letter to the Editor

Diabetic retinopathy (DR) is a common complication of diabetes and may lead to blindness through vision-threatening complications, such as diabetic macular edema (DME) and proliferative retinopathy (PDR). Several studies have established that certain systemic factors have associations with incidence and progression of DR, namely glycemic control, arterial hypertension, high cholesterol and hyperlipidemia, obesity, inflammatory markers, sleep-disordered breathing and exercise (1,2). In addition to systemic factors, there are ocular factors that should be considered since they may identify the eyes at risk (2).

We here report a five-year prospective longitudinal observational cohort study that investigates the risk of both systemic and ocular factors that may play a role in the development of DME and PDR, the vision-threatening complications of DR.
This observational cohort study included eyes/patients with mild NPDR, Early Treatment Diabetic Retinopathy Study (ETDRS classification) grades 20 and 35 (3), which were followed for a period of five years or until the time of development of center-involved macular edema (CIME), clinically-significant macular edema (CSME) or PDR. A total of 212 patients were included, men and women with diagnosed adult-onset type 2 diabetes, aged 42 to 82 years, with a maximum baseline HbA1c value of 10% (86 mmol/mol). Exclusion criteria included any laser treatment or intravitreal injections or any other comorbidity that could affect the retina. Excluded were also subjects with uncontrolled systemic hypertension above 210 mmHg and history of ischemic heart disease.

A complete eye examination, which included BCVA, slit-lamp examination, intraocular pressure measurement, digital seven-field color fundus photography (CFP) and optical coherence tomography (OCT) was performed annually. Additionally, 45/50º field-2 images were obtained for microaneurysm turnover (MAT) analyses using the RetmarkerDR (Retmarker SA, Coimbra, Portugal). Of the 212 eyes included in the study, 172 eyes of persons with type 2 diabetes, one eye per person, completed the study. Fourteen eyes developed CSME (8%) and ten developed CIME (6%), whereas four eyes developed PDR (2%) with one of these eyes showing both CSME and PDR (3).

Analysis of descriptive data of demographic and systemic characteristics determined that patients that developed CSME and/or PDR, had lower age ($p<0,001$), lower BMI ($p<0,032$) and higher HbA1c values ($p<0.015$).

Regarding ocular characteristics and their relationship with vision-threatening outcomes, it was possible to identify statistically higher values of MAT in patients that developed CSME (p=0.001) or PDR (p=0.007) and higher central retinal thickness (CRT) values in patients that developed CIME or CSME (both p<0.001).

The Cox hazard regression confirmed the importance of the ocular markers in the risk of development of CSME (table 1). After adjustment for systemic characteristics, MAT presented an HR of 1.03 (95% CI: 1.01-1.06; p=0.018). CRT presented an HR of 1.08 (95% CI: 1.03-1.14; p=0.003) and ganglion cell layer + inner plexiform layer (GCL+IPL) thickness an HR of 1.13 (95% CI: 1.04-1.22; p=0.002). Among the systemic factors used for adjustment of the risk of each ocular marker, age was consistently a significant confounder, with risk reduction of 11-17% per unit increase (hazard ratios 0.83-0.89). BMI was also associated with risk reduction in association with MAT and GCL+IPL thickness. For CIME, only the baseline CRT and GCL+IPL thickness were associated with risk increase (table 1).

Table 1: Univariate and Multivariate Cox Proportional Hazards Regression of Progression to CSME and CIME by different types of ocular markers

CSME					
Ocular markers	**Univariate**		**Multivariate*** HR (95% CI, p)		
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**Significant confounders**
MA turnover	1.04 (1.02 - 1.05)	**<0.001***	1.03 (1.01 - 1.06)	**0.018***	Age 0.88 (0.79 - 0.97, 0.015); BMI 0.84 (0.73 - 0.97, 0.015)
MA formation rate	1.08 (1.05 - 1.11)	**<0.001***	1.06 (1.01 - 1.12)	**0.018***	Age 0.87 (0.79 - 0.97, 0.009); BMI 0.84 (0.73 - 0.97, 0.015)
MA disappearance rate	1.07 (1.04 - 1.11)	**<0.001***	1.06 (1.01 - 1.12)	**0.027***	Age 0.88 (0.79 - 0.97, 0.014); BMI 0.85 (0.74 - 0.97, 0.017)
CRT	1.06 (1.03 - 1.10)	**0.001***	1.08 (1.03 - 1.14)	**0.003***	Age 0.89 (0.80 - 0.98, 0.023)
Δ CRT V1_Vlast	1.03 (1.02 - 1.04)	**<0.001***	1.08 (1.04 - 1.11)	**<0.001***	Age 0.83 (0.73 - 0.94, 0.004); HDL 0.83 (0.73 - 0.94, 0.003)
GCL+IPL CSF thickness	1.12 (1.05 - 1.11)	**<0.001***	1.13 (1.04 - 1.22)	**0.002***	Age 0.88 (0.79 - 0.98, 0.022); BMI 0.85 (0.74 - 0.98, 0.028)
Δ GCL+IPL CSF V1_Vlast	0.99 (0.90 - 1.09)	0.864	1.01 (0.91 - 1.12)	0.886	Age 0.85 (0.77 - 0.94, 0.001); HDL 0.88 (0.79 - 0.97, 0.013); BMI 0.86 (0.76 - 0.98, 0.024)
GCL+IPL InRing	1.11 (1.04 – 1.18)	**0.001***	1.05 (0.97 – 1.12)	0.230	Age 0.86 (0.78 - 0.95, 0.003); HDL 0.89 (0.80 - 0.99, 0.029; BMI 0.85 (0.76 - 0.96, 0.006)
ΔGCL+IPL InRing V1_Vlast	1.05 (0.92 – 1.19)	0.483	1.07 (0.93 – 1.24)	0.340	Age 0.85 (0.77 - 0.94, 0.001); HDL 0.87 (0.79 - 0.97, 0.015); BMI 0.86 (0.75 - 0.98, 0.025)

CIME					
Ocular markers	**Univariate**		**Multivariate*** HR (95% CI, p)		
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**Significant confounders**
MA turnover	0.96 (0.85 - 1.08)	0.529	0.99 (0.87 - 1.12)	0.827	
MA formation rate	0.71 (0.43 - 1.17)	0.177	0.82 (0.52 - 1.30)	0.398	
MA disappearance rate	1.01 (0.88 - 1.15)	0.934	1.07 (0.87 - 1.19)	0.844	
CRT	1.17 (1.08 - 1.27)	**<0.001***	1.04 (1.02 - 1.07)	**<0.001***	
ΔRT V1_Vlast	1.02 (1.00 - 1.04)	**0.047***	1.03 (1.01 - 1.07)	0.020*	
GCL+IPL CSF thickness	1.18 (1.09 - 1.28)	**<0.001***	1.27 (1.11 - 1.46)	**<0.001***	Systolic BP 0.95 (0.91 - 1.00, 0.038)
ΔGCL+IPL CSF V1_Vlast	0.97 (0.87 - 1.07)	0.525	0.96 (0.85 - 1.09)	0.511	
GCL + IPL InRing	1.05 (0.97 – 1.14)	0.257	1.03 (0.96 – 1.12)	0.393	
ΔGCL+IPL InRing V1_Vlast	1.05 (1.02 – 1.08)	**0.002***	1.05 (1.01 – 1.10)	**0.018***	

Boldface type indicates statistical significance where P , 0.005. CRT: BP, blood pressure; CSF, central subfield; MA, microaneurysm; RT V1_Vlast, retinal thickness visit 1_last visit. *Multivariate analysis adjusted for age, duration of diabetes, sex, HbA1c, total cholesterol, HDL, LDL, triglycerides, systolic blood pressure, and BMI.

ROC curves show that MAT, CRT, GCL+IPL CSF thickness and GCL+IPL InRing are good predictors of the development of CSME (AUC of 0.87, sensitivity 85.7% and specificity 83.4%). For CIME, the predictive value of these markers is higher (AUC of 0.97, sensitivity 90.0% and specificity 91.7%).

Our results show that development of macular edema, either CSME or CIME, and PDR are associated with ocular risk markers such as baseline MAT, CRT and GCL+IPL thickness metrics. They can help better predict the development of complications than systemic markers of metabolic control.

Eyes with mild retinopathy in type 2 diabetes individuals with MAT lower than 6 and with HbA1c measurements below 8% (64 mmol/mol) showed a very low likelihood of developing CSME or PDR (3 of 88; 3%) in a period of five years. On the other hand, an eye with mild retinopathy in a patient with type 2 diabetes, with MAT equal or higher than 6, and with HbA1c equal or above 8% (64 mmol/mol), showed high likelihood of developing CSME and PDR (9 of 25; 36%).

In summary, ocular risk markers (MAT, CRT and GCL+IPL thickness) are good predictors of the development of CSME with an AUC of 0.87. For CIME, the predictive value of the ocular markers is even higher with an AUC of 0.97. When considering CIME, CSME and PDR, the ocular risk markers remain determinant.

Limitations of this study include the fact that the study population is a relatively small and with a small number of eyes that developed the endpoints of interest, possibly because it was a well-controlled group which was selected based on exclusion criteria such as excessive HbA1c levels and uncontrolled blood pressure.

In conclusion, ocular risk markers are more informative than systemic risk markers to predict in eyes of well-controlled patients with mild retinopathy which ones are at risk of developing vision-threatening complications.

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Chapter 5

Multimodal Imaging of the Initial Stages of Diabetic Retinopathy: Different Disease Pathways in Different Patients

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Research Question

In our previous research we have identified 3 different phenotypes of NPDR, with different risk of progression to vision threatening complications and different risk of DR severity worsening.

Such phenotypes are characterized by the predominance of one of three main disease pathways occurring in diabetic retinal disease: neurodegeneration, edema and ischemia.

How are these pathways distributed in different ETDRS severity levels of NPDR? Are the different patterns of disease present in the same patients? What is the relationship between the presence of neurodegeneration, edema and ischemia and the severity of the retinopathy?

In this paper we could reinforce the idea that neurodegeneration is present since the initial stages of DR, even before the development of visible vascular lesions (present in 24% of eyes ETDRS 10-20). Edema and ischemia were also present in this initial stages and were independent of neurodegeneration. The metrics of vessel density, indicating ischemia, were the ones associated with the severity of the ETDRS level. Neither edema nor neurodegenerative changes correlate with the severity of the retinopathy given by ETDRS score, being underestimated if we only consider the ETDRS levels as the severity marker.

Abstract

The objective of this study was to evaluate the prevalence of different disease pathways (ischemia, neurodegeneration and edema) in the initial stages of diabetic retinopathy. In this retrospective cross-sectional study, eyes were grouped by diabetic retinopathy severity using the 7-field Early Treatment Diabetic Retinopathy Study (ETDRS) protocol (levels 10–20, 35, and 43–47). Neurodegeneration was identified by thinning of the retinal nerve fiber layer and/or ganglion cell layer. Edema was identified by thickening of the inner nuclear layer, outer plexiform layer, or full retina. Ischemia was identified by metrics of retinal vessel density. Imaging was performed in 142 eyes from 142 patients (28% women) aged 52–88 years. Vessel density (ischemia) was significantly different between the ETDRS groups ($P < 0.020$). On multivariate regression analysis, it remained significantly different between stages of the disease and showed associations with age ($P < 0.001$), sex ($P = 0.028$), and metabolic control ($P = 0.034$). No significant differences between ETDRS groups were found in retinal thinning (neurodegeneration) or retinal thickness (edema). Eyes with the same ETDRS retinopathy grading from different patients with diabetes showed that the prevalence of different disease pathways varies between patients, even within the same severity group. Ischemia (capillary dropout) is the only disease pathway that shows correlation with retinopathy severity and metabolic control.

Introduction

Diabetic retinopathy (DR) is the leading cause of blindness among working adults in the United States (1). Furthermore, Narayan *et al.* (2) demonstrated that the prevalence of diabetes in the United States is expected to increase dramatically. Our group has proposed three phenotypes of mild non-proliferative DR (NPDR) with different risks for development of vision-threatening complications (3). These phenotypes may be characterized by the predominance of one of three main disease pathways occurring in diabetic retinal disease: neurodegeneration, edema, and ischemia(4).

A forward-looking approach consistent with the concept of personalized medicine would be to develop quantitative assessments of these different disease pathways to enable early and individualized treatment (5).

We therefore used a noninvasive, multimodal imaging approach to examine the prevalence of different disease pathways in the initial stages of the disease. Neurodegeneration can be identified by thinning of the retinal tissue (retinal nerve fiber layer [RNFL] and ganglion cell layer plus inner plexiform layer [GCL–IPL]). Retinal edema may be characterized by increases in retinal thickness in the full retina and in individual layers by using spectral domain optical coherence tomography (SD-OCT). Finally, ischemia may be identified by vessel density metrics using OCT angiography (OCTA).

For this study, we examined eyes of patients with type 2 diabetes showing the initial stages of NPDR. All eyes underwent 7-field color fundus photography for classification by Early Treatment Diabetic Retinopathy Study (ETDRS) criteria, as well as SD-OCT and OCTA to quantify the prevalence of changes in retinal layer thickness and vessel density.

Methods

In this retrospective cross-sectional study of data from an observational study (ClinicalTrials.gov identifier: NCT03010397), participants with diabetes in the initial stages of NPDR were included from February 2016 to April 2018. The tenets of the Declaration of Helsinki were followed, and approval was obtained from the National Ethics Committee for Clinical Research (and Institutional Ethical Review Board). Written informed consent to participate in the study was obtained from all individuals after all procedures were explained. All participants underwent a full ophthalmological examination, SD-OCT and OCTA imaging using Cirrus HD-OCT AngioPlex (Carl Zeiss Meditec, Dublin, CA, USA) and CFP. A total of 142 eyes from 142 patients with diabetes, one eye per patient, were classified into three groups according to the ETDRS severity level: no or minimal NPDR, ETDRS levels 10-20 (54 eyes); mild NPDR, ETDRS level 35 (54 eyes); and moderate NPDR, ETDRS levels 43-47 (34 eyes). The DR severity level was determined by two independent graders within the context of an experienced reading center and was based on the 7-field protocol using the ETDRS classification. The eye showing the more advanced ETDRS grading in any given patient was chosen to be the study eye.

Age, duration of diabetes, and hemoglobin A1c (HbA1c) level, as an indicator of diabetic metabolic control, were collected for each participant from their patient records. Visual acuity (VA) was measured for each eye using the ETDRS protocol and Precision Vision charts at 4 meters.

Exclusion criteria included any previous laser treatment or intravitreal injections, or presence of age-related macular degeneration, glaucoma, or vitreomacular disease and high ametropia (spherical equivalent greater than -6 and +2 DPT), or any other systemic disease that could affect the eye, with special attention for uncontrolled systemic hypertension (values outside normal range: systolic 70-210 mmHg and diastolic 50-120 mmHg) and history of ischemic heart disease. A control group of 106 eyes from healthy control subjects (36 women and 25 men; mean [SD] age 47.6 [12.8] years) was used to compare with the study group.

Thinning and thickening of the retina layers (Neurodegeneration and Edema)

The Macular Cube 512 x 128 acquisition protocol, consisting of 128 B-scans with 512 A-scans each, was used to assess the subjects' central retinal thickness (CRT) and the average thickness value of the GCL-IPL, collected from the standard Cirrus examination reports. Segmentation of retinal layers was performed on the SD-OCT data using a segmentation software implemented by the Association for Innovation and Biomedical Research on Light and Image (AIBILI) (6). Segmentation results were reviewed by a masked grader. None of the eyes in this series of patients were observed

to have cystoid macular edema or disorganization of the retinal inner layers that could have influenced the segmentation analysis.

Eyes with subclinical and clinical macular edema (center-involving macular edema) were identified following the reference values established by the DRCR.net for Cirrus SD-OCT (7,8).

Capillary Dropout (Ischemia)

OCTA data were collected by the AngioPlex device using the Angiography 3x3 mm acquisition protocol. This acquisition protocol consists of a set of 245 clusters of B-scans repeated 4 times, where each B-scan consisted of 245 A-scans over a 3x3x2 mm^3 volume in the central macula. The AngioPlex eye tracking algorithm was used to reduce the effect of eye motion artifacts. Images were evaluated at the time of acquisition for quality, as signal strength greater than 7 was required, with minimal motion artifacts and no evidence of defocus or blur.

Vessel density metrics for the entire 3x3 mm^2 central macular area were computed for the superficial retinal plexus (SRP) and deep retinal plexus (DRP) by a proprietary automated software (Carl Zeiss Meditec, Inc., version 10.0.0.12787). Area and circularity index of the foveal avascular zone (FAZ) for the SRP were also computed using the same software.

Statistical Analysis

Statistical analysis was performed with Stata 12.1 (Stata Corp. LP, College Station, TX, USA), and a P-value ≤ 0.05 was considered to be statistically significant.

Reference values were taken considering the mean and standard deviation (SD) values of the healthy control population taking the patient's sex into consideration.

Changes were considered "absent" if the values were within the normal range of the healthy control groups, "possible" if different by more than 1 SD and "definite" if different from normal values by more than 2 SD.

Differences from the control population were categorized by ETDRS severity groups, and the corresponding 95% CI was computed using the Clopper-Pearson method.

Comparison of the ETDRS groups for categorical outcomes was conducted using the $\chi 2$ test. Continuous outcomes were compared using an ANOVA test. To explore correlations, the Pearson coefficient and the respective significance were computed. ANCOVA of the study outcomes was performed, adjusted for baseline characteristics.

Results

The distribution of characteristics of eyes/patients for each of the stages of the disease is presented in Table 1. No statistically significant differences were found between eyes/patients within different stages of the disease except for HbA1c levels that reflected an association between poorer metabolic control and more severe stages of the disease (Table 1).

Table 1: Distribution of characteristics of eyes/patients considering distinct ETDRS stages of the disease

Characteristic	EDTRS 10-20 (N=54)	EDTRS 35 (N=54)	EDTRS 43-47 (N=34)	p*
Age, y				
Mean (SD) [range]	68.4 (6.3) [53-80]	68.5 (6.3) [56-88]	67.6 (7.9) [52-83]	0.839
Median (IQR)	69 (64-72)	68 (65-73)	68 (64-73)	
Sex, No./Total No. (%) of participants				
Female	19/54 (35.2)	9/54 (16.7)	12/34 (35.3)	
Male	35/54 (64.8)	45/54 (83.3)	22/34 (64.7)	0.058
BCVA, letters				
Mean (SD) [range]	82.1 (5.3) [65-90]	82.3 (4.8) [70-94]	81.0 (6.1) [55-90]	0.507
Median (IQR)	83.5 (80-85)	85 (80-85)	82 (80-85)	
Diabetes duration, y				
Mean (SD) [range]	17.4 (7.5) [7-41]	18.7 (7.6) [1-35]	19.8 (6.6) [6-34]	0.295
Median (IQR)	15.5 (11-20)	19.5 (13-24)	19.5 (16-24)	
Hemoglobin A_{1c}, %				
Mean (SD) [range]	6.9 (0.9) [4.8-9.6]	7.3 (0.9) [4.2-9.5]	8.0 (1.5) [5.7-11.8]	**<0.001**
Median (IQR)	6.9 (6.2-7.3)	7.3 (6.8-7.8)	7.4 (7.0-8.7)	

Abbreviations: BCVA: best-corrected visual acuity; IQR: interquartile range. *P-value for ANOVA model for comparison between ETDRS groups

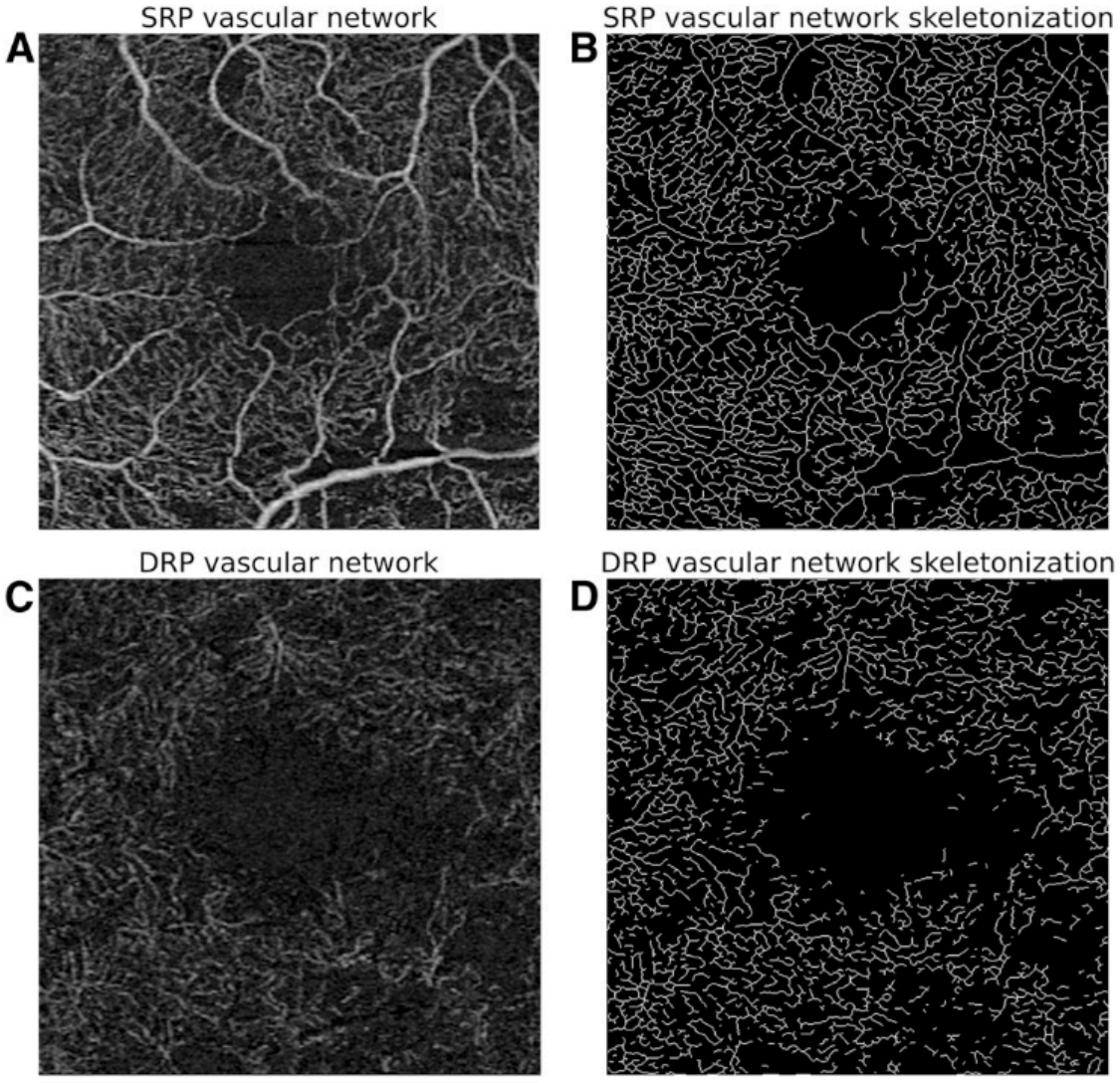
The measurements of neurodegeneration, edema, and capillary dropout for each of the stages of the disease are presented in Table 2.

Table 2: Descriptive statistics of the metrics measured considering the distinct ETDRS stages of the disease

	ETDRS 10–20 (n = 54)			ETDRS 35 (n = 54)			ETDRS 43–47 (n = 34)			
	Mean (SD)	95% CI	Mean (SD) difference from the control group adjusted by sex (%)	Mean (SD)	95% CI	Mean (SD) difference from the control group adjusted by sex (%)	Mean (SD)	95% CI	Mean (SD) difference from the control group adjusted by sex (%)	P*
Vessel density										
SRP (mm^{-1})	19.7 (1.5)	19.3–20.1	−4.7 (7.4)	19.1 (1.6)	18.7–19.5	−8.1 (7.7)	18.4 (1.8)	17.7–19.0	−11.3 (8.8)	**0.001**
DRP (mm^{-1})	15.2 (2.2)	14.7–15.8	−7.9 (14.3)	14.6 (2.3)	14.0–15.2	−13.2 (13.7)	13.8 (2.4)	13.0–14.6	−16.6 (14.3)	**0.020**
FAZ circularity index	0.60 (0.15)	0.56–0.64	−2.6 (25.1)	0.62 (0.13)	0.59–0.66	2.1 (20.9)	0.53 (0.17)	0.47–0.59	−13.7 (28.1)	**0.010**
RNFL thickness (μm)	6.3 (3.2)	5.5–7.2	−14.2 (45.2)	6.5 (3.2)	5.7–7.4	−16.8 (43.2)	6.2 (3.6)	5.0–7.4	−16.3 (51.1)	0.907
Average GCL-IPL thickness (μm)	79.9 (7.9)	77.7–82.0	−3.2 (9.6)	78.2 (7.8)	76.1–80.2	−5.0 (9.2)	80.9 (7.5)	78.4–83.5	−1.9 (9.0)	0.236
INL thickness (μm)	22.3 (6.1)	20.7–24.0	26.9 (33.8)	23.5 (7.2)	21.6–25.4	32.5 (39.5)	24.6 (8.2)	21.8–27.3	39.9 (47.0)	0.334
OPL thickness (μm)	26.5 (5.5)	25.1–28.0	17.2 (24.3)	28.6 (13.6)	24.9–32.2	24.6 (58.9)	26.7 (5.9)	24.8–28.7	18.1 (26.0)	0.487
Full retina thickness (μm)	264.0 (24.0)	257.6–270.4	0.3 (8.7)	271.4 (26.9)	264.2–278.5	2.2 (9.7)	268.5 (25.3)	60.0–277.0	2.0 (8.9)	0.318

*P value for ANOVA model for comparison between the ETDRS groups. The bold P values are statistically significant ($P < 0.05$).

In univariate analysis, vessel density in the SRP and DRP was significantly different between ETDRS groups, with eyes in a more severe stage of the disease more likely to have reduced vessel density (P = 0.001 in the SRP and P = 0.020 in the DRP) (Fig. 1). The FAZ circularity index was also significantly reduced in ETDRS group 43–47 (P = 0.010).



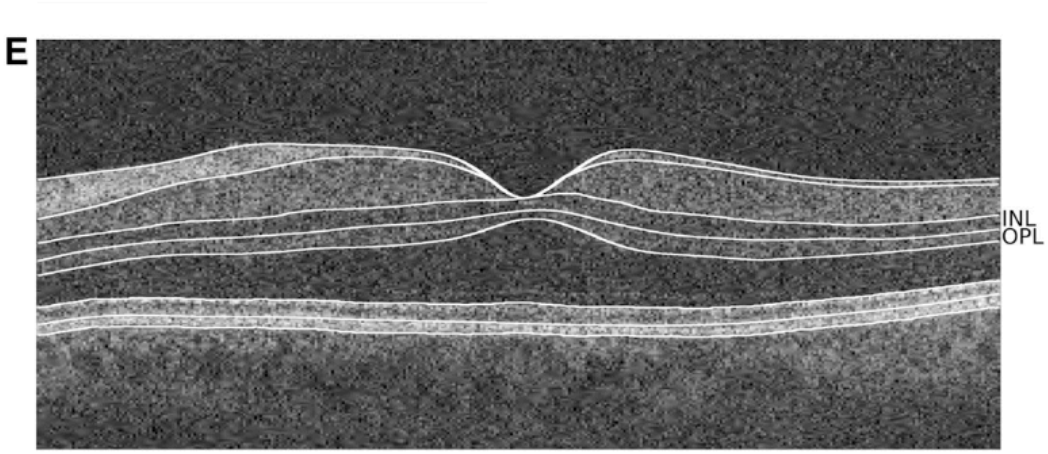


Figure 1: Examples for capillary dropout and edema. a) to d) Superficial Retinal Plexus (SRP) and Deep Retinal Plexus (DRP) images and skeletonization for vessel density measurements, from a 71 year-old male patient of the ETDRS severity level 43-47 group, showing definite capillary dropout on the SRP (17.2 mm^{-1}) and on the DRP (11.4 mm^{-1}). e) A B-Scan in the central macular area, from a 77 year-old male patient of the ETDRS severity level 35 group, with central retinal thickness of 348 μm (center-involving macular edema) showing definite macular thickening in the central subfield for the inner nuclear layer (47.8 μm) and outer plexiform layer (37.0 μm).

