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UNIVERSIDADE D
COIMBRA

Cláudia Virgínia Louro Farinha

AGE-RELATED MACULAR DEGENERATION IN PORTUGAL

Prevalence, Incidence and Risk Factors in the Era of Multimodal Imaging

Tese no âmbito do Programa de Doutoramento em Ciências da Saúde – ramo de Medicina, orientada pelo Professor Doutor Rufino Martins Silva e pela Professora Doutora Maria da Luz Beja Cachulo Damasceno e apresentada à Faculdade de Medicina da Universidade de Coimbra

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This thesis is built upon the Coimbra Eye Study, which encompasses the Epidemiological Study of the Prevalence of Age-Related Macular Degeneration in Portugal and the Five-year Incidence of Age-related Macular Degeneration in the Central Region of Portugal (AMDIncidencePT). They are registered in the public accessible database - Clinicaltrials.gov: Epidemiological Study - NCT01298674; AMD Incidence Study - NCT027048824.

The sponsor of the Coimbra Eye Study was AIBILI – Association for Innovation and Biomedical Research on Light and Image.

The Five-year Incidence of Age-related Macular Degeneration in the Central Region of Portugal (AMDIncidencePT) received financial support by NOVARTIS.

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

The Study was reviewed and approved by the Ethics Committee of AIBILI, Comissão de Ética para a Saúde da AIBILI.

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

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Sem eles nada disto valeria a pena. Com eles tudo ganha significado e sentido.

Table of Contents

List of Abbreviations

AMD	Age-Related Macular Degeneration
ARMS2	Age-Related Maculopathy Susceptibility 2
AREDS	Age-Related Eye Disease Study
A2E	N-retinyl-N-retinylidene ethanolamine
BMI	Body Mass Index
BrM	Bruch's Membrane
BDES	Beaver Dam Eye Study
BMES	Blue Mountains Eye Study
CAM	Classification of Atrophy Meeting
CFI	Complement Factor I
CFH	Complement Factor H
CFP	Color Fundus Photography
C3	Complement Component 3
C9	Complement Component 9
CNV	Choroidal Neovascularization
cRORA	Complete Retinal Pigment Epithelium and Outer Retinal Atrophy
DNA	Deoxyribonucleic Acid
E3	European Eye Epidemiology Consortium
ELM	External Limiting Membrane
FA	Fluorescein Angiography
FAF	Fundus Autofluorescence
HDL	High-density Lipoprotein
HTRA1	High Temperature Requirement A Serine Peptidase 1
IAMDGC	International Age-related Macular Degeneration Genomics Consortium
ICGS-ARM/AMD	International Classification and Grading System for Age-Related Maculopathy and Age-Related Macular Degeneration
ICGA	Indocyanine Green Angiography
IL	Interleukin
iRORA	Incomplete Retinal Pigment Epithelium and Outer Retinal Atrophy
GWAS	Genome-Wide Association Study

GRS	Genetic Risk Score
GA	Geographic Atrophy
HRF	Hyperreflective Foci
MAC	Membrane Attack Complex
MNV	Macular Neovascularization
MAF	Minor Allele Frequency
MESA	Multi-Ethnic Study of Atherosclerosis
MMP	Metalloproteinases
MCI	Multicolor Imaging
MMI	Multimodal Imaging
nvAMD	Neovascular AMD
NIR	Near-Infrared Reflectance
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
OCT	Optical Coherence Tomography
OCTA	Optical Coherence Tomography Angiography
OR	Odds Ratio
PED	Pigment Epithelial Detachment
PEDF	Pigment Epithelium-Derived Factor
RCT	Randomized Clinical Trial
RNA	Ribonucleic Acid
RPE	Retinal Pigment Epithelium
RS	Rotterdam Study
SNP	Single Nucleotide Polymorphism
SD-OCT	Spectral-Domain Optical Coherence Tomography
SS-OCT	Swept-Source Optical Coherence Tomography
SDD	Subretinal Drusenoid Deposits
VEGF	Vascular Endothelial Growth Factor
TIMP	Tissue Inhibitors of Metalloproteinases
UWF	Ultra-Widefield
WARMGS	Wisconsin Age-Related Maculopathy Grading System

Resumo

A degenerescência macular da idade (DMI) é a principal causa de perda de visão central nas populações idosas de países industrializados, esperando-se que a sua prevalência e incidência venham a aumentar em virtude do envelhecimento global da população.

A DMI é uma doença multifatorial influenciada por fatores de risco demográficos, ambientais, de estilo de vida, clínicos e genéticos. Os fatores de risco modificáveis mais consistentemente associados à DMI são o tabagismo e a dieta. Por outro lado, várias variantes genéticas comuns e raras desempenham um papel proeminente no risco para a doença. No entanto, a interação entre esses fatores é complexa e, até o momento, a compreensão de todos os mecanismos fisiopatológicos subjacentes à DMI ainda não é completa.

A caracterização fenotípica da DMI está em constante evolução e a imagiologia multimodal (IMM) é atualmente considerada o método padrão no diagnóstico e seguimento da doença. Diferentes tecnologias de imagem fornecem informação complementar e consequentemente melhoram a compreensão da fisiopatologia a um nível estrutural quase celular. Além disso, os vários biomarcadores imagiológicos obtidos através de IMM podem ser essenciais na previsão da progressão da doença e na tomada de decisões terapêuticas no contexto da medicina personalizada. Adaptar os sistemas de estadiamento da DMI a esta nova realidade é um passo necessário para melhorar a correlação entre fenótipo e a interação dos vários fatores de risco.

Nesta Tese apresentamos os resultados do Estudo da Incidência da DMI (NCT027048824), parte do estudo epidemiológico *Coimbra Eye Study*, a que se seguirá a análise do impacto em dados epidemiológicos da transição para imagiologia multimodal na classificação de imagens em DMI. Em seguida, exploramos os biomarcadores imagiológicos obtidos com essa abordagem multimodal e a sua associação com a gravidade da doença. O passo seguinte consiste na caracterização genética desta população para a DMI, incluindo a análise de variantes comuns e raras, a aplicação do s*core* de risco genético para a DMI e a pesquisa de diferenciais genéticos de distrofias maculares mimetizadoras da DMI. Finalmente, reportamos novas associações genótipo-fenótipo, nomeadamente sobre o efeito da presença de variantes raras no gene do fator H do complemento (*CFH)* no fenótipo dos nossos doentes com DMI.

O Capítulo 1 corresponde à introdução geral e nele se apresenta uma revisão da epidemiologia e fisiopatologia da DMI, dos principais fatores de risco genéticos e não genéticos, as principais características clínicas e de imagem multimodal, os sistemas de classificação e estadiamento mais relevantes, e as terapêuticas mais atuais bem como as perspetivas futuras.

No Capítulo 2 são apresentados os resultados do Estudo da Incidência da DMI, no qual esta Tese se baseia. Neste, descobrimos que a incidência da DMI precoce na cidade costeira de Mira na região centro de Portugal é semelhante àquela descrita em grandes estudos epidemiológicos de populações com ascendência europeia; no entanto, também verificamos que a incidência de DMI tardia foi menor do que o esperado.

No Capítulo 3, exploramos o impacto nos dados epidemiológicos da introdução de uma abordagem multimodal na classificação de imagens e estadiamento da DMI. Comparamos a classificação obtida apenas com retinografia com aquela obtida com IMM, e avaliamos as mudanças no estádio de DMI e, consequentemente, nas taxas de prevalência e de incidência nesta população. Os nossos resultados mostraram que a classificação e o estadiamento da DMI são mais corretos com a abordagem de imagem multimodal, sendo isto especialmente verdadeiro nos casos de DMI tardia, com impacto significativo na informação epidemiológica.

No Capítulo 4, exploramos o potencial da IMM em desvendar novos biomarcadores de gravidade da doença na nossa população em análise. Apesar do foco predominante nos biomarcadores localizados na retina externa e coróide na progressão da DMI, as alterações degenerativas na retina interna parecem estar presentes nos estádios iniciais da doença. Neste estudo demonstramos que um estádio mais avançado de DMI precoce está associado à atrofia progressiva de várias camadas neurorretinianas, tanto internas como externas, apoiando a noção de que existe neurodegenerescência progressiva na DMI em toda a retina. Além disso, os depósitos drusenóides sub-retinianos estão possivelmente associados a um padrão de neurodegenerescência mais proeminente e rápida. As espessuras das camadas da retina neurosensorial podem, assim, ser usadas como biomarcadores quantitativos de progressão da doença na DMI precoce.

No Capítulo 5 apresentamos os resultados do primeiro estudo numa população portuguesa sobre o risco genético para a DMI. Determinamos a contribuição de variantes genéticas comuns e raras no desenvolvimento da doença, exploramos o efeito cumulativo de variantes raras deletérias e calculamos as diferenças entre o *score* de risco genético de doentes com DMI em comparação com participantes sem DMI. O efeito de variantes genéticas raras foi explorado pois sabe-se que estas têm forte impacto no desenvolvimento e progressão da DMI. Várias variantes comuns e raras foram identificadas em associação à DMI na nossa população, mas foi uma variante rara no gene *CFH* que conferiu o maior risco de doença. Por outro lado, três variantes de risco consideradas major tiveram uma frequência alélica menor do que o esperado na nossa população oriunda de uma região geográfica com menor prevalência e incidência de DMI. O *score* de risco genético foi maior nos doentes com DMI, mas verificamos existir sobreposição significativa com os controlos sem doença. Por fim, variantes raras deletérias no gene *CFH* foram cumulativamente mais comuns nos casos de DMI.

No Capítulo 6 exploramos a presença de variantes raras no gene *CFH* e a sua associação com as características fenotípicas obtidas com IMM nos nossos doentes com DMI. Nesta análise final, encontrámos de facto diferenças fenotípicas entre os portadores e os não-portadores das variantes raras no gene *CFH*. Os portadores apresentaram-se com doença mais grave, ou seja, com maior área de drusen maculares na retinografia e, a nível da tomografia de coerência ótica, com características como a presença de descolamentos do epitélio pigmentar da retina, focos hiperrefletivos e retinas mais finas. Além disso, outro achado relevante foi o facto de que os portadores com depósitos drusenóides sub-retinianos compartilhavam todos a mesma variante rara no *CFH*, à exceção de um caso, e provavelmente apresentam risco aumentado de progressão da DMI.

No último capítulo é apresentada uma discussão abrangente com o resumo dos principais resultados desta Tese, juntamente com os próximos passos a desenvolver nesta pesquisa.

Em conclusão, os resultados que aqui apresentamos contribuem para o aprofundar do conhecimento da fisiopatologia da DMI, porque tanto os biomarcadores de imagem como os biomarcadores genéticos são as próximas revoluções no âmbito da medicina personalizada. Será graças a eles que novas estratégias preventivas assim como novos tratamentos dirigidos às diferentes vias fisiopatológicas nos estádios mais iniciais da doença serão desenvolvidos. Estes objectivos são mais do que nunca necessários.

Abstract

Age-related macular degeneration (AMD) is the leading cause of central vision loss in the elderly populations of industrialized countries, and its prevalence and incidence are expected to increase due to the significant aging of the population.

AMD is a multifactorial disease, influenced by demographic, environmental-lifestyle, clinical, and genetic risk factors. The most consistent modifiable risk factors are smoking and diet, while several common and rare genetic variants have been found to play a prominent role. The interplay between these factors is however complex and to date, the full picture of the involved pathophysiologic mechanisms and interactions is still not completely understood.

The phenotypic characterization of AMD is also continuously evolving, and Multimodal Imaging (MMI) is currently the standard in AMD diagnosis and management. Different imaging technologies provide complementary information and improve the understanding of pathophysiology at the structural, almost cellular, level. In addition, imaging biomarkers obtained can further help in predicting progression and in guiding treatment decisions in the context of personalized medicine. Adapting staging systems of AMD to this new reality is a necessary step to improve the correlation between the phenotype and the interplay of risk factors.

In this Thesis, the results of the AMD Incidence Study (NCT027048824), a part of the umbrella epidemiologic Coimbra Eye Study, will be presented. This will be followed by an analysis of the impact of transitioning to multimodal imaging in epidemiologic data. We then aimed to explore imaging biomarkers obtained with this multimodal approach and their association with disease severity. The next step was to extensively characterize the genetics of AMD in this population including common and rare variants analysis, to apply the genetic risk score for AMD to a Portuguese population, and to investigate genetic differentials of mimicking macular dystrophies. Finally, we set to uncover new genotypic-phenotypic correlations, namely the effect of rare variants in complement factor H (*CFH)* gene on the phenotype of our AMD patients.

Chapter 1 corresponds to the general introduction and presents a review of the epidemiology and pathophysiology of AMD, main genetic and non-genetic risk factors, clinical and multimodal imaging main features, most relevant classification and staging systems, and current therapeutics and future management perspectives.

In Chapter 2 the results of the AMD Incidence Study, upon which this Thesis is based, are presented. In this report we found that early AMD incidence in a coastal town of central Portugal,

Mira, was similar to that described in major epidemiological studies of European-descent populations; however, the incidence of late AMD was lower than expected.

In Chapter 3 we explored the impact in epidemiologic data of introducing a multimodal imaging approach in AMD grading and staging. We compared grading with color fundus photography only to grading with MMI in the Mira cohort from the Incidence Study and evaluated the consequent changes in AMD stage, prevalence, and incidence rates in this population. Our results showed that AMD grading and staging are more accurate with a multimodal imaging approach, and this is especially relevant for late AMD, significantly impacting our epidemiological data.

In Chapter 4 the potential of Multimodal Imaging in unraveling new biomarkers of disease severity in our cohort from Mira is explored. Despite the extensive focus on the features located in the external retina and choroid as biomarkers of AMD progression available in the literature, degenerative changes in the inner retina seem to be present in the early stages of AMD. We found that a higher stage in early AMD is associated with thinning of several inner and outer neuroretinal layers, supporting the existence of progressive neurodegeneration. In addition, the presence of subretinal drusenoid deposits is possibly associated with more prominent and faster neurodegeneration. Neuronal retinal layer thicknesses might thus be used as quantitative biomarkers of disease progression in AMD.

In Chapter 5 we present the results of the first genetic analysis in a Portuguese population on genetic risk for AMD. We determined the contribution of common and rare genetic variants in the development of disease, explored the burden of pathogenic rare variants, and calculated differences between the genetic risk score of AMD patients compared to non-AMD participants. Rare variants were explored since they are known to have a strong impact due to high penetrance and may predispose to more severe disease. Both common and rare variants were associated with AMD, but a *CFH* rare variant conferred the highest risk of disease while three major risk variants had a lower-than-expected allele frequency in our population from a geographic region with a lower rate of AMD. The genetic risk score was higher in AMD patients, but with significant overlap with non-AMD cases. Damaging rare variants in the *CFH* gene were cumulatively more common in AMD cases.

In Chapter 6 we explored the presence of rare variants in the *CFH* gene and their association with the phenotypic features obtained with multimodal imaging from our AMD patients of the Mira Cohort. In this final analysis, phenotypic differences were found between carriers and noncarriers of rare variants in the *CFH* gene in AMD patients. Carriers had more severe disease, namely superior drusen burden, and features such as the presence of pigment epithelial detachments, hyperreflective foci, and thinner retinas in Optical Coherence Tomography. Carriers with

subretinal drusenoid deposits shared the same rare variant in all cases except one, and they probably are at increased risk of progression.

In the last chapter a comprehensive discussion with a summary of the main overall results is presented, along with the forthcoming developments of this research.

Our findings reported in this Thesis have the potential to contribute to AMD pathophysiology knowledge, as both imaging biomarkers and genetic biomarkers are the next revolutions in AMD, where personalized medicine strategies, as well as targeted treatments at different pathways in the earlier stages of disease, are more than ever needed.

List of Publications

1. **Incidence of Age-Related Macular Degeneration in the Central Region of Portugal: The Coimbra Eye Study – Report 5**.
   Farinha C, Cachulo ML, Alves D, Pires I, Marques JP, Barreto P, Nunes S, Costa J, Martins A, Sobral I, Laíns I, Figueira J, Ribeiro L, Cunha-Vaz J, Silva R.
   Ophthalmic Res. 2019;61(4):226-235.
   doi: 10.1159/000496393

2. **Age-Related Macular Degeneration Staging by Color Fundus Photography vs. Multimodal Imaging – Epidemiological Implications. The Coimbra Eye Study – Report 6.**
   Farinha C, Cachulo ML, Coimbra R, Alves D, Nunes S, Pires I, Marques JP, Costa J, Martins A, Sobral I, Barreto P, Laíns I, Figueira J, Ribeiro L, Cunha-Vaz J, Silva R.
   J. Clin. Med. 2020 May 2;9(5):E1329.
   doi: 10.3390/jcm9051329

3. **Retinal Layer Thicknesses and Neurodegeneration in Early Age-Related Macular Degeneration: Insights from the Coimbra Eye Study.**
   Farinha C, Silva AL, Coimbra R, Nunes S, Cachulo ML, Marques JP, Pires I, Barreto P, Madeira MH, Cunha-Vaz J, Silva R.
   Graefes Arch Clin Exp Ophthalmol. 2021 Sep;259(9):2545-2557.
   doi: 10.1007/s00417-021-05140-0

4. **Common and Rare Genetic Risk Variants in Age-related Macular Degeneration and Genetic Risk Score in the Coimbra Eye Study.**
   Farinha C, Barreto P, Coimbra R, Iutis A, Cachulo ML, Cunha-Vaz J, Lechanteur YTE, Hoyng CB, Silva R.
   Acta Ophthalmol. 2022 Aug 29. *Online ahead of print.*
   doi: 10.1111/aos.15232

5. **Phenotypic Expression of *CFH* Rare Variants in Age-Related Macular Degeneration Patients in the Coimbra Eye Study.**
   Farinha C, Barreto P, Coimbra R, Iutis A, Cachulo ML, Cunha-Vaz J, Lechanteur YTE, Hoyng CB, Silva R.
   Invest Ophthalmol Vis Sci. 2022 Aug 2;63(9):5.
   doi: 10.1167/iovs.63.9.5

CHAPTER 1.
GENERAL INTRODUCTION

1.1. Background

Age-related macular degeneration (AMD) is the leading cause of central vision loss in the elderly populations of industrialized countries. However, it is well recognized that global population aging is expected in the next decades, which will cause a significant rise in the number of patients affected by AMD, not only in developed countries but also in developing countries.(1, 2)

AMD is a degenerative disease that primarily affects the complex of photoreceptors, retinal pigment epithelium (RPE), Bruch's membrane (BrM), and choroid. Drusen are the hallmark lesion of disease, and their accumulation leads to the progressive degeneration of photoreceptors and RPE. The most pronounced pathological changes affect the macula, resulting in loss of central vision, however, it is recognized that the disease is not limited to this area.(3)

Clinically, AMD is broadly classified into early, intermediate, and late forms. Early and intermediate forms are often and generically aggregated into early "dry" AMD and correspond to about 80% of all cases. They are associated with the presence of drusen and/or pigmentary changes in the retina and are usually not perceived by patients, who are asymptomatic or only refer to mild visual symptoms. Late AMD is however responsible for severe visual loss and may present in two subtypes: geographic atrophy (GA) and neovascular AMD (nvAMD), also known as "wet" AMD or exudative AMD, in which development of macular neovascularization (MNV) takes place. In either case, visual compromise can be profound. Atrophic late AMD is currently a non-treatable condition and in neovascular AMD the significant initial gains obtained with antiangiogenic treatment may be lost in the long term when neuroretinal atrophy and fibrosis ensues.(4) Thus, significant effort is currently being made to develop treatments capable of halting disease progression before vision loss occurs and in developing monitoring strategies that can accurately predict an individual's risk and/or rate of such progression. To achieve these goals a profound understanding of the pathophysiology is essential.

