

INTERMEDIATE AGE-RELATED MACULAR DEGENERATION – NONINVASIVE MULTIMODAL ANALYSIS AND LONGITUDINAL STUDY

RITA MARIA RIO PEDRO FLORES

Tese para obtenção do grau de Doutor em Ciências da Saúde

na Especialidade de Medicina

na Faculdade de Ciências Médicas | NOVA Medical School da Universidade NOVA de Lisboa

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“Vision isn’t just about sight. It’s about the ability to see beyond the here and now.”

Tim Reddish

Às minhas filhas, Maria e Francisca

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Thesis outline

The structure of this thesis is detailed in the following paragraphs.

Firstly, in the Abstract, the author presents a summary of the Thesis.

In Chapter I, *Introduction*, the author explores the existing literature and evidence regarding prevalence, risk factors, genetics, pathophysiology, diagnosis, and classification of age-related macular degeneration. Then we explore the known biomarkers of disease progression, and finally we reflect about the priorities for AMD research clarifying the gaps and needs on current knowledge.

In Chapter II, the author details the *Rationale and Aims* of this Thesis based on the previously presented motivations presenting, in Chapter III, the *Methods*.

In Chapter IV, V and VI the different steps of the research protocol are detailed, as well as the achieved results. These chapters globally explore three lines of clinical investigation: IV - *Fundus autofluorescence findings*; V - *Chorioretinal vascular findings*; VI - *Identification of other disease progression biomarkers*. Imaging aspects of the macula of intermediate AMD patients are evaluated and some hypotheses are raised related to physiopathology and with disease progression.

In Chapter VII, the author presents a comprehensive and global *Discussion* with a summary of the main findings of this Thesis, along with the overall results, limitations, and future perspectives.

Abstract

Age-related Macular Degeneration (AMD) is an important cause of irreversible visual impairment, secondary to degenerative macular disorder. AMD can compromise central vision, essential for reading and for recognizing small details.

Currently we recognize three stages in AMD: early, intermediate and late stage. Early AMD is characterized by the presence of medium ($\geq 63\mu\text{m}$ and $\leq 125\mu\text{m}$) drusen with no associated pigmentary abnormalities and intermediate AMD (iAMD) is characterized by the presence of large ($>125\mu\text{m}$) drusen associated or not with pigmentary abnormalities. Advanced or late AMD can be atrophic or neovascular, with death of photoreceptors associated or not with macular neovascularization. Intermediate stage is a common pathway to more advanced forms of the disease and has, recently, been arousing special interest as a field of AMD investigation.

Although some progress is being made towards a treatment for atrophic AMD no subsequent visual improvement can yet be obtained. Early and intermediate AMD stages have no preventive effective treatment besides general recommendations as eating healthfully, taking vitamin supplements, and not smoking. On the other hand, anti-vascular endothelial growth factor (anti-VEGF) therapies with repeated intravitreal administrations are a proven therapeutic option to control exudative disease.

AMD pathophysiology has some known factors (age, genetics, smoke and nutrition) and some others are still under debate. There is a documented genetic background with genes encoding complement system, high-density lipoprotein metabolism, extracellular matrix remodeling, angiogenesis and cell survival. High risk genetic variants linked to AMD are found in the genes related complement system proteins (complement factor I, complement C2, C3, C9, and CFH). Other possible implicating factors identified as predictors for AMD development are human high-temperature requirement A-1 (HTRA1) and Age-related Maculopathy Susceptibility 2 (ARMS2) as well as other rare genetic variants. It seems possible that still other unknown elements may be involved not only in AMD pathophysiology but also in its progression.

In this study we focus the issue on possible factors involved in AMD pathophysiology or in its

progression.

We propose to study iAMD patients, with follow-up visits every six months for two consecutive years, in a non-interventional prospective way. These patients will undergo a complete ophthalmologic evaluation and retinal imaging including Color Fundus Photography (CFP), Fundus Autofluorescence (FAF), Spectral Domain Optical Coherence Tomography (SD-OCT), OCT-Angiography (OCT-A) using Spectralis OCT with OCT Angiography Module (Heidelberg Eng. GmbH, Germany) at the baseline and during the follow-up period. Multimodal retinal imaging is used to explore biomarkers of disease progression. We studied imaging aspects of the macula of iAMD patients and hypothesized their relationship with the disease pathophysiology and with progression to advanced disease. The study develops in three lines of clinical investigation: fundus autofluorescence findings, chorioretinal vascular findings and finally identification of other disease progression biomarkers.

Fundus autofluorescence findings - Drusen autofluorescence was correlated with drusen type and morphology and with additional outer retinal structural changes on SD-OCT. We found a statistically significant correlation between drusen autofluorescence (AF) and homogeneity, hyperreflective foci (HRF), external limiting membrane (ELM) integrity, ellipsoid zone (EZ) integrity, and choroidal hypertransmission (CH). All correlations were weak or moderate and no strong correlations were found with any parameter. These findings support the concept that drusen and lipofuscin emerge from biologic processes directly unrelated, both meaning RPE metabolic overload, and raise the hypothesis that FAF can be useful in monitoring iAMD patients. Further we found a statistically significant difference in the rate of progression to advanced AMD between patients with hypo and/or hyperautofluorescent drusen and patients with only isoautofluorescent drusen. Moreover, progression to geographic atrophy (GA) alone was significantly superior in patients with at least one hypo or hyperfluorescent drusen, meaning a more benign prognosis for isoautofluorescent drusen.

Chorioretinal vascular findings - A non-interventional prospective study was conducted to evaluate choroidal and retinal vascular parameters in iAMD patients. We found significant differences concerning total subfoveal choroidal area, luminal area and choriocapillaris flow density

in iAMD compared with age matched control eyes. No apparent relationship was found between those quantitative vascular parameters and progression to late AMD.

Identification of other disease progression biomarkers - Prospective non-interventional study to investigate and characterize drusen, HRF and other OCT features (presence of incomplete retinal pigment epithelial and outer retinal atrophy, and EZ status) as biomarkers of disease progression. A risk prediction model for progression to late AMD was calculated, with estimates of odds ratio of each significant variable. The combination of incomplete retinal pigment epithelial and outer retinal atrophy (iRORA) and EZ disruption constitute a high risk of progression to complete retinal epithelial and outer retinal atrophy (cRORA) within two years. We further perform a retrospective analysis of data from a longitudinal study concerning nonexudative macular neovascularization and progression to exudative AMD; its occurrence was found in 23.5% in the follow-up period, most frequently in younger patients, in eyes with foveal NE membranes and presenting exudative AMD in the fellow eye.

The findings reported in this Thesis have the potential to contribute to AMD pathophysiology knowledge, exploring and understanding imaging retinal or choroidal biomarkers. This information could inspire investigators and clinicians in the prevention of disease progression and in the identification of potential treatment pathways, effective in early stages of AMD.

Sumário

A Degenerescência Macular da idade (DMI) é uma causa importante de diminuição irreversível da acuidade visual, associada a alterações degenerativas da área macular. É uma doença que compromete a visão central, essencial para a capacidade de leitura e reconhecimento de pequenos detalhes. Actualmente reconhecem-se três estadios da doença: precoce, intermédia e avançado ou doença tardia. A DMI precoce é caracterizada pela presença de drusen de tamanho médio ($\geq 63\mu\text{m}$ and $\leq 125\mu\text{m}$), sem migrações pigmentares associadas e a doença intermédia é definida pela presença de drusen de grandes dimensões ($>125\mu\text{m}$) associados ou não a anomalias pigmentares. A doença avançada ou tardia poderá ser atrófica ou neovascular, com morte dos fotorreceptores associada ou não a neovascularização macular. A DMI intermédia representa assim, no trajecto evolutivo destes doentes, um estadio comum e heterogéneo, razão pela qual tem despertado interesse científico e é atualmente um importante campo de investigação.

Apesar de recentemente terem surgido alguns progressos na abordagem terapêutica da DMI atrófica, não há ainda nenhum tratamento que promova recuperação ou melhoria da acuidade visual. A DMI precoce e intermédia não apresenta ainda tratamento efectivo para além de recomendações de ordem geral relacionadas com a alimentação, estilo de vida saudável, suplementos vitamínicos e abstinência tabágica. Pelo contrário, as injeções intravítreas repetidas de fármacos antiangiogénicos são uma opção terapêutica aprovada que permite, na maioria dos casos, controlar a doença exsudativa.

A fisiopatologia da DMI contempla alguns factores etiológicos conhecidos (idade, genética, consumo de tabaco) e outros que são ainda matéria de debate. Reconhece-se um substracto genético conhecido com genes que codificam o sistema do complemento, o metabolismo das HDL, a matriz extracelular, a angiogénese e a apoptose. As variantes genéticas de alto risco para DMI são relacionadas com sistema do complemento (factor I do complemento, C2, C3, C9 e CFH). Outros factores possivelmente implicados são o HTRA1 (*human high-temperature requirement A-1*) e ARMS 2 (*Age-related Maculopathy Susceptibility*), bem como variantes genéticas mais raras. Outros intervenientes poderão ainda estar envolvidos não só na fisiopatologia da doença inicial, mas também na sua progressão.

Neste estudo focamos a “grande questão” nos possíveis factores envolvidos na fisiopatologia da doença e na sua progressão para estadios mais avançados.

Propomos seguir um grupo de doentes com DMI intermédia, com intervalos de follow-up semestrais durante pelo menos dois anos consecutivos, num estudo prospectivo não intervencional, com avaliações clínicas e imagiológicas em abordagem multimodal. Os doentes realizam fotografia do fundo ocular, autofluorescência do fundo, tomografia de coerência óptica e OCT-angiografia, na baseline e ao longo do tempo de follow-up, sendo que os dados desta avaliação retiniana multimodal serviram de base para a pesquisa de biomarcadores de progressão. Múltiplos parâmetros imagiológicos foram analisados e investigou-se a sua possível relação com a fisiopatologia da doença, bem como com a sua progressão para estadios avançados. Neste trabalho desenvolvemos três linhas de investigação: autofluorescência do fundo ocular, vascularização coriorretiniana e identificação de outros biomarcadores de progressão.

Autofluorescência do fundo ocular – correlação da autofluorescência dos drusen com o seu tipo e morfologia, bem como com alterações estruturais das camadas externas da retina (diagnosticadas pelo SD-OCT). Encontrámos a existência de correlação estatisticamente significativa entre a autofluorescência dos drusen e a sua homogeneidade, presença de focos hiperreflectivos, integridade da membrana limitante externa e linha elipsóide e presença de hipertransmissão coroideia. Todas estas correlações revelaram-se fracas ou moderadas e nenhuma correlação forte foi assinalada. Estes achados apoiam o conceito de que os drusen e a lipofucsina se associam a processos biológicos tradutores de sobrecarga metabólica do epitélio pigmentado, não directamente relacionados. Subsequentemente verificou-se a existência de uma diferença estatisticamente significativa na progressão para estadios avançados em doentes que apresentam drusen hipo ou hiperautofluorescentes, comparativamente aqueles que somente apresentam drusen isoautofluorescentes. A progressão para atrofia geográfica foi significativamente superior em doentes que apresentavam pelo menos um drusen hipo ou hiperautofluorescente.

Vascularização coriorretiniana – estudo prospectivo não intervencional que avaliou parâmetros vasculares coroideus e retinianos em doentes de DMI intermédia. Registaram-se diferenças estatisticamente significativas na área coroideia total subfoveal, na área luminal e na densidade da coriocapilar em olhos com DMI intermédia comparativamente a olhos saudáveis (grupo controle).

Não foi encontrada relação estatisticamente significativa entre os parâmetros vasculares estudados e a progressão da doença para estádios avançados.

Identificação de outros biomarcadores de progressão – estudo prospectivo não intervencional que investigou e caracterizou os drusen, os focos hiperreflectivos e outros parâmetros tomográficos (presença de atrofia incompleta do epitélio pigmentado e das camadas externas da retina – iRORA - e disrupção da linha elipsóide) como possíveis marcadores de progressão. Um modelo de predição de risco foi calculado, bem como as estimativas de razão de probabilidade de risco para cada variável em estudo. A coexistência de iRORA e de disrupção da elipsóide traduziu um elevado risco de progressão aos dois anos. Foi ainda realizada uma análise retrospectiva do estudo longitudinal, referente a presença e evolução de membranas neovasculares não exudativas. Detectámos uma percentagem de conversão exsudativa de 23.5%, mais frequente em doentes jovens, em membranas não exudativas de localização subfoveal e associadas a estadio exudativo no olho adelfo.

Os achados relatados nesta Tese têm o potencial de contribuir para uma melhor compreensão da fisiopatologia da doença, explorando e melhor compreendendo os biomarcadores de imagem da retina e coróideia. Estas informações poderão inspirar investigadores e clínicos na compreensão de mecanismos capazes de prevenir a progressão da doença para estádios avançados, bem como na identificação de potenciais linhas de tratamento dirigidas para estádios precoces e intermédios.

List of thesis publications

The core struture of this Thesis contains the following published manuscripts (described by order of appearance):

- Flores R, Carneiro A, Serra J, Gouveia N, Pereira T, Mendes J, Coelho P; Tenreiro S, Seabra M: Correlation study between drusen morphology and fundus autofluorescence. *Retina* 2021; 41: 555-562. doi: 10.1097/IAE.0000000000002881
<https://pubmed.ncbi.nlm.nih.gov/32604342>
- Flores R, Carneiro A, Vieira M, Tenreiro S, Seabra M, C: Age-Related Macular Degeneration: Pathophysiology, Management, and Future Perspectives. *Ophthalmologica* 2021;244:495-511. doi: 10.1159/000517520
<https://pubmed.ncbi.nlm.nih.gov/34130290/>
- Flores R, Carneiro A, Tenreiro S, Seabra MC. Retinal Progression Biomarkers of Early and Intermediate Age-Related Macular Degeneration. *Life* (Basel). 2021; 12(1):36. doi:10.3390/12010036qa
<https://pubmed.ncbi.nlm.nih.gov/35054429/>
- Flores R, Carneiro A, Nery G, Quenderra B, Barata M, Tenreiro S, Seabra M. Choroidal vascular impairment in intermediate AMD patients. *Diagnostics*. 2022; 12, 1290. doi:10.3390/12051290
<https://pubmed.ncbi.nlm.nih.gov/35626445/>
- Flores R, Fradinho A, Pereira R, Mendes J, Seabra M, Tenreiro S, Carneiro A. Identifying imaging predictors of intermediate Age-Macular Degeneration progression. *Translational vision science and technology*. *Translational Vision Science & Technology* 2023, 12, doi: 10.1167/tvst.12.7.22
<https://pubmed.ncbi.nlm.nih.gov/37490304/>

Additional publications

Some of the results were first presented as poster or oral presentations in national or international symposiums. The list of published abstracts is detailed below:

- Flores R, Basilio AL, Moura-Coelho N, Marques M, Vieira L, Carneiro A, Tenreiro S, Seabra M. Novel disease progression biomarkers in non-exudative age-related macular degeneration: an association study between drusen type and morphology and Fundus Autofluorescence. *Investigative Ophthalmology & Visual Science* 60 (9), 1148-1148, 2019
<https://iovs.arvojournals.org/article.aspx?articleid=2741677&resultClick=1>
- Flores R, Carneiro A, Nery G, Tenreiro S, Seabra M. Impairment of chorioretinal vasculature in patients with intermediate age-related macular degeneration. *Investigative Ophthalmology & Visual Science* 62 (8), 93-93, 2021
<https://iovs.arvojournals.org/article.aspx?articleid=2772734>

In addition, the author was an editor of the following book or monography:

- Ângela Carneiro, Rita Flores, António Campos, OCTA in Ophthalmology, 1st Edition, 2021, Lisbon, Nº198, ISBN: 978-989-33-2449-3
https://www.researchgate.net/publication/356977909_OCTA_in_Ophthalmology

And published the following book chapter related with the Thesis subject:

- Rita Flores, Early and Intermediate AMD, Editors: Ângela Carneiro, Rita Flores, António Campos, OCTA in Ophthalmology, 1st Edition, Lisbon 2021: 105-107. ISBN: 978-989-33-2449-3.
https://www.researchgate.net/publication/356977909_OCTA_in_Ophthalmology

Finally, we add an additional publication related to the subject:

- Gomes N, Meireles A, Carneiro Â, Silva R, Campos A, Duarte L, Flores R, Marques-Neves C; Portuguese RAMD consensus group. A Delphi study on the clinical management of age-related macular degeneration. *Int Ophthalmol*. 2022 Jun;42(6):1799-1809. doi: 10.1007/s10792-021-02177-2
<https://pubmed.ncbi.nlm.nih.gov/35149923/>

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List of abbreviations

AF	Autofluorescence
AMD	Age-related macular degeneration
AREDS	Age-related eye disease study
BCVA	Best corrected visual acuity
BM	Bruch's membrane
CAM	Consensus International Atrophy Meeting
CC	Choriocapillaris
CEDOC	Chronic Diseases Research Center
CFD	Choriocapillaris flow density
CFP	Color fundus photography
CH	Choroidal hypertransmission
CHULC	Centro Hospitalar Universitário de Lisboa Central
CI	Confidence Interval
CMT	Central Macular Thickness
cORA	Complete outer retinal atrophy
cRORA	Complete retinal pigment epithelial and outer retinal atrophy
CRU-NMS/FCM	Clinical Research Unit Nova
CT	Choroidal Thickness
CVI	Choroidal vascularity index
DCP	Deep Capillary Plexus
DVP	Deep Vascular Plexus
eCRF	Electronic case report form
ELM	External limitant membrane
EZ	Ellipsoid zone
FAF	Fundus autofluorescence
FAZ	Foveal Avascular Zone
FD	Flow Density
GA	Geographic atrophy

GRS	Genetic risk score
GWAS	Genome-wide association study
HIRD	Heterogeneous internal reflectivity material within drusen
HRF	Hyperreflective foci
HTD	Hypertransmission defects
iAMD	Intermediate age-related macular degeneration
IAMDGC	International Age-related Macular Degeneration Genomics Consortium
ILM	Internal limiting membrane
INL	Inner nuclear layer
iORA	Incomplete outer retinal atrophy
IPL	Inner plexiform layer
IQR	Interquartil range
IR	Infrared reflectance
iRORA	Incomplete retinal pigment epithelial and outer retinal atrophy
LA	Luminal area
LE	Left eye
MCI	Multicolor Imaging
nAMD	Neovascular age related macular degeneration
NE-MNV	Non exudative macular neovascularization
NIR	Near Infrared reflectance
NOVA-IMS	NOVA Information Management School
OCT	Optical Coherence Tomography
OCT-A	Optical Coherence Tomography Angiography
ODS	Optical Coherence Tomography reflective drusen substrutres
ONL	Outer nuclear layer
OPL	Outer plexiform layer
PED	Pigment epithelium detachment
RAT	Retinal pigment epithelium drusen complex abnormal thinning
RCG	Retina Ganglion Cells
RE	Right eye
RNFL	Retinal Nerve Fiber Layer

ROC curves	Receiver operating characteristics curves
RPE	Retinal pigment epithelium
RPEDC	Retinal pigment epithelium drusen complex
SCP	Superficial retinal capillary plexus
SCP	Superficial Capillary plexus
SD	Standard Deviation
SDD	Subretinal drusenoid deposits
SD-OCT	Spectral Domain Optical Coherence Tomography
SD-OCT EDI	Spectral Domain Optical Coherence Tomography with enhanced depth imaging
SIRE	Shallow irregular retinal pigment epithelium elevation
SS	Swept Source
SVP	Superficial Vascular Plexus
TCA	Total choroidal area
YO	Years old

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CHAPTER I. GENERAL INTRODUCTION

CHAPTER I. GENERAL INTRODUCTION

To better clarify the existing literature on the topic, we systematize the available data in the two following review manuscripts:

AGE-RELATED MACULAR DEGENERATION: PATHOPHYSIOLOGY, MANAGEMENT, AND FUTURE PERSPECTIVES.

Flores R, Carneiro Â, Vieira M, Tenreiro S, Seabra MC

Ophthalmologica 2021;244:495-511.

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Impact Factor: 3.25, Q1 in Medicine, Ophthalmology, Sensory Systems

<https://pubmed.ncbi.nlm.nih.gov/34130290/>

RETINAL PROGRESSION BIOMARKERS OF EARLY AND INTERMEDIATE AGE-RELATED MACULAR DEGENERATION

Flores R, Carneiro A, Tenreiro S, Seabra MC.

Life (Basel). 2021; 12(1):36.

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Impact Factor: 3.31, Q2 in Biology

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CHAPTER I. GENERAL INTRODUCTION

1. BACKGROUND

Age-related macular degeneration (AMD) is a progressive, vision-threatening disease that affects older adults and is a leading cause of irreversible blindness in Western countries. AMD-associated visual loss predominantly occurs in later stages of the disease and manifests as deterioration of high-resolution central vision owing to several processes ¹.

The earliest histopathological manifestation of the disease are lipids deposits within Bruch's membrane (BM), possibly from failure of the retinal pigment epithelium (RPE) to process the cellular debris associated with outer segment turnover ². These deposits in the extracellular matrix are known as basal linear and laminar deposits and, as they evolve, thickening of the Bruch's membrane (BM) collagenous layers with degeneration of elastin and collagen will predispose to drusen formation, the earliest visible clinical sign of AMD ³. Their accumulation leads to progressive degeneration of photoreceptors and RPE.

In AMD, staging of earlier disease states relies on specific sizes, types, and location of drusen and pigmentary changes, and criteria vary among classification systems. In contrast, diagnosis and staging of late AMD is more straightforward and consistent. The two clinical forms of advanced disease are geographic atrophy (GA) and neovascular AMD (nAMD) (Figure 1).

Typical drusen appear as focal whitish yellow excrescences, located between the RPE and BM, varying in number, size, shape and distribution⁴.

Drusen can be classified as hard, soft, cuticular and reticular pseudodrusen. Hard drusen are present in 80% of the general population over 30 years of age and appear as round, discrete yellow-white spots, measuring less than 63 μm , currently considered as a physiological sign of ageing ^{5,6}.

Soft drusen are ill defined, with non-discrete borders measuring more than 63 μm ; large, soft and confluent drusen are associated with a higher risk for development of advanced AMD ^{5,6}.

Cuticular drusen or basal laminar drusen are yellow deposits located bellow the basal membrane of RPE, usually in clusters and measuring 50-75 μm . They tend to coalesce over time,

but they can also disappear and may be associated with acquired vitelliform lesions.

Reticular pseudo-drusen or subretinal drusenoid deposits (SDD) are a yellow faint interlacing network, first identified on blue-light fundus photography, believed to be present in around 11% of AMD patients. Their presence entails a worse prognostic, being associated with a more aggressive disease^{7,8} and their location is different from other drusen as they stay above RPE (Figure 2).

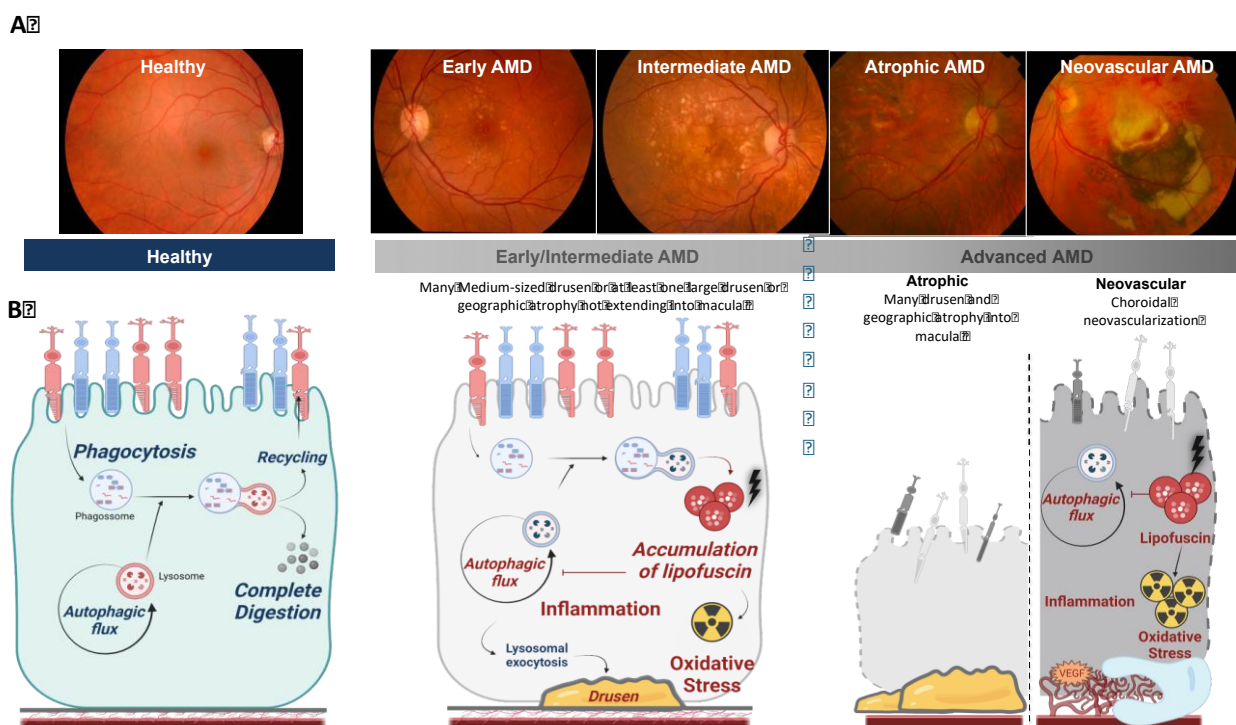


Figure 1 – AMD stages.

A) CFP images of healthy eye and of AMD stages (early, intermediate and late or advanced AMD) with the features that characterise each stage and B) the corresponding cellular and molecular events (phagocytosis, recycling, lipofuscin accumulation, inflammation, oxidative stress) known to occur in the RPE with deposits formation (drusen) and further cellular death or neovascularization.

The retina exhibits an inherent autofluorescence (AF) that mainly arises from lipofuscin in RPE cells. For many cell types, lipofuscin originates internally (autophagy) but in the RPE, lipofuscin derives primarily from phagocytosed photoreceptor outer segments^{9,10}. Fundus autofluorescence (FAF) assessment relies on native fluorescence from physiological retinal components, providing an indication of healthy function of RPE cells.

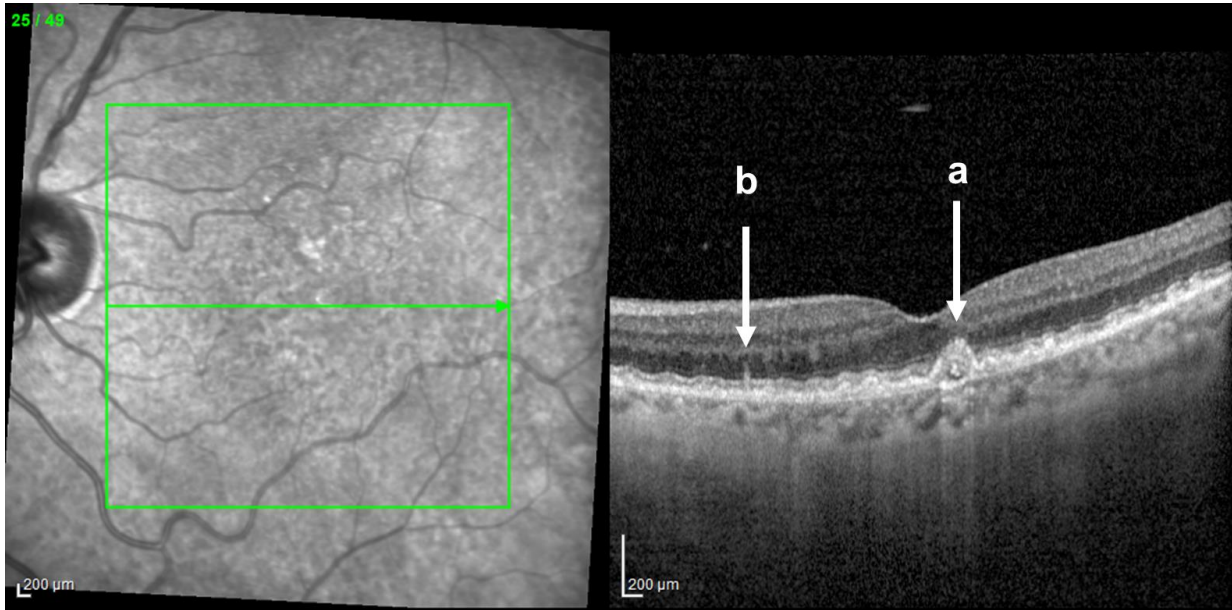


Figure 2 – SD-OCT depicting drusen types.

SD-OCT depicting drusen types: a) below the RPE (soft drusen) and b) above the RPE (SDD)

Lipofuscin is potentially toxic and, as a byproduct of photoreceptor metabolism, can accumulate in lysosomal compartments of RPE cells. Beyond critical concentrations, lipofuscin seems to interfere with normal cell function and ultimately can lead to cells death^{9,10}, but definitive proof of lipofuscin toxicity is still missing. Drusen deposits and lipofuscin accumulation reflect metabolic overload of the RPE, and increased lipofuscin accumulation is assumed to be an important factor in the pathogenesis of AMD, although direct evidence for this hypothesis is missing. It seems that drusen and lipofuscin are important biologic processes yet directly unrelated¹¹. Exploring the relationship between drusen and lipofuscin could clarify the finding of any common pathophysiological pathway and could highlight the role of FAF in monitoring intermediate AMD patients.

Progressive degeneration of macular photoreceptors is not a unique event in AMD. Juxtaposed to the photoreceptor layer is the RPE, whose apical extensions intercalate with the dense layer of photoreceptor outer segments, and whose basal layer resides on Bruch's membrane, separating the outer retina from the choroid. With the focus on the RPE as the principal cell layer in determining the fate of the retina in AMD, the hemodynamic theory of AMD pathogenesis has received far less attention from cell and molecular biologists. However, emerging data collectively suggest that local hemodynamics may play a crucial role in the manifestation of AMD pathologies.

The concept that anomalous choroidal blood flow is a pathogenic stimulus for AMD dates back to at least 1905, when Possek proposed atherosclerotic changes to the ocular blood supply as a cause of what is now named neovascular AMD ^{12,13}.

The modern hemodynamic theory of AMD proposed by Friedman in 1997 ¹⁴, and updated over the next decade¹⁵, hypothesizes that AMD arises due to alterations in choroidal blood flow resulting from increased stiffening of Bruch's membrane and sclera. Stiffening of these structures is thought to be due to the accumulation of lipoproteins in a similar manner to lipid deposition in arterial walls during atherosclerosis ¹⁴. Such mechanism increases choroidal vascular resistance, resulting in either an increase in choriocapillaris hydrostatic pressure or a decrease in choroidal perfusion.

Drusen may act as a hydrophobic barrier, preventing passage of fluid and nutrients between the choroid and the outer retina, resulting in relative ischemia. These mechanisms can promote secondary changes in retinal capillary plexus, namely in deep retinal capillary plexus (located at inner nuclear layer) or in the superficial retinal capillary plexus (located at ganglionar cells layer). Once the disease begins, anatomic transformation of the choriocapillaris causes further hemodynamic alterations in choroidal vascular layer and retinal capillary plexus which, in turn, can exacerbate disease progression.

An increased knowledge concerning chorioretinal vascular features of intermediate AMD patients, and the documented changes over the follow-up period, can provide some important physiopathology data and clues regarding the disease progression. Those vascular insights can, eventually, create and expand areas of investigation of novel therapeutic options for such a disabling and prevalent disease.

Until the early 2000s, no effective treatment was available for atrophic or exudative AMD. Treatment with intravitreal injected inhibitors of vascular endothelial growth factor (VEGF) has transformed patient care, restoring or stabilizing vision in those with the exudative form of disease. Those treatments involve frequent intravitreal injections and follow-up visits that pose significant burdens in clinicians, patients, and caregivers. For patients with atrophic AMD, slowly worsening vision remains the rule. Although integrated in a multifactorial pathogenesis, the dysregulation of the complement system appears to play a major role and new treatments promoting complement inhibition are being approved for therapeutic intervention in GA.

Intermediate AMD (iAMD) is a heterogeneous common pathway to more advanced forms of the disease. This is one of the reasons why intermediate stage has been arousing special interest as a field of research, studying and evaluation progression biomarkers and exploring possibilities of intervention before visual function deteriorates.

CHAPTER I. GENERAL INTRODUCTION

2. PREVALENCE AND RISK FACTORS

2.1. PREVALENCE

As populations age globally, diseases of older patients are becoming more prevalent. Based on projections from a 2014 systematic review, the estimated 2020 global prevalence of AMD was 196 million and was expected to reach 288 million by 2040 ¹⁶. In 2015, AMD was a leading cause of moderate or severe vision impairment, affecting 8.4 million individuals globally ^{17,18}. Early and late AMDs in Europe were estimated in a meta-analysis of 42,080 patients aged 40 years or older in the European Eye Epidemiology (E3) consortium ¹⁹.

The prevalence of early AMD increased with age from 3.5% in those aged 55–59 years to 17.7% in patients aged >85 years; for late AMD, these values were 0.1 and 9.8%, respectively. Regarding neovascular AMD (nAMD), the authors identified a decrease in the prevalence simultaneous with an increase in visual acuity, which could be attributed to the effects of anti-VEGF therapy ¹⁹.

The incidence of early or late AMD is projected to stabilize or decrease in Europe by 2040 with the adoption of healthier lifestyles, particularly avoidance of smoking, but the aging population is expected to yield a modest increase in AMD prevalence ¹⁹.

Joachim et al. ¹⁸ showed that the 15-year incidence of geographic atrophy (GA) was 3.6% among 2,503 at-risk patients in the Blue Mountains Eye study, which took place west of Sydney, Australia.

The long-term incidence of GA was associated with common AMD risk factors, such as age, smoking, high-risk CHF (i.e., complement factor H), and ARMS2 genotypes. However, characteristics of early AMD lesions – including type and location – also were predictive of GA risk, independent of these common risk factors ¹⁸.

Results of numerous population-based studies of AMD worldwide have indicated racial and ethnic differences in disease prevalence, including greater prevalence of early AMD in Europe than in Asia, but similar prevalence of late AMD on these continents ¹⁶. AMD prevalence among those of

Asian or African descent has been found to be similar.

Europeans had a higher prevalence of geographic atrophy subtype than Africans, Asians and Hispanics. Some data suggest that Asian individuals were more likely to develop exudative or nAMD than Caucasians, possibly because the higher incidence of polypoidal choroidal vasculopathy (PCV) as a cause of exudative AMD ¹⁶. Because PCV occurs at a higher prevalence in Asians than in Europeans, data on prevalence of late AMD in Asians may be influenced by this fact ¹⁶.

In the Comparison of AMD Treatments Trials (CATT), investigators found that reticular pseudodrusen or subretinal drusenoid deposits (SDD) were associated with incident late AMD (i.e., nAMD or GA); hence, they concluded that SDD represented a risk factor for AMD progression ²⁰. This relationship varies with the type of SDD: dot lesions are associated with nAMD, whereas confluent SDD are more associated with GA ²⁰.

In the Coimbra Eye Study of AMD, prevalence in a coastal town in Portugal was as follows: 15.5% of individuals aged 55 years or older had early AMD, and 0.7% had late-stage AMD, including 0.4% with nAMD and 0.3% with GA ²¹. In a subsequent report, the age and gender adjusted prevalence of any AMD was 12.5% (late AMD, 1.2%) and included individuals from a coastal town and an inland town. The AMD rate was higher in the inland population by multivariate analysis ²². Investigators then evaluated the 6.5-year incidence and progression of AMD in a coastal region in Portugal and determined the cumulative incidence to be 10.7% for early AMD and 0.8% for late AMD ²³. Disease progression occurred in 17.2% of patients ²³.

Staging of AMD lesions and evaluation AMD progression is affected by the grading methodology (CFP only or with multimodal imaging). In CFP-based grading, the prevalence was 14.11% for early AMD and 1.05% for late AMD. Using multimodal grading, the prevalence increased to 14.60% for early AMD and 1.61% for late AMD. AMD staging was more accurate with the multimodal approach and this was especially relevant for late AMD.²⁴

It should be noted that epidemiologic estimates of AMD must be interpreted with caution. Inter-study comparability is limited for several reasons, including different AMD classification systems, the age range of the patient population, and the technology applied to evaluate disease e.g., color fundus photography (CFP) or optical coherence tomography (OCT) ²⁵.