No statistically significant differences between these three initial ETDRS groups were found in retina thickness (thinning or increased thickness) (Table 2).

In multivariate regression analysis, considering a model adjusted for age, sex, HbA1c, visual acuity and diabetes duration, vessel density remained significantly different between ETDRS groups and showed significant associations with age (P<0.001), sex (P=0.028) and metabolic control (P=0.034). No significant differences between ETDRS groups were found in RNFL and GCL-IPL thinning (neurodegeneration) or in inner nuclear layer (INL) and outer plexiform layer (OPL) thickness (edema) (Fig. 1).

Analysis of the variables representative of capillary dropout, retinal neurodegeneration, and retinal edema showed a wide range of values in each ETDRS grade, demonstrating that there are very different degrees of capillary dropout, neurodegenerative changes, and edema in eyes from different patients within the same retinopathy grade. The results are summarized in Supplementary tables.

Retinal Thinning (Neurodegeneration)

Definite RNFL or GCL-IPL thinning (2 SD) was found in 24%, 28%, and 21% of the eyes in groups 10–20, 35, and 43–47, respectively (Table 3 and Supplementary Tables).

Table 3: Number of patients with definite capillary dropout (≤2 SD), neurodegeneration (≤2 SD), and edema (≥2 SD) by ETDRS severity groups

		ETDRS 10-20 (n=54)		ETDRS 35 (n=54)		ETDRS 43-47 (n=34)	
		n	**% (95% CI)**	**n**	**% (95% CI)**	**n**	**% (95% CI)**
Capillary Dropout	VD SRP 3x3	9	16.7% (6.7 - 26.6)	14	25.9% (14.2 - 37.6)	17	50.0% (29.9 - 62.0)
	VD DRP 3x3	11	20.4% (9.6 - 31.1)	21	38.9% (25.9 - 51.9)	13	38.2% (21.9 - 54.6)
	VD SRP 3x3 or VD DRP 3x3	12	22.2% (11.1 - 33.3)	24	44.4% (31.2 - 57.7)	20	58.8% (42.3 - 75.4)
	FAZ circularity	4	7.4% (0.4 - 14.4)	2	3.7% (0 - 8.7)	6	17.6% (4.8 - 30.5)
Neurodegeneration	RNFL Thinning	5	9.3% (1.5 - 17.0)	6	11.1% (2.7 - 19.5)	3	8.8% (0 - 18.4)
	GCL-IPL Thinning	10	18.5% (8.2 - 28.9)	11	20.4% (9.6 - 31.1)	4	11.8% (0.9 - 22.6)
	RNFL or GCL-IPL Thinning	13	24.1% (12.7 - 35.5)	15	27.8% (15.8 - 39.7)	7	20.6% (7.0 - 34.2)
Edema	INL Thickness	13	24.1% (12.7 - 35.5)	14	25.9% (14.2 - 37.6)	11	32.4% (16.6 - 48.1)
	OPL Thickness	7	13.0% (4.0 - 21.9)	10	18.5% (8.2 - 28.9)	5	14.7% (2.8 - 26.6)
	Full RT	44	7.4% (0.4 – 14.4)	4	7.4% (0.4 – 14.4)	4	11.8% (0.9 - 22.6)
	INL or OPL Thickness	19	35.2% (22.4 - 47.9)	19	35.2% (22.4 - 47.9)	13	38.2% (21.9 - 54.6)

Retinal Thickening (Edema)

Increases in retina thickness were predominantly located in the INL or in the OPL. Definite increases were noted in 35% (19 of 54), 35% (19 of 54), and 38% (13 of 34) for the ETDRS groups 10–20, 35, and 43–47, respectively. These increases were significantly correlated with increases in retinal thickness in the full retina (INL: r=0.77; P<0.001; OPL: r=0.48; P<0.001) (Table 3 and Supplementary Tables).

Retinal Vessel Density (Ischemia)

When analyzing the data for the three ETDRS severity groups, definite (2 SD) decreases in retinal vessel density were present in 22% (12 of 54) of the eyes in ETDRS group 10–20, in 44% (24 of 54)in ETDRS group 35, and in 59% (20 of 34) in ETDRS group 43–47 (Table 3 and Supplementary Tables). The vessel density in the SRP showed a strong correlation with the vessel density in the DRP (r=0.77; P<0.001) and a weakly positive correlation with FAZ circularity (r=0.23; P=0.006).

Correlations between Retinal Vessel Density, Retinal Thinning and Retinal Edema

When we examined the data for correlations between the parameters that represent capillary dropout, retinal thinning, and edema, no association was found between these different alterations.

Discussion

Analysis of the initial stages of diabetic retinal disease shows retinal neurodegeneration in one-quarter of patients. It appears to occur independently of the presence of edema and capillary dropout (9). Edema is present in approximately one-third of the eyes, possibly associated with an alteration of the blood-retinal barrier in the DRP (10,11). Capillary dropout, demonstrated by decreased vessel density occurring in both SRP and DRP, correlates with increases in severity of DR, as judged by ETDRS criteria. It may provide major clinical value by indicating the eyes at increased risk for progression to more severe stages of retinopathy. Previous reports by our group have indicated that metrics of vessel density detected changes in DR earlier in SRL than DRL (12). However, this study shows that the DRL is involved as much as the SRL. Correlation with severity of retinopathy was also found with HbA1c, but not with age and sex, confirming that metabolic control is relevant for retinopathy progression.

Our findings may have major implications for the management of DR. Our observation that different eyes in the same ETDRS severity level show different predominant disease pathways confirms previous studies suggesting that eyes from different patients may have different phenotypes of disease progression (13).

This study shows that there are eyes with retinal neurodegeneration present from the earliest stages that do not show evidence of edema or ischemia. Other eyes show the presence of edema without neurodegeneration and in the absence of ischemia. Finally, there are eyes with evidence of ischemia identified by decreased vessel density, without neurodegeneration and edema. This phenotype, characterized by the presence of ischemia, appears to be the only one associated with increase in retinopathy severity.

One limitation of this study is the use of automated retinal layer segmentation analyses. These, however, were performed in retinas that remained structurally preserved with no evidence of cystoid changes, and they were reviewed by a masked grader.

In conclusion, eyes in the initial stages of diabetic retinal disease may show neurodegeneration, edema and decreases in vessel density. These changes, however, occur to different degrees in different eyes, indicating that the predominant mechanism of retinal disease may be different in different patients. Retinal thinning is an early finding that appears to occur independently of the presence of edema or capillary dropout. Edema may also be identified at the earliest stages of diabetic retinal disease, but its prevalence does not correlate with severity of the disease. Finally, decreases in vessel density involving both the DRP and SRP appear to be the only feature that shows clear association with increase in retinopathy severity, as identified by ETDRS level. Our findings support the concept that capillary dropout is a critical process in DR (14).

Metrics of retinal vessel density obtained with OCTA in a noninvasive manner, allowing repeated examinations, appear to identify the eyes/patients at a higher risk for increase in severity, thus promoting a larger role for precision medicine in the management of DR in individual patients.

Supplementary Table 1: Results for the ETDRS group 10-20. The ⇊ (⇈) corresponds to the case when the values of the metrics were lower (higher) when compared to the mean value of the healthy controls population minus (plus) two standard deviations (SD). The ⇓ (⇑) corresponds to the value of the metric minus (plus) more than one SD when compared to the mean value of the healthy control population. The ↓ (↑) corresponds to the case when the value is lower (higher) when compared to the mean of the control population. For the full thickness values the framed values correspond to cases with subclinical macular edema.

Id	Age [y]	Gender	ETDRS Level	HbA1C [%]	Visual Acuity (letters)	Diab. Dur. [y]	VD 3x3 SRP [mm^{-1}]	VD 3x3 DRP [mm^{-1}]	FAZ Circularity	RNFL Thick [µm]	Zeiss Avg.GCL+IPL [µm]	Ret. Thick [µm]	INL Thick [µm]	OPL Thick [µm]
1	78	M	20	8.3	80	14	⇊	⇊	⇈	↓	↑	257	↑	↑
2	76	M	10	7.2	80	17	↓	⇑	⇊	⇓	⇑	**306**	⇈	↑
3	74	M	10	7.2	85	10	⇊	⇊	↓	⇓	↑	284	⇈	↑
4	62	M	14A	7.8	85	15	⇓	⇊	↑	⇓	↑	228	↑	↓
5	78	M	15	6.9	80	10	⇊	⇊	↑	⇓	⇓	239	↑	⇑
6	65	M	15	4.8	90	16	↑	↓	⇈	↓	↑	300	⇈	⇑
7	73	M	12	6	80	8	⇊	⇓	⇑	↓	⇓	289	⇈	↑
8	70	M	10	7.3	70	11	↓	↓	⇑	⇊	↑	268	⇑	↑
9	75	M	10	7	83	20	⇓	↓	⇓	↑	⇈	280	⇑	↑
10	76	M	20	5.8	83	21	⇓	⇓	↑	↓	⇓	265	⇑	⇈
11	76	F	10	6.4	75	16	↑	↓	↑	↑	⇊	**297**	↑	↑
12	72	M	15	6.5	65	29	⇓	↓	⇓	⇓	⇊	268	↑	↑
13	70	M	20	7.3	66	27	↓	↓	⇑	↓	⇓	271	⇑	⇑
14	69	M	20	6.8	80	18	↑	⇑	⇈	↓	⇑	281	⇑	⇑
15	61	M	20	7.7	90	10	↑	↑	↓	⇑	↓	288	⇈	⇑
16	69	M	10	6	85	24	↑	↓	↓	↓	⇓	281	⇈	↑
17	65	M	10	6.4	88	41	⇓	↓	↑	↓	↓	280	⇈	⇈
18	67	F	20	6.1	85	7	↓	⇓	↑	↓	↑	226	⇓	⇓
19	65	M	10	6.5	90	13	↓	⇓	⇈	↓	⇑	257	↑	⇑
20	64	M	10	5.6	79	15	↑	↓	↓	⇑	↑	275	↑	↓
21	64	M	14B	6.1	85	10	↑	↑	⇑	⇑	↓	282	⇑	↓
22	72	M	15	6	80	13	↓	⇓	↑	⇊	⇊	276	⇑	↑
23	54	F	12	7.2	85	11	⇑	⇑	⇓	↓	⇑	261	↑	⇈
24	70	F	12	6.9	80	15	↑	↑	↑	↓	⇊	249	↑	↑
25	80	M	10	7.8	85	29	⇊	⇊	↑	↑	↑	221	↓	↓
26	74	M	15	6.8	86	15	⇊	⇊	↓	⇓	⇊	230	↑	⇑
27	60	M	15	5.8	90	15	↓	⇓	⇑	↑	⇈	265	↑	↑
28	66	M	10	7.8	80	19	⇓	⇊	⇑	↓	⇓	270	⇈	↑
29	63	M	20	8.1	85	10	↑	↓	⇈	⇊	⇑	250	↑	⇑
30	62	M	10	5.7	80	8	⇓	⇓	⇑	⇊	↑	260	⇑	⇈
31	68	M	10	9.6	85	19	↓	⇓	↑	⇊	⇊	273	⇑	↑

Id	Age [y]	Gender	ETDRS Level	HbA1C [%]	Visual Acuity (letters)	Diab. Dur. [y]	VD 3x3 SRP [mm^{-1}]	VD 3x3 DRP [mm^{-1}]	FAZ Circularity	RNFL Thick [µm]	Zeiss Avg.GCL+IPL [µm]	Ret. Thick [µm]	INL Thick [µm]	OPL Thick [µm]
32	67	M	15	7.1	85	14	↓	↓	↑	↑	↓	256	↑	↓
33	66	M	20	6.7	80	20	↓	↓	↓	↑	↓	**311**	↑↑	↑
34	77	M	15	7.8	70	19	↓	↓	↓↓	↑	↓	**307**	↑↑	↑
35	71	M	20	8.7	85	10	↓	↓↓	↓	↓	↑	247	↑	↑
36	71	F	20	7.2	84	20	↓↓	↓↓	↑	↓	↓	264	↑	↑
37	72	M	15	6.2	85	8	↓	↓	↓	↑	↓	285	↑	↑
38	58	F	10	7	80	13	↑	↑	↑	↓	↓	241	↓	↑↑
39	68	M	15	7.5	76	30	↓↓	↓↓	↓	↓	↓↓	271	↑↑	↓
40	68	F	10	6.5	85	18	↓	↓	↓	↑	↓↓	259	↑	↑↑
41	64	F	10	6.9	85	25	↑	↑	↑	↑↑	↑	274	↑	↑
42	70	M	10	6.7	83	20	↓↓	↓↓	↓↓	↓	↓↓	259	↑	↓
43	71	F	10	7.4	80	35	↓	↓	↓	↓	↓	215	↓	↓
44	63	F	15	6.7	80	11	↓	↑	↓	↓	↓	259	↑	↑
45	79	F	15	5.9	85	19	↓	↓	↓	↑	↓↓	288	↑↑	↑
46	77	F	10	6.4	80	8	↓	↓	↑	↓	↓	241	↑	↑
47	62	F	15	7	85	17	↓	↑	↑	↓	↓	255	↑	↑
48	71	F	10	6.9	85	24	↓	↓	↑	↓	↑	263	↑	↓
49	70	F	15	6.9	85	13	↓	↓	↓	↓	↑	238	↓	↑↑
50	60	F	20	8.5	80	34	↑	↑	↑	↓	↓	212	↓	↓
51	58	M	10	6.2	80	25	↓	↓	↑	↓	↓	264	↑	↑
52	53	F	20	6.2	85	19	↑	↑↑	↓	↑	↑	287	↑↑	↑
53	71	F	10	6.1	83	14	↓↓	↓↓	↓↓	↑	↓	210	↓	↓
54	67	F	12	6.6	85	15	↑	↑	↓	↓	↑	271	↑	↑

Supplementary Table 2: Results for the ETDRS group 35. The ↓↓ (↑↑) corresponds to the case when the values of the metrics were lower (higher) when compared to the mean value of the healthy control population minus (plus) two standard deviations (SD). The ↓ (↑) corresponds to the value of the metric minus (plus) more than one SD when compared to the mean value of the healthy population. The ↓ (↑) corresponds to the case when the value is lower (higher) when compared to the mean of the control population. For the full thickness values the framed values correspond to cases with subclinical macular edema and with bold to clinical macular edema.

Id	Age [y]	Gender	ETDRS Level	HbA1C [%]	Visual Acuity (letters)	Diab. Dur. [y]	VD 3x3 SRP [mm^{-1}]	VD 3x3 DRP [mm^{-1}]	FAZ Circularity	RNFL Thick [µm]	Zeiss Avg.GCL+IPL [µm]	Ret. Thick [µm]	INL Thick [µm]	OPL Thick [µm]
1	67	M	35C	6.9	85	16	↓	⇓	↓	↑	⇓	288	⇑	⇑
2	63	M	35C	5.3	85	15	⇑	↓	↓	↓	↓	269	⇑	↑
3	68	M	35C	6.8	82	17	⇓	⇓	↑	⇓	↑	289	⇑	↑
4	66	M	35C	7.8	85	29	⇓	⇓	⇓	↑	↑	268	↑	⇑
5	56	M	35C	8.3	83	24	⇓	⇓	↓	↑	↓	260	↓	⇓
6	69	M	35C	8.5	80	12	↓	↓	↑	↑	↓	253	↑	↓
7	69	M	35C	8.4	85	22	⇓	⇓	↑	⇓	⇓	268	↑	↑
8	74	M	35C	6.7	80	18	⇓	⇓	↑	↑	⇓	290	⇑	↓
9	65	M	35C	7	90	22	⇓	⇓	↓	↓	⇓	293	⇑	↑
10	65	M	35F	8	85	8	↑	↓	⇑	⇑	⇓	277	⇑	⇑
11	71	M	35C	6.9	75	31	⇓	⇓	⇑	↓	⇓	289	⇑	⇑
12	61	M	35C	8.6	85	18	⇓	↓	↑	⇓	⇓	257	⇑	⇑
13	76	M	35E	6.8	87	20	⇓	⇓	⇓	⇓	⇓	265	↑	↓
14	73	F	35E	6.7	85	16	↓	↑	↑	↑	↑	259	↓	↓
15	65	M	35C	7.1	94	25	↑	↑	⇑	⇑	⇑	302	⇑	↑
16	71	M	35C	7.5	85	26	↑	⇑	↑	↑	↓	281	⇑	⇑
17	70	M	35C	6.5	90	31	↓	↓	⇑	⇓	↓	287	⇑	↑
18	78	M	35D	6.2	85	15	⇓	⇓	↑	↑	↓	260	↑	⇑
19	67	M	35C	8.2	80	29	⇓	⇓	⇑	↓	⇓	255	↑	⇑
20	68	M	35D	7.1	75	20	⇓	⇓	⇑	⇓	⇓	245	⇑	↓
21	61	M	35D	7.2	85	26	⇓	⇓	⇑	⇓	↓	260	↑	↓
22	69	M	35D	6.8	85	12	↓	⇓	↑	⇓	↑	239	↓	↑
23	68	M	35C	6.6	85	20	⇓	↓	⇑	↓	↑	257	↓	↓
24	69	F	35E	6.8	81	20	↓	↓	⇓	↑	⇑	272	⇑	↑
25	88	M	35E	8.7	-	34	⇓	⇓	↓	⇓	↑	280	⇑	⇑

Id	Age [y]	Gender	ETDRS Level	HbA1C [%]	Visual Acuity (letters)	Diab. Dur. [y]	VD 3x3 SRP [mm^{-1}]	VD 3x3 DRP [mm^{-1}]	FAZ Circularity	RNFL Thick [µm]	Zeiss Avg.GCL+IPL [µm]	Ret. Thick [µm]	INL Thick [µm]	OPL Thick [µm]
26	57	M	35C	7.6	85	10	⇓	↑	⇑	⇑	↓	**325**	⇑⇑	⇑⇑
27	77	M	35D	6.8	80	27	⇓	↓	⇓⇓	↓	⇓	283	⇑⇑	↓
28	66	M	35C	6.5	83	13	⇓	⇓⇓	⇑	↑	↓	256	↓	↓
29	65	M	35C	7.1	85	20	⇓⇓	↓	↑	↑	↑	**320**	⇑⇑	⇑⇑
30	58	M	35C	7.3	80	20	⇓	↓	↑	⇓	⇓	241	↓	↓
31	72	M	35C	6.3	80	16	⇓⇓	⇓⇓	↑	⇓	↓	271	⇑	↓
32	69	M	35C	7.8	80	9	⇓	⇓	⇑	⇓⇓	⇓	262	↑	↓
33	72	M	35C	7.3	75	7	⇓⇓	⇓⇓	⇓	⇓	⇓⇓	265	⇑	↓
34	76	M	35C	8.9	80	16	⇓⇓	⇓⇓	⇓	⇓	⇓	240	↑	⇑⇑
35	76	M	35C	8	70	25	⇓	⇓	⇓	⇓	↓	274	↑	⇑
36	64	F	35C	7.4	80	10	↑	⇑	↑	↑	⇓	273	⇑	↓
37	77	M	35C	6.8	85	29	↓	↑	↑	↑	↑	**348**	⇑⇑	⇑⇑
38	69	F	35C	7.5	78	12	⇓	⇓	↑	↓	⇓	228	↓	↑
39	63	M	35C	7.3	85	15	↓	⇓	↓	⇓	↓	266	⇑	↑
40	73	M	35C	7.6	80	20	↑	↑	↓	↓	↑	301	⇑	⇑
41	68	F	35C	7.5	85	20	⇓	⇓⇓	↑	↓	↑	244	↑	⇑⇑
42	57	F	35D	7.7	85	1	↓	↓	⇑	⇑	↓	254	↑	↑
43	56	F	35F	6.3	85	12	↓	⇓	⇑	↓	⇑	264	↑	↑
44	74	M	35C	7.8	-	23	⇓⇓	⇓⇓	⇑⇑	⇓⇓	⇓⇓	227	⇓	⇓
45	77	M	35C	6.8	80	24	⇓⇓	⇓⇓	↓	⇓	⇓⇓	261	↑	↑
46	67	M	35C	4.2	80	3	↓	↓	↓	↑	⇓	301	⇑⇑	↓
47	76	M	35C	9	70	35	↓	↓	↑	↓	↑	238	↓	↑
48	68	F	35C	7.7	85	9	↓	↓	↓	⇑	↑	246	↓	↑
49	66	M	35C	7.6	85	20	↓	⇑	↑	↓	↓	276	⇑⇑	⇑
50	60	M	35C	8.5	85	19	⇓⇓	⇓	⇓	⇓	↓	255	↑	⇑
51	74	F	35C	7	80	10	⇓	⇓⇓	↓	⇓	↑	258	↑	⇑⇑
52	70	M	35C	9.5	70	17	⇓	↓	⇑⇑	↓	⇓⇓	283	↑	↑
53	66	M	35D	8.1	84	16	↓	⇓⇓	⇑	↓	⇓⇓	264	⇑	⇑⇑
54	68	M	35D	7.2	85	25	⇓	⇓⇓	↓	↓	⇓	**366**	↑	⇑⇑

Supplementary Table 3: Results for the ETDRS group 43-47. The ↓↓ (↑↑) corresponds to the case when the values of the metrics were lower (higher) when compared to the mean value of the healthy controls population minus (plus) two standard deviations (SD). The ↓ (↑) corresponds to the value of the metric minus (plus) more than one SD when compared to the mean value of the healthy control population. The ↓ (↑) corresponds to the case when the value is lower (higher) when compared to the mean of the control population. For the full thickness values the framed values correspond to cases with subclinical macular edema.

Id	Age [y]	Gender	ETDRS Level	HbA1C [%]	Visual Acuity (letters)	Diab. Dur. [y]	VD 3x3 SRP [mm-1]	VD 3x3 DRP [mm-1]	FAZ Circularity	RNFL Thick [µm]	Avg.GCL+IPL Zeiss [µm]	Ret. Thick [µm]	INL Thick [µm]	OPL Thick [µm]
1	67	F	43A	6.6	82	12	↓	↓	↑	↓	↑	223	↓	↓
2	73	M	43A	7	75	20	↓↓	↓↓	↑	↓	↓	273	↑	↑
3	68	M	43A	9.7	85	22	↓	↓	↑	↓	↓	265	↑↑	↑
4	67	M	43A	7	82	22	↓	↓↓	↓↓	↓	↑	248	↑	↑↑
5	60	M	47A	6.6	-	10	↓	↓	↓	↑	↓	313	↑	↑
6	56	M	43B	7.1	85	17	↑	↓	↑	↓	↑	261	↑	↑
7	74	F	43A	6.4	80	8	↓	↓	↓	↓	↑	276	↑	↑
8	83	F	43A	8.4	80	28	↓↓	↓	↓	↑	↓	251	↑	↑
9	68	M	43A	6.8	80	16	↓↓	↓	↓↓	↑	↓	269	↑	↑
10	68	F	43A	8.8	75	21	↓↓	↓↓	↑	↑	↓	232	↓	↑
11	54	M	43A	7	85	6	↑	↑	↓	↓	↑↑	294	↑	↑
12	52	M	47A	9.8	87	15	↓	↓	↑	↓	↓	252	↑	↓
13	67	M	43A	7.4	80	15	↓	↓	↓	↓	↓	300	↑↑	↑
14	70	M	43A	9.9	55	19	↓	↓	↓	↓	↓	308	↑↑	↑
15	67	M	43A	7.9	82	27	↓	↓↓	↑	↓	↓	280	↑	↑
16	75	M	47D	8.1	80	27	↓↓	↓	↓↓	↓↓	↓	260	↑↑	↑
17	64	M	43A	5.7	85	24	↓	↓↓	↓	↓↓	↑	249	↑	↑
18	65	M	43A	7.1	85	23	↓	↑	↓	↑	↑	315	↑↑	↑↑
19	68	M	43A	8.7	85	17	↓↓	↓↓	↓	↓	↓	255	↑	↑
20	70	M	43A	7.1	80	19	↓↓	↓	↓	↓	↓↓	297	↑↑	↑
21	77	M	43A	6.8	80	34	↓↓	↓↓	↑	↓↓	↑	299	↑↑	↑
22	76	F	43A	8.4	80	30	↓	↓	↑	↓	↓	243	↑	↑
23	64	M	47A	11.8	85	18	↓↓	↓↓	↑	↑	↑	243	↑	↓
24	83	F	43A	6.9	85	21	↓	↓	↓↓	↓	↑	261	↑↑	↑↑
25	69	M	43A	7.3	75	23	↓↓	↓↓	↓	↑	↓	287	↑↑	↑
26	58	F	43A	11.8	85	31	↓↓	↓↓	↓	↓	↑	257	↑	↓
27	72	F	47A	8.2	85	20	↓↓	↓↓	↑	↓	↓↓	257	↑	↑
28	68	M	43A	7.4	75	17	↓↓	↓	↓	↓	↓	267	↑	↓
29	71	M	43A	10.6	85	19	↓↓	↓↓	↓	↑	↓↓	271	↑	↑↑
30	59	M	47A	8.2	90	16	↓	↓	↓	↑	↑	271	↑	↑
31	74	F	43A	7.3	80	28	↓↓	↓	↓	↑	↑	273	↑↑	↑
32	54	F	43B	7.4	85	14	↑	↑	↓	↓	↑	240	↑	↓
33	59	F	47C	10.4	80	11	↓↓	↓	↓↓	↓	↑↑	314	↑↑	↑↑
34	80	F	43A	6.6	75	24	↓↓	↓↓	↓↓	↑	↓↓	226	↓	↓

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Chapter 6

Characterization of disease progression in the initial stages of retinopathy in diabetes type 2. A two-year longitudinal study.

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Research Question

In the previous paper we have characterized a cohort of the population according to the presence of the three main disease pathways occurring in diabetic retinal disease: neurodegeneration, edema and ischemia and described their distribution in different ETDRS severity levels of NPDR.

We reported that ischemia was the only disease pathway that showed correlation with retinopathy severity and metabolic control.

What is the predictive value of these structural changes in the development of DR complications? How is the evolution of these 3 pathways in a two-year period? Are the 3 pathways related to each other and to the DR ETDRS severity changes?

In this prospective study capillary drop out showed a good correlation with the ETDRS level since the baseline visit, while edema and neurodegeneration did not. We verify that, in a two-year period, very few patients increased the DR ETDRS severity score by 2 steps, the limit accepted as clinically meaningful. However, vessel density metrics demonstrating capillary drop out increased in 35% of the eyes in the same period. Edema and neurodegenerative alterations remained stable during the 2 years of follow up.

Capillary dropout present a better detection capacity of DR progression when compared to ETDRS worsening and will likely be an informative biomarker of DR progression.

Abstract

Purpose: To characterize two-year changes occurring in neurodegeneration, edema and capillary dropout in nonproliferative diabetic retinopathy (NPDR)

Methods: Two-year prospective longitudinal observational cohort of eyes/patients with diabetes type-2 using spectral domain-optical coherence tomography (SD-OCT) and optical coherence tomography angiography (OCTA). Eyes were examined three times with intervals of one year. Thickness of the full retina and layer by layer measurements were used to identify edema or neurodegeneration. OCTA vessel density maps of the retina were used to identify capillary dropout. ETDRS classification was performed using the 7 field ETDRS protocol.

Results: A total of 62 eyes from 62 patients with diabetes were followed for two years. After verification for image quality, a total of 44 eyes from 44 patients (30% women) aged 52-80 years were retained for data analysis. There were 18 eyes with ETDRS grades 10-20, 17 eyes with ETDRS grade 35 and 9 eyes with ETDRS grades 43-47. During the 2-year follow-up period there was a progressive increase in capillary dropout, whereas edema and neurodegeneration remained stable. In multivariate analysis, considering a model adjusted for age, gender, HbA1c, visual acuity and diabetes duration, vessel density remained significantly different between DRSS groups (Wilks' lambda=0.707; p=0.015) showing association with disease progression.

Conclusions: Capillary dropout increased in a period of 2-years in eyes with minimal, mild and moderate DR, whereas the presence of edema and neurodegeneration remained stable.