AMD is a complex multifactorial disease, influenced by demographic, environmental, clinical, and genetic risk factors. The most consistent modifiable risk factors are smoking and diet, and variants in the complement factor H (*CFH*) gene and age-related maculopathy susceptibility 2 (*ARMS2*) / high-temperature requirement A serine peptidase 1 (*HTRA1*) genes confer the highest risk of AMD.(5-9) The interplay of all these factors is however complex and to date, the full picture of pathophysiologic mechanisms and interactions is still not completely understood. This is the complex puzzle that we must solve in the forthcoming years if personalized management of our AMD patients is to be achieved and to halt the irreversible vision loss associated with this disease and the associated personal and societal overwhelming costs.

1.2. Epidemiology

Age-related macular degeneration is the leading cause of central vision loss in the elderly populations of industrialized countries and accounts for 8.7% of legal blindness globally.(1, 2) This burden is expected to increase as population aging becomes a global phenomenon, although much more pronounced in industrialized countries. Epidemiologic studies on the prevalence and incidence of AMD are therefore cornerstones for planning for demand in health care systems and for establishing prevention measures.

Prevalence of AMD. In the last three decades, several populational studies conducted across the globe focused on the estimation of early and late AMD prevalence. The Beaver Dam Eye Study (BDES) in the USA, the Blue Mountains Eye Study (BMES) in Australia, and the Rotterdam Study (RS) in Europe were among the first and most important studies that used standardized and certified methodology to estimate AMD prevalence in large cohorts.(10-12) Prevalence of late AMD was found to be similar in the three populations that share common European ancestry (1.6% in the BDES, 1.9% in the BMES, 1.7% in the RS). Since then, other populational studies have been carried out in different regions of the globe. More recently, a comprehensive meta-analysis of population-based studies conducted by Wong *et al* (2) estimated the global prevalence of AMD to be 8.69% (95% credible intervals (CrI) 4.26–17.40%) for any AMD. The authors further estimated the prevalence of early and late AMD to be, respectively, 8.01% (95% CrI 3.98–15.49%) and 0.37% (95% CrI 0.18–0.77%). Considering late AMD, the prevalence of GA was 0.44% (95% CrI 0.15–1.36%) and that of nvAMD was 0.46% (95% CrI 0.18–1.08%).(2) This worldwide estimate reported a projected number of 288 million affected individuals by 2040, and a doubling of late AMD cases, which corresponds to an increase of 9 million individuals.(2)

In Europe, Coljin *et al.*(1) investigated the prevalence of both early and late AMD using summary data of population-based cohort studies from the European Eye Epidemiology (E3) consortium. They projected the estimated number of AMD-affected persons until the year 2040 based on 2 different scenarios: 1) based on stable prevalence, and 2) following the trend of declining prevalence, which was based on data from the meta-analysis of the E3 consortium regarding the 2006-2013 time period. The latter approach was perceived by the group as more realistic since it used E3 historic data and a decelerating slope. The first scenario suggested that the absolute number of persons with early AMD will increase from 15.0 million in 2013 to 21.5 million in 2040, and the number of people with late AMD from 2.7 million to 4.8 million (a 1.5-times increase). The second scenario (based on declining prevalence rates) predicted only a small increase in the number of people with early AMD, from 14 million in 2016 to 14.9 million by 2040, but a larger relative increase in the number of people with late AMD, from 2.7 million in 2016 to 3.9 million by 2040. Because of the declining rates of smoking and the implementation of healthier diets in elderly

persons in the last decades, the second projection was deemed more accurate by the authors, with the modest increase in prevalence mainly driven by the aging of the population.(1)

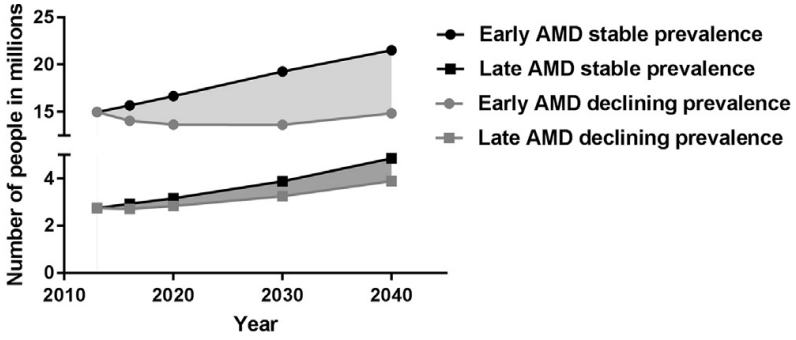

Figure 1: Predicted number of persons with AMD in years from 2013 to 2040 as a function of 2 prevalence scenarios. From "Prevalence of Age-Related Macular Degeneration in Europe: The Past and the Future." by Colijn JM *et al*, 2017, Ophthalmology, 124(12), 1753-1763. Copyright 2017 by the American Academy of Ophthalmology. This is an open access article under the CC BY-NC-ND license (http://?creativecommons.org/licenses/by-nc-nd/4.0/).(1)

Another recent European meta-analysis involving patients above 60 years old described an overall prevalence of 25.3% (95% CI, 18.0–34.4%) for early and intermediate AMD and a prevalence of 2.4% (95% CI, 1.8–3.3%) for any late AMD. For early and intermediate AMD, it ranged from 9.3% (95% CI, 4.4% to 18.5%) in those ≤64 years-old to 26.9% (95% CI, 16.7% to 40.3%) in those above 75 years-old. For any late AMD, prevalence ranged from 0.3% (95% CI, 0.1% to 0.5%) to 6.4% (95% CI, 5.2% to 8.0%). NvAMD was 1.4 times more prevalent than GA (1.4%; 95% CI, 1.0% to 1.9% *vs* 1.0%; 95% CI, 0.7% to 1.5%).(13) The same group estimated that for the year 2050 more than 77 million individuals in the European Union will be affected by AMD, compared to 67 million in 2015. The largest increase would be expected in those aged 75 years and older due to population aging (15%, 50 to 57.6 million). For any late AMD, the increase until 2050 was estimated at 20% (from 10 to 12 million individuals).(13)

The discrepancies observed in prevalence estimates reported in the literature are essentially explained by differences in the included studies, but most importantly, by the use of different AMD classification systems. In this respect, Brandl *et al* (14) compared the Beckman Clinical Classification to the Harmonized Three Continent AMD Consortium Severity Scale (3CACSS) and found important differences, namely in the prevalence of early and intermediate AMD. Early/ intermediate AMD was much more prevalent when using the Beckman Classification compared to the 3CACSS (which is more similar to the Rotterdam classification). Another factor hampering comparability, is the fact that the classification systems used so far are based only on color fundus photography (CFP), instead of multimodal imaging (MMI), making staging less accurate, which can be especially true for incipient late AMD. All these limitations are important when analyzing epidemiological data and evidence the need for an international consensus to establish a truly global AMD classification system.

Incidence of AMD. While prevalence measures the proportion of disease in the population, calculation of the incidence has added value because it allows planning for demand in health care systems, and therefore aids in establishing adequate preventive measures. After initially reporting on prevalence, the major populational studies estimated AMD incidence, and some on different time-points. The BMES reported a cumulative incidence of 8.7% for early AMD and 1.1% for late AMD at 5 years, which increased to 14.1% and 3.7% at 10 years, and 22.7% and 6.8% at 15 years of follow-up, respectively.(15-17) The BDES reported a cumulative incidence of 8.2% for early AMD and 0.9% for late AMD at 5 years, increasing to 12.1% and 1.2% at 10 years, and to 14.3% and 3.1% at 15 years.(18-20). A meta-analysis conducted in the USA by Rudnicka *et al* (21) estimated the annual incidence of late AMD in American whites 50 years or older to be 3.5 per 1.000 persons (95% CrI, 2.5–4.7 per 1000), which was equivalent to 293.000 new cases per year (95% CrI, 207.000-400.000). The annual incidence of GA was estimated to be 1.9 per 1000 and nvAMD was 1.8 per 1000.(21)

In Europe, the RS reported a 5-year risk of early AMD of 7.9% and 0.9% for late AMD. The incidence of GA was 0.4% and nvAMD was 0.5%.(22) In the French ALIENOR Study the cumulative incidence at 5 years was much superior: 32.9% for early AMD and 8.9% for late AMD. However, this study included older subjects (≥73 years old), and the definition of early AMD was broader as they included patients with stage 1 Rotterdam classification.(23) The German Gutenberg Health Study recently reported an AMD prevalence of 8.5% (95% CI, 7.9–9.2%) at baseline, which increased to 10.3% (95% CI, 9.6–11.1%) at follow-up. This represented a cumulative 5-year incidence of only 2.0% (1.7–2.4%), which was attributable by the authors to the younger age groups included in this study (from 35 to 74 years at baseline).(24) Finally, the recent European meta-analysis conducted by Li *et al* (13) estimated the pooled annual incidence of any late AMD in Europe to be 1.4 per 1000 individuals (95% CI, 0.8 to 2.6). Furthermore, they projected that the incidence of late AMD will increase from the current 400.000 per year to 700.000 per year in 2050.(13)

Comparability between the multiple incidence studies available in the literature is once again limited. Heterogeneity in study designs, implementation of different classification systems, and true populational and/ or ethnic differences in analyzed cohorts are again the main causes limiting the comparability of epidemiological data across populations.

AMD epidemiology in Portugal. Until recently there were no studies on AMD prevalence and incidence in Portugal. The **Coimbra Eye Study** aimed to address this gap and comprises two studies: the *Epidemiological Study* (NCT01298674) and the *AMD Incidence Study* (NCT027048824), which provide important information on AMD prevalence and incidence, respectively. The included populations are from the central region of the country, one from an inland town (Lousã) and another from a coastal town (Mira). Furthermore, reports that

comprehensively address the impact of risk factors, including demographic, environmental, and lifestyle-related (such as diet and physical activity) were published in recent years.(25-30) A brief overview of the Epidemiologic Study followed by the full report of the Incidence Study, which is the basis of this Thesis, will be addressed in detail in **Chapter 2**.

1.3. Risk Factors

Older age, northern European ancestry, and family history are well-known non-modifiable risk factors for advanced AMD. The main modifiable risk factor of advanced AMD development is smoking, and smokers are 2 to 4 times more likely to experience AMD. Diet also seems to play an important role in AMD development and progression. Other factors such as hyperlipidemia and hypertension may be risk factors, but the level of evidence for these is lower.(5, 31, 32)

1.3.1. Non-Modifiable Risk Factors

Age. Age is the strongest risk factor for AMD. The age at onset varies greatly but AMD typically begins above 55 years old. The prevalence increases with age, especially that of late AMD (OR of 4.2 per decade).(21, 33) Wong *et al* (2) reported in their global meta-analysis that the prevalence of both early and late disease increased with age in each one of the ethnic groups and regions included. Furthermore, in the populations with European ancestry, the prevalence of late disease increased most rapidly after the age of 75 years, and with a similar trend in Europe and Oceania.(2) Regarding incidence all populational studies and meta-analyses conducted so far demonstrate a significant increase with age.(17, 20, 22)

Race. Although the prevalence of drusen and early stages of AMD seem similar in whites and non-whites, more advanced forms of the disease seem more prevalent in whites.(5) The Multi-Ethnic Study of Atherosclerosis (MESA) longitudinal study, reported on the prevalence and incidence of 4 racial groups, and found an overall prevalence ranging from 2.4% in African Americans to 4.2% in Hispanics, 4.6% in Chinese, and 5.4% in Whites.(34) The same group later described that the incidence of early and late AMD was highest in whites (5.3% and 4.1% respectively), followed by Chinese (4.5% and 2.2%) and Hispanic (3.3% and 0.8%) populations, and lowest in Blacks (1.6% and 0.4%). Plus, blacks had a 67% lower risk of developing early AMD after multivariable adjustment than whites.(35) **Iris color** has also been shown to be associated with AMD risk in some studies. Patients with lighter colored irides were shown to have a two-fold higher incidence of AMD compared to those with darker irides.(5) However, data is conflicting, and more definitive research is necessary.

Gender. Concerning gender current evidence is conflicting. Many studies have concluded that there is no significant increased risk for developing AMD based on gender alone.(5) Colijn *et al* (1) reported that in the oldest age category (85 years and older), women seemed to have a higher prevalence of late AMD, but a more detailed analysis showed that this was mostly owing to the imprecision of the estimate in men, caused by a lower number of men in that age group.

1.3.2. Modifiable Environmental Risk Factors

Smoking. Multiple studies confirm smoking as a strong risk factor for the development of AMD. There is also evidence of an association between the risk of developing late AMD and the number of cigarettes smoked over a lifetime. The mechanism is thought to be caused by its pro-inflammatory effect, promoting oxidative stress and raising the levels of inflammatory cytokines, along with compromise in blood flow, coagulation, and lipoprotein pathways.(3, 32) In the pooled data of the Beaver Dam Eye Study, Rotterdam Study, and Blue Mountains Eye Study there was a threefold risk of any AMD associated with current smoking (odds ratios (OR) of 2.4, 3.1, 4.2, respectively), and the risk magnitude was higher for nvAMD (OR, 4.6) compared with GA (OR, 2.6).(36) Seddon *et al.* (37) further reported that past smokers still had a 1.7-fold increased risk of AMD. However, after 20 years of cessation, the risk appears to be similar to non-smokers.(3, 5)

Lifestyle - Diet & Physical Activity. A well-balanced Diet is increasingly recognized to have a benefic effect on AMD development and progression. Higher consumption of foods containing lutein and zeaxanthin, such as spinach and dark green leafy vegetables, and increased consumption of fish oils or ω-3 long-chain polyunsaturated fatty acids (e.g. docosahexaenoic acid and eicosapentaenoic acid), are associated with decreased risk of late AMD.(38) Furthermore, research showed that supplements with high doses of oral antioxidants (vitamin C, vitamin E, and carotenoids lutein and zeaxanthin), in addition to zinc, reduced progression from intermediate to advanced AMD by 25% over 5 years.(39, 40) Supplements based on this formulation are now routinely recommended in clinical practice for individuals with intermediate AMD.

The **Mediterranean Diet** in particular, as part of a healthy lifestyle, was recently recognized by several study groups as a “diet model”, contributing to decreasing AMD risk and progression to late AMD.(38) The traditional Mediterranean diet is characterized by high consumption of plant foods, moderate consumption of fish and wine, low consumption of dairy and meat, and intake of monounsaturated fatty acids as the primary fat source. The pooled data from the Rotterdam Study and ALIENOR Study reported by the EYE-RISK consortium showed that higher adherence to the Mediterranean diet was associated with a 41% reduced risk of incident advanced AMD.(9) In the Portuguese Coimbra Eye Study we reported similar findings with a protective effect of this type of

diet in AMD development. Our group also described a beneficial effect of physical activity in reducing the risk of AMD, in the context of a healthier lifestyle.(29, 30)

Other environmental and systemic risk factors. Inconsistent associations to AMD are the impact of cataract surgery and sunlight exposure.(3, 5) The rationale behind cataract surgery worsening AMD status, and particularly hampering the treatment of neovascular AMD, derives from post-surgical related intra-ocular inflammation. Some linked surgery to an increased risk of progression to late AMD, but these findings, however, were not supported by other groups. Not only there was no increased risk of progression, but the benefits of functional improvement outweighed the putative risks.(41, 42)

As for systemic risk factors, the association of cardiovascular risk factors including hypertension, atherosclerosis, high body mass index (BMI), and metabolic dysfunction such as diabetes mellitus and hyperlipidemia, remains controversial because of contradictory reports by different study groups.(3, 5) Concerning lipid metabolism, a meta-analysis from the Three Continent Age-Related Macular Degeneration Consortium found no associations of cholesterol measures, history of statin use, or lipid pathway genes to the incidence and progression of AMD. They conclude that their findings add to inconsistencies in earlier reports showing weak associations, no associations, or inverse associations of high-density lipoprotein (HDL) cholesterol and total cholesterol with AMD.(43) More recently, however, the EYE-RISK and European Eye Epidemiology Consortia explored the relationships between systemic lipids levels, lipid genes, and AMD in a large European dataset. They found that HDL cholesterol, and particularly extra-large HDL subfractions, was associated with increased risk of AMD (mostly through the association with drusen), while triglycerides were negatively associated. The group acknowledged that whether systemic lipids directly influence AMD or "just" represent lipid metabolism in the retina remained unanswered.(44) Studies on plasma metabolomics also support the hypothesis that lipid homeostasis is altered in patients with AMD.(45) In this line, an open-label prospective clinical study explored the use of high-intensity atorvastatin (80 mg daily), and reported regression of drusen in some patients with improvement in visual acuity and no progression to advanced exudative AMD. The authors thus suggested a possible role for statins and other lipid-lowering medications such as fibrates in AMD management, and the need for future large randomized controlled trials and prospective cohort studies.(46)

1.3.3. Genetic Risk factors

Several genetic risk variants have been identified in association with AMD in the last two decades, and recently a landmark genome-wide association study (GWAS) conducted by the International Age-related Macular Degeneration Genomics Consortium (IAMDGC) established 52 variants at

34 genomic regions as independently associated with AMD. Forty-five of these variants were common variants and 7 were rare variants (this is, a minor allele frequency (MAF) < 1%) (Table1).(7)

Table 1. Identification of 52 independent AMD risk variants in 34 loci.(7)

Signal Number [a]	Locus name	Index variant [c]	Chr:Position	Major/minor allele
1.1	*CFH*	rs10922109	1:196,704,632	C/A
1.2	*CFH*	rs570618	1:196,657,064	G/T
1.3	*CFH*	rs121913059 [d]	1:196,716,375	C/T
1.4	*CFH*	rs148553336 [d]	1:196,613,173	T/C
1.5	*CFH*	rs187328863	1:196,380,158	C/T
1.6	*CFH (CFHR3/CFHR1)* [b]	rs61818925	1:196,815,450	G/T
1.7	*CFH*	rs35292876 [d]	1:196,706,642	C/T
1.8	*CFH*	rs191281603 [d]	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
4.1	*COL8A1*	rs140647181	3:99,180,668	T/C
4.2	*COL8A1*	rs55975637	3:99,419,853	G/A
5.1	*CFI*	rs10033900	4:110,659,067	C/T
5.2	*CFI*	rs141853578 [d]	4:110,685,820	C/T
6	*C9*	rs62358361 [d]	5:39,327,888	G/T
7	*PRLR/SPEF2*	rs114092250	5:35,494,448	G/A
8.1	*C2/CFB/SKIV2L*	rs116503776	6:31,930,462	G/A
8.2	*C2/CFB/SKIV2L*	rs144629244	6:31,946,792	G/A
8.3	*C2/CFB/SKIV2L (PBX2)* [b]	rs114254831	6:32,155,581	A/G
8.4	*C2/CFB/SKIV2L*	rs181705462	6:31,947,027	G/T
9	*VEGFA*	rs943080	6:43,826,627	T/C
10	*KMT2E/SRPK2*	rs1142	7:104,756,326	C/T
11	*PILRB/PILRA*	rs7803454	7:99,991,548	C/T
12	*TNFRSF10A*	rs79037040	8:23,082,971	T/G
13	*MIR6130/RORB*	rs10781182	9:76,617,720	G/T
14	*TRPM3*	rs71507014	9:73,438,605	GC/G
15	*TGFBR1*	rs1626340	9:101,923,372	G/A
16	*ABCA1*	rs2740488	9:107,661,742	A/C
17	*ARHGAP21*	rs12357257	10:24,999,593	G/A
18	*ARMS2/HTRA1*	rs3750846	10:124,215,565	T/C
19	*RDH5/CD63*	rs3138141	12:56,115,778	C/A
20	*ACAD10*	rs61941274	12:112,132,610	G/A
21	*B3GALTL*	rs9564692	13:31,821,240	C/T
22.1	*RAD51B*	rs61985136	14:68,769,199	T/C
22.2	*RAD51B*	rs2842339	14:68,986,999	A/G
23.1	*LIPC*	rs2043085	15:58,680,954	T/C
23.2	*LIPC*	rs2070895	15:58,723,939	G/A
24.1	*CETP*	rs5817082	16:56,997,349	C/CA
24.2	*CETP*	rs17231506	16:56,994,528	C/T
25	*CTRB2/CTRB1*	rs72802342	16:75,234,872	C/A
26	*TMEM97/VTN*	rs11080055	17:26,649,724	C/A
27	*NPLOC4/TSPAN10*	rs6565597	17:79,526,821	C/T
28.1	*C3*	rs2230199	19:6,718,387	C/G
28.2	*C3*	rs147859257 [d]	19:6,718,146	T/G
28.3	*C3 (NRTN/FUT6)* [b]	rs12019136	19:5,835,677	G/A
29	*CNN2*	rs67538026	19:1,031,438	C/T
30.1	*APOE*	rs429358	19:45,411,941	T/C
30.2	*APOE(EXOC3L2/MARK4)*[b]	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

a. Independent signals within loci that were detected by sequential forward selection are indexed by their corresponding signal number, jointly analyzed regions (10 Mb regions) were sorted by position, while the signals (within each of the 10 Mb regions) are sorted according to their discovery by the sequential forward selection; b. The peak of this independent association signal is located closer to genes in parenthesis than to the locus-defining genes; c. dbSNPID of the signal index variant; d. Rare variants (MAF<1%).