2.2. RISK FACTORS

AMD is a complex, multifactorial disease for which numerous modifiable and nonmodifiable risk factors have been identified. The main modifiable risk factor of advanced AMD development is smoking; smokers are 2-4 times more likely to experience AMD ²⁶. Older age (≥ 65 years), northern European ancestry, and family history are nonmodifiable risk factors for advanced AMD ²⁷. Additionally, AMD in 1 eye increases the likelihood that AMD will develop in the fellow eye. Generally, men and women experience AMD at similar rates ¹⁷. Hyperlipidemia and hypertension also may be risk factors, but the level of evidence for these is lower ²⁸.

Nutritional factors have been linked to AMD development and progression. Diets containing dark green vegetables, fish oils or long-chain polyunsaturated fatty acids (high levels of docosahexaenoic and eicosapentaenoic acid) are associated with decreased risk of late AMD. Supplementation with high doses of oral antioxidants (vitamin C, vitamin E and carotenoids lutein and zeaxanthin) and zinc reduced progression from intermediate to late AMD in approximately 25% over 5 years ^{29,30}.

The mediterranean diet (with high consumption of vegetables, moderate consumption of fish and wine, and low consumption of meat) has been recognized by several groups as a model option, contributing to decreased AMD risk and progression to late AMD ³¹. Studies had reported that higher adherence to mediterranean diet was associated with a 41% reduced risk of progression to late AMD ³². In Coimbra Eye Study similar findings were reported with a protective role of this diet in AMD development ^{33,34}.

Physical activity, sunlight exposure and cataract surgery are other factors that have been implicated in AMD development with inconsistent association ³⁵.

2.3. GENETICS

In a study of elderly twin men, genetic factors were found to explain 46–71% of the variance in AMD severity ³⁶. AMD-associated genes are likely to be particularly involved in complement system modulation, the extracellular matrix, and lipid metabolism. Investigators leading a large genome-wide association study (GWAS) identified 52 coding variants, distributed in 34 loci that occurred more frequently in patients with AMD ³⁷, as shown in Table 1. This GWAS was conducted by an

international consortium – the International Age-related Macular Degeneration Genomics consortium (IAMDGC).

In this genome wide association study of more than 12 million variants, authors identified 52 common and rare variants at 34 loci ³⁷. Although AMD subtypes have similar allelic patterns, these authors were the first to detect a genetic variant specifically predictive of neovascular AMD identified near MMP9. They also identified very rare coding variants in CFH, CFI, and TIMP3 and a splice variant in SLC16A8, supporting the hypothesis that these genes are involved in the etiology of AMD ³⁷. The 52 variants explained 27.2% of the variability in disease, with rare variants contributing 1.4% ³⁷.

The authors then computed effect-size weighted sums of the risk associated alleles for the 52 variants and divided them by the sum of all the effect sizes to arrive at a genetic risk score (relative and absolute) for each individual ³⁷. This work represents progress toward personalized and precision medicine, although it has yet to be applied in clinical practice. No other study has yielded results of genetic determinants strongly associated with AMD risk ^{38,39}.

Table 1– Identification of 52 independent AMD risk variants in 34 loci (Addapted from Fritsche ²⁹).

Signal number	Locus name	Index variant	Chr: Position	Major / minor allele
1.1	CFH	rs10922109	1:196,704,632	C/A
1.2		rs570618	1:196,657,064	G/T
1.3		rs121913059	1:196,716,375	C/T
1.4	CFH (CFHR3/CFHR1)	rs148553336	1:196,613,173	T/C
1.5	CFH	rs187328863	1:196,380,158	C/T
1.6		rs61818925	1:196,815,450	G/T
1.7		rs35292876	1:196,706,642	C/T
1.8		rs191281603	1:196,958,651	C/G
2	COL4A3	rs11884770	2:228,086,920	C/T
3	ADAMTS9-AS2	rs62247658	3:64,715,155	T/C

Signal number	Locus name	Index variant	Chr: Position	Major / minor allele
4.1	<i>COL8A1</i>	rs140647181	3:99,180,668	T/C
4.2		rs55975637	3:99,419,853	G/A
5.1	<i>CFI</i>	rs10033900	4:110,659,067	C/T
5.2		rs141853578	4:110,685,820	C/T
6	<i>C9</i>	rs62358361	5:39,327,888	G/T
7	<i>PRLR/SPEF2</i>	rs114092250	5:35,494,448	G/A
8.1	<i>C2/CFB/SKIV2L</i>	rs116503776	6:31,930,462	G/A
8.2		rs144629244	6:31,946,792	G/A
8.3	<i>C2/CFB/SKIV2L (PBX2)</i>	rs114254831	6:32,155,581	A/G
8.4	<i>C2/CFB/SKIV2L</i>	rs181705462	6:31,947,027	G/T
9	<i>VEGFA</i>	rs943080	6:43,826,627	T/C
10	<i>KMT2E/SRPK2</i>	rs1142	7:104,756,326	C/T
11	<i>PILRB/PILRA</i>	rs7803454	7:99,991,548	C/T
12	<i>TNFRSF10A</i>	rs79037040	8:23,082,971	T/G
13	<i>MIR6130/RORB</i>	rs10781182	9:76,617,720	G/T
14	<i>TRPM3</i>	rs71507014	9:73,438,605	GC/G
15	<i>TGFBR1</i>	rs1626340	9:101,923,372	G/A
16	<i>ABCA1</i>	rs2740488	9:107,661,742	A/C
17	<i>ARHGAP21</i>	rs12357257	10:24,999,593	G/A
18	<i>ARMS2/HTRA1</i>	rs3750846	10:124,215,565	T/C
19	<i>RDH5/CD63</i>	rs3138141	12:56,115,778	C/A
20	<i>ACAD10</i>	rs61941274	12:112,132,610	G/A
21	<i>B3GALT1</i>	rs9564692	13:31,821,240	C/T
22.1	<i>RAD51B</i>	rs61985136	14:68,769,199	T/C
22.2		rs2842339	14:68,986,999	A/G
23.1	<i>LIPC</i>	rs2043085	15:58,680,954	T/C
23.2		rs2070895	15:58,723,939	G/A
24.1	<i>CETP</i>	rs5817082	16:56,997,349	C/CA
24.2		rs17231506	16:56,994,528	C/T
25	<i>CTRB2/CTRB1</i>	rs72802342	16:75,234,872	C/A
26	<i>TMEM97/VTN</i>	rs11080055	17:26,649,724	C/A
27	<i>NPLOC4/TSPAN10</i>	rs6565597	17:79,526,821	C/T

Signal number	Locus name	Index variant	Chr: Position	Major / minor allele
28.1	C3	rs2230199	19:6,718,387	C/G
28.2				
28.3	C3 (NRTN/FUT6)	rs147859257	19:6,718,146	T/G
29	CNN2	rs12019136	19:5,835,677	G/A
		rs67538026	19:1,031,438	C/T
30.1	APOE	rs429358	19:45,411,941	T/C
30.2	APOE(EXOC3L2/MARK4)	rs73036519	19:45,748,362	G/C
31	MMP9	rs142450006	20:44,614,991	TTTTC/T
32	C20orf85	rs201459901	20:56,653,724	T/TA
33	SYN3/TIMP3	rs5754227	22:33,105,817	T/C
34	SLC16A8	rs8135665	22:38,476,276	C/T

Current data suggest that clinical characteristics, such as baseline best corrected visual acuity (BCVA) and neovascularization lesion size, are superior to genetic findings for predicting AMD progression and making treatment decisions ^{27,40}.

Nevertheless, research to identify and characterize AMD-associated genes is ongoing. Lipid genes have been implicated because of an association between AMD and elevated high-density lipoprotein (HDL) levels. A cross sectional comparison of CFP images and lipid subfraction data revealed a link between AMD and HDL, particularly the extra-large HDL particles, such that an increase in HDL level from the 25th to the 75th percentile corresponded to an AMD risk increase of 20% ⁴¹.

The association of AMD with elevated lipid levels could potentially explain the accumulation of lipid-rich drusen, which characterizes early and intermediate disease stages.

Associations of VEGF genetic variants with treatment outcomes have been investigated^{40–42}, and in a 1-year analysis of a Comparison of AMD Treatments Trials study cohort, no AMD-implicated genotype was found to be associated with any clinical measure of response to VEGF inhibition, including visual acuity gains, OCT findings, vessel leakage, mean lesion size, or mean number of injections ⁴³.

Certain complement pathway genes have been identified as potential therapeutic targets in AMD because dysregulation of the complement cascade is implicated in AMD pathogenesis ⁴⁴. Several AMD-associated genetic polymorphisms involve the complement cascade, and drusen has been found to comprise CFH and C3 gene products, along with other complement effectors, in patients with AMD ³⁵. AMD-associated CFH and C3 variants are regarded as strong genetic risk factors, constituting an estimated 50 and 22%, respectively, of the attributing disease risk ³⁵. Certain polymorphisms in ARMS2 and HTRA1 also are considered to confer risk of AMD ³⁵. Compared with control patients awaiting cataract surgery, patients with nAMD were significantly more likely to have certain polymorphisms in CFH (CC genotype), ARMS2 (TT), HTRA1 (AA), and C3 (CG), and these associations were stronger among women than among men ³⁹. Other investigators found that fewer than half of CFH variants were associated with AMD visual outcomes on anti-VEGF agents ⁴². Moreover, efforts to slow enlargement of GA area by systemically inhibiting complement component 5 ³⁸ or complement factor D ⁴⁵ have been unsuccessful. More recently, local complement C3 inhibition with pegcetacoplan resulted in statistically significant reduction in the growth of GA compared with sham treatment ^{44,46}.

Researchers applied a knowledge-driven pathway analysis to search for driver genes in the largest available database of advanced AMD cases ⁴⁷. They found that only PLCG2, which encodes the signaling enzyme phospholipase C gamma 2, was a key player in pathways from the KEGG, Reactome, GO, and NetPath data-bases and remained significant in sensitivity analyses ⁴⁷. Functional studies are underway to better characterize PLCG2 as a candidate AMD gene.

In addition to individual gene-disease associations, genetic data can be used and “pooled” in the calculation of an individual’s genetic risk score (GRS). Since the heritability of AMD is estimated to be 45% to 70%, AMD risk scores based solely on genotype have the potential to predict a substantial amount of risk. The GRS calculation comprehends both protective and risk-conferring genetic variants and represents the genetic susceptibility to AMD for any individual. In other words, it represents the cumulative genetic risk for developing AMD based on the person’s sequenced genotype regarding variants known to be associated with the disease. The calculated GRS showed that individuals in the highest genetic risk had a 44-fold increased risk of advanced AMD compared to those in the lowest. ³⁷.

Recently, groups such as the EYE-RISK consortium used this GRS model in European populations, showing that there was a significantly higher GRS in individuals with late AMD compared to patients with early or intermediate AMD and comparing them to healthy controls. They found, however, an overlap between the groups, meaning that the GRS alone was unable to completely distinguish between them ⁴⁸. The same group calculated pathway-based GRS, considering the risk-conferring and risk-protective alleles for each biological pathway (e.g., complement, extracellular matrix, lipids, etc).

The GRS can be further used in multifactorial prediction models accounting for other interacting environmental/ demographic risk factors. This is of special interest in the progression towards more personalized medicine in AMD management ⁴⁸.

CHAPTER I. GENERAL INTRODUCTION

3. PATHOPHYSIOLOGY AND DISEASE PROGRESSION

Aging of the eye is accompanied by the buildup of uncleared cellular debris that originates from the retinal pigment epithelium (RPE) and accumulates where the RPE interfaces with Bruch's membrane (BM) and the neurosensory retina.

These deposits, known as drusen, typically are the first ophthalmoscopic sign of AMD, appearing before visual function is appreciably affected. Drusen are composite structures, primarily consisting of lipids as well as proteins and carbohydrates that can be visualized as small white or yellowish deposits on the macula. Drusen deposition, concomitant with other structural and biochemical changes associated with AMD pathogenesis (including persistent activation of the complement cascade, oxidative stress and chronic inflammation), lead to thickening and decreased permeability of BM⁴⁹ (Figure 1), with subsequent obstruction of both nutrient transport to the retina and waste exchange to the choroid and thinning of the choroidal vasculature. Combined with neurodegenerative changes within the photoreceptor-RPE complex, result in pigmentary abnormalities of the RPE, including hypo or hyperpigmentation, in early or intermediate stages of disease⁵⁰. This combination of factors results in impaired RPE and photoreceptor function⁴⁹.

AMD is defined as the presence, within two-disc diameters of the fovea, of those acellular lipid-rich deposits beneath the retinal pigment epithelium sometimes associated with pigmentary abnormalities. Besides AMD there are other diseases characterized the presence of drusen or drusen-like deposits that should be considered in the diferencial diagnosis: several inherited retinal conditions, inflammatory or infiltrative diseases, renal diseases (membranoproliferative glomerulonephritis and Alport syndrome), vitamin A deficiency, pseudoxanthoma elasticum and drug deposits.

Being chronic and progressive, AMD lesions sometimes progress to late-stage, vision-threatening complications, particularly towards atrophic pathway in geographic atrophy (also known informally as dry AMD) or towards exudative pathway in neovascular AMD (also known informally as wet AMD)⁵¹.

3.1. DRY AMD

Dry AMD is characterized by a slowly progressive loss of visual function, owing to deterioration of the choriocapillaris, atrophic loss of the outer retina, and disruption and eventually death of the photoreceptor layer. GA is the advanced form of dry AMD⁵⁰. In a study of patients with GA and 20/50 vision or better at baseline, the cumulative risk of legal blindness was 27% by 4 years⁵².

Drusen aggregate and undergo morphologic changes during AMD progression. Small, well-defined drusen particles, referred to as hard drusen, are common in younger individuals and are not a sign of AMD risk⁵³. Soft drusen are less demarcated and grow over time, coalescing into confluent drusen, and co-occurring with AMD. Eventually, soft drusen mature to a crystalline form also known as refractile drusen, which is predictive of GA⁵³. Drusen are not static structures. They can aggregate, coalesce and regress, concurrent with the transition from intermediate to advanced disease⁵⁴.

Dry AMD is characterized by unrelenting expansion of atrophic areas. Investigators in the global Proxima trials demonstrated a 2-year mean increase in GA lesion size of 3.87 mm² among patients with bilateral GA at baseline⁵⁵. One-third of patients with unilateral GA at baseline had bilateral GA by year 1 of follow-up⁵⁵.

3.2. WET AMD

Although neovascular disease affects only about 20% of patients with AMD, this clinical form is responsible for approximately 90% of severe central vision loss caused by AMD²⁷.

The pathogenesis of wet AMD involves recruitment of immune cells to the damaged macula and secretion of proinflammatory and proangiogenic cytokines, particularly VEGF. Several retinal cell types, including RPE cells, also can express and secrete VEGF⁵⁶. VEGF stimulates endothelial cell proliferation and migration and leads to angiogenesis and increased vascular permeability^{57,58}. The newly growing blood vessels leak fluid, disrupting and damaging the layer of photoreceptors and impairing vision.

Although the abnormal vessels of wet AMD typically arise from the choroidal circulation,

neovascularization and leakage also can emanate from the retinal vasculature ⁵⁹. Left untreated, this subretinal exudation leads to fibrosis and atrophy of the macula and irreversible loss of central vision in as little as a few months or even sooner.

In AMD, neovascularization may start in the outer retina or in the choroid; therefore, the term choroidal neovascularization (CNV) is not always appropriate, although it is widely accepted ⁶⁰. Recently, a group of experts convened to address nAMD nomenclature and proposed the term macular neovascularization (MNV) to denote neovascular disease of the macula, independent of origin or location ⁶⁰.

Type 1 MNV was defined as ingrowth of vessels initially from the choriocapillaris into and within the sub-RPE space, type 2 MNV denoted neovascularization originating from the choroid that traverses Bruch's membrane and the RPE monolayer and then proliferates in the subretinal space, and type 3 MNV entailed neovascularization originating from the retinal circulation (typically the deep capillary plexus) and extending toward the outer retina and choroid ⁶⁰.

MNV can occur without exudation ⁶¹. Nonexudative (also named subclinical) MNV with stability beyond six months are designed as quiescent and investigators have hypothesized that those seemingly quiescent lesions may afford some protection against atrophy ⁶². These eyes do not have substantial visual loss and do not respond to anti-VEGF treatment, however they present, particularly in the first six months, a considerable risk of progressing to exudative AMD and should be monitored closely ^{61,62}. Nonexudative MNV does not appear to contribute to deterioration of visual function and has no indication for anti-VEGF treatment ⁶¹.

CHAPTER I. GENERAL INTRODUCTION

4. DIAGNOSIS AND CLASSIFICATION

The diagnostic signs of early and intermediate AMD are drusen and pigmentary changes or abnormalities. Patients with early or intermediate AMD constitute 85–90% of all patients with AMD²⁷. Staging of AMD disease traditionally is determined from CFP images of the macula. Staging of earlier disease states relies on specific sizes, types, and locations of drusen and pigmentary changes, and criteria vary among classification systems.

There are several classification schemes that utilize drusen characteristics and pigmentation to differentiate early and intermediate AMD. These include the Rotterdam system⁶³ (Table 2), the 9-step severity scale based on drusen and pigmentary abnormalities, reported in the Age-Related Eye Disease Study (AREDS)⁶⁴ and the simplified AREDS severity scale⁶⁴ (Table 3).

Table 2 – Rotterdam staging system.

Stage	Definition
0	a No signs of age-related maculopathy at all b Hard drusen (<63 μm) only
1	a Soft distinct drusen ($\geq 63 \mu\text{m}$) only b Pigmentary abnormalities only, no soft drusen ($\geq 63 \mu\text{m}$)
2	a Soft indistinct drusen ($\geq 125 \mu\text{m}$) or reticular drusen only b Soft distinct drusen ($\geq 63 \mu\text{m}$) with pigmentary abnormalities
3	Soft indistinct drusen ($\geq 125 \mu\text{m}$) or reticular drusen with pigmentary abnormalities
4	Atrophic or neovascular AMD

The AREDS classification of AMD lesions refers to both eyes (second eye must have the same category or below) and was the basis for the analysis in the AREDS prospective studies on AMD, which also evaluated the efficacy of antioxidant and other dietary supplements in delaying the onset of advanced AMD ⁶⁴.

Table 3 – AREDS clinical classification.

Stage	Clinical characteristics
No AMD	No or small drusen (<63 µm)
Category 1	Total drusen area <125 µm diameter No pigmentary abnormalities
Early AMD	<u>Presence of one or more of the following:</u> Small drusen (<63 µm) or medium size drusen (≥63 µm and <125 µm)
Category 2	Total drusen area ≥125 µm diameter Pigmentary abnormalities
Intermediate AMD	<u>Presence of one or more of the following:</u> Large drusen (≥125 µm)
Category 3	Soft, indistinct drusen, drusen size ≥63 µm and drusen area >0.241 disk diameter Soft, indistinct drusen, drusen size ≥63 µm and drusen area >0.439 disk diameter No central geographic atrophy (outside 500 µm radius from the foveal center)
Late AMD	<u>Presence of one or more of the following:</u> Geographic atrophy in the central subfield with at least questionable involvement of the center of the macula
Category 4	Evidence of neovascular AMD lesions: - fibrovascular/serous pigment epithelial detachment - serous/hemorrhagic sensory retinal detachment - subretinal/sub-retinal pigment epithelial detachment - subretinal fibrous tissue (or fibrin) - photocoagulation for AMD

More recently, the evidence-based clinical classification system was described by Ferris et al.⁵⁹ (Table 4). Each step corresponds to an incremental increase in risk of 5-year progression to late AMD in patients 55 and older: from 0.5% for eyes classified as normal aging to 50% for the intermediate risk group^{59,65}.

The clinical classification proposed by the Classification Committee of the Beckman Initiative for Macular Research is now commonly used in clinical and research work. This classification was created in 2013 and requires only clinical examination or colour fundus images to ensure its universal accessibility and applicability⁵¹.

In this classification system, AMD is defined as the presence, within two-disc diameters of the fovea of medium or large drusen; pigmentary abnormalities of the RPE associated with at least medium drusen; or lesions associated with late-stage (Table 4). Age-related macular degeneration pigmentary abnormalities are defined as any definite hyperpigmentary or hypopigmentary abnormalities associated with medium or large drusen but not associated with other known disease entities.

Table 4 – Beckman classification of AMD.

Stage	Clinical characteristics (CFP)
No ageing changes	No drusen, and No pigmentary abnormalities
Normal ageing changes	Small drusen (<63 µm), and No pigmentary abnormalities
Early AMD	Medium drusen (≥63 µm and ≤125 µm), and No pigmentary abnormalities
Intermediate AMD	Large drusen (>125 µm), or Any pigmentary abnormalities, or both
Late AMD	nAMD, or GA, or both

These classification systems were used in clinical practice but also, in clinical trials and epidemiologic studies, based on CFP alone. Today, multimodal imaging provides much more detailed and complete information and is superior in lesion detection and characterization. OCT is a noninvasive test that enables visualization of retinal and choroidal structures in cross section. OCT is more sensitive than CFP, from which most epidemiologic data on AMD are derived. AMD diagnosis and disease staging by means of OCT represents an emerging paradigm shift ⁶⁵.

In 2018, an international group of experts described criteria for staging AMD-associated atrophy based on OCT findings ⁶⁶. The group identified the following stages of atrophic progression based on involvement of specific retinal layers ⁶⁶:

- (1) incomplete outer retinal atrophy (iORA),
- (2) complete outer retinal atrophy (cORA),
- (3) incomplete RPE + outer retinal atrophy (iRORA), and
- (4) complete RPE + outer retinal atrophy (cRORA).

The last stage, cRORA, is equivalent to the traditional terminology of GA, but GA term should only be used when cRORA is present without any evidence of an associated neovascular membrane. In addition, the authors set forth criteria for diagnosis of complete retinal pigment epithelium and outer retinal atrophy (cRORA), including ⁶⁶:

- Hypertransmission $\geq 250 \mu\text{m}$.
- Attenuation or disruption of the retinal pigment epithelium band $\geq 250 \mu\text{m}$.
- Evidence of overlying photoreceptor degeneration (outer nuclear layer thinning, loss of the external limiting membrane, or loss of the ellipsoid zone or interdigitation zone).

These criteria cannot be used in the presence of a retinal pigment epithelium tear.

The same group set the criteria for incomplete retinal pigment epithelium and outer retinal atrophy (iRORA) ⁶⁶:

- Hypertransmission $< 250 \mu\text{m}$.
- Attenuation or disruption of the retinal pigment epithelium band $< 250 \mu\text{m}$.
- Evidence of overlying photoreceptor degeneration (outer nuclear layer thinning, loss of the external limiting membrane, or loss of the ellipsoid zone or interdigitation zone).

These criteria cannot be used in the presence of a retinal pigment epithelium tear.

Finally, the concept of Nascent geographic atrophy (nGA) should be used as long as we find at least one of the following signs on SD-OCT:

- Subsidence of the outer plexiform layer and inner nuclear layer.
- Development of a hyporeflective, wedge-shaped band within the outer plexiform layer.

The E3 consortium has sought to establish a new classification system for macular diseases based on SD-OCT. The authors have proposed standardized terminology and a grading scheme for structural evaluations of macular diseases, to be applied in future epidemiologic studies ⁶⁷. OCT is crucial in the diagnosis and monitoring of AMD patients and may be combined with other retinal studies in a multimodal approach, especially in cases of differential diagnosis, unusual disease evolution, or poor response to treatment ^{68,69}.

CHAPTER I. GENERAL INTRODUCTION

5. BIOMARKERS OF DISEASE PROGRESSION

The five-year progression from early and intermediate AMD to advanced disease is known to range from 0.4% to 53% depending on disease severity stage ⁷⁰. This wide variation in AMD risk progression reflects the population heterogeneity and explains the particular interest in exploring different phenotypes and searching predictive biomarkers. Since 2017, the Consensus International Group (CAM—Classification of Atrophy Meeting) has been discussing and publishing on this subject, whose main objective is to provide standardized biomarkers for future interventional clinical trials ^{66,71–74}.

Predictive knowledge is also an important goal in clinical practice, supporting the identification of patients at increased risk of late AMD changes. Evaluation of drusen in CFP was, in the past, the main clinical standard for assessing intermediate AMD patients. There seems to be a positive correlation between the number, area, and extent of drusen observed in CFP and the risk of progression in more than two years ⁷⁵. Nevertheless, this method has limited value for predicting individual AMD progression because it is unreliable in the short term ^{75,76}.

More advanced experimental predictive strategies including a combination of the aforementioned drusen classification in CFP combined with genetic, demographic, and environmental factors, such as smoking, light exposure, or diet have been proposed ⁷⁷. Some authors have proposed an online risk calculation considering age, family history, smoking habits, fundoscopy, and optional genetic data (Advanced AMD Risk Calculator, <http://caseyamdcalc.ohsu.edu> accessed on 26 December 2021).

While these methods show promising results, they do not explore a variety of quantitative features of drusen and adjacent structures that can be useful predictors of AMD progression ^{76,78}.

The recent widespread use of non-invasive approaches (Fundus Autofluorescence, Optical Coherence Tomography and OCT-angiography) in early and intermediate AMD retinal imaging has provided important tools in the identification of biomarkers of disease progression. With the aid of

such multimodal retinal imaging modalities, complemented with automated algorithms, clinicians and researchers are suggesting a wide range of under recognized image biomarkers of AMD progression that can be useful and promising in clinical practice.

5.1. COLOR FUNDUS PHOTOGRAPHY

Drusen characteristics explored in CFP are drusen size, area, and associated pigmentary changes. Natural history studies have demonstrated that the most important risk factors are advanced AMD in the fellow eye, the presence of large, soft, and confluent drusen as well as AMD-related pigmentary abnormalities ⁷⁰.

Therefore, some strategies of risk calculation have been proposed based on drusen size, pigmentary abnormalities, and fellow eye status ^{70,77}. The proposed AREDS simplified scoring system assigns to each eye one risk factor for the presence of 1 or more large drusen ($\geq 125 \mu\text{m}$) and one risk factor for the presence of any pigment abnormality. The summation of risk factors in both eyes yields the 5-year risk of developing advanced AMD ⁷⁰.

Drusen area has been associated with the development of advanced or late AMD ⁷⁹. This relationship may result from loss of the overlying photoreceptors and the outer retina layers due to focal disruption or local compression induced by the drusen itself ^{80,81}. Other studies found that the most important risk factor is the presence of advanced disease in the fellow eye, reporting less significance in drusen size and total drusen area, particularly in the risk of conversion to neovascular AMD ⁸².

Drusen area can be calculated from manually tracing drusen or by semiautomatic drusen analysis from CFP. Those estimates are considerably subjective, depending on aspects related to the observer and related to the patient (opacities, pigmentation, and variability of drusen appearance). Studies comparing drusen area calculation obtained by CFP and by OCT showed only a fair agreement ^{83,84}. Drusen area measurements tend to be larger with CFP than with OCT. This difference can be attributed to an underestimation of small and flat drusen on OCT measurements.

The Age related Eye Disease Study (AREDS) group reported that drusen regression is also a risk

factor, as 82% of the eyes that developed significant atrophic changes had preceding drusen regression and hypopigmentary changes^{77,79–81}. Other researchers further reported that in patients with early and intermediate AMD, drusen regression occurred in 44% of the cases, and preceded the progression to advanced AMD (both GA and nAMD)⁸².

Refractile drusen, with calcified nodules, are marked by RPE loss and may represent a possible step before regression and subsequent conversion to advanced stages of the disease⁸⁵. Their glistening appearance results from calcium-containing spherules. In the past, it was supposed that drusen regression caused RPE death, but recent research supports the opposite hypothesis (RPE dies as the drusen collapse)⁸⁶.

Drusenoid pigment epithelium detachment (PED) results from soft drusen coalescence, reaching diameters >350 μm . The natural history of drusenoid PED presents some overlapping features with the drusen: size and confluence are related to progression. AREDS study reported 19% progressing to GA and 23% to nAMD in 5-year follow-up⁷⁷.

5.2. FUNDUS AUTOFLUORESCENCE

Fundus autofluorescence (FAF) assessment relies on native fluorescence from physiological retinal components, providing an indication of healthy function of RPE cells. FAF with blue light excitation (488 nm) is currently the most used in the clinical setting. The high contrast retinal images clearly show the areas with preserved RPE and the areas affected by RPE dysfunction and atrophy: loss of RPE and fluorophores in atrophic areas is associated with a decreased FAF signal (hypoautofluorescence) while dysfunctional areas present with an increased signal (hyperautofluorescence).

The more important FAF findings to highlight in early and intermediate AMD are FAF patterns of drusen, of SDD, and those associated with pigmentary changes. SD-OCT imaging largely overcomes conventional FAF as it adds insight into the condition of the neurosensory layers and associated alterations at the level of the RPE and choroid⁸⁷. FAF imaging modalities are more influenced by opacities than SD-OCT but, in cases of AMD-mimicking macular dystrophies, are helpful in the differential diagnosis.

Drusen FAF patterns were related to histologic examination, in the first direct clinico- pathologic study correlation. Authors described four FAF stages as follows ⁸⁷:

Stage 1 is characterized by isoautofluorescent drusen (visible on CFP);

Stage 2, with mildly uniform hyperautofluorescence corresponding to discrete photoreceptors suffering and complete coverage by RPE;

Stage 3, where a ring of hyperautofluorescence is visible around an hypoautofluorescence center corresponding to early RPE atrophy (RPE gaps);

Stage 4, with an uniform hypoautofluorescence corresponding to absence of RPE (complete RPE atrophy), and which includes a high proportion of refractile drusen (82%) with many calcific nodules, visible on CFP.

Stages 3 and 4 are, indeed, related to atrophic suffering, sometimes occurring over individual drusen with subsequent dysfunction and loss of RPE and concomitant outer nuclear layer thinning, external limiting membrane and ellipsoide zone disruption, as defined by the CAM group ^{66,73,74}.

Reticular pseudodrusen are identified in FAF imaging as an area of isoautofluorescence on the center of each reticular pseudodrusen surrounded by halos of reduced autofluorescence, responsible for the “target aspect”, around the macula and, especially, along the superior vascular arcades ⁸⁸.

Pigmentary abnormalities also promote FAF changes whose patterns depend on the presence of hypo or hyperpigmentation and on the status of RPE cells.

FAF patterns are largely studied in atrophic AMD, particularly in the junction zone ⁸⁹, but the use of this imaging modality in intermediate AMD has not defined a particular FAF pattern exclusively associated with conversion or progression ⁹⁰.

Concerning atrophic AMD different FAF patterns in junctional zone, some authors demonstrated that those patterns have prognostic implications and are associated with the rate of GA enlargement ^{89,91,92}. GA progression is significantly higher in eyes with banded and diffuse FAF pattern compared to eyes without FAF abnormalities or with focal FAF pattern. The diffuse trickling pattern is associated to an even greater progression of GA when compared to the other diffuse patterns ⁹².

The possibility of using FAF imaging in monitoring GA patients and in quantifying GA area is valued, as an important advantage, by Food and Drug Administration. Data obtained by FAF quantifying changes in GA lesion size over time, is used, as primary outcome, in several clinical trials⁷¹.

5.3. OPTICAL COHERENCE TOMOGRAPHY

The main OCT biomarkers related to progression to advanced AMD include drusen volume, OCT-reflective drusen substructures (ODS), reticular pseudodrusen or subretinal drusenoid deposits (SDD), hyperreflective foci (HRF), hyper-transmission defects (HTD) and incomplete retinal pigment epithelial and outer retinal atrophy (iRORA)^{66,71–74,93–95}.

5.3.1. DRUSEN VOLUME

In line with previous studies based on CFP, several investigators using SD-OCT in patients with early and intermediate AMD reported that drusen area, height, and length predicted progression to advanced stages⁷⁶. Eyes with higher baseline drusen volume have an increased risk of progression to either nAMD or GA⁷⁹, increase in drusen height is an important risk factor for progression to late atrophic AMD, and drusen length seems to have a positive correlation with the risk of conversion to nAMD⁹⁶.

More recent SD-OCT volume studies provided more comprehensive measures^{97–102}. Quantitative automatic determinations are available in some devices whose role can be useful in clinical practice. It is possible to automatically distinguish normal eyes from eyes with early and intermediate AMD by identifying total retinal volumes and RPE-drusen complex volumes.

Several studies have used automatic RPE-drusen complex (RPEDC) calculations with SD-OCT devices. Advanced RPE analysis in Cirrus HD-OCT platform provides the clinician with a rapid and reproducible quantitative approach for following disease progression in AMD patients (Advanced RPE analysis with RPEDC abnormal thickening and thinning)^{103,104}. The RPEDC abnormal thickening and RPEDC abnormal thinning (RAT) volumes were generated by semiautomated segmentation

within a 5 mm diameter macular field: macular OCT drusen volumes and RAT volumes increased significantly in AMD eyes over 2 years ¹⁰⁴. These quantitative SD-OCT biomarkers predict 2-year AMD progression and may serve as useful biomarkers for disease progression. Abnormal thinning of the RPE layer is believed to be an early precursor of atrophic lesions, as well as a marker of progression at the margins of expanding GA ¹⁰⁴. The RAT volume measurement improves specificity in distinguishing eyes with early precursors of GA from healthy control eyes, because control eyes did not demonstrate significant change in this parameter over 2 years ¹⁰⁴.

Swept Source (SS) technology, with its longer wavelength and faster acquisition, provides better penetration below the RPE and therefore better visualization of the external layers of the retina and choroid. Improved visualization of external layers with SS-OCT devices, provides better automated segmentation performances, and more concordance in manual and automated measurements of drusen volume. Even more, some authors found no significant differences in drusen volume prior to and after manual correction in SS-OCT compared with a significant difference in SD-OCT devices ¹⁰⁵.

5.3.2. OCT-REFLECTIVE DRUSEN SUBSTRUCTURES

Variation in the OCT structure and properties of drusen has also been proposed to represent an increased risk of AMD progression. Drusen usually appears as a smooth, dome shaped RPE elevation with homogeneous medium internal reflectivity and without HRF. These characteristics occur in about 47% of all drusen and only in 50% of the so-called soft drusen in CFP ⁷⁸. However, varying morphology, reflectivity, and internal homogeneity may be observed in other cases and these findings are important in monitoring intermediate AMD patients (Figure 3C).

A large multicenter study investigated such variations in patients with intermediate AMD enrolled in the AREDS2 SD-OCT study ⁵⁴. The authors found drusen substructures in 24% of intermediate AMD patients and described four phenotypic subtypes of variations or ODS: (a) low-reflective core (L type); (b) split-reflective core (S type); (c) high-reflective core (H type); and (d) conical debris (C type). Presence of ODS at baseline in eyes with intermediate AMD was associated with progression to GA, but not to nAMD ⁵⁴. Low reflective cores may consist primarily of lipids,

whereas high reflective cores may consist primarily of proteins and hydroxyapatite. Split drusen may simply be a heterogeneously composed drusen, with disease-associated molecules that have not seeded onto spherules, hence the split designation ⁵⁴. Conical debris may be calcified deposits caused by a shattering of high reflective cores. Conical debris (C type) is the subtype more closely related to atrophic changes, followed by H type, S type, and finally by L type ⁵⁴.

In more recent studies, authors analyzed the heterogeneous internal reflectivity material within drusen (HIRD) and concluded it is formed by multilobular nodules composed of hydroxyapatite and that they are quite different from the spherules or refractile drusen, another refractile feature found in the retina of patients with AMD ¹⁰⁶. Unlike spherules, which are small, refractile on CFP and reflective on OCT, nodules are large, refractile on CFP and nonreflective on OCT. Nodules are not simply aggregations of spherules and the heterogeneous HIRD should be called “calcified nodules” instead of the alternative terms “hyperreflective pyramidal structures” or conical debris ¹⁰⁶. These findings suggest that hydroxyapatite nodules may be indicators of progression to advanced AMD and that multimodal retinal imaging can help us to find outcome measures in AMD progression ¹⁰⁶.

5.3.3. RETICULAR PSEUDODRUSEN OR SUBRETINAL DRUSENOID DEPOSITS

These subretinal collections of granular, interlacing, hyper-reflective material located above RPE are commonly found in the superior macula or close to superotemporal arcades (Figure 3B). Observable by OCT, reticular pseudodrusen or SDD undergo a characteristic lifecycle of growth, invasion into the adjacent ellipsoid zone, and finally regression. Advanced stages in the SDD lifecycle are related to RPE degeneration and photoreceptor loss ¹⁰⁷.