Introduction

Optical coherence tomography (OCT) is a noninvasive imaging modality that allows detailed structural visualization of the retina. Optical coherence tomography angiography (OCTA) is a functional extension of OCT that detects motion or blood flow contrast. OCTA imaging has emerged as a noninvasive strategy to visualize the retinal and choroidal microvasculature without the use of an intravenous dye injection (1,2). It offers the possibility of quantification of features of interest. In particular, vessel density in the macula and the size of the foveal avascular zone (FAZ) are known to be affected by presence of diabetic retinopathy (DR) (2), allowing to discriminate between different eyes of different patients and correlate with clinically relevant measures such as stage of disease and visual acuity (3,4). Structural OCT has also shown in diabetic eyes the presence of neurodegeneration evidenced by thinning of the retinal nerve fiber layer (RNFL) or ganglion cell and inner plexiform layers (GCL-IPL) and the occurrence of edema evidenced by full retina thickening and localized thickening of the inner nuclear layer (5,6). OCT and OCTA offer, therefore, the possibility of quantifying the occurrence of retinal neurodegeneration, retinal edema and capillary dropout (reduced vessel density), in the initial stages of DR. The fundus abnormalities seen in DR can be split into three categories: those findings resulting from increased apoptosis (neurodegeneration); those findings resulting from leaking microvasculature (retinal edema, hemorrhages) and those findings resulting from microvascular changes (decreased perfusion, ischemia) (7–9).

In a recent study we have shown that the prevalence of different disease pathways varies between patients, even within the same severity group. Ischemia (capillary dropout) was the only disease pathway that showed correlation with retinopathy severity and metabolic control (10).

For this analysis, we examined eyes of patients with diabetes type 2 and initial NPDR three times with intervals of one-year (baseline, one-year and two-year visits). All eyes underwent 7-field fundus photography for ETDRS classification as well as spectral domain OCT (SD-OCT) and OCTA, looking for the occurrence of microvascular alterations, retinal neurodegeneration or retinal edema. Presence of these alterations and their progression were analyzed during a 2 year period.

Methods

In this prospective longitudinal observational cohort (ClinicalTrials.gov identifier: NCT03010397) participants with diabetes with initial stages of NPDR were analyzed from January 2016 to November 2018. The tenets of the Declaration of Helsinki were followed, and the approval was obtained from the National Ethics Committee for Clinical Research (and Institutional Ethical Review Board) and written informed consent to participate in the study was obtained from all individuals after all procedures were explained. An age-matched population of individuals without diabetes or other retinal diseases were used as controls including 84 eyes (45 women and 39 men; mean [SD] age, 69.2 [4.5] years).

All participants underwent a full ophthalmological examination, SD-OCT and OCTA imaging. Seven-field color fundus photography (CFP) was performed on diabetic participants for DR severity grading.

Exclusion criteria included any previous laser treatment or intravitreal injections, or presence of age-related macular degeneration, glaucoma, or vitreomacular disease and high ametropia (spherical equivalent greater than -6 and +2 DPT), or any other systemic disease that could affect the eye, with special attention for uncontrolled systemic hypertension (values outside normal range: systolic 70-210 mmHg and diastolic 50-120 mmHg) and history of ischemic heart disease. Eyes with central thinning and values identifying center-involved macular edema (CIME) but without any evidence of cysts and no indication for treatment were included.

Age, duration of diabetes, hemoglobin A_{1C} (HbA1c) and blood pressure level were collected for each participant from their patient records. The DR severity level was determined within the context of an experienced reading center and was based on the 7-field protocol using the ETDRS classification. Visual acuity (VA) was measured for each eye using the ETDRS protocol and Precision Vision charts at 4 meters. A total of 62 eyes from 62 patients with diabetes were examined three times with intervals of one-year (baseline, one-year and two-year visits). The eyes were classified in three groups according to their DR ETDRS severity scale (DRSS): 10-20, 35 and 43-47. Of these eyes, only 44 eyes from 44 patients with diabetes, passed the quality checks established for the three points of data collection performed at one-year intervals. For data analysis there were 18 eyes/patients with level 10-20, 17 eyes/patients for level 35 and 9 eyes/patients for level 43-47.

Thinning and thickening of the retina layers (Neurodegeneration and Edema)

The Macular Cube 512x128 acquisition protocol, consisting of 128 B-scans with 512 A-scans each, was used to assess the subjects' central retinal thickness (CRT) and the average thickness value of the GCL-IPL collected from the standard Cirrus examination reports.

CRT was used to identify eyes with (sub)clinical macular edema following the reference values established by the DRCR.net for Cirrus SD-OCT (11,12). Clinical macular edema is defined as retinal thickness (RT) greater than or equal to 290 μm in women and greater than or equal to 305 μm in men and subclinical macular edema is defined as RT between 260 μm and 290 μm in women and between 275 μm and 305 μm in men.

Retinal layers segmentation for layer thickness calculation was performed on the structural OCTA using the segmentation software implemented by AIBILI (Santos T et al. IOVS 2015; 43: ARVO E-Abstract 5953). Automated analysis results were reviewed by a masked grader.

RNFL and/or GCL-IPL thickness decreases were considered to identify neurodegeneration(13) whereas full retina thickness increases were considered to identify edema (6).

Capillary Dropout (Ischemia)

OCTA data were collected by the CIRRUS™ HD-OCT 5000 with AngioPlex® OCT Angiography (Carl Zeiss Meditec, Dublin, CA, USA) device using Angiography 3x3 mm^2 acquisition protocol. To calculate the perfusion density and the vessel density a thresholding algorithm was applied to the superior capillary plexus (SCP), deep capillary plexus (DCP) an full retina (FR) *en-face* images to create a binary slab that assigns to each pixel a 1 (perfused) or 0 (background). From this slab a skeletonized slab was created, representing vessels with a trace of 1 pixel in width. We define the perfusion density as the total area of perfused vasculature per unit area in a region of measurement, calculated by taking the mean of the binary slab within a desired region of interest. We define the vessel density as the total length of perfused vasculature per unit area in a region of measurement. A similar length-based metric has been used as a measurement of road density(4). We calculate the vessel density by taking the mean of the skeletonized slab within a desired region of interest and scaling the result by the distance between pixels (in this case, 245 pixels per 3 mm). The mean of skeletonized slab is only a first-order estimate of the length of perfused vasculature. The Angiography 3x3 mm^2 acquisition protocol consists of a set of 245 clusters of B-scans repeated 4 times, where each B-scan consists of 245 A-scans over a 3x3x2 mm^3 volume in the central macula. The CIRRUS eye tracking algorithm was used to reduce the effect of eye motion artifacts. For quality check all OCTA acquisitions were reviewed by a masked grader. Only eyes that had OCTA examinations with signal strength greater or equal to 7, minimal motion artifacts and no evidence

of defocus or blur in the three examinations were included in this analysis. This is particularly relevant in a longitudinal study where data from different examinations are compared. From the total number of scans examined, 17% were excluded. As a result, from the total number of patients followed in the study 29% were excluded from the data analysis because they did not meet the set of quality criteria in the three examinations leading to the final number of 44 patients that fulfilled the quality criteria in the three examinations.

Vessel density metrics for the entire 3x3 mm^2 central macular area were computed for the SCP, DCP and FR by the automated Carl Zeiss Density Exerciser software (version 10.0.0.12787). Area and circularity index of the FAZ for the SCP are also computed using the same software. The FAZ circularity index follows the $4\pi A/P^2$ ratio, with A being the area and P the perimeter. To account for potential projection artifacts, particularly when examining the DCP, we also used vessel density metrics of the FR.

Capillary dropout was therefore identified by decreased vessel density measured in the SCP, DCP and FR.

Statistical Analysis

Statistical analysis was performed with Stata 12.1 (Stata Corp. LP, College Station, TX, USA), and a p-value ≤ 0.05 was considered statistically significant.

Reference values were taken considering the mean and standard deviation (SD) values of the healthy control population taking the patient's gender into consideration.

Variables were summarized for each DRSS group, 10-20, 35 and 43-47, using the following statistics: mean, standard deviation, confidence interval and normalized mean (standard deviation) difference from the control adjusted by gender.

To measure the association between two categorical variables the Chi-square test was used. Continuous variables were compared between groups using the ANOVA test. To explore correlations, the Pearson's coefficient and the respective significance were computed. Mann-Whitney U-tests were performed to assess the statistical significance difference between the measurements of healthy controls and diabetic patients, while to perform similar assessment within the same group of patients for the different visits the Wilcoxon signed rank test was used.

ANOVA and MANOVA was used to perform multivariate analysis to test associations between the study features and progression in disease severity adjusting for baseline characteristics

Results

The demographic and baseline systemic and ocular characteristics of eyes/patients for each of the stages of the disease are presented in Table 1 and 2, respectively. No statistically significant systemic differences were found between eyes/patients within different ETDRS stages of the disease (Table 1).

Table 1: Baseline Characteristics for Patients' Systemic Data Considering the Distinct DRSS Stages of the Disease

Characteristic	Controls (N=84)	EDTRS 10-20 (N=18)	EDTRS 35 (N=17)	EDTRS 43-47 (N=9)	p* (three Groups) DRSS
Age, y					
Mean (SD) [range]	69.2 (4.5) [59-84]	66.4 (6.6) [52-79]	64.9 (6.0) [54-78]	62.4 (8.5) [54-80]	0.368
Median (Q1-Q3)	68 (66-72)	66.5 (61.5-70.2)	65.6 (60.2-67.3)	59.3 (56.0-66.7)	
Sex, No./Total No. (%)					
Female	45/84 (53.6)	8/18 (44.4)	2/17 (11.8)	3/9 (33.3)	
Male	39/84 (46.4)	10/18 (55.6)	15/17 (88.2)	6/9 (66.7)	0.102
Diabetes duration, y					
Mean (SD) [range]	-	17.9 (7.5) [6.8-34.8]	16.1 (5.9) [3.0-24.3]	16.1 (5.5) [6.8-24.1]	0.690
Median (Q1-Q3)	-	18.2 (12.3-24.4)	17.9 (11.4-19.8)	16.5 (14.7-20.1)	
Hemoglobin A_{1c}, %					
Mean (SD) [range]	-	6.7 (0.9) [4.8-8.5]	6.9 (1.1) [4.2-8.6]	7.6 (1.2) [6.6-10.5]	0.070
Median (Q1-Q3)	-	6.7 (6.0-7.1)	6.9 (6.6-7.4)	7.4 (7.0-7.8)	
BCVA, letters					
Mean (SD) [range]	-	82.8 (5.2) [65-90]	84.1 (3.2) [75-90]	82.8 (4.4) [75-90]	0.614
Median (Q1-Q3)	-	85 (80-85)	85 (85-85)	85 (80-85)	

Q1-Q3, first to third quartiles; BCVA: best-corrected visual acuity.
*p-value for the Chi-square test and the ANOVA test for comparison between the 3 DRSS groups.

Only ocular changes such as vessel density in the SCP and FAZ circularity were significantly different between DRSS groups, reflecting an association between retinal capillary dropout and different severity stages of the disease (Table 2, Figure 1).

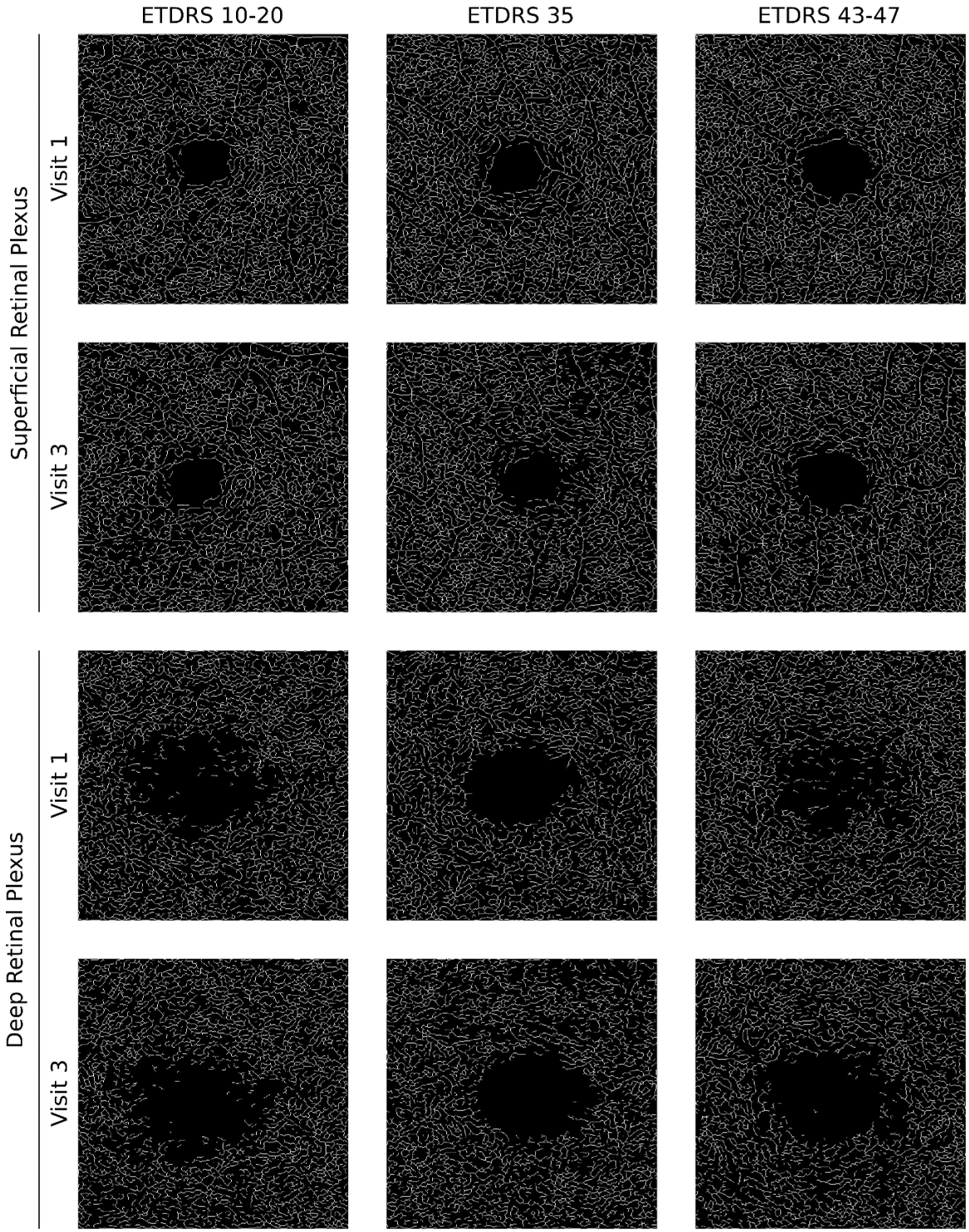

Figure 1: Examples of changes in vessel density of the superficial and deep retinal plexi during the 2-year follow-up period of three eyes, one from the DRSS 10 to 20 group, one from the DRSS 35 group, and another from the DRSS 43 to 47 group.

In each DRSS group, values for capillary dropout (reduced vessel density), edema and neurodegeneration covered a wide range, identifying different levels of damage in different eyes as shown by the maximum and minimum values found for each of the variables (Table 2).

Table 2: Baseline Characteristics of Eyes/Patients Considering Distinct DRSS Stages of the Disease

Characteristic	Controls (N=84)	EDTRS 10-20 (N=18)	EDTRS 35 (N=17)	EDTRS 43-47 (N=9)	p* (three Groups)	DRSS
VD SRP, mm^{-1}						
Mean (SD) [range]	21.1 (0.7) [20.2-22.8]	20.5 (1.0) [18.3-21.9]	19.4 (1.6) [16.1-22.3]	19.5 (1.8) [15.8-21.9]	**0.047**	
p value†		0.051	**<0.001**	**0.005**		
VD DRP, mm^{-1}						
Mean (SD) [range]	16.1 (1.8) [12.2-19.8]	16.0 (1.5) [13.8-19.5]	15.5 (2.1) [10.5-19.8]	14.8 (2.9) [9.1-17.9]	0.386	
p value†		0.632	0.257	**0.005**		
VD FR, mm^{-1}						
Mean (SD) [range]	22.4 (0.6) [21.2-24.2]	22.2 (0.8) [20.4-23.3]	21.4 (1.5) [18.2-24.0]	21.1 (1.6) [17.6-23.3]	0.072	
p value†		0.510	**<0001**	**0.006**		
FAZ Area, mm^2						
Mean (SD) [range]	0.24 (0.11) [0.04-0.54]	0.27 (0.12) [0.10-0.55]	0.23 (0.12) [0.07-0.43]	0.23 (0.08) [0.14-0.37]	0.440	
p value†		0.309	0.717	0.907		
FAZ Circularity Index						
Mean (SD) [range]	0.65 (0.07) [0.39-0.87]	0.68 (0.06) [0.58-0.79]	0.62 (0.09) [0.39-0.75]	0.60 (0.10) [0.37-0.70]	**0.019**	
p value†		0.104	0.260	0.887		
RNFL Thickness, µm						
Mean (SD) [range]	7.0 (3.4) [0.7-13.6]	6.3 (2.9) [2.5-13.7]	7.1 (3.4) [2.3-15.1]	6.3 (1.8) [4.6-9.9]	0.691	
p value†		0.449	0.889	0.556		
GCL–IPL Thickness, µm						
Mean (SD) [range]	82.7 (5.5) [71-94]	82.0 (6.6) [72-92]	76.2 (7.4) [58-90]	81.3 (8.4) [69-97]	0.056	
p value†		0.878	**0.001**	0.484		
Full Retinal Thickness, µm						
Mean (SD) [range]	260.6 (18.3) [218-299]	261.6 (25.2) [212-300]	270.4 (30.1) [225-320]	267.0 (27.0) [223-304]	0.565	
p value†		0.669	0.365	0.919		

VD, vessel density. Values in bold indicate statistical significance.
*p-value for the Mann Whitney test for comparison between the control and DRSS groups.
†p-value for the ANOVA test for comparison between the 3 ETDRS groups.

The presence of capillary dropout, evidenced by reduced vessel density in SCP, DCP and FR, showed statistically significant differences at one and two-year intervals (Table 3). Moreover, in the SCP, capillary dropout was found to be significant in all ETDRS levels examined after the two-year period.

Vessel density in the SCP, DCP and FR showed strong positive correlations (SCP vs DCP: r=0.77; p<0.001; SCP vs FR: r=0.96; p<0.001; DCP vs FR: r=0.84; p<0.001). Also, vessel density decreased progressively, during the 2-year follow-up period, when analyzing the SCP, DCP and FR. Still, the decreases were more apparent in SCP, particularly in the initial stages of DR (Table 3, supplementary S1).

Table 3: Progression in Capillary Dropout, Neurodegeneration, and Edema After 1- and 2-Year Intervals in Different DRSS Groups

		ETDRS 10-20 (N=18)				
		Visit 1	Visit 2	Visit 3	*p-value V2-V1	*p-value V3-V1
		Mean(SD)	Mean(SD)	Mean(SD)		
Vessel Density, mm^{-1}	SCP	20.5 (1.0)	19.4 (1.3)	18.8 (1.5)	**0.002**	**<0.001**
	DCP	16.0 (1.5)	14.7 (2.1)	13.9 (1.9)	**0.012**	**0.001**
	FR	22.2 (0.8)	21.3 (1.1)	20.7 (1.2)	**0.003**	**0.001**
FAZ (Circularity index)		0.7 (0.1)	0.7 (0.1)	0.6 (0.1)	0.102	**0.031**
RNFL (Thinning, µm)		6.3 (2.9)	6.0 (2.8)	6.0 (3.0)	0.948	0.647
GCL-IPL (Thinning, µm)		82.0 (6.6)	81.4 (6.1)	81.7 (6.6)	**0.024**	0.209
Full Retina (Thickening, µm)		261.6 (25.2)	261.7 (26.5)	261.8 (26.2)	0.742	0.660
		ETDRS 35 (N=17)				
		Visit 1	Visit 2	Visit 3	*p-value V2-V1	*p-value V3-V1
		Mean(SD)	Mean(SD)	Mean(SD)		
Vessel Density, mm^{-1}	SCP	19.4 (1.6)	18.6 (2.1)	17.9 (1.7)	0.062	**0.001**
	DCP	15.5 (2.1)	14.8 (2.5)	13.7 (2.3)	**0.028**	**<0.001**
	FR	21.4 (1.5)	20.5 (2.0)	20.0 (1.6)	**0.015**	**0.001**
FAZ (Circularity index)		0.6 (0.1)	0.6 (0.1)	0.6 (0.1)	0.089	0.754
RNFL (Thinning, µm)		7.1 (3.4)	7.1 (3.9)	6.0 (3.0)	0.981	0.136
GCL-IPL (Thinning, µm)		76.2 (7.4)	76.5 (6.7)	76.5 (7.2)	0.391	0.337
Full Retina (Thickening, µm)		270.4 (30.1)	270.5 (29.2)	271.1 (29.9)	0.226	0.115
		ETDRS 43-47 (N=9)				
		Visit 1	Visit 2	Visit 3	*p-value V2-V1	*p-value V3-V1
		Mean(SD)	Mean(SD)	Mean(SD)		
Vessel Density, mm^{-1}	SCP	19.5 (1.8)	18.9 (1.6)	17.9 (2.4)	0.110	**0.015**
	DCP	14.8 (2.9)	14.1 (2.9)	13.3 (2.6)	0.173	0.066
	FR	21.1 (1.6)	20.7 (1.6)	19.5 (2.1)	0.110	**0.015**
FAZ (Circularity index)		0.6 (0.1)	0.6 (0.1)	0.5 (0.1)	0.953	0.069
RNFL (Thinning, µm)		6.3 (1.8)	6.9 (2.4)	6.7 (4.5)	0.767	0.767
GCL-IPL (Thinning, µm)		81.3 (8.4)	81.7 (8.2)	81.7 (8.3)	0.257	1.0
Full Retina (Thickening, µm)		260.7 (27.0)	262.7 (29.0)	263.3 (28.5)	0.372	0.169

		All patients				
		Visit 1	Visit 2	Visit 3	*p-value V2-V1	*p-value V3-V1
		Mean(SD)	Mean(SD)	Mean(SD)		
Vessel Density, mm^{-1}	SCP	19.9 (1.5)	19.0 (1.7)	18.3 (1.8)	**<0.001**	**<0.001**
	DCP	15.6 (2.1)	14.6 (2.4)	13.7 (2.2)	**<0.001**	**<0.001**
	FR	21.7 (1.3)	20.9 (1.6)	20.2 (1.6)	**<0.001**	**<0.001**
FAZ (Circularity index)		0.6 (0.1)	0.6 (0.1)	0.6 (0.1)	**0.034**	**0.025**
RNFL (Thinning, µm)		6.6 (2.9)	6.6 (3.2)	6.1 (3.3)	0.944	0.294
GCL-IPL (Thinning, µm)		79.6 (7.6)	79.6 (7.1)	79.7 (7.5)	0.628	0.884
Full Retina (Thickening, µm)		264.8 (27.3)	265.3 (27.7)	265.7 (27.8)	0.126	0.065

*p-value calculated by Wilcoxon signed rank test. Values in bold indicate statistical significance.

Progression in capillary dropout occurred after one and two-year intervals in DRSS groups 10-20 and 35 but in group 43-47 progression in capillary dropout, represented by decreased vessel density, was only identified after two years of follow up (table 3). In each DRSS group examined there was a subgroup of eyes/patients that showed more marked decreases in vessel density (Table 4). We established a 10% decrease as relevant, as it equals a decrease of 3 standard deviations in the value of the control reference (healthy eyes; VD = 21.10.7).

Table 4: Eyes Showing Decreases in Vessel Density of More Than 10% in the 2-Year Period

Visit	**Vessel Density** (No./Total No.)	**EDTRS 10-20** (No./Total No.)	**EDTRS 35** (No./Total No.)	**EDTRS 43-47** (No./Total No.)
V3-V1	**SCP**	27.8% (5/18)	41.2% (7/17)	22.2% (2/9)
	DCP	50.0% (9/18)	58.8% (10/17)	55.6% (5/9)
	FR	22.2% (4/18)	41.2% (7/17)	22.2% (2/9)

SCP: superficial capillary plexus; DCP: deep capillary plexus; FR: full retina.

In multivariate analysis, considering a model adjusted for age, gender, HbA1c, visual acuity and diabetes duration, vessel density remained significantly different between DRSS groups (Wilks' lambda=0.707; p=0.015) showing association with disease progression.

The differences between visits in full retina and layer thickness showed that neurodegeneration and edema remained generally stable during the two-year period (RNFL: p=0.504; GCL+IPL: p=0.777; CRT: p=0.480) (Figure 2). Neurodegeneration was better identified in the GCL+IPL than RNFL.

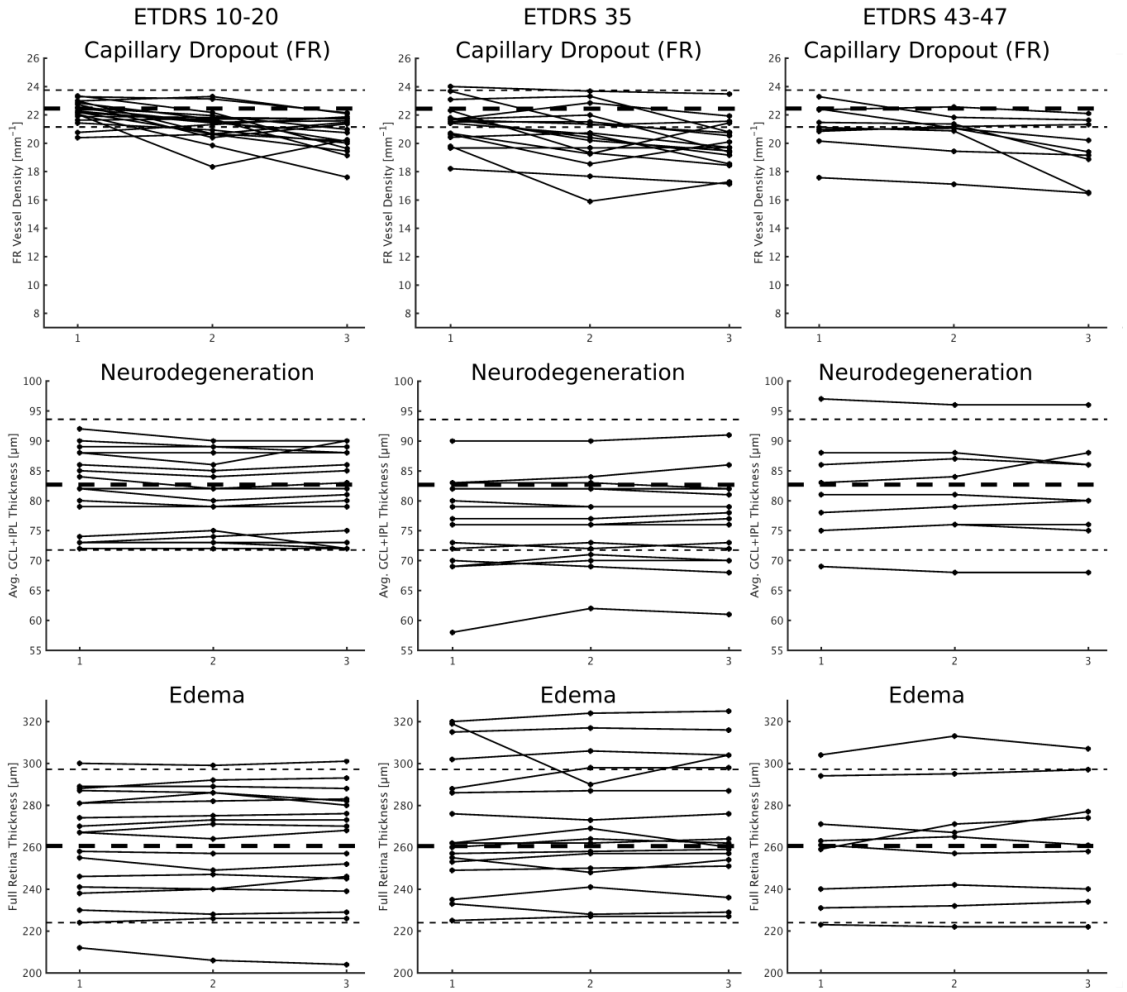

Figure 2: Individual changes over time, by ETDRS level at baseline, of vessel density metrics (SCP, DCP, and FR) for capillary dropout. Horizontal black dashed lines represent the average of the healthy control population (thicker) and the standard deviations from the mean (thinner)

When analyzing ETDRS level changes in the 2-year period of follow up, there was only 1 eye in group 10-20 (2%) with a two-step worsening change (10 to 35). (Supplementary S1). All other ETDRS changes where one-step changes, 10 eyes with worsening changes and 5 eyes with improvement changes. In the DRSS 10-20 group, there was one-step worsening in 8 eyes and one-step improvement in 1 eye. In DRSS 35 group, there was one-step worsening in 1 eye and one-step improvement in 1 eye. In DRSS 43-47 group, there was one-step worsening in 1 eye and one-step improvement in 3 eyes. Although there was higher percentage of decrease in vessel density in the eyes that showed DRSS worsening, a clear correlation between clinical progression (change in DRSS grade) and change in vessel density could not be identified, due to the small number of cases with worsening and improvement in each grade.