Most of the implicated genes belong to the following pathways: complement system, high-density lipoprotein metabolism, extracellular matrix (ECM) remodeling, angiogenesis, and cell survival. A large risk effect has been reported for genetic variants located at the *CFH* gene on chromosome 1 and in two neighboring genes *ARMS2/HTRA1*, on chromosome 10.(8, 47, 48) However, the *ARMS2* and *HTRA1* variants are in high *linkage disequilibrium* (this is, the non-random association of alleles at different loci), and thus statistical genetic approaches cannot distinguish the effects attributable to one or the other. All other loci have low attributable risk, because of low prevalence or weak association.

Many high-risk genetic variants linked to AMD are found in genes encoding complement system regulatory proteins (*CFH,* complement factor I [*CFI*], complement component 3 [*C3*], complement component 9 [*C9*], complement factor B/ complement component 2 [*FB/C2*]). Two common variants in the *CFH* gene alone, the rs1061170 (Y402H) and the intronic rs1410996 explain over 17% of AMD liability.(49) Nevertheless, the exact mechanisms by which the common variant rs1061170 (Y402H) in *CFH* increases AMD susceptibility are not clear. Studies have suggested uncontrolled activation of the complement system and reduced binding affinity of the *CFH* H402 variant to heparan sulfate chains leading to lipoprotein accumulation in the BrM. Plus, it alters the binding affinity of CFH to C reactive protein and the subretinal clearance of mononuclear phagocytes. Endogenously produced CFH in RPE cells also protects them from oxidative stress.(3) Regarding the *ARMS2/HTRA1*, their relation to AMD pathophysiology is more elusive and to this date, it is not fully understood. Studies suggest a function for *ARMS2* gene product in the mitochondrial outer membrane, whereas others suggest that it encodes an extracellular protein. *HTRA1* encodes a heat shock serine protease and is involved in regulating ECM deposition, angiogenesis and TGFβ signaling, as well as in subretinal inflammation, by controlling monocyte elimination.(3)

The full set of the 52 SNPs identified by the IAMDGC account for 27% of late AMD risk, which is >50% of the total genomic heritability. This puts in evidence the so-called *missing heritability* in AMD.

Despite the importance of common variants in global risk assessment, Fritsche *et al* (7), noted that a significant burden of rare variants was present in the *CFH*, *CFI, TIMP3,* and *SLC16A8* genes, and other groups confirmed that rare genetic variants located in the complement genes conferred high risk for AMD. These rare variants might complete the puzzle on AMD heritability.(50-52)

Rare Variants. The development of AMD can be influenced not only by common variants (MAF > 5%) but also by low frequency (MAF between 1 and 5%) and rare (MAF < 1%) genetic variants. Fritsche *et al* (5) showed in their GWAS study that seven rare variants were independently associated with AMD: *CFH* rs121913059 (Arg1210Cys), *CFI* rs141853578 (Gly119Arg), *C3* rs147859257 (Lys155Gln), *C9* rs34882957 (Pro167Ser) and three non-coding variants in or near the *CFH* (rs148553336, rs35292876, rs191281603). Until now, more than 100 rare variants from different pathways have been implicated in AMD pathophysiology in case-control studies from different populations and in family aggregation studies or twin studies.

Approximately 20% to 30% of the patients have a positive family history of AMD. A positive family history also has been associated with earlier age at the disease onset. Although the heritability of late AMD is estimated to be up to 71%, the clustering of known common genetic risk factors does not fully explain the number of affected family members in large, densely affected families.(3) Thus, it was hypothesized that rare but highly penetrant genetic variants may account for the clustering of AMD in these families and lead to more severe disease.

Highly penetrant rare variants have indeed been identified in families with AMD in the last decade, thus confirming the hypothesis of familial clustering. Rare variants are more likely to be deleterious, as their effect sizes tend to be much larger conferring up to a 20-fold increased risk, compared with up to 3-fold risk for common variants.(32) Furthermore, rare variants seem to act in an autosomal dominant manner. This means that if they cause, for example, haploinsufficiency of the cofactor protein (Factor H) or of the necessary protease (Factor I), necessary to inactivate the complement component C3b, this will be sufficient to allow for excessive inflammation, which is critical in the pathogenesis of AMD.(51, 52)

Understanding the contribution of these rare variants to the clinical characteristics of AMD is important because carrying these variants may have diagnostic, predictive, and therapeutic consequences for carriers.(53) Many of these rare variants are located in genes of the complement system, which plays a major role in the pathogenesis of AMD. Two rare AMD-associated variants in the *CFH* gene (rs121913059 [Arg1210Cys] and rs35292876) were found to deviate significantly in frequency among different geographic regions. The highly penetrant *CFH* variant Arg1210Cys was first reported in a case-control study from the United States and confers the strongest genetic risk for AMD in this population (OR > 20).(32) Several studies replicated this finding, but other studies on Asian and White populations were unable to find such a strong association. Another example is the highly heterogeneous distribution of *CFI* variant rs141853578 (Gly119Arg).(54) This emphasizes the importance of identifying population-specific rare variants by performing sequencing studies in populations from distinct geographic distributions.

Functional Impact of Genetic Variation. Genetic variants, and especially rare variants, that usually are highly penetrant and more deleterious, may have important functional implications.

However, most of the identified rare variants lack description in the literature regarding their functional impact on metabolic and inflammatory pathways related to AMD. More knowledge in this regard is necessary before classifying a variant as deleterious or benign. This is commonly assumed in genetic studies based only on the association to phenotype in case/control analysis, but without a full understanding of the underlying pathophysiology.

The complement system has been the pathway most extensively analyzed from this perspective. Seddon and coworkers (32) found that variants in *CFI* led to lower serum factor I (FI) levels and low FI levels were associated with a higher risk of AMD. Plus, rare variants in the *CFH* were associated with reduced serum levels of Factor H in advanced AMD. Geerlings *et al* (55) analyzed the functional effect of rare variants in *CFH, CFI, C9,* and *C3*. Carriers of *CFI* variants (Gly119Arg; Leu131Arg) had decreased FI levels, and *C9* Pro167Ser carriers had elevated C9 serum levels. Carriers of *CFH* (Ser193Leu, Arg175Gln) and *CFI* rare variants had a reduced ability to degrade C3b, but in the case of *CFH* this was independent of FH serum levels, which were not altered. Variants group into 2 major mutation types: type 1 causes low protein levels from misfolding or degradation, and type 2 causes reduced protein functionality with normal levels.(55) Their results thus support that these carriers are less able to inhibit the complement activation. The significance of such results is important, as these patients might benefit more from complement inhibiting therapies or gene-targeted therapies, making their identification in different populations important in selection for clinical trials, for example.(56)

Genetic risk score **(GRS).** In addition to individual gene-disease associations, genetic data can be used and "pooled" in the calculation of an individual's 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 IAMDGC, which reported in their GWAS 52 independent variants across 34 loci, provided an updated genetic risk score weighted by the effect size of the variants. The calculated GRS showed that individuals in the highest decile of genetic risk had a 44-fold increased risk of advanced AMD compared to those in the lowest decile.(7) 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 to healthy controls. They found, however, an overlap between the groups, meaning that the GRS alone was unable to completely distinguish between them. 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). This approach aids in identifying which pathways

are more predominant in different patients, contributing to understanding differences in phenotype and eventually, response to therapeutic interventions.(8, 48)
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, 57)

Genotype and Phenotype. Another concept to bear in mind is that genetic risk factors that influence AMD development and progression might be different in the different stages of the disease. For example, the genetic pathways involved in early or intermediate AMD development are probably different from those driving the evolution to late AMD, and to whether GA or nvAMD develops. Seddon and colleagues (32) showed that the *ARMS2* locus conferred an increased risk for both advanced AMD subtypes, but was the only one (among 115 SNPs evaluated) that conferred greater risk for nvAMD compared with GA.
It is known that specific phenotypic features, such as the presence of subretinal drusenoid deposits (SDD), hyperreflective foci (HRF), and drusen burden, are associated with different AMD phenotypes and different rates of disease progression. Several groups found an association of rare variants to increased drusen burden and drusen extending outside the vascular arcades and nasal peripapillary area. These patients could be at increased risk of progression making their identification important to individualize management.(58-60) The presence of other features such as peripheral retinal drusen beyond the posterior pole and peripheral retinal reticular pigment changes were phenotypic characteristics found to be associated with AMD. Two variants in the gene *CFH* (Y402H and intronic rs1410996), conferred a 2-fold higher risk of having these peripheral retinal phenotypes, but similar associations were not seen for the genes *CFB, C2,* or *C3*.(32, 61) Thus, it is clear that different genetic factors must contribute to different phenotypes and to different rates of disease progression in the complex disease model that is AMD.

1.3.4. Prediction Models in AMD – Interplay of Multiple Risk Factors

The increased knowledge of genetic and non-genetic risk factors led several groups to explore multifactorial models capable of better predicting disease development and progression. The field is however constantly expanding and a universal, comprehensive model, valid across different populations, is not yet available.
Genetic variants not only determine the phenotypic and clinical characteristics of AMD *per se*, but they influence the impact of environmental and lifestyle risk factors in the disease's pathophysiology. This genetic *modulation* or *interplay* has been the subject of comprehensive multifactorial risk analyses. For example, Merle and coworkers (38) report that higher adherence to the alternate Mediterranean diet (a validated score used to estimate adherence to the

Mediterranean diet in the USA population), was associated with a reduced risk of progression to advanced AMD, but this effect was *modified* by genetic susceptibility. There was a protective effect of the diet among subjects carrying the *CFH* Y402H non-risk (T) allele, but the effect was lost in subjects homozygous for the risk (C) allele. The same group further reported that progression in drusen size was influenced by both genetics, as quantified by the *genetic risk score*, and by the alternate Mediterranean Diet score. Genetic predictors and diet quality were, however, independently related to an increase in drusen size.(62) Among antioxidant and zinc supplement users, patients with the non-risk genotype in *CFH* (TT) had a lower risk of progression to advanced AMD. However, there was no significant effect in those homozygous for the *CFH* risk allele (CC). A protective effect was observed among patients with the high-risk *ARMS2* (TT) genotype.(32)
Numerous predictive models have been tested, but so far there is substantial variability in the risk factors included, study designs, and outcomes measured. For AMD risk scores to be implemented in the context of clinical practice and personalized medicine they must account for all the variability and must be valid across a wide spectrum of patients or populations. For this purpose, prediction algorithms of disease progression should account not only for the genetic, environmental, and socio-demographic data but imaging and clinical features are a major part that should be included. Multimodal imaging, instead of color fundus photography alone (which has been the basis of AMD classification systems), provides constantly new biomarkers that should be accounted for inclusion in predictive modeling.

Machine learning algorithms based on retinal imaging alone proved to be accurate in the short-medium term, and the association of both genetic and socio-demographic features added little value to prediction in some studies.(63) Others reported instead that genetic information quantified by the GRS added value over clinical characteristics in predicting AMD progression, but only in patients with less severe disease stages.(64) For now, it seems that imaging features should be the basis for assessing risk in regular clinical practice, and only those who are suspicious of having more deleterious variants based on the clinical picture and/or family history should be directed toward genetic analysis, instead of routinely genotype all AMD patients for risk stratification.

In conclusion, for now, genetic testing is not recommended as part of routine clinical care by professional entities such as the American Academy of Ophthalmology (USA) or the National Institute for Health and Care Excellence (UK). The justification lies in the absence of interventions capable of decreasing AMD risk development in those with high genetic risk; the absence of approved genotype-dependent treatments; and, as stated above, the relatively good accuracy of risk assessment based on phenotype through multimodal imaging, with only marginal gain when adding genotype in most cases.(63) It is however important to pursue genotyping at the research level, not only in densely affected families or in individuals with severe disease and early onset, but also in

large populations and cohorts, to better apprehend AMD pathophysiology and to support the development of new targeted treatments.

1.4. Pathophysiology

AMD can be thought of as a disruption in the normal homeostatic mechanisms of the retina, where aging changes coupled with genetic risk, chronic inflammation, oxidative stress, lipid and lipoprotein deposition, and impaired extracellular matrix maintenance, lead to an imbalance, that ultimately promotes the development of the disease and its progression towards advanced stages (Figure 2).(3) Comprehension of the known pathophysiology of AMD and to further expanding this knowledge are the key factors for the development of new preventive and therapeutic strategies that are still lacking in the present day.

Age-related changes. Age is the strongest risk factor for AMD, and age-related changes are the basis on which AMD subsequently develops. These changes include:

- *Lipid and lipoprotein deposition in the RPE and Bruch's membrane*. An important feature of the aging BrM is the accumulation of drusen or basal linear deposits and basal laminar deposits. These deposits have a high lipid concentration and are believed to contribute to the thickening of BrM and its decreased function with age. They also exacerbate inflammatory responses such as cellular infiltration and accumulation of cellular debris.(65)
- *Oxidative and metabolic stress* causing cell damage and consequent apoptosis and/ or necrosis. Affected cells respond by producing proinflammatory cytokines and chemokines with overactivation of the local immune system.
- *Parainflammation*. If the insult continues and the local reparative capacity is overcome, the systemic immune system is activated, which includes innate pathways such as the complement system, in an effort of inducing tissue repair and remodelling.
- *Microangiopathy affecting the choriocapillaris* is a major effect of aging, there is progressive rarefaction and loss of the vascular network. This contributes to an ischemic environment that ultimately affects the nutrition of both RPE and photoreceptors, while diminishing the clearance of lipoproteins from the RPE and BrM, contributing to retinal degeneration.

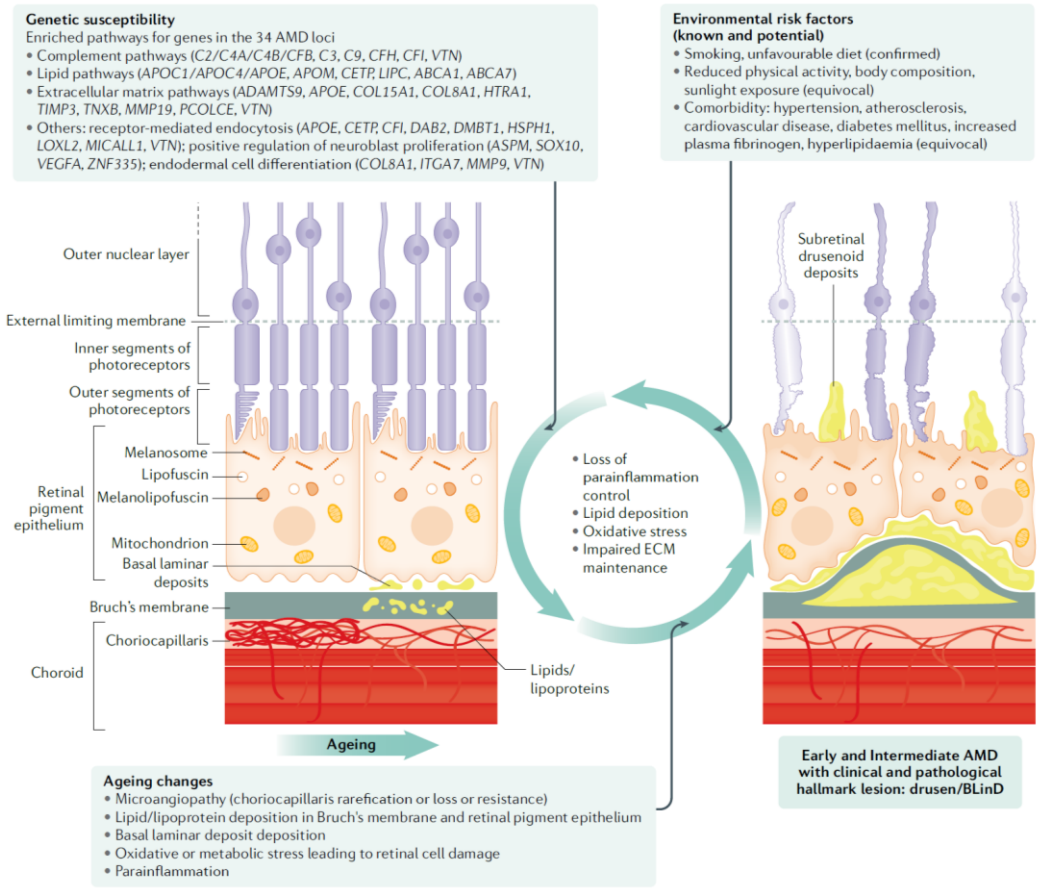

Figure 2: Model of AMD pathogenesis. AMD affects the complex of photoreceptors-RPE-BrM-choriocapillaris and is a multifactorial disease caused by a complex interplay of aging, genetic and environmental factors. Chronic inflammation, oxidative stress, altered lipid and lipoprotein deposition, and impaired ECM maintenance, lead to the formation of drusen, the hallmark lesion of AMD. From "Age-related macular degeneration" by Fleckenstein M *et al*, 2021, Nat Rev Dis Primers, 7(1),31. Copyright 2021 by Springer Nature. Reprinted with permission.(3)

Chronic inflammation. Immune activation in AMD includes extracellular deposit formation, localized immune response, and subretinal and choroidal recruitment of microglia or macrophages. The exact pathogenic sequence remains, however, largely unknown. A two-level hypothesis was suggested by Rozing *et al*. (65) in which AMD can be considered the consequence of the age-related accumulation of molecular damage with subsequent local inflammatory activation, a stimulus which, if continued, elicits a systemic inflammatory host response.