Infrared reflectance (IR) and SD-OCT appear to be particularly relevant methods to diagnose SDD. Using multimodal imaging, the prevalence of SDD appears higher than previously reported in studies based on retinal photography only. Regarding imaging techniques, the prevalence of SDD is lowest when diagnosed by CFP (6.7%) and fundus autofluorescence FAF (9.5%) and reaches the higher percentages when diagnosed with IR (18.1%) or SD-OCT (17.4%) ⁸⁸.

Reticular pseudodrusen are found to be present in approximately 9% to 58% of patients with

intermediate AMD, depending on the population studied ^{8,108}. In other studies, reticular pseudodrusen were present in 4.6% of eyes without AMD, 13.0% with early AMD, 62.6% with intermediate AMD, 34.6% with atrophic AMD, and 8.1% with neovascular AMD ^{8,88}. Researchers reported that the presence of reticular pseudodrusen is associated with an additional 2 to 6-fold increased risk of progression to nAMD or central GA, with the risk of progression higher for reticular pseudodrusen located outside the macula ⁸.

5.3.4. HYPER-REFLECTIVE FOCI

HRF are well defined, highly backscattering lesions within the neurosensory retina; they can be located adjacent to the drusen edge or apex (Figure 3A) or in the inner neurosensory retina. They are described as representing extracellular pigment granules and outer segment debris (outer HRF) or displacement and clumping of degenerated RPE cells or microglia (inner HRF). Curcio et al. hypothesized that anteriorly migrating RPE constitute one population of HRF and posteriorly migrating microglia the other ¹⁰⁹.

RPE tends strongly to migrate anteriorly, suggesting either attractive signals from the retina or repellent signals from the drusen or both. One explanation is that cells at the druse apex are at maximum distance from the choriocapillaris and thus migrate into retina to seek oxygen from retinal vessels¹⁰⁹. Another hypothesis considers that the high mechanical tension on the RPE layer at the HRF can appear as single or in clusters. They are relatively stable structures and considered a strong predictor of AMD progression ⁹⁴.

The number and distribution of HRF across retinal layers are factors with predictive value in development of GA and nAMD, the latter being more associated with inner location and cluster distribution ⁹⁴. An extension of the AREDS2 study using SD-OCT demonstrated that patients with HRF on OCT at baseline had 5 times increased risk of progression to GA at 2 years when compared with controls ⁹⁴.

Correlation with neovascular evolution is not so well demonstrated. Nevertheless, in nAMD, the regression of HRF after anti-VEGF treatment is considered a good functional prognostic sign. It was demonstrated that one month after the first injection, there was a statistically significant regression

of HRF ($p < 0.04$) and an even more significant regression at the 3-month follow-up examination ($p < 0.01$). In the same study, there was a statistically significant reduction of HRF in the good and moderate visual acuity subgroups (respectively, $p < 0.01$ and $p < 0.02$). The persistence of HRF correlated significantly with the poor visual acuity group ($p < 0.02$)¹¹⁰.

5.3.5. HYPER-TRANSMISSION DEFECTS

A commonly described OCT feature associated with atrophy is the presence of increased transmission of signal below the level of the RPE and into the choroid, resulting from loss of scatter or attenuation from overlying RPE and neurosensory retina. A variety of terms were proposed including sub-RPE illumination, choroidal hyperreflectivity, narrow columns of hyper-reflectivity, and hyper-transmission defects. CAM participants agreed that the term hyper-transmission defects was the preferred term because it reports to the cause for the observed phenomenon and includes all cases even those with tall pigment epithelial detachments (PEDs), where the hyper-transmission may not always penetrate to the underlying choroid^{66,71–74}.

Another common feature was a persistent hyperreflective line within the atrophy area, but significantly thinner than the adjacent RPE Bruch's membrane band. This line is supposed to represent the so called "persistent basal laminar deposit" corresponding to a few dissociated RPE cells, as well as RPE granules, processes of Müller cells, and avascular fibrosis¹¹¹.

Finally, we can observe narrow columns of hyper-reflectivity under the RPE that suggest deficiencies in the RPE layer (Figure 3C)¹¹². Prevalence is still undescribed but some authors described sub-RPE hyper-reflective columns in 27% of eyes that progressed to nAMD or GA by at least three months¹¹². These hyper-reflective columns are essentially hypertransmission in narrow strips, and their potential different meaning is not well defined.

5.3.6. INCOMPLETE RETINAL PIGMENT EPITHELIAL AND OUTER RETINAL ATROPHY

The term iRORA refers to the localized loss of tissue in the outer layers of retina without definite RPE loss. iRORA represents the new nomenclature obtained by the Consensus International Group (CAM—Classification of Atrophy Meeting) and has replaced the so called "Nascent GA"^{66,71–74}.

Nascent GA and iRORA have not the same meaning, one of the reasons is because nascent GA should be only used in the absence of current or prior macular neovascularization.

iRORA is defined on OCT as: (1) a region of signal hypertransmission into the choroid $<250\ \mu\text{m}$.; (2) a corresponding zone of attenuation or disruption of the RPE $<250\ \mu\text{m}$.; and (3) evidence of overlying photoreceptor degeneration. The term iRORA should not be used when there is an RPE tear ⁷². iRORA is strongly associated with impending GA as an average of 11 months elapsed between early signs of nascent GA and the development of GA ¹¹³. Nascent GA represents a subsidence of the outer plexiform layer and the inner nuclear layer with a hypo-reflective wedge (Figure 3C) ¹¹⁴, tomographic signs of photoreceptor degeneration. Other signs of photoreceptor degeneration, also included in iRORA definition, are outer nuclear layer thinning, external limiting membrane and ellipsoid zone disruption. iRORA is a finding found in 22% of eyes with intermediate AMD and in 7% of all AMD patients ¹¹³.

Recognizing that photoreceptor atrophy can occur prior to RPE atrophy, and that atrophy can undergo different stages from outer retinal layer to RPE involvement, two previous stages were described in the atrophic progression: incomplete outer retinal atrophy (iORA) and complete outer retinal atrophy (cORA). This new classification opens novel reflection data in the atrophic pathologic development with progressive and complementary involvement of RPE and outer retinal layers cells. In this new nomenclature, the term complete RPE and outer retinal atrophy (cRORA) was proposed as an end point for atrophy that occurred in the presence of drusen and was defined by the following criteria: (1) a region of hypertransmission of at least $250\ \mu\text{m}$ in diameter; (2) a zone of attenuation or disruption of the RPE of at least $250\ \mu\text{m}$ in diameter; and (3) evidence of overlying photoreceptor degeneration, all occurring in the absence of signs of an RPE tear ⁶⁶. The CAM group believed that the term GA, well known in the AMD nomenclature and with several current CFP definitions, should remain and continue to be used as a term applied to the subset of cRORA occurring in the absence of current or past macular neovascularization ⁶⁶.

Multimodal retinal imaging allows earlier, and precise diagnosis of atrophic AMD progression compared with isolated CFP. OCT identification of these different precursor lesions is an important issue in understanding the evolution to atrophic late stages¹¹⁵.

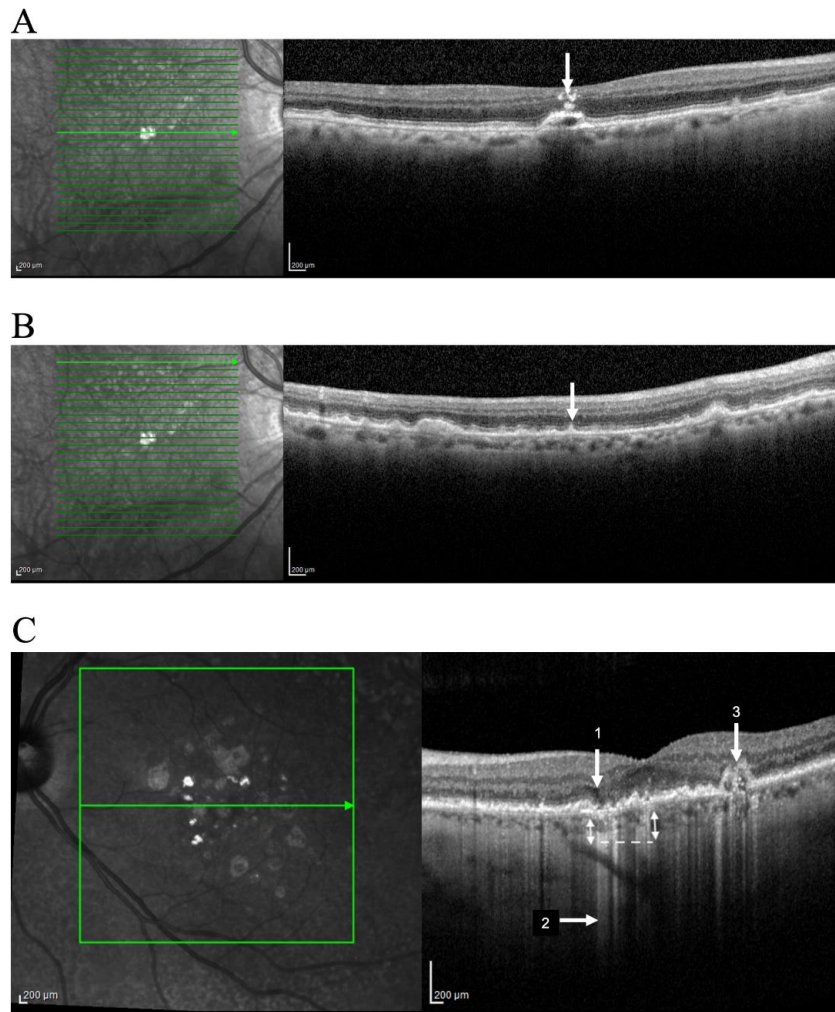


Figure 3 – Identification of OCT progression biomarkers of AMD in cross sectional SD-OCT scan.

(A) Hyperreflective foci (arrows); (B) Subretinal drusenoid deposits (arrow); (C) iRORA (1); Hyper-transmission defects (2); OCT-reflective drusen substructures (3). Representative images acquired using OCT Spectralis.

Table 5 – Progression biomarkers in iAMD.

^aOdds Ratio; ^bNot Determined; ^cRPE Abnormal Thinning; ^dOuter plexiform layer; ^eInner nuclear layer.

Biomarker	Imaging findings	Mechanism(s)	Prevalence in iAMD	Expected progression (ora)
Drusen Volume	Baseline drusen volume	Displacement or deterioration of photoreceptor layer	ND ^b	1.31 risk of progression to nAMD (for each 0.1 mm ³ of drusen increase) ¹⁰⁴
RPE-Drusen complex Advanced analysis	RAT ^c	RPE suffering and drusen regression	ND ^b	1.32 risk of developing central GA (for each 0.001 mm ³ increase in RAT volume) ¹⁰⁴
ODS	Internal heterogeneity	Metabolic instability	24% in soft drusen	5.6 risk of progression to new atrophy onset ¹¹⁶
SDD	Small yellow or white deposits; reticular, ribbon-like or interdigitated	Dysfunctional cholesterol homeostasis, retinoid processing or choroidal hypoxia (110)	32% a 76% in AMD patients	2.24 to 3.4 risk of progression to advanced disease ^{8,59}
HRF	Punctate hyperreflective lesions accompany pigmentary abnormalities	Anterior migration of fully pigmentated RPE cells, inflammatory or microglia cell debris and calcification	50% in AMD	5-fold higher risk of 2-year progression to GA ⁹⁴
HTD	Columns or strips of hyperreflectivity	Deficiencies within RPE layer	27% in AMD patients ¹⁰⁴	ND ^b
iRORA	Subsidence of OPL ^d and INL ^e with a hyporeflective wedge	New onset of atrophy (nascent atrophy)	7% in iAMD patients ¹¹⁷	5.2 risk of progression to GA ¹¹³
Nonexudative macular neovascularization	Neovascular lesion without fluid	Protective mechanism against ischemia	6.25 to 27% in the fellow eye of exudative AMD ⁶¹	1.21 risk of progression to exudative AMD at 1 year ⁶⁸

5.4. OPTICAL COHERENCE TOMOGRAPHY-ANGIOGRAPHY

OCT angiography (OCT-A) provides a non-invasive method by which clinicians and researchers can diagnose and monitor exudative and nonexudative neovascularization in AMD eyes^{118–120}.

Nonexudative neovascularization demonstrated ill-defined hyperfluorescence with no leakage on fluorescein angiography, hypercyanescence in a plaque configuration on indocyanine green angiography, and a shallow, irregular pigment epithelial detachment with moderate hyperreflectivity on OCT^{68,69}. The detection of nonexudative neovascularization increased significantly with OCT-A imaging and actually evidence of nonexudative neovascularization occurs in about 6.25% to 27% in the fellow eye of exudative AMD⁶¹.

Naïve patients with nonexudative neovascularization are phenotypic intermediate AMD patients and can be classified as subclinical or quiescent neovascularization (stability beyond six months). They carried an increased risk of conversion to exudative AMD compared with patients who did not have nonexudative neovascularization (21.1% vs. 5.4% at one-year follow-up)^{61,68,119}.

Early detection of macular neovascularization vessels should result in better early monitoring of patients with intermediate AMD who are at high risk of conversion to late neovascular AMD. Until data from natural history studies are more consistent, eyes with nonexudative neovascularization should be followed up more closely than eyes without neovascularization because of the presumed increased risk of exudation and vision loss⁶⁸. The possibility to include OCT-A as an imaging technique to be performed at baseline and at follow-up visits of intermediate AMD should be considered in those cases and some limitations that preclude the use of OCT-A in daily practice will, certainly, be minimized in the near future. Anti-VEGF treatment is not recommended until the appearance of signs of exudative activity⁶⁸. Some studies even consider the possibility of a potential protective effect on overlying neurosensory retina, probably related with improved oxygenation^{60,62,118,121}.

Investigators are interested in exploring anatomic changes related to near-term exudation. The onset of exudation seems to be correlated with lesions with less vascularity and smaller PED volume. Other parameters as macular neovascularization area, choroidal thickness, choroid vascularity index, and choriocapillaris flow deficits were also studied, with no proven relationship with new

exudation¹²².

Nonexudative neovascularization needs differentiation from the so-called inactive neovascularization. Inactive neovascularization is defined by absence of exudative activity (6 months with no fluid on OCT) in a previously treated neovascular lesion. As opposed to quiescent or subclinical neovascularization, those lesions had been active and treated in the past. So, this term (inactive neovascularization) does not apply to naïve neovascularization and is documented in OCT-A as a skeletonized net^{68,78,123}.

5.5. EVIDENCE AND RISK

Although routine ocular imaging in AMD clinical assessment is useful and indispensable, there is still no accurate biomarker, single or in combination, that can reliably identify those eyes that will progress from those that will not. Nevertheless, as AMD progression carries a significant negative visual prognosis, its detection has important clinical implications. Not only do predictive biomarkers may provide insights into AMD progression, but they might also prove to be valuable in managing and monitoring patients with this disease.

The ideal risk calculator should integrate a combination of demographic, historical, genetic, functional, and structural risk factors accumulated over time. Theoretically, it should be able to provide a clinically meaningful and accurate absolute risk score. Analogous to the experience with risk prediction in cardiovascular disease, these tools should require greater scrutiny of predictive performance, internal and external validation, and head-to-head comparison; unfortunately, they are still not available for practical use.

Clinicians must take into consideration the level of associated risk and the strength of the evidence of each particular risk factor (Table 6). Although there is no current consensus strategy for identifying patients at greatest risk of progression, we can use the available evidence to better stratify and manage patients with intermediate AMD.

In this prognostic evaluation, we should consider the information obtained by still useful clinical criteria: presence of late AMD fellow eye, large drusen, and pigmentary abnormalities. Despite its

potential high clinical usefulness, the ability to predict progression from intermediate to late AMD requires full patient phenotyping obtained by multimodal imaging including CFP, FAF, OCT, and OCT-A¹¹³.

Important and recent studies have documented significant and interesting correlation between histology and OCT findings in AMD^{117,124,125}. The association of OCT with other retinal image techniques increases diagnostic precision even more.

Regarding OCT progression biomarkers, the main OCT features associated with increased risk of progression to late AMD are HRF, SDD, and drusen volume. As for progression to nAMD, only HRF and SDD were clearly associated¹²⁶.

Table 6 – Prognostic biomarkers for progression in iAMD.

¹Odds Ratio; ²Confidence Interval; ³Not Determined.

		Strenght of evidence	
		High	Low
Strenght of risk	High		ODS ORa=5.61 for new atrophy onset, 6 months 95% CI ² : 1.277-24.67, p<0.001 ¹¹⁶
		HRF OR ¹ =4.72 to central GA, 2 years 95% CI ² : 2.43-9.80, p<0,001 ¹⁰⁹	iRORA+HRF OR ¹ =10 to cRORA, 2 years 95% CI ² : 2.36-42.20, p=0.002 ^{113,127}
		SDD OR ¹ =2.64 to late AMD, 2 years 95% CI ² : 1.07-6.49, p=0.034 ⁸	iRORA+SDD OR ¹ =1.9 to cRORA, 2 years 95% CI ² : 0.60-6.2, p=0.263 ^{115,127} (108, 124)
	Low	Drusen volume OR ¹ =1.31 to late AMD, 2 years 95% CI ² : 1.06-1.63, p=0.013 ⁷⁴	Hypertransmission OR ¹ ND ³ ¹¹²
		RPE Drusen Complex OR ¹ =1.31 to central GA, 2 years 95% CI ² : 1.14-1.53, p<0.001 ⁹⁴	Ellipsoid zone disruption OR ¹ ND ³ ¹²⁸

Some authors developed a simple, clinical, and practical OCT-based scoring system for early and intermediate AMD to prognosticate risk progression to late AMD; those algorithms still need validation ^{126,129}. Machine learning and artificial intelligence are promising tools to image guided prediction of AMD progression. Large data and instant analysis would accelerate and contribute to identifying new biomarkers of disease progression ^{130–132}.

Interventions that stop or delay the transition from early and intermediate to late AMD are acutely needed. Randomized clinical trials are being conducted to investigate future treatments, potentially useful in early and intermediate stages of the disease. Until effective therapies are recommended with probed efficacy, management plans should be tailored to the level of risk patients with a higher probability of progression, considering more regular surveillance, smoking cessation, self-monitoring strategies, and nutritional supplements.

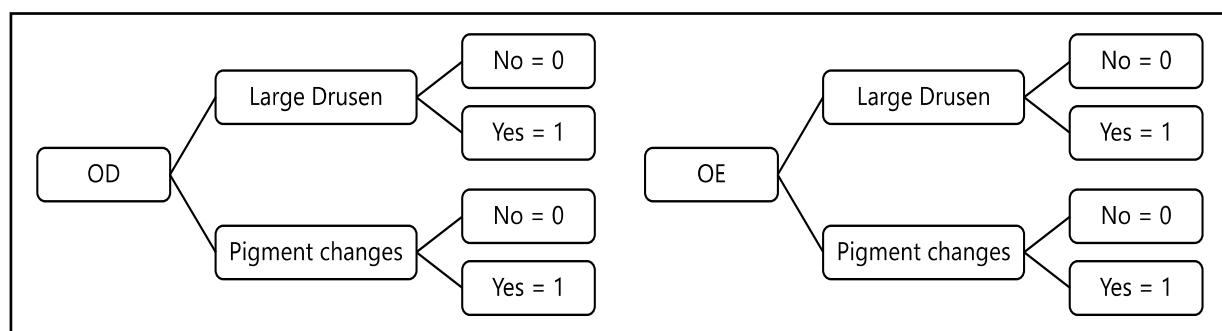
CHAPTER I. GENERAL INTRODUCTION

6. UNDERSTANDING AND PREVENTING PROGRESSION

The 5-year progressing risk from intermediate to advanced or late AMD ranges from 0.4 to 53% (mean 18%)⁷⁰. This wide range illustrates the heterogeneity of the patient population and emphasizes the need for AMD progression-related biomarkers. In studies addressing the natural history of AMD, certain features have been identified as most associated with disease-progression risk. These include advanced AMD in the fellow eye; large, soft, and confluent drusen; and AMD-related pigmentary abnormalities⁷⁰. Several risk calculation strategies have followed from these findings^{70,77}.

In the AREDS simplified scoring system⁷⁰, 1 risk factor is assigned per eye for 1 or more large ($\geq 125 \mu\text{m}$) drusen, and 1 risk factor is assigned per eye for any pigment abnormality; these factors are summed to yield a 5-step severity scale (0–4)⁷⁰. On this scale, the 5-year risk of developing advanced AMD in at least 1 eye is estimated as follows: 0 factors, 0.5%; 1 factor, 3%; 2 factors, 12%; 3 factors, 25%; and 4 factors, 50%. For eyes with no large drusen, the presence of medium-sized drusen in both eyes is counted as 1 risk factor (Table 7)⁷⁰.

Table 7 – Simplified scoring scale for intermediate AMD.



Conventional tests of visual function, such as BCVA under high-luminance and high-contrast conditions, are not sensitive enough to detect the subtle deficits in visual function that embody early and intermediate AMD, in part because of the predominant loss of rods versus cones¹⁰⁸.

In a study of visual function tests, Pondorfer et al.¹³³ found that many widely utilized tests, including BCVA, have inadequate performance in intermediate AMD, even after controlling for age and gender. The authors found that 2-test combinations – contrast sensitivity coupled with either low-luminance visual acuity or Moorfields Vanishing Optotypes Acuity Charts-Visual Acuity – allowed for the best discrimination between patients with intermediate AMD and controls¹³³. Work is ongoing by the MACUSTAR consortium to validate a study protocol and statistical test based on predictive modeling to follow disease progression in AMD¹³⁴. These investigators demonstrated that visual function assessments in a low-contrast and low-luminance setting enabled discrimination of AMD severities within the intermediate stage and of progression to exudative late disease.

Evaluation of drusen in CFP represents the current clinical practice standard for assessing patients with intermediate AMD. Employing CFP, researchers have found that the number, area, and extent of drusen are positively correlated with risk of progression in >2 years⁷⁵. However, this strategy has limited predictive value for AMD progression risk because it is relatively crude and unreliable in the short term. Other authors have proposed a more advanced predictive approach that incorporates the aforementioned CFP-based drusen classification with genetic, demographic, and environmental factors, such as age, smoking, family history, genetic variants, and diet; the risk assessment tool is available as a web application⁷⁷.

Multicolor imaging (MCI) is a non-invasive retinal imaging modality available in the Spectralis platform that simultaneously acquires three reflectance images of the retina using three individual lasers of different wavelengths (blue 848nm, green 515 nm, infrared 820 nm) with different depth penetration. MCI is an excellent tool for measurement and location of atrophic areas (infrared images). Refractive drusen are hyper-reflectant in green and blue images, whereas soft drusen appear mildly hyper-reflectant. Reticular pseudo drusen or SDD are detected more frequently than with CFP (87.1% vs 33.1%)¹³⁵.

As a prerequisite to reliably identifying iAMD before it progresses, the bounds of disease severity that constitute the intermediate stage must be standardized¹³⁴. Farsiu et al.¹³⁶ proposed an SD-OCT-based classification system for distinguishing intermediate AMD by means of semi-automated thickness mapping of the retinal structures. The authors developed a regression model based on total retina volume, RPE-drusen complex (DC) volume, and RPE-DC thickened or thinned volume

and showed that they could discern intermediate AMD- affected eyes from control eyes with 99% precision ¹³⁶. Sitniska et al. ¹³⁷ aimed to define a phenotypic profile of eyes with early or intermediate disease at increased risk of progression to late AMD. They found that patient age, CFH variant rs1061170, pigment abnormalities, drusenoid PED, and HRF were associated with progression to late AMD in 5.9 years in a multivariable model ¹³⁷. Another research group demonstrated that eyes with HRF at baseline, especially copious HRF or HRF tending toward the inner retinal layers, were more likely to have GA at 2 years ⁹⁴.

Topographic and sensitivity disruptions that occur in iAMD could potentially be used to categorize disease severity. Researchers applied mesopic and scotopic fundus-controlled perimetry in combination with SD-OCT in a series of eyes with intermediate AMD ¹³⁸. They found significantly reduced sensitivity levels within the central 4° of the macula in eyes with intermediate AMD versus unaffected controls and a correlation between decreased sensitivity and localized structural Age-Related Macular Degeneration Review changes, including increasing RPE-DC thickness and decreasing outer nuclear layer and photoreceptor layer thicknesses ¹³⁸. In recent years, progress has been made on characterizing biomarkers that portend advanced AMD. The widespread use of noninvasive approaches (FAF, OCT and OCT-A) in the diagnosis and monitoring of intermediate AMD has resulted in the identification of numerous imaging biomarkers of disease progression ^{108,137,139}.

With the aid of such retinal imaging modalities, an array of previously under recognized risk factors are being further investigated (Figure 4). Drusen load, drusen regression, HRF, and SDD are biomarkers that are encountered relatively frequently in eyes with intermediate AMD and are predictive of higher risk of advanced disease ¹⁰⁸.

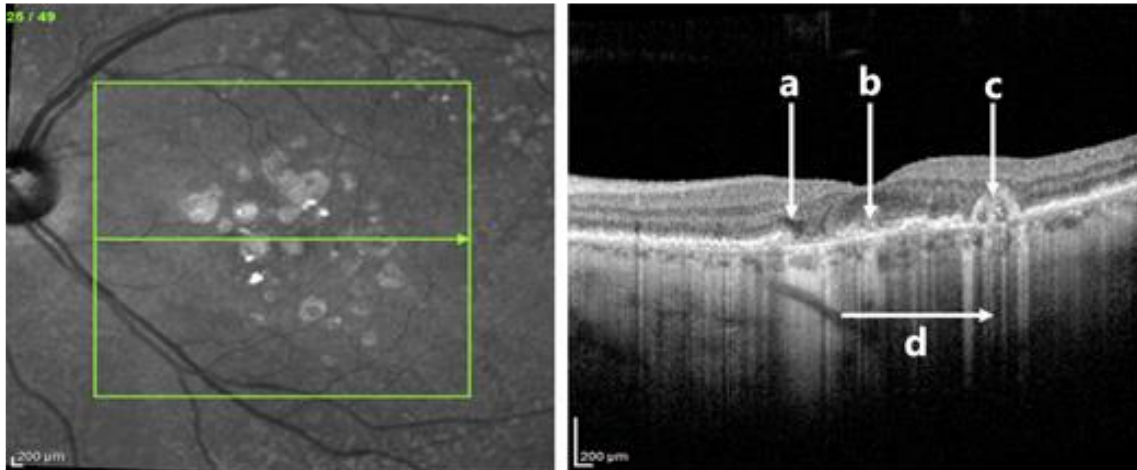


Figure 4 – SD-OCT image showing progression biomarkers.

SD-OCT image showing progression biomarkers: a) wedge-shaped band of hyporeflectivity in the OPL; b) HRF; c) ODS; d) hypertransmission/ sub-RPE hyperreflective columns.

Some others SD-OCT signs are highly predictable of atrophic progression such as regression of the outer plexiform layer and inner nuclear layer and a wedge-shaped band of hyporeflectivity in the outer plexiform layer¹¹³. Analogously, narrow columns of hyper-reflectivity seen under the RPE suggest deficiencies in the RPE layer and precede onset of advanced disease. Confluent large drusen with accompanying fluid-filled subretinal spaces beneath the neurosensory retina on SD-OCT have been posited as subclinical MNV but also could be a product of mechanical strain posed by drusen¹⁴⁰.

Defining biomarkers of disease progression is even more relevant in neovascular subtype evolution. Approximately 10–15% of patients with AMD will experience progression to advanced exudative disease¹⁴¹. In eyes with iAMD, early detection and routine monitoring are crucial. Patients lose an estimated 3 to 5 lines of visual acuity in the interim from onset of nAMD to its diagnosis⁴⁰. MNV lesions have been found to grow more rapidly at lesion onset than in advanced disease⁴⁰, potentially resulting in substantial unrecoverable vision loss before nAMD is diagnosed. Additionally, visual acuity at pretreatment baseline has been shown to modify the amount of improvement that is possible on VEGF inhibitors. Patients with better visual function at baseline have higher on-treatment visual acuity, even if they gain fewer letters than do patients with worse visual function at baseline⁴⁰.

Certain findings in the fellow eye also are predictive of AMD progression risk. Cachulo et al.⁹⁰ demonstrated that in patients with unilateral exudative AMD, abnormalities on fundus autofluorescence in the fellow eye were predictive of MNV development in that eye with 93% sensitivity and 37% specificity, although no specific autofluorescence abnormality was associated with disease progression. In eyes with early or intermediate AMD, progression to GA is more common in patients with late AMD in the fellow eye¹⁴². In AREDS, 26.4% of eyes with medium drusen progressed to advanced AMD by 10 years when the fellow eye had advanced AMD¹⁴³.

Besides clinical and imaging biomarkers nongenetic molecular biomarkers are being investigated. Results of a comprehensive review of nongenetic molecules implicated in AMD progression indicated that those involved in the oxidative stress pathway, the complement system, and lipid metabolism appear to show the greatest utility as biomarker candidates¹⁴⁴.

Hypothesis-free studies on proteomics, metabolomics, and microRNA profiling may yield promising biomarker candidates, but these works still need to progress and mature for AMD screening purposes^{35,145,146}.

Despite all this wide and deep research, no single biomarker or set of biomarkers has been found to provide a reliable, sensitive, and specific measure of whether disease progression will occur in an eye with AMD¹⁰⁸. Although more than 100 potential AMD biomarker compounds have been considered in the scientific literature, few are consistently associated with disease progression, and even few are currently used in the clinic¹⁴⁴.

Clinical trials testing early interventions could be interesting but not practical or even efficient at short term, because they need large sample and longer follow-up. Interventional or noninterventional clinical trials conducted in the intermediate AMD stage are more promising and, as previously explained, more efficient.

One important way of progressing in this knowledge is identifying early biomarkers and surrogate, robust anatomical and functional, endpoints to facilitate early intervention studies. Identification of such early endpoints will be important in developing trials for interventions to slow disease progression and to continue the research of such a challenging disease⁵¹.

CHAPTER I. GENERAL INTRODUCTION

7. PRIORITIES FOR AMD RESEARCH

Age-related macular degeneration is a successful research area that has taken giant strides in the last decades. Recently several new, potentially long-acting anti-VEGF agents have been approved for use in exudative AMD and the first treatment for geographic atrophy has received regulatory approval, with the aim of slowing down the progression of the disease. Furthermore, trials of gene therapies for both geographic atrophy and neovascular age-related macular degeneration are well underway and these could be, in the near future, some new and promising therapeutic modalities.

However, there are still areas of unmet need that have been identified by relevant professional and patient stakeholders and should be a priority for clinical and pharmacologic research ⁵¹.

These include implementing strategies to:

- a) develop a deeper understanding of the cause and drivers of disease progression;
- b) slow the progression of earlier stages of age-related macular degeneration;
- c) identify anatomical and functional early endpoints for early intervention trials;
- d) integrate artificial intelligence (AI) into screening and decision-support tools;
- e) develop increasingly advanced, home-based monitoring tools;
- f) develop services designed to prioritise marginalised and vulnerable groups, including those from low-income countries.

CHAPTER II. RATIONALE AND AIMS

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1. RATIONALE

Having those unmet needs in mind, and worried about its resolution (or, at least, its mitigation) we developed this thesis project, particularly directed to the first three of them:

- a) developing a deeper understanding of the cause and drivers of disease progression;
- b) slowing the progression of earlier stages of age-related macular degeneration;
- c) identifying anatomical and functional early endpoints for early intervention trials.

2. AIMS AND TASKS

The general aim of this research project is to explore if FAF findings and vascular factors are related to intermediate AMD progression and to look for other image biomarkers of disease progression. To accomplish the aimed objectives, we developed work in three lines of clinical investigation (specific aims):

A. FUNDUS AUTOFLUORESCENCE FINDINGS

- 1. Analyse the correlation between drusen morphology and FAF findings;
- 2. Analyse the correlation between outer retinal layers morphology and FAF findings;
- 3. Assess FAF related biomarkers of disease progression.

1. Drusen type and morphology were analysed using CFP and SD-OCT. FAF was obtained using AF module associated in the SD-OCT device. A standardized imaging protocol was performed. The SD-OCT was acquired in combination with FAF, and images were overlaid. The morphological characteristics of the analyzed drusen were size, shape, internal reflectivity, homogeneity and presence or absence of associated HRF.

2. Additional structural analysis on SD-OCT, such as ELM, EZ, RPE-BM and CT were performed. Those morphological characteristics and alterations were evaluated, specifically and independently of one another, at the site of each analyzed drusen and correlated with FAF findings.

3. Assess FAF biomarkers of disease progression in a longitudinal study, every year in a two years period. In this study we analyzed the correlation between drusen morphology and lipofuscin distribution in intermediate AMD patients and follow those findings during the follow-up period, in a longitudinal way.

B. CHORIORETINAL VASCULAR FINDINGS

1. Test if choriocapillaris perfusion is disturbed in Intermediate AMD patients;
2. Test if retinal capillary plexus perfusion is disturbed in Intermediate AMD patients:
 - 2.1. Analyze superficial retinal capillary plexus (SCP),
 - 2.2. Analyze deep retinal capillary plexus (DCP);
3. Assess if chorioretinal vascular changes are related with disease progression.

1. Choroidal perfusion can be studied using a common ophthalmological noninvasive diagnostic examination (Spectral Domain Optical Coherence Tomography with enhanced depth imaging: SD-OCT-EDI mode) that scan the retina and choroid in the macula area. Subfoveal choroidal thickness was measured manually and choroidal binarization was performed resorting to Image J software. The resulting black and white images were interpreted as representing lumen and stroma, respectively. Vascular indexes were obtained relating those measurements to its ratio.

2. Retinal Capillary plexus perfusion can be studied using the Optical Coherence Tomography – Angiography: OCT-A, a noninvasive ophthalmological examination that gives us an information about the vascularization using an algorithm whose information is related with red blood cells movement inside the vessels. Scans with the highest quality were further analyzed using the same binarization software. Retinal superficial and deep vascular plexus (SCP and DCP, respectively) were analyzed in terms of quantitative data. The same methodology was performed for the choriocapillary layer.

3. Patients undertook imaging one and two years later to explore a possible relationship between vascular features and AMD progression. In this study, the vascular aspects of the

chorioretinal complex of intermediate AMD patients (choroidal vascular layer, superficial and deep retinal capillary plexus) were evaluated and compared with a control group. These vascular features can be considered as a trigger or as adjuvant factors for AMD progression and their impact in the evolution of the disease, since the early stages of AMD to more advanced form of the disease, were analysed.

C - IDENTIFICATION OF OTHER DISEASE PROGRESSION BIOMARKERS

AMD patients usually accomplish a long journey in their disease progression, sometimes evolving three or more decades and occasionally never reaching the late stage of the disease. That is one reason why intermediate stage is a common field for investigation: those patients are in particular risk of progression (and the risk can be calculated by some already published algorithms based on drusen number and size, and presence of pigmentary abnormalities), but currently there are no specific anatomical biomarkers for disease progression that can reliably predict individual risk of conversion to late AMD.

1. In this study we followed a cohort of iAMD patients for 24 months and we explored some markers of pathological retinal ageing that can predict individual progression to advanced stages. Particularly, we investigated and characterized drusen, hyperreflective foci and other OCT features (presence of incomplete retinal pigment epithelial and outer retinal atrophy, and ellipsoid zone status) as biomarkers of disease progression and we calculated a risk prediction model for progression to late AMD, with estimates of sensitivity, specificity, and the receiver operating characteristic (ROC) curve analysis.

2. OCT-A data and nonexudative macular neovascularization was also investigated in our cohort, with the aim of evaluating the natural history of those intermediate AMD patients with documented nonexudative macular neovascularization (NE-MNV).

CHAPTER III. METHODS

CHAPTER III. METHODS

To accomplish those aims we have proposed a study protocol that included patients selected from the Medical Retinal Section of the Centro Hospitalar Universitário de Lisboa Central (CHULC) Ophthalmology Department.

Patients were enrolled in a non-interventional prospective study if they met inclusion criteria and upon informed consent after detailed explanation of the study. The study protocol and the informed consent forms were designed and approved by the local ethics committees and followed all good practices, involving anonymous data collection, not including sensitive data or processing of genetic information. Participants of both genders were included and invited to sign the informed consent.

INCLUSION CRITERIA: Subjects over 55 years old with drusen secondary to intermediate AMD based on Beckman classification^{59,70}. We considered soft, cuticular and reticular pseudo-drusen. Hard drusen were excluded as they are considered non-pathological.