When using the 10% vessel density decrease as a threshold, progression in the severity of diabetic retinopathy was identified in 9 of the 26 eyes/patients examined (35%), showing that vessel density has more than 4 times detection capacity than the ETDRS one-step worsening.

Discussion

The results here reported confirm that eyes in the initial stages of retinopathy in patients with diabetes type 2 show evidence of neurodegenerative changes, edema and capillary dropout and that these changes are present in different degrees in different patients. Furthermore, the metrics of these changes show a wide range of values. Definite neurodegeneration, edema or capillary dropout can occur very early in the disease process but are not present in every patient and, when present, not at the same time nor with the same degree.(10)

In this study we have followed, for a period of two years, eyes categorized as minimal, mild and moderate retinopathy using 7-field ETDRS grading. Of the three different disease pathways, only ischemia (capillary dropout), identified by metrics of vessel density using OCTA, showed significant progression. This progression in capillary dropout appears to be driven by a subgroup of eyes/patients that showed changes in vessel density of 10% or more from the baseline. These findings reinforce previous observations suggesting that increased capillary dropout (ischemia) is associated with increased severity of the retinopathy(4).

The decrease in vessel density occurs in both retinal capillary plexuses, superficial and deep, but the changes occurring in the superficial retinal capillary plexus are more reliably detected, evidenced by the lower standard deviation of the measurements performed in the SCP. To account for projections artifacts that may mask the detection of vessel density changes in the DCP we also measured vessel density in the FR, detecting SCP and DCP changes simultaneously. The results obtained confirm earlier detection of capillary dropout in the SCP (4). This finding confirms that one of the earliest changes associated with diabetic retinopathy is a decrease in retinal blood flow (14,15).

It is of major relevance that metrics of neurodegeneration and edema do not show progression over the two-year period of follow-up. These disease pathways do not appear to be associated with increased severity of the retinopathy, even in eyes that showed one step progression in ETDRS level. To identify retinal neurodegeneration, ganglion cell and inner plexiform layer measurements were more informative in this study than retinal nerve fiber layer measurements.

This study shows that only capillary dropout (ischemia) appears to be associated with increased severity of the retinopathy and two-year progression of the retinal disease. It is noteworthy that, in a two-year period of follow-up, minimal, mild and moderate retinopathy show decreases in vascular density. During this 2-year follow up study, there were mainly one-step changes in ETDRS grading which are not generally accepted as clinically meaningful. Therefore, when using ETDRS level changes a two-year period of follow-up is clearly too short as shown in this study. Metrics of

vessel density identifying capillary dropout may offer a viable alternative to identify a specific phenotype associated with increased risk of progression (16,17).

A strict quality check on OCTA vessel density measurements is particularly necessary when comparing different exams performed in the same patient in longitudinal studies. In this study, with exams performed by experienced technicians, data from 17% of the examinations performed could not be included in the data analysis because they did not pass the final quality check. Future longitudinal studies using OCTA for measurement of vessel density need to set strict standards for image quality that need to be checked at the end of each examination.

We have identified the presence of capillary dropout in the initial stages of diabetic retinal disease and that capillary dropout is associated with retinopathy severity and two-year progression. Initially, the DCP, unlike the SCP, did not show capillary dropout, probably due to limitations in the measurement methodology. In this study, the decrease in retinal blood flow is better identified in the SCP, and well identified also when measuring full retina vessel density metrics. The methods used for calculating DCP capillary density metrics may need to be refined, as suggested by Rosen *et al* (18,19).

Limitations of this study are the number of eyes included in the study and the use of automated layer segmentation analyses for measurements of retinal thinning (neurodegeneration) and increased retinal thickness (edema). These procedures, however, were performed in retinas in the initial stages of retinal disease that remained structurally preserved with no evidence of cystoid changes and were reviewed by a masked grader.

In conclusion, eyes with minimal, mild or moderate DR followed with repeated visits, for a period of two years, show evidence of neurodegeneration, edema and ischemia, distributed over a wide range of values in different eyes/patients. Only capillary dropout (ischemia) showed evidence of progression over the two-year period. This progression was particularly clear in a subgroup of eyes/patients showing higher rates of reduction in vessel density. This group of patients should receive particular attention, needing earlier diagnosis and closer follow-up. Metrics of vessel density obtained with OCTA in a non-invasive manner, allowing repeated examinations and close follow-up, will most likely be an informative biomarker of diabetic retinopathy progression and thus create the adequate environment for a larger role of precision medicine in the management of DR.

Supplementary Table S1

ID	Gender	Age [yrs]	Diab. Dur. [yrs]	Visit	HbA1c [%]	Blood Pressure [mmHg] S.	Blood Pressure [mmHg] D.	BVCA	ETDRS	VD SRP [mm^{-1}]	VD SRP %	VD DRP [mm^{-1}]	VD DRP %	VD FR [mm^{-1}]	VD FR %	FAZ Area [mm^2]	FAZ Area %	FAZ Circ [au]	FAZ Circ %	RNFL Thickness [µm]	RNFL Thickness %	GCL-IPL Thickness [µm]	GCL-IPL Thickness %	Full Retina Thickness [µm]	Full Retina Thickness %
1	Male	70	27	V1	7.3	147	75	65	20	20.1	--	14.6	--	22.1	--	0.2	--	0.6	--	6.8	--	73	--	267	--
				V2	7.3			66	20	19.6 *	-2.3	16.4	12.6	21.6	-2.4	0.2	15.6	0.7	22.1	7.8	13.4	74	1.4	271	1.5
				V3	7.4			85	35C	18.6 *	-7.2	16.9	15.7	20.1 *	-9.1	0.2	-2.4	0.6	0.1	7.1	3.2	75	2.7	270	1.1
2	Female	76	7	V1	5.8	142	71	80	10	21.1	--	15.4	--	22.3	--	0.4	--	0.7	--	2.7	--	82	--	241	--
				V2	6.4			80	10	19.9	-5.8	14.3	-7.3	21.4	-3.7	0.4	2.1	0.7	-2.5	1.9	-27.6	82	0.0	240	-0.4
				V3	6.4			85	35C	19.1 *	-9.3	12.6	-18.2	20.7 *	-6.9	0.4	-4.7	0.7	0.3	3.2	22.3	82	0.0	239	-0.8
3	Female	63	25	V1	6.9	147	81	85	10	21.6	--	17.4	--	23.3	--	0.2	--	0.7	--	13.7	--	84	--	274	--
				V2	6.9			85	10	19.6 *	-9.1	15.1	-13.3	22.2	-5.0	0.2	1.2	0.8	0.9	5.6	-59.3	82	-2.4	275	0.4
				V3	7.6			85	10	17.5 *	-18.6	12.5 *	-28.5	19.6 *	-15.9	0.3	9.0	0.7	-7.6	5.9	-56.8	83	-1.2	276	0.7
4	Female	52	19	V1	6.2	140	80	85	20	21.5	--	19.5	--	23.0	--	0.2	--	0.6	--	11.4	--	92	--	287	--
				V2	6.2			85	35C	21.6	0.4	19.6	0.5	23.3	1.4	0.2	2.8	0.7	6.2	12.5	10.1	90	-2.2	286	-0.3
				V3	6.2			85	35C	20.9	-2.8	18.7	-4.4	22.1	-3.9	0.2	1.9	0.6	-1.1	6.7	-41.0	90	-2.2	282	-1.7
5	Male	63	12	V1	6.5	136	67	85	10	21.0	--	16.2	--	22.5	--	0.3	--	0.7	--	6.7	--	88	--	258	--
				V2	6.5			90	10	20.5	-2.3	14.8	-8.6	21.9	-2.5	0.2	-6.4	0.8	12.2	5.8	-13.4	88	0.0	257	-0.4
				V3	6.5			85	10	19.1 *	-8.9	13.6	-16.2	21.0 *	-6.9	0.3	17.7	0.5	-22.4	6.2	-8.3	88	0.0	257	-0.4
6	Male	69	24	V1	6	125	67	85	10	21.8	--	16.3	--	23.3	--	0.3	--	0.6	--	7.1	--	74	--	281	--
				V2	6.7			85	10	21.5	-1.1	17.1	4.7	23.1	-0.7	0.3	6.0	0.6	-5.7	8.5	20.0	75	1.4	286	1.8
				V3	6.7			80	20	20.8	-4.3	15.2	-6.9	22.1	-4.9	0.2	-3.7	0.7	22.3	7.2	1.2	72	-2.7	280	-0.4
7	Female	62	17	V1	7	150	89	85	20	19.6 *	--	17.1	--	22.0	--	0.2	--	0.7	--	5.3	--	79	--	255	--
				V2	7			85	35D	17.3 *	-11.6	13.9	-18.6	19.8 *	-10.0	0.2	2.6	0.6	-5.9	7.2	37.2	79	0.0	249	-2.4
				V3	7.4			85	20	15.1 *	-22.6	12.1 *	-29.1	17.6 *	-20.2	0.1	-42.8	0.5	-26.5	7.3	37.8	79	0.0	252	-1.2
8	Male	61	15	V1	7.1	140	75	85	20	20.7	--	16.1	--	22.2	--	0.3	--	0.7	--	3.8	--	86	--	230	--
				V2	7.8			85	20	18.6 *	-10.1	12.6	-21.6	20.6 *	-6.9	0.4	6.4	0.6	-14.3	3.4	-10.4	85	-1.2	228	-0.9
				V3	7.8			88	20	19.7 *	-4.8	14.7	-8.5	21.5	-3.2	0.3	0.0	0.7	3.2	4.7	24.9	86	0.0	229	-0.4
9	Female	66	7	V1	6.5	142	72	85	20	20.2	--	13.8	--	21.6	--	0.5	--	0.7	--	6.2	--	85	--	224	--
				V2	6.1			85	20	20.4	0.7	13.9	1.1	21.7	0.4	0.4	-1.5	0.7	-1.0	5.0	-20.0	84	-1.2	226	0.9
				V3	6.4			85	35C	17.2 *	-14.8	12.1 *	-12.2	19.1 *	-11.5	0.4	-15.3	0.5	-35.1	0.7	-88.8	85	0.0	226	0.9
10	Male	73	9	V1	6	144	74	80	20	18.3 *	--	15.3	--	20.4 *	--	0.2	--	0.7	--	7.9	--	73	--	289	--
				V2	6.6			80	20	18.5 *	1.6	13.3	-13.5	20.6 *	1.2	0.2	3.5	0.6	-9.7	5.1	-35.0	73	0.0	289	0.0
				V3	6.7			80	10	17.2 *	-5.5	13.9	-9.4	19.4 *	-4.7	0.2	4.6	0.6	-9.2	7.3	-7.8	73	0.0	288	-0.3
11	Female	60	35	V1	8.5	145	77	80	20	20.5	--	15.7	--	22.0	--	0.6	--	0.7	--	4.2	--	80	--	212	--
				V2	8.4			80	20	16.4 *	-19.8	11.1 *	-28.9	18.3 *	-16.8	0.5	-2.5	0.6	-6.7	1.5	-64.4	79	-1.3	206	-2.8
				V3	8.8			85	20	18.3 *	-10.6	11.3 *	-28.1	20.2 *	-8.1	0.5	-16.1	0.4	-35.0	2.4	-42.1	80	0.0	204	-3.8

ID	Gender	Age [yrs]	Diab. Dur. [yrs]	Visit	HbA1c [%]	Blood Pressure [mmHg]		BVCA	ETDRS	VD SRP		VD DRP		VD FR		FAZ Area		FAZ Circ		RNFL Thickness		GCL-IPL Thickness		Full Retina Thickness	
						S.	D.			[mm^{-1}]	%	[mm^{-1}]	%	[mm^{-1}]	%	[mm^2]	%	[au]	%	[µm]	%	[µm]	%	[µm]	%
12	Male	65	17	V1	4.8			90	20	21.4	--	17.1	--	22.8	--	0.2	--	0.7	--	8.3	--	86	--	300	--
				V2	4.8	114	74	85	35C	20.1	-6.0	16.0	-6.3	21.7	-5.0	0.2	4.9	0.7	-2.4	9.0	8.6	85	-1.2	299	-0.3
				V3	5.3			73	35C	19.8	-7.5	15.9	-7.0	21.8	-4.7	0.2	0.7	0.8	3.9	14.8	79.0	86	0.0	301	0.3
13	Male	67	20	V1	7.8			80	10	18.9 *	--	13.9	--	21.4	--	0.2	--	0.7	--	6.0	--	73	--	270	--
				V2	7.8	145	80	85	35C	18.1 *	-4.1	13.3	-4.5	20.9 *	-2.3	0.2	19.9	0.6	-18.9	4.2	-30.1	73	0.0	273	1.1
				V3	7.8			85	10	17.2 *	-9.1	12.9	-7.5	20.0 *	-6.6	0.2	29.3	0.7	-6.0	7.1	17.8	72	-1.4	273	1.1
14	Male	71	10	V1	7.9			85	20	21.9	--	15.3	--	22.7	--	0.3	--	0.7	--	3.8	--	90	--	246	--
				V2	8.7	143	71	85	20	19.4 *	-11.5	11.0 *	-28.2	20.4 *	-10.3	0.3	16.7	0.5	-21.3	4.2	9.7	89	-1.1	247	0.4
				V3	8.6			85	20	20.3	-7.6	11.9 *	-21.7	21.4	-6.0	0.3	7.6	0.5	-21.0	4.7	23.8	88	-2.2	245	-0.4
15	Female	79	19	V1	5.9			85	20	20.3	--	15.5	--	22.5	--	0.1	--	0.6	--	7.0	--	72	--	288	--
				V2	5.9	136	62	85	20	19.4 *	-4.2	14.3	-7.8	21.5	-4.4	0.1	21.8	0.5	-20.3	9.3	32.1	72	0.0	292	1.4
				V3	5.9			85	35C	19.4 *	-4.2	15.7	1.2	21.6	-4.0	0.1	42.7	0.5	-8.1	7.9	12.0	72	0.0	293	1.7
16	Male	69	19	V1	6.8			80	20	21.4	--	18.7	--	23.0	--	0.2	--	0.8	--	6.5	--	89	--	281	--
				V2	6.8	146	65	83	20	18.9 *	-11.8	16.1	-14.0	20.7 *	-10.1	0.2	8.8	0.7	-13.2	7.1	9.0	89	0.0	282	0.4
				V3	6.9			82	20	18.4 *	-14.0	13.7	-26.5	20.2 *	-12.3	0.2	10.3	0.5	-31.4	7.6	16.2	89	0.0	283	0.7
17	Female	70	14	V1	6.9			85	20	19.6 *	--	15.5	--	20.8 *	--	0.5	--	0.6	--	2.5	--	88	--	238	--
				V2	8.5	136	67	70	20	20.0	2.3	14.6	-6.0	21.3	2.6	0.5	0.3	0.7	10.9	5.6	123.2	86	-2.3	240	0.8
				V3	7.2			80	35C	20.5	4.5	13.6	-11.9	21.9	5.3	0.5	0.6	0.8	21.9	2.2	-12.3	90	2.3	246	3.4
18	Male	57	25	V1	6			85	10	20.1	--	15.1	--	21.9	--	0.3	--	0.7	--	3.4	--	82	--	267	--
				V2	6.2	121	56	80	10	19.9	-0.7	16.9	11.9	21.8	-0.5	0.2	-1.3	0.7	-3.7	4.6	35.0	80	-2.4	264	-1.1
				V3	6			80	20	20.0	-0.7	13.8	-8.6	21.7	-0.9	0.3	7.8	0.7	-8.8	5.0	46.2	81	-1.2	268	0.4

ID	Gender	Age [yrs]	Diab. Dur. [yrs]	Visit	HbA1c [%]	Blood Pressure [mmHg] S.	D.	BVCA	ETDRS	VD SRP [mm^{-1}]		VD DRP [mm^{-1}]		VD FR [mm^{-1}]		FAZ Area [mm^2]		FAZ Circ [au]		RNFL Thickness [µm]		GCL-IPL Thickness [µm]		Full Retina Thickness [µm]	
											%		%		%		%		%		%		%		%
19	Male	67	3	V1	4.2	127	75	80	35C	20.0	--	16.1	--	22.3	--	0.1	--	0.6	--	10.7	--	73	--	302	--
				V2	5.4			80	43A	17.1 *	-14.4	13.9	-14.0	19.3 *	-13.4	0.1	-56.6	0.5	-4.7	14.1	31.0	72	-1.4	306	1.3
				V3	6.6			85	35E	16.5 *	-17.4	13.8	-14.1	18.4 *	-17.4	E	E	E	E	7.6	-29.5	73	0.0	304	0.7
20	Male	71	21	V1	7.3	132	90	85	35C	18.1 *	--	13.4	--	19.8 *	--	0.4	--	0.7	--	4.8	--	70	--	225	--
				V2	7.2			76	35C	14.4 *	-20.1	10.8 *	-19.8	15.9 *	-19.6	0.4	2.4	0.6	-4.2	1.5	-68.5	69	-1.4	227	0.9
				V3	7.8			76	35C	15.5 *	-14.4	10.5 *	-21.7	17.3 *	-12.7	0.0	-99.8	0.9	39.2	2.0	-59.3	68	-2.9	227	0.9
21	Male	60	19	V1	8.5	128	78	85	35C	18.1 *	--	15.4	--	20.6 *	--	0.4	--	0.5	--	3.5	--	82	--	255	--
				V2	8.5			85	35E	16.0 *	-11.3	13.5	-12.8	18.5 *	-10.1	0.4	-12.0	0.4	-20.4	1.5	-58.1	82	0.0	248	-2.7
				V3	6.4			83	35C	17.2 *	-4.8	13.8	-10.4	20.1 *	-2.6	0.4	-4.9	0.6	26.5	2.6	-27.0	82	0.0	254	-0.4
22	Male	60	18	V1	8.6	162	65	85	35C	18.0 *	--	15.6	--	20.7 *	--	0.3	--	0.6	--	4.5	--	69	--	257	--
				V2	7.9			85	35C	17.0 *	-5.5	13.9	-11.1	19.3 *	-7.0	0.1	-60.0	0.5	-25.8	4.0	-12.5	71	2.9	258	0.4
				V3	7.2			90	20	18.6 *	3.4	14.0	-10.4	21.4	3.5	0.3	1.6	0.6	0.5	2.8	-39.0	70	1.4	259	0.8
23	Male	54	8	V1	7.4	131	71	90	35C	21.6	--	19.8	--	23.7	--	0.1	--	0.7	--	15.1	--	77	--	320	--
				V2	7.6			80	35C	18.8 *	-12.6	18.1	-8.9	21.3	-10.1	0.1	-0.9	0.6	-7.9	13.3	-11.8	77	0.0	324	1.3
				V3	7.6			85	35C	19.2 *	-11.0	18.2	-8.3	21.6	-8.9	0.1	-5.8	0.7	4.2	12.2	-19.1	78	1.3	325	1.6
24	Male	66	21	V1	7.7	147	73	85	35C	20.0	--	14.7	--	21.7	--	0.1	--	0.6	--	9.0	--	76	--	286	--
				V2	7.7			65	35C	19.0 *	-4.9	14.0	-4.6	20.4 *	-6.0	0.1	-1.8	0.6	-3.2	8.6	-4.6	76	0.0	287	0.3
				V3	7.3			75	43A	17.5 *	-12.7	11.6 *	-21.1	19.2 *	-11.7	0.2	99.4	0.5	-20.9	10.9	20.9	76	0.0	287	0.3
25	Male	59	24	V1	6.6	142	83	85	35C	19.5 *	--	15.9	--	21.5	--	0.3	--	0.7	--	9.5	--	82	--	262	--
				V2	6.7			90	35C	19.7 *	1.4	14.5	-8.7	21.5	0.0	0.3	-5.2	0.7	4.3	13.0	37.9	82	0.0	262	0.0
				V3	7.2			85	35D	18.0 *	-7.3	13.8	-13.5	20.5 *	-4.5	0.3	-4.6	0.7	5.1	3.7	-60.6	81	-1.2	264	0.8
26	Male	57	19	V1	7.3	125	75	85	35C	19.2 *	--	16.1	--	21.7	--	0.3	--	0.6	--	5.2	--	72	--	235	--
				V2	7.3			80	35C	19.3 *	0.2	16.8	4.8	22.0	1.3	0.3	-2.0	0.6	-2.1	3.9	-25.6	73	1.4	241	2.6
				V3	8.7			85	35C	16.9 *	-12.2	12.3 *	-23.1	19.4 *	-10.7	0.3	-5.7	0.6	-8.6	4.3	-17.1	72	0.0	236	0.4
27	Male	63	15	V1	5.3	161	83	85	35C	22.3	--	18.7	--	24.0	--	0.1	--	0.6	--	6.2	--	80	--	262	--
				V2	5.3			87	35C	22.3	0.0	16.9	-9.8	23.7	-1.4	0.1	5.5	0.6	-3.9	7.2	15.4	79	-1.3	269	2.7
				V3	5.3			85	35D	22.0	-1.3	17.4	-7.2	23.5	-2.2	0.1	7.9	0.6	-11.0	7.2	15.2	79	-1.3	260	-0.8
28	Male	67	20	V1	6.6	138	79	85	35C	19.5 *	--	15.6	--	21.7	--	0.3	--	0.7	--	2.3	--	83	--	253	--
				V2	6.6			85	35C	19.5 *	-0.1	15.9	2.0	21.3	-1.5	0.3	-5.6	0.7	-7.4	6.7	189.4	84	1.2	257	1.6
				V3	8			85	35C	18.7 *	-3.9	15.0	-3.6	20.7 *	-4.2	0.3	-14.0	0.5	-28.1	4.2	81.8	86	3.6	257	1.6
29	Male	66	24	V1	7.1	140	60	85	35C	19.8	--	17.3	--	21.8	--	0.1	--	0.6	--	11.4	--	83	--	315	--
				V2	7.1			83	35C	18.3 *	-7.5	19.0	9.9	20.2 *	-7.4	0.1	-4.0	0.6	5.1	7.4	-35.0	83	0.0	317	0.6
				V3	7.6			85	35C	17.3 *	-12.6	15.4	-11.2	19.5 *	-10.8	0.1	-2.6	0.7	11.1	5.6	-50.8	82	-1.2	316	0.3

ID	Gender	Age [yrs]	Diab. Dur. [yrs]	Visit	HbA1c [%]	Blood Pressure [mmHg] S.	Blood Pressure [mmHg] D.	BVCA	ETDRS	VD SRP [mm^{-1}]	VD SRP %	VD DRP [mm^{-1}]	VD DRP %	VD FR [mm^{-1}]	VD FR %	FAZ Area [mm^2]	FAZ Area %	FAZ Circ [au]	FAZ Circ %	RNFL Thickness [µm]	RNFL Thickness %	GCL-IPL Thickness [µm]	GCL-IPL Thickness %	Full Retina Thickness [µm]	Full Retina Thickness %
30	Female	63	9	V1	6.7	145	86	80	35C	20.3	--	16.4	--	21.7	--	0.2	--	0.7	--	6.4	--	76	--	276	--
				V2	7.4			80	35C	21.3	5.3	17.6	7.3	22.9	5.4	0.1	-26.9	0.7	3.6	6.6	3.1	76	0.0	273	-1.1
				V3	7			80	35C	20.4	0.7	15.8	-3.9	21.9	1.1	0.1	-19.0	0.7	4.3	7.9	23.4	77	1.3	276	0.0
31	Female	68	11	V1	7.5	146	72	85	35D	18.7 *	--	13.9	--	20.4 *	--	0.4	--	0.7	--	2.3	--	76	--	233	--
				V2	7.5			78	35C	19.1 *	1.9	13.2	-5.2	20.7 *	1.5	0.4	-7.5	0.7	4.0	5.4	132.5	76	0.0	228	-2.1
				V3	7.5			80	35E	18.0 *	-3.8	11.4 *	-17.9	19.7 *	-3.5	0.4	-4.7	0.6	-1.7	3.5	51.4	76	0.0	229	-1.7
32	Male	67	16	V1	6.9	160	70	85	35C	19.8	--	14.6	--	21.4	--	0.1	--	0.6	--	8.5	--	69	--	288	--
				V2	6.9			83	35C	18.9 *	-4.8	12.7	-12.9	20.6 *	-3.6	0.1	1.1	0.5	-7.1	8.3	-2.6	70	1.4	298	3.5
				V3	6.9			85	35E	17.0 *	-14.0	10.8 *	-26.1	18.5 *	-13.4	0.1	1.8	0.5	-18.4	8.6	0.7	70	1.4	298	3.5
33	Male	73	18	V1	6.7	110	54	75	35C	16.1 *	--	10.5 *	--	18.2 *	--	0.2	--	0.4	--	6.0	--	58	--	319	--
				V2	6.7			80	35C	15.5 *	-4.2	10.6 *	1.4	17.7 *	-3.0	E	E	E	E	10.1	69.3	62	6.9	290	-9.1
				V3	7.3			76	35D	14.8 *	-8.4	10.8 *	2.7	17.1 *	-5.9	0.1	-64.4	0.5	29.5	8.6	44.6	61	5.2	304	-4.7
34	Male	62	10	V1	6.5	146	73	85	35C	21.4	--	16.5	--	23.1	--	0.2	--	0.7	--	6.0	--	90	--	249	--
				V2	8.1			85	20	21.4	0.1	16.4	-0.7	23.3	1.0	0.2	0.5	0.7	-0.3	2.9	-52.6	90	0.0	250	0.4
				V3	7.5			85	35C	19.4 *	-9.3	15.5	-6.1	20.8 *	-10.0	0.2	3.2	0.7	-8.8	3.7	-38.9	91	1.1	251	0.8
35	Male	78	16	V1	6.2	133	72	85	35D	17.6 *	--	13.7	--	19.7 *	--	0.2	--	0.6	--	9.0	--	79	--	260	--
				V2	6			82	20	17.9 *	1.7	13.1	-4.6	19.7 *	0.1	E	E	E	E	6.4	-28.9	79	0.0	264	1.5
				V3	5.9			80	35C	18.2 *	3.3	12.4 *	-9.2	19.7 *	0.1	0.3	19.6	0.6	-5.7	6.7	-26.1	79	0.0	262	0.8