Components of drusen (cellular debris, lipids, lipoproteins, amyloid deposits) and lipofuscin (by-products of photoreceptor outer-segment degradation, including N-retinylidene-N-retinylethanolamine [A2E]), as well as other products of oxidative stress such as advanced glycation end products, are thought to initiate inflammation by multiple pathways, like the complement cascade and the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome.(66) The NLRP3 is an intracellular sensor that detects a broad range of microbial

motifs, endogenous danger signals, and environmental irritants, resulting in the formation and activation of the NLRP3 inflammasome. Assembly of the NLRP3 inflammasome leads to caspase 1-dependent release of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18, as well as to gasdermin D-mediated pyroptotic cell death.

The Complement System. The complement system is part of the innate immune system and when properly working assists in favorable immune responses. Increased immune activity through the complement is advantageous in reducing, for example, the risk of infections. The complement system also has an important role in maintaining healthy tissues by clearance of apoptotic cells via phagocytosis, and non-immunological roles such as neuronal synapse remodelling, lipid metabolism, and bone remodelling.(66) Tight regulation of this system is, however, needed to protect the body's cells from tissue damage. Chronic dysregulation and overactivity of the complement can be damaging to vulnerable cells such as those in the retina, which will contribute to AMD development and progression in susceptible individuals.(32)

The complement cascade consists of 3 different converging pathways, each activated by different factors: classic (antigen-antibody complexes), lectin (polysaccharides on microorganisms), and alternative (pathogen cell surfaces). In addition, the alternative pathway continuously undergoes spontaneous self-low-level activation and acts as an amplification loop for the classical and lectin pathways. The central component of the complement system is C3, which is cleaved into C3b and C3a. C3b is a crucial component of both C3 and complement component 5 (C5) convertases that catalyze further steps in the cascade. On host cells, endogenous factors shut down the cascade, but on pathogens, the cascade continues with the cleavage of complement factor C5. The final step of this system is the formation of the membrane attack complex (MAC), which includes several copies of C9. Factor H (FH) is one of the main inhibitors of complement through binding of C3b and aiding its degradation by serine protease Factor I (FI) (Figure 3).(55) Factor H thus leads to decreased complement activity and helps in preventing its overactivation. The compromise of this regulatory function tilts the balance towards amplification and excessive activation, leading to a pro-inflammatory state. Deleterious *CFH* gene mutations have been associated with a broad spectrum of pathological conditions including membranoproliferative glomerulonephritis, atypical hemolytic uremic syndrome, and of course AMD. The same holds for Factor I. (50, 60) CFI is a rate-limiting enzyme of complement termination and *in vitro* studies showed that increasing CFI concentration by 25% can effectively shut down activation of the alternative pathway. Thus, a small change in CFI activity or abundance caused by genetic variants may have significant effects on complement activity.(66) In fact, several mutations in complement genes (including *C3* and *C9* besides *CFH* and *CFI*) that lead to ongoing activation of the complement system and loss of regulatory activity were identified in association with AMD.(7) Analyses of donor eye tissue further revealed the presence of complement proteins such as C3 and C5 in drusen of patients with

AMD, and post-mortem studies showed that MAC accumulates in the choriocapillaris and the BrM with increasing age and in AMD.(3)

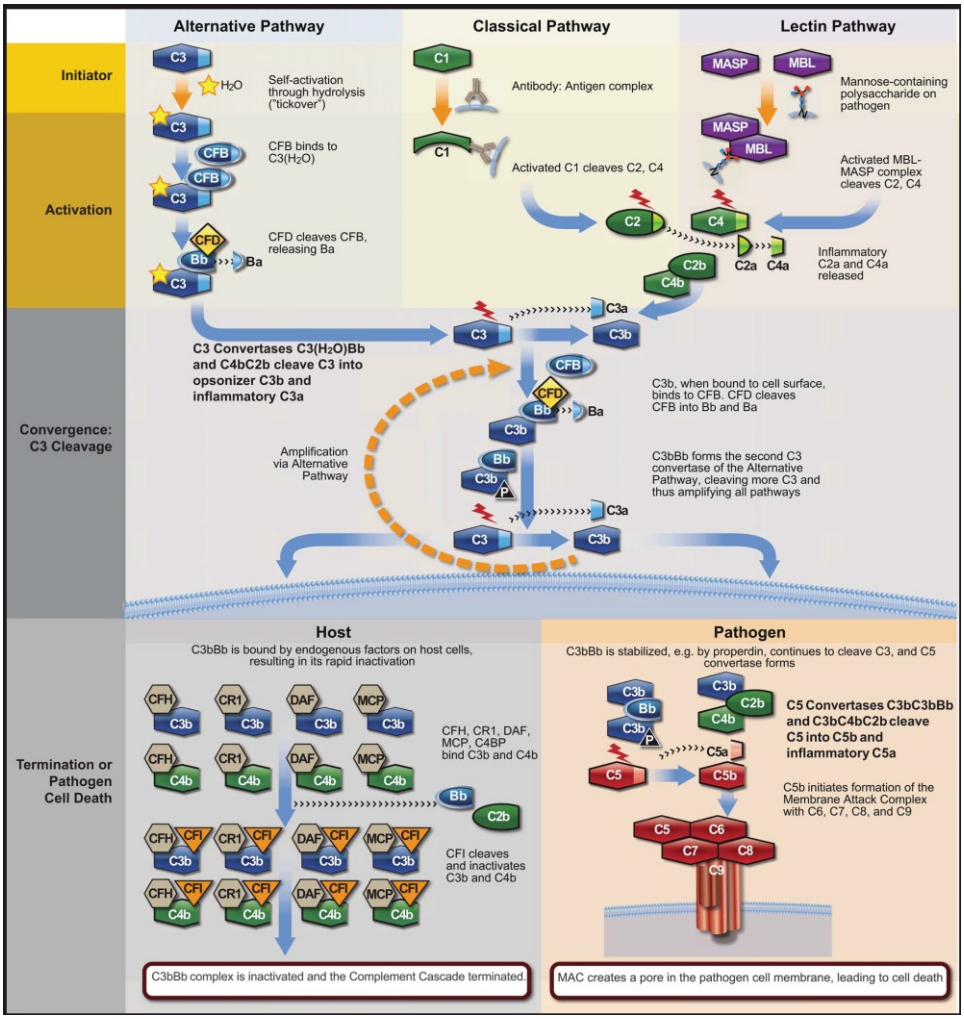

Figure 3: Complement cascade. CFB, complement factor B; CFD, complement factor D; CFH, complement factor H; CFI, complement factor I; CR1, complement receptor 1; DAF, decay accelerating factor; MAC, membrane attack complex; MASP, MBL-associated serine protease; MBL, mannose binding lectin; MCP, membrane cofactor protein. From "THE PATHOPHYSIOLOGY OF GEOGRAPHIC ATROPHY SECONDARY TO AGE-RELATED MACULAR DEGENERATION AND THE COMPLEMENT PATHWAY AS A THERAPEUTIC TARGET." by Boyer DS *et al*, 2017, Retina, 37(5), 819-835. Copyright 2017 by Wolters Kluwer Health, Inc. Reprinted with permission.(66)

Oxidative Stress. Reactive oxygen species are by-products of cellular respiration, and their production is counterbalanced by antioxidants. Oxidative stress results from disruption of this equilibrium and can lead to damage of lipids, proteins, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) molecules.(65)

The retina is a highly metabolically active tissue, an ideal environment for the excessive generation of reactive oxygen species, and consequently is exposed to oxidative stress. These characteristics include high oxygen consumption by the outer retina and RPE, high levels of irradiation, abundance

of photosensitizers, and polyunsaturated fatty acid-rich photoreceptor outer segment membranes susceptible to oxidation, which can initiate a cytotoxic chain reaction.(3)

Lipid and Lipoprotein deposition. The RPE accumulates cholesterol from the ingestion of lipoproteins from the circulation or phagocytosis of photoreceptor outer segments.(3) The secreted lipids from the RPE cells are called "lipid-like particles" and with age, they accumulate and contribute to the thickening of the BrM. The reason for the age-related decline of clearance of lipids from the BrM is not fully understood but macrophages seem to play a role. The continuity of this process eventually leads to the accumulation of debris and drusen formation. Lipids are indeed a main constituent of drusen, and their major ultrastructural component is large ApoB- and ApoE-containing cholesterol-rich lipoproteins secreted by the RPE. Interestingly, the composition of lipids in the BrM is consistent with dietary sources, rather than photoreceptor origin, emphasizing the nutritional role in AMD.(65)

Soft drusen or basal linear deposits are the result of the deposition of metabolites in the subRPE–basal lamina space because of impaired clearance across the BrM–choriocapillaris endothelium. The progression of drusen involves oxidation of lipoproteins and deposition of hydroxyapatite that becomes coated with lipids and inflammatory proteins.(3)

Variants in several lipid-related genes, including *LIPC, CETP, ABCA1,* and *APOE*, are associated with AMD risk, supporting the role of lipid transport pathways in the pathogenesis.(7)

The pathophysiological role of accumulated lipids in AMD is thus unquestionable. For this reason, the modulation of lipid pathways might be a potential target in treatment, either nutritionally or by using lipid-lowering drugs such as statins. Some studies reported encouraging findings with this latter approach, but larger, randomized clinical trials are needed to establish a clear association and benefit. For now, statins are not recommended in the treatment of AMD.(32, 65)

Drusen formation and subretinal drusenoid deposits. Drusen are the hallmark lesion of AMD and the number one intraocular risk factor for severe vision loss. Drusen are usually classified based on their morphology in fundoscopy in hard drusen (small and well-defined), soft drusen (less distinct borders, with size ranging from small to large), and cuticular drusen (numerous, punctate, densely packed).(65) They are composed of lipids, proteins, and minerals. The largest volumetric component are lipids (>40%) including esterified cholesterol, unesterified cholesterol, and phosphatidylcholine. Compositional studies also revealed the presence of carbohydrates, zinc, and nearly 150 proteins including vitronectin, apolipoproteins E and B, and numerous components of the complement system.(67) Drusen typically increase in size and number as the disease progresses, a process that is dynamic, with subsequent regression or reabsorption, that potentially leads to atrophy of the complex photoreceptor-RPE.

Subretinal drusenoid deposits, or reticular pseudodrusen, are a different deposit lesion that was more recently described in association with AMD, as well as with other clinical entities. SDD are located above the RPE cells and beneath the photoreceptors, and their pathophysiology is yet poorly understood. Their composition is also different, with a higher concentration of non-esterified cholesterol, vitronectin, opsins, and agglutinin, among others.(68, 69) In fact, studies demonstrated in SDD the presence of non-esterified cholesterol but undetectable esterified cholesterol, whereas drusen contained both.(67) Further differences between SDD and drusen lipid composition is related to mineralization. In this regard, lipid pools, refractile spherules of hydroxyapatite and calcific nodules presumed to be hydroxyapatite were found in drusen but not in SDD, meaning that mineralization occurs frequently in the former but not in the latter. Finally, vitronectin is a prominent and quantitative marker of SDD.(67) SDD are usually associated with a phenotype of thin choroid and with increased risk of disease progression, with late-stage AMD, and with functional deficits most pronounced within the scotopic range.(3, 65, 70-73)

Impaired ECM maintenance. The BrM is an extracellular matrix mainly composed of elastin and collagen, that provides support for the RPE, serves as a barrier to cellular migration, and facilitates the flow of nutrients and waste products between the RPE and the choriocapillaris.
In AMD there is progressive damage of this ECM with changes in elastin and collagen composition at different stages of the disease. Metalloproteinases (MMP) and the tissue inhibitors of metalloproteinases (TIMP) regulate the dynamic metabolism of the ECM, and the dysregulation of specific MMP or TIMP complexes contributes to pathological changes in AMD.(3) Genes that regulate the ECM structure and function such as *HTRA1*, *MMP9* and *TIMP3* are implicated in the pathophysiology in GWAS, and importantly, most of the risk variants are associated with late AMD.(7)

Choroidal changes in AMD. The highly metabolically active photoreceptors located in the avascular outer retina and the RPE are dependent on the choroidal vasculature for oxygen, nutrients, and waste removal.(74) The thinning of the choroid with age is well documented. In AMD choroidal thinning was found as a risk factor for disease, and marked thinner choroids are usually associated with the presence of subretinal drusenoid deposits.(65, 71)
The role of the choroid in AMD, however, has been controversial. Vascular insufficiency might contribute to the loss of photoreceptors and RPE and accumulation of debris in the BrM, contributing to drusen formation. Lutty *et al* (74) found a linear relationship between the loss of choriocapillaris and the loss of RPE in GA. They reported a 50% reduction in vascular area in regions of complete RPE atrophy in GA, but the areas were never completely devoid of choriocapillaris. The remaining viable capillaries were, however, extremely constricted. The borders of the RPE defects were delineated and overlapped with the areas of decreased vascular

density. Since there were areas with RPE loss at the borders of GA that had a normal choriocapillaris pattern, they suggested that RPE loss occurred before choriocapillaris death.(74)
High levels of TIMP3, a peptidase involved in the degradation of the ECM, are present in thinned choroids of eyes with GA. In addition, there is an increase of MAC in the choroid of AMD patients, but not in the retina or RPE, an important feature considering the role of the complement system in this disease.(65) MAC were found to be even more abundant in the choriocapillaris if the patient had the risk Y402H *CFH* polymorphism, with high-risk histidine homozygotes having over 60% elevated MAC compared to low-risk tyrosine homozygotes, suggesting that an even more aggressive inflammation takes place in the choroid of these cases.(75) The choroid also expresses intercellular adhesion molecule (ICAM)-1, or CD54, which facilitates the recruitment of inflammatory cells. While these mechanisms might be beneficial for the clearance of debris in healthy eyes, they could become detrimental in an AMD scenario, where the removal capacity of accumulated debris is compromised, leading to an excessively pro-inflammatory environment.(65)

Photoreceptor degeneration. All above-described pathophysiological processes disrupt the photoreceptor metabolism, outer segment morphogenesis, and phototransduction, which culminates in photoreceptor cell death, this is neurodegeneration. Both in aging and AMD, rods start to degenerate before cones, suggesting a greater vulnerability of these cells.(3, 74)
The photoreceptor metabolism is maintained by the RPE cells, which also degenerate in AMD. Some authors suggested that loss of photoreceptors precedes loss of the RPE, and others supported this premise when reporting the presence of apoptotic photoreceptors and RPE cells at the borders of expanding GA.(65)

RPE in AMD. The RPE is composed of a single layer of epithelial cells overlying the BrM and in close relation to the overlying photoreceptor outer segments. The RPE is central to the visual cycle, provides metabolic support to photoreceptors, secretes growth factors, and forms the outer blood-retinal barrier.
The RPE cells appear to contribute to the lipid component of drusen. The drusen life cycle of increase in size and number, and subsequent regression, is accompanied by pathological alterations in the RPE, such as detachment of individual RPE cells from their basement membrane and migration into the neurosensory retina. This process manifests as funduscopically visible foci of hypopigmentation and/or hyperpigmentation and heralds the progression toward GA.(3)
Regarding inflammation, a complement regulatory system seems to exist in the complex photoreceptors-RPE-choroid, with complement components including C1qb, C1r, C2, C3, C4, CFB, and CFH. In addition, complement expression is regulated in the RPE by cytokines and chemokines, such as tumor necrosis factor α, interferon-γ, and IL-27.(65)

Geographic atrophy. The pathogenic pathways that cause degeneration of the photoreceptors-RPE-BrM-choriocapillaris complex ultimately may lead to GA development. GA is an advanced form of AMD with loss of well-demarcated areas of photoreceptors, RPE, and choriocapillaris that progress over time causing irreversible loss of visual function. GA is usually bilateral although asymmetric, with half of the patients progressing towards the involvement of the second eye within seven years.(66) GA is often multifocal with a growth rate superior to unifocal cases. The growth pattern is typically foveal sparing, suggesting that the fovea is less susceptible to atrophy than extrafoveal areas. In addition, it was demonstrated that GA progression toward the periphery is almost three times faster than toward the fovea. This supports the concept that rods are preferentially affected in AMD and GA.

The pathogenesis of GA is multifactorial and driven by intrinsic factors (genetic, inflammatory, etc.) and extrinsic factors such as smoking. Dysregulation of the complement cascade appears to play a major role in GA, leading to the idea of "complement inhibition" as a potential efficient therapeutic intervention.(76, 77) Other pathways, such as inflammasome activation, have also been implicated. Activation of the NLRP3 inflammasome can lead to caspase-mediated processing of IL-1b and IL-18, which are mediators of innate and adaptive immunity, and cleavage of gasdermin D that drives pyroptosis, a lytic type of cell death.(66) The interaction between complement and inflammasome pathways, and the roles they play in the pathophysiology of GA, is, however, yet to be fully understood.

Macular neovascularization (MNV). The occurrence of neovascularization in AMD appears to be a trophic response to degenerative photoreceptors and RPE cells, and choriocapillaris rarefaction, in a pro-inflammatory and ischemic environment. However, since this response is "nonspecific", at some point it becomes pathological with fluid exudation into the retina, hemorrhage and fibrosis, and consequent functional impairment with metamorphopsia, scotomata, and rapid visual deterioration. Without treatment, exudative MNV typically results in extensive fibrosis with severe central vision loss.