EXCLUSION CRITERIA: Patients were excluded if it is not possible to obtain a good quality of CFP, SD-OCT, OCT-A images, if refractive error is $\geq \pm 6$ D or if there is any evidence of accumulation of extracellular fluid, hemorrhage, exudates, or fibrosis. Additional exclusion criteria included any history of retinal surgery including laser treatment, signs of diabetic retinopathy, history of retinal vascular occlusion, history of anti-VEGF treatment in the study eye or any signs or history of hereditary retinal or macular dystrophy.

These patients underwent a complete ophthalmologic evaluation, including best corrected visual acuity (BCVA), intraocular pressure (IOP), slit lamp biomicroscopy and 90D indirect fundoscopy and have undergone a retinal imaging protocol that included Color Fundus Photography (CFP), Spectral Domain Optical Coherence Tomography (SD-OCT), OCT-Angiography (OCT-A) using Spectralis OCT with OCT Angiography Module (Heidelberg Eng. GmbH, Germany). A standardized imaging protocol was performed in all patients.

We included 150 eyes of 150 patients. If both eyes of the same patient were eligible, we also

evaluated and followed the second eye but, for most of the statistical evaluation, we considered only one eye per patient.

To some analysis described in this thesis we also considered a control group. The control group consisted of age matched healthy eyes recruited from the refraction department at the same hospital, and without any sign of AMD on CFP, FAF and OCT scans. We included 50 healthy control eyes, from 50 patients; control group was age and gender matched with patient group.

Starting at baseline at intermediate AMD stage (recruitment began in 2019), patients were reevaluated every six months during the follow-up period and reclassified according to their progression: stable or progression to late AMD.

Clinical Research Unit Nova (CRU-NMS/FCM) and NOVA Information Management School (NOVA-IMS) collaborated in this study conducted in the CHULC Ophthalmology Department (recruiting patients and capturing images) and Chronic Diseases Research Center (CEDOC), NMS/FCM (processing images and algorithm protocol and validation).

CRU-NMS/FCM or NOVA-CRU developed an electronic Case Report Form (eCRF). This platform has allowed the collection of large amounts of data, with standard procedures, preparing the future task of organizing and performing statistical analysis and minimizing bias, according to the requirements of the clinical protocol. NOVA Information Management School (NOVA-IMS) was responsible for conducting or coordinating statistical data analysis.

Sample size was calculated to detect significant differences between patients predicted with high and low risk of AMD evolution at a 1% significance level, being the power of the test not smaller than 80%. We expect that the difference between the proportion of patients with AMD progression will be around 25%, comparing high risk patients and low risk patients for significant AMD evolution. More precisely we postulated that progression would occur in 40% of high-risk patients and in 15% of low risk patients. This hypothesis test was reassessed after preliminary results to adjust those proportion differences and NOVA-IMS confirmed the recommended sample size.

CHAPTER IV. FUNDUS AUTOFLUORESCENCE FINDINGS

CHAPTER IV. FUNDUS AUTOFLUORESCENCE FINDINGS

Related to FUNDUS AUTOFLUORESCENCE FINDINGS we have addressed three tasks:

TASK 1. Analyze the correlation between drusen morphology and FAF findings

TASK 2. Analyze the correlation between outer retinal layers morphology and FAF findings

TASK 3. Assess FAF related biomarkers of disease progression

The work considered in TASK 1 and TASK 2 was included in the following manuscript:

CORRELATION STUDY BETWEEN DRUSEN MORPHOLOGY AND FUNDUS AUTOFLUORESCENCE

Flores R, Carneiro A, Serra J, Gouveia N, Pereira T, Mendes J, Coelho P,
Tenreiro S, Seabra M

Retina. 2021 Mar 1;41(3):555-562
doi: 10.1097/IAE.0000000000002881
PMID: 32604342

Impact Factor: 3.649, Q1 in Medicine, Ophthalmology

<https://pubmed.ncbi.nlm.nih.gov/32604342/>

CHAPTER IV. FUNDUS AUTOFLUORESCENCE FINDINGS

1. CORRELATION STUDY BETWEEN DRUSEN MORPHOLOGY AND FUNDUS AUTOFLUORESCENCE

ABSTRACT

Purpose: To correlate drusen morphology and outer retinal status with autofluorescence imaging in patients with intermediate age-related macular degeneration (AMD).

Methods: Drusen type and morphology were analyzed using Color Fundus Photography (CFP) and Spectral Domain Optic Coherence Tomography (SD-OCT) while Fundus Autofluorescence (FAF) was used for drusen autofluorescence evaluation. Additional structural changes on SD- OCT such as disruption of external limiting membrane (ELM), ellipsoid zone (EZ) and Retinal Pigment Epithelium/Bruch's membrane complex (RPE-BM), as well as the presence of choroidal hypertransmission (CH) at correspondent locations were also evaluated and correlated with FAF findings. Spearman's correlation coefficient was used to analyze the correlation between SD- OCT morphological characteristics of drusen and autofluorescence appearance of the corresponding drusen. Strength of correlation was calculated (r) and p -value < 0.05 was considered statistically significant.

Results: 228 drusen from 53 eyes of 53 patients were analyzed: 130 soft drusen (57.02%) and 98 cuticular drusen (42.98%). Sixty percent of the drusen were isoautofluorescent ($n=136$), 35% hyperautofluorescent ($n=80$) and 5% hypoautofluorescent ($n=12$). We found positive correlation between drusen autofluorescence and hyperreflective foci ($r=0.4$). Outer retinal layers morphology (ELM and EZ status and hypertransmission) also correlates with autofluorescent findings ($r=0.3$).

Conclusions: Multimodal imaging reveals a broad spectrum of ultrastructural changes, which may reflect different stages in the evolution of drusen. Our results suggest that drusen morphological characteristics and autofluorescence findings are correlated but other factors or cofactors may be involved. The described correlations will help us understand new progression biomarkers of non-exudative AMD.

1.1 INTRODUCTION

Drusen are the hallmark of Age-related Macular Degeneration (AMD), the most important acquired retinal disease in the elder population. Drusen are yellowish-whitish deposits of extracellular debris, usually located between the basal lamina of the retinal pigment epithelium (RPE) and the inner collagenous layer of Bruch's membrane ^{147–149}, and their presence is one of the earliest sign of this degenerative disease ⁹.

AMD can be classified in three different stages: early, intermediate and advanced ¹⁴⁸. Early AMD is characterized by the presence of medium-sized ($\geq 63 \mu\text{m}$ and $\leq 125 \mu\text{m}$) drusen with no associated pigmentary abnormalities and intermediate AMD is characterized by the presence of large ($>125 \mu\text{m}$) drusen associated or not with pigmentary abnormalities ^{147,148}. There are several types of drusen (hard, soft, cuticular and pachydrusen), and their morphology can be studied using Color Fundus Photography (CFP) and Spectral Domain Optical Coherence Tomography (SD-OCT). Hard drusen are considered non pathologic. Although the ultrastructural characteristics of cuticular drusen appear more similar to those of hard drusen, their lifecycle and macular complications are more comparable with those of soft drusen ¹⁵⁰. Soft and cuticular drusen can grow, coalesce and progress into advanced forms of atrophic or neovascular maculopathy ^{6,9}.

The retina exhibits an inherent autofluorescence (AF) that originates mainly from lipofuscin in retinal pigment epithelium cells ¹⁵¹. For many cell types, lipofuscin originates internally (autophagy), but in the RPE lipofuscin derives primarily from phagocytosed photoreceptor outer segments ^{9,10}. Fundus Autofluorescence (FAF) assessment relies on native fluorescence from physiological retinal components, providing an indication of healthy function of the retinal pigment epithelium cells. Lipofuscin is potentially toxic and, as a byproduct of photoreceptor metabolism, can accumulate in the lysosomal compartments of RPE cells. Beyond critical concentrations, lipofuscin toxicity interferes with normal cell function and ultimately leads to cell death ^{9,10}. Nevertheless, lipofuscin toxicity is still a matter of debate. Rudolf and collaborators showed that hyperautofluorescence at the borders of atrophy (junctional GA zone) is attributable to superimposition of cells and cellular fragments, rather than increased accumulation of lipofuscin at this location ¹⁵². In another study, Ablonczy and collaborators proved that A2E, an important component of lipofuscin, is found in high concentration in retinal periphery and in low concentration in human macula ¹⁵³. In those molecular

assay methods, the authors did not find correlation between the spatial distribution of A2E and lipofuscin fluorescence in humans. More recently, highly differentiated human fetal RPE Cultures seem to be resistant to the accumulation and to the toxicity of lipofuscin-like material ¹⁵⁴.

Changes in the normal FAF appearance may be among the first signs of retinal disease, even before they are evident in fundoscopy. These changes are widely studied, and their usefulness is accepted in monitoring several diseases, particularly AMD patients with geographic atrophy and macular dystrophies. FAF imaging in monitoring drusen in early and intermediate AMD patients is less studied and less understood ^{9,10}. Lipofuscin is the main fluorophore contributing to notable FAF changes but other fluorophore types located in the sub-RPE space or in Bruch's membrane (autofluorescent sub-RPE deposits) may be the source of FAF changes ^{155–157}. Although lipofuscin-containing RPE cells are considered to be the main source of FAF, other retinal cells, including cone photoreceptors, were shown several decades ago to contain lipofuscin, in small amounts ^{157–161}.

Drusen deposits and lipofuscin accumulation reflect metabolic overload of the RPE ¹⁶². Increased lipofuscin accumulation is assumed to be an important factor in the pathogenesis of AMD, although direct evidence for this hypothesis is missing ^{148,159}. Recent studies related with soft drusen suggest that their major ultrastructural component is large cholesterol-rich lipoproteins containing apolipoprotein B, E secreted by the RPE. RPE offload unneeded lipids of dietary and outer segment origin to create an atherosclerosis-like progression in the subRPE-basal lamina space ¹⁵⁷. It seems that drusen and lipofuscin are important biologic processes, yet directly unrelated. However, a lot of questions remain unanswered: do they share common pathophysiological pathways? Can FAF be useful in monitoring intermediate AMD patients? Do extracellular deposits like drusen have any relationship with intracellular lipofuscin accumulation? Do extracellular deposits like drusen promote autofluorescent changes in adjacent RPE cells? The purpose of the current study was to explore the relationship between drusen morphology and autofluorescence findings.

1.2. METHODS

Study design and study population

This is a prospective and non-interventional study. Inclusion criteria were subjects over 55 years old with drusen secondary to AMD in the intermediate stage of the disease. Patients were selected among the population of Ophthalmology Department of “Centro Hospitalar Universitário de Lisboa Central” (CHULC), a tertiary hospital in Lisbon-Portugal, attending the Medical Retinal Consultation, and were enrolled in the study if they met inclusion criteria. Patients were excluded if it was not possible to obtain a good quality of SD-OCT, CFP or FAF images, if refractive error was $\geq \pm 6D$ or if there was any evidence of accumulation of extracellular fluid, hemorrhage, exudates or fibrosis. Additional exclusion criteria included any history of retinal surgery including laser treatment, signs of diabetic retinopathy, history of retinal vascular occlusion, history of anti-VEGF treatment in the study eye or any signs or history of hereditary retinal or macular dystrophy, and also patients that were unable to consent their participation.

Ethics Issues

Participants that fulfill the inclusion criteria were invited to participate, and after detailed explanation of the scope of the study they gave written informed consent. The protocol adhered to the tenets of the Declaration of Helsinki and was approved by the Ethic Commission of CHULC.

Image analysis and measurements

Soft and cuticular drusen were considered but not hard or reticular pseudodrusen (subretinal drusenoid deposits). Only one eye per patient was considered, even if both eyes were potentially eligible. Studied drusen were large ($>125 \mu m$), soft or cuticular, and located in the 20° centered by the fovea. No more than 5 drusen per patient were considered and we choose those that could be found on color, OCT, and FAF, simultaneously. Drusen were manually evaluated and characterized by two independent ophthalmologists. In the case of discrepancy, they discussed each case and agreed upon the final decision. Drusen type and morphology were analyzed using CFP (Topcon

Imagenet) and SD-OCT (Spectralis, Heidelberg). FAF (Spectralis OCT with Autofluorescence Module, Heidelberg) was used for drusen autofluorescence evaluation. A standardized imaging protocol was performed in all patients, which included acquisition of combined FAF images ($\lambda=488\text{nm}$, emission 500-700 nm, field of view $30^\circ \times 30^\circ$, image resolution 768×768 pixels) and SD-OCT ($\lambda=870$ nm, acquisition speed 40,000 A-scans per second, $20^\circ \times 20^\circ$, dense program, 512×49 scans, spacing $120 \mu\text{m}$). The SD-OCT was acquired in combination with FAF and images were overlaid. Thereafter we could correlate the SD-OCT morphological characteristics of the drusen with the FAF findings observed at the corresponding location (Figure 5).

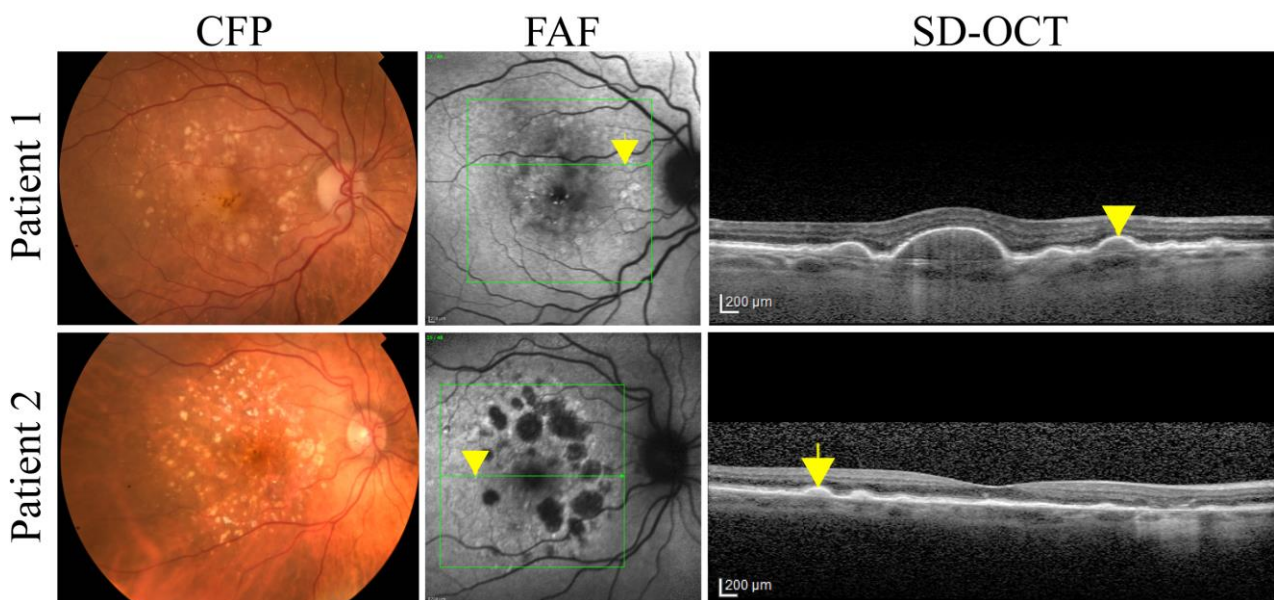


Figure 5 – Images of CFP, the corresponding FAF and SD-OCT of two different iAMD patients.
Yellow arrows indicate the analyzed drusen in FAF and, corresponding, SD-OCT scans.

The morphological characteristics of the analyzed drusen were size, shape, internal reflectivity, homogeneity and presence or absence of overlaying hyperreflective foci. Size was calculated using two perpendicular dimensions (length and height) of each particular druse. Length was measured in the drusen base and height was measured from the apex to the base in a perpendicular way (Figure 6A). Simultaneous scanning laser ophthalmoscopy images and overlaid fundus images were used to ensure that the scan went through the center of an individual druse. Only drusen which had been crossed in their center were included for the analysis. If the cross-section was off-center, those drusen were not included for the analysis.

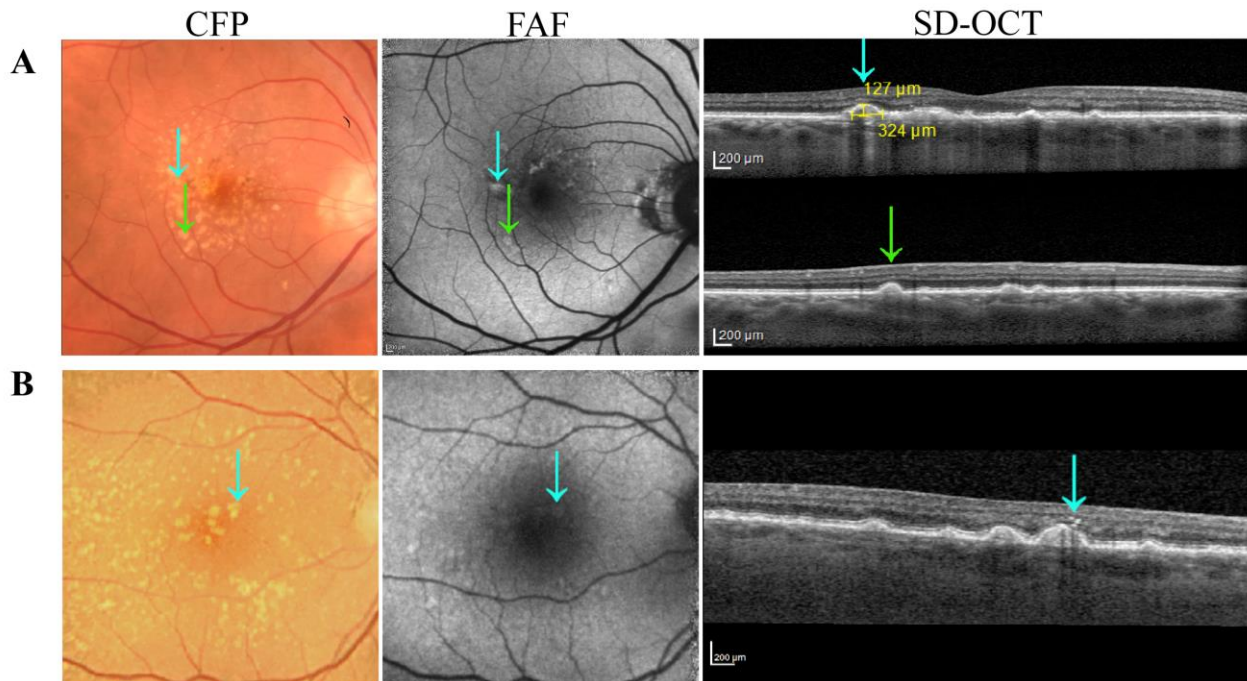


Figure 6 – Examples of images of CFP, the corresponding FAF, and SD-OCT.

(A) showing measuring drusen size—length and height (blue arrow) and hyperautofluorescent drusen (green arrow);
 (B) Images showing HRF.

Additional structural analyses on SD-OCT such as integrity of the external limiting membrane (ELM), the ellipsoid zone (EZ) and the RPE-Bruch's membrane (RPE-BM) membrane as well as the presence of choroidal hypertransmission (CH) were also considered. Those morphological characteristics and alterations were evaluated, specifically and independently of one another, at the site of each analyzed drusen (Table 8).

Considering FAF, studied drusen were classified as hypoautofluorescent, normal autofluorescent (isofluorescent) or hyperautofluorescent. The FAF signal in the area of the selected drusen was classified in comparison to the background signal (reference area in the superior temporal corner of the square $20^\circ \times 20^\circ$ overlaid in FAF image (area below the superior temporal arcade). Recorded FAF changes are located in perfect correspondence with the drusen but, as we compare an enface image (FAF) with a scan image (SD-OCT), they can be related to any structure in the same cross-sectional location of the drusen (inner or outer) and not the drusen itself.

Table 8 – Drusen Morphological Characteristics evaluated by SD-OCT.

Parameters	Classification
Size	>125 μm $\leq$ 250 μm , >250 μm
Shape	Convex, Triangular, Mixed
Internal Reflectivity	Low , Medium, High
Homogeneity	Heterogeneous; Homogeneous
Hyperreflective foci (HRF)	No HRF; With HRF
Status of external limiting membrane (ELM)	ELM integrity; ELM disruption
Status of ellipsoid zone (EZ)	EZ integrity; EZ disruption
Status of RPE-Bruch membrane (RPE-BM)	RPE-BM integrity; RPE-BM disruption
Choroidal hypertransmission (CH)	No CH; With CH

In the central area, we considered the physiological hypoautofluorescent attenuation by RPE melanin and by macular pigment, centered in the fovea and extending 7° - 12° around it (we take a medium value of 10°). Two independent ophthalmologists classified drusen according to FAF (RF and AC). In case of discrepancy, they discussed each case and agreed upon the final classification in a similar manner to the one described regarding SD-OCT drusen morphological characterization.

Large soft drusen and some cuticular drusen present peripheral slightly hyperautofluorescent ring, related to thickening of RPE at the drusen base, and slightly hypoautofluorescent center (Figure 5A)^{85,149}. In this study those rings were not considered in drusen autofluorescence classification and we consider mainly the drusen core. Anteriorly migrating RPE that appear above some drusen, and calcified nodules that appear in refractile drusen, were considered as hyperreflective foci (Figure 6B)^{85,163}. Those drusen, associated with hyperreflective foci, had usually some irregular hypoautofluorescent boundaries or present minimal changes^{106,164}. They may represent a stage of drusen regression marked by loss of RPE, thus contributing to the development of geographic atrophy. Those autofluorescent findings, precursors of geographic atrophy, are similar to those considered in a post-hoc analysis of FAF images from the Age-related Eye Disease Study 2¹⁶⁴.

Statistical Analyses

Spearman's correlation coefficient was used to analyze the correlation between SD-OCT morphological characteristics of drusen and autofluorescence appearance of the corresponding drusen. Strength of correlation is perfect (-1 or +1) when the considered variables are in perfect relationship with each other. The statistical significance of the correlation was done on the basis of a p-value less than 0.05. Strength of correlation (r value) was rated as follows: 0.0-0.2 (very weak to negligible correlation); 0.2-0.4 (weak, low correlation - not very significant); 0.4-0.7 (moderate correlation); 0.7-0.9 (strong, high correlation), and 0.9–1.0 (very strong correlation).

1.3. RESULTS

A total of 228 drusen were considered from 53 eyes of 53 patients over 55 years old with drusen secondary to intermediate AMD. Patients (23 male and 30 female) ranged from 63 to 95 years old (80 +/- 8DP) (Figure 7). Inter-observer agreement was substantial for both SD-OCT measured parameters and AF classification (greater than 80%, except for RPE-BM status). A total of 228 drusen were analyzed and classified, 130 soft drusen (57.02%) and 98 cuticular drusen (42.98%); in total, 60% were isoautofluorescent (n=136), 35% were hyperautofluorescent (n=80) and 5% were hypoautofluorescent (n=12).

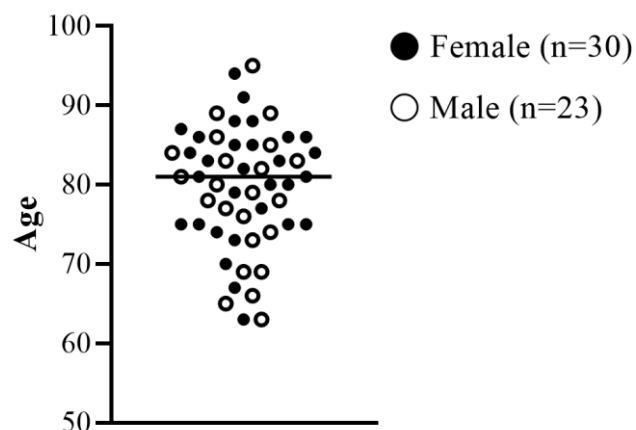


Figure 7 – Age and sex distribution of the patients with drusen secondary to iAMD included in the study.

Drusen with homogeneous interiors are more frequently isofluorescent. ($r=0.2$; $p<0.05$). Hyperreflective foci are more frequently associated with hypo or hyperautofluorescent cases ($r=0.4$; $p<0.0001$). Concerning integrity of retinal external layers, we find that ELM and EZ integrity tend to occur in isofluorescent cases and disruption was more frequent in hypo or hyperautofluorescent cases ($r=0.3$; $p<0.0001$). Finally, CH tend to be absent in isofluorescent cases ($r=0.3$; $p<0.0001$).

The strength of correlation was weak or moderate. No strong correlation was found with other parameters (Table 8), namely no correlation was found between drusen autofluorescence and type of drusen (soft or cuticular), nor between drusen autofluorescence and shape or internal reflectivity.

Concerning drusen size (length and height), this study suggested differences between changes in autofluorescence and drusen size, which were not statistically significant. We found that all hypo-autofluorescent drusen were $>250\ \mu\text{m}$ in length. Larger drusen were also more prevalent among hyperautofluorescent than isoautofluorescent cases, but the difference was not statistically significant (Table 9).

Parameters showing overall correlations with autofluorescence were analysed further. Isoautofluorescence is more prevalent in homogenous drusen and also when there is absence of hyperreflective foci (Table 9). Changes in autofluorescence are more prevalent when homogeneity is absent and when hyperreflective foci are present.

Table 9 – Correlation between SD-OCT morphological characteristics and autofluorescence of the same drusen.

Morphological characteristics	Spearman correlation coefficient	r value	p-value
Size	-0.087	0.1	0.20432
Shape	0.004	0.0	0.95295
Internal reflectivity	0.021	0.0	0.75696
Homogeneity	-0.157	0.2	0.02077
Hyperreflective foci	0.351	0.4	<0.0001
ELM Integrity	-0.295	0.3	<0.0001

Morphological characteristics	Spearman correlation coefficient	r value	p-value
Ellipsoid Zone (EZ) Integrity	-0.286	0.3	<0.0001
RPE- BM Integrity	-0.242	0.3	0.00033
Choroidal hypertransmission	0.267	0.3	<0.0001

The three morphological parameters that studied external layers of retina (namely disruption of the ELM, EZ and RPE-BM) showed more frequent disruption in hyper or hypoautofluorescent cases. Conversely, the reverse (integrity) is found primarily associated with isoautofluorescence cases. Finally, choroidal hypertransmission (CH) is more prevalent in hyper or hypoautofluorescent cases (Table 10).

Table 10 – Morphological characteristics of drusen and autofluorescent findings evaluated by SD-OCT and FAF.

	Hyperautofluorescence N=80 Absent (%) / Present (%) Relative (%)	Isoautofluorescence N=136 Absent (%) / Present (%) Relative (%)	Hypoautofluorescence N=12 Absent (%) / Present (%) Relative (%)
Homogeneity	24.88 / 11.98 (67)/(33)	27.65 / 31.80 (47)/(53)	2.76 / 0.92 (75)/(25)
Hyperreflective foci	17.05 / 19.82 (46)/(54)	51.15 / 8.29 (86)/(14)	1.84 / 1.84 (50)/(50)
Choroidal hypertransmission	16.59 / 20.28 (45)/(55)	47.93 / 11.52 (81)/(19)	0.92 / 2.76 (25)/(75)
Disrupt / Intact			
ELM Integrity	29.03 / 7.83 (79)/(21)	23.96 / 35.48 (40)/(60)	3.22 / 0.46 (88)/(13)
Ellipsoid zone Integrity	31.80 / 5.07 (86)/(14)	30.88 / 28.57 (52)/(48)	3.22 / 0.46 (88)/(13)
RPE-BM Integrity	24.42 / 12.44 (66)/(34)	23.04 / 36.41 (39)/(61)	1.84 / 1.84 (50)/(50)
>125 and ≤250 μm / >250 μm			
Size	0.46 / 36.41 (1)/(99)	0 / 59.45 (0)/(100)	0 / 3.68 (0)/(100)

1.4. DISCUSSION

The main objective of this work was to correlate lipofuscin-related autofluorescence changes with pathological findings in intermediate AMD.

Multimodal evaluation is a recommended approach in the study of several retinal diseases. CFP, SD-OCT and FAF are three complementary imaging techniques commonly used in AMD patients. Recorded FAF changes are located in perfect correspondence with the drusen but, as we compare an enface image (FAF) with a scan image (SD-OCT), they can represent any structure in the same location above or below the drusen.

As RPE cells are the main source of lipofuscin and short-wavelength (blue-light – 488nm) emitting fluorophores, FAF changes can represent the status of RPE cells in precise relationship with this particular drusen instead of the druse itself; melanin pigment can also play a role, namely hyperpigmentation or hypopigmentation of the adjacent area. So, when we discuss drusen autofluorescence we mean FAF findings in the drusen neighborhood.

We found a statistically significant correlation between drusen autofluorescence and homogeneity, hyperreflective foci, ELM integrity, EZ integrity and CH. Of those, the more significant correlations were found with hyperreflective foci ($r=0.4$), ELM integrity ($r=0.3$) and EZ integrity ($r=0.3$) although all correlations were weak or moderate and no strong correlations were found with any parameter.

The lack of strong correlation with the analysed parameters appears different from reports in previous publications^{9,10}. Landa⁹ found strong correlation with only two items, namely drusen size and the status of the EZ (inner segment-outer segment (IS-OS) layer) above the drusen ($r = 0.78$, $p < 0.001$; $r = 0.58$, $p < 0.001$, respectively). Our results show an identical trend but with less or no statistical significance, particularly drusen size (length and height). This is possibly due to the fact that our patient population exhibited almost exclusively large drusen. Gobel¹⁰ looked for alterations of the FAF signal in early and intermediate AMD and found that they are not necessarily associated with corresponding fundus or angiographic visible changes, including drusen. Researchers have speculated that FAF findings may therefore represent an independent measure for disease progression. In agreement with our findings, Einbock¹⁵⁸ and coworkers clearly illustrated a wide range of FAF findings in eyes with drusen, as they observed increased, normal or decreased FAF at

the site of different single drusen. However, there was a tendency toward more pronounced changes in larger drusen.

The correlation of outer retinal layers status (ELM, EZ) with FAF findings needs some reflection. We know the importance of these layers in the prognosis of multiple retinal diseases and their disruption usually means worse visual performance. Our results showed that cases of disruption (ELM, EZ) were significantly related to FAF changes (hypo or hyperautofluorescent). We conclude that drusen related to FAF changes (hypo or hyperautofluorescent) are associated with signs of worse prognosis in the outer retinal layers.

The findings in outer retinal layers (ELM, EZ and RPE-BM) and autofluorescent changes were similar but did not overlap. They represent different stages of the atrophic process and reflect the fact that photoreceptor atrophy can occur without retinal pigment epithelium atrophy. This knowledge is well documented in the recent Classification of Atrophy Consensus Group (cRORA and iRORA criteria) ^{66,72}.

The correlation was more evident with EZ than with ELM or with RPE-BM. This fact was quite unexpected because RPE-BM disruption, by itself, promotes direct FAF alteration. Interpretation of RPE-BM integrity showed some variability, possibly related to reported difficulties in distinguishing thinning of RPE in drusen apex from disruption. This parameter exhibited the highest discrepancy between the two selected ophthalmologists (approximately 73%). Its interpretation should have this bias in mind and this fact may explain the unexpected weaker correlation.

CH also showed a statistically significant correlation with FAF changes. CH is a consequence of RPE atrophy and therefore it was reassuring to observe a more evident relationship with hypoautofluorescent cases. In other studies, hypertransmission into the choroid (pinstripe hypertransmission) has also been considered as a reliable indicator of loss of RPE, namely in geographic atrophy or associated with degeneration of RPE lying external to subretinal drusenoid deposits ¹⁰⁷.

Another interesting conclusion is the relevance of other factors aside from the RPE in autofluorescence findings. Drusen homogeneity and hyperreflective foci seem to have statistically significant importance in the autofluorescence features of the studied drusen and this relationship is not dependent on RPE status.

One weakness of this work is the fact that it did not use the Quantitative Autofluorescent Technique^{151,159,160,165}. Quantitative AF could give us more accurate results, but this technology is currently restricted to some centers. Nevertheless, recent references showing non-correlation of quantitative FAF signal with AMD presence, severity, and drusen load have discouraged us from using this quantitative approach^{166–168}.

In the last few years some studies using SD-OCT and FAF have been used together in patients with late dry AMD to classify the margins of geographic atrophy, as a means of predicting progression^{169,170}. Similarly, we propose that investigation of the correlation between SD-OCT and FAF appearance of drusen may contribute to a better understanding of intermediate AMD. The described correlations may help to understand new progression biomarkers for intermediate AMD.

CHAPTER IV. FUNDUS AUTOFLUORESCENCE FINDINGS

2. CORRELATION STUDY OF DRUSEN AUTOFLUORESCENCE AND AMD PROGRESSION

Considering TASK 3 we performed a prospective study to correlate different patterns of drusen autofluorescence in FAF with progression from intermediate to late AMD. This work was presented as an oral communication at 65th Congress of Portuguese Society of Ophthalmology, 2022.

2.1. INTRODUCTION

AMD hallmark is the presence of drusen, deposits of extracellular debris located between the basal lamina of the retinal pigment epithelium (RPE) and the inner collagenous layer of the Bruch's membrane. The RPE exhibits inherent autofluorescence due to the presence of lipofuscin granules and fundus autofluorescence (FAF) that can, therefore, provide an indication of healthy function of the RPE cells¹⁷¹. Changes in FAF may be one of the earliest signs of retinal disease. Multimodal imaging can reveal a broad spectrum of ultrastructural changes that may reflect different stages of disease progression. The purpose of this work was to study differences in progression from intermediate to late AMD in eyes with different patterns of drusen autofluorescence on FAF.

2.2. METHODS

Starting from the previous study (228 drusen from 53 patients) we performed a further prospective evaluation, after a follow-up period of 24 months. We lost 5 patients during the follow-up. Patients received, at the baseline, a comprehensive ophthalmologic evaluation including Color Fundus Photography (CFP), Fundus Autofluorescence (FAF), Optical Coherence Tomography (OCT) and OCT-Angiography (OCT-A) imaging. Baseline OCT scans and FAF were evaluated for the characteristics of drusen and drusen were classified as iso, hypo or hyperautofluorescent (Table 11). After 24 months, patients were classified according to progression to: no progression or progression to advanced AMD, which included progression to geographic atrophy (GA) or neovascular AMD. The central 10° were evaluated and the larger drusen (up to 5 per patient) were included in the analysis.

All OCT analysis were performed by two investigators and disagreements were settled by the senior investigator.

2.3. RESULTS

48 eyes, 48 patients with intermediate AMD were evaluated. 206 drusen were included in the analysis. After 24 months, progression to advanced AMD was detected in 18 patients (37.5%), with 14 eyes progressing to GA and four eyes progressing to neovascular AMD. When looking at the different patterns of drusen autofluorescence, we found a statistically significant difference in the rate of progression to advanced AMD between patients with at least one hypo and/or hyperautofluorescent drusen and patients with only isoautofluorescent drusen (48.5% versus 13.3%, respectively; $p=0.02$). Moreover, progression to GA alone was significantly superior in patients with at least one hypo or hyperfluorescent drusen ($p=0.039$) (Table 12). Differences in age, sex, drusen type (serous or mixed) and presence of baseline areas of iRORA were not statistically significant between patients groups.

Table 11 – Baseline characteristics of patients to prospective correlation FAF study.

Baseline characteristics	48 Eyes from 48 Patients
Age, years	82.4 ± 9.9 YO
Female gender, n (%)	26 (54.2%)
Drusen type, n (%)	
• Serous	36 (75%)
• Serous and cuticular	12 (25%)
Areas of iRORA on baseline, n (%)	15 (31.3%)
Drusen autofluorescence, n (%)	
• Only Isoautofluorescent drusen	15 (31.3%)
• At least one hypo ou hyperautofluorescent drusen	33 (68.8%)

Table 12 – Drusen autofluorescence and disease progression.

	Advanced disease p = 0.02	Neovascular AMD	Atrophic AMD p = 0.039
Only isofluorescent drusen n (%)	2 (13.3%)	2 (13.3%)	0 (0%)
At least one hypo or hyperfluorescent drusen n (%)	16 (48.5%)	2 (6.1%)	14 (42.4%)

2.4. DISCUSSION

In a previous study from the same set of patients, the authors found that drusen morphological characteristics and autofluorescent findings are correlated but other factors or cofactors may be involved. In the present study, the authors found that the presence of hypo and/or hyperautofluorescent drusen on FAF was positively associated with progression to advanced disease and GA in particular.