ID	Gender	Age [yrs]	Diab. Dur. [yrs]	Visit	HbA1c [%]	Blood Pressure [mmHg] S.	Blood Pressure [mmHg] D.	BVCA	ETDRS	VD SRP [mm^{-1}]	VD SRP %	VD DRP [mm^{-1}]	VD DRP %	VD FR [mm^{-1}]	VD FR %	FAZ Area [mm^2]	FAZ Area %	FAZ Circ [au]	FAZ Circ %	RNFL Thickness [µm]	RNFL Thickness %	GCL-IPL Thickness [µm]	GCL-IPL Thickness %	Full Retina Thickness [µm]	Full Retina Thickness %
				V1	6.6			75	43A	15.8 *	--	9.1 *	--	17.6 *	--	0.3	--	0.4	--	9.9	--	69	--	223	--
36	Female	80	24	V2	7	119	60	75	35C	15.5 *	-1.9	8.9 *	-2.4	17.1 *	-2.6	0.3	26.9	0.6	53.6	4.6	-53.9	68	-1.4	222	-0.4
				V3	7.4			80	35C	14.7 *	-6.9	8.6 *	-5.7	16.5 *	-6.2	0.3	0.5	0.3	-26.3	2.4	-76.0	68	-1.4	222	-0.4
				V1	11			80	43A	19.9	--	16.6	--	21.5	--	0.1	--	0.7	--	7.4	--	75	--	263	--
37	Male	67	21	V2	9.7	150	85	85	43A	19.4 *	-2.6	16.0	-3.4	21.3	-0.5	0.1	3.9	0.6	-9.1	7.6	2.9	76	1.3	265	0.8
				V3	12			85	43A	16.9 *	-15.0	13.8	-16.9	18.9 *	-12.1	0.1	1.1	0.6	-17.3	8.0	8.0	75	0.0	261	-0.8
				V1	7.1			85	43B	20.9	--	14.9	--	22.4	--	0.2	--	0.6	--	5.1	--	88	--	261	--
38	Male	56	17	V2	7.1	137	77	80	35E	21.1	0.6	16.2	8.6	22.6	0.9	0.2	0.5	0.7	9.2	4.1	-19.8	88	0.0	257	-1.5
				V3	7.1			85	35C	20.5	-1.8	13.2	-11.3	22.1	-1.2	0.2	-0.2	0.6	2.0	4.0	-22.0	86	-2.3	258	-1.1
				V1	7.6			80	43A	18.9 *	--	16.1	--	21.1 *	--	0.2	--	0.6	--	6.9	--	78	--	271	--
39	Male	67	16	V2	7.4	150	65	76	43A	18.3 *	-2.8	14.1	-12.5	20.9 *	-1.2	0.2	-2.3	0.6	0.6	7.8	12.8	79	1.3	267	-1.5
				V3	7.4			75	43A	14.1 *	-25.3	11.2 *	-30.6	16.5 *	-21.8	E	E	E	E	4.8	-30.6	80	2.6	277	2.2
				V1	6.6			85	43A	18.7 *	--	15.3	--	21.0 *	--	0.2	--	0.6	--	4.7	--	81	--	304	--
40	Male	59	10	V2	6.6	140	77	85	47A	19.3 *	3.4	15.9	4.1	21.1 *	0.4	0.2	-0.2	0.6	-3.2	10.7	130.8	81	0.0	313	3.0
				V3	6.8			88	43A	17.8 *	-4.9	13.5	-11.8	19.4 *	-7.5	0.1	-14.5	0.4	-36.7	5.8	25.4	80	-1.2	307	1.0
				V1	7			85	43A	21.1	--	17.9	--	22.4	--	0.2	--	0.6	--	4.6	--	97	--	294	--
41	Male	54	7	V2	5.9	139	85	85	35C	19.4 *	-8.0	14.9	-16.9	21.1 *	-6.0	0.2	13.2	0.5	-8.2	5.3	16.4	96	-1.0	295	0.3
				V3	6.2			90	35C	19.1 *	-9.5	16.3	-9.0	20.2 *	-9.8	0.2	4.8	0.5	-8.1	8.0	75.7	96	-1.0	297	1.0
				V1	7.8			80	43A	18.6 *	--	11.2 *	--	20.1 *	--	0.3	--	0.7	--	7.8	--	75	--	231	--
42	Female	67	20	V2	8.8	150	70	75	43A	17.7 *	-4.8	9.6 *	-14.7	19.4 *	-3.6	0.3	-0.4	0.7	1.3	7.1	-8.6	76	1.3	232	0.4
				V3	8.8			75	47A	17.5 *	-6.2	11.2 *	0.3	19.1 *	-5.0	0.3	-5.8	0.7	-2.4	5.8	-25.5	76	1.3	234	1.3
				V1	8.2			90	47A	19.3 *	--	14.5	--	20.8 *	--	0.3	--	0.7	--	4.9	--	83	--	259	--
43	Male	57	15	V2	8.2	146	84	90	47A	19.5 *	1.0	14.9	3.0	21.2	1.6	0.3	5.6	0.6	-15.6	10.1	105.0	84	1.2	271	4.6
				V3	7.8			90	47A	20.1	4.0	16.6	14.8	21.3	2.3	0.3	1.0	0.6	-13.1	17.6	258.8	88	6.0	274	5.8
				V1	7.4			85	43B	21.9	--	17.8	--	23.3	--	0.4	--	0.6	--	5.4	--	86	--	240	--
44	Female	55	15	V2	7	136	77	85	43B	20.1	-8.2	16.4	-7.9	21.8	-6.2	0.4	1.4	0.6	5.5	4.6	-15.2	87	1.2	242	0.8
				V3	7.8			85	43B	20.2	-8.0	15.2	-14.3	21.6	-7.1	0.4	4.0	0.6	15.2	3.5	-35.0	86	0.0	240	0.0

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Chapter 7

OCTA metrics predict severity progression of Diabetic Retinopathy – 3-year longitudinal study

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SUBMITED TO RETINA

Research Question

In the previous paper we have characterized, in 44 eyes, the 2-year progression of neurodegeneration, edema and ischemia in diabetic patients with NPDR. Capillary dropout presented a better predictive value for DR progression when compared to ETDRS worsening and is an informative biomarker of DR progression. Edema and neurodegenerative alterations remained stable during the 2 years of follow up.

What is the behavior of this patterns, capillary closure and neurodegeneration, in longer follow up periods studying a higher number of subjects?

For that we performed a three-year prospective study in 78 diabetic eyes. We observed that neurodegeneration (ND) and capillary closure (CC) features were already present since the initial DR stages and tended to increase in the more advanced DR ETDRS severity level. These 2 pathways appear to be independent, with no eyes presenting both definite characteristics in the very initial stages.
During the follow up period, the vessel density decreased in all retinal plexuses, particularly in the superficial capillary plexus, and was significantly higher in eyes with worsening in ETDRS level comparing to eyes that maintained DR severity. The GCL+IPL thickness also decreased during the follow up in both groups, but the difference between the groups did not reach the statistically significance. However, in terms of percentage of patients presenting neurodegenerative features, it increase with the higher severity of disease.

Phenotype C, with higher MA turnover, present higher degrees of capillary closure and GCL-IPL thinning comparing to the other phenotypes.

There was also a suspicion that comes out from that study that the capillary closure present in these initial stages can be reversible, at least partially, opening new hope in a treatment strategy capable of reverting ischemic signs.

Abstract

Background: To examine retinal vessel closure metrics and neurodegenerative changes occurring in the initial stages of nonproliferative diabetic retinopathy (NPDR) and severity progression in a three-year period.

Methods: Three-year prospective longitudinal observational cohort of individuals with type 2 diabetes (T2D), one eye per person, using spectral domain-optical coherence tomography (SD-OCT) and OCT-Angiography (OCTA). Eyes were examined four times with one-year intervals. OCTA vessel density maps of the retina were used to quantify vessel closure. Thickness of the ganglion cell + inner plexiform layer (GCL + IPL) was examined to identify retinal neurodegenerative changes. Diabetic retinopathy ETDRS classification was performed using the seven-field ETDRS protocol.

Results: A total of 78 eyes/patients, aged 52 to 80 years, with T2D and ETDRS grades from 10 to 47 were followed for 3 years with annual examinations. A progressive increase in retinal vessel closure was observed. Vessel density (VD) showed higher decreases with retinopathy worsening demonstrated by step-changes in ETDRS severity scale ($p < 0.001$). No clear correlation was observed between neurodegenerative changes and retinopathy progression.

Conclusions: Retinal vessel closure in NPDR correlates with DR severity progression. Our findings provide supporting evidence that OCTA metrics of vessel closure may be used as a surrogate for DR severity progression.

Introduction

Diabetic retinopathy (DR) is a major complication of type 2 diabetes (T2D) and a leading cause of visual impairment and blindness, and its incidence tends to increase [1].

Early and accurate identification of retinal changes and individual rates of progression are paramount to guide treatment decisions and determine prognosis and may help in the prevention of vision loss. In the initial stages, diabetic eyes may show neurodegeneration (ND), edema, and increases in vessel closure (VC). These changes occur to different degrees in different eyes, indicating that different mechanisms of retinal disease may predominate in different patients [2]. Our group has investigated DR biomarkers for progression profiles in different individuals, having proposed three phenotypes of progression [3,4], with different prognoses for progression to vision-threatening complications [5] and DR severity [6]. Indeed, the use of non-invasive imaging approaches has gained much relevance in the identification of DR biomarkers of staging and progression. Multimodal imaging approaches, such as OCT and OCT-Angiography (OCTA), facilitate the identification of different pathways of DR, namely ND, edema and ischemia [2,7]. Hence, OCTA has emerged as an innovative non-invasive tool to investigate quantitatively and qualitatively the retinal blood flow and capillary networks. It is a functional extension of structural OCT, that uses repeated B-scans to detect motion contrast, allowing the visualization of retinal microvasculature without intravenous dye injection [8]. Importantly, OCTA provides depth-resolved information on retinal circulation facilitating the evaluation of the individual's retinal capillary plexus.

Taking advantage of OCTA, we have shown in a 2-year longitudinal study, that retinal VC increased with retinopathy progression, in contrast with edema and ND, which remained relatively stable [8]. There is, therefore, a clear need for evaluation of the OCTA metrics prognostic value in DR progression in longitudinal studies over longer periods. We present here a 3-year follow-up study, with data from a cohort of T2D individuals with non-proliferative diabetic retinopathy (NPDR), in which we have investigated the relationship between quantitative OCTA metrics, ND and DR severity progression.

Methods

This study is a 3-year prospective longitudinal study designed to analyze 90 eyes. The study was designed to analyze individuals with T2D and with NPDR (ETDRS grades 10 to 47), who have completed four visits in a period of 3-years of follow-up. The tenets of the Declaration of Helsinki were followed, approval was obtained from the AIBILI'S Ethics Committee for Health and written informed consent to participate in the study was obtained from all individuals after all procedures were explained.

Exclusion criteria included any previous laser treatment or intravitreal injections, presence of other retinal disease (e.g., age-related macular degeneration, glaucoma, or vitreomacular disease), high ametropia (spherical equivalent greater than −6 and +2 diopters), or any other systemic disease that could affect the eye, with special attention to uncontrolled systemic hypertension (values outside normal range: systolic 70–210 mmHg and diastolic 50–120 mmHg) and history of ischemic heart disease.

A total of 90 eyes of individuals with T2D and ETDRS levels between 10 and 47 were included, with a maximum glycated hemoglobinA1c (HbA1c) value of 10%. A population of 84 eyes of individuals without diabetes, in the same age range, or other retinal diseases was used as a control group to set normal values and identify abnormal deviations between diabetic and non-diabetic control populations. No follow-up of non-diabetic individuals was performed. Age, duration of diabetes, HbA1c, and blood pressure levels were collected for each participant at the baseline visit.

All participants underwent a full ophthalmologic examination, including visual acuity, 7-field color fundus photography (CFP), SD-OCT, and OCTA imaging, at baseline and at the 1-year, 2-year and 3-year follow-up visits.

Eighty-four healthy control eyes (one eye per subject), from an age-matched population, were imaged in a single visit within the scope of the screening program, using SD-OCT and OCTA and were used as a reference control.

Seven-field Color Fundus Photography

The 7-fields CFP were acquired using the Topcon TRC 50DX camera (Topcon Medical Systems, Tokyo, Japan), at 30/35 . The DR severity score was determined at baseline and at every annual visit by 2 independent graders in a context of an experienced reading center (Coimbra Ophthalmology Reading Center—CORC, Coimbra, Portugal) using a modified Airlie House classification scheme according to the ETDRS Protocol [9,10]. The observed agreement between the 2 graders was 97%. All disagreement cases were resolved by mutual agreement [11]. Step changes in the ETDRS retinopathy severity scale were used to describe worsening or improvement of the retinopathy [9,12].

Optical Coherence Tomography

Optical coherence tomography (OCT) was performed using the Cirrus HD-OCT 5000 (Carl Zeiss Meditec, Inc., Dublin, CA, USA). The Macular Cube 512 × 128 acquisition protocol, consisting of 128 B-scans with 512 A-scans each, was used to assess the subjects' CRT. The average thickness values at the inner ring of the Ganglion Cell Layer + Inner Plexiform Layer (GCL+IPL) were gathered with Zeiss Cirrus standard reports.

Decreases in GLP+IPL were considered to identify ND, whereas CRT increases were considered to identify edema.

OCT-Angiography

OCT-Angiography data were collected by the Cirrus HD-OCT 5000 device using the Angiography 3 × 3 mm2 acquisition protocol, which consists of a set of 245 clusters of 4 B-scans repetitions, where each B-scan consists of 245 A-scans, over a 3 × 3 × 2 mm3 volume in the central macula.

The Carl Zeiss Meditec Density Exerciser (version:10.0.12787; Carl Zeiss Meditec, Inc., Dublin, CA, USA) was applied to calculate perfusion density (PD) and vessel density (VD) [8,13].

Vascular density metrics (VD and PD) for the Inner Ring region were analyzed for the SCP, DCP and FR. The circularity index of the Foveal Avascular Zone (FAZ) detected on the SCP was also computed. We also calculated the VD metrics of the FR, since those should be independent of projection artifacts that may affect the examination of the DCP.

All OCTA examinations underwent a quality check and normalization of signal strength as previously described [13,14]. Likewise, from 90 eyes included in this study, 13.3% were excluded

as they did not meet the set of quality criteria in the first or last visits, leading to a final number of 78 eyes that allowed analysis of the 3-year progression. Included in the data set there is a small number of eyes with poor imaging quality in the second 13 (17%) and third visits 14 (18%) (Figure 1).

Capillary closure was identified by decreased VD or PD metrics measured in the SCP, DCP and FR.

Statistical Analysis

Variables were summarized for each diabetic retinopathy severity scale (DRSS) group, 10–20, 35, and 43–47, using mean and SD.

The $\chi 2$ test for categorical variables and the Kruskal–Wallis-H test for continuous variables were performed for comparison between the 3 ETDRS groups. To assess statistically significant differences between the measurements of healthy controls and each ETDRS group, the $\chi 2$ test was used for categorical variables and the Mann–Whitney *U* test was used for continuous variables.

For comparison of the progression of the disease in terms of VD and GCL + IPL layer Mann–Whitney U tests were used. Multiple regression analysis was performed to identify factors associated with VD. Correlation between changes of VD and GCL + IPL thickness from the first to the last visits was assessed by the Spearman's rank correlation coefficient. Statistical analysis was performed with Stata 16.1 (StataCorp LLC, College Station, Texas, USA), and a p value ≤ 0.05 was considered statistically significant. When performing the comparison of VD metrics with ETDRS severity worsening, between the distinct ETDRS groups a Bonferroni correction was applied to correct for multiple comparisons.

Results

Of the 78 eyes analyzed, 24 (31%) were graded at ETDRS levels 10–20 at baseline (with 16 being level 10 and 8 level 20), 31 (40%) as ETDRS 35 and 23 as ETDRS 43–47 (29%). Demographic and baseline systemic and ocular parameters of 84 healthy control eyes and 78 T2D eyes included in the study are presented in Table 1. Of the systemic variables only HbA1c shows statistically significant differences between the DR severity groups (p = 0.005). The ocular parameters, VD and PD, both representing VC (i.e., decreased perfusion of red blood cells) and FAZ circularity were significantly different between ETDRS groups, reflecting an association between retinal capillary non-perfusion and different severity grades of the retinopathy (Table 1). Across the different ETDRS levels examined, OCTA was able to detect differences in VC compared with changes in PD.

Presence of VC and ND varied widely within each of the three main ETDRS groups examined (Figure 1). There were eyes with definite VC and ND (≥2 standard deviation [2 SD] vs healthy controls) and eyes with minimal or no change.

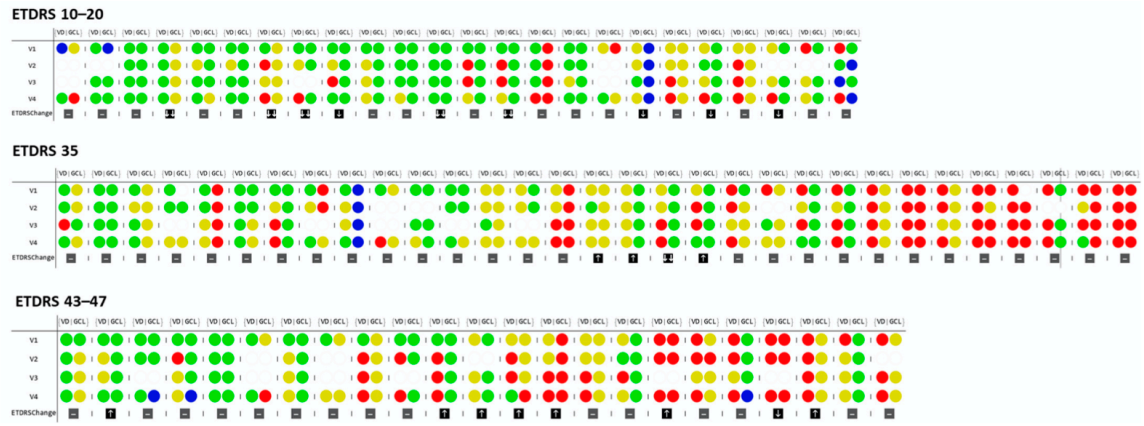

Figure 1: Schematic representation of individual vessel density values in the SCP inner ring and thinning of GCL + IPL and its progression over the four visits, presented according to differences and variation in VD across ETDRS groups. The values are given in relation to the control group: Values within a normal range are depicted in green; 2 SD decrease is depicted in red; 1 SD decrease is depicted in yellow; and 2 SD increase are shown in blue. Circles without color indicate that reliable measurements could not be obtained in that specific visit due to insufficient image quality. Arrows indicate ETDRS step progression: one or two increase (↑), maintenance (-) or decrease (↓). VD: Vessel density; GCL: Ganglion cell layer and Inner plexiform layers. V1: Baseline visit; V2: 1 year visit; V3: 2-year visit; V4: Last visit (3 years).

The presence and degree of VC is shown to be related with the severity of the retinopathy as identified by ETDRS grade (Figure 1). At the last visit, decreases in VD larger than 1 or 2 SD in comparison with healthy controls were registered in 58% of the eyes in groups 10–20 (14 of 24), in 67% in group 35 (21 of 31) and 74% in group 43–47 (17 of 23). The presence of ND was detected less

frequently. At the last visit, GCL + IPL thinning of more than 1 or 2 SD compared to healthy controls was identified in 33% of the eyes in group 10–20 (8 of 24), 69% in group 35 (20 of 31) and 52% in group 43-47 (12 of 23).

Table 1. Baseline characteristics for healthy controls and T2D individuals considering distinct DRSS stages of the disease.

	Healthy Controls	ETDRS 10–20	ETDRS 35	ETDRS 43–47	*p*-Value (Between the Three ETDRS Groups) **
	(*n* = 84)	(*n* = 24)	(*n* = 31)	(*n* = 23)	
Sex, Male/female	39/45	16/8 *0.08*	25/6 ***0.001***	17/6 ***0.019***	*0.499*
Age, years	69.2 ± 4.5	69.5 ± 5.9 *0.706*	65.4 ± 5.5 ***0.002***	66.5 ± 7.2 *0.213*	*0.064*
Diabetes duration, years	—	18.2 ± 7.1	16.5 ± 6.8	17.6 ± 5.8	*0.577*
BCVA, letters	—	85.5 ± 4.1	85.3 ± 4.4	86.8 ± 3.4	*0.528*
HbA1c, %	—	6.9 ± 1.1	7.3 ± 1.1	8.0 ± 1.2	***0.005***
VD, SCP, inner ring, mm^{-1} *(p-value *)*	22.3 ± 0.89	21.7 ± 1.1 0.058	20.9 ± 1.1 **$p < 0.001$**	21.2 ± 1.3 **$p < 0.001$**	***0.033***
VD, DCP, inner ring, mm^{-1} *(p-value *)*	17.0 ± 2.14	*17.2 ± 1.97* *0.918*	*16.2 ± 2.2* *0.078*	*16.4 ± 2.2* *0.310*	*0.324*
VD, FR, inner ring, mm^{-1} *(p-value *)*	23.7 ± 0.90	23.5 ± 1.1 *0.958*	22.6 ± 1.1 **$p < 0.001$**	22.9 ± 1.1 ***0.010***	***0.009***
PD, SCP, inner ring, mm^{-1} *(p-value *)*	0.398 ± 0.02	0.403 ± 0.02 *0.416*	0.390 ± 0.02 ***0.006***	0.400 ± 0.02 *0.073*	***0.038***
PD, DCP, inner ring, mm^{-1} *(p-value *)*	0.315 ± 0.04	0.332 ± 0.03 *0.535*	0.312 ± 0.04 *0.119*	0.318 ± 0.04 *0.491*	*0.221*
PD, FR, inner ring, mm^{-1} *(p-value *)*	0.416 ± 0.02	0.429 ± 0.02 *0.051*	0.414 ± 0.02 *0.059*	0.426 ± 0.02 *0.176*	***0.011***
FAZ circularity index *(p-value *)*	0.687 ± 0.07	0.638 ± 0.2 *0.636*	0.607 ± 0.1 *0.205*	0.560 ± 0.1 **$p < 0.001$**	***0.004***
GCL + IPL, Inner Ring, µm (58 healthy controls) *(p-value *)*	82.7 ± 5.5	82.8 ± 9.1 *0.710*	77.6 ± 8.1 ***0.003***	78 ± 6.8 ***0.007***	*0.087*

Data presented as Mean ± SD. * χ^2 test (categorical variables) and Mann–Whitney U-test (continuous variables) for comparison between Healthy Controls and each ETDRS group. ** χ^2 test (categorical variables) and Kruskal–Wallis test (continuous variables) for comparison between the three ETDRS groups. BCVA: Best corrected visual acuity; HbA1c: Glycated hemoglobin; VD: Vessel density; PD: Perfusion density; SCP: Superficial capillary plexus; DCP: Deep capillary plexus; FR: Full retina; FAZ: Foveal avascular zone; GCL + IPL: Ganglion cell + Inner plexiform layer.

When analyzing only definite VC and ND (i.e., 2 SD changes related to normal healthy controls) in the 3-year follow-up visit, definite VC was identified in 33.3% of the eyes in group 10–20, 42% of the group 35 and in 47.8% of the group 43–47, with definite ND identified only in 8.3% of the eyes in group 10–20, 24.1% of the group 35 and 21.7% of the group 43–47 (Table 2).

Table 2. Percentage and correlation of changes of vessel closure and neurodegenerative changes ≥ 2 SD at baseline and last visit.

≥2D Changes	ETDRS 10–20 (*n* = 24)	ETDRS 35 * (*n* = 31)	ETDRS 43–47 (*n* = 23)
Visit 1			
Vessel closure (VC)	8.3%	38.7%	30.4%
Neurodegeneration (ND)	4.2%	22.6%	13.0%
VC and ND in the same eye	0.0%	9.6%	8.7%
Visit 4			
Vessel closure (VC)	33.3%	41.9%	47.8%
Neurodegeneration (ND)	8.3%	24.1%	21.7%
VC and ND in the same eye	16.0%	16.1%	8.7%
VD (SCP) and GCL + IPL thickness correlation change (V4 − V1)	0.07 ($p = 0.739$)	0.03 ($p = 0.872$)	−0.25 ($p = 0.246$)

Percentage of eyes presenting 2 SD decreases in vessel closure (vessel density decrease) and neurodegeneration (GCL + IPL thinning), relative to normal healthy control eyes in the baseline visit (V1) and last visit of the 3-year follow-up (V4), in ETDRS groups 10–20, 35 and 43–47. * ETDRS group 35 has $n = 29$ for eyes assessed for neurodegeneration. Spearman's rank correlation coefficients between the change (V4–V1). VC: Vessel closure, ND: Neurodegeneration.

Presence of definite VC and ND (≥2 SD changes) in the same eyes were not observed at the baseline visit in ETDRS group 10–20 but were present in 16% of the eyes in the last visit of the same group. In group 35, definite VC and ND were present in the same eyes in 9.6% at the baseline visit and 16% in the last visit. Finally, in group 43–47, definite VC and ND were present in the same eyes only in 8.7% of the eyes in both baseline and last visit, showing that dissociation between definite VC and definite ND predominates during the 3-year follow-up period (Table 2). Furthermore, no statistically significant correlations were found between the changes (first to last visits) of VD and GCL + IPL thickness, except for a weak correlation found on ETDRS 35 group when considering the DCP layer ($\varrho = 0.37$, $p = 0.045$).

Concerning ETDRS level changes at 3 years of follow-up ten eyes (12.8%) presented a one-step improvement and 11 eyes showed worsening (14.1%), with one step-worsening found in 5 eyes (6.4%) and two-step worsening in the other 6 eyes (7.7%). When comparing VD between groups of ETDRS grade changes during the three-year follow-up period, significant differences were identified. The eyes with one-or two-step worsening showed a higher decrease in VD than the eyes that maintained their ETDRS grade or showed improvement during the three-year follow-up (Table 3). Comparison of VD Inner Ring metrics with disease progression showed statistical significance in all layers assessed, namely SCP, DCP and FR (respectively: $p = 0.014$, $p = 0.048$ and $p = 0.047$). No significant differences were observed in the GCL + IPL thinning between eyes that showed worsening of ETDRS severity grade and the eyes that maintained or improved during the 3-year period of study (Table 3).

Table 3. Vessel density and ganglion cell layer + inner plexiform layer thickness comparison between T2D individuals that improved and worsened 1 and 2 steps in DRSS stage, at the end of the three-year follow-up.

ETDRS Change		Vessel Density Inner Ring mm^{-1}						Layer Thickness Inner Ring, µm	
		SCP		DCP		FR		GCL + IPL	
		V1	V4 − V1	V1	V4 − V1	V1	V4 − V1	V1	V4 − V1
Worsening (1 and 2 steps) ($n = 11$)	AVG	21.5	−1.3	16.7	−2.1	23.1	−1.3	81.9	−0.5
	SD	0.9	1.0	1.7	1.5	0.9	1.1	8.7	1.2
	Min	20.0	−2.9	14.6	−4.8	21.3	−3.2	65.0	−2.0
	Max	22.5	−0.3	19.7	−0.1	24.3	−0.1	98.0	1.0
No change + improving ($n = 67$)	AVG	21.2	−0.4	16.6	−1.0	23.0	−0.4	78.9	−1.1
	SD	1.2	1.0	2.2	1.7	1.2	1.0	8.3	5.6
	Min	18.5	−1.8	11.4	−4.4	20.3	−1.9	58.0	−26.0
	Max	24.9	3.1	22.1	2.9	26.5	3.1	110.0	18.0
Statistical Diff V4 − V1 (*p*-value)		**0.014 ***		**0.048**		**0.047**		0.810	

Vessel density (mm^{-1}) for the Inner Ring were collected using the Cirrus HD-OCT 5000 with AngioPlex module. The average thickness values at the inner ring of the GCL + IPL were collected from Zeiss Cirrus standard reports. Comparison was performed by Mann–Whitney U-test, followed by Bonferroni correction for multiple comparisons. Significant *p*-values ($p < 0.005$) are highlighted in bold. * indicates a significance level of 0.017 obtained after Bonferroni correction. AVG: Average; SD: Standard deviation; Min: Minimum value; Max: Maximum value; SCP: Superficial capillary plexus; DCP: Deep capillary plexus; FR: Full retina; GCL + IPL: Ganglion cell + Inner plexiform layers. V1: Baseline visit; V4: Last visit (3 years).

When looking for factors associated with baseline VD on a univariate regression analysis, MAT and GCL+IPL were found to be significantly associated with VD ($p = 0.003$ and $p = 0.011$, respectively). These variables were then considered for a multivariate regression analysis along with demographic and systemic features. In this model, only MAT (ß = −0.096, 95%CI: −0.168 to −0.024, $p = 0.009$) and GCL + IPL (ß = 0.035, 95%CI: 0.003 to 0.067, $p = 0.032$) showed a statistically significant association with baseline VD.

Discussion

The results here reported confirm that eyes in the initial stages of retinopathy in T2D patients show evidence of VC and neurodegenerative changes and that these changes are present in different degrees in different patients even when classified as belonging to the same ETDRS severity grade. Moreover, the metrics of these changes show a wide range of values. Definite ND and VC (i.e., ≥2 SD changes) can occur very early in the disease process but are not present in every patient and, when present, they do not have the same rate of progression.

In this study, we have followed, for a period of 3 years, eyes categorized as no retinopathy, minimal, mild, and moderate retinopathy using seven-field ETDRS grading. Only VC (ischemia) showed significant progression during the 3-year period of follow-up. Furthermore, during this period, one- or two-steps worsening of retinopathy severity showed higher degrees in VD, confirming previous observations [8].