This 'rescue' mechanism became more apparent in recent years with the introduction of Optical Coherence Tomography Angiography (OCTA) in clinical practice, which allows for non-invasively detecting and follow non-exudative neovascular membranes.(78) Non-exudative MNV was first reported as "quiescent", and defined as type 1 MNV (or sub-RPE MNV) without intraretinal and/or subretinal exudation on optical coherence tomography (OCT) for at least 6 months. "Subclinical" was another term, simply defined as type 1 MNV in intermediate AMD without fluid, a concept that contrary to quiescent MNV had no minimum follow-up without exudation for definition. Subclinical MNV can therefore represent a pre-exudative stage in the development of "normal" exudative type 1 MNV. Besides, quiescent MNV showed low-rate growth over time (monthly rate, 0.64% to 1.07% of the baseline area), low perfusion density in

OCTA, and low rate of activation (monthly rate, 1.17%), a stability pattern that rendered the assumption that they are indeed a different clinical entity in comparison to exudative/subclinical type 1 MNV. Quiescent MNV is probably more "arterialized", with large branches and trunks, and without branching of vascular sprouts, an important factor in providing nutrition to photoreceptors and RPE and preventing degeneration and atrophy.(79)

Capuano *et al* (80) observed with OCTA that quiescent MNV in the context of GA bordered the atrophic areas in all cases, leading to the assumption that the development of quiescent MNV in GA patients could be a protective factor regarding the development and/or further progression of atrophy. These membranes would supply oxygen to the hypoxic outer retina and RPE. They further suggested that these cases should not be treated to preserve this nutritional role. In the same way, Pfau *et al* (81) reported that there was markedly reduced RPE atrophy progression in areas co-localizing with quiescent and exudative type 1 MNV, which was compatible with a potential protective effect of type 1 MNV on the RPE and overlying neurosensory retina. It thus seems that type 1 MNV, this is, MNV in the sub-RPE space, and the most common type in AMD, can slow down photoreceptor and RPE degeneration, by mimicking the role of the choriocapillaris.

At the pathophysiological level, oxidative changes in lipoprotein debris from pro-angiogenic signals lead to vascular growth. This activates microglia to release cytokines which mediate VEGF expression in RPE cells, which in turn, promotes choroidal endothelial cell activation and migration. Conversion from quiescent or subclinical MNV to exudative MNV may be mediated by signaling pathways such as changes in pericyte coverage, and the local balance of vascular trophic factors (VEGF and pigment epithelium-derived factor (PEDF)), or modulation of complement function.(3)

The stimulus leads to the growth of abnormal neovessels, which typically arise from the choroidal vasculature, but can also originate from the retinal vasculature. Recently the Consensus on Neovascular AMD Nomenclature (CONAN) Study Group defined the term macular neovascularization, instead of choroidal neovascularization (CNV), to consider both origins.(82) Type 1 MNV is defined as an ingrowth of vessels from the choriocapillaris into and within the sub-RPE space, type 2 MNV reports to a choroidal origin that traverses the BrM and RPE into the subretinal space, and type 3 MNV is neovascularization originating from the deep retinal capillary plexus (former retinal angiomatous proliferation).(31, 82) Polypoidal choroidal vasculopathy (PCV) or aneurysmal type 1 neovascularization, is an important subtype of type 1 MNV. PCV is defined by a branching neovascular network and nodular vascular dilations, called polypoidal lesions.(83) Traditionally regarded as more common in Asian patients, and clinically distinct from classic AMD, they share, however, some phenotypic and genotypic features.(84, 85)

Implications to treatment. Of all pathogenic pathways, the complement cascade appears to be the most important, however, the first attempts to target this pathway in clinical trials were

unsuccessful.(76) In addition, it is now recognized that the proportion to which different pathways contribute to AMD is variable in different individuals. Pathway-based GRS are generated by weighted summation of the risk alleles for each biological pathway.(8) This approach helps to understand whether different AMD phenotypes represent different predominance of pathways, and potentially identify patients that might require different targeted treatments. The pathways' "weight" was also found to be different in the early and late stages of AMD. For example, the ECM pathway predominates in progression to late AMD. Thus, the specific pathway that is targeted and the treatment provided could be different and dependent on the disease timeline for a given patient. Targeting treatments according to the patients' major contributing pathways or according to specific susceptibility genotypes is probably part of the solution to achieving better results in clinical trials. This point of view underscores the need for genetically characterize different populations, in order to target those who could benefit more from this personalized approach, and for the development of treatments pertaining the most relevant pathways. In fact, there are already phase II clinical trials using a specific genotype in the complement pathway as a criterion for the inclusion of subjects.(56, 77)

1.5. Clinical Presentation, Imaging, and Biomarkers of Progression

AMD is a degenerative disease that primarily affects the photoreceptors, RPE-BrM, and choroid. As shown in the previous section, the pathological changes lead to the formation of drusen (the hallmark lesion of AMD) and pigmentary changes in the macula in early stages, and the development of geographic atrophy and/or macular neovascularization in advanced stages. Plus, SDD or reticular pseudodrusen, a more diffuse form of deposits located above the RPE, is frequently found in association with AMD.(69) The most pronounced pathological changes affect the macula, leading to loss of central vision in advanced disease, however, it is now recognized that they are not limited to this area, and features such as extramacular drusen and reticular pigmentary changes extend into the retinal periphery.(61)

Clinically, the diagnosis is commonly made by fundoscopy alone or, preferably, with the support of imaging exams, based on the presence of typical AMD lesions and associated sequelae in cases of MNV, such as macular edema, hard exudates, intra/sub-retinal or sub-RPE hemorrhages and fibrosis.

Drusen are classified according to size and morphology, and their physical properties and molecular components translate into different characteristics in MMI:

- ***Hard drusen***: extracellular deposits located below the RPE, with less than 63 μm, a punctiform appearance and yellowish-white color in fundoscopy. In fluorescein angiography (FA) and fundus autofluorescence (FAF) no significant changes are usually observed. However, when there is attenuation of the RPE at the apex of the drusen, there is punctiform hyperfluorescence due to window defect in FA and hypoautofluorescence in FAF. In OCT they have a prolate appearance but correlation to fundoscopy is not absolute, as there may be drusen seen in the fundus without evident structural change in OCT. Hard drusen are considered a sign of normal aging and are not associated with an increased risk of progression to advanced forms of AMD (0.4%).(68)

- ***Soft drusen:*** are located between the RPE basement membrane and the inner collagenous layer of BrM, have a dome-shaped appearance, and a diameter equal to or superior to 63 μm. Intermediate drusen (63 to 124 μm), and large drusen (≥ 125 μm) in the macula are characteristic of AMD.(86) They can be further classified as distinct or indistinct. Distinct soft drusen have a nodular shape and well-defined boundaries while indistinct soft drusen have less well-defined limits.(87) In ophthalmoscopy they have a yellow-gray color and may show confluence leading to drusenoid pigment epithelial detachments (PED). In FA, there is minimal hyperfluorescence from staining in the late phase, although this depends on their composition. In FAF, discrete hyperautofluorescence is observed, especially at the edges of the drusen. The typical OCT image is that of a dome-shaped elevation of variable reflective content.(68, 88)

  Soft drusen are associated with an increased risk of GA and MNV. According to the classification proposed by the Age-Related Eye Disease Study (AREDS), the risk of progression to advanced forms depends on the size and laterality of the lesions.(89)

- ***Cuticular drusen (or basal laminar drusen):*** they were recently considered as part of the AMD spectrum and share high-risk SNPs, including rs1410996 (*CFH*), rs10490924 (*ARMS2*), rs4151667 (*CFB*), rs9332739 (*C2*), rs2230199 (*C3*), rs7412 (*APOE E2*), and rs429358 (*APOE E4*).(90) Cuticular drusen, like soft drusen, are deposits located between the RPE basement membrane and the inner collagenous zone of the BrM. They appear as multiple, small (50-75 μm), rounded yellowish-white lesions, scattered throughout the posterior pole and symmetrically distributed in both eyes. They tend to coalesce over time, but they can also disappear and may be associated with acquired vitelliform lesions. FA shows the classic "starry sky" appearance with speckled hyperfluorescence resulting from the apexes of the drusen. In FAF they are characterized by a central hypoautofluorescence surrounded by a ring of hyperautofluorescence. In OCT the "sawtooth RPE" is characteristic, as is the "barcode sign" due to linear posterior hypertransmission from attenuation of the RPE at the apex of the drusen.(88, 91)

In the context of AMD, they are associated with a 25% risk of GA and a 12% risk of MNV. The risk of progression increases if there are associated lesions: soft drusen (45%), vitelliform lesions (24%), or pigmentary changes (47%).(91) In young people, it is important to assess renal function to exclude associated membranoproliferative glomerulonephritis type II.(90)

Subretinal drusenoid deposits or reticular pseudodrusen: these deposits are located above the RPE at the level of the subretinal space, unlike other drusen, hence the name pseudodrusen.
In ophthalmoscopy, they appear as whitish lesions that are difficult to visualize, usually in the superotemporal macula. They may appear as individual round lesions or as extensive reticular networks. In FA they are either not visible or can sometimes be hypofluorescent. In FAF, individual "target"-like lesions with a hypoautofluorescent center and isoautofluorescent border are observed, in a slightly hyperautofluorescent background. Near-infrared reflectance (NIR) is the imaging modality where they are best identified as hyporeflective round lesions. OCT shows conical hyperreflective deposits above the RPE, which may evolve, causing disruption in the photoreceptors layer and the external limiting membrane (ELM).(69)
SDD are an independent risk factor for progression to advanced stages of AMD. They confer an increased risk of developing GA, and GA in multi-lobular pattern and with a higher rate of progression.(92) They are also a risk factor for the development of type 2 and 3 MNV, and a risk factor for the progression of atrophy during anti-VEGF treatment.(72, 93)

Other deposit lesions such as acquired vitelliform lesions and drusenoid PEDs might be found in AMD.

- ***Acquired vitelliform lesions*** are located in the subretinal space and appear as yellow-orange deposits with indistinct limits, mainly in the fovea. FA shows increasing hyperfluorescence without leakage, a pattern that can be mistaken for poorly defined (type 1) choroidal neovascularization. FAF shows intense hyperautofluorescence due to the high lipofuscin content. In OCT, a heterogeneous hyperreflective deposit is observed between the photoreceptors and the RPE. These lesions evolve from growth to subsequent resorption. The risk of vision loss is high in the short-medium term (5-7 years) due to the collapse of the lesion by resorption of the subretinal material, with consequent development of atrophy of the outer retina and RPE (35%), or development of type 1 MNV (10%).(94)
- ***Drusenoid PEDs*** result from the coalescence of soft drusen in one lesion usually larger than 350 μm in the narrowest diameter as defined in the AREDS study (despite the absence of global consensus on the size limit).(95) In ophthalmoscopy, drusenoid PEDs are generally seen as well-defined, pale yellow or white large mounds, preferentially located in the central macula, and frequently with secondary hyperpigmentation. FA shows progressive

hyperfluorescence without leakage. FAF shows iso or hyperautofluorescence, surrounded by a halo of hypoautofluorescence. In OCT, an elevation of the RPE with a generally dome-shaped morphology is observed, which can be "bumpy", with heterogeneously reflective content, and commonly with overlying hyperreflective foci. Subretinal fluid can also be found in association with drusenoid PEDs without MNV, as a result of RPE decompensation and pump failure.(96, 97) There is a high risk of progression to advanced stages of AMD when drusenoid PEDs are present. After 5 years of follow-up, atrophy was observed in 19%, and MNV in 23%.(97)

In AMD pigment abnormalities are seen as **RPE hyperpigmentary changes,** which correspond to areas of dark brown or black pigment on fundoscopy, and/or as **RPE hypopigmentary changes**, which are sharp areas of depigmentation without visible choroidal vessels.(86) Focal areas of hyperpigmentation correspond to localized areas of RPE cell hypertrophy in histology, sometimes together with clumps of hyperpigmented cells in the sub-RPE and subretinal space, or migrating into the outer retina. In this respect, Spectral-Domain Optical Coherence Tomography (SD-OCT) confirmed *in vivo* the deposits' location, which have moderate to high hyperreflectivity, and are commonly seen above drusen and even in more anterior retinal layers.(68) They may also harbor incipient neovascularization, corresponding to type 3 MNV. Focal areas of hypopigmentation correlate with attenuated, depigmented RPE cells, and SD-OCT shows an attenuated signal from the RPE.(68)

Late AMD is defined by the appearance of **GA** and/or **MNV** (previously, choroidal neovascularization).

- **Geographic atrophy** was defined by Bird *et al* (86) almost 30 years ago as the presence of one or more round areas with well-defined boundaries of hypopigmentation, depigmentation, or apparent loss of RPE, with a diameter of $\geq$175 μm measured using a 30º or 35º fundus camera, and with increased visualization of underlying choroidal vessels.(86) More recently the Classification of Atrophy Meeting (CAM) Group reviewed this definition following new insights provided by multimodal imaging, with SD-OCT becoming the basis of RPE (and outer retina) atrophy classification.(98) GA is now considered as a subset of the more comprehensive term *complete RPE and outer retinal atrophy* (cRORA) when MNV is not present.(98)
- **Macular Neovascularization,** the hallmark feature of exudative AMD, refers to the presence of neovascularization arising from the choroid or retina. Based on multimodal imaging there are three types of macular neovascularization: type 1, type 2, and type 3. Type 1 corresponds to a neovascular membrane that grows from the choriocapillaris and proliferates in the sub-

RPE space and in type 2 the neovessels grow further into the subretinal space. Type 3 MNV or Retinal Angiomatous Proliferation, refers to a down growth of vessels from the retinal circulation, namely the deep capillary plexus, toward the outer retina and sub-RPE space.(82, 99) Polypoidal Choroidal Vasculopathy is an important sub-type of Type 1 MNV and is characterized by a branching neovascular network and nodular vascular dilations called polypoidal lesions.(83) Recently the term aneurysmal Type 1 neovascularization has been proposed as an alternative to PCV. Nevertheless, a consensus does not exist if polypoidal lesions are simple aneurysms or more complicated vascular structures.(82)

According to the international classification of the disease, nvAMD is characterized by the presence of at least one of the following findings: subretinal or sub-RPE neovascular membranes, RPE detachments which may be associated with neurosensory retinal detachments, subretinal hemorrhages, hard exudates in the macula, and scar/glial tissue or fibrin-like deposits.(86) Clinically, the first signs of MNV more commonly include hemorrhages, intra or subretinal fluid, and hard exudates. The MNV may appear as a grey lesion located deep in the retina. In cases of Type 3 MNV, parafoveal location with retinal edema, hard exudates, and small spotted intraretinal hemorrhages are common, sometimes with visible deepening of the dilated anastomotic vessel at 90° orientation over a PED. Cases presenting with large sero-hemorrhagic PEDs are more frequent in PCV cases.

Large soft drusen, presence of pigmentary abnormalities, presence of noncentral GA, and presence of advanced AMD in the fellow eye are all well-recognized risk factors for progression to advanced AMD.(89)

From the **functional perspective**, the visual symptoms in AMD vary from mild in early stages, mainly noticed under scotopic conditions, to profound vision loss in advanced disease. Deficits in visual acuity under reduced illumination (low luminance visual acuity) may precede vision loss under normal light conditions. Delayed dark adaptation has been shown to correlate with age, worse visual acuity, presence of SDD, AMD severity, and subfoveal choroidal thinning. Changes are even present before the disease is clinically apparent, and dark adaptometry showed high sensitivity and specificity for diagnosing AMD.(100-102) Microperimetry also reveals greater visual deficits under scotopic conditions in the early stages of AMD.(103) This happens in part because of the predominant loss of rods versus cones in AMD.(31)

In late AMD, the patient with nvAMD may refer to metamorphopsia, reduction in visual acuity, and/or scotomas but a vast majority present with serious and irreversible structural and functional alterations at the time of diagnosis. In GA, large central scotomas develop culminating in legal blindness when they progress to the fovea. The same is true in cases of nvAMD that result in

disciform scarring. In both scenarios, central fixation is lost and an extrafoveal preferred fixation locus may develop.(66)

Imaging in AMD. Nowadays, several exams are available allowing more accurate diagnosis, staging, and prognosis in AMD. This concept defined as **Multimodal Imaging** is now considered standard in AMD, and efforts have been made to implement this methodology in routine clinical practice to improve outcomes, as well as in research and clinical trials.(82, 98, 104)
Historically, color fundus photography was the basis for documenting the clinical diagnosis, and the only method used for AMD staging in large, pivotal, epidemiologic studies, with obvious limitations. Fluorescein angiography is still the gold standard for MNV diagnosis and for classification in subtypes such as classic or occult CNV that had prognostic value, and Indocyanine Green Angiography (ICGA) is the gold standard for PCV diagnosis. In recent years, however, these die-based invasive exams have been progressively superseded by ground-breaking technologies such as the OCT and OCTA. This happened because of their non-invasive nature coupled with relatively fast image acquisition, reliability, and layer-by-layer near-histologic morphological analysis of the retina and choroid, turning them into the mainstay in AMD clinics for routine diagnosis and management.
In addition, with multimodal imaging an extensive amount of morphologic ultra-structural data became easily available, which allowed for the identification of several imaging biomarkers predictive of disease progression and of treatment response in cases of nvAMD.

Color Fundus Photography. Is the historical standard and the most identical to fundoscopy. CFP allows for the detection of AMD lesions such as drusen, pigmentary changes, calcified drusen, crystalline deposits, atrophy, fibrosis, and features related to MNV such as macular edema, hard exudates, and hemorrhages. It has important shortcomings, such as limited value in atrophy diagnosis and in documenting its progression.(104) The initial definition of GA by the International Age-Related Maculopathy Epidemiological Study Group was based on CFP alone but it has been recently updated by the CAM study group, through MMI with OCT serving as the basis for classification.(98) Other important features like SDD, are more difficult to detect with CFP. Bats *et al* (105) reported that the sensitivity for the detection of SDD was only 33,1% in CFP, compared to 99.3% with SD-OCT, 84.6% with NIR, 87.1% with Multicolor Imaging (MCI), and 73.2% with FAF. Hogg *et al* (72) also compared the ability of the different imaging methods to demonstrate SDD. They were visualized best in the FAF or NIR images, while the sensitivity of CFP was only 36%.(72) Gil and colleagues (73) also shown that the most sensitive image modality for SDD was NIR (93%), followed by FAF (92%) and OCT (74%), and the lowest was CFP (29%). However, CFP is still essential in clinical practice and AMD clinical trials to accurately diagnose AMD lesions such as hemorrhages and focal pigmentary changes, for example.

Important biomarkers of progression derived from systematic CFP analysis include: the number, area, and extent of drusen and the presence of pigmentary changes.

Spectral-Domain-Optical Coherence Tomography. OCT is now a widely available and indispensable technology for AMD diagnosis and management. It allows the high-resolution cross-sectional and *en-face* morphologic visualization of the retina and choroid, in close correlation to histology. OCT became the basis for the new consensus on disease definitions in atrophic and nvAMD, instead of the historical CFP.(82, 98) In early AMD, it allows the accurate identification of features like drusen, SDD, and hyperreflective foci (that correlate with hyperpigmentation in CFP) which have prognostic value, and is essential to monitor the eventual progression towards atrophy and/or the development of MNV.

Differences in the reflectivity of drusen in OCT suggest their variable composition and possibly different patterns of progression. These reflective patterns within drusen are termed **Optical Coherence Tomography–reflective Drusen Substructures.** Veerappan *et al* (106) have identified four subtypes: low-reflective cores, high-reflective cores, conical debris, and split drusen. They also found that split drusen and low-reflective core ODS mostly transitioned to high-reflective cores, which then progressed into conical debris, before finally regressing and disappearing. They herald the development of atrophy (but not of nvAMD), and the conical debris is associated with the most rapid progression to GA, followed by high-reflective cores, split drusen, and low-reflective cores. No focal color photographic correlate for conical debris was identified.(106)

Hyperreflective foci located above the RPE and identified by OCT are important to recognize because it was shown that their presence also predicts progression to GA.(107, 108) Hyperreflective foci are thought to represent RPE cells migrating into the inner nuclear and inner plexiform layers of the retina, or macrophages or microglial cells that ingested RPE organelles, thus taking on their reflective properties.(97, 107) OCT also definitely established the location of reticular pseudodrusen as **subretinal drusenoid deposits** above the RPE, and it has high sensitivity and specificity in the detection of these lesions which have prognostic implications, as explained above.(69)

Improved segmentation algorithms currently allow for thickness measurement of almost all retinal layers, providing quantitative biomarkers of retinal degeneration associated with AMD progression.(109) This function, however, is still hampered by the distortion of the retinal anatomy in AMD cases, making manual adjustments needed in most cases of "automated segmentation".