The results support the idea that changes in the RPE reported in fundus autofluorescence may be an early sign of progression of AMD and further strengthen the importance of multimodal imaging when following patients with AMD, in order to detect the earliest signs of progression of the disease.

CHAPTER V. CHORIORETINAL VASCULAR FINDINGS

CHAPTER V. CHORIORETINAL VASCULAR FINDINGS

Related to VASCULAR FINDINGS we have designed three tasks.

TASK 1. Test if choriocapillaris perfusion is disturbed in Intermediate AMD patients;

TASK 2. Test if retinal capillary plexus perfusion is disturbed in Intermediate AMD patients:

2.1. Analyze superficial retinal capillary plexus (SCP),

2.2. Analyze deep retinal capillary plexus (DCP);

TASK 3. Assess if chorioretinal vascular changes are related with disease progression.

Considering TASK 1 and TASK 2 we published the following paper:

CHOROIDAL VASCULAR IMPAIRMENT IN INTERMEDIATE AMD PATIENTS

Flores R, Carneiro A, Nery G, Quenderra B, Barata M, Tenreiro S, Seabra MC.

Diagnostics. 2022; 12, 1290.

doi: 10.3390/diagnostics12051290.

PMID: 35626445;

Impact Score: 3.706, Q2 in Medicine, General and Internal

<https://pubmed.ncbi.nlm.nih.gov/35626445/>

CHAPTER V. FUNDUS AUTOFLUORESCENCE FINDINGS

1. CHOROIDAL VASCULAR IMPAIRMENT IN INTERMEDIATE AGE-RELATED MACULAR DEGENERATION

ABSTRACT

Purpose: Age-related macular degeneration (AMD) is a multifactorial disease, whose complete pathogenesis is still unclear. Local hemodynamics may play a crucial role in its manifestation and progression.

Methods: To evaluate choroidal and retinal vascular parameters, a total of 134 eyes were analyzed, 100 with intermediate AMD and 34 age matched healthy controls. 131 eyes of 104 patients were eligible for complete image assessment and 3 eyes were excluded for insufficient image quality: Group 1: intermediate AMD (n = 97) and Group 2: healthy controls (n = 34). Spectral domain optic coherence tomography (SD-OCT) with enhanced depth imaging (EDI) and optic coherence tomography angiography (OCT-A) were acquired using Spectralis (Heidelberg Engineering). Choroid and retinal capillary plexus were evaluated and image binarization was used to obtain quantitative data.

Results: Mean age was 77.67 years old (YO) and 67.2% were women. Total subfoveal choroidal area and luminal area were significantly reduced in Group 1 compared with Group 2 (0.88 mm^2 and 0.40 mm^2 vs. 1.24 mm^2 and 0.55 mm^2 , respectively) ($p < 0.05$). Regarding choriocapillary flow density, AMD eyes recorded reduced values (34.83%) compared with controls (36.25%) ($p < 0.05$).

Conclusion: Chorioretinal vasculature is impaired in intermediate AMD patients and vascular parameters could be attractive new prognostic biomarkers. Future therapeutic approaches may target this vascular dysfunction and delay disease progression.

1.1. INTRODUCTION

Age-Related Macular Degeneration (AMD) is a chronic and progressive disorder of the macula, being the leading cause of irreversible visual impairment among older adults, affecting 30–50

million individuals worldwide ^{16,148,172}. AMD is defined by a progressive degeneration of the photoreceptors, retinal pigment epithelium (RPE) and choriocapillaris, leading to loss of central vision ¹⁴⁸. Although its pathogenesis is still unclear, there is a proven genetic background, with polymorphisms within genes encoding complement factor H, human high-temperature requirement A-1 (HTRA1), age-related maculopathy susceptibility 2 (ARMS2), complement factor B (CFB), complement C2 and C3, identified as potential predictors for AMD development ^{6,173}. Environmentally, the most important risk factor is smoking, and advanced age is the strongest risk factor ¹⁴⁸.

The earliest histopathological manifestation of the disease are lipid deposits within Bruch's membrane (BM), possibly from reduced effectiveness of the RPE to digest the cellular debris associated with photoreceptor outer segments turnover ². These deposits are known as basal linear or laminar deposits and, as they evolve, thickening of the BM collagenous layers with degeneration of elastin and collagen will predispose drusen formation, the first visible clinical sign of AMD ³. Typical drusen appear as focal, whitish yellow excrescences, located between the RPE and BM, varying in number, size, shape, and distribution ⁴.

Hard drusen are present in 80% of the general population over 30 years of age and appear as round, discrete yellow-white spots, measuring less than 63 μm , currently considered as a physiological sign of ageing ⁶. Soft drusen are poorly defined, with non-discrete borders measuring more than 63 μm , being large, soft and confluent drusen associated with a higher risk for development of advanced AMD ⁶. They are also associated with aging, affecting 26% of individuals over the age of 70. Cuticular drusen are yellow deposits located below the basal membrane of RPE, triangular shape, small diameter (50-75 μm) and usually distributed in clusters. Reticular pseudodrusen or subretinal drusenoid deposits (SDD) are yellow faint and interlacing network in the apical region of the RPE and their presence entails a worst prognostic, being associated with a more aggressive disease ^{5,7,8,174}.

Previous bibliography and published articles refer AMD as a difficult issue with respect to its etiology and pathogenic mechanisms. AMD was first described in the late 19th century, and since then several hypotheses have been proposed: an inflammatory, a degenerative disease or a result of choroidal hemodynamic disturbances and ischemia. Ischemic processes have been frequently

suggested to have a central role in its development and progression ¹⁷⁵.

Progressive degeneration of macular photoreceptors is not the unique event in AMD with RPE being an important target in this pathogenic process. RPE apical extensions are in close proximity to the photoreceptor's outer segments and the basal cellular pole next to BM, separating the outer retina from the choroid. Although cellular and molecular theories of AMD have been targets of particular interest in the last decades, emerging data suggests that local hemodynamic factors may have a significant role in AMD pathologies. In fact, RPE and photoreceptors are metabolically related cells, and such a close relationship is necessary to achieve a healthy status, which in turn depends primarily on choroidal blood flow as a source of oxygen and nutrients.

Changes in choroidal blood flow have been proposed as a pathogenic trigger for AMD, in the past. Possek hypothesized that atherosclerotic changes in ocular blood vessels could be a factor subjacent to neovascular AMD ^{12,13}. Friedman and other authors developed this hemodynamic theory further, proposing that BM and sclera stiffening due to accumulations of lipoproteins, increases choroidal vascular resistance that subsequently promotes an increase in hydrostatic pressure or a decrease in choroidal perfusion ^{14,15,176,177}. Such mechanisms have some resemblance to atherosclerosis due to lipid deposition in arterial walls.

In the healthy outer retina, 85–90% of oxygen is delivered via the choriocapillaris to the outer layers, while the remaining 10–15% is obtained from the deep retinal capillary plexus ^{178,179}. Consequently and concerning vascular etiology for AMD, the primordial pathological changes are likely to occur in the choroidal vasculature retinal ¹⁷⁹. Macular area is consistently exposed to light with potential toxicity and consequently overload of oxidative stress; these factors explain the small tolerance for any homeostatic disturbance, like BM thickening or drusen deposition ^{178,179}. The primary insult could occur at the level of the RPE, which leads to secondary choriocapillaris degeneration, and drusen formation could be affected by choriocapillaris dysfunction. Whether those microvascular events are a cause or a consequence of drusen remains to be determined.

Optic coherence tomography (OCT) with enhanced-depth imaging (EDI) improvement and OCT angiography (OCT-A) are noninvasive techniques that provide information concerning choroid ¹⁸⁰ and retinal ¹⁷⁹ circulation. Quantitative data can be obtained directly in some OCT and OCT-A devices or indirectly, using image processing tools as binarization ^{180,181} method, that allows relative

quantification of choroid and retina vascular component. Nevertheless, there is still some misunderstanding about the best parameter to study choroidal vascular disease but relationship between vascular and stroma components (choroidal vascularity index) seem to be a promising tool^{180–182}. Impact of subretinal drusenoid deposits in total choroidal thickness and in choroidal vascularity index has been studied in nonexudative AMD patients¹⁸² but the authors did not specifically evaluate these factors in intermediate stages.

In this study, we describe and analyse vascular changes of the choroid and retina in intermediate AMD patients that could become new prognostic biomarkers.

1.2. METHODS

Study Design, Study Population and Ethics Issues

Cross sectional, prospective and noninterventional study conducted in outpatient Medical Retina Clinic of the Ophthalmology department of the Central Lisbon University Hospital Center (Centro Hospitalar Universitário de Lisboa Central-CHULC), a tertiary hospital in Lisbon, Portugal.

The study complied with the tenets of the Declaration of Helsinki and was approved by Institutional review board and ethics committee. All participants were asked to sign an informed consent.

Inclusion criteria consider intermediate AMD patients, based on Beckman classification^{59,148,179}, recruited during the period of January 2019 and December 2021. This clinical classification system is based on fundus lesions assessed within 2 disc diameters of the fovea in persons older than 55 years. Persons with large drusen ($\geq 125 \mu\text{m}$) or with pigmentary abnormalities associated with at least medium drusen (≥ 63 and $< 125 \mu\text{m}$) were considered to have intermediate AMD. If two eyes of the same patient were eligible we included both in the study.

Patient underwent a complete ophthalmologic evaluation, including best-corrected visual acuity (BCVA), intraocular pressure (IOP), slit lamp biomicroscopy and 90D indirect fundus- copy. Color fundus photography (CFP), fundus autofluorescence (FAF), near infrared reflectance (NIR), optic coherence tomography (OCT) with enhanced-depth imaging (EDI) improvement and OCT angiography (OCT-A) were performed in all patients. The presence of SDD was determined by

identification of characteristic dot/ribbon shaped lesions on NIR and FAF, with further confirmation on OCT.

The control group consisted of age matched healthy subjects recruited from the refraction department at the same hospital, and without any sign of AMD (on CFP, FAF and OCT scans). The exclusion criteria included patients with less than 55 years old, with refractive error above 6 diopters (positive or negative spherical equivalent), with previous documented or known retinal disease, previous retinal surgery or laser, history of glaucoma or optic neuropathy and history of systemic illness or medication with potential retinal toxicity. Any significant media opacities and inappropriate fundus imaging were also excluded. Previous cataract surgery was not considered an exclusion criterion.

Image Analysis and Measurements

Macular spectral domain OCT (SD-OCT) with enhanced depth imaging (EDI) and OCT-A, were acquired using OCT Spectralis (Heidelberg Engineering, Heidelberg, Germany). The choroid was imaged using the SD-OCT-EDI mode¹⁸³. The macular region was scanned with a wavelength of 870 nm, acquisition speed of 40.000 A-scans per second, 20°×20° centered on the fovea, dense program, 512×49 scans. Each scan was spaced 120 µm apart from each other. Subfoveal choroidal thickness was measured manually using the calliper tool available in the software, using the 1:1 µm viewing mode, in the horizontal EDI scan passing through the center of the fovea (Figure 8). Choroidal thickness was defined as the vertical distance from the hyperreflective line of the Bruch's membrane (BM): lower boundary of Retinal Pigment Epithelium (RPE) to the choriocleral interface. If the hyperreflective line of the BM was not separated from the RPE, the choroid was measured from beneath the outermost hyperreflective line of RPE/BM layer to the choriocleral interface¹⁸⁴. Total choroidal area (TCA), luminal area (LA), stromal area (SA) and choroidal vascularity index (CVI=LA/TCA) of the subfoveal 6000µm, were obtained after choroidal binarization performed resorting to Image J software using the scan passing through the fovea.

Only this scan was selected as the region of interest, as we suppose it is a representative segment of the macular region due to the segmental nature of choroidal blood supply. Binarization

imaging converts grey scale into black and white pixels images. Black pixels images in the choroid correspond to vascular lumen and white pixels images to stroma.

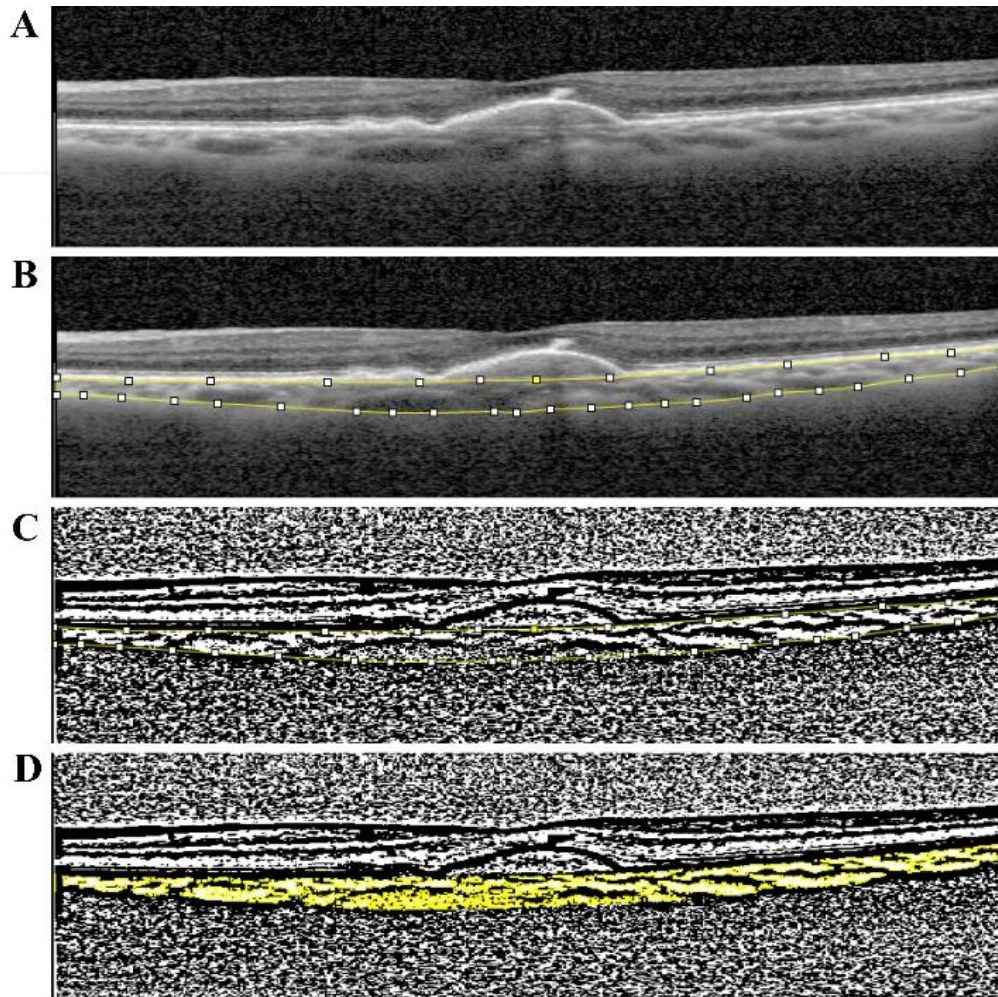


Figure 8 – Examples of image binarization of the Choroid.

(A) Macular SD-OCT-EDI centered on fovea and 6000 μm centered section in the fovea.

(B) Manual measurement of the choroidal thickness. Yellow lines delimit inner boarder—Bruch's membrane and outer boarder— escleral transition.

(C) Image binarization—converting grey scale in binarized images.

(D) Studied area and image soft-ware: black pixels images (vascular lumen) and white pixels images (stroma).

Additionally, the OCT-A was performed with a wavelength of 870 nm, acquisition speed 40.000 A-scans per second, $20^\circ \times 20^\circ$ centered on the fovea, 512×512 scans, spacing 11 μm .

The scan with the highest quality was selected and further analysed using the same binarization software. Those binarized “enface” images of superficial capillary plexus (SCP), deep capillary plexus (DCP) and choriocapillaris area (CC) allowed to calculate vascular area (white pixels images) for each particular layer.

OCT-A en face projections were defined for SCP as the projection from automatic segmented internal limiting membrane (ILM) to inner plexiform layer (IPL); DCP as the projection from automatic segmented IPL to outer plexiform layer (OPL) and CC as the projection from automatic segmented BM with a thickness of 20 μm centered 10 μm below BM automatic segmentation line. In those images, total measured area is constant (studied area corresponding to $20^\circ \times 20^\circ$ centered on the fovea), and flow density (FD) of each particular layer represents the ratio between vessel area and total measured area (Figure 9).

Imaging artefacts were assessed by two experimented ophthalmologists in this area.

Motion artefacts were considered present when a white-line was detectable on the OCT-A image with concomitant lateral displacement on B-scan images.

Projection artefacts as well as motion artefacts, displacement artefacts (discontinuous blood vessels), shadowing (attenuation of the signal), vessel doubling (two copies of a blood vessel), and white line artefacts were assessed on sequential OCT-A images. After each examination, the images were checked, and those with clearly visible retinal vessels and no artefacts were selected.

Statistical Analyses

Normality of the test was analyzed in Q-Q box and with Shapiro Wilk test, and the sample showed a normal distribution in most but not in all the variables. The group comparison was performed with known parametric test t-student for most of the studied variables and with nonparametric Mann-Whitney U test (Wilcoxon rank-sum test for clustered data) and Kruskal-Wallis test for TCA, LA and CVI, which showed non normal distribution. SPSS software (Version 26, Statistical Package for Social Science IBM) was used for statistics analysis and to generate box-and whisker plots; values of $p < 0.05$ were considered statistically significant.

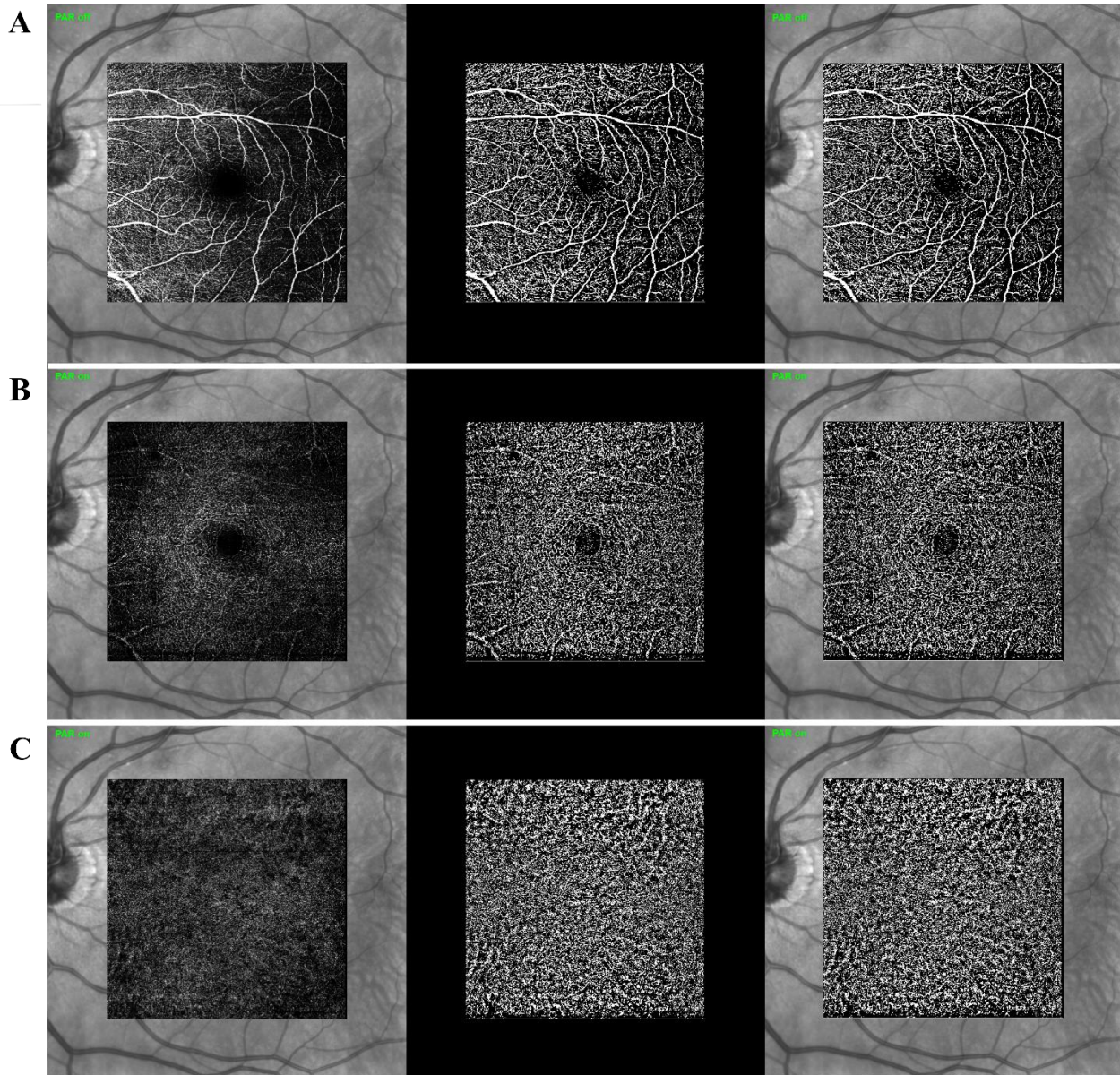


Figure 9 – Examples of image binarization of Retinal Capillary Plexus (SCP, DCP) and Choriocapillaris (CC).

(A) First row images: Near-infrared reflectance (NIR) and overlap composition with “enface” OCT-A images of SCP; SCP image binarization; Near-infrared reflectance (NIR) and overlap composition with SCP image binarization.

(B) Second row images: Near-infrared reflectance (NIR) and overlap composition with “enface” OCT-A images of DCP; DCP image binarization; Near-infrared reflectance (NIR) and overlap composition with DCP image binarization.

(C) Third row images: Near-infrared reflectance (NIR) and overlap composition with “enface” OCT-A images of Choriocapillaris; CC image binarization; Near-infrared reflectance (NIR) and overlap composition with CC image binarization.

Further data analysis was performed in Python using the application Jupyter Note-books version 6.4.5 from *Anaconda*.

Linear regression equations and respective coefficients of determination (R-squared) were calculated to assess the linear relationship and the strength of the model, respectively, between total choroidal and luminal areas of healthy and AMD patients. Additionally, Spearman's rank correlation coefficient (ρ) was calculated between choriocapillary flow density and the luminal choroidal area for patients and controls.

This statistical measure indicated the strength of a monotonic relationship between the paired data and statistical significance (p-value) was calculated for each correlation coefficient.

1.3. RESULTS

Study Population Characteristics

Demographic characteristics of study population are presented in Table 13. A total of 134 eyes were recruited in this study, 100 with intermediate AMD and 34 healthy controls.

Poor image quality for accurate analysis, motion and projection artefacts in OCT-A images preventing reliable evaluation were excluded.

A total of 131 eyes of 104 patients were eligible for binarization and were included in the final analysis (3 eyes were excluded for insufficient image quality). Groups were divided according to drusen characteristics: 57 eyes of 52 patients presented soft or cuticular drusen (Group 1A) and 40 eyes of 28 patients presented association with SDD (Group 1B). The mean age in years in the study sample was 77.67.

Groups 1 and 2 were age matched: Group 1: 78.62 ± 8.59 and Group 2: 75.72 ± 8.30 . The majority of patients were women (67.2%).

Table 13 – Demographic characteristics of study population.

M: Male; F: Female; Drusen: soft or cuticular drusen; Drusen + SDD: drusen in association with subretinal drusenoid deposits.

	Total	AMD Group 1	Drusen Group 1A	Drusen + SDD Group 1B	Control Group 2
N	131	97	57	40	34
%	100%	74.0%	43.5%	30.5%	26.0%
Gender (M/F)	(43/88)	(39/58)	(22/37)	(18/22)	(5/29)
Age	77.67 ± 8.59	78.62 ± 8.59	78.58 ± 7.38	78.67 ± 8.25	75.72 ± 8.30

Choroid Evaluation in Intermediate AMD

We studied OCT images with EDI mode for all recruited patients and considered choroid vascular parameters after image binarization. We found a mean TCA of $0.88 \pm 0.50 \text{ mm}^2$ in Group 1 and $1.24 \pm 0.47 \text{ mm}^2$ in Group 2. Luminal area (LA) was also reduced in Group 1 ($0.40 \pm 0.22 \text{ mm}^2$) compared with Group 2 ($0.55 \pm 0.19 \text{ mm}^2$). Mann-Whitney non-parametric test (Wilcoxon rank-sum test for clustered data) confirmed statistically significant differences in TCA and LA between Group 1 and Group 2 ($p < 0.05$). CVI obtained from the ratio of LA/TCA revealed lower values in Group 1 (45.3%) than in Group 2 (45.6%) with no statistical significance.

Concerning subgroup analysis, the comparison between 1A versus 1B using Mann-Whitney or Wilcoxon nonparametric test showed significant differences in choroidal evaluation studied by TCA and LA with significant lower values in drusen group (Group 1A) than in those associated with SDD (Group 1B) (Figure 10 and Supplementary Table 1 and 2 in Appendix).

Retinal Capillary Plexus and Choriocapillaris Evaluation in Intermediate AMD

Superficial and deep retinal capillary plexus presented no significant differences in FD between Group 1 and Group 2 (Figure 11 and Supplementary Table 1 and 2 in Appendix). We recorded reduced choriocapillaris flow density in Group 1 (0.34 ± 0.02) compared with healthy controls (0.36 ± 0.02), with statistical significance in t-student test ($p = 0.040$). Group and subgroup analysis with ANOVA

unidirectional test ($p < 0.05$) found statistically differences in choriocapillaris flow density between groups and in subgroups (Figure 11 and Supplementary Table 1 and 2 in Appendix). Group 1A presented the lowest values in choriocapillaris flow density.

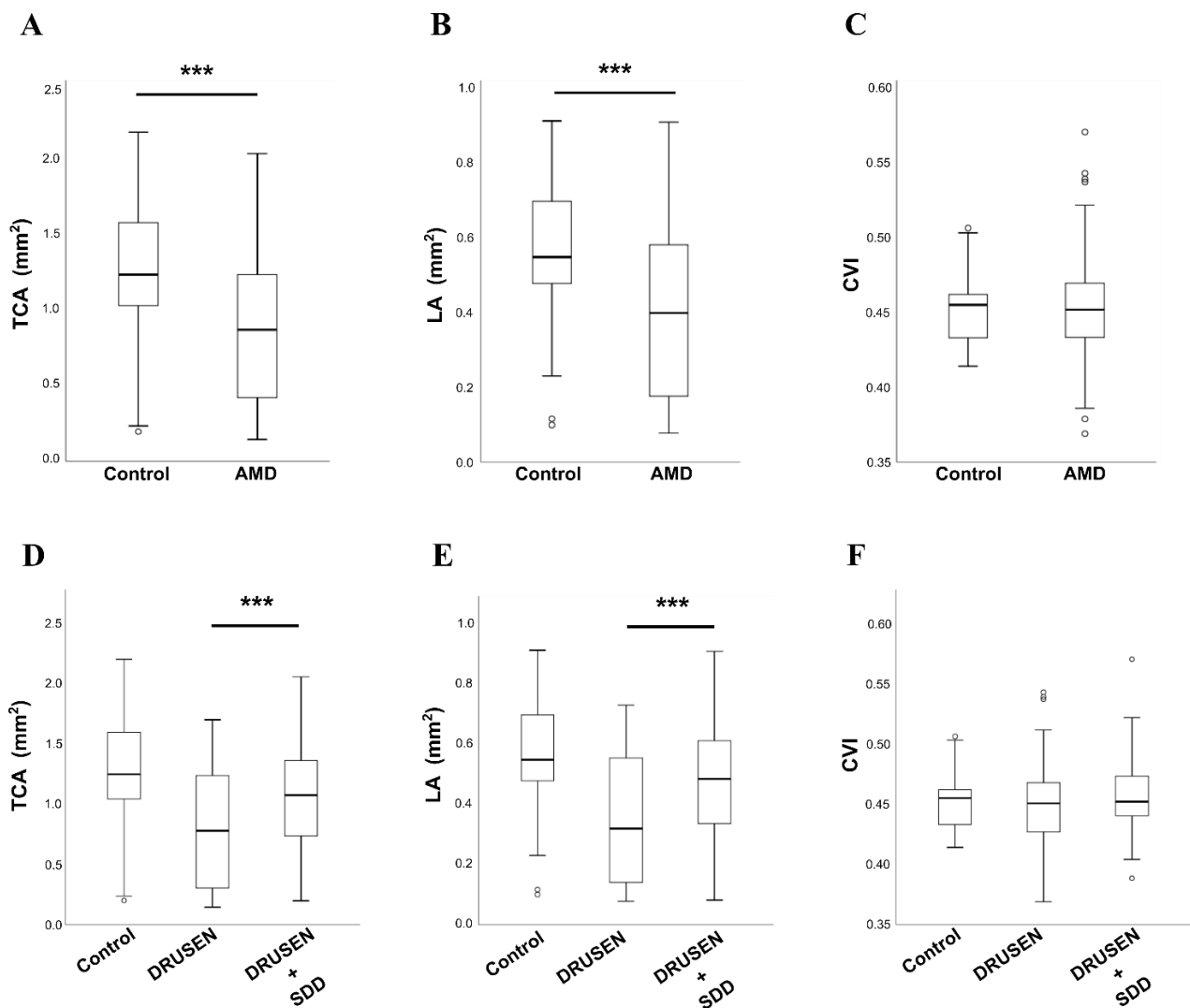


Figure 10 – Choroid evaluation in intermediate AMD.

(A–C) Graphical distribution of total choroidal area (TCA), luminal area (LA) and choroidal vascularity index (CVI), respectively, among control and AMD groups through box plots. (D–F) Box plots displayed the distribution of TCA, LA and CVI, respectively, among the control group and the two sub-groups (i.e., DRUSEN and DRUSEN + SDD) defined among AMD patients. Box-and whisker plots show median, first and third quartile, the whiskers extend to 1.5 times the interquartile range (IQR) (values exceeding this are plotted as individual points). TCA: Total choroidal area; LA: Luminal area; CVI: Choroidal vascularity index; DRUSEN: soft or cuticular drusen; DRUSEN + SDD: drusen in association with subretinal drusenoid deposits. (***) p -value ≤ 0.001 .

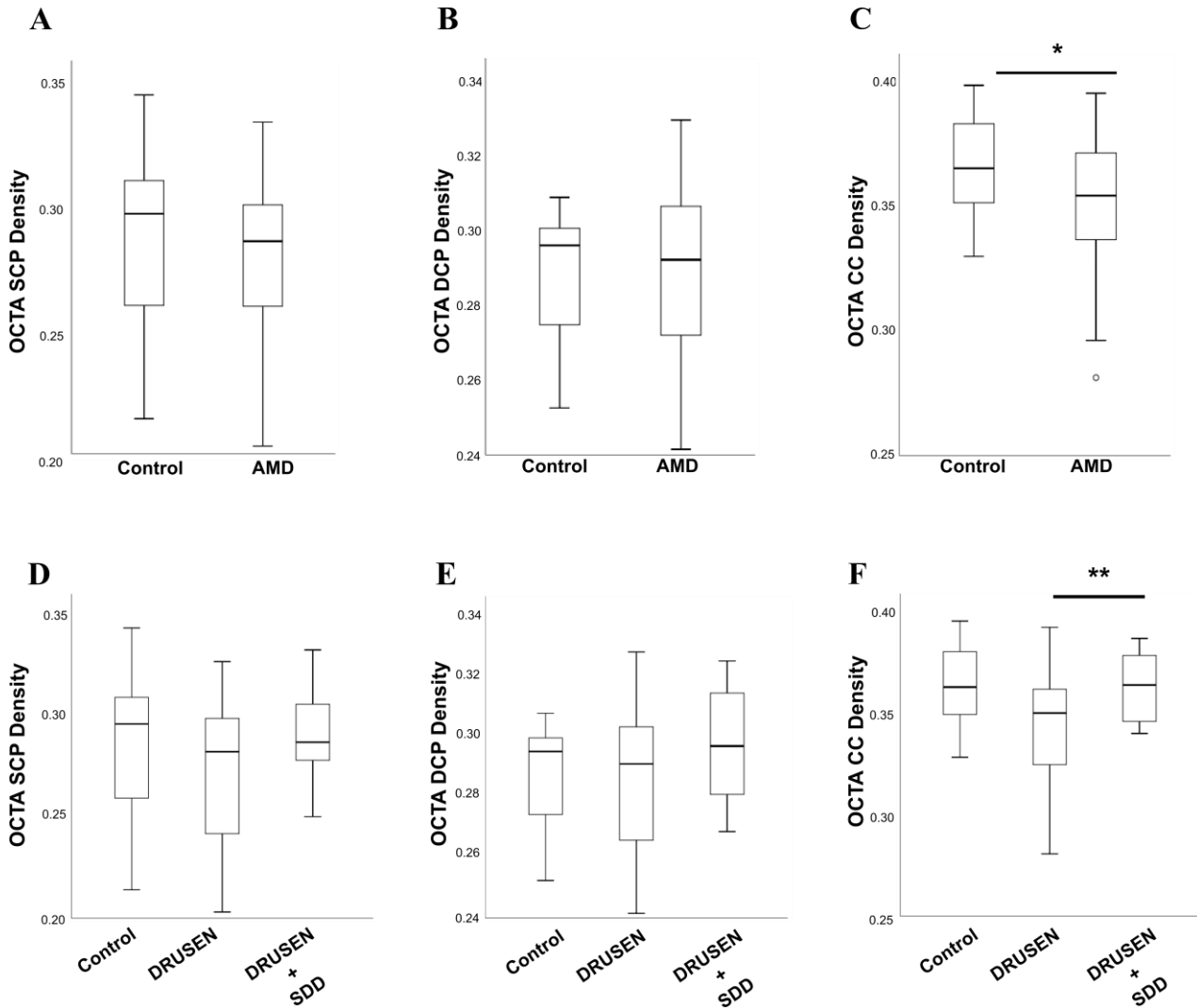


Figure 11 – Retinal Capillary Plexus and Choriocapillaris evaluation in intermediate AMD.

(A–C) Graphical distribution of SCP, DCP and CC flow density obtained by OCTA among control and AMD groups through box plots, respectively. (D–F) Box plots showed the distribution of SVP, DVP and CC flow density among the control group and the two sub-groups (i.e., DRUSEN and DRUSEN + SDD) defined among AMD patients, respectively. Box-and whisker plots show median, first and third quartile, the whiskers extend to 1.5 times the interquartile range (IQR) (values exceeding this are plotted as individual points). SCP: Superficial capillary plexus; DCP: Deep capillary plexus; CC: Choriocapillaris; FD: Flow density; DRUSEN: soft or cuticular drusen; DRUSEN + SDD: drusen in association with subretinal drusenoid deposits (* p-value <0.05; ** p-value ≤ 0.01).

1.4. DISCUSSION

One main conclusion of this work is that total subfoveal choroidal area (TCA) and luminal area (LA) were reduced in intermediate AMD, compared with age matched healthy controls, supporting the concept that the choroidal vascular network is impaired in AMD patients.

The significant differences in TCA and LA between healthy and AMD patients corroborate the possibility of reduced choroidal perfusion and insufficient nourishing of the RPE in intermediate AMD¹⁷⁷. In our cohort, CVI was also decreased in AMD patients but with no statistical significance, meaning that the decrease in luminal area was not so meaningful as the decrease in TCA. Besides, it was found that TCA and LA were linearly correlated in all groups (i.e., controls, group 1A and group 1B), evidenced by the high coefficient of determination (R-squared) of 0.99 (Figure 12A). Additionally, the slopes of the regression lines were very similar among controls and intermediate AMD patients (either in group 1A or group 1B), which can explain why no statistically significant difference was found in CVI calculations for controls and AMD patients, even though a decrease in the CVI of AMD patients was perceived. Note that CVI is the ratio between the two variables plotted, therefore the slope of the regression equation can be a rough indicator of the CVI mean for each group. Such fact could indicate that the decrease in luminal area was not so meaningful as the decrease in TCA in diseased groups.

Other authors have also studied those parameters in patients with geographic atrophy (GA) and found significant lower values of TCA, LA and CVI in those patients and CVI worsened over the follow-up period¹⁸⁵. In our intermediate AMD sample choroidal vascular status was not so disturbed as in this GA population and consequently CVI was not so decreased compared with age matched control group.

No significant differences in retinal circulation were found in intermediate AMD patients as compared with age-matched healthy controls. Our findings should be distinguished from other studies that found reduced vascular density in the superficial capillary plexus but a nonsignificant reduction in the deep capillary plexus in intermediate AMD patients compared with normal eyes¹⁸⁶.