Our study confirms the presence of VC in the initial stages of DR, with earlier detection in the SCP, suggesting that one of the earliest changes associated with DR is reduced VD, possibly due to a decrease in retinal blood flow in selected capillaries [15,16]. The reduced capillary flow in the diabetic retina is most probably related to a decrease in the number of capillaries that carry red blood cells, instead of changes in capillary diameter, because skeletonized VD is the metric that better detects diabetic VC. The increase in VC due to the number of closed capillaries to red blood cell flow is compatible with the development of preferential channels or arteriovenous shunts which have been observed on histologic and trypsin-digest preparations of diabetic retinas [16–18]. We have reported VC results from SCP and DCP as well as from the full retina; although calculation of VD for full retina decreases the impact of projection artifacts, the overlap of vessels of SCP and DCP may affect its sensitivity.

Our study confirms that VC occurs initially in the perifoveal retina, progressing rapidly in the ETDRS levels 10, 20 and 35 but the changes in the perifovea appear to plateau at levels 43–47. This may be associated with a shift in the location of the VC to the midperiphery of the retina [19].

Our study shows that VC identified with OCTA reaches different degrees in patients with the same ETDRS severity grade and might be a tool to monitor DR progression. This observation suggests that different patients with T2D have different microvascular responses with some patients being able to maintain a viable retinal circulation and showing minimal changes, whereas others respond by poor capillary recruitment and progressive VC. Indeed, our results are in

agreement with recent studies that suggest the predictive role of OCTA on DR progression and development of vision-threatening complications in diabetic individuals [20–22].

It is this variability of the retinal microvascular changes in T2D, regarding both their initiation and progression in relatively initial stages of NPDR that we consider a most relevant finding of this study. Some patients show steady and progressive worsening whereas others show a variable course and evidence of reversibility of their changes, as well demonstrated in Figure 1. These observations offer two important messages. First, the reversibility of the VC opens the door for early intervention with the possibility of stopping disease progression. Second, each patient should be followed closely, and a variety of risk factors should be considered to determine a specific risk profile for that patient. It demonstrates the complexity of diabetic retinal disease and indicates that multiple genes and environment factors may be involved, creating different subtypes of progression.

Neurodegeneration identified by GCL + IPL thinning has been proposed as playing a major role in the development and progression of DR. This study shows that ND association with VC was present at baseline in this cohort, but this association appears to uncouple as the disease progresses, as shown in Figure 1.

A strict quality check of OCTA vessel metrics is particularly important and necessary when comparing different examinations performed in the same patient in longitudinal studies and to identify disease progression. In this study, with examinations performed by experienced technicians, data from 17% of the examinations did not pass the final quality check and had to be excluded from the data analysis.

A limitation of this study is the number of eyes included in the study. Other limitations include the lack of use of a projection removal algorithm that can increase sensitivity, and the limited scan field (3 × 3 mm), which prevents a more detailed overview of the VC differences between ETDRS levels observed with Swept-Source OCTA (15 × 9 mm) [13]. However, measurements of retinal thinning were performed in retinas that remained structurally preserved with no evidence of cystoid changes. Of special value, a strict quality check was performed by a masked grader and normalization of OCTA metrics based on signal strength was performed.

In conclusion, OCT-angiography metrics of retinal VC, more specifically VD measure- ments based on skeletonized images, obtained in a noninvasive manner that allow repeated examinations and close follow-up, are particularly promising candidates as biomarkers of DR severity progression and are expected to impact disease management.

Conclusions

Eyes with initial stages of retinopathy in T2D individuals followed by OCT and OCTA during a 3-year period demonstrate progressive increase in vessel closure. Vessel density showed higher decrease in eyes with retinopathy worsening demonstrated by step changes in ETDRS severity scale. Neurodegenerative changes, although associated with vessel closure at baseline, did not uniformly progress during the 3-year period of follow up.

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Chapter 8

Swept source OCTA quantification of capillary closure predicts ETDRS severity staging of NPDR

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Research Question

In the previous paper we have characterized the 3-year progression of neurodegeneration, edema and ischemia in diabetic patients with NPDR. Capillary dropout, determined by VD parameters in the inner ring, was the most informative biomarker of DR progression. Neurodegeneration also increased during the 3 years of follow up, in a subset of patients.
Previous studies have shown that the progression of diabetic retinopathy may be related to the development of retinopathy lesions in specific retinal areas. Recent widefield OCTA imaging allowed to study a wider retinal area and may change the actual knowledge.

Is there a single or composite set of OCTA parameters that better predict NPDR evolution and that may be routinely used in clinical practice?

We performed a cross sectional study of 105 diabetic eyes with or with no DR and in 38 healthy individuals. The swept source OCT PlexElite 9000 (ZEISS) with both 15x9 mm and 3x3 mm protocols was used.

In eyes presenting mild NPDR, retinal capillary closure was mainly located in the macular area, in the parafoveal retinal circulation (inner ring), whereas in moderate to severe DR the predominant capillary closure was observed in the retinal midperiphery, with a higher damage in DCP, showing that the combination of the 2 OCTA macular protocols are the most accurate in prediction of DR severity changes.

Abstract

Purpose: To test whether a single or composite set of parameters evaluated with optical coherence tomography angiography (OCTA), representing retinal capillary closure, can predict nonproliferative diabetic retinopathy (NPDR) staging according to the gold standard ETDRS grading scheme.

Methods: 105 patients with diabetes, either without retinopathy or with different degrees of retinopathy (NPDR up to ETDRS grade 53) were prospectively evaluated using Swept-Source OCTA (SS-OCTA, PlexElite, Carl Zeiss Meditec) with 15x9 mm and 3x3 mm angiography protocols. Seven-field photographs of the fundus were obtained for ETDRS staging. Eyes from age-matched healthy subjects were also imaged as control.

Results: In eyes of patients with type 2 diabetes without retinopathy or ETDRS levels 20 and 35, retinal capillary closure was in the macular area, with predominant alterations in the parafoveal retinal circulation (inner ring). Retinal capillary closure in ETDRS stages 43-53 becomes predominant in the retinal midperiphery with vessel density average values of 25.2 ± 7.9 (p=0.001) in ETDRS 43 and 23.5 ± 3.4 (p=0.001) in ETDRS 47-53, when evaluating extended areas of 15x9 protocol. Combination of acquisition protocols 3x3 mm and 15x9 mm, using SS-OCTA allows discrimination between eyes with mild NPDR (ETDRS 10, 20, 35) and eyes with moderate to severe NPDR (ETDRS grades 43-53).

Conclusions: Retinal capillary closure, quantified by SS-OCTA, can identify NPDR severity progression. It is located mainly in the perifoveal retinal capillary circulation in the initial stages of NPDR, whereas the retinal midperiphery is predominantly affected in moderate to severe NPDR.

Introduction

Diabetic retinopathy (DR) is one of the leading causes of blindness worldwide and the prevalence of vision-threatening DR is expected to double in the next decade [1,2] . Considering that more than 90% of the cause of vision loss can be prevented [3], accurate staging and classification of DR is fundamental to guide treatment decisions and identify progression.

The Early Treatment of Diabetic Retinopathy Study (ETDRS) grading scheme is the gold-standard for DR staging [4]. However, it is labour-intensive with low throughput and, thus, has limited applicability in daily practice.

Optical Coherence Tomography Angiography (OCTA) has gained a critical position in the study of DR[5]. It is a functional extension of structural OCT, that uses repeated B-scans to detect motion contrast, allowing a dye-less visualization of retinal microvasculature [6]. Furthermore, it provides non-invasive three-dimensional mapping of the retinal microvasculature, allowing the identification and quantification of retinal capillary closure, which is the feature that has been shown to better correlate with the clinical features of nonproliferative diabetic retinopathy (NPDR) in its main stages and progression [7] .

There are also indications that information on regional distribution of retinal capillary changes may be particularly relevant in the more advanced stages of the retinopathy[8]. Studies with widefield fluorescein angiography have shown that midperipheral and peripheral changes in retinal progression need to be considered and may be valuable for determining retinopathy progression.

The recent development of commercially available Swept Source OCT (SS-OCT) instruments enables OCTA visualization of retinal vasculature over larger fields of view, 12 x 12 mm or 15 x 9 mm [9]. Also, SS-OCT-imaging provides advantages in the speed of acquisition and better penetration, with enhanced resolution and improved sensitivity due to the density of A-scans, compared with conventional Spectral Domain OCT (SD-OCT) imaging [10].

This study applies these recent technological advances to examine the value of commercially available SS-OCTA in the characterization of retinal capillary nonperfusion in NPDR and the contribution of macular and widefield imaging to improve characterization of the different ETDRS stages of NPDR.

Methods

In this prospective cross-sectional study, 105 individuals with diabetes with or without retinopathy and 38 individuals without diabetes were recruited from AIBILI - Association for Innovation and Biomedical Research on Light and Image screening programs and underwent OCTA imaging and a full ophthalmological examination. The tenets of the Declaration of Helsinki were followed, approval was obtained from the Institutional Ethical Review Board, and written informed consent to participate in the study was obtained from all individuals after the procedures were explained.
The study exclusion criteria comprised the presence of age-related macular degeneration, glaucoma, vitreomacular disease, high ametropia (spherical equivalent greater than -6 and +2 dioptres), any previous laser treatment or intravitreal injections or any patient comorbidity likely to affect the eye and not related with diabetes or cardiovascular disease. Excluded were also people with T2D with uncontrolled systemic hypertension (values outside normal range: Systolic 70-120mmHg and diastolic 50-120 mmHg), haemoglobin A1c (HbA1c) levels above 10% and history of ischemic heart disease.

Optical Coherence Tomography Angiography Imaging

Study subjects were imaged by the swept-source OCT PlexElite 9000 (ZEISS, Dublin, CA, USA). The acquisition protocols used were the "Angio 15 mm x 9 mm" (834 x 500 x 1536 voxels with 2 B-Scan repetitions) and the "Angio 3 mm x 3 mm" (300 x 300 x 1536 voxels with 4 B-Scan repetitions) protocols.

Acquired data from the PlexElite was processed by the "Density Quantification v0.3.5" algorithm available on the Advanced Retina Imaging (ARI) portal which uses multi-layer segmentation and calculates vascular density metrics for the Superficial Capillary Plexus (SCP), Deep Capillary Plexus (DCP) and Full Retina (FR). For retinal mid-periphery areas, to take advantage of the wide-field capabilities of the 15x9 mm protocol, 3 extra concentric rings were added to the standard 9 ETDRS areas with 31, 42 and 52 degrees of field of view (Extended 1, Extended 2 and Extended 3). Vessel Density (VD) is calculated by applying a threshold algorithm to the SCP, DCP and FR angiography *en face* images, resulting in a binary image where each pixel corresponds to a perfused or non-perfused area, 0 or 1, respectively. These images are then skeletonized to represent vessels by their centrelines (traces with 1-pixel width). VD is the average of the skeletonized image over the region of interest scaled by the distance between pixels.

The VD algorithm is expected to perform more poorly when the pixel sampling is lower as is the case for larger fields of view. For this reason, we do not expect the results from the swept-source

and spectral domain 3x3 mm scans to give identical results (different wavelengths, bandwidths, and sampling densities) [7], and we do not expect the density measured in the inner retinal area of the 15 x 9 mm scans to match what is measured in the more densely sampled 3 x 3 mm area. Data from the most densely sampled scan should always be expected to be closer to ground truth, but in the absence of adaptive optics we do not expect any commercial device to give a truly accurate estimate of the overall microvascular density. However, VD has been found to correlate with stage of disease even for less densely sampled scans, so we expect the measurements to be useful in a relative manner – data can be compared across populations and longitudinally, but should always be matched for the same scan and instrument type. Results obtained with Spectral domain OCTA (AngioPlex) and Swept Source OCTA (PlexElite) for the same eyes and area showed a Spearman correlation of 0.7 (Supplementary table 1).

All OCTA examinations underwent a quality check to discard acquisitions having a Signal Strength (SS) lower than 7, motion artifacts or evidence of defocus or blur in more than 25% of the area under analysis.

Retinal Thickness and layer segmentation

The structural data from the same SS-OCTA scanning protocols was also analysed by the "ETDRS Retinal Thickness v0.1" algorithm available on the ARI portal to calculate total retinal thickness values for each ETDRS standard areas. The average thickness values for the Ganglion Cell Layer + Inner Plexiform Layer (GCL+IPL) were calculated with "Multilayer Segmentation v0.3" algorithm also available on the ARI portal.

Colour Fundus Photography

Colour fundus photography was performed according to the ETDRS protocol using a Topcon TRC 50DX camera (Topcon Medical Systems, Tokyo, Japan). The DR severity score was determined by 2 independent graders in a context of an experienced reading centre (Coimbra Ophthalmology Reading Centre – CORC, Coimbra, Portugal) using a modification of the Airlie House classification scheme according to the Early Treatment Diabetic Retinopathy Study Protocol [4]. This severity scale comprises 8 severity levels of the retinopathy.

Statistical Analysis

Statistical analysis was performed using Stata 12.1 (Stata Corps. LP, College Station, TX, USA). Variables were summarized for healthy controls and for each ETDRS group, 10, 20, 35, 43 and 47 to 53 using mean and standard deviation. The Mann-Whitney U-test was performed to compare the statistically significant differences between VD metrics and retinal thickness for each of the

defined groups. Bonferroni correction was applied to VD comparisons setting the statistical significance to $p<0.0167$, otherwise $p<0.05$.

Results

One hundred and five diabetic patients, one eye per patient, either without retinopathy or with different degrees of retinopathy, were prospectively imaged for this study, using two acquisition protocols, 3x3 mm and 15x9 mm from the PlexElite.

Of the 105 eyes of diabetic patients, 16 eyes had no clinical evidence of retinopathy, 18 eyes were classified as ETDRS grade 20, 39 eyes were identified as grade 35, 17 eyes as grade 43 and 15 eyes were identified as grades 47-53. Thirty-eight healthy subjects were evaluated as control age-matched population. A diagram of the retinal locations examined, and representation of the areas sampled is depicted in Figure 1.

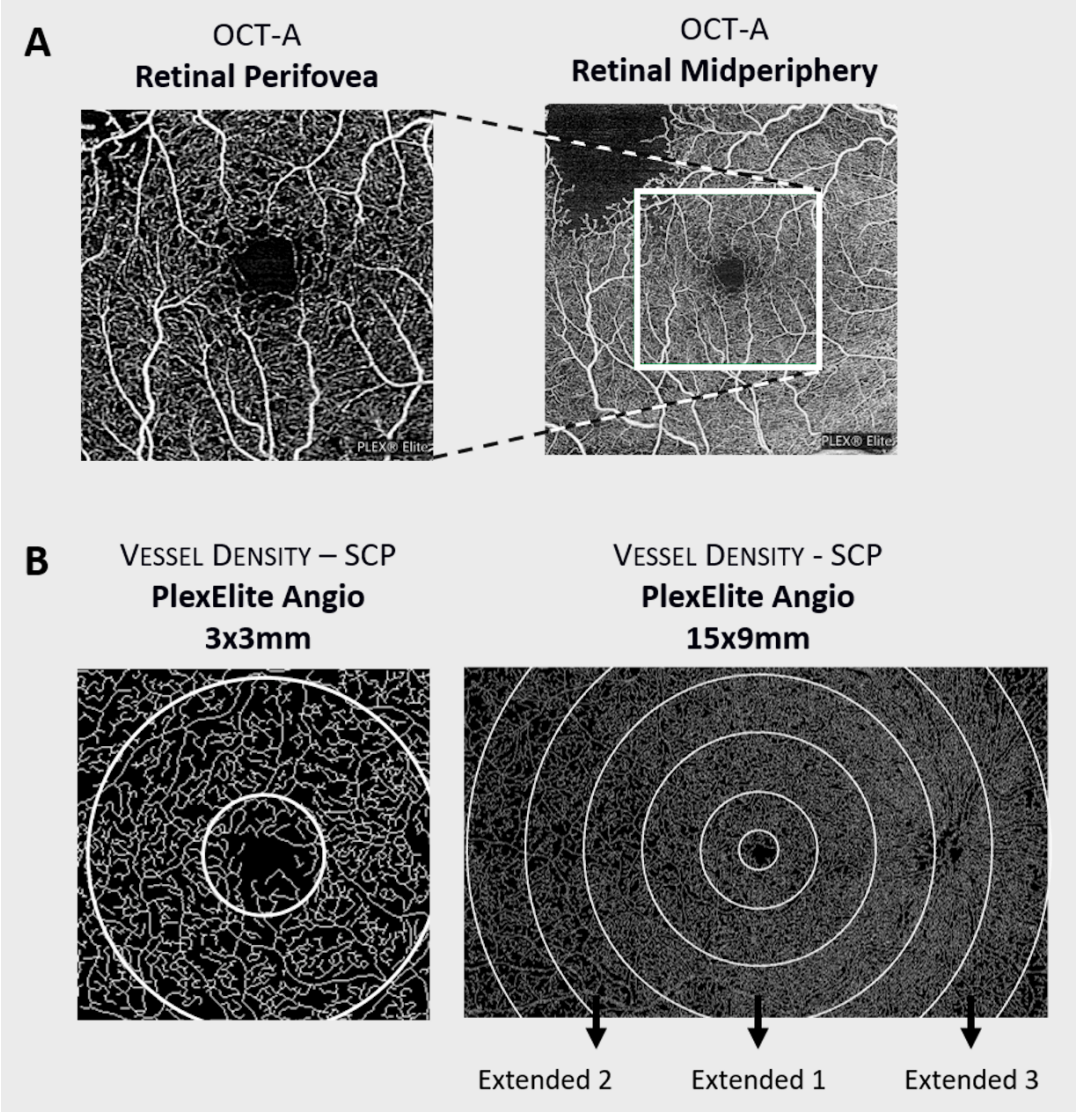


Figure 1: Representative diagram depicting retinal locations examined with OCT-A (perifovea vs mid-periphery) **(A)** and the sample areas acquired with PlexElite Angio 3x3mm and 15x9mm **(B)**.

Demographics of the population considering the distinct ETDRS levels and OCTA VD metrics are summarized in Table 1. The OCTA VD metrics are presented as the mean values (±SD) at inner ring quantified by PlexElite, using 3x3 mm acquisition protocol. The VD metrics in the superficial retinal capillary plexus (SCP), deep retinal capillary plexus (DCP) and full retina (FR) identify decrease in VD in all retinal capillary layers in individuals with diabetes. In the macular area, the best correlation between ETDRS grade of retinopathy and VD changes are obtained with the inner ring metrics, representing the parafoveal retinal capillary microvasculature. No significant differences were observed in the total retinal thickness, both at central and inner ring areas, between the different ETDRS grades.

Table 1: Baseline demographic characteristics and Angio 3x3mm vessel density metrics considering distinct DRSS stages of the disease

Demographics		**Healthy**	**ETDRS 10**		**ETDRS 20**		**ETDRS 35**		**ETDRS 43**		**ETDRS 47-53**	
N		38	16		18		39		17		15	
Age (years)		65.8 ± 10.2	68.8 ± 4.8		64.8 ± 7.0		66.9 ± 9.5		66.6 ± 7.9		65.4 ± 7.5	
Sex (Male/female)		20/18	9/7		10/8		28/11		13/4		10/5	
Diabetes duration (years)		--	18.5 ± 11.3		20.8 ± 13.8		21.6 ± 10.7		21.7 ± 5.9		16.2 ± 8.8	
HbA1c (%)		6.2 ± 1.0	7.1 ± 1.1		7.4 ± 1.3		77.6 ± 0.8		7.5 ± 1.3		8.1 ± 1.1	
BCVA (letters)		83.0 ± 4.3	83.4 ± 4.4		84.3 ± 6.3		84.2 ± 4.5		82.4 ± 6.8		80.1 ± 8.0	
Vessel Density SCPm mm^{-1}	(Inner Ring [5. 10]º)	14.7 ± 1.3	12.6 ± 2.5	**p=0.009**	13.2 ± 1.7	**p=0.005**	12.1 ± 2.0	**p=0.000**	12.2 ± 1.9	**p=0.010**	12.0 ± 1.3	**p=0.001**
Vessel Density DCP, mm-1	(Inner Ring [5. 10]º)	14.5 ± 1.5	11.9 ± 2.8	**p=0.003**	13.0 ± 1.6	**p=0.012**	12.9 ± 2.3	**p=0.014**	12.3 ± 2.5	p=0.045	11.6 ± 1.2	**p=0.001**
Vessel Density FR, mm^{-1}	(Inner Ring [5. 10]º)	17.9 ± 1.1	15.7 ± 2.8	**p=0.009**	16.6 ± 1.3	**p=0.008**	16.0 ± 2.0	**p=0.000**	15.4 ± 2.0	**p=0.010**	15.2 ± 1.4	**p=0.000**
Thickness Retina, µm	(Central SF [0. 5]º)	271.4 ± 20.6	276.3 ± 29.6	p=0.675	277.4 ± 38.3	p=0.952	282.2 ± 29.0	p=0.201	274.8 ± 23.4	p=0.677	305.0 ± 49.6	p=0.132
	(Inner Ring [5. 10]º)	332.8 ± 17.7	330.1 ± 12.7	p=0.646	331.7 ± 14.5	p=0.971	328.1 ± 17.2	p=0.509	333.4 ±17.8	p=0.850	346.3 ± 30.3	p=0.338

Data is presented as mean±SD. Vessel Density values, obtained using PlexElite 3mmx3mm, and Retinal thickness values were calculated using Zeiss MultiLayer segmentation algorithm. Comparisons between diabetic individuals and healthy controls were performed using Mann-Whitney U-test. Bonferroni correction was applied to Vessel Density comparisons setting the statistical significance to $p<0.0167$, otherwise $p<0.05$. Statistical significance highlighted in bold.
BCVA: Best Corrected Visual Accuity; HbA1c: Glycated Haemoglobin; VD: Vessel Density; IR: inner ring (5-10º); CSF: Central subfield (0-5º); SCP: Superficial Capillary Plexus; DCP: Deep Capillary Plexus; FR: Full Retina

Retinal midperiphery analysis of VD performed using the 15x9 mm protocol with the SS-OCTA PlexElite showed relevant information identifying involvement of more peripheral regions of the retina in the more advanced ETDRS stages of NPDR. Measurements with the 15x9 mm protocol were particularly discriminatory as the retinopathy progressed to the 43-53 stages. Particularly, in the extended regions 2 and 3 the ETDRS 43 and 47-53 groups presented average VD values that are lower that ETDRS 20-35 groups (Table 2). The thickness of GCL+IPL remained with normal range in the different retina regions examined along the different ETDRS severity stages of NPDR.

Table 2: Comparison between ETDRS levels using PlexElite 15x9 mm protocol

Metric	Acq. Protocol	Area	Healthy	ETDRS10		ETDSR20		ETDRS35		ETDRS43		ETDRS47-53	
Vessel Density SCP, mm^{-1}	PlexElite 15mm x 9mm	Inner Ring [5. 10]º	26.2 ± 5.9	23.2 ± 7.3	0.150	21.3 ± 8.6	0.053	22.1 ± 4.8	**0.000**	18.2 ± 10.0	**0.003**	22.9 ± 5.1	0.023
		Outer Ring [10. 21]º	29.1 ± 4.5	26.1 ± 6.2	0.030	25.3 ± 7.3	0.110	26.3 ± 3.5	**0.000**	22.4 ± 8.1	**0.001**	27.0 ± 4.7	0.063
		Extended 1 [21. 31]º	28.2 ± 3.1	25.5 ± 5.8	0.051	26.1 ± 5.5	0.333	26.9 ± 2.9	**0.016**	23.7 ± 6.5	**0.004**	26.9 ± 3.1	0.108
		Extended 2 [31. 42]º	23.2 ± 2.4	20.7 ± 5.6	0.150	21.3 ± 4.6	0.200	21.8 ± 3.3	0.063	19.6 ± 5.0	**0.005**	21.0 ± 2.7	**0.014**
		Extended 3 [42. 52]º	16.7 ± 3.7	16.2 ± 6.4	0.922	16.1 ± 6.3	0.857	16.9 ± 4.0	0.919	13.7 ± 6.1	0.134	15.1 ± 3.0	0.114
Vessel Density DCP, mm^{-1}	PlexElite 15mm x 9mm	Inner Ring [5. 10]º	23.8 ± 7.5	19.9 ± 8.2	0.097	17.9 ± 10.5	0.053	20.5 ± 6.0	**0.013**	16.7 ± 10.4	**0.016**	19.5 ± 6.2	**0.014**
		Outer Ring [10. 21]º	24.9 ± 7.2	20.8 ± 9.3	0.118	19.4 ± 10.6	0.079	20.8 ± 6.5	**0.011**	16.7 ± 10.1	**0.007**	19.4 ± 5.4	**0.005**
		Extended 1 [21. 31]º	22.4 ± 6.7	18.6 ± 9.3	0.164	18.1 ± 9.7	0.224	18.5 ± 6.4	**0.017**	14.4 ± 8.5	**0.003**	15.8 ± 5.0	**0.001**
		Extended 2 [31. 42]º	20.6 ± 6.0	17.5 ± 9.0	0.354	17.0 ± 9.3	0.242	17.1 ± 6.3	0.060	13.4 ± 7.8	**0.007**	13.6 ± 4.9	**0.000**
		Extended 3 [42. 52]º	17.3 ± 7.0	15.5 ± 9.3	0.643	15.0 ± 9.3	0.444	14.9 ± 6.7	0.269	11.2 ± 7.3	**0.014**	10.0 ± 4.0	**0.001**
Vessel Density FR, mm^{-1}	PlexElite 15mm x 9mm	Inner Ring [5. 10]º	30.8 ± 6.4	27.3 ± 8.2	0.067	25.2 ± 10.2	0.019	27.7 ± 5.2	**0.000**	22.4 ± 11.7	**0.003**	28.0 ± 5.3	**0.013**
		Outer Ring [10. 21]º	32.3 ± 4.7	29.1 ± 7.3	0.019	28.0 ± 8.1	0.038	30.1 ± 3.9	**0.000**	25.0 ± 9.7	**0.001**	30.3 ± 4.7	0.027
		Extended 1 [21. 31]º	31.0 ± 3.5	27.6 ± 6.7	0.020	28.2 ± 6.3	0.200	29.5 ± 3.4	**0.014**	25.2 ± 7.9	**0.001**	28.7 ± 3.2	**0.006**
		Extended 2 [31. 42]º	27.2 ± 3.3	24.1 ± 6.9	0.150	24.2 ± 5.9	0.105	25.5 ± 4.1	0.082	22.2 ± 6.4	**0.003**	23.5 ± 3.4	**0.001**
		Extended 3 [42. 52]º	20.9 ± 4.8	20.2 ± 7.6	0.884	18.9 ± 8.2	0.653	20.6 ± 5.3	0.909	16.6 ± 7.3	0.064	17.5 ± 3.6	0.020
Thickness GCL+IPL, µm	PlexElite 15mm x 9mm	Inner Ring [5. 10]º	91.5 ± 7.2	88.6 ± 10.5	0.407	90.6 ± 9.1	0.875	87.7 ± 9.3	0.151	87.0 ± 13.2	0.399	85.9 ± 9.1	0.059
		Outer Ring [10. 21]º	62.0 ± 4.3	60.5 ± 5.8	0.435	64.2 ± 5.9	0.301	62.7 ± 5.6	0.703	61.7 ± 7.5	0.981	61.6 ± 6.9	0.980
		Extended 1 [21. 31]º	43.7 ± 3.8	44.0 ± 3.6	0.678	45.1 ± 4.1	0.322	45.0 ± 3.3	0.147	45.0 ± 6.5	0.779	45.5 ± 4.9	0.177
		Extended 2 [31. 42]º	41.0 ± 4.1	43.8 ± 5.9	0.150	42.5 ± 3.7	0.163	44.3 ± 4.4	**0.003**	43.8 ± 8.1	0.223	41.5 ± 5.1	0.799
		Extended 3 [42. 52]º	35.3 ± 3.1	35.4 ± 5.1	0.542	37.4 ± 6.2	0.192	37.1 ± 3.1	0.058	36.3 ± 6.2	0.870	37.6 ± 5.1	0.161

Vessel Density and GCL+IPL thickness values were calculated using Zeiss PlexElite MultiLayer segmentation algorithm. Comparisons between diabetic individuals and healthy controls were performed using Mann-Whitney U-test. Bonferroni correction was applied to Vessel Density comparisons setting the statistical significance to $p<0.0167$, otherwise $p<0.05$. Statistical significance highlighted in bold and in grey cells.
SCP: Superficial Capillary Plexus; DCP: Deep Capillary Plexus; FR: Full Retina; GCL+IPL: Ganglion Cell Layer and Inner Plexiform Layer.