Important information such as the presence of the **double-layer sign** (when the RPE band elevates from the BrM), which may represent **non-exudative MNV**, is given only by OCT. Narita *et al* (110) reported in this regard that when the double-layer sign presented as an RPE elevation with the greatest transverse linear dimension ≥1000 μm, an irregular RPE layer, a height of

predominantly <100 μm, and non-homogenous internal reflectivity, non-exudative MNV was probable. They termed these collective features as Shallow, Irregular RPE Elevation, this is SIRE.(110) In these cases, adding OCTA is very helpful in demonstrating the MNV flow non-invasively.

In **late AMD OCT** is essential to the accurate diagnosis of both atrophy and MNV. In nvAMD, fluid presence and location are easily assessed, documenting exudation and indication to treat. Monitoring and retreatment decisions are quick and easily supported by OCT/OCTA. The presence of PED and their nature (serous, fibrovascular, hemorrhagic), retinal hemorrhages location relative to the neuroretina and RPE, presence of subretinal hyperreflective material, and fibrosis are other features identified with high accuracy with OCT, improving treatment decisions and establishing prognosis.

OCT also became important to differentiate **nvAMD subtypes**, replacing the historical FA-based classification of classic/ occult CNV. Type 1, 2, and mixed MNV is defined by the location of the fibrovascular component below and/or above the RPE band, respectively.(82, 99) Type 3 MNV is suggested when there is a punctate or focal intraretinal hyperreflective lesion located in the outer retina above the external limiting membrane, overlying an area of outer nuclear layer thinning and photoreceptors loss or overlying a PED. It is sometimes possible to observe the focal break in the RPE band of the PED through which a continuous hyperreflective band connects the intraretinal component to the sub-RPE component. Another distinctive feature is the significant intraretinal fluid extension in the outer plexiform layer compared to the amount of subretinal fluid (which in contrast is more abundant in type 1 and 2 MNV).(111) As for PCV the gold standard for diagnosis is still ICGA, but several features were identified in OCT in a consensus study group to reduce the need for ICGA or at least to identify which cases should undergo the invasive dye-based exam. These features include the presence of notched PEDs or sharp and peaked PEDs, complex and multilobular PEDs, round or ovoid ring-like lesions under and attached to the RPE, and the double-layers sign, among others.(83)

In **atrophic AMD or GA,** OCT is now used as the basis for a new classification system from the CAM Group.(98) GA became a subset of the more comprehensive term cRORA, but now restricted to the cases of atrophy in the absence of a contiguous MNV (present or previous), and cRORA encompassing macular atrophy both with and without associated MNV.(98) On the other hand, ***nascent GA***, a biomarker of progression to GA, was redefined as incomplete retinal pigment epithelium and outer retinal atrophy (iRORA) (again in the absence of MNV). Presence of nascent GA or iRORA requires subsidence of the inner nuclear layer and outer plexiform layer, or a hyporeflective wedge-shaped band within the Henle fiber layer as evidence of photoreceptor loss (plus, RPE change and hypertransmision, thought they are not mandatory).(112)

Finally, the **Enhanced Depth Imaging** acquisition technique with SD-OCT and the introduction of **Swept-Source OCT** (SS-OCT), which has higher scanning rates and wider scan areas and uses

a longer wavelength light source with better tissue penetration, provide a detailed analysis of choroidal features in AMD. Choroidal thinning for example is many times observed in association with advanced AMD and SDD, while PCV is many times associated with pachychoroid.(71, 113, 114)

Optical Coherence Tomography Angiography. OCTA is especially relevant in AMD in the context of suspected neovascularization. In clinical practice, it has already replaced conventional FA/ICGA in many situations, although the latter remains the gold standard. MNV presents in OCTA as a well-circumscribed high-flow network with larger feeder vessel trunks, fine arborizing vessels in the periphery, and anastomotic loops or arcades at vessel termini, producing patterns described as "lacy wheel," "sea-fan," or "medusa head"; or poorly circumscribed, irregular vascular networks, said as "filamentous," "tangled," or "dead tree".(115) This noninvasive novel technology may allow for early detection of MVN flow even before exudation takes place, aiding in the detection and monitoring of *quiescent* and *subclinical* MNV, and providing at the same time information on retinal structure in conventional OCT B-scans. Analysis of the MNV network complexity and morphology during treatment provides clues on the level of activity, with a set of changes occurring before reactivation is observed in conventional SD-OCT, although not yet fully validated.(116) Routine use of OCTA also allowed for the identification of a subset of MNV visible both in OCT and OCTA, with a limited amount of subretinal fluid, but without leakage on FA. These cases were termed *subthreshold MNV*, and correspond to a more indolent, mature, and stable neovascular network, better managed by observation.(117)

OCTA has shown value in diagnosis and monitoring of non-exudative AMD. Reduction in choriocapillaris flow signal was observed in early/intermediate AMD cases, and the areas surrounding GA were found to have a significantly higher flow impairment compared to more peripheric areas. This supported previous pathophysiologic findings of GA development on a background of choroidal microvasculopathy and supports the use of OCTA-derived biomarkers in monitoring GA progression.(3)

Limitations of OCTA in nvAMD include the lack of information on vascular integrity (e.g., leakage), which, however, is many times counterbalanced by prompt visualization of intraretinal/ subretinal fluid in the accompanying structural OCT scans.(104)

Fluorescein angiography and Indocyanine green angiography. FA is still the gold standard for the assessment of MNV and provides information on the choriocapillaris in atrophic AMD. FA is the basis for MVN classification used in clinical practice and clinical trials as classic, occult, or mixed choroidal neovascularization, with dye leakage in the late phase as a marker of activity or exudation. FA is still important in differential diagnosis, but it is an invasive method with the risk

of anaphylaxis, time-consuming, and despite providing information on vascular integrity, leakage may obscure lesion boundaries.(104, 118, 119)
As for ICGA, it allows for improved visualization of sub-RPE vascular pathology, supports the differential diagnosis, aids in the detection of nvAMD type 3 MNV, and is still essential for the definitive diagnosis of PCV. However, like FA, it is invasive, there is risk of anaphylaxis, and is time-consuming.(104, 119)

Fundus Autofluorescence. 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 since the loss of RPE and fluorophores in atrophic areas is associated with a decreased FAF signal while dysfunctional areas present with an increased signal. This advantage in accurately quantifying GA led the Food and Drug Administration to approve FAF as a primary outcome measure to quantify changes in GA lesion size over time in clinical trials.(104) Furthermore, different FAF patterns surrounding GA have prognostic implications and are associated with the rate of GA enlargement.(120) 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.(121)
FAF is also useful in the detection of SDD (along with near-infrared imaging and SD-OCT), and in the differential diagnosis of AMD-mimicking macular dystrophies.

Near-Infrared Reflectance. Used in the context of MMI mainly with SD-OCT and FAF, NIR is minimally absorbed by media opacities and macular pigments, thus aiding in foveal assessment (e.g., foveal-involving atrophy). It is also of great value in the detection of SDD with high sensitivity.

Ultra-Widefield (UWF) Imaging. The introduction of UWF allowed for a more frequent visualization of concomitant peripheral lesions in AMD. Extramacular drusen and extramacular pigmentary changes occur in AMD with higher frequency compared to healthy controls.(122) In addition, Seddon *et al* (61) found that peripheral retinal drusen and reticular pigment are associated with AMD and with *CFH* Y402H and *CFH* rs1410996 genotypes. The presence of peripheral reticular pigmentary changes was found to be independently associated with a prolonged time to dark-adapt, a typical functional feature of AMD. Available data thus points to the concept that AMD may be more than a macular pathology and involves the entire retina. Future studies of peripheral changes in AMD with UWF imaging are needed for a better understanding of AMD pathogenesis.(70, 122)

AMD lesions in Multimodal Imaging:

A. Early/ Intermediate AMD

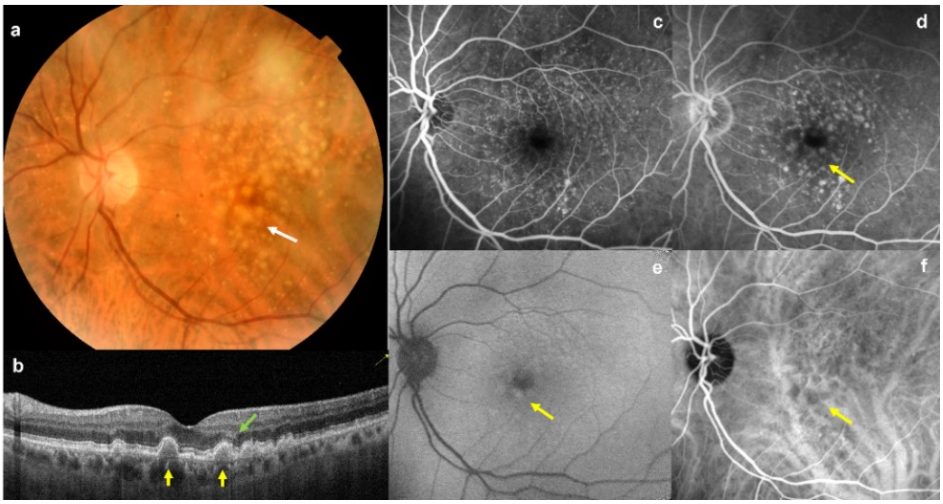

Figure 4. Multimodal imaging of Intermediate AMD. (a) CFP shows soft drusen as yellowish lesions with ill-defined borders in the central macula with a diameter of ≥ 125 µm, some of them are confluent, and hard drusen are also present; (b) On SD-OCT, dome-shaped lesions are visible located below the RPE band, with moderate homogeneous hyperreflectivity (yellow arrows), HRF are seen immediately above some drusen (green arrow); (c) and (d) On fluorescein angiography, soft drusen are initially hypofluorescent (c), with staining in the late phases (d), but with low hyperfluorescence; (e) On FAF, soft drusen are seen as moderately hyperautofluorescent lesions while smaller drusen in the superior macula are hypoautofluorescent; (f). Indocyanine green angiography (ICGA) shows hypofluorescent lesions.

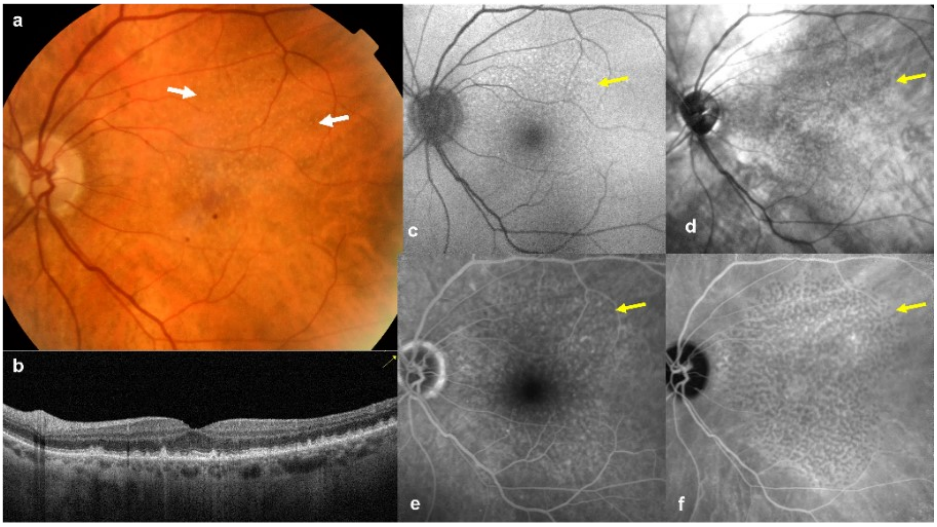

Figure 5. Multimodal imaging of SDD or reticular pseudodrusen. (a) CFP shows whitish lesions that are difficult to visualize, more prominent in the superotemporal macular region (arrows); (b) On SD-OCT, conical hyperreflective deposits are visible on the RPE, some of them causing disruption in the ellipsoid zone and external limiting membrane; (c) and (d) FAF and NIR shows hypoautofluorescent and hyporeflective dot-like lesions, respectively, their distribution is better visualized compared to retinography; (e) In FA they are hypofluorescent through the angiogram; (f) The same happens with ICGA where they are clearly seen.

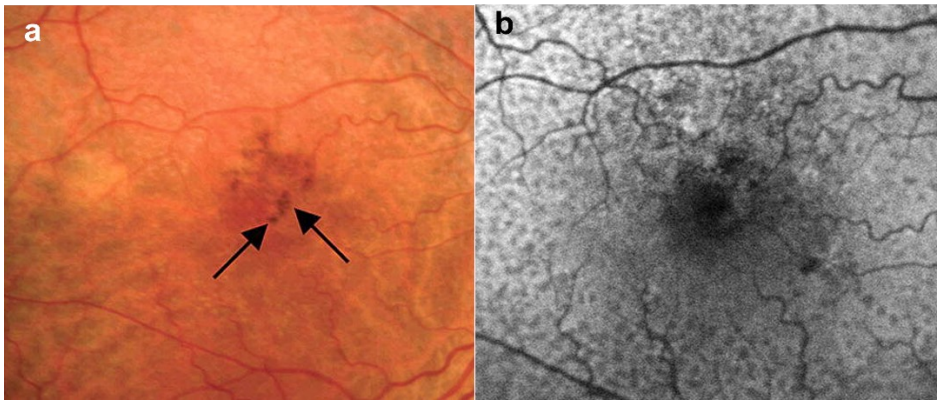

Figure 6. Multimodal imaging of SDD and hyperpigmentary changes. (a) CFP shows the hyperpigmentary changes in the central macula (arrows), while in FAF (b) they are seen as hypoautofluorescent focal lesions. FAF allows to clearly visualize SDD, which are barely seen in CFP.

B. Late AMD – Exudative / Neovascular form

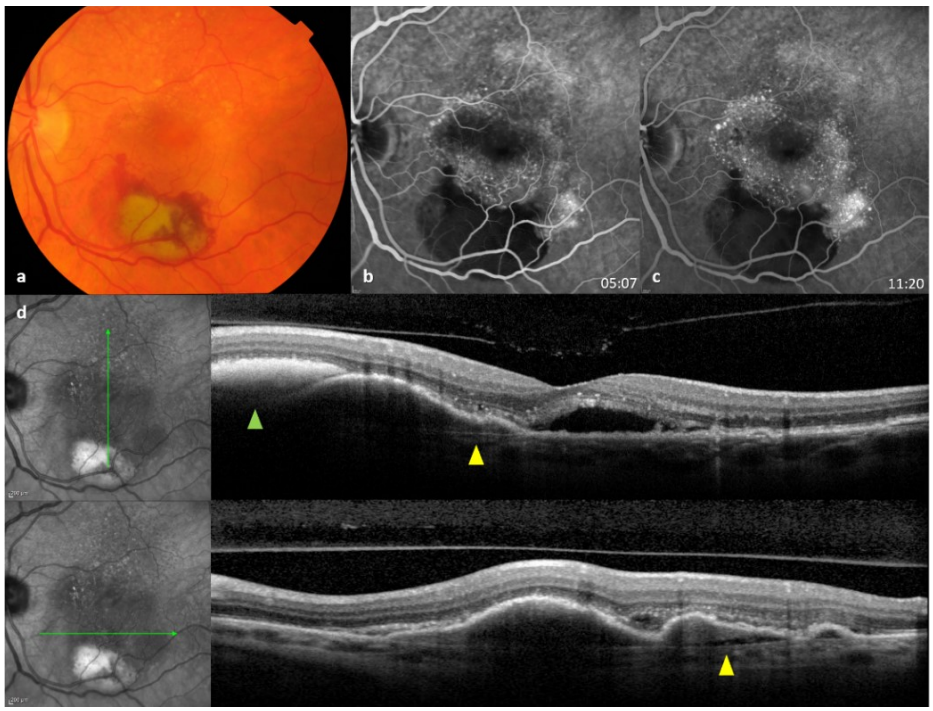

Figure 7. Multimodal imaging of Type 1 MNV. (a) CFP shows the presence of a PED in the central macula with soft drusen and subretinal fluid, and an inferior subretinal hemorrhage with dehemoglobinized blood; (b, c) FA shows occult CNV with fibrovascular PED pattern as an irregular area of stippled hyperfluorescence in intermediate transit phase, with persistent staining and discrete leakage in late phase; there is also blocked fluorescence of the choroid from the sub-retinal blood; (d) SD-OCT scans show the presence of the fibrovascular PED corresponding to type 1 MNV, seen as irregularly elevated RPE with underlying heterogenous laminar tissue, separating from the Bruch's membrane; there is a fluid cleft beneath the fibrovascular tissue (triple layer sign) (yellow arrows). Subretinal fluid involving the foveal center and a subretinal hemorrhage, that is hyperreflective and blocks visualization of the RPE and choroid (green arrow), are also seen.

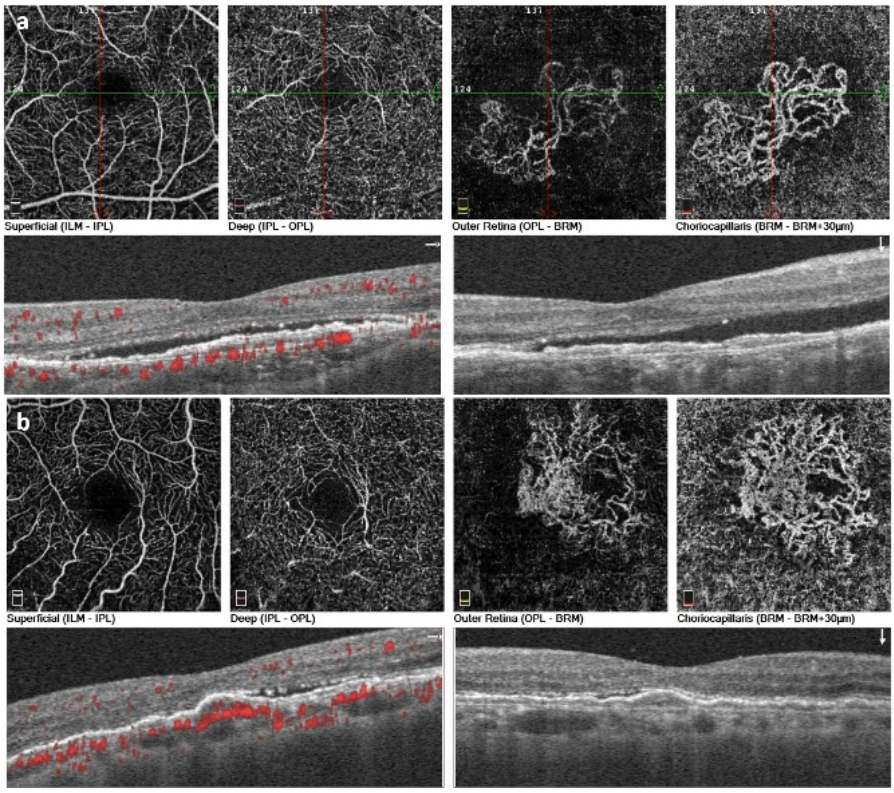

Figure 8. OCTA 3x3mm *en-face* and B-scans with superimposed flow (in red) of two cases of AMD related type 1 MNV. (a, b) In both cases the *en-face* reconstruction shows more clearly the neovascular network in the choriocapillaris slab segmented bellow the RPE; the simultaneous B-scan allows for identification of the double layer sign, corresponding to the MNV and of subretinal fluid, a marker of lesion activity. *En-face* images show the MNV typical lacy pattern with peripheral arcades and loops, and surrounding hyposignal halo, which are probably biomarkers of activity status.