Interestingly, choriocapillaris layer had significant lower density in AMD patients compared with controls and we found the smallest values in Group 1A; the coexistence of SDD in Group 1B was associated with improved choriocapillary density, compared with group 1A. Furthermore, the correlations between CC flow density and choroidal luminal area are very distinct in all groups. Among healthy subjects, a strong positive monotonic correlation was observed ($\rho = 0.60$, $p < 0.01$), whereas in Group 1A and 1B, the correlation between these parameters was negligible or weak and

not statistically significant (Figure 12B). The CC flow density accompanies the increase in the choroidal luminal area for control subjects, which it is not observed in intermediate AMD groups, i.e., an increase in luminal choroidal area is not followed by increase in the CC flow density in AMD patients. Considering such, one may hypothesize that AMD affects in a more significant manner the choriocapillaris vascularization than the entire choroid. This consideration and graphic relationship between CC flow density and choroidal luminal area is an interesting tool and its representativeness may constitute a challenging novel concept.

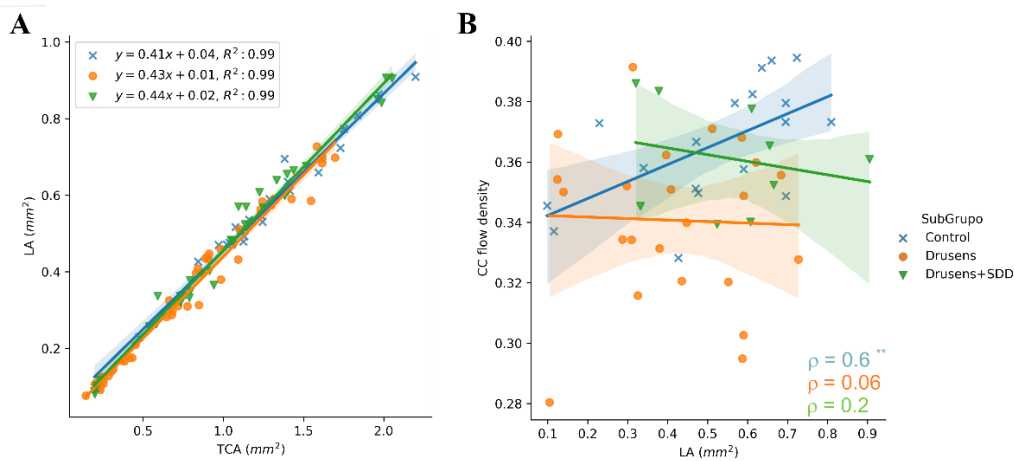


Figure 12 – Correlation analysis with Scatter plots.

Correlation analysis with (A) Scatter plots with linear regression lines and coefficient of determination (R-squared) calculated for the total choroidal area and luminal area among control, 1A (Drusens) and 1B (Drusens + SDD) groups. Linear regression lines (blue—control; orange—group 1A and green—group 1B) were calculated and represented using stats' linregress and seaborn's Implot functions, respectively. Light blue-, orange-, and green-coloured regions indicate the 95% confidence interval for each regression line; (B) Correlation scatter plots between choriocapillary (CC) flow density and luminal area (LA) (mm²) in control, 1A (Drusens) and 1B (Drusens + SDD) groups. Best-fitted lines (blue—control; orange—group 1A and green—group 1B) were represented and visualized through seaborn's Implot function. Light blue-, orange-, and green-coloured regions indicate the 95% confidence interval for each regression line. Spearman's rank correlation coefficient (ρ) for each group is in the lower right corner. The correlation coefficients were: 0.6 (control), -0.06 (group 1A) and -0.2 (group 1B). The asterisks represent the p-value of the statistical test: ** p-value < 0.01.

We hypothesize that drusen may act as a hydrophobic barrier, impeding the passage of fluid and nutrients between the choroid and the outer retina, resulting in relative ischemia¹⁷⁷. Once the disease begins, anatomic transformation of the choriocapillaris and retinal capillary plexus causes further hemodynamic alterations, which in turn exacerbate disease progression. These vascular features can be considered as coadjuvant factors for AMD progression or eventually trigger factors,

being the choriocapillaris dysfunction the first step for the subsequent pathogenic process.

In the healthy retina, oxygen diffusion from the choroidal vasculature to photoreceptors is supposed to be perpendicular to the retinal axes. BM thickening and drusen deposition in macular area, are changes that induce oxidative stress in such a region, consistently exposed to light ^{178,179}. Some models have proposed that drusen can decrease oxygen levels directly above, however allowing lateral diffusion down the concentration gradient ^{178,187}. This lateral transport explains why tall drusen can fail to induce severe hypoxia if they are relatively narrow to enable lateral diffusion; on the contrary thinner drusen can induce more complete hypoxia if they are large enough in lateral dimensions ¹⁸⁸. This appears to be the case for SDD whose coalescence in an interlacing network might prevent lateral oxygen diffusion and worsening final hypoxia, and this could be one reason for their worse prognosis ^{7,8}. Oxygen diffusion from outer to inner layers or on lateral direction can, as a compensation mechanism, modify the final impact of ischemia and influence our findings in vasculature impairment. Choroidal vascular and stromal characterization studies in eyes with SDD have showed some confusing results that need further clarification. Velaga et al. found thinner choroid and higher CVI in eyes with SDD but the real meaning of those parameters in final choroidal vascularity are not clear ¹⁸². In this study, patients with SDD presented significantly higher CVI values compared to those with nonneovascular AMD patients without SDD, and healthy patients presented the lowest values. These results are quite different from those obtained in our study although the study populations do not have completely overlapping characteristics.

Functional vascular studies were already the subject of previous reports. Laser Doppler flowmetry and interferometry have been used to study foveolar choroidal blood volume, velocity, and flow ¹⁷⁷, in the fovea. Flow in the non-exudative AMD is, on average, lower than in age-matched controls. Similar methodology showed a marked disturbance of macular retinal capillary blood flow in patients with advanced AMD ¹⁸⁹, indicating a reduced macular perfusion in the patients with the disciform end stage of late AMD. Previous published studies have documented a decreased vascular perfusion in drusen surrounding areas ¹⁸⁸ and demonstrated, in smaller samples, that both superficial and deep retinal plexus are changed among patients affected by the disease. These findings bring further evidence that pathological AMD-induced changes may not be limited to the outer retina but involve the inner retina as well. Nevertheless, the possible association between macular perfusion and AMD does not clarify any potential cause-effect relationship.

The present study demonstrated certain limitations. Although the sole focus of the analysis was structural parameters, a further structure-function assessment would potentially provide additional valuable insights into our understanding of vascular role in AMD pathophysiology. *In vivo* imaging of the choriocapillaris can be achieved with OCT-A, but the presence of artifacts in the outer layers continues to be a relevant limiting factor. Furthermore, diurnal variation and hydration status may have impact in the reliability of the obtained results. Finally, the inherent subjectivity associated with manual measurements and the downsides of image binarization techniques, such as under or overestimation of hypo and hyper reflective areas, must also be considered as limiting factors¹⁹⁰. The possibility of some bias in the inclusion of two eyes of the same patient, in AMD and healthy control group, can also be considered a potential weakness. Largest subgroup samples could eventually allow more solid and significant conclusions.

Studying AMD vascular pathophysiology is a relevant issue because future therapeutic approaches may target this vascular dysfunction and prevent or delay disease progression in intermediate AMD stages. Additionally, since morphological scores for predictive AMD prognosis are under investigation⁶⁵, retinal and choroidal vascular parameters could be attractive new parameters to evaluate. Choroidal vascularity index, as a measure of choroidal vascular status, has been proposed as surrogate marker for monitoring atrophic progression in AMD patients¹⁸⁵.

In the present study total subfoveal choroidal area, luminal area and choriocapillary flow density, seem to reveal themselves as attractive vascular parameters in intermediate AMD. Further studies are needed to evaluate their role as prognostic biomarkers, to propose other more and to compare them with existing prognostic factors.

CHAPTER V. FUNDUS AUTOFLUORESCENCE FINDINGS

2. RETINAL AND CHOROIDAL VASCULAR IMPAIRMENT AND AMD PROGRESSION

Considering TASK 3, we have conducted a subsequent study to evaluate the association of vascular factors with AMD progression. This work was presented as a poster at the 7th International Scientific meeting of the Portuguese Retina Study Group, 2023 and was accepted as an Audio Narrated Free Paper to be presented at the 23th Euretina Congress, 2023.

2.1. INTRODUCTION

In the previous study vascular changes of the choroid and the retina in intermediate AMD showed that total subfoveal choroidal area (TCA), luminal area (LA) and choriocapillaris flow density were significantly reduced compared with healthy controls. Details of the image analysis and measurements was presented previously.

2.2. METHODS

The same population was clinically evaluated for progression to late AMD at least 18 months after the first analysis. Progression was considered when patients presented, at the end of follow-up period, late disease status (geographic atrophy or neovascular AMD). Evolution to incomplete retinal pigment epithelial and outer retinal atrophy (iRORA) or iRORA enlargement was not considered as progression.

In this cohort study we considered a total of 85 eyes from the 97 previously studied eyes. 12 patients were lost to follow-up. Choroid vascular parameters (TCA, LA, and choroidal vascularity index (CVI), calculated as $CVI = LA/TCA$), and retinal capillary plexus and choriocapillaris flow density were correlated with AMD progression.

2.3. RESULTS AND DISCUSSION

A total of 26 eyes (30.59%) showed progression to late stages: 11 eyes (12.9%) to neovascular AMD (nAMD) and 15 eyes (17.6%) to complete RPE and outer retinal atrophy (cRORA). Mean age was 78.48 years old (YO) and 72.1% were women (nAMD group age was 80.3 ± 5.9 YO and cRORA group age was 79.8 ± 8.4 YO).

There were no significant differences in TCA, LA and CVI between eyes with no progression and with progression to late AMD. Furthermore, all 3 parameters were similar between nAMD and cRORA. (Tables 14, 15 and 16)

Table 14 – Total Choroidal Area (TCA) among “No progression” and “Progression” Groups.

	No progression n = 59, 69.41 %	Progression n = 26, 30.59 %	Total N = 85	p-value (test)
TCA	71.08 %	28.92 %		
Mean (SD)	0.88 (0.527)	0.97 (0.453)	0.9 (0.506)	0.348 (Wilcoxon)
Median (IQR)	0.83 (0.85)	0.95 (0.71)	0.89 (0.83)	

Table 15– Luminal area (LA) among “No progression” and “Progression” Groups.

	No Progression n = 59, 69.41 %	Progression n = 26, 30.59 %	Total N = 85	p-value (test)
LA	71.08 %	28.92 %		
Mean (SD)	0.39 (0.232)	0.43 (0.195)	0.4 (0.222)	0.369 (Wilcoxon)
Median (IQR)	0.37 (0.41)	0.45 (0.32)	0.4 (0.39)	

Table 16 – Choroidal Vascularity Index (CVI) among “No progression” and “Progression” Groups.

	No Progression n=59, 69.41 %	Progression n = 26, 30.59 %	Total N = 85	p-value (test)
CVI	71.08 %	28.92 %		
Mean (SD)	0.46 (0.042)	0.45 (0.02)	0.45 (0.037)	0.483 (t-test)
Median (IQR)	0.46 (0.05)	0.45 (0.02)	0.45 (0.04)	

Superficial and deep retinal capillary plexus and choriocapillary presented no significant differences in flow density between groups (Table 17, 18 and 19). Once again, all 3 parameters were similar between nAMD and cRORA.

Table 17 – Choriocapillary (CC) FD among “No progression” and “Progression” Groups.

	No Progression n = 59, 69.41 %	Progression n = 26, 30.59 %	Total N = 85	p-value (test)
CC FD	75.76 %	24.24 %		
Mean (SD)	0.35 (0.026)	0.35 (0.028)	0.35 (0.026)	0.855 (t-test)
Median (IQR)	0.35 (0.03)	0.36 (0.04)	0.35 (0.03)	

Table 18 – Superficial capillary plexos (SCP) FD among “No progression” and “Progression” Groups.

	No Progression n = 59, 69.41 %	Progression n = 26, 30.59 %	Total N = 85	p-value (test)
DCP FD	75.76 %	24.24 %		
Mean (SD)	0.28 (0.035)	0.27 (0.033)	0.28 (0.034)	0.328 (t-test)
Median (IQR)	0.28 (0.04)	0.28 (0.02)	0.28 (0.04)	

Table 19 – Deep capillary Plexus (DCP) FD among “No progression” and “Progression” Groups.

	No Progression n=59, 69.41%	Progression n=26, 30.59%	Total N=85	p-value (test)
DCP FD	75.76%	24.24%		
Mean (SD)	0.29 (0.023)	0.28 (0.018)	0.29 (0.022)	0.328 (t-test)
Median (IQR)	0.29 (0.03)	0.28 (0.02)	0.29 (0.03)	

A logistic regression model for progression after stepwise selection did not identify any statistically significant association between the studied variables and AMD progression. Although chorioretinal vasculature is impaired in intermediate AMD patients, vascular changes of the choroid and retina did not appear to be related to disease progression to late AMD.

CHAPTER VI. IDENTIFICATION OF DISEASE PROGRESSION BIOMARKERS

CHAPTER VI. IDENTIFICATION OF DISEASE PROGRESSION BIOMARKERS

Considering this aim, and regarding the identification of disease progression biomarkers, we have studied OCT and OCT-A biomarkers (TASK 1 and TASK 2 respectively).

Considering TASK 1 we published the following paper:

*IDENTIFYING IMAGING PREDICTORS OF INTERMEDIATE AGE-RELATED MACULAR
DEGENERATION PROGRESSION*

Flores R, Fradinho A, Pereira R, Mendes J, Seabra M, Tenreiro S, Carneiro A.
Translational Vision Science & Technology 2023, 12,
doi: 10.1167/tvst.12.7.22
PMID: 37490304

Impact Score: 3.048,Q1 in Ophthalmology

<https://pubmed.ncbi.nlm.nih.gov/37490304/>

CHAPTER VI. IDENTIFICATION OF DISEASE PROGRESSION BIOMARKERS

1. IDENTIFYING IMAGING PREDICTORS OF INTERMEDIATE AGE-RELATED MACULAR DEGENERATION PROGRESSION

ABSTRACT

Purpose: Intermediate AMD (iAMD) is a risk factor for progression to advanced stages, but rates of progression vary between individuals. Predicting individual risk is advantageous for programing timely and effective treatment and for patient stratification into future clinical trials.

Methods: Prospective and non-interventional study following iAMD patients for 24 months. OCT parameters related with drusen, hyperreflective foci (HRF), presence of incomplete retinal pigment epithelial and outer retinal atrophy (iRORA) and ellipsoid zone (EZ) status were explored at the baseline. Patients were reclassified at the end of the follow-up period and divided according to their progression. A risk prediction model for progression to late AMD was developed.

Results: A total of 135 patients were enrolled in the study and 30.4% developed late disease. A multivariate logistic regression model was created using those OCT parameters, further optimized by backward feature elimination. Parameters offering the best fit in prediction progression were presence of iRORA, EZ status, drusen area and presence of HRF. iRORA is the feature that provides a higher probability of developing late AMD (OR = 12.91, $p = 0.000$), followed by EZ disruption status (OR = 3.54, $p = 0.0018$). The area under the ROC curve calculated for the testing set was 0.77 (95% CI 0.56–0.98).

Conclusion: The combination of iRORA and EZ disruption constitute a high risk of progression to cRORA within two years.

Translational Relevance: We propose a practical and useful model to help clinicians in their daily practice in predicting individual progression to advanced AMD.

1.1. INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible severe vision loss in individuals over 55 years old in the developed world ^{59,191}. Intermediate AMD (iAMD) is a risk factor for progression to advanced stages, but rates of progression vary between individuals. Inter-individual progression rates of iAMD to advanced forms are extremely variable and currently there are no specific anatomical markers for disease progression that can predict individual risk of conversion to late AMD reliably ^{192,193}. If it was possible to preview progression to late AMD and to identify who might progress faster, this metric could be advantageous for programming timely and effective anti-VEGF treatment for neovascular AMD (nAMD), for patient stratification into clinical trials for atrophic AMD and, in the longer term, to allow better personalized medicine if novel treatments emerge in the future.

Evaluation of drusen in color fundus photographs (CFP) was, in the past, the main clinical standard for assessing patients with iAMD. There seems to be a positive correlation between the number, area, and extent of drusen observed in CFP and the risk of progression in more than 2 years ⁷⁵. Nevertheless, this method has limited value for predicting individual progression because it is unreliable in the short term ^{75,76}.

The recent widespread use of other noninvasive approaches in AMD retinal imaging has provided important tools in the identification of biomarkers of disease progression. With the aid of such multimodal retinal imaging modalities, clinicians and researchers are recognizing a wide range of image biomarkers of AMD progression.

Optical coherence tomography (OCT) indicates different types of drusen that may coexist in a patient but their significance in AMD progression is still unknown¹⁹⁴. Other OCT biomarkers related to progression to advanced AMD include drusen volume, hyperreflective foci (HRF), reticular pseudo-drusen or subretinal drusenoid deposits (SDD), incomplete retinal pigment epithelial and outer retinal atrophy (iRORA), hypertransmission defects and OCT-reflective drusen substructures (ODS)^{93,94}.

Several investigators using spectral-domain OCT (SD-OCT) in patients with early and intermediate AMD reported that drusen area, height, and length predicted progression to advanced

stages.⁶ Baseline drusen volume is reported as an important factor of increased risk progression to either nAMD or geographic atrophy (GA)⁷⁹. Drusen height seems to have a positive correlation with progression to late atrophic AMD and drusen length with conversion to nAMD⁹⁶. More recent SD-OCT volume studies provide more comprehensive measures^{97–102}. Quantitative automatic determinations are available in some devices (Cirrus, Zeiss) whose role can be useful in clinical practice. Other devices have no automatic volume determinations (Spectralis, Heidelberg) and drusen quantification volumes are dependent on OCT layer predictions and freely available non approved algorithms^{195,196}.

HRF are discrete, well-defined lesions, with isorefectivity or higher reflectivity than the retinal pigment epithelium (RPE). They are highly backscattering lesions located within the neurosensory retina, usually adjacent to the drusen edge or apex or in the inner neurosensory retina layers. They are described as representing extracellular pigment granules and outer segment debris (outer HRF) or displacement and clumping of degenerated RPE cells or microglia (inner HRF)¹⁰⁹. HRF are associated with increased risk for progression to advanced AMD^{93,126,197} and the presence of HRF is the strongest individual predictor for AMD progression.²² In previous works the number of HRF showed correlation with progression to late AMD,²² in some studies with geographic atrophy⁷⁹ and in others with macular neovascularization^{93,94}.

Reticular pseudodrusen or subretinal drusenoid deposits (SDD) are subretinal collections of granular, hyper-reflective interlacing material located above the RPE. They are usually found in the superior macula or next to superotemporal arcades. Near infrared reflectance (NIR) and SD-OCT seem to be particularly relevant and effective methods for diagnosing SDD. Depending on the studied population, reticular pseudodrusen are present in approximately 9% to 58% of patients with iAMD^{8,108}. The presence of reticular pseudodrusen is associated with an additional two- to six-fold increased risk of progression to advanced AMD, with the risk of progression being higher for reticular pseudodrusen located outside the macula⁸.

Incomplete Retinal Pigment Epithelial and Outer Retinal Atrophy (iRORA) has replaced the so-called nascent GA (nGA) in the new nomenclature obtained by the Consensus International Group (Classification of Atrophy Meeting)^{66,71–73}. nGA corresponds with a localized loss of tissue in the outer retina layers without definite RPE loss and is defined, on OCT images, as a subsidence of the

outer plexiform layer and the inner nuclear layer with a hypo-reflective wedge ¹¹⁴. iRORA, by definition, is related to the presence of hypertransmission (less than 250 μm), RPE attenuation or disruption, and photoreceptor degeneration. This finding was found in 22% of eyes with iAMD and in 7% of all AMD patients. iRORA is strongly associated with impending GA, an average of 11 months elapsed between early signs of nascent GA and the development of GA ¹¹³. Hyper-transmission defects (the presence of increased transmission of signal below the RPE and into the choroid) and OCT-reflective drusen substructures (variation in the OCT drusen structure and properties) has also been proposed to be associated with an increased risk of AMD progression ⁵⁴. Their real prevalence in patients with iAMD and the associated risk of progression is still unknown. Finally, ellipsoid zone (EZ) disruption and focal RPE disruptions have also been associated with progression to advanced AMD and neovascularization ¹²⁸.

In this study, we followed a cohort of patients with iAMD for 24 months and we explore some markers of pathological retinal ageing that can predict individual progression to advanced stages. Particularly, we investigate and characterize drusen, HRF and other OCT features (presence of iRORA, and EZ status) as biomarkers of disease progression and we calculate a risk prediction model for progression to late AMD, with estimates of sensitivity, specificity, and the receiver operating characteristic (ROC) curve analysis.

1.2. METHODS

Study design

This is a prospective, noninterventional study, with a follow-up of 24 months. Inclusion criteria at baseline were subjects older than 55 years with iAMD, selected from the Retina Clinic of Ophthalmology department of “Centro Hospitalar Universitário de Lisboa Central”, a tertiary hospital in Lisbon, Portugal. iAMD was defined based on Beckman classification^{59,179,198} based on fundus lesions assessed within 2 disc diameters of the fovea. Eyes presenting large drusen (≥ 125 μm) or with pigmentary abnormalities associated with at least medium drusen (≥ 63 and < 125 μm) were considered to have iAMD. Only one eye per patient was considered, even if both eyes were potentially eligible.

Patients were excluded if it was not possible to obtain a good image quality, if the refractive error was more than ± 6 diopters or if there was any evidence of accumulation of extracellular fluid, hemorrhage, exudates, or fibrosis. Additional exclusion criteria included previous retinal surgery, laser treatment, history of glaucoma, signs of diabetic retinopathy, retinal vascular occlusion, hereditary retinal or macular dystrophy and anti-vascular endothelial growth factor treatment in the study eye. In this prospective study, patients underwent a complete ophthalmologic evaluation, including best-corrected visual acuity (BCVA), intraocular pressure (IOP), slit-lamp biomicroscopy and 90D indirect fundoscopy. Color fundus photography (CFP), fundus autofluorescence (FAF), near infrared reflectance (NIR), spectral domain optic coherence tomography (SD-OCT) and OCT angiography (OCT-A) were performed in all patients and monitoring was performed every 6 months, during the follow-up period.

A standardized imaging protocol was performed in all patients. For this cohort analysis, we included acquisition of combined (Figure 13, 14 and 15):

a) NIF images ($\lambda = 815$ nm, emission 700-1400 nm, field of view of $30^\circ \times 30^\circ$, and image resolution of 768 x 768 pixels);

b) SD-OCT ($\lambda = 870$ nm, acquisition speed 40,000 A-scans per second, $20^\circ \times 20^\circ$, dense program, 512 x 49 scans, and spacing of $120\mu\text{m}$). The SD-OCT was acquired in combination with NIR allowing image overlapping.

Starting at the baseline at iAMD stage, patients were reclassified, at the end of the follow-up period, and divided according to their progression: stable or progression to late AMD (complete RORA [cRORA] or nAMD).

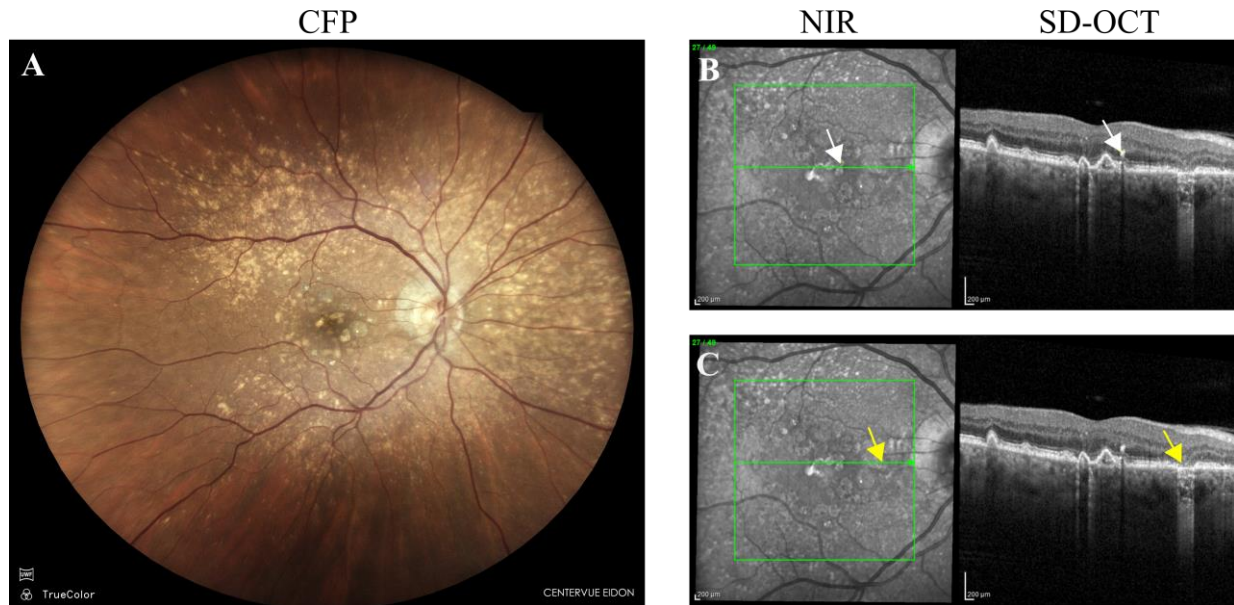


Figure 13 – Representative images of the indicated AMD features.

- (A) CFP, and the corresponding NIR and SD-OCT from an AMD patient in intermediate stage of the disease;
 (B) Example of HRF (white arrows) and
 (C) iRORA with hypertransmission, indicating RPE disruption and photoreceptor degeneration (yellow arrows).

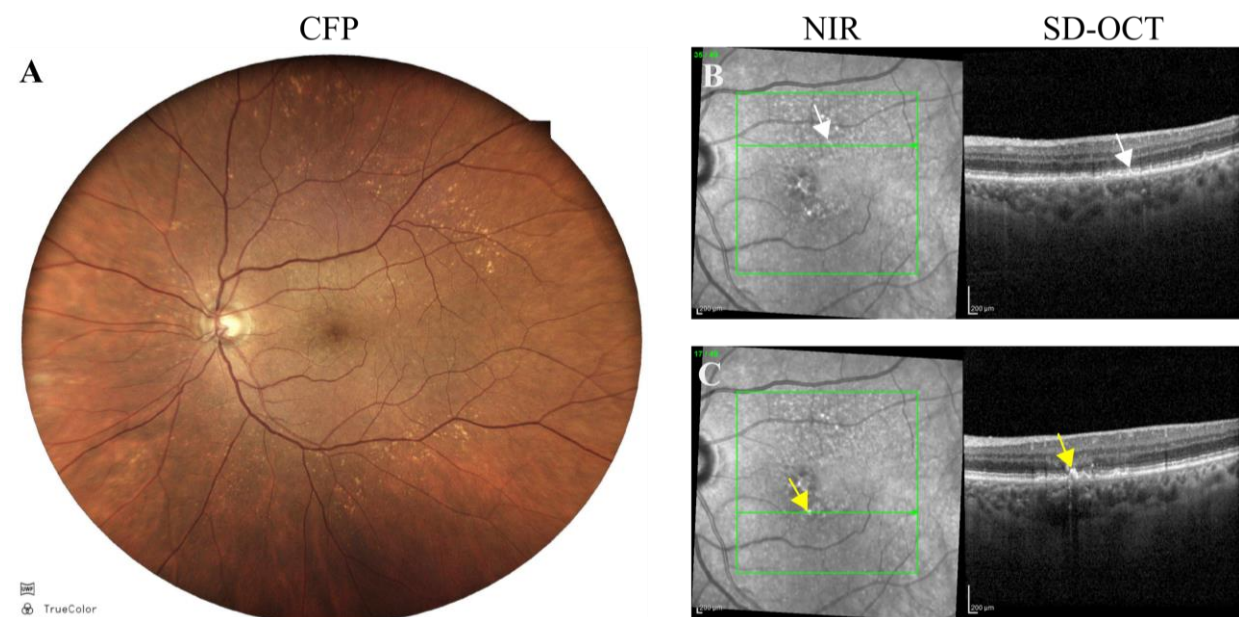


Figure 14 – Representative images of the indicated AMD features.

- (A) CFP, and the corresponding NIR and SD-OCT from an AMD patient in intermediate stage of the disease;
 (B) Example of SDD (white arrows) and
 (C) Hyperreflective drusen and adjacent HRF (yellow arrows).

Ethics issues

Participants who fulfilled the inclusion criteria were invited to participate and gave written informed consent after detailed explanation of the scope of the study. The protocol adhered to the tenets of the Declaration of Helsinki and was approved by the Ethic Commission of the “Centro Hospitalar Universitário de Lisboa Central”.

Image analysis and measurements

For this investigational study, we manually analyzed OCT images (SD-OCT Spectralis, Heidelberg, Germany) at the baseline and at the end of the follow-up period. The SD-OCT was acquired in combination with NIR allowing image overlapping.

Drusen, HRF, and the other predefined OCT features in the outer retinal layers (presence of iRORA and EZ status) were analyzed. Subjective imagiologic features were evaluated and classified by two independent ophthalmologists. In the case of discrepancy, they discussed each dubious case and agreed on the final decision. The graders were masked as to the patient’s clinical data not related with image analysis.

The drusen area was calculated using two perpendicular dimensions (length and height). Length was measured in the drusen base, and height was measured from the apex to the base in a perpendicular manner. Simultaneous scanning laser ophthalmoscopy images and overlaid enface NIR fundus images were used to ensure that the scan went through the center of an individual druse. Only those drusen that had been crossed in their center were included for the analysis. If the cross-section was off-center, those drusen were not included in the analysis. We only considered for analysis the $10^\circ \times 10^\circ$ area centered around the fovea and included in the analysis the drusen with the biggest area and the sub foveal one if present (approximately 500 μm).

Beside the drusen areas, we also studied drusen morphology, internal reflectivity, homogeneity, and the integrity of the EZ) around the selected drusen. Assessment of incomplete RPE and outer retinal atrophy (iRORA) was considered. Concerning HRF, we analyzed their presence in the area

10° x 10° around the fovea, their number, location within the central 500µm and association with drusen. Such metrics were also assessed at the follow-up OCT.

Statistical analyses

Statistical analysis was performed in Python using the application Jupyter Notebooks version 6.4.5 from Anaconda®. For each OCT feature, a comparison between groups (no disease progression/disease progression) was performed by nonparametric Mann-Whitney *U* test.

The association between all variables, including the target variable ('0' [no progression] and '1' [progression to late AMD]), was assessed using Spearman's rank correlation coefficient (ρ). In this way it was possible to assess if any of the SD-OCT features were correlated among each other and with the outcome variable (i.e., disease progression). Among SD-OCT variables highly correlated ($\rho \geq 0.7$), only one of them was selected to be present in the multivariate logistic regression analysis. Additionally, it was applied k-fold cross-validation method (it was defined as $k=5$) to resample our data and therefore evaluate the influence of each categorical variable within different subsets of our dataset. The importance of categorical variables within the dataset to the target variable was evaluated by Chi Squared test of independence, which tests whether two categorical variables are independent. Each independence test used a significance level of 5%.

Statsmodels is a Python module that provides Logit() function for performing logistic regression and statistical tests in the respective summary. A logit function models the probability of an event taking place by having its log-odds ratio as linear combination of one or more independent variables. Adjusted-R², Akaike's information criteria AIC), and Bayesian information criterion (BIC) were used to evaluate the performance between logistic regressions. A multivariate logistic regression with the optimal subset of predictors was created using forward stepwise selection. Forward stepwise starts with a model that has no predictors and then adds predictors to it, one at a time. The variable that provides the greatest additional improvement to the fit was added to the model at each step. Statistical significance was considered for *P* value of less than 0.05.

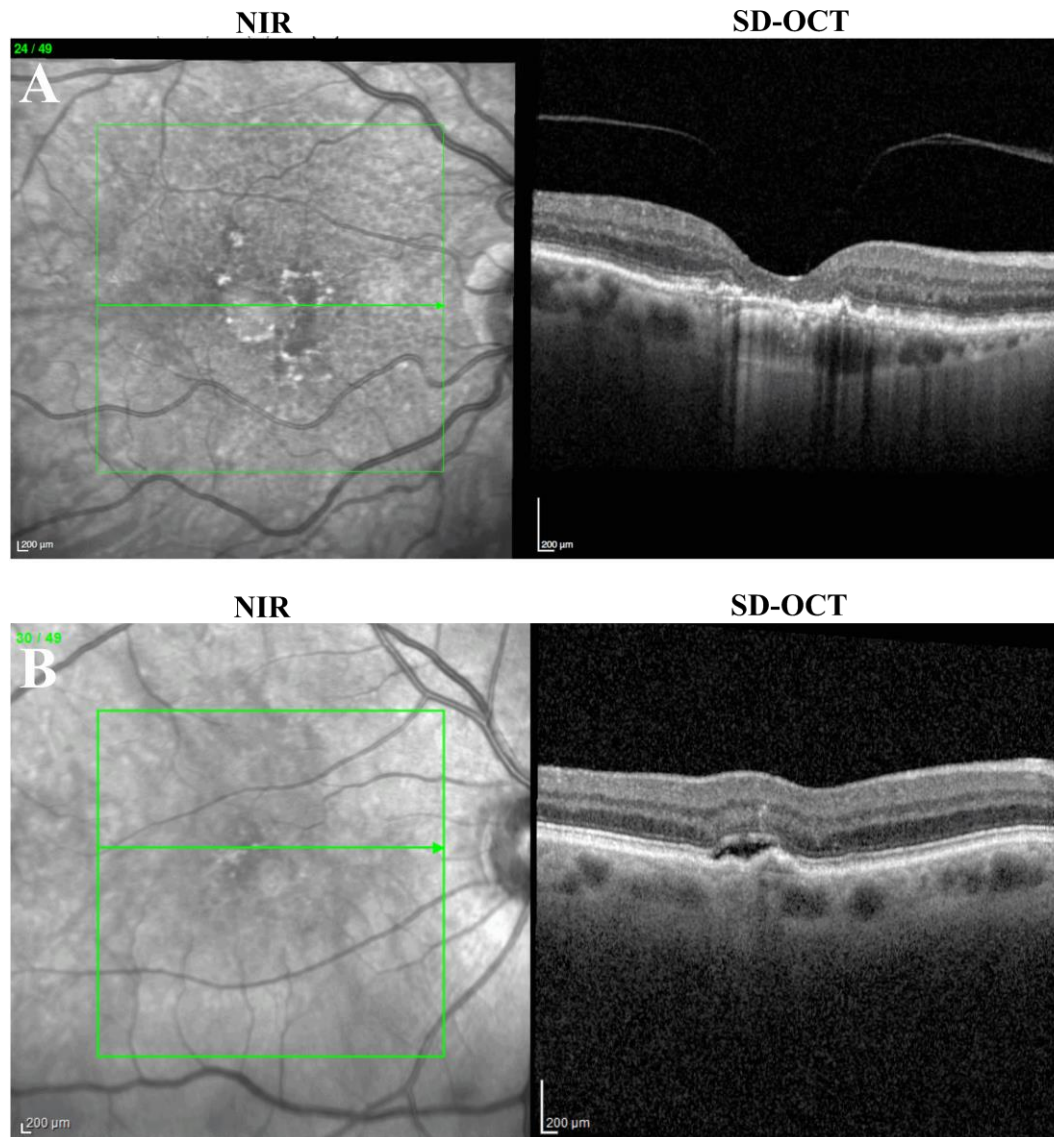


Figure 15 – Representative images of NIR and SD-OCT from patients in advanced or late AMD.

(A) An example of cRORA and

(B) An example of nAMD.

To develop a logistic regression classifier to predict disease progression, a training and a testing set were randomly selected (80% and 20% of the data set, respectively). For the logistic regression classifier, a different approach was considered for selecting the best subset of variables. It used backward feature selection, whose main purpose is to examine the multivariate model and sequentially eliminate predictors that do not significantly impact the final outcome. Beginning with all potential predictors in the multivariate model, backward elimination sequentially keeps or discards variables according to a predetermined significance criterion (in our analysis F1-score was the evaluation measure for model selection). To quantify the performance of the logistic regression

classifier with the selected variable predictors, the training dataset was divided using a stratified five-fold cross-validation, allowing to ensure that each of the five considered folds had the same proportion of observations. Additionally, several other evaluation metrics for the classification model were computed to assess model performance: (i) accuracy, (ii) balanced accuracy, (iii) precision, (iv) recall (or sensitivity), and (v) specificity.