Combining the results of the protocols 3x3 mm and 15x9 mm, it was possible to distinguish two major groups of NPDR representing two major stages of NPDR progression: Group A including eyes with diabetes and either no ophthalmological signs of retinopathy (ETDRS 10) and eyes with mild retinopathy (ETDRS 20-35), and Group B, including eyes with ETDRS grades 43 to 53, showing increased retinal capillary closure in the retina midperiphery (Table 3). The increase in capillary closure of the more peripheral regions of the retina in the more advanced ETDRS stages involves predominantly the DCP.

Table 3: Comparison between ETDRS groups assembled by severity level

Metric	Acq. Protocol	Area	Healthy	Healthy vs ETDRS10-35	ETDRS10-35	ETDRS10-35 vs ETDRS43-53	ETDRS43-53
Vessel Density SCP, mm^{-1}	PlexElite 3mm x 3mm	Inner Ring [5, 10]º	14,7 ± 1,3	**0,000**	12,6 ± 1,9	**0,017**	11,9 ± 1,5
	PlexElite 15mm x 9mm	Extended 1 [21, 31]º	28,2 ± 3,1	0,018	26,4 ± 4,3	0,304	24,9 ± 5,5
		Extended 2 [31, 42]º	23,2 ± 2,4	0,047	21,5 ± 4,2	0,137	20,2 ± 4,3
		Extended 3 [42, 52]º	16,7 ± 3,7	0,976	16,5 ± 5,2	0,059	14,3 ± 5,1
Vessel Density DCP, mm^{-1}	PlexElite 3mm x 3mm	Inner Ring [5, 10]º	14,5 ± 1,5	**0,000**	12,7 ± 2,2	**0,003**	11,6 ± 1,6
	PlexElite 15mm x 9mm	Extended 1 [21, 31]º	22,4 ± 6,7	0,023	18,4 ± 7,9	0,046	15,1 ± 7,4
		Extended 2 [31, 42]º	20,6 ± 6,0	0,071	17,2 ± 7,6	0,032	13,6 ± 6,8
		Extended 3 [42, 52]º	17,3 ± 7,0	0,288	15,1 ± 7,9	**0,015**	10,9 ± 6,2
Vessel Density FR, mm^{-1}	PlexElite 3mm x 3mm	Inner Ring [5, 10]º	17,9 ± 1,1	**0,000**	16,1 ± 2,0	**0,002**	15,2 ± 1,4
	PlexElite 15mm x 9mm	Extended 1 [21, 31]º	31,0 ± 3,5	**0,009**	28,8 ± 5,0	0,062	26,5 ± 6,6
		Extended 2 [31, 42]º	27,2 ± 3,3	0,042	24,8 ± 5,3	0,030	22,7 ± 5,4
		Extended 3 [42, 52]º	20,9 ± 4,8	0,861	20,1 ± 6,6	**0,016**	17,0 ± 6,1
Thickness GCL+IPL, µm	PlexElite 3mm x 3mm	Inner Ring [5, 10]º	93,7 ± 8,2	0,324	88,2 ± 11,6	0,747	84,0 ± 21,4
	PlexElite 15mm x 9mm	Extended 1 [21, 31]º	43,7 ± 3,8	0,184	44,8 ± 3,6	0,897	45,3 ± 5,9
		Extended 2 [31, 42]º	41,0 ± 4,1	0,007	43,7 ± 4,6	0,188	43,1 ± 7,1
		Extended 3 [42, 52]º	35,3 ± 3,1	0,167	36,8 ± 4,5	0,787	37,0 ± 5,9

Data is presented as mean±SD. Vessel Density and GCL+IPL thickness values were calculated using Zeiss PlexElite MultiLayer segmentation algorithm. Comparisons between diabetic individuals and healthy controls were performed using Mann-Whitney U-test. Bonferroni correction was applied to Vessel Density comparisons setting the statistical significance to $p<0.0167$, otherwise $p<0.05$. Statistical significance highlighted in bold and in grey cells. SCP: Superficial Capillary Plexus; DCP: Deep Capillary Plexus; FR: Full Retina; GCL+IPL: Ganglion Cell Layer and Inner Plexiform Layer.

Discussion

In previous reports we have demonstrated that SD-OCTA is able to identify and quantify retinal capillary closure in the initial stages of diabetic retinal disease [7,11,12]. These studies were focused on the initial stages of DR identifying changes in the parafoveal retinal circulation. Now, in this study, retinal capillary closure was analysed in diabetic eyes of different disease severity grades using also widefield (15x9 mm) SS-OCTA images. Our results show that there is a statistically significant increase in the retinal capillary closure in more peripheral regions of the retina as DR severity increases, particularly in ETDRS grades 43-53, an observation that confirms the findings of a recent study using 12x12 mm SS-OCTA images [9]. Retinal capillary closure is measured with OCTA skeletonized vessel density and interpreted as representing individual capillaries that are either absent or have red cell blood flow below the threshold of detection.

Retinal capillary closure was found to be increased in the eyes of persons with type 2 diabetes evaluated in this study, even in eyes with no retinal changes detected by ophthalmoscopy, i.e., ETDRS grade 10, confirming previous reports [13]. In eyes with DR retinal capillary closure increases in parallel with retinopathy severity [12], and this progressive increase is better identified in the perifoveal region of the macula in the initial stages of the retinopathy. Involvement of the retinal midperiphery becomes dominant only in more advanced stages of the retinopathy, ETDRS grades 43-53. Fluorescein angiography was not performed in this study because it has been shown in previous work by our group than is clearly less effective in determining capillary closure that OCTA [14].

Relevant and valuable information about the presence of retinal capillary closure in NPDR is, therefore, obtained by collecting data with the SS-OCTA, which allows data sampling using both protocols, 3x3 mm and 15x9 mm, and thus obtaining information from the parafoveal region and retinal midperiphery.

Our study suggests that capillary closure occurs very early in diabetic retinal disease and is initiated in the macula. It is only later on, as the disease progresses with remodelling of the retinal circulation and an altered retinal blood flow distribution probably through preferential arteriovenous preferential channels that capillary closure develops in more peripheral regions of the retina [15].

Our study shows that the use of SS-OCTA and a combined protocol, 3x3 mm and 15x9 mm allows discrimination between different ETDRS grades, identifying two main stages of NPDR, mild versus severe.

The ETDRS classification is based on the presence of different features, as microaneurysms, hard and soft exudates, venous loops, etc, identified by fundus photography. These alterations are considered to be related to the presence of capillary closure and ischemia [16]. It is, therefore, reasonable to think that capillary closure, if reliably quantified and measured, may be an appropriate indicator of changes identified by fundus photography and become an alternative to the ETDRS classification.

Considering that only 2-step changes in ETDRS severity grading have been shown to be clinically significant, we propose that ETDRS groups assembled using in group A, grades 10-35 and in group B, grades 43-53, corresponding to no and mild retinopathy in group A and moderate to severe in group B, is a promising approach and may represent objectively and more accurately the real progression of NPDR. The use of SS-OCTA, with combined protocols, capable of detecting early capillary closure in the perifoveal region and later capillary closure in the midperiphery, may offer, therefore, a simple to use alternative to the laborious and demanding ETDRS grading process which classifies a complex set of parameters. The results here reported give, indeed, a practical basis for the International Classification of Diabetic Retinopathy [17]. Hence, we propose a numerical threshold to separate mild NPDR from moderate and severe NPDR. Skeletonized VD in the SCP (Inner Ring protocol, 3x3mm) lower than 13 (mean of healthy controls less 1SD) and higher than 10 (mean of healthy controls less 1SD) in the DCP midperiphery (protocol 15x9, extended area 3) identifies mild NPDR. On the other hand, skeletonized VD lower than 13 in the SCP (Inner Ring protocol, 3x3 mm) and lower than 10 in the DCP midperiphery (protocol 15x9, extended area 3) identifies moderate and severe NPDR (respectively AUC: 0.83; 0.63; supplementary figure 1). This identification is particularly relevant for clinical practice as mild NPDR has a five-year incidence of vision-threatening complications of approximately 10%[18] much lower than the five-year incidence of moderate and severe NPDR[19].

This study confirms that regional distribution of the DR lesions in the retina may reflect risk factors and may be important in defining the stage of diabetic retinopathy[20,21]. Capillary closure in the midperiphery in a diabetic retina is indicative of an advanced stage of retinopathy, whereas capillary closure limited to the perifovea suggests a milder stage of the disease. Furthermore, regional distribution of DR lesions has led Hove et al [8] to suggest that future improvements to

existing grading systems should focus on the quantification of overall retinopathy lesions in the early stages and the regional distribution of retinopathy lesions in the more advanced stages retinopathy. Red blood cell flow in retina and brain capillaries has been shown to be redistributed between thoroughfare channels, capillaries with high resting velocity and exchange capillaries, capillaries characterized by low resting velocity [22]. Pre-existing preferential arteriovenous connections may act as shunts that bypass the occluded retinal microcirculation [23–25]. These concepts are in line with our findings, again emphasizing the potential of OCTA nonperfusion metrics to identify DR progression.

The location of retinal capillary closure in the different retinal capillary layers in the initial stages of DR has been a matter of controversy [7,11]. Some authors have found evidence for initial retinal non-perfusion changes in the SCP, whereas others found that the DCP is the first retinal capillary plexus to show nonperfusion [26]. The division and separation of the retinal capillary network in two, three or four layers has also been a matter of controversy [27,28]. In this study retinal capillary closure predominates initially in the SCP with progressive later involvement of the DCP. The involvement of the DCP becomes predominant as the DR progresses in severity. This finding may have important implications to our understanding of the pathophysiology of DR. It must be taken into account, however, that the actual segmentation of the retina in different OCTA equipment is different which may explain the different findings of different research groups. It may be that for clinical purposes, the best option is to quantify in a way that is independent of the segmentation, i.e. using the full retina instead of some definition of the superficial or deeper layers.
The GCL+IPL shows similar degrees of thinning i.e., neurodegeneration, in eyes with different degrees of nonperfusion. It is of particular interest to register that the increase in nonperfusion present in the more peripheral regions of the retina, in more severe stages of the retinopathy occurs without increased thinning of the GCL+IPL demonstrating that the nonperfusion is associated with an ischemic situation and is not the end result of retinal tissue atrophy.

Our study has several limitations. The sample size is relatively small with relatively few patients having severe NPDR, in contrast with a previous report using widefield images with SS-OCTA[9]. Also, the 15x9mm field of view, while widefield in the context of previously reported OCTA imaging is still a relatively small field compared with widefield fluorescein angiography imaging, and more information may therefore be obtained when more peripheral retina images are collected. However, our study has also important advantages over previous reports. The distribution of eyes is balanced across the different ETDRS grades. The eyes examined are naïve eyes that had not been previously submitted to any local treatment such as laser or steroid or anti-VEGF intravitreal

injection. We also examined the different retinal capillary plexus, superficial and deep, as well as the full retina, to account for projection artifacts.

In conclusion, we have performed a study combining regional examination of the retinal circulation, including the macula region and the retinal midperiphery, in patients with type 2 diabetes in eyes with no retinopathy and different ETDRS grades of NPDR. The study shows that retinal capillary closure is an early finding in the macular area of the diabetic retina and that this alteration increases as the retinopathy progresses in severity involving later more peripheral regions of the retina. Furthermore, SS-OCTA metrics of retinal capillary closure, allowing measurements to be performed in the macula and in more peripheral regions of the retina, may offer an objective and easier to perform alternative to ETDRS severity grading.

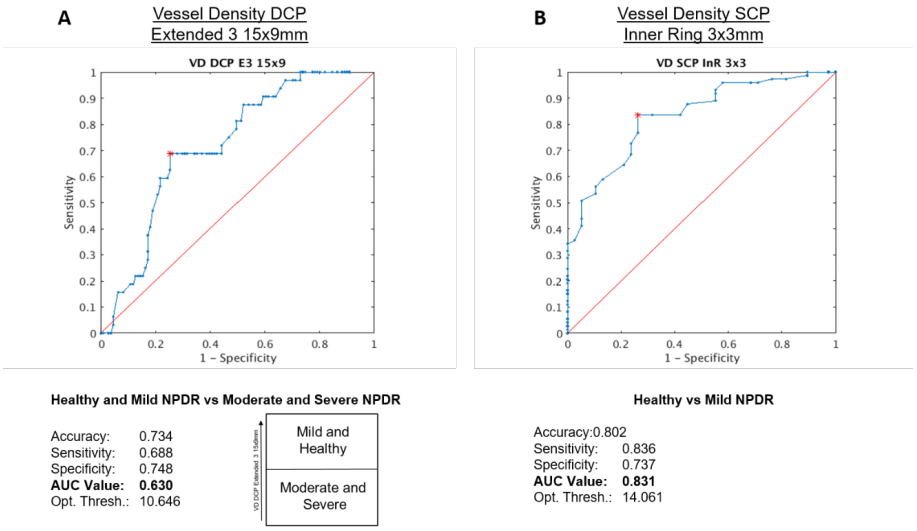
Supplementary table 1: Spearman correlation coefficient between AngioPlex and PlexElite Vessel Density measurements in all individuals and NPDR.

			ALL NPDR			ETDSR20			ETDRS35			ETDRS43		
			Central SF [0, 5]º	Inner Ring [5, 10]º	Circle 3mm [0, 10]º	Central SF [0, 5]º	Inner Ring [5, 10]º	Circle 3mm [0, 10]º	Central SF [0, 5]º	Inner Ring [5, 10]º	Circle 3mm [0, 10]º	Central SF [0, 5]º	Inner Ring [5, 10]º	Circle 3mm [0, 10]º
Vessel Density	SCP	Spearman Coef.	**0,70**	**0,73**	**0,72**	0,59	**0,78**	**0,71**	**0,77**	**0,66**	**0,68**	**0,65**	**0,92**	**0,92**
		P-value	0,00	0,00	0,00	0,05	0,00	0,01	0,00	0,00	0,00	0,24	0,03	0,02
	DCP	Spearman Coef.	0,54	0,55	0,53	**0,60**	0,22	0,28	0,56	0,59	0,57	0,02	**0,78**	**0,74**
		P-value	0,0009	0,0006	0,001	0,05	0,52	0,40	0,01	0,01	0,01	0,97	0,12	0,15
	FR	Spearman Coef.	**0,76**	**0,72**	**0,72**	**0,72**	0,54	0,49	**0,88**	**0,75**	**0,77**	0,26	**0,95**	**0,94**
		P-value	0,0	0,0	0,0	0,01	0,09	0,12	0,00	0,00	0,00	0,68	0,01	0,02

Correlations between AngioPleX and PlexElite Vessel Density measurements were performed using Spearman Coefficient. Statistical significance ($p<0.05$) highlighted in bold. Strong and Very Strong correlations in grey cells. SCP: Superficial Capillary Plexus; DCP: Deep Capillary Plexus; FR: Full Retina

Supplementary Figure 1: ROC curve for Vessel Density values in the DCP and SCP.

A. Represents VD values in the DCP extended region area 3 using protocol 15x9 mm, correlating Health and Mild NPDR with Moderate and Severe NPDR.
B. Represents VD values in the SCP Inner Ring using protocol 3x3 mm, correlating Health and Mild NPDR.



Values for accuracy, sensitivity, Specificity, AUC and Optimal Threshold are depicted.
DCP: Deep Capillary Plexus; SCP: Superficial Capillary Plexus; NPDR: Non proliferative diabetic retinopathy; AUC: Area under the curve

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Chapter 9

Discussion and General Considerations

The estimated number of people (20–79 years) living with diabetes has increased by 62% during the past 10 years (1). It is thought that, for type 2 diabetes, which accounts for approximately 90% of the total of cases, this rising trend can be attributed to ageing, a rapid increase in urbanization and obesogenic environments (2). Fifty percent of diabetic patients are unaware of their condition (3).

Diabetic retinopathy (DR) remains a major cause of blindness among working-age population and diabetes prevalence is expected to double between 2000 and 2030. Diabetic Retinopathy affects around 30% of the diabetic population. Data collected from 22.896 individuals from 35 studies in the US, Australia, Europe and Asia, showed that the overall age-standardized prevalence of any DR was 34.6%, proliferative DR was 7.0%, diabetic macular edema was 6.8% and vision threatening DR was 10.2% (4). The economic and social impact of visual impairment is enormous, also because 3 in 4 people suffering from visual impairment are in the working-age group (5).

Diabetic retinopathy has been considered as a microvascular disease, where vascular lesions begun in the small vessels, with endothelium proliferation, pericyte damage and microaneurysm formation. Clinically, microaneurysms and small hemorrhages are the first lesions to be seen in the fundoscopic examination.

According to some studies, at the time lesions are seen on clinical examination, there is already damage of the neuroglial tissue, responsible for maintenance of the normal equilibrium and adaptation of the retina to the changes of the diabetic microenvironment (6). At that time, patients may already experience some symptoms related to the neural retinal dysfunction, such as loss of chromatic discrimination, contrast sensitivity and dark adaptation, detected on electrophysiological studies (7).

The disease progresses slowly over time and goes unnoticed until the development of sight threatening complications, clinically significant macular edema (CSME), the most frequent, and proliferative diabetic retinopathy (PDR). The evolution of DR occurs at different rates in different individuals and only a small proportion of them develop significant visual impairment. The current treatment regimens, going from anti-VEGF intravitreal injections, corticosteroids or laser treatment, are only indicated when those complications arise. Furthermore, at this advanced stages, some eyes, for unknown reasons, tend to have a poor response to treatment.

Identifying the eyes/patients at risk of progression to sight-threatening complications and visual loss was the main objective of this thesis. Understanding the systemic risk factors that influence the development of the retinopathy is of utmost importance in order to stratify the patients according

to the risk of developing such complications. The reason why only few patients develop retinal disease and progress to vision loss is the crucial question that needs to be answered in order to fully understand the evolution of disease. The identification of retinal imagiological biomarkers that allows the clinician to predict the eyes that will evolute faster is fundamental for appropriate follow up of patients and earlier detection of complications, in order to avoid vision loss. Furthermore, the comprehension of the disease pathways and the pathophysiology behind the retinal damage and their progression may open the way for new treatments, in the very early phases of DR, delaying the progression of the disease before complications occur.

Current non-proliferative DR classifications are limited, with no sufficient discriminative power to predict the individual risk of developing sight-threatening complications. The standard stepwise changes on DR severity scale and best-corrected visual acuity evaluation, even when stratified by traditional systemic risk factors (such as HbA1c levels, diabetes duration, hypertension and hyperlipidemia), are not enough to characterize the full picture and predict future developments.

Natural history studies, with long follow up and with a good morphological and functional characterization of the disease evolution are essential to fully characterize these non-proliferative diabetic retinopathy profiles.

Diabetic Retinopathy is a multifactorial disease. Different factors or different disease pathways may predominate in different subjects. The incidence of DR increases with the duration of diabetes and with a poor metabolic control (8)(9). Other factors have been identified with more conflicting results. A variety of imaging biomarkers have been identified.
Microaneurysm turnover (MAT) has been validated as a prognostic biomarker of development of clinically significant macular edema. Subclinical macular edema (SCME), identified by OCT, appears to be also a good candidate as biomarker of DR (10).
Three different DR phenotypes (phenotype A, B and C) were characterized by our group, with different dominant retinal alterations and different risks of progression to vision-threatening complications (11).

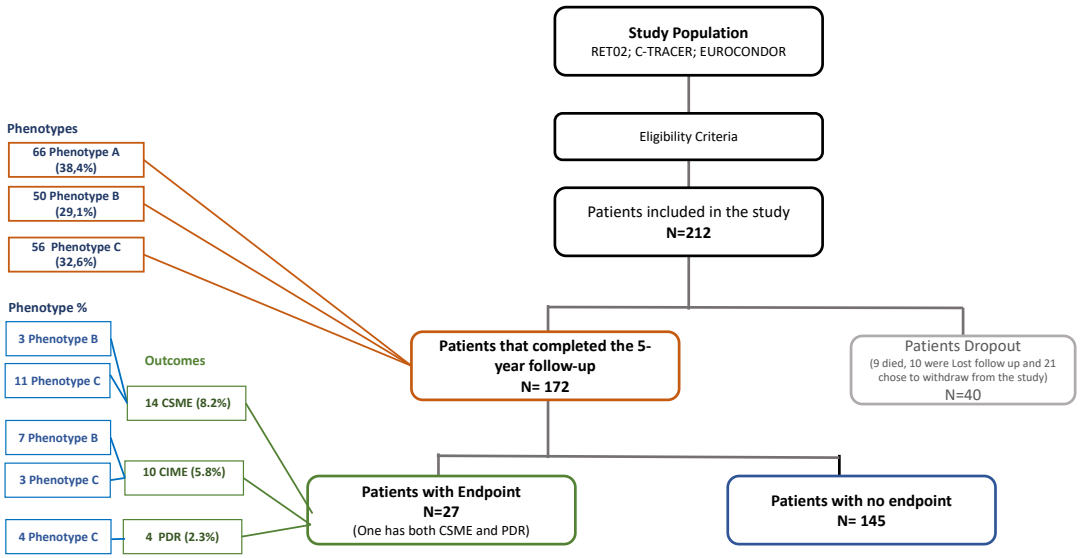
In this work we characterized a cohort of two hundred and twelve patients with type 2 diabetes and no or mild NPDR in a prospective longitudinal observational study (ClinicalTrials.gov identifier: NCT03010397), with annual examinations, for 5 years of follow up or until the development of vision threatening complications, at AIBILI, Coimbra.

The inclusion criteria were: Type 2 *diabetes mellitus*, aged > 35 years old at baseline, no or mild NPDR (levels 10-20 or 35, according to ETDRS classification), best corrected visual acuity (BCVA) ≥ 75 letters.

The exclusion criteria included: inadequate ocular media or pupil dilation that does not allow good quality fundoscopic images, any previous laser treatment or intravitreal injections, or presence of age-related macular degeneration, glaucoma, vitreomacular disease or high ametropia (spherical equivalent greater than -6 and +2 DPT); HbA1c > 10% or any other systemic disease that could affect the eye, with special attention to uncontrolled systemic hypertension. Eyes with central retinal thickness (CRT) values identifying center involved macular edema (CIME) based on DRCR.net criteria (12) at baseline were also excluded from the analysis.

A complete ophthalmologic examination with BCVA was performed annually, as well as 7 field and Field 2 at 50º Color Fundus Photography (Topcon TRC 50DXTM®)) for DR ETDRS level determination and MA turnover (MAT) assessment (RetMarkerDR®)), spectral domain Optical Coherence Tomography (Cirrus HD-OCT 5000, Carl Zeiss Meditec, Dublin, CA, USA) and OCT-Angiography (Cirrus HD-OCT 5000 AngioPlex®, Carl Zeiss Meditec, Dublin, CA, USA), from the moment that the machine was available. Phenotype classification were performed at 6 months of follow up, based on the CRT on SD-OCT and on MAT. Briefly, Phenotype A is characterized by low MAT (< 6) and normal CRT; Phenotype B by low MAT (< 6) and increased CRT and Phenotype C by higher MAT (≥ 6) with or without increased CRT.

Figure 1: CONSORT flowchart



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

1. Phenotype C identifies eyes at higher risk for development of CSME and PDR, whereas phenotype A identifies eyes at very low risk for vision-threatening complications.

In our first paper (CHAPTER 2), we could confirm the prognostic value of the previous described phenotypes in a 5-year follow up study (11). One hundred and seventy two eyes from the 212 included eyes/patients completed the 5 years of follow-up or developed vision threatening complications.

From the included population, 66 (38%) eyes were categorized as phenotype A, 50 (29%) eyes identified as phenotype B and 56 (33%) eyes classified as phenotype C. Higher levels of HbA1c and triglycerides were present in Phenotype C patients at baseline, when compared to phenotypes A and B. Microaneurysm turnover were significantly increased in phenotype C and CRT was increased in phenotype B when compared to the others phenotypes. Phenotype A is the phenotype associated with the increased thinning of the ganglion cell layer-inner plexiform layer (GCL - IPL) in the central subfield. Phenotype C was associated with more advanced stages of retinal disease at baseline (97% of cases were ETDRS level 35).

Twenty-seven eyes (16%) developed one study outcome: 14 patients with clinically significant macular edema (CSME), 10 with center involved macular edema (CIME), 4 with proliferative diabetic retinopathy (PDR), one eye with both CSME and PDR. The risk of developing such complications varies widely according to the phenotype group. Phenotype C, which comprises near 30% of the included subjects, was the phenotype with higher rate of complications, including 79% of the eyes that developed CSME and all of the cases that developed PDR. Phenotype C presents, in a multivariate analysis, an OR of 17.4 for CSME development, when compared to the other phenotypes. Phenotype A showed no association with development of vision-threatening complications.

Increased values of MAT and phenotype C, independent of CRT values, appear to identify the eyes that will progress and develop vision-threatening complications such as CSME and PDR, which are not expected to improve without intervention.

The association of increased values of MAT with CSME highlight to the potential relevance of capillary closure and ischemia in the process of CSME development. CIME, on the contrary, may not be necessarily a predictive factor for CSME and doesn't share the same risk factors. Its higher

frequency in phenotype B is probably related to the defined baseline CRT characteristics of this phenotype group.

The study suggests that specific non-proliferative retinopathy patterns may influence the individual risk of DR progression, independently of the systemic characterization and individual characteristics. This is a step forward in the knowledge about the retinal diabetic disease.

2. Phenotype C is associated with increased HbA1c values and presents a higher risk of worsening of the ETDRS severity score, with 23% patients worsening 2-or-more-steps.

The incidence of the considered clinically significant endpoints (e.g. PDR or CSME) is very low in patients with mild NPDR, making clinical trials very long in time and needing a very high number of participants. Other clinically meaningful measures have been proposed as primary endpoints for disease progression, such as 2 or 3-step progression on the ETDRS DR severity scale. These endpoints will allow to find a higher number of patients with progression.

In the second paper presented in the thesis (CHAPTER 3), we confirm the significant differences in diabetic retinopathy (DR) progression between the different identified phenotypes and their predictive value for worsening of DR severity level. From the eyes included in the study, 58 (27%) were graded as ETDRS level 20 and 154 (73%) graded as ETDRS level 35. During the 5 years of follow up, the vast majority of eyes maintained the ETDRS classification or change level in a 1-step interval (n=145, 84%). Of the 63 participants with phenotype A only 2 eyes (3%) presented 2-or-more-step worsening and none of the 53 participants characterized as phenotype B, whereas 13 of the 56 eyes (23.2%) characterized as phenotype C had 2-or-more-steps worsening at five years of follow up. Phenotype C presents a 16-fold higher risk for 2-or-more-steps worsening (OR 15.94; 95% CI: 3.45-73.71; $p<0.001$) and a higher sensitivity, correctly identifying 86.7% of cases at risk (AUC: 0.84 95% CI: 0.72-0.96; $p<0.001$).

HbA1c values present a continuous increase in values from 2-steps improvement to 2-or-more-steps worsening. No other demographic or systemic clinical characteristics appear to influence the DR ETDRS level progression. Ocular factors such as MAT, MA formation and disappearance rates, seems to be good indicators of DR progression, with higher values present in the group of 2-or-more-steps worsening. However, MAT could not increase the sensitivity of the systemic markers alone model.

Vessel density (VD) obtained from the OCTA at 5 years of follow up, representing capillary closure, showed a progressive decrease in all ETDRS levels and was also associated with DR severity progression.

A large group of eyes/patients, phenotypes A and B, which combined represent 70% of the entire cohort, are at a very low risk for 2-or-more-steps ETDRS severity score worsening (3%). This observation is relevant for appropriate planning and management of patients.

3. The risk of developing vision-threatening complications was associated with ocular risk markers such as MAT and CRT whereas HbA1c remained the most relevant systemic marker identified.