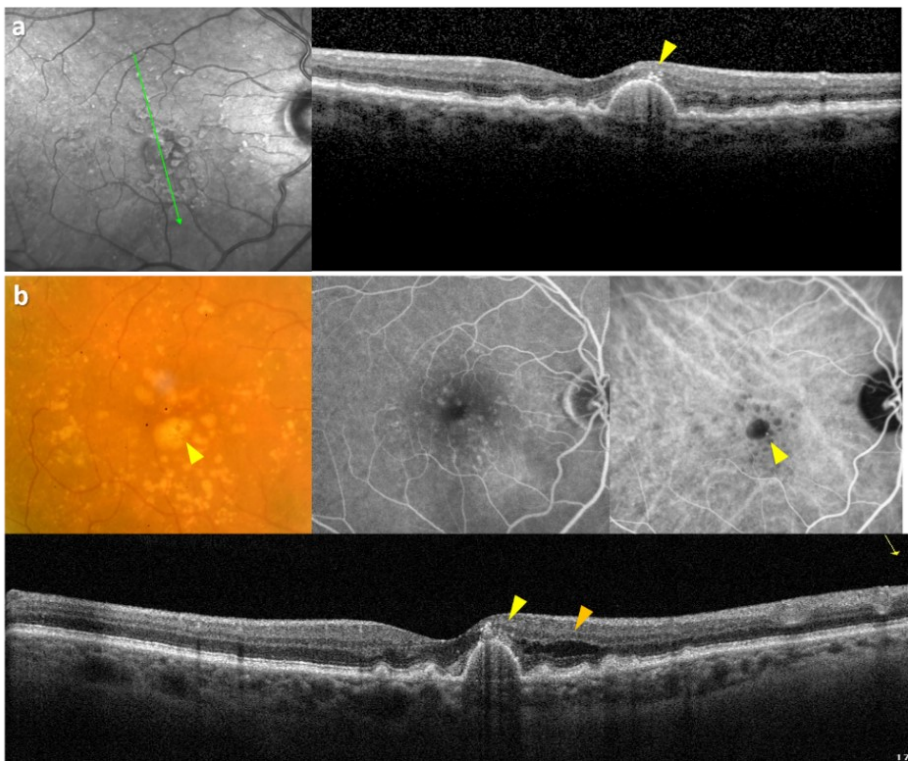

Figure 9. Multimodal imaging of stage 1 Type 3 MNV. (a) At baseline the SD-OCT scan shows drusenoid-like PED with relatively homogenous content and HRF overlying the RPE (yellow arrow). (b) after 2 months, one can see in the same orientation SD-OCT scan the disruption of the RPE below the HRF, with an ill-defined band of increased reflectivity extending to the RPE, accompanied by the development of cystoid spaces from intraretinal fluid (orange arrow, bottom row); *upper row:* CFP shows only increased pigmentation in the area above the soft drusen and ICGA (top, right) shows a small hot spot representing the incipient RAP lesion (yellow arrow).

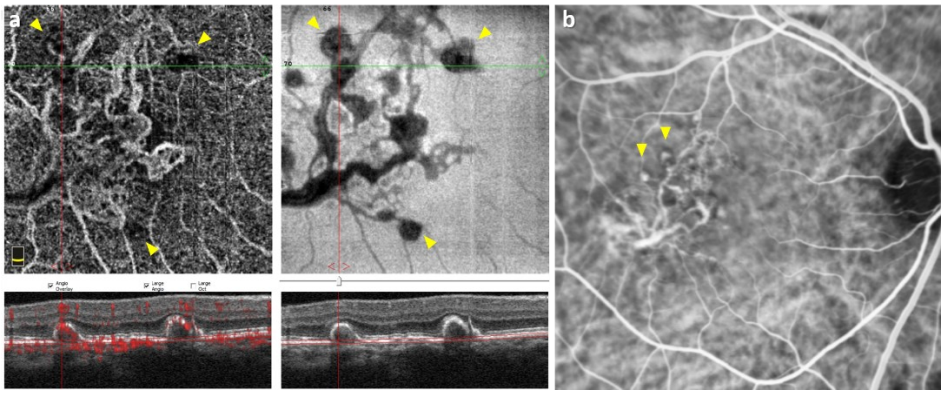

Figure 10. Macular PCV lesion in OCTA and ICGA. OCTA *en-face* image in the left (a) shows the branch neovascular network or type 1 MNV with more detail compared to ICGA (b), however the polypoidal lesions are devoid of flow in the choriocapillaris slab where the MNV is best seen, and appearing as round hyposignal structures (yellow arrows); in the B-scan image with super-imposed flow (a, bellow, in the left) one can appreciate the presence of flow (in red) in the top of the peaked RPE elevations corresponding to the polypoidal lesions in ICGA (b). The *en-face* structural OCT (a, above, in the right) shows very nicely the branching neovascular network and the polypoidal lesions in close correspondence with the observed in ICGA (b).

C. Late AMD – Geographic Atrophy

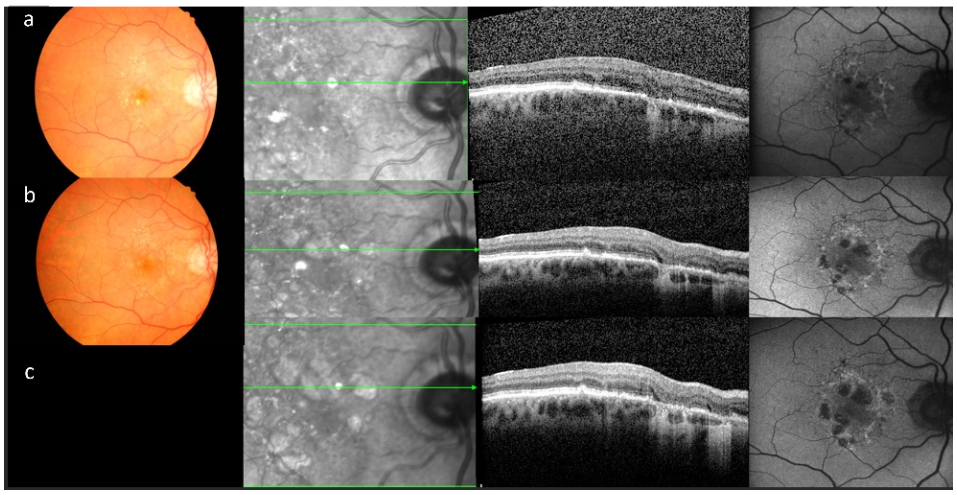

Figure 11. GA evolution in CFP, SD-OCT, NIR and FAF imaging. GA atrophy progression during follow-up is clearly depicted with accuracy with MMI, namely with SD-OCT where cRORA development and progression is detected (a,b,c); FAF clearly shows the progressive enlargement of atrophy and the surrounding hyper-hypo FAF reticular pattern is predictive of future progression. NIR is useful in depicting atrophy that involves the fovea and is masqueraded in FAF due to macular pigments.

1.6. Classification of AMD Lesions and Staging Systems

Through the years several classification systems were developed to assess AMD severity. These were designed by different study groups around the world, and usually they are not completely interchangeable, although some attempts at uniformization have been made.

Drusen size and presence of pigmentary changes are usually the features used to define early and intermediate AMD stages, while the presence of nvAMD and/or GA determines the most advanced stage of late AMD. The size of drusen was defined as the basis of classification in early/ intermediate AMD since it is an objective, quantifiable feature, and the risk of developing late AMD was shown to be greatest if drusen were large in area and associated with pigmentary changes.(20, 22, 123, 124) These classification systems used in clinical practice but also, in clinical trials and epidemiologic studies were, however, developed based in CFP alone. This is currently a major handicap, as MMI provides a much more detailed and complete information on AMD pathophysiology and is superior on lesion detection and characterization (e.g., SDD, geographic atrophy, MNV type).

The establishment of a standardized methodology for AMD classification started with the development of the *Wisconsin Age-Related Maculopathy Grading System* (WARMGS), followed by the *International Classification and Grading System for Age-Related Maculopathy and Age-Related Macular Degeneration* (ICGS-ARM/AMD), which were an important milestone in epidemiological studies.(86, 125) Based on these two classifications, several disease severity scales were elaborated, such as those used in the AREDS and Rotterdam Study, for example.(22, 126)

The AREDS classification of AMD lesions refers to both eyes 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. In the AREDS classification the characteristics of early and intermediate AMD (size, area and type of drusen and pigmentary anomalies), their definition, and the levels of severity for each characteristic were very similar to the WARMGS and ICGS-ARM/AMD classifications. This severity scale of AMD lesions comprises 9 stages and is very detailed and complex, which led to the subsequent development of a simplified 5-level severity scale to facilitate the use in clinical practice and to ease the calculation of risk of late AMD.(89) Finally, the AREDS defined a four-step AMD severity scale for participants, ranging from no or minimal age-related macular degeneration to advanced AMD, as part of the study eligibility criteria.(126)

In AREDS a special review of eyes that appeared to have late AMD features that regressed in longitudinal evaluation was conducted. Researchers found that in 71 eyes differences in photography across visits and/or grader error caused the discrepancy. Of these 71 eyes, 41 were considered “false positives” for advanced AMD at the earlier visit and 30 were considered “false negatives” at the later visit.(126) This finding is very relevant as it demonstrates not only the

inherent possibility of error in grading from normal intergrader and intragrader variations, but also that these mistakes are probably enhanced by using CFP alone, instead of the full range of information provided by MMI. So far, the impact of using MMI to support existing AMD classifications and staging systems has not been addressed. This would be of great interest to explore to increase accuracy in AMD grading and to avoid errors that impact clinical trials and epidemiologic data.

In the Rotterdam Study, a major European epidemiologic study, another severity scale was developed for staging of AMD lesions. Early AMD was defined as the presence of either soft distinct drusen (≥63 μm) with hyperpigmentation and/or hypopigmentation of the RPE or soft indistinct or reticular drusen with or without pigmentary irregularities. To study the progression, and to enhance clinical application the fundus signs were stratified into 5 mutually exclusive stages. Stages 2 and 3 correspond with early disease while stage 4 is equal to advanced disease (atrophic or neovascular AMD).(22) The Rotterdam severity scale and its definitions on early and late AMD was the system used in the Coimbra Eye Study for the estimation of AMD prevalence in the central region of Portugal and later in the subsequent Incidence study.(25-27)

Recently, in the hopes of establishing a unified classification easier to adopt in clinical practice and with prognostic value regarding progression, the Beckman Initiative for Macular Research Classification Committee presented a modified clinical classification. Major updates with this new AMD classification were: small drusen (<63 μm) were defined as "*drupelets"* to distinguish normal ageing-associated changes (with no increased risk of progression to AMD) from actual pathological drusen; SDD were excluded; the presence of at least 1 drusen ≥ 63 μm is always necessary for the pigmentary changes to be considered AMD-related; and any GA within two optic disc diameters around the fovea was considered as late AMD.(123)

These classification systems are presented in detail in Tables 2, 3 and 4.

Table 2. AREDS classification (126)

AREDS classification	
• **No AMD Category 1**	- No or small drusen (<63 μm) - Total drusen area <125 μm diameter - No pigment abnormalities
• **Early AMD Category 2**	*Presence of one or more of the following:* - Small (<63 μm) or medium-sized (≥63; <125 μm) drusen - Total drusen area ≥125 μm diameter - Pigment abnormalities consistent with AMD, defined as one or more of the following, in the center or inner subfields: a. depigmentation. b. increased pigment ≥125 μm. c. increased pigment present and depigmentation at least questionable.
• **Intermediate AMD Category 3**	*Presence of one or more of the following:* - Large drusen (≥125 μm)

	- Soft, indistinct drusen, drusen size ≥63 μm and drusen area > 0.241 disk diameter (I-2) - Soft, distinct drusen, drusen size ≥63 μm and drusen area > 0.439 disk diameter (O-2) - Non-central geographic atrophy (outside 500 μm radius from foveal center).
• **Advanced AMD Category 4**	*Presence of one or more of the following:* -Geographic atrophy in central subfield with at least questionable involvement of the center of the macula - Evidence of neovascular AMD: a. fibrovascular/serous pigment epithelial detachment b. serous (or hemorrhagic) sensory retinal detachment c. subretinal/sub-retinal pigment epithelial hemorrhage d. subretinal fibrous tissue (or fibrin) e. photocoagulation for AMD.
Note: Second eye must be same category or bellow.	

Table 3. Rotterdam Study Severity Scale (22)

Classification of Mutually Exclusive Stages of ARM	
Stage	**Definition**
0	a No signs of ARM at all b Hard drusen (< 63 μm) only
1	a Soft distinct drusen (≥63 μm) only b Pigmentary abnormalities only, no soft drusen (≥63 μm)
2	a Soft indistinct drusen (≥125 μm) or reticular drusen only b Soft distinct drusen (≥ 63 μm) with pigmentary abnormalities
3	Soft indistinct (≥125 μm) or reticular drusen with pigmentary abnormalities
4	Atrophic or neovascular AMD
Abbreviations: AMD, age-related macular degeneration; ARM, age-related maculopathy.	

Table 4. AMD Clinical Classification (123)

Clinical Classification	
Classification of AMD	**Definition (Lesions assessed within 2-disc diameters of fovea in either eye)**
No apparent aging changes	No drusen and No AMD pigmentary abnormalities*
Normal aging changes	Only drupelets (small drusen <63 μm) and No AMD pigmentary abnormalities*
Early AMD	Medium drusen (≥ 63 μm and ≤125 μm and No AMD pigmentary abnormalities*
Intermediate AMD	Large drusen > 125 μm and/or Any AMD pigmentary abnormalities*
Late AMD	Neovascular AMD and/or Any geographic atrophy
AMD = age-related macular degeneration. **AMD pigmentary abnormalities = any definite hyper- or hypopigmentary abnormalities associated with medium or large drusen but not associated with known disease entities.*	

In the **Coimbra Eye Study,** the Epidemiological Study (NCT01298674) and the AMD Incidence Study (NCT027048824) provided information on AMD prevalence and incidence. In both studies classification of digital color fundus photographs was performed in a platform (RetmarkerAMD

Research, Retmarker SA, Coimbra, Portugal) developed to assist manual grading of lesions according to the *International Classification and Grading System for Age-related Maculopathy and Age-related Macular Degeneration* from the International ARM Epidemiological Study Group.(86) After the classification of images with this software, AMD staging was performed according to the *Rotterdam stratification system* of AMD signs.(22)

Advanced imaging-based AMD classification systems. With the introduction of high-resolution imaging such as SD-OCT and OCTA and of *en-face* imaging such as FAF, refinement in the classification of AMD lesions such as atrophy or neovascular disease emerged as new pathophysiologic mechanisms were uncovered. This task was actively pursued by several groups of retina experts around the world, from which important classification consensus were established in the last decade. This was done to incorporate new information regarding pathophysiology and for accuracy in staging and in predicting progression of AMD. In addition, consensus in the concepts and terminology for phenotypic features is essential to compare outcomes across research and in clinical trials.

Classification of Atrophy Meeting Group (CAM) consensus on dry AMD. Recently, a new classification for dry AMD based in OCT but comprising multimodal assessment including CFP, FAF and NIR imaging for complementary and confirmatory information, was developed by an international group of experts. They considered that photoreceptor atrophy can occur without RPE atrophy, and that atrophy could undergo through different stages. Four terms and histologic candidates were proposed: complete RPE and outer retinal atrophy (cRORA), incomplete RPE and outer retinal atrophy (iRORA), complete outer retinal atrophy (cORA), and incomplete outer retinal atrophy (iORA). Specific OCT criteria to diagnose cRORA were: (1) a region of hypertransmission of at least 250 μm in diameter, (2) a zone of attenuation or disruption of the RPE of at least 250 μm in diameter, (3) evidence of overlying photoreceptor degeneration, and (4) absence of scrolled RPE or other signs of an RPE tear. Plus, cRORA encompasses GA, but the GA term should only be used when cRORA is present without any evidence of an associated neovascular membrane.(98, 112)

Consensus on Neovascular AMD Nomenclature (CONAN) Group. This group composed by an international panel of retina specialists, imaging and image reading center experts, and ocular pathologists, arrived at a consensus nomenclature to define and classify neovascular AMD and its subtypes based mainly on OCT and OCTA and within multimodal evaluation. This group also introduced concepts such as the change from the term “choroidal neovascularization” to “macular neovascularization” to better incorporate neovascular membranes arising from the retinal circulation. Despite, neovascularization in AMD may also occur in the extramacular retina.(82)

The E3 classification for OCT in macular pathology. The E3 consortium developed a new classification system for macular diseases based on SD-OCT. The authors have proposed a complete and standardized terminology and a grading scheme for structural evaluation of macular diseases, including AMD, to be applied in epidemiologic studies.(127)

Limitations in AMD classification and future needs. Despite attempts of uniformization, to date, there is no widespread classification or staging system for AMD universally adopted, and thus comparing epidemiological studies on AMD is still hampered by the differing approaches used. However, important steps were taken, first by the development of the Three Continent AMD Consortium Severity Scale, which was developed by harmonizing the grading of the Rotterdam Study, the Beaver Dam Eye Study, the Los Angeles Latino Eye Study, and the Blue Mountain Eye Study; and afterward by the introduction of the Clinical Classification by the Beckman Initiative for Macular Research Classification Committee.(123, 128)

Another unanswered need is the fact that none of these classifications use MMI, and thus accurate identification of lesions and grading is limited to the observation of color fundus images. Newer classification systems based on OCT were recently introduced but their use in clinical practice and in research, including epidemiologic studies, is still limited.(129) Introduction of multimodal imaging is of paramount importance and probably the next step to develop truly homogenous and highly reproducible grading systems in AMD. Such classification systems will have a higher predictive power regarding progression and in evaluating response to therapies in the pipeline.

1.7. Preventing Strategies, Therapeutic Approaches and Future Directions

AMD is a disease for which no medical treatment is available, except for the late neovascular form since the revolutionary introduction of anti-VEGF agents. No approved therapeutics exist to halt progression in its earliest stages, nor to stop the progression of late atrophic AMD or geographic atrophy.