Furthermore, the area under the curve of the ROC was calculated for the testing set never seen by the classifier. Note that, this testing set has the same proportion of patients who progressed versus those who did not progress, as the entire dataset. The optimal cut-off point for late AMD predictions was estimated as the value that warranted the highest of true positive rate (sensitivity), while maintaining a low false positive rate (specificity).

1.3. RESULTS

A total of 135 eyes of 135 patients with iAMD were considered in this study. Patients (55 men and 80 women) ages ranged from 57 to 98 years. Image analysis was performed by two experts and interobserver agreement was substantially favorable (>90%) for SD-OCT measured parameters related with drusen and with HRF; classification of progression was also congruent among both ophthalmologists.

Considering disease progression, 30.4% of the patients ($n = 41$) developed cRORA or nAMD and the remaining 69.6% ($n = 94$) did not progress to late AMD forms. We analysed 238 drusen and classified them considering size, morphology, internal reflectivity, homogeneity, and integrity of EZ around the selected drusen. At baseline, drusen areas ranged from 23481.25 μm^2 to 62010.25 μm^2 (median [Q1-Q3]). Of the patients, 31.9% presented subfoveal drusen (located within central 500 μm) at the baseline. Regarding HRF, they were found in 52.6% at baseline of our cohort and most of those patients presented two to five HRF in the predefined area.

These imaging features were assessed at baseline in the two groups of patients (no progression vs. progression to late AMD). The overall assessment is shown in Table 1.

At the baseline, Mann–Whitney test revealed statistically significant differences in the following features: “iRORA”, “EZ disruption”, “Number of HRF”, “HRF”, “HRF association to drusen”, between

the group of patients that developed late AMD and the group that did not progress to advanced forms of AMD (Figures 16 and 17).

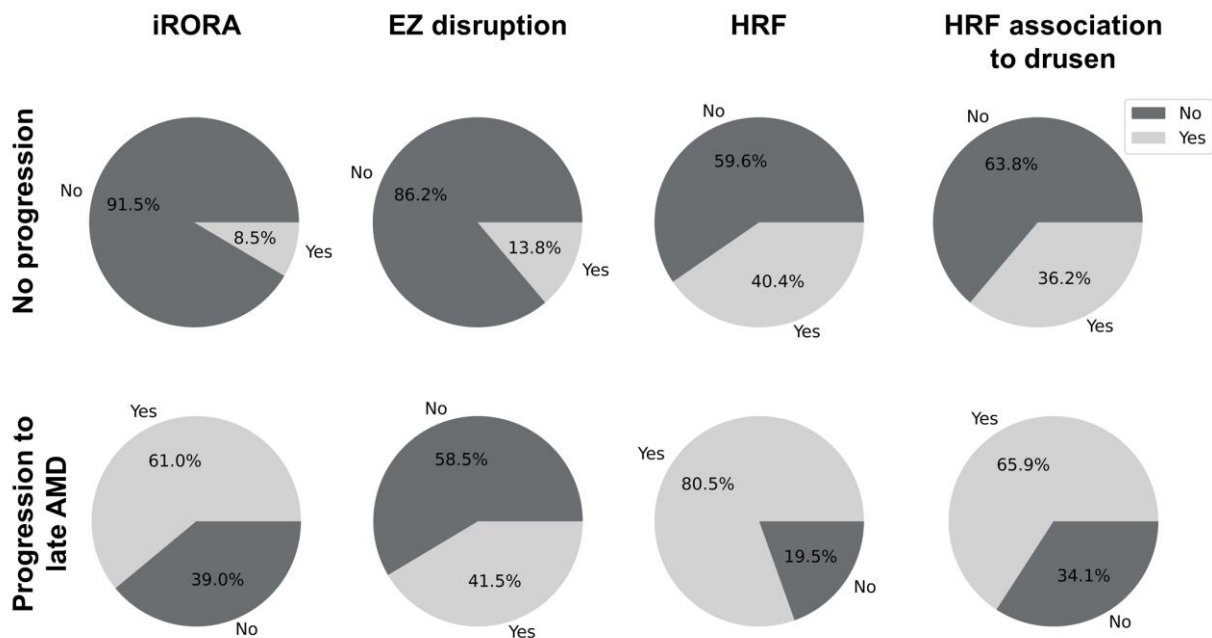


Figure 16 – Pie charts of binary SD-OCT variables.

Represented are the variables which revealed a statistically significant difference between the groups of patients that progressed to late AMD vs. those that remained in intermediate AMD.

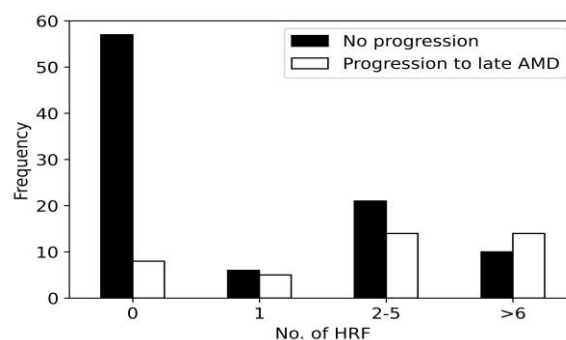


Figure 17 – Bar plot of the HFR.

The graphic shows the number of HRF found at the baseline in groups where disease progression was observed (white) and where AMD remained in the intermediate phase (black).

Table 20 – SD-OCT features at baseline.

Features for the groups of patients where no progression was observed vs. where evolution to late AMD was found Mann-Whitney test, level of significance was set at $p < 0.05$. Significant values are in bold.

SD-OCT features	No Progression	Progression to late AMD	<i>p</i> -value
iRORA	No – 91.5% Yes – 8.5%	No – 61.0% Yes – 39.0%	1×10^{-10}
Drusen morphology	Serous – 59.6% Reticular or both - 40.4%	Serous – 58.5% Reticular or both - 41.5%	0.913
Central drusen (within 0.5mm)	Yes – 42.6% No – 57.4%	Yes – 46.3% No – 53.7%	0.686
Subfoveal drusen area (μm^2)	39559.5 (21027.3 – 100811.5)	26002 (17314 – 53435)	0.166
Other drusen area (μm^2)	35967.5 (23946 – 57165.8)	35853 (27894.3 – 57000.8)	0.516
Drusen reflectivity	Low – 6.4% Intermediate – 69.1% High – 24.5%	Low – 7.3% Intermediate – 75.6% High – 17.1%	0.377
Drusen homogeneity	Homogeneous – 84.0% Heterogeneous – 16.0%	Homogeneous – 75.6% Heterogeneous – 24.4%	0.249
Ellipsoid zone disruption	No – 86.2% Yes – 13.8%	No – 58.5% Yes – 41.5%	0.0004
Hyperreflective foci	No – 59.6% Yes – 40.4%	No – 19.5% Yes – 80.5%	0.00002
Number of hyperreflective foci	0 – 60.6% 1 – 6.4% 2-5 – 22.3% ≥ 6 – 10.6%	0 – 19.5% 1 – 12.2% 2-5 – 34.1% ≥ 6 – 34.1%	0.000007
Hyperreflective foci Location (within 500 μm disc area)	No – 86.2% Yes – 13.8%	No – 82.9% Yes – 17.1%	0.630
Hyperreflective foci association to drusen	No – 63.8% Yes – 36.2%	No – 34.1% Yes – 65.9%	0.002

Filter methods (Spearman correlation and Chi-square test of independence) provided an initial approach to the data to assess the correlation among SD-OCT variables. “HRF”, “Number of HRF” and “HRF association to drusen” were very strongly correlated among each other ($\rho \geq 0.8$). The Chi Squared test showed that categorical variables, “iRORA”, “EZ disruption”, “HRF”, “Number of HRF” and “HRF association to drusen” were important for the prediction model. Additionally, univariate logistic regressions were performed for each SD-OCT feature to determine which candidate predictors significantly correlate with the outcome. The SD-OCT features whose coefficients showed statistical significance were “iRORA”, “EZ disruption”, “HRF”, “Number of HRF” and “HRF association to drusen” ($p < 0.05$) in agreement with Table 1.

The 12 models created by forward stepwise selection can be seen in Supplementary Table 3. The best models were composed by 3 (“iRORA”, “EZ disruption”, “Drusen area”) using AIC criteria (Figure 18A) or 2 (“iRORA”, “EZ disruption”) predictor variables using BIC criteria (Figure 18B), respectively. However, the model with 4 predictor variables (“iRORA”, “EZ disruption”, “Drusen area”, “HRF”) evidenced the highest adjusted-R² (0.3558) (Figure 18C), i.e., this set of predictors was the best one to explain the variation in the outcome variable (disease progression), without considerably increasing AIC and BIC, as observed in Figure 18.

A multivariate logistic regression with the aforementioned SD-OCT variables was created to ascertain the effects of each variable (while holding the remaining variables at a fixed value) on the likelihood that patients would remain in iAMD or progress to late AMD forms (Table 2). The fitted model indicated that the odds of developing late AMD for patients who have iRORA over the odds of patients that do not exhibit atrophy is 12.91 ($P = 0.000$). The odds for patients with iRORA constitute approximately a 12-fold increase in comparison to the odds for patients who do not have it. Similarly, the odds of evolving to advanced AMD is 3.54 for patients whose EZ is disrupted versus those whose EZ integrity is maintained, indicating a 2.5-fold increase in developing late AMD for patients who have a disruption in the EZ ($P = 0.018$).

Table 21 – Results of logistic regression analysis. Significant values are in bold.

SD-OCT variables	b_r (estimator coefficients)	p -value	Odds ratio
iRORA	2.5576	0.00002	12.905
EZ disruption	1.2627	0.018	3.535
Drusen area	1.5137	0.216	4.544
HRF	0.6295	0.243	1.878

Concerning the development of a logistic regression classifier, through backward feature selection, the subset with the highest F1-score was composed by 6 variables, i.e., “Drusen morphology”, “iRORA”, “Drusen area”, “EZ disruption” “HRF” and “HRF in central location”, (supplementary Figure 1). The F1-score of the designated model with the 5-fold cross validation was 0.707+/-0.02 in the training set and 0.731+/-0.08 in the validation set. On the testing set, the F1-score was 0.67.

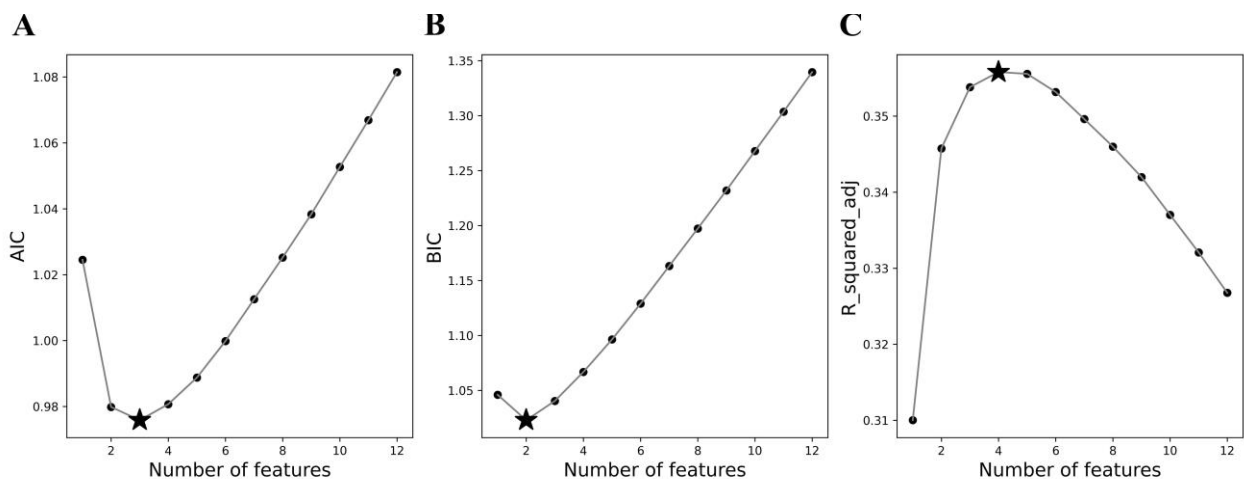


Figure 18 – The logistic regression models created by forward stepwise selection.

Models were evaluated with

(A) AIC (Akaike's Information Criteria),

(B) BIC (Bayesian Information Criterion) and

(C) adjusted-R². The best model according to each criterion is marked with a star.

The evaluation metrics averages for the training and validation datasets and the exact values for the testing subset is presented in the Supplementary Table 4. The area under the ROC curve calculated for the testing set was 0.77 (95% CI 0.56–0.98) (Figure 19). The optimal cut-off value was 0.68 (sensitivity: 0.75, specificity: 0.79), i.e. if the predicted probability of developing late AMD is greater than 0.68, that observation is classified as ‘1’, i.e., as a patient whose iAMD will evolve to late AMD.

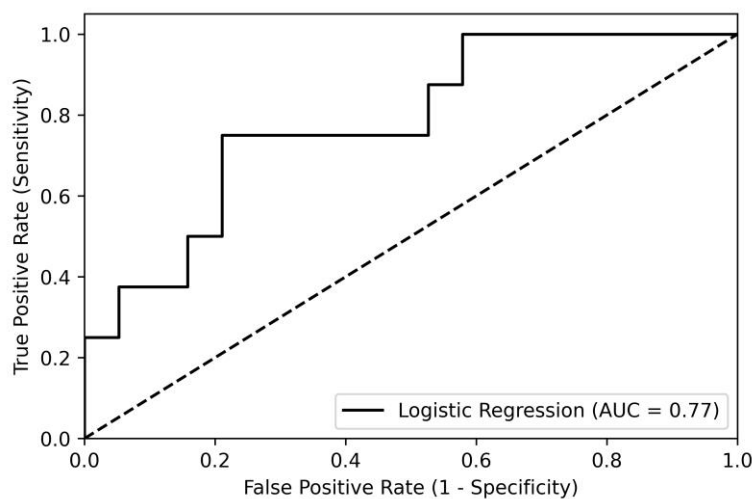


Figure 19 – ROC curve for disease prediction.

The model performance using “Drusen morphology”, “iRORA”, “Drusen area”, “EZ disruption” “HRF” and “HRF in central location”, parameters. ROC curve (solid line) was plotted using the testing dataset and the area under the curve was calculated: AUC=0.77). The dashed diagonal line serves as an imaginary reference line representing a non-discriminatory test.

1.4. DISCUSSION

Here we present a practical and useful clinical model to help clinicians in their daily practice in predicting individual progression to advanced AMD. The main conclusion of our study is that some key imaging parameters are able to predict AMD progression.

In this study, 30.4% of our patients progressed to advanced disease, a percentage higher than expected considering similar studies¹⁹⁹. One possible reason for this finding is the patients’ mean age of our cohort: 80.23 years old (YO), median 81 YO, SD 8.17. It is a particularly elderly population

with a known increased risk of progression. Another important consideration is the percentage of patients with advanced disease in the fellow eye, in our study at the baseline (25.9%). Previous studies have also documented that the presence of advanced AMD in the fellow eye at baseline increased the rates of progression to advanced AMD in study eyes ¹⁴³. Finally, all our intermediate AMD patients are under AREDS vitamin supplementation, but we noticed that it was not the rule before the reference to our center.

In this study, the presence of iRORA and the disruption of the EZ were SD-OCT features that emerged as having a significant association in predicting disease progression over a period of two years, even in the multivariate analysis. The individual predictor, iRORA, was found to account for approximately 31% of the variability observed in the dependent variable (disease progression). This finding suggested that iRORA was the most influential predictor variable. The addition of three more variables, specifically the EZ disruption variable, contributed to a 5% increase in the overall explanatory power of the model, which led to more accurate predictions. The addition of more variables should be considered when they are not highly correlated with the already existing variables in the model, to avoid the risk of multicollinearity and the consequent misleading interpretation of beta estimators. However, if they increase the explanatory power of the model, their addition should be considered. Still, it was important to assess the trade-off between model complexity (i.e., many variables) and predictive power of the model when selecting whether to incorporate more variables or not. Having this balance in mind, the model that reflected the best trade-off was the one shown for the multivariate analysis. In this model, the odds of patients presenting with iRORA developing late AMD were 12-fold higher than the odds for those who did not. Additionally, the odds for patients with EZ disrupted were 254% higher than the odds for those whose EZ integrity was maintained (2.5-fold higher).

These results are understandable if we report to features required to define iRORA definition, which include choroidal hypertransmission, disruption of the RPE, and degeneration of photoreceptors. One of the signs of photoreceptors degeneration is, really, EZ disruption but others can be considered in iRORA definition as external limiting membrane disruption, outer nuclear layer disruption, and subsidence of the outer plexiform layer and the inner nuclear layer with a hypo-reflective wedge.

Furthermore, the presence of HRF, and their number or association to drusen, showed statistically significant correlation with disease progression. Owing to multicollinearity issues, only the presence of HRF was selected to be present in the multivariate logistic regression. Interestingly, when iRORA, EZ disruption and drusen are were held constant, the presence of HRF no longer predicted the outcome significantly. One may hypothesize that the presence of HRF were only predictive because of its associations with other predictors or because the other parameters are more significantly related with disease progression.

The area under the ROC curve calculated for the testing set in our study was 0.77 (95% confidence interval, 0.56 - 0.98), which is considered a good discrimination for predicting disease progression. The inclusion and analysis of other imaging parameters, such as vascular parameters obtained by OCT-A, in a multimodal approach could possibly increase the performance of the model.

We also analyzed whether the study could predict progression towards subtypes of advanced AMD (cRORA or nAMD). The multiclass logistic regression model correctly identified four of five patients whose disease had progressed to cRORA but failed to correctly identify (0/3) nAMD patients in a randomly selected testing set. One may speculate that the SD-OCT variables collected in this study are more related with atrophic features, and, hence the correct prediction of cRORA development, compared with the relative weakness in predicting strictly neovascular progression. Therefore, the addition of vascular parameters evaluated by OCT-A (nonexudative macular neovascularization) may not only increase the robustness of the prediction model, but also predict development of nAMD correctly.

A recent study provided support to the hypothesis that iRORA could be considered a common risk factor in eyes with intermediate AMD patients for development of atrophic late disease²⁰⁰. We still need more data to understand its definite value, as a risk factor for predicting AMD progression from intermediate to advanced atrophic stage, particularly when considered alongside other well-known fundoscopic risk factors, such as large drusen and pigmentary abnormalities.

The MACUSTAR clinical study is a low interventional clinical multicentric study that was designed to develop and evaluate functional, structural, and patient-reported outcome measures, that will improve our understanding of natural history and prognostic markers of iAMD progression, with

aims to be used in future clinical trials. It included a longitudinal and cross-sectional arm, beginning in September 2017, with the end date programed in February 2024^{134,201,202}. One other important study, the PINNACLE, has been designed as a large-scale retrospective and prospective study, noninterventional and multicenter, and is still running in multiple sites in United Kingdom, Austria, and Switzerland²⁰³. Its main goal is the identification of prediction biomarkers and to report the natural history of progression of iAMD with multimodal retinal imaging. This study also combines both machine learning and retrospective and prospective AMD patient data²⁰³.

Our study shares similarities with MACUSTAR and PINNACLE concerning primary and secondary outcomes, although it is mainly prospective, with a smaller sample and shorter follow-up, and image analyses were not obtained by automatic procedures such as deep or machine learning models. It also does not include functional or patient-reported outcomes data. It will be interesting, in the near future, to compare our results with larger and longer studies, as those obtained by MACUSTAR and PINNACLE^{134,201–203}.

This study has some considerable limitations: those related with follow-up period and sample size are important issues, particularly in such a chronic and prevalent disease as AMD. Another important weakness was the inclusion of SD-OCT images, excluding the potential value of swept source OCT technology. The exclusive manual analysis used in this study is also an important limitation and automatic image analysis methods, as those obtained by artificial intelligence methods, should add interesting data and a tool to be considered in the near future.

Future work includes the need to extend the sample and the follow-up period as well as considering the analysis of more image parameters in a multimodal approach for this cohort. Additionally, the human observation used here may soon be substituted by artificial intelligence methods. Deep learning models generally achieve high performances comparable with that of individual specialists^{204,205}. Those models reveal significant potential to improve the quality and accessibility of ophthalmic care but they use parameters that sometimes are difficult to apply in clinical practice and need further external validation to be performed in the near future.⁴⁶

In summary, we believe the results of this study are robust enough to allow us to propose a practical and useful clinical model to guide clinicians in daily practice, using simple tomographic parameters. The present study suggests that the combination of iRORA and EZ disruption constitute

a high risk of progression to cRORA within two years. We aim to continue to develop a scoring system that may facilitate general ophthalmologists in the identification of patients with iAMD with a high risk of progressing to advanced stage of disease. Quantifying markers of pathological retinal ageing predictive of individual progression to advanced AMD is an appealing goal toward personalized risk assessment in AMD.

CHAPTER VI. IDENTIFICATION OF DISEASE PROGRESSION BIOMARKERS

2. NONEXSUDATIVE MACULAR NEOVASCULARIZATION

Other important considerations regarding disease progression biomarkers are those obtained by longitudinal OCT-A information. We conducted an additional study, reporting OCT-A data in our intermediate AMD cohort, with the particular aim of evaluating the natural history of intermediate AMD patients with documented nonexudative macular neovascularization (NE-MNV). This work was presented as an oral communication at 65th Congress of Portuguese Society of Ophthalmology, 2022.

2.1. INTRODUCTION

Patients with phenotypically intermediate AMD subtyped as nonexudative (subclinical or quiescent) neovascular AMD or nonexudative macular neovascularization (NE-MNV) have an increased risk of conversion to exudative disease.

Detection of nonexudative macular neovascularization is suspected when we found some OCT signs. Double layer sign (low lying irregular RPE detachment with reflective material in sub-RPE space, which is above the highly reflective layer identified as BM) firstly described and more recently the SIRE (Shallow/Irregular/RPE/Elevation) sign, related to RPE elevations with a greatest transverse linear dimension of 1000 μm or more and height of less than 100 μm ^{206–208}.

FA and ICGA enable detection of nonexudative neovascularization. OCT angiography (OCT-A) is a newer technology that permits rapid, noninvasive visualization of retinal vasculature and is expected to become a mainstay in the diagnosis and monitoring of patients with this kind of neovascularization ^{206,207,209}.

Nonexudative macular neovascularization does not cause significant visual loss but it is a significant predictor of development of exudative AMD. There is a great variability in the incidence of exudation among studies, depending of the follow-up period: 22.2% in 6 months ²⁰⁸(148), 24% in 12 months ¹²⁰, 20% in 15 months ²⁰⁶, 34.5% in 24 months ¹¹⁹ and 80% in 24 months ²⁰⁷.

Early detection of MNV before leakage occurs should allow for improved monitoring of these at-risk patients⁶⁸. Anti-VEGF treatment is not recommended until signs of exudative activity occur⁶⁸.

The presence of subclinical or quiescent neovascularization in the context of drusenoid elevation is a diagnostic challenge. Growth and regression of drusen are influenced by opposing forces of material deposition and macrophage activity^{68,69,209}. Regressing drusen, evident as multilaminar sub-RPE hyper-reflectivity, are difficult to distinguish from subclinical or quiescent neovascularization⁶⁸ (61). Vascularized tissue also can occur in the context of drusenoid deposits, and its identification is not straight-forward with OCT alone⁶⁸. Quiescent neovascularization grows in length (horizontally), and vascularized drusen enlarge in height (vertically and in a multilaminar pattern)⁶⁸. This represents another instance in which multimodal evaluation and natural history data can improve the accuracy of differential diagnosis. Neovascularization also can be considered inactive, meaning that exudative activity has ceased (no fluid on OCT) but had previously occurred and was managed with anti-VEGF treatment⁶⁸. This setting differs from naïve nonexudative neovascularization (subclinical or quiescent nAMD) and is evident on OCT-A as a skeletonized net^{68,69,209}.

Inclusion of OCT-A imaging at baseline and at follow-up should be considered for cases of intermediate AMD – if not always in daily practice, at least in trials enrolling patients with nonexudative AMD^{68,69,209}.

2.2. MATERIAL AND METHODS

We retrospectively evaluated eyes with nonexudative MNV documented by OCT-A, in our iAMD cohort with a follow-up ≥ 6 months (retrospective analysis of data from a longitudinal study). Demographic and clinical data, BCVA (ETDRS letters), dilated fundus biomicroscopy description, OCT and OCT-A data were recorded. All patients were treatment naïve.

All OCT analysis were performed by two investigators and disagreements were settled by the senior investigator (Table 22). 17 eyes of 17 patients with NE-MNV were included and further analysed. Progression during the follow-up was studied.

Table 22 – OCT and OCT-A baseline characteristics of NE-MNV eyes.

Baseline imaging characteristics	%
OCT double layer sign	88.2%
OCT SIRE	73.5%
SDD	35.3%
Foveal NE- MVC (OCT-A)	64.7%

We only considered SIRE when RPE elevation was greater than 1000 μm (transverse linear dimension) and height of less than 100 μm . Concerning double layer signs, the average measures of RPE elevation were: mean width 1460.80 μm (SD 840,23) and mean height 118,80 μm (SD 62,80). Considering SIRE sign the average measures were 1325.00 μm (SD 559.66) and mean height 84,25 μm (SD 11,62).

2.3. RESULTS

Membrane growth in OCT-A was evident in 52.9% of the cases. Progression from NE-MNV to exudative was recorded in 23.5% of the cases during the follow-up period.

Average progression occurred 24.18 months after the diagnosis of NE-MNV. 75% of progression cases occurred in patients that presented exudative AMD in the fellow eye. 100% of progression cases occurred in foveal location membranes. Patients that progressed to exudative AMD were younger than those whose NE-MNV remained stable (60.50 vs 80.77 years; $p=0.017$).

Concerning BCVA variability, eyes with NE-MNV that did not progress to exudative MNV lost 0.7 ETDRS letters at the end of the follow-up period. In those eyes that progressed with exudative MNV, we recorded lost of 3.0 ETDRS letters when exudation was detected and lost of 1.8 letters at the end of the follow-up period.

2.4. CONCLUSION

The average NE-MNV AMD patient in our study had an asymptomatic eye with intermediate AMD and an exudative AMD in the fellow.

Concerning NE-MNV diagnosis we concluded that BCVA was not a useful tool for the screening of NE-MNV, OCT signs, such as double layer sign and SIRE, were highly prevalent and their recognition should prompt the need for an OCT-A.

Progression to exudative AMD was common, mostly in younger patients, in eyes with foveal NE membranes, frequently presenting exudative AMD in the fellow eye.

CHAPTER VII. DISCUSSION AND FUTURE PERSPECTIVES

CHAPTER VII. DISCUSSION AND FUTURE PERSPECTIVES

1. GENERAL DISCUSSION AND CONCLUDING REMARKS

1.1. MAJOR FINDINGS AND CONCLUSIONS

The present thesis aimed to evaluate the implication of FAF findings and chorioretinal vascular factors in AMD pathophysiology and to explore their correlation in intermediate AMD progression. We further progress looking for other image biomarkers of disease progression. To accomplish the aimed objectives, we developed work in three lines of clinical investigation and its major findings were:

A. FUNDUS AUTOFLUORESCENCE FINDINGS

The first purpose was to explore the relationship between drusen morphology and fundus autofluorescence findings. Additional structural analyses on SD-OCT, such as integrity of the ELM, EZ, and RPE-BM as well as the presence of CH were also considered.

We found a statistically significant correlation between drusen AF and homogeneity, HRF, ELM integrity, EZ integrity, and CH. Of those, the more significant correlations were found with HRF ($r = 0.4$), ELM integrity ($r = 0.3$), and EZ integrity ($r = 0.3$), although all correlations were moderate, and no strong correlations were found with any parameter.

One important conclusion from FAF study is that drusen morphological characteristics and autofluorescent findings are correlated but other factors or cofactors may be involved. As RPE cells are the main source of lipofuscin and short wavelength (blue light-488 nm) emitting fluorophores, FAF changes can represent the status of RPE cells in precise relationship with a particular drusen instead of the drusen itself; melanin pigment can also play a role, namely hyperpigmentation or hypopigmentation of the adjacent area. So, when we discuss drusen AF we mean FAF findings in the drusen neighborhood.

The findings in outer retinal layers (ELM, EZ, and RPE-BM) and autofluorescent changes were similar but did not perfectly overlap. They represent different stages of the atrophic process and

reflect the fact that photoreceptor atrophy can occur without RPE atrophy. This knowledge is well documented in the recent Classification of Atrophy Consensus group (cRORA and iRORA criteria) ⁶⁶.

Another interesting consideration is the relevance of other factors aside from the RPE in AF findings. Drusen homogeneity and HRF seem to have statistically significant importance in the autofluorescence features of the studied drusen and this relationship is not directly dependent on the RPE status.

We progress further, performing a prospective study with the aim to correlate different patterns of drusen autofluorescence in FAF with progression from intermediate to late AMD.

In this prospective study, we found a statistically significant difference in the rate of progression to advanced AMD between patients with hypo and/or hyperautofluorescent drusen and patients with only isoautofluorescent drusen (48.5% versus 13.3%, respectively; $p=0.02$). Those differences in autofluorescence may reflect different stages in drusen evolution as hypo or hyperautofluorescence are positively associated with progression to advanced disease. The presence of hypo and/or hyperautofluorescent drusen on FAF was positively associated with progression to advanced disease and GA in particular. Moreover, progression to GA alone was significantly superior in patients with at least one hypo or hyperfluorescent drusen ($p=0.04$).

B - CHORIORETINAL VASCULAR FINDINGS

In this study, the vascular aspects of the chorioretinal complex of iAMD patients (choroidal vascular layer, superficial and deep capillary plexus) were evaluated and compared with a control group.

One main conclusion of this study was that total subfoveal choroidal area, luminal area and choriocapillaris flow density were reduced in iAMD, compared with age matched healthy controls, supporting the concept that the choroidal vascular network is impaired in AMD patients. No significant differences in retinal circulation were found in iAMD patients as compared with age-matched healthy controls. Interestingly, choriocapillaris layer had significantly lower density in AMD patients compared with controls.

Concerning choroidal and retinal vascular impairment in AMD, there is a general reduction in

choriocapillaris density in iAMD patients, compared with age matched normal controls, with some focal areas of choriocapillaris loss or flow impairment. Those areas appear as dark patches at choriocapillaris level and we consider that displacement of the larger choroidal vessels can also occur into the space previously occupied by the choriocapillaris. These results are in line with histopathological studies demonstrating that areas with increased drusen density seem to correspond to decreased vascular density in choriocapillaris ¹⁸⁶.

Drusen and RPE changes frequently cause attenuation shadowing in SD-OCT technology that can simulate lack of blood flow. These areas of shadowing can be distinguished from areas of decreased perfusion by simultaneously observing enface OCT-A image and cross-sectional OCT intensity image: areas of shadowing will appear dark in both OCT intensity image and angiographic image, whereas areas of decreased flow will appear normal in OCT intensity image but dark in en face OCT-A image.

In our study no significant differences in retinal circulation (superficial and deep capillary plexus) were found in intermediate AMD patients as compared with age-matched healthy controls. Our findings should be distinguished from other studies that found reduced vascular density in the superficial capillary plexus but a nonsignificant reduction in the deep capillary plexus in iAMD patients compared with normal eyes¹⁸⁸. In another previous study, authors used OCT-A to evaluate the superficial and deep vessel density in AMD patients. They found a statistically significant difference in both superficial and deep capillary plexus between controls and dry AMD patients, although no significant differences between early AMD and intermediate AMD patients ^{186,188}.

We further progress with a logistic regression model for AMD progression using those choroidal and retinal vascular parameters after stepwise selection. We did not identify any statistically significant association between the studied variables and AMD progression.

Although choroidal vasculature is impaired in intermediate AMD patients, vascular changes of the choroid and retina did not appear to be related to disease progression to late AMD, at least with the used methodology and during the considered follow-up period. We considered the hypothesis that our results could be enhanced with a longer -up period with SS-OCT imaging.

C - IDENTIFICATION OF OTHER DISEASE PROGRESSION BIOMARKERS

Drusen, HRF and the other predefined OCT features in the outer retinal layers (presence of incomplete retinal pigment epithelial and outer retinal atrophy – iRORA - and ellipsoid zone status) were evaluated and classified by two independent ophthalmologists.

A total of 135 eyes of 135 patients were enrolled in the study, 30.4% (41 patients) developed cRORA or nAMD and the remaining 69.6% (94 patients) did not progress to late AMD forms.

A rate of 30.4% of progression to advanced disease during the 2 years follow-up is a percentage higher than expected, considering similar studies¹⁹⁹. One possible reason for this finding is the fact that the patients' mean age is 80.23 years old (median 81, SD 8.17). It's a particularly elderly population with known increased risk of progression. Another important consideration is the considerable percentage of patients with advanced disease in the fellow eye, at the baseline, in our cohort (25.9%). Previous studies have also documented that the presence of advanced AMD in the fellow eye at baseline increased the rates of progression to advanced AMD in study eyes¹⁴³.

The main results of our study are related with the possibility of predicting AMD progression using parameters such as presence of iRORA, disruption of ellipsoid zone and presence of HRF. The other studied tomographic variables didn't show relevant significance in predicting progression to late disease. The combination of iRORA and EZ disruption constitutes a high risk of progression to cRORA within two years.

Odds ratio of each significant variable, while holding the remaining variables at a fixed value, were calculated. In terms of percent change, the odds for patients with iRORA are 1191% higher than the odds for patients that do not have it (OR 12.91, $p=0.000$). Similarly, the odds of evolving to advanced AMD forms for patients whose EZ is disrupted versus those whose EZ integrity is preserved is 3.54, indicating an increase of 254% in developing late AMD for patients who have a disruption in the EZ ($p=0.018$).

Concerning the identification of other biomarkers of AMD progression, we concluded that, in our longitudinal study, the presence of iRORA and disruption of EZ were the SD-OCT features that emerged as having a significant association in predicting disease progression over a period of two years. The odds of the patients presenting with iRORA developing late AMD were 12-folds higher

than the odds for those who did not. Additionally, the odds for patients with EZ disruption were 2.5-folds higher than odds when EZ was maintained.

Futhermore, the presence of HRF, their number or association to drusen, showed statistically correlation with disease progression. In multivariate logistic regression the selected variable (presence of HRF) no longer predicted the outcome significantly.

NE-MNV, suspected by OCT and diagnosed by OCT-A, was also revealed as an important image biomarker of AMD progression. Concerning prevalent or incident NE-MNV was recorded in 11.3% (17 from 150 iAMD eyes) of our iAMD cohort and 23.5% (4 eyes) progressed to exudative AMD stage during the follow-up period. Membrane growth in OCT-A was evident in 52.9% of the cases. Average progression occurred 24.18 months after the diagnosis of NE-MNV. Three quarters of cases progression occurred in patients that presented exudative AMD in the fellow eye and 100% of those cases occurred in foveal membranes. Patients that progressed to exudative MNV were younger than those whose NE-MNV remained stable (60.50 vs 80.77 YO). BCVA was not a useful tool for the screening of NE-MNV, OCT signs, such as double layer sign and SIRE, were highly prevalent and their recognition should reinforce the need for OCT-A imaging.

Those patients should not be treated but carefull monitoring is advised and prompt treatment is recommended when exudation occurs.

1.2. INTEGRATING OUR RESULTS WITH LITERATURE DATA

The slow progression of AMD, from early to advanced disease, requires the use of surrogate end points in clinical trials. Currently, no outcome measures are clinically validated and accepted as clinical endpoints by regulatory agencies for drug development in iAMD.

This lack of clinical endpoints capable of determining the effectiveness of potential treatments under development is a real limitation. The possible end points categories could be functional, structural or a combination of both and MACUSTAR Consortium, a public-private research group funded by the European Innovative Medicines Initiative, intends to close or reduce this knowledge gap¹³⁴.

The MACUSTAR clinical study is a low interventional clinical multicentric study that was designed to develop and evaluate functional, structural, and patient-reported outcome measures. It includes

a cross-sectional and a longitudinal arm, beginning in September 2017, with the end scheduled for February 2024 (more than 6 years of follow-up). The participants are recruited at 20 clinical sites from seven European countries (Denmark, France, Germany, Italy, Netherlands, Portugal and UK). Objectives are intended to characterize the functional deficit of iAMD and to develop and validate functional, structural and patient reported outcome measures for iAMD. In addition, risk factors for progression from iAMD to late AMD will also be investigated.