In the third paper presented in the thesis (CHAPTER 4), we further explored the risk of both systemic and ocular factors in the development of CSME, CIME and PDR, the vision-threatening complications of DR.

Analysis of the demographic and systemic characteristics revealed that patients that developed CSME and/or PDR, presented lower age, lower body mass index (BMI) and higher HbA1c values. Regarding the ocular characteristics, statistically higher values of MAT were identified in patients that developed CSME and PDR and higher CRT values were present in patients that developed CIME and CSME. Among these risk factors only HbA1c is correlated specifically with one phenotype, phenotype C.

In a multivariate analysis including all the relevant demographic and systemic characteristics, the risk factors found to be important in development of CSME were MAT, CRT and GCL-IPL thickness in the central subfield (CSF). For the development of CIME, the important risk factors were CRT and GCL-IPL thickness in the central subfield.

Among the systemic factors used for adjustment of the risk of each ocular marker, age was consistently a significant confounder, with risk reduction of 11-17% per unit increase. Body mass index was also associated with risk reduction in association with MAT and GCL-IPL CSF thickening. For CIME, only the baseline CRT and GCL+IPL thickening were associated with risk increase.

The positive correlation of complications' development and HbA1c and negative correlation found with BMI are in agreement with the literature which depicts the benefits of good glycemic and blood pressure control and found an inverse correlation with obesity (8)(13)(14).

We did not find an increased neurodegeneration (represented by GCL-IPL thinning) in eyes that developed complications. Actually, GCL-IPL thickening was observed in those eyes. We hypothesize that this fact could be justified by the increased thickness of the total retina by the

accumulation of fluid frequent in the more advanced stages, that may mask some decreases in the most inner retinal layers.

The study shows that eyes with mild retinopathy in persons with T2D, identified as phenotypes A and B, with MAT lower than 6 and with HbA1c equal or lower than 7%, present a very low likelihood of developing vision-threatening complications, CSME or PDR, in a period of 5 years. On the other hand, an eye with mild retinopathy in a patient with T2D, identified as phenotype C, with MAT equal or higher than 6, indicating increased microvascular disease activity, shows high likelihood of development of vision-threatening complications such as CSME and PDR.

Our study shows, once again, the limited role of systemic risk markers in prediction of development of vision-threatening complications of DR, in type 2 diabetes patients.

The study population here presented is relatively small and with a small number of eyes that developed the endpoints of interest, which makes the finding of significant risk factors difficult. Furthermore, given the pre-specified exclusion criteria, such as excessive HbA1c levels and uncontrolled blood pressure, this cohort represents a well-controlled and uniform population.

There has been an attempt to objectively identify DME and consider that both CIME and CSME represent basically the same disease process, with edema resulting from excessive accumulation of extracellular fluid in the retina (15). Our findings, in this 5-year study, suggest that actually CIME and CSME represent different disease processes and that they should not be added up when referring to DME. CIME and CSME are associated with different retinopathy phenotypes and with different ocular risk markers, with a distinct evolution. Retinal thickness measurements, indicating the presence of edema, are the only risk marker that is present in both CIME and CSME. We think that CIME can actually resolve or stabilize, not confirming previous observations that it may be a predictor for development of clinically significant macular edema (16).

4. Eyes within the same ETDRS level of retinopathy present different disease pathways (ischemia, neurodegeneration and edema). Ischemia (capillary dropout) is the only disease pathway that shows correlation with retinopathy severity and metabolic control.

In the fourth paper (CHAPTER 5), we presented a cross-sectional analysis of 142 eyes from 142 patients in the initial stages of diabetic retinopathy. In this study, eyes were grouped by diabetic ETDRS levels 10-20, 35 and 43-47. We evaluated the prevalence of different disease pathways (ischemia, neurodegeneration and edema) by SD-OCT (Cirrus HD-OCT 5000, Carl Zeiss Meditec, Dublin, CA, USA) and OCTA (Cirrus HD-OCT 5000 AngioPlex®, Carl Zeiss Meditec, Dublin, CA, USA). Ischemia was identified by decreased retinal vessel density (VD) metrics, in the superficial (SRP), deep (DRP) retinal vascular plexuses and/or full retina (FR). Edema was identified by thickening of the inner nuclear layer (INL), outer plexiform layer (OPL) or FR. Neurodegeneration was identified by thinning of the retinal nerve fiber layer (RNFL) and/or ganglion cell-inner plexiform layer (GCL-IPL). Concerning the baseline characteristics of eyes in each ETDRS level, the only significant difference was in HbA1c levels, reflecting an association between poorer metabolic control and more advanced stages of the disease. In univariate analysis, vessel density in the SRP and DRP was significantly different between ETDRS groups, with eyes in a more severe stages of the disease more likely to have reduced vessel density. The foveal avascular zone (FAZ) circularity index was also significantly reduced in ETDRS group 43-47. This remains significant even after having adjusted for age, sex, HbA1c, visual acuity and diabetes duration, showing significant associations with age, sex and metabolic control.

No significant differences were found in RNFL and GCL-IPL thinning (neurodegeneration) or in inner nuclear layer (INL) and outer plexiform layer (OPL) thickness (edema) between ETDRS groups. Analysis of the variables representative of capillary dropout, retinal edema and retinal neurodegeneration showed a wide range of values in each ETDRS grade, demonstrating that there are very different degrees of these changes in eyes from different patients within the same retinopathy severity grade. Definite RNFL or GCL-IPL thinning was found in 24%, 28% and 21% of the eyes in groups 10-20, 35 and 43-47, respectively; definite increases in INL or OPL thickness were noticed in 35%, 35% and 38% for the ETDRS groups 10-20, 35, and 43-47, respectively; and definite decreases in retinal VD were present in 22%, 44% and 59% of the eyes in groups 10-20, 35 and 43-47, respectively. Only microvascular measurements showed correlation with disease severity.

Furthermore, the different disease pathways appear to occur independently, with no association or correlation found between these different structural alterations.

This conclusion supports the concept of three major pathways of disease occurring in type 2 diabetes patients.

Similar findings were also reported by Durbin *et al* (Figure 2) in a smaller sample size (17).

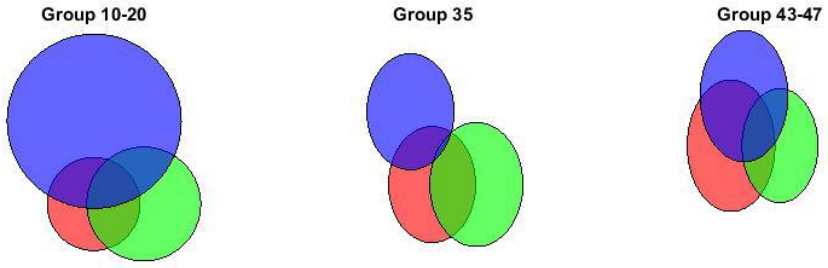


Figure 1: Venn diagrams for eyes with possible RNFL loss (blue), likely VD loss (red), and likely edema (green) (13)

At levels 10-20, capillary closure was seen in 18% of eyes, compared to 40% in group 35 and 67% in group 43-47, demonstrating the generalized occurrence of capillary closure at 43-47 level. Likely edema was seen in 27% of eyes in group 10-20, 40% in group 35 and 33% in group 43-47. There were no eyes with RNFL thinning, but 64% of eyes showed possible thinning in group 10-20, compared to 35% in group 35 and 33% in group 43-47. The categories did not correlate with each other, as seen in the Venn diagrams in Figure 1. Only microvascular measurements showed correlation with disease severity (13).

Capillary closure may, indeed, contribute to the relative decrease in the occurrence of retinal edema in the more advanced ETDRS grades. Thinning of retinal nerve fiber layer (RNFL), compatible with neurodegeneration, was detected in a relatively large percentage of eyes since initial DR ETDRS levels, but it does not appear to increase in parallel with retinopathy severity. Retinal neurodegeneration appears independently of the microvascular changes, although it may still have a role as a trigger to the microvascular pathology, as it is described by other groups (18). The definite evidence of capillary closure, with the metrics available, was registered in both SRP and DRP but was detected earlier in the SRP, contradicting the findings reporting in other studies that described a more pronounced alteration in the DRP metrics, preceding the changes in the SRP (19). This fact can be explained by the utilization of different OCTA machines, using different algorithms and segmentation slabs, with Zeiss Cirrus Angioplex® presenting a better discriminative value for superficial capillary retinal plexus and Optovue RTVue XR Avanti® being more discriminative for the deep capillary retinal plexus.

Our observation that different eyes in the same ETDRS severity level show different predominant disease pathways confirms previous studies suggesting that eyes from different patients may have different phenotypes of disease progression (20).

5. Capillary dropout increased in a period of 2-years in eyes with minimal, mild and moderate DR, whereas the presence of edema and neurodegeneration remained stable.

In the fifth paper presented (CHAPTER 6), we performed a longitudinal follow up study of 62 eyes from 62 patients with diabetes, for two years of follow up. Forty four eyes had sufficient quality for being considered in the analysis. Eighteen eyes were classified as ETDRS grades 10-20, 17 eyes as ETDRS grade 35 and 9 eyes as ETDRS grades 43-47. No statistically significant systemic differences were found between eyes/patients within different ETDRS stages of the disease, except for HbA1c, which present higher levels in eyes with ETDRS level 43-47. The vessel density in the SCP and the FAZ circularity index were significantly different between ETDRS groups, with a decrease in more advanced disease. The FAZ area, as reported in other previous studies (21), was not sufficiently discriminative to show any differences between groups. Vessel density remained significantly different between ETDRS groups in multivariate analysis adjusted for age, gender, HbA1c, visual acuity and diabetes duration. This finding confirms that one of the earliest changes associated with diabetic retinopathy is a decrease in retinal blood flow (22). During the 2-year follow-up there was a progressive increase in capillary dropout, whereas edema (CRT) and neurodegeneration (RNFL and GCL-IPL thickness) remained stable.

In each ETDRS group, values for capillary dropout (reduced vessel density), edema and neurodegeneration covered a wide range, identifying different levels of damage in different eyes. This fact was already reported in our previous paper (CHAPTER 5).

The presence of capillary dropout was evidenced by a reduction in vessel density in SCP, DCP and FR and showed statistically significant differences at one and two-year intervals, with a progressive decrease during the follow up. The capillary dropout was more apparent and reliable in the SCP, particularly in the initial stages of DR. It is possible that capillary drop out begin at SCP level and generalize with time to the DCP, like Durbin and colleagues have suggested (23). Other possible explanation is the presence of limitations in the measurement methodology of the DCP with projection artifacts affecting this deep layer. Possibly the methods used for calculating DCP capillary density metrics may need to be refined, as suggested by Rosen *et al* (24)(25). The analysis of the full retina and layer thicknesses showed that neurodegeneration and edema remained

generally stable during the two-year of follow up. Neurodegeneration was identified by RNFL and/or GCL-IPL thickness decrease, using the segmentation software implemented by our group in order to improve retinal layers segmentation and layers thicknesses calculation, performed on the structural OCTA. The use of automated layer segmentation analyses for measuring retinal thicknesses could be a limitation of the study. However, the almost normal thickness of the studied eyes with no major morphologic abnormalities makes the automatic segmentation less prone to segmentation errors and artifacts and is a more robust measurement in comparison to manual procedures.

There was a higher percentage of decrease in vessel density in the eyes that showed ETDRS worsening but no correlation could be found between VD and clinical progression (change in ETDRS level). The small number of cases with worsening and improvement in each ETDRS group can be an explanation for that. This study showed that 10% vessel density decrease used as a threshold has more than 4 times detection capacity for progression in the severity of diabetic retinopathy compared to the ETDRS one-step change.

6. Capillary dropout in NPDR correlates with DR severity progression in 3 years of follow up. OCTA metrics may be used as a surrogate for DR severity progression.

The sixth paper here presented (CHAPTER 7) included 78 eyes from 78 diabetic patients followed for 3 years of follow up. Of the 78 eyes analyzed, 24 (31%) were graded at ETDRS levels 10-20 at baseline, with 16 being level 10 and 8 level 20, 31 (40%) as ETDRS 35 and 23 as ETDRS 43-47 (29%). Of the systemic variables only HbA1c correlates positively with the DR severity.

The ocular parameters, VD and PD in the inner ring, representing capillary closure, and FAZ circularity index, were significantly different between ETDRS groups, reflecting an association between retinal capillary non-perfusion and different severity grades of the retinopathy, which was already explored in CHAPTER 6, and were decreased since the ETDRS level 35. The GCL-IPL thinning, reflecting neurodegeneration, was also apparent since ETDRS level 35, in comparison to the healthy population, but no significantly different between ETDRS groups was found.

Neurodegeneration (ND) and capillary closure (CC) features tended to increase in the more advances DR ETDRS severity levels. However, there is a clear dissociation between both disease pathways in every ETDRS group during the 3 years of follow-up. No eyes presented both definite characteristics in the very initial stages and the occurrence of both characteristics in higher levels simultaneously is low (<16%).

In this study, as in the 2-year study previously reported, capillary closure was the most important retinal marker of development of complications, namely worsening in the ETDRS severity grade. The eyes that showed one-or-two-steps ETDRS worsening had significantly more capillary closure than eyes with no changes or improvement in ETDRS grade during the three-year follow-up period. This was true for VD metrics in the inner ring for all layers assessed, namely SCP, DCP and FR, however more significative for the SCP, as suggested in our previous researches. Despite the observation that GCL+IPL thickness decreased during the follow up in both groups (with worsening and with maintenance/improvement in ETDRS level), the difference was not significant between them. The thinning of GCL-IPL was not correlated with ETDRS changes. There were no differences observed in the RNFL or GCL-IPL thinning, representing neurodegeneration, between those patients that improved or maintained the ETDRS score during the 3 years of follow up.

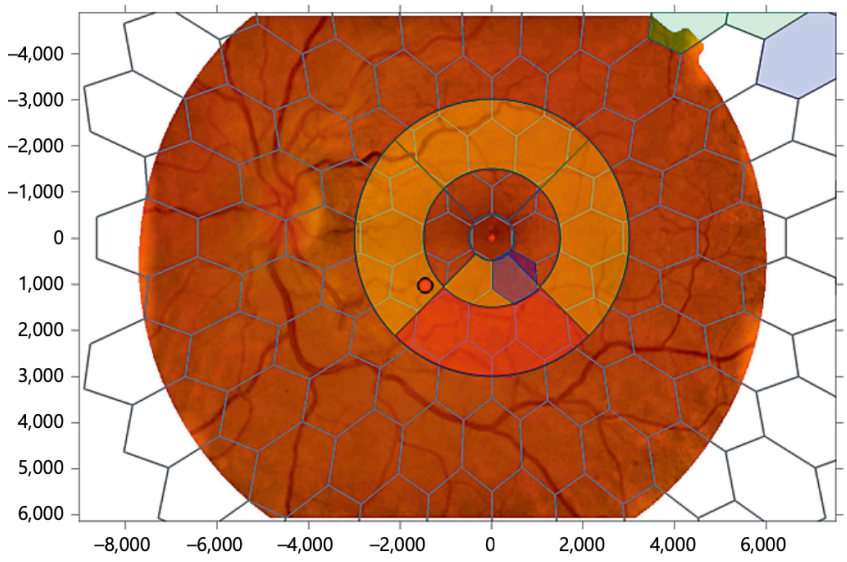
When considering phenotype classification at baseline, phenotype C presented significant lower baseline VD, either in the SCP, DCP and FR and was also associated with increased GCL+IPL thinning at baseline.

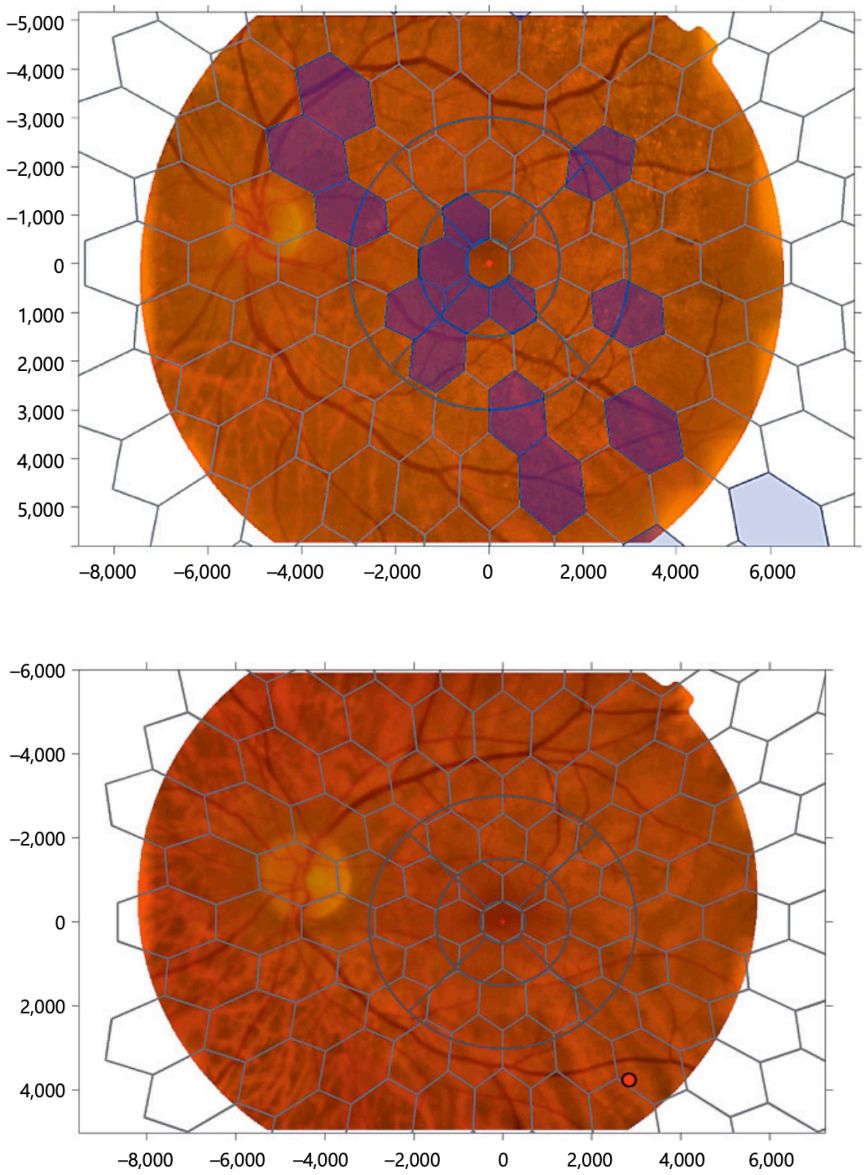
This study shows that ND does not appear to be directly associated with CC and shows no correlation with DR severity progression in a 3 years of follow-up, contradicting some studies that refer that neurodegeneration plays a major role in the development and progression of DR and may occur before visible capillary abnormalities (26)(27). Signs of ND and ischemia are distributed over a wide range in different patients. Some patients show steady and progressive worsening whereas others show a variable course and evidence of reversibility of their changes. An important finding in this analysis was that vessel closure presented in these initial stages appears to be reversible, at least partially, opening new hope in a possible treatment capable of reverting the disease.

This finding may open perspectives concerning the therapeutic approach in the initial stages of the disease, capable of reverting ischemic signs. Each patient should be followed closely, considering a variety of predictive risk factors to determine a specific risk profile for each patient.

A cross-sectional analysis of the baseline characteristics of patients included in a large clinical trial (EUROCONDOR Study) was performed in order to evaluate the relationship between the functional and structural measurements of neurodegeneration in the initial stages of diabetic retinopathy (ETDRS < 35) and also the presence of neurodegeneration and early microvascular impairment (28). Multifocal electroretinogram (mfERG) abnormalities compatible with

neurodysfunction (delayed implicit time, IT (P1) and lower amplitude (P1) compared to controls) were found in 58% of diabetic patients with no visible microvascular lesion and in 66% of diabetic patients with ETDRS 20–35. The most important structural damage of the retina detected by SD-OCT was thinning of the GCL-IPL and RNFL layers, although in few eyes in agreement with the mfERG abnormalities. Those findings support the concept that functional impairment related to neurodegeneration is an early event in the diabetic retina. Although, in 82 of 256 (32%) diabetic patients with early microvascular impairment (ETDRS 20–35), mfERG abnormalities were not found, confirming the presence of a primarily microvascular or a microangiopathic phenotype. Therefore, it seems that in some of these patients, retinal neurodegeneration does not play an essential role in the development of DR, at least when assessed by mfERG. Multimodal analysis of the eyes, superimposing the sites of mfERG alterations, presence of abnormally increased retinal thickness and location of MAs, shows that these alterations occur in most cases in the initial stages of DR, independently and without any apparent association (Figures 3-5).





Figures 2-4: Multimodal image presenting the functional measurement using mfERG (hexagons grid), the retinal thickness measured using SD-OCT (nine ETDRS grid), and the microaneurysms detected in the CFP (red circle).

In a sub analysis of data from 73 patients of the EUROCONDOR study performed at AIBILI, Sílvia Simão reported that, after 24 months of follow-up, a decrease in the GCL (p=0.001) and in the INL (p=0.013), as well as a decrease in the mfERG implicit time of P1 wave and an increase in its amplitude (p>0.05), were observed in diabetics without and with mild DR comparing to controls (29).

7. Retinal capillary closure quantified by SS-OCTA can identify DR ETDRS severity progression. In the initial stages of NPDR, CC is mainly located in the perifoveal retinal capillary circulation, whereas in moderate to severe NPDR the retinal midperiphery is predominantly affected.

In the last paper presented in the thesis (CHAPTER 8), 105 patients with diabetes, either without retinopathy or with NPDR (up to ETDRS grade 53) were evaluated using wide field Swept-Source OCTA (SS-OCTA, PlexElite, Carl Zeiss Meditec) with 15x9 mm and 3x3 mm angiography protocols and compared to an age-matched healthy subjects. The Swept-Source OCTA is a recently development software allowing larger fields of view, 12x12 mm or 15x9 mm, to be analysed. We wanted to study the contribution of macular and widefield imaging to improve characterization of the different ETDRS stages of NPDR. Of the 105 eyes of diabetic patients, 16 eyes had no clinical evidence of retinopathy, 18 eyes were classified as ETDRS grade 20, 39 eyes were identified as grade 35, 17 eyes as grade 43 and 15 eyes were identified as grades 47-53. The VD metrics identify decrease in VD in all retinal capillary layers in individuals with diabetes. In the macular area, the best correlation between ETDRS retinopathy level and VD changes were obtained with the inner ring metrics, representing the perifoveal retinal capillary microvasculature. Using the 15x9 mm protocol, which permits the analysis of retinal midperiphery, VD showed relevant information identifying involvement of more peripheral regions of the retina in the more advanced ETDRS stages of NPDR, and were particularly discriminatory as the retinopathy progressed to the 43-53 stages. These increase in capillary closure of the more peripheral regions of the retina appears to involve predominantly the DCP.

The thickness of GCL-IPL remained within the normal range in the different retina regions examined along the different ETDRS stages of NPDR, again reinforcing our previous findings.
Combining the results of the OCTA protocols 3x3 mm and 15x9 mm, it was possible to distinguish two major groups of NPDR progression: Group A, including eyes with diabetes and either no ophthalmological signs of retinopathy (ETDRS 10) and eyes with mild retinopathy (ETDRS 20-35), showing increased retinal capillary closure in the central parafoveal area, and Group B, including eyes with ETDRS grades 43 to 53, showing increased retinal capillary closure in the retina midperiphery.

The importance of quantitative analysis of retinal nonperfusion on wide-field OCTA have been demonstrated in recent studies, allowing early detection and monitoring of disease in patients with DR (30).

Our study confirms the presence of CC in the initial stages of DR, with earlier detection in the SCP, with progressive later involvement of the DCP, suggesting that one of the earliest changes associated with DR is reduced VD. This capillary closure appears to occur initially in the perifoveal retina, progressing rapidly in the ETDRS levels 10, 20 and 35, but the changes in the perifovea seems to be less significant in more advanced stages of retinopathy. This may be associated with a shift in the location of the CC to the midperiphery of the retina in more advanced cases. Capillary closure in the midperiphery in a diabetic retina is indicative of an advanced stage of retinopathy, whereas capillary closure limited to the perifovea suggests a milder stage of the disease.

The GCL-IPL shows similar degrees of thinning in eyes with different degrees of nonperfusion. The increase in nonperfusion present in the more peripheral regions of the retina observed in severe stages of the retinopathy occurs without increased thinning of the GCL-IPL demonstrating that the nonperfusion is associated with a primary vascular alteration and is not the end result of retinal tissue atrophy.

The observations here reported offer promising perspectives for personalized management of DR. After diagnosis of NPDR, and still in the initial stages of retinal disease, three different phenotypes can be identified through colour fundus photography, including MAT evaluation, and OCT/OCTA, with CRT determination. These examinations are easy to perform and can be repeated easily without major inconvenience to the patient. The phenotypic classification, as well as the inclusion of vessel density metrics, improve the predictive value of current monitoring strategies and classifications. Furthermore, the identification of different pathways of disease, with different prevalence in distinct patients, may provide a clue to investigate new target therapies, directed to a specific disease pathway.

The findings reported in this thesis contribute to improve management strategies of NPDR patients, allowing personalized follow-up and a timely and targeted treatment, before clinically significant complications occur.

Conclusions

1. Phenotype C comprises near 30% of the NPDR patients and was the phenotype with the higher rate of complications, including 79% of the eyes that developed clinically significant macular edema (CSME) and all the cases that developed PDR. This phenotype is also associated with increased HbA1c.
2. Phenotype A identifies eyes that are at a very low risk of development of vision-threatening complications
3. MA turnover and capillary closure, representing microvascular damage, reflects DR severity and predict the development of sight threatening complications in NPDR patients.
4. The ocular risk markers seem to be more predictive of DR severity progression and development of sight threatening complications than the systemic risk markers. However, HbA1c remained the most relevant systemic marker identified.
5. Some eyes present neurodegeneration already in the early phases of DR, which occur independently of capillary closure.
6. The neurodegeneration, evaluated by OCT retinal layers analysis, seems to be stable during the 3 years of follow up and not related to progression of DR.
7. Edema, evaluated by OCT thickening, occurs independently of the ETDRS level and may be present at any stage of DR.
8. Capillary dropout (ischemia) increases with retinopathy severity and appears to be associated with increased DR severity and metabolic control during the three years of follow up.
9. Combination of acquisition protocols 3x3 mm and 15x9 mm, using SS-OCTA, allows discrimination between eyes with mild and moderate to severe NPDR. Retinal capillary closure predominates in the perifoveal retinal capillary circulation in the initial stages of NPDR, whereas the retinal midperiphery becomes predominantly affected in moderate to severe NPDR.
10. Capillary closure evaluated by OCTA metrics seems to be reversible in the early phases of the retinopathy.
11. OCTA offers a promising option to identify retinopathy progression.

Future Perspectives/ Developments

1. Analysis of the five-year data focusing on retinal degeneration evaluated by layer-by-layer OCT analysis and its correlation with microvascular events and disease severity progression.
2. Correlation of structural retinal neurodegeneration markers with other systemic neural involvement in diabetic disease.
3. Correlation of structural retinal neurodegeneration markers with other functional visual parameters (VA, ERG, dark adaptation, contrast sensitivity).
4. Investigation of retinal and choroidal vascular indices, with new imaging methodologies, as Swept Source OCTA or Ultra-Wide Field Imaging, that may be more informative in predicting disease outcomes.
5. Further validation of OCT-Leakage software as predictive biomarker of diabetic edema and blood retinal barrier function.
6. Development and validation of a DR risk score that allows the identification of individuals at risk of progression. Combine a robust qualitative classification with quantitative and objective metrics assessed by OCT and OCTA may permit a better stratification of diabetic patients in order to improve our monitoring strategy.
7. Exploration of new functional metrics, like fixation stability, reading speed, stereoacuity, color vision, scotopic sensitivity, as adjunctive methodologies to follow diabetic retinal disease and which may be used as surrogate markers of efficacy for new therapeutic agents.
8. Validation and standardization of additional functional tests to assess disease evolution, others than BCVA, that can be used in clinical studies to evaluate the impact of the disease in visual function and vision-related quality of life.
9. Development of new therapies to be used in the initial DR stages, capable of act in the reversible phases of disease, and specifically targeting the affected disease pathway identified using the methodologies developed in our research.

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