It is supported with considerable funding and grant agreement by the Innovative Medicines Initiative, a partnership between the European Union and the European pharmaceutical industry.

In the cross sectional arm, a total of 300 people were examined (50 with early AMD, 150 with iAMD and 50 with late AMD, plus 50 healthy controls with comparable ages to AMD patients). Longitudinal study is following more than 700 patients to assess the progression of AMD to late stage of the disease.

In our study we focus the issue on structural parameters of an iAMD cohort, selected among the Retina Clinic Unit of Ophthalmology department of “Centro Hospitalar Universitário de Lisboa Central”, a tertiary hospital in Lisboa, Portugal.

We conducted a prospective, non-interventional study, with a follow-up of 24 months, performing cross sectional and longitudinal data analysis.

We used the event of progression to advanced AMD as a clinical end point because it is the one that is currently accepted by regulatory authorities and health technology assessment bodies ²¹⁰. We consider that knowledge of the prevalence and distribution of structural iAMD biomarkers is crucial to identify reliable outcome measures for disease progression and included longitudinal analyses because those data are needed to evaluate prognostic value and risk for conversion to advanced disease stages, thereby avoiding many of the pitfalls of the retrospective analysis we have seen over the years.

It was designed with 150 iAMD eyes of 150 patients and 50 age matched controls.

Our study had limited resources and reduced funding, mainly centered in standard ophthalmology evaluation, usually performed in clinical practice. No other examination was considered in our study protocol than routine functional and structural evaluation, with the aim to help clinicians in predicting individual progression in their daily setting.

In our study we found that 30.4% of our iAMD patients progressed to advanced disease, a

percentage higher than expected from similar studies ¹⁹⁹. Possible reasons for this finding is the high patients' mean age of our cohort (80.23 YO, median 81 YO, SD 8.17) and the considerable percentage of patients with advanced disease in the fellow eye at the baseline (25.9%). Those facts possible account for the higher rate of progression to advanced AMD compared with the results of previous studies ¹⁴³. Finally, even our iAMD patients are under AREDS vitamin supplementation that was not the rule before the reference to our center.

Some results, already released by MACUSTAR study, have reported that besides large drusen and pigmentary abnormalities, the most prevalent structural feature in iAMD study eyes were HRF (51.8%). Quiescent neovascularization (nonexudative MNV with stability beyond six months) were observed in 2.4% of the sample and direct precursor lesions for manifest retinal atrophy were detected in 15.9%, being 10.7% (iRORA) and 4.2% (cRORA) ²⁰².

In our study drusen areas at the baseline ranged from 0.0235 mm² to 0.0620 mm² (median) and 31.9% of the patients presented subfoveal drusen (located within central 500 µm) at the baseline. Regarding hyperreflective foci (HRF), they were found in 52.6% at baseline and most of those patients presented 2 to 5 HRF in the predefined area. Precursor atrophic lesions were found at the baseline in 19.5%, a slightly higher percentage than reported in Macustar study. Nonexudative MNV were observed in 11.3% (17 from 150 iAMD eyes) of our iAMD cohort and 23.5% (4 eyes) progressed to exudative AMD stage during the follow-up period.

Concerning ellipsoid zone disruption, MACUSTAR reported that quantification of the relative ellipsoid zone reflectivity might be a structural surrogate parameter for an early disease progression in the context of AMD. Correlation studies have analyzed the association of AMD stage and AMD associated high-risk features including presence of pigmentary abnormalities, SDD, volume of RPEDC with the relative EZ reflectivity. Relative EZ reflectivity decreased significantly in the intermediate and even further in the late AMD group. This study showed an association of relative EZ reflectivity with increasing disease severity and the presence of iAMD high-risk features. Further studies are necessary to evaluate this parameter as a novel biomarker for AMD and disease progression ²¹¹.

Ellipsoid zone disruption, evaluated in our cohort, may have equivalent meaning compared to this novel parameter (relative ellipsoid zone reflectivity). Indeed, we found that as individual

predictor, iRORA, was able to account for approximately 31% of the variability observed in the dependent variable (disease progression). The addition of 3 more variables, specifically the EZ disruption variable, contributed to a 5% increase in the overall explanatory power of the model, which led to even more accurate predictions. Odds were calculated: presence of iRORA represented a risk 12 folds higher and EZ disruption a risk 2.5 fold higher in disease progression.

Although some important differences reported between MACUSTAR and our study, it will be interesting to be able to validate some of our results with near future. Comparing results must take into account discrepancies related with sample dimension, study design, used methodology and follow-up period.

During the last years inumerous retrospective and longitudinal studies have been conducted with the same objective: identification of reliable outcome measures for iAMD progression. One recent retrospective published study had the purpose to determine the frequency of multiple OCT biomarkers of iAMD and to explore their relationship with the development of cRORA after 2 year follow-up. At month 24, 16.36% of iAMD eyes developed cRORA. In this study, beside the known and previously documented image biomarkers (HRF, SDD, ODS, HTD) OCT double layer sign (OR 4.517, $p=0.006$) and cRORA in the fellow eye (OR 7.184, $p=0.003$) were associated with progression to late atrophyc disease.²¹²

One other important and also recent longitudinal study examined the association between iRORA on OCT imaging and the subsequent risk of developing GA (defined by convencional CFP) in an iAMD cohort of patients with large bilateral drusen²⁰⁰. Retrospective analysis of data from that longitudinal study recorded a total of 58 eyes (21%) with iRORA at the baseline, and a further 87 eyes (31%) with iRORA developing during the follow-up period of 36 months. Time-to-event analysis demonstrated that prevalent or incident iRORA was associated with an increase rate of GA development. In this study, the presence and development of iRORA were associated with a 14-fold increased risk of developing GA when compared with eyes without iRORA, a risk quite similar from the obtained in our study. Even so, the end point evaluated in both studies is not perfectly the same (GA vs cRORA) and we recognize that cRORA seen on OCT imaging is likely to be detected earlier than GA seen on CFP. This fact associated by the baseline patients characteristics (bilateral large drusen vs Beckman intermediate AMD definition) certainly explain iRORA odds ratio differences

obtained in both studies.

Wu et al. considered nGA at the baseline as an exclusion criteria in their study, but its development during the follow-up period was accepted and proved to be a considerable risk factor for progression (even stronger than iRORA). In our study we only considered iRORA evaluation (not differentiated from nGA) but it is worth noting to clarify that iRORA and nGA are similar but not completely overlapping concepts. iRORA can be, but is not always, present when nGA is detected because the signs fulfilling the original definition of nGA (the subsidence of the ONL and IPL, or a hyporeflective wedge-shaped band within the limits of Henle's fiber layer) do not require choroidal signal hypertransmission and RPE attenuation or disruption to be present. On the other hand nGA can also be, but is not always, present in all cases of iRORA because other less-specific signs of photoreceptor degeneration, apart from those used to define nGA, could be used to define the presence of iRORA. These other signs include the thinning of the outer nuclear layer (ONL) or the disruption of the ellipsoid zone (EZ) or external limiting membrane (ELM). Although the risk of disease progression when nGA is present has been characterized in eyes of individuals with intermediate AMD, the understanding of the risk of progression associated or not with iRORA needs to be further explored.

PINNACLE study is one of the first studies in iAMD to combine both machine learning, retrospective and prospective AMD patient data with the goal of identify biomarkers of progression and to report the natural history of progression of intermediate AMD with multimodal retinal imaging. The aim of this project is to utilise advanced statistical modelling and artificial intelligence as innovative approaches to explore novel features and accurately quantify markers of pathological retinal ageing that can individualise progression to advanced AMD ²⁰³.

Deep phenotyping of iAMD with both retrospective and prospective parts, conducted in multiple sites in UK, Austria and Switzerland, recording visual function tests, multimodal retinal imaging and genotyping. With a large dataset featuring eyes with AMD at various stages and healthy controls, investigators aim to identify imaging biomarkers for disease progression. The primary outcome will be the sensitivity and specificity of the OCT imaging biomarkers. Secondary outcomes include sensitivity and specificity of novel multimodal imaging characteristics at predicting disease progression, ROC curves, time from development of imaging change to development of these

endpoints, structure-function correlations, structure-genotype correlation and predictive risk models. At this present moment, here is no available preliminary data concerning PINNACLE study

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Our study has some similarities with MACUSTAR and PINNACLE concerning primary and secondary outcomes, although is only prospective, has a smaller sample and shorter follow-up, and image analysis were not obtained by automatic procedures such as deep or machine learning models. Comparing some of our results with larger and longer studies, as those obtained by MACUSTAR or PINNACLE, could be an important opportunity of expected external validation.

1.3. CONCLUDING REMARKS

With the aim of resolving or mitigating some of previous considered unmet needs, we developed strategies to:

- a) develop a deeper understanding of the cause and drivers of disease progression;
- b) slow the progression of earlier stages of age-related macular degeneration;
- c) identify anatomical and functional early endpoints for early intervention trials.

Besides known AMD risk factors, already presented and discussed in the introduction of this thesis, we explored the possibility of including lipofuscin metabolism and autofluorescence data as etiological factors as well as chorioretinal vascular features, particularly those related with choroidal vascular impairment (choroid and choriocapilaris perfusion). Those findings were analysed in a cross sectional and longitudinal way, attempting to find any relationship with disease progression. We found significant correlation between some of autofluorescence and chorioretinal vascular findings and AMD development but poor evidence as potential drivers of progression to advanced stage of disease, particularly in those related with chorioretinal vascular issues. Considering FAF data, we found a statistically significant difference in the rate of progression to advanced AMD between patients with hypo and/or hyperautofluorescent drusen comparing them with patients with only isoautofluorescent drusen. Those differences in autofluorescence may reflect different stages in drusen evolution, or even different kinds of drusen composition, as hypo or hyperautofluorescence are positively associated with progression to advanced disease. We know that drusen are different

from each other in their morphology, meaning and behavior; they reflect metabolic overload in RPE and are directly linked with the disease progression. As long as our knowledge about their biochemical and histopathology composition will improve many doubts in this regard will be clarified.

It seems reasonable to consider the coparticipation of an hemodynamic substrate in AMD pathophysiology, and decreased choroidal and choriocapillaris perfusion may be involved in these events, adopting the role of trigger or just collaborating as facilitator. Progression to advanced stages of the disease seems to be dependent on other than hemodynamic factors, at least with the methodology used in this study. Extending the sample and follow-up period and including SS-OCT technology in image analysis could be important game changers in this challenging matter.

AMD is a complex and multifactorial disease and no definitive treatment with curative intent is still available. If the cure of AMD is still a goal far from being achieved slowing the progression of earlier stages could be a possibility more easily attainable. Identifying anatomical and functional early endpoints for initial intervention trials is crucial and recognizing patients that are at particularly risk of progression is a primordial and major step towards individualized medicine.

In our study iRORA and disruption of EZ (as a sign of photoreceptor degeneration) seem to be significant risk factors for AMD progression (from intermediate to late stage), as well as HRF and NE-MNV. Those parameters could become important surrogate endpoints to be considered in future clinical trials.

1.4. LIMITATIONS

As AMD is a degenerative and chronic disease, two years of follow-up can be quite a short period to detect unquestionable progression. In addition to this important limitation, considering largest subgroup samples in a multicentric approach, could eventually allow more solid and significant conclusions.

In addition to this observation, some other limitations can be addressed related to the used methodology in some of the present studies. One possible weakness of the FAF related investigation is the fact that we did not use quantitative autofluorescent technique. Recent references showing

non-correlation of quantitative FAF signal with AMD presence, severity, and drusen load have also discouraged us from using this quantitative approach. Nevertheless, quantitative AF could give us more accurate results, but this technology is currently strictly restricted to some investigation centers and is not available in ours.

Considering the chorioretinal vascular findings, although the sole focus of the analysis were structural parameters, a further structure-function assessment would potentially provide additional valuable insights into understanding of vascular role in AMD pathophysiology. The inherent subjectivity associated with manual measurements and the downsides of image binarization techniques, such as under or overestimation of hypo and hyperreflective areas, must also be considered as limiting factors. Manual measurements make volumetric evaluations unfeasible or difficult to accomplish, limiting many of our records to two-dimensional calculations.

In vivo imaging of the choriocapillaris can be achieved with OCT-A, but the presence of artifacts in the outer layers continues to be a relevant limiting factor: not only the attenuation ones but also the projection artifacts in which superficial vessels are projected onto deeper layers of the retina and choroid. High resolution modules and swept source devices (SS-OCT-A), with its longer wavelength and faster acquisition, provides better penetration below the RPE and therefore better visualization of the choroid. Its deeper penetration and less prevalent attenuation artifacts would promote much easier to assess to choriocapillaris flow and more significant results concerning its diffuse or focal flow loss.

Furthermore OCT-A can only provide information concerning vascular status at a single time point and cannot demonstrate dynamic vascular leakage as it is possible with FA or ICG, nor can document flow below a certain threshold. Blood flow determination and velocities measurements in non-exudative neovascular lesions could be important in predicting the onset of exudation²¹³, using the OCT angiography capillary velocimetry technique²¹⁴. Once these technologies become available to clinicians (using an image technique known as variable interval scan time analysis, that requires a SS-OCT, and is not presently commercially available) the ability to measure blood flow velocities with a neovascular lesion could prove usefulness in predicting the near-term onset of exudation. Further this technology could be extended to measure the blood flow velocity within the choriocapillaris and choroid.

Diurnal variation and hydration status may have impact in the reliability of the obtained results. Our OCT-A evaluations were all performed during the morning period but not always, in the same clock hour time, expecting variations among patients and among follow-up examinations.

Concerning the identification of other biomarkers of AMD progression, the most relevant weakness of our longitudinal study, besides the need to extend the sample and the follow-up period, is the potential added value of including complementary functional data in the final analysis. Considering more image parameters in a multimodal approach and their inclusion in the multivariate logistic regression would certainly increase the performance of future models in predicting progression to late AMD.

CHAPTER VII. DISCUSSION AND FUTURE PERSPECTIVES

2. FUTURE PERSPECTIVES

Future work needs to extend the sample and the follow-up period as well as consider the analysis of more image parameters in a multimodal approach. Using high resolution OCT and OCT-A images or OCT swept source technology could highlight the results and should be considered in the future. Inclusion of functional and “patient reported outcomes” data should also be considered.

Additionally artificial intelligence methods of image analysis is another importante goal²⁰⁵. Deep learning models generally achieve high performances comparable with that of individual specialists. Those models reveal significant potential to improve the quality and accessibility of ophthalmic care, but they use parameters sometimes difficult to apply in clinical practice and need some further external validation to be performed in the near future.

We are particularly excited to progress further developing a scoring system that may facilitate general ophthalmologists in the identification of iAMD patients in high risk of progressing to advanced stage of disease. This scoring system should be an easy tool to be able to help clinician in the identification of high risk patients. It should include demographic, functional and structural parameters (or even others) that, considered together and multiplied by a respective coefficient, could classified iAMD patients with an individual score representing the real risk of progression to late stage disease, at this particular moment. Quantifying markers of pathological retinal ageing predictive of individual progression to advanced AMD is an appealing goal towards personalized risk assessment in AMD.

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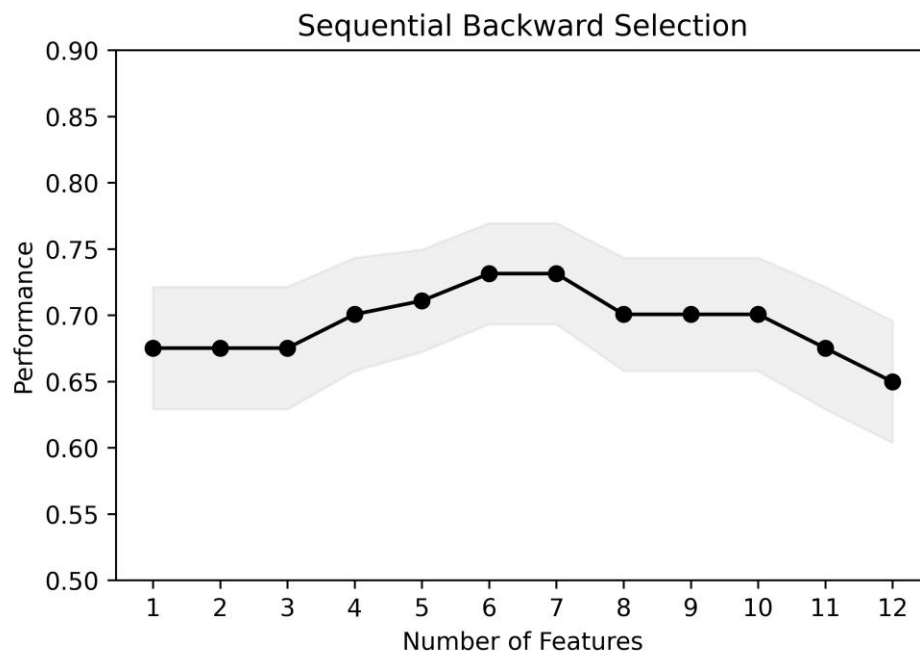
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APPENDIX

SUPPLEMENTARY FIGURES



Supplementary Figure 1 – Sequential backward selection.

The sequential backward selection algorithm aims to reduce the dimensionality of the initial feature subspace from N to K features with minimal model performance loss in order to increase computational efficiency and decrease generalization error. Sequentially removing features from a given features list of N features will yield the list of K features. We increased the performance of the algorithm, which in our case was a logistic regression, by including six dataset parameters, i.e., “Drusen morphology”, “iRORA”, “Drusen area”, “EZ disruption” “HRF” and “HRF in central location”.

SUPPLEMENTARY TABLES

Supplementary Table 1 - Detailed information of the iAMD cohort: Group 1 (AMD).

Gender 1. Female; Gender 2. Male

SubGroup A. Drusen B. Drusen+SDD

Group 1										
N	Gender	SubGroups	Eye	Age	TCamm	LAmm	CVI	OCT-A SVP Density	OCT-A DCP Density	OCT-A CC Density
35	1	A	LE	81	0.3833	0.1660	0.4331			
36	1		RE	83	0.6456	0.2821	0.4370			
37	1		LE	84	0.6834	0.2978	0.4358	0.2844	0.2707	0.3521
38	2		LE	81	0.2842	0.1280	0.4503			
39	2		LE	84	0.2286	0.1240	0.5425	0.2964	0.3259	0.3542
40	2		LE	83	0.9113	0.4475	0.4911	0.2421	0.2665	0.3399
41	1		LE	64	1.6154	0.6837	0.4233	0.2702	0.2903	0.3557
42	1		RE	78	0.8409	0.4081	0.4853	0.2978	0.2899	0.3510
43	2		LE	77	0.2076	0.1043	0.5026	0.2073	0.2414	0.2804
44	1		LE	78	1.4043	0.6213	0.4424	0.3066	0.3007	0.3598
45	2		LE	77	0.4171	0.1752	0.4202			
46	2		LE	82	0.9070	0.4326	0.4769			
47	2		LE	79	0.2273	0.0967	0.4255			
48	2		RE	85	1.6162	0.6955	0.4303			

Group 1										
N	Gender	SubGroups	Eye	Age	TCamm	LAmm	CVI	OCT-A SVP Density	OCT-A DCP Density	OCT-A CC Density
49	2	A	LE	90	1.2325	0.5630	0.4568			
50	1		RE	82						
51	1		RE	93	0.9834	0.4596	0.4674			
52	1		RE	85	1.0928	0.4322	0.3955			
53	1		LE	68				0.2914	0.3038	0.3805
54	2		RE	68	0.8482	0.3128	0.3688	0.3220	0.3171	0.3915
55	2		LE	79	0.6912	0.3173	0.4590			
56	1		RE	88	0.9850	0.3799	0.3857	0.2206	0.2568	0.3314
57	1		LE	87	0.2255	0.1028	0.4557			
58	1		RE	75	1.6966	0.6981	0.4115			
59	1		RE	85	0.6627	0.3251	0.4905	0.2372	0.2593	0.3158
60	1		LE	73	1.3223	0.5871	0.4440	0.3129	0.2953	0.2949
61	1		LE	70	1.4791	0.6545	0.4425			
62	1		LE	88	0.1433	0.0769	0.5366			
63	1		LE	83	0.2132	0.0970	0.4552			
64	1		RE	90	1.2330	0.5517	0.4474	0.2686	0.2834	0.3203
65	1		RE	75	1.3131	0.5896	0.4490	0.2030	0.2474	0.3027
66	1		RE	75	1.5462	0.5855	0.3787	0.2828	0.2930	0.3681
67	1		RE	75	0.7184	0.3105	0.4322			
68	1		LE	84						
69	1		LE	75	1.2433	0.5840	0.4697			
70	2		LE	73	0.9003	0.4395	0.4882			
71	1		RE	85	1.4253	0.5900	0.4140	0.2401	0.2637	0.3488
72	1		RE	81	0.8662	0.3978	0.4593			

Group 1										
N	Gender	SubGroups	Eye	Age	TCamm	LAmm	CVI	OCT-A SVP Density	OCT-A DCP Density	OCT-A CC Density
73	1	A	RE	77	0.8290	0.3964	0.4782	0.2580	0.2861	0.3624
74	1		LE	78	1.5826	0.7272	0.4595	0.2141	0.2521	0.3278
75	1		RE	82						
76	2		RE	68	1.0999	0.5114	0.4649	0.3247	0.3041	0.3710
77	2		LE	59	0.6790	0.2873	0.4231	0.2856	0.3027	0.3343
78	1		RE	66	0.8872	0.4350	0.4903	0.2790	0.2835	0.3206
79	1		RE	81	0.4526	0.2094	0.4626			
80	2		RE	81	0.3148	0.1454	0.4618			
81	2		RE	84	0.2454	0.1255	0.5112	0.2996	0.3040	0.3693
82	2		RE	77	0.3013	0.1392	0.4621	0.2907	0.2946	0.3501
83	2		RE	77	0.4325	0.1756	0.4060			
84	2		RE	77	0.2521	0.1076	0.4268			
85	1		LE	82	1.2993	0.5744	0.4421			
86	1		RE	87	0.2337	0.0917	0.3923			
87	1		LE	92	0.2309	0.1015	0.4394			
88	1		RE	88	0.2009	0.1082	0.5385			
89	1		RE	83	0.3698	0.1675	0.4530			
90	1		LE	83	0.2556	0.1191	0.4660			
91	2		RE	59	0.7766	0.3099	0.3990	0.2609	0.2896	0.3343
92	2	B	RE	80	2.0528	0.9052	0.4410	0.2612	0.2799	0.3609
93	1		LE	85	0.5383	0.2576	0.4786			
94	2		RE	78	0.5835	0.2634	0.4514			
95	2		LE	81	0.7343	0.3205	0.4365	0.2868	0.2999	0.3860
96	2		LE	89	1.3334	0.6392	0.4794			
97	2		LE	82	0.9146	0.4029	0.4405			
98	1		LE	74	2.0164	0.9060	0.4493			

Group 1										
N	Gender	SubGroups	Eye	Age	TCamm	LAmm	CVI	OCT-A SVP Density	OCT-A DCP Density	OCT-A CC Density
99	2	B	RE	82	0.5911	0.3368	0.5699			
100	2		LE	54	1.0713	0.4713	0.4399			
101	1		RE	81	1.1364	0.5237	0.4608	0.2493	0.2679	0.3394
102	1		RE	88	0.1958	0.0885	0.4518			
103	1		LE	79	0.9410	0.3653	0.3882			
104	1		RE	93	0.5423	0.2512	0.4632			
105	2		LE	76	1.9862	0.8416	0.4237			
106	2		LE	65	1.4384	0.6651	0.4624	0.2971	0.3090	0.3524
107	2		LE	77	1.4026	0.6101	0.4350	0.2767	0.2792	0.3777
108	2		LE	77	1.0477	0.4828	0.4608			
109	2		RE	72	1.1420	0.5698	0.4990			
110	2		RE	76	1.0577	0.4815	0.4552			
111	1		RE	78	1.3981	0.6552	0.4686	0.3304	0.3230	0.3654
112	1		RE	82						
113	1		LE	93	1.0943	0.5704	0.5213			
114	1		RE	86						
115	2		LE	79	1.2486	0.5681	0.4550			
116	2		LE	83	0.7315	0.3362	0.4596			
117	1		LE	73	0.7926	0.3779	0.4768	0.3040	0.3126	0.3835
118	1		RE	86	0.7888	0.3319	0.4208	0.2842	0.2911	0.3454
119	1		LE	87	0.2122	0.1021	0.4809			
120	1		RE	75	0.7961	0.3503	0.4400			
121	1		RE	83				0.2795	0.2821	0.3742
122	2		RE	54	1.3842	0.5987	0.4326			
123	1		LE	88	0.1993	0.0805	0.4039			
124	2		RE	65	1.5157	0.6762	0.4462			

Group 1										
N	Gender	SubGroups	Eye	Age	TCamm	LAmm	CVI	OCT-A SVP Density	OCT-A DCP Density	OCT-A CC Density
125	1	B	LE	78	1.9635	0.8505	0.4332			
126	1		LE	82						
127	1		RE	93	1.2256	0.6075	0.4957	0.3155	0.3164	0.3401
128	1		LE	86	1.1518	0.5095	0.4424			
129	2		RE	83	1.1834	0.5302	0.4481			
130	1		RE	87	0.2313	0.1175	0.5081			
131	1		RE	90						

Supplementary Table 2 - Detailed information of the iAMD cohort: Group 2 (Control).

Gender 1. Female; Gender 2. Male

Group 2									
N	Gender	Eye	Age	TCamm	LAmm	CVI	OCT-A SVP Density	OCT-A DVP Density	OCT-A CC Density
1	2	LE	76	1.0764	0.5172	0.4805			
2	1	LE	66	0.9715	0.4698	0.4836	0.2583	0.2734	0.3512
3	1	LE	88	1.0210	0.4715	0.4617	0.2683	0.2880	0.3667
4	1	LE	74	1.7308	0.7231	0.4178	0.3038	0.3023	0.3946
5	1	RE	85				0.2137	0.2586	0.3393
6	2	RE	82	1.5936	0.6596	0.4139	0.2944	0.3003	0.3936
7	1	LE	75	1.2871	0.5900	0.4584	0.3094	0.2958	0.3577
8	2	RE	82	1.2486	0.5681	0.4550			
9	2	RE	77	0.4753	0.2290	0.4817	0.3050	0.2938	0.3729
10	1	LE	77	1.7529	0.7733	0.4412			
11	1	RE	82						
12	1	LE	79	0.1973	0.0985	0.4993	0.2852	0.2856	0.3455
13	1	RE	76	0.8441	0.4270	0.5059	0.2448	0.2618	0.3282
14	1	RE	70	1.2486	0.5681	0.4550	0.3411	0.3061	0.3796
15	1	RE	45	1.1093	0.5098	0.4596			
16	1	RE	83	1.0382	0.4761	0.4586	0.2819	0.2808	0.3497
17	1	LE	63	1.9686	0.8637	0.4388			
18	1	RE	71	1.3824	0.6951	0.5028	0.3074	0.2951	0.3732
19	1	RE	79	1.1612	0.5362	0.4617			
20	1	LE	81				0.2141	0.2521	0.3278
21	1	LE	81	0.7466	0.3395	0.4547	0.2812	0.2756	0.3581
22	1	LE	69	1.2357	0.5461	0.4419			

Group 2									
N	Gender	Eye	Age	TCamm	LAmm	CVI	OCT-A SVP Density	OCT-A DVP Density	OCT-A CC Density
23	1	LE	84	1.4140	0.6120	0.4328	0.3161	0.3049	0.3825
24	1	RE	80	1.1316	0.4922	0.4349			
25	2	LE	82	1.6162	0.6955	0.4303	0.2257	0.2718	0.3487
26	1	LE	82						
27	1	RE	79	0.2346	0.1151	0.4906	0.2555	0.2579	0.3371
28	1	LE	76	1.1245	0.4791	0.4261			
29	1	LE	70	1.6162	0.6955	0.4303	0.3411	0.3061	0.3796
30	1	LE	83	1.2453	0.5301	0.4257			
31	1	RE	63	2.1973	0.9093	0.4138			
32	1	LE	71	1.8372	0.8090	0.4404	0.3074	0.2951	0.3732
33	1	RE	81				0.3098	0.2981	0.3622
34	1	RE	69	1.4027	0.6353	0.4529	0.2993	0.2976	0.3912

Supplementary Table 3 – Performance evaluation of the logistic regression models formed by forward stepwise selection.

N variables	OCT variables	AIC	BIC	R ²	Adjusted R ²
1	["iRORA"]	1.0245	1.0460	0.3152	0.3100
2	["iRORA", "EZ disruption"]	0.9799	1.0229	0.3555	0.3457
3	["iRORA", "EZ disruption", "Drusen area"]	0.9758	1.0404	0.3683	0.3538
4	["iRORA", "EZ disruption", "Drusen area", "HRF"]	0.9807	1.0668	0.3750	0.3558
5	["iRORA", "EZ disruption", "Drusen area", "HRF", "HRF in central location"]	0.9888	1.0964	0.3796	0.3556
6	["iRORA", "EZ disruption", "Drusen area", "HRF", "HRF in central location", "Drusen reflectivity"]	0.9999	1.1290	0.3821	0.3532
7	["iRORA", "EZ disruption", "Drusen area", "HRF", "HRF in central location", "Drusen reflectivity", "HRF association to drusen"]	1.0125	1.1632	0.3836	0.3496
8	["iRORA", "EZ disruption", "Drusen area", "HRF", "HRF in central location", "Drusen reflectivity", "HRF association to drusen", "Drusen homogeneity"]	1.0252	1.1974	0.3850	0.3460
9	["iRORA", "EZ disruption", "Drusen area", "HRF", "HRF in central location", "Drusen reflectivity", "HRF association to drusen", "Drusen homogeneity", "Central drusen"]	1.0383	1.2320	0.3862	0.3420
10	["iRORA", "EZ disruption", "Drusen area", "HRF", "HRF in central location", "Drusen reflectivity", "HRF association to drusen", "Drusen homogeneity", "Central drusen", "Drusen morphology"]	1.0527	1.2679	0.3865	0.3370

N variables	OCT variables	AIC	BIC	R ²	Adjusted R ²
11	["iRORA", "EZ disruption", "Drusen area", "HRF", "HRF in central location", "Drusen reflectivity", "HRF association to drusen", "Drusen homogeneity", "Central drusen", "Drusen morphology", "No. HRF"]	1.0669	1.3036	0.3869	0.3321
12	["iRORA", "EZ disruption", "Drusen area", "HRF", "HRF in central location", "Drusen reflectivity", "HRF association to drusen", "Drusen homogeneity", "Central drusen", "Drusen morphology", "No. HRF", "Subfoveal drusen area"]	1.0815	1.3397	0.3871	0.3268

Supplementary Table 4 – Evaluation metrics of the training, validation, and testing datasets.

Accuracy, Balanced Accuracy, Precision, Recall, and F1-score for 5-Fold Cross-Validation for the training and validation datasets (summed composed 80% of the data). The mentioned evaluation metrics were applied to a testing set, obtained on the remaining 20% of the data.

Evaluation metrics	Train	Validation	Test
Accuracy	0.845	0.861	0.78
Balanced Accuracy	0.780	0.79	0.75
Precision	0.836	0.883	0.60
Recall	0.614	0.638	0.75
Specificity	0.9474	0.960	0.79
F1-score	0.707	0.731	0.67



DECLARAÇÃO

Para os devidos efeitos declaro que o projeto de investigação intitulado “*INTERMEDIATE AGE RELATED MACULAR DEGENERATION – MULTIMODAL ANALYSIS AND LONGITUDINAL STUDY*” (nº 85/2020/CEFCM), submetido para apreciação pela Dra. Rita Maria Rio Pedro Flores, foi desenhado e conduzido de acordo com as normas éticas exigidas pela Comissão de Ética da NOVA Medical School | Faculdade de Ciências Médicas da Universidade Nova de Lisboa (CEFCM).

Lisboa, 26 de outubro de 2020

O Presidente da Comissão de Ética,

A handwritten signature in black ink, appearing to read "Diogo Pais".
(Professor Doutor Diogo Pais)

TO WHOM IT MAY CONCERN

I declare that the research project entitled “*INTERMEDIATE AGE RELATED MACULAR DEGENERATION – MULTIMODAL ANALYSIS AND LONGITUDINAL STUDY*” (nr. 85/2020/CEFCM), submitted for evaluation by .Rita Maria Rio Pedro Flores, MD, was designed and conducted according to the ethical guidelines of the NMS|FCM-UNL’s Ethics Research Committee (CEFCM).

Lisbon, November 26th, 2020

The Chairman of the Ethics Research Committee,

A handwritten signature in black ink, appearing to read "Diogo Pais".
(Diogo Pais, MD, PhD)



COMISSÃO DE ÉTICA PARA A SAÚDE

Parecer

Data: 17.05.2018
Processo n.º 581/2018

Título: "Correlação entre o tipo e a morfologia das drusas musculares (avaliadas por retinografia e tomografia de coerência óptica-OCT) com os achados de Autofluorescência e OCT-Angiografia em doentes c/ degenerescência macular relacionada com a idade, forma seca"

Relator: João Alves, Dr

Investigadora principal: Rita Flores, Dra

Local: Serviço de Oftalmologia, CHLC

Parecer:

1. É um estudo observacional, prospectivo, cujo objectivo principal consiste em analisar a relação entre o tipo e a morfologia das drusas, avaliadas pela Retinografia a Cores e pela Tomografia de Coerência Óptica em Spectral Domain (SD-OCT), com as alterações correspondentes da Autofluorescência e da Tomografia de Coerência Óptica-Angiografia (OCT-A) em doentes com Degenerescência Macular da Idade (DMI), comparar estes achados com um grupo controle de indivíduos idosos (>55 anos) e sem alterações fundoscópicas ou retinográficas e analisar a evolução destas alterações e suas correlações um ano depois.
2. É um estudo de iniciativa do investigador. O investigador principal é a Dr.ª Rita Flores, do Serviço de Oftalmologia do CHLC. Prevê-se a inclusão de 90 doentes no grupo de estudo e 50 doentes no grupo controle.
3. Serão incluídos doentes com drusas associadas a DMI, acompanhados na Consulta de Retina Médica do Serviço de Oftalmologia do CHLC, após consentimento informado.
4. Os doentes seleccionados serão submetidos a uma observação oftalmológica completa, com cálculo da melhor acuidade visual corrigida, tonometria de aplanção, biomicroscopia e oftalmoscopia indirecta. A aquisição multimodal de imagens inclui: Retinografia a Cores ou CFP, NIR (Near Infrared Image), FAF (Fundus Autofluorescence), SD-OCT e OCT-A. Os cortes tomográficos são sobrepostos de forma precisa sobre a imagem de FAF, com a imagem de NIR e "en face". Desta forma, as características morfológicas das drusas obtidas por SD-OCT podem correlacionar-se, de forma precisa, com os achados de Autofluorescência, exactamente na mesma posição. As características morfológicas das drusas são analisadas através da Retinografia e SD-OCT e incluem: tamanho da drusa, integridade da camada elipsóide adjacente à drusa, forma da drusa, reflectividade interna, homogeneidade e presença ou ausência de focos hiperreflectivos adjacentes. Serão ainda obtidas imagens referentes às várias camadas (plexo retiniano superficial, plexo retiniano profundo, avascular, coriocapilar e corioideia) que são posteriormente analisadas. O exame oftalmológico e os exames de imagem serão repetidos após 12 meses. As informações recolhidas durante o estudo serão introduzidas numa base de dados informatizada, apenas através de um número de código do doente, garantindo a confidencialidade.
5. Os formulários de consentimento informado incluem informação sobre os objectivos do estudo, procedimentos, potenciais riscos, inconvenientes e benefícios, garantia da confidencialidade dos dados e contactos dos investigadores.
6. Não estão previstas quaisquer despesas para a instituição.
7. Foi obtido parecer favorável do responsável da especialidade de Oftalmologia.

Conclusão:

O presente estudo não levanta questões do ponto de vista ético, respeitando as normas de boa prática clínica e encontra-se de acordo com a Declaração de Helsínquia e posteriores actualizações, pelo que se entende emitir parecer favorável à sua realização.

O Presidente da Comissão de Ética

(Gonçalo Cordeiro Ferreira)

Secretariado - Manuela Ricca e Nunes - António Couto
e-mail - comissao.etica@chlc.min-saude.pt - Tel. 213514410