Prevention. In respect to preventive measures, several studies addressed the effect of both environmental and lifestyle factors, and it is now recommended that adopting a healthy lifestyle, including avoiding smoking, having a balanced diet with Mediterranean-like nutritional composition, practicing physical exercise, etc., to be key factors in delaying the onset of AMD as well as its progression.(32) In respect to nutrition a high consumption of fish, nuts, lutein and zeaxanthin, and the unsaturated omega-3 fatty acids DHA and EPA, is associated with a reduced risk of AMD, whereas the consumption of red meat and trans fats is associated with an increased risk.(32, 37, 130)

The AREDS was a multicenter randomized clinical trial initiated in 1991 on dietary supplements containing vitamin C, vitamin E, zinc, and beta-carotene. In people with unilateral late AMD or bilateral intermediate AMD (including non- central GA), the combination of supplements reduced progression from intermediate to advanced neovascular AMD by 25% over 5 years. The subsequent AREDS2 included supplements with lutein, zeaxanthin, and omega-3 fatty acids, but found no evidence for the latter in reducing AMD progression. Plus, it confirmed that beta-carotene should be excluded because of increased risk of lung cancer. The role of lutein and zeaxanthin in delaying progression was suggested in secondary exploratory analyses.(39, 40, 131)
The AREDS and AREDS2 results ultimately led to the widespread recommendation of supplements containing vitamin C, E, zinc, as well as lutein and zeaxanthin for patients with unilateral late AMD or bilateral intermediate AMD.(131)

Treatment in AMD, the present and the future:

- **Anti-VEGF therapy –** NvAMD accounts for approximately 10–20% of total cases of AMD but is responsible for 80% of cases of severe vision loss.(132) However, since the introduction of anti-VEGF treatments visual impairment due to nvAMD was significantly reduced, by around 50%.(133)

  The Vascular Endothelial Growth Factor or VEGF is a growth factor that acts in the regulation of angiogenesis and vascular permeability. Development of anti-VEGF biologic agents such as bevacizumab, ranibizumab, aflibercept, and more recently, brolucizumab and faricimab were introduced with success and irreversible vision loss was avoided in many cases, but not for all. Bevacizumab is a full-length humanized monoclonal VEGF antibody, and its derivative ranibizumab is a humanized monoclonal antibody fragment that binds all VEGF isoforms. Aflibercept is a recombinant fusion protein that acts as a soluble decoy receptor and is a competitive inhibitor of VEGF, and also binds placental growth factors 1 and 2. Brolucizumab is a humanized single-chain antibody fragment that allows higher molar dosing than the previous anti-VEGF agents. Faricimab is the most recently approved by the *Food and Drug Administration* and the *European Medicines Agency,* and is the first bispecific monoclonal antibody, targeting both VEGF A and angiopoietin 2 (Ang-2).(3, 31, 134) These agents usually need to be frequently injected, from 4 to 16 weeks interval.

  Real-world data consistently showed that despite their success, outcomes in clinical practice are usually inferior to those reported in major clinical trials, especially in the long term.(135-137) This is mainly attributed to suboptimal treatment and the development of complications such as macular atrophy and/or fibrosis.(135, 138, 139) Long-term treatment with multiple intravitreal injections and almost life-long follow-up are needed to maintain visual gains achieved with anti-

VEGF treatment, and the consequence is a significant burden put upon patients, their relatives, doctors, and institutions.

In conclusion, there is an unmet need to optimize treatment to improve outcomes and alleviate the treatment burden in nvAMD. The development of new long-acting drugs, of long-duration drug delivery systems, and of promising new therapies in the pipeline such as genetic therapy might be the answer.(140, 141)

- **Complement-targeted therapies –** As referred in the previous sections the complement pathway plays a key role in the pathogenesis of AMD. However, complement based therapies, such as lampalizumab, were so far unsuccessful, and failed to halt progression of late atrophic AMD.(76) More recently randomized clinical trials (RCT) showed promising results in respect to targeting C3 and C5 of the complement pathway.(142-144)

  The safety and efficacy of avacincaptad pegol ("ACP", Zimura, IVERIC bio Inc, New York, NY), a C5 inhibitor, was assessed in participants with GA in RCTs. Intravitreal treatment led to a significant reduction of GA growth in eyes with AMD over a 12-month period (27.4% for the 2 mg cohort and 27.8% for the 4 mg cohort compared with their corresponding sham cohorts). Plus, because C5 inhibition theoretically preserves C3 activity, it may have safety advantages.(143)

  The intravitreal administration of pegcetacoplan (APL-2, Apellis Pharmaceuticals), which targets the C3, also slowed the progression of GA lesions and this was observed in Phase 3 studies. GA growth at 12 months was reduced by 17% with monthly treatments and by 14% with every-other-month (EOM) treatments, compared with sham, in the pooled analysis. Plus, pegcetacoplan demonstrated greater efficacy in patients with extrafoveal lesions at baseline. A tradeoff, however, was that the rate of new active nvAMD was superior in the treatment arms compared to sham (6% in monthly, 4.1% in EOM, and 2.4% in sham groups).(144)

  Considering their mechanism of action, it has been proposed that the key for success of these complement-targeting therapies is the adequate selection of patients. This may imply, for example, selecting patients with abnormal systemic complement activation or who have specific risk genotypes that promote complement overactivation. Functional studies correlating genotype variations with functional levels of complement and studies focusing on genetic characterization of different populations are thus important in this setting.

- **Genetic therapies –** Genetic therapy in nvAMD and in GA is currently under intense investigation, and the preliminary data available is promising.(77, 140) Gene therapy intervention is focused on the sustained expression of antiangiogenics in nvAMD and of anticomplement proteins in GA.

  In the treatment of nvAMD, the aim is to achieve long-term sustained delivery of anti-VEGF agents through gene therapy-mediated intraocular expression. Hopefully this will significantly

alleviate burden related to conventional anti-VEGF therapy, and/or improve efficacy by targeting additional angiogenic pathways (e.g., PDGF signaling and Angiopoietin-Tie2 signaling).(140)

In dry AMD the objective has been targeting the complement pathway.(77) GT005 (Gyroscope Therapeutics) is a recombinant non-replicating adeno-associated vector therapy that aims to control complement overactivation by increasing the production of CFI. Two Phase II clinical trials, EXPLORE and HORIZON are currently evaluating the safety and efficacy of GT005 administered as a single subretinal injection in subjects with GA. In addition, in the EXPLORE study eligible subjects must have a rare genetic variant in the *CFI* gene (thus with increased risk of having complement overactivation as discussed previously).(56)

Therefore, in this context, targeting treatments according to the patients' major contributing pathways or according to specific susceptibility genotypes could be part of the solution to achieve better results in clinical trials in atrophic AMD and in new therapeutics in nvAMD.(56, 77) In addition, directing treatment to the pathways most affected in early and intermediate AMD, in a ultra-personalized medicine approach, could be another direction to pursue in order to treat AMD before it reaches late stages.

We are now at the verge of a new treatment paradigm shift in AMD, and the most profound and complete the knowledge on AMD pathophysiology the most likely new targeted treatments will be a success, changing the life of millions affected by this devastating disease worldwide.
The importance of contributing to this knowledge, and specifically to the understanding of genotype-phenotype interactions, is thus evident, and is a main objective of this Thesis.

1.8. Unmet Needs and Main Objectives

1. We will briefly describe the population-based Epidemiologic Coimbra Eye Study and present in detail the subsequent AMD Incidence Study, upon which this thesis is based.
   This will be presented in Chapter 2.

2. The AMD Incidence Study is one of the few epidemiologic studies in which multimodal imaging was performed.
   An important question arises: if MMI truly impacts AMD grading and to what extent, and if this translates to real change in AMD epidemiology.
   We will compare how staging of AMD lesions is affected by grading with conventional color fundus photography only or with multimodal imaging grading, and the impact of the consequent changes in AMD prevalence and incidence rates in the CES.

The results of this comparison are presented in Chapter 3.

3. The need for new imaging biomarkers of progression and treatment response in AMD is well-recognized and necessary to achieve true personalized medicine in the future.
   Based in Multimodal Imaging data available from the Incidence Study phenotypic features will be explored in association with AMD. New imaging biomarkers of AMD severity are to be identified in this population-based study. Our findings regarding this topic are presented in Chapter 4.

4. Besides extensive phenotypic characterization, genotypic analysis was pursued.
   We will characterize the population from the Incidence Study from a genetic perspective, in respect to the presence of common and rare variants in pathways associated to AMD, and variants associated to AMD-mimicking macular dystrophies.
   This will be the first time that a Portuguese population will be genetically characterized in respect to AMD.
   This research is presented in Chapter 5.

5. Few studies are available on genotypic-phenotypic associations in AMD, and even fewer use multimodal imaging. Plus, rare variants are known to be associated with high risk of disease and with more severe phenotypes.
   As a final step, we will establish genotypic-phenotypic associations in AMD patients from the Incidence Study who carry rare variants in the complement pathway, namely in the *CFH*, as these patients are more susceptible to complement overactivation.
   Our findings are presented in Chapter 6.

Finally, in the last chapter, a brief discussion will be made with a summary of the overall results and of the future directions of this research.

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CHAPTER 2.
THE COIMBRA EYE STUDY

INCIDENCE OF AGE-RELATED MACULAR DEGENERATION IN THE CENTRAL REGION OF PORTUGAL: THE COIMBRA EYE STUDY – REPORT 5

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2.1. Background

The **Coimbra Eye Study** (CES) is a population-based study developed to address an important scientific gap: *the lack of epidemiologic data on AMD in Portugal*. It comprises two studies: the ***Epidemiological Study*** (NCT01298674) and the ***AMD Incidence Study*** (NCT027048824), which provide important information on AMD prevalence and incidence, respectively.

The ***Epidemiologic Study*** was conducted in 2009/2013. In this first study, 5996 subjects from two primary Portuguese healthcare units (Mira and Lousã) were phenotypically characterized and risk factors for AMD were assessed. In brief, in the Epidemiologic Study, the age- and sex-adjusted prevalence of any AMD for the Portuguese population was 12.48% (95% CI: 11.61–13.33) with late AMD accounting for 1.16% (95% CI: 0.85–1.46). Neovascular AMD and geographic atrophy prevalence rates were 0.55% (95% CI: 0.36–0.75) and 0.61% (95% CI: 0.37–0.84), respectively. This was found to be similar to reports from other large-scale population-based studies. However, when analyzing the Mira cohort alone, the authors found that late AMD prevalence was inferior in this subpopulation to that reported in those large epidemiologic studies.(1, 2)

Another important finding of the Epidemiologic Study was that when the authors analyzed the data by location, they found that the crude prevalence of early and late AMD was 6.99 and 0.67%, respectively, for the coastal town of Mira and 15.39 and 1.29% for the inland town of Lousã. In addition, after adjusting for age, sex, family history, smoking history, hypertension, diabetes, and body mass index, the subjects from the inland town presented a significantly higher risk of early and late AMD than subjects from the coastal town (OR = 2.57, 95% CI: 2.12–3.12, $p < 0.001$ for early AMD, and OR = 2.06, 95% CI: 1.07–3.95, $p = 0.029$ for late AMD). This unexpected finding led the authors to recognize that further analysis would be needed to understand the underlying causes for this difference in AMD prevalence by geographic location. Perhaps the two populations had specific environmental or even genetic characteristics affecting AMD risk in opposite ways.(1, 2) Lifestyle-related factors such as diet and physical activity were explored in subsequent reports of the CES, but no genetic analysis was pursued until now.(3, 4)

The ***AMD Incidence Study*** was conducted in 2016/2018. In this study, 1617 participants from the original cohort of Mira were included. In addition to the procedures performed in the Epidemiologic Study, all participants underwent multimodal imaging evaluation with the objective of extensive phenotypic characterization, and blood samples were collected for genetic assessment. The following section presents in detail the first report of the AMD Incidence Study, which focuses on the incidence of AMD in the coastal town of Mira and is the basis for the development of this Thesis.

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2.2. The AMD Incidence Study

ABSTRACT

Purpose: To describe the 6.5-year incidence and progression of age-related macular degeneration (AMD) in a coastal town of central Portugal.

Methods: Population-based cohort study. Participants underwent standardized interviews and ophthalmological examination. Color fundus photographs were graded according to the International Classification and Grading System for AMD and ARM. The crude and age-standardized incidence of early and late AMD was calculated, and progression was analyzed.

Results: The 6.5-year cumulative incidence of early AMD was 10.7%, and of late AMD it was 0.8%. The incidence of early AMD was 7.2, 13.1 and 17.7% for participants aged 55–64, 65–74 and 75–84 years ($p < 0.001$). The late AMD incidence was 0.3, 0.9 and 2.8% for the corresponding age groups ($p = 0.003$). The age-standardized incidence was 10.8% (95% CI, 10.74–10.80%) for early and 1.0% (95% CI, 1.00–1.02%) for late AMD. The incidence of both neovascular AMD and geographic atrophy was 0.4%. Progression occurred in 17.2% of patients.

Conclusion: The early AMD incidence in a coastal town of central Portugal was found to be similar to that of major epidemiological studies of European-descent populations; however, the incidence of late AMD was lower, and further analysis on risk factors will be conducted.

Key Words: Age-related macular degeneration · Incidence · Epidemiology · Early age-related macular degeneration · Late age-related macular degeneration.

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible visual impairment and blindness in the elderly in developed countries [1]. It is well recognized that global population ageing is expected in the next decades, and therefore, the number of patients affected by AMD is expected to increase significantly not only in developed countries, but also in developing countries. Recent estimates point to a projected number of people with AMD in 2020 of 196 million, and of 288 million in 2040 [2, 3]. In this respect, and while prevalence measures the proportion with disease in the population, the incidence adds value because it allows to plan for demand in health care systems and for establishing preventive measures [4].

To date several population-based studies have provided important information on the incidence for AMD [5– 14]. However, in Europe fewer reports on AMD incidence are available, and none is focused on the southern European countries [3, 15–19]. These countries are known to have distinct sociocultural and economic aspects. For example, subjects from these countries mostly follow a Mediterranean diet, with higher consumption of fish, fruit and vegetables, compared to northern European countries (OECD 2012) [20], and these environmental factors may impact AMD prevalence and incidence [20–22].

Our group previously reported on AMD prevalence in a coastal town and in an inland town of the central region of Portugal [20, 23]. Globally, the prevalence of early and late AMD in Portugal was found to be comparable to other Western and Asian countries [20]. In the subanalysis of the coastal town the early forms of the disease had a similar prevalence to that of other large-scale population-based cohorts, but late AMD was less frequent [23].

The purpose of this first report is to describe the incidence of early and late AMD, as well as the progression over 6 years in the population-based Coimbra Eye Study in Portugal, for the coastal town of Mira.

METHODS

Study Design and Population

Information on the identification and description of the baseline study population has appeared in previous reports [20, 23]. Briefly, the Epidemiologic Coimbra Eye Study is a single-center population-based study whose cohort included two geographically distinct Portuguese populations aged ≥55 years: one from a coastal town (Mira) and the second from an inland town (Lousã), both in the central region of Portugal. Subjects who participated in the baseline analysis for the estimation of AMD prevalence in Portugal (NCT01298674) and recruited from the primary health care unit in Mira between August 2009 and April 2011 were identified, and the surviving cohort was invited to participate in the present follow-up analysis (NCT02748824).

Participants were contacted between June 2016 and December 2017. To ensure a good response rate, two contacts were made by telephone: one to explain the purpose of the new study and to invite individuals to participate and the second one to schedule the appointment at the primary health care center in Mira if they agreed to participate. Subjects who did not attend were offered another date. At the appointment, the study purpose was again carefully explained, and a signed informed consent was obtained.

This study adhered to the tenets of the Declaration of Helsinki (2008) and was approved by the Association for Innovation and Biomedical Research on Light and Image (AIBILI) Ethics Committee.

Data Collection

A detailed questionnaire-based interview was administered to all included subjects by a trained interviewer. The following information was collected: sociodemographic data, medical history (general and ophthalmological), family history of AMD, current medication, nutritional and lifestyle habits (including smoking habits, alcohol consumption, practice of physical exercise). A nurse from the primary health care center evaluated the blood pressure and heart rate, height and weight were obtained using standardized techniques and equipment, with subsequent calculation of body mass index (weight/squared height). Available laboratorial data from the subjects' medical records were obtained, namely the lipid profile and glycated hemoglobin. Blood samples were collected for further analysis.

Ophthalmic Examination

All participants underwent complete bilateral ophthalmological examination. Best-corrected visual acuity was tested in each eye separately using the Early Treatment Diabetic Retinopathy Study chart, and refraction assessment was performed with an autorefractor. Tonometry was performed in all subjects with a noncontact air puff tonometer.

Images from color fundus photography (CFP) were obtained after pharmacological mydriasis. Fields 1M (centered on the optic disk), 2 (centered on the macula) and 3M (temporal to the macula), acquired at 45° for both eyes, were recorded using a digital nonmydriatic Topcon® fundus camera (TRC-NW8; Topcon Corp., Tokyo, Japan). Fundus reflex photographs were taken to document media opacities. Optical coherence tomography (OCT), fundus autofluorescence (FAF) and infrared (IR) imaging of both eyes were also performed for posterior analysis.

Grading Methods

CFP images were exported in the TIFF format, and the OCT, FAF and IR exams were exported as a Heidelberg E2E file to a centralized reading center (Coimbra Ophthalmology Reading Center, AIBILI). All graders were ophthalmologists certified by the reading center according to the study protocol. All exams were analyzed and graded in a step-wise manner.

A general analysis of all included participants was carried out to identify major retinal pathology and to identify suspected AMD cases. This analysis was independently performed by the graders, and reports based on the findings were sent to the primary care physician and the participant.

After this preliminary grading, 4 senior graders (C.F., M.L.C., I.P., J.P.M.) performed a differential analysis and graded the suspected AMD cases using the International Classification and Grading System [24]. Differential grading was performed by grading CFP images alone to allow for comparability with our previous prevalence study of AMD in this population, whose grading was based on CFP only [20, 23]. CFP image quality was adjusted using color balance software, an algorithm developed to automatically standardize the brightness, contrast and color balance of digital color fundus photographs in AMD grading [25]. The differential grading was supported by software developed to visualize digital color fundus photographs and to grade AMD retinal lesions – *RetmarkerAMD Research* (Retmarker SA, Coimbra, Portugal). This platform was developed to assist manual grading of lesions, according to the International Age-Related Macular Epidemiological Study Group Classification [24], and automatically calculates the number of lesions, their size, area and location, as previously
described by our group [26].

At the end of each differential analysis, the graders classified the signs of AMD into 5 exclusive stages (stage 0–4) using the Rotterdam staging system (Table 1) [15, 16]. Staging of an individual participant was based on the eye with severer status if both eyes were gradable, and on the gradable eye if only one eye was gradable.

Table 1. Classification of mutually exclusive stages of AMD according to the Rotterdam classification.

Stage	Definition
0	
a	No signs of AMD at all.
b	Hard Drusen (< 63µm) only
1	
a	Soft distinct drusen (≥ 63µm) only
b	Pigmentary abnormalities only, no soft drusen (≥ 63µm)
2	
a	Soft indistinct drusen (≥ 125µm) or reticular drusen only
b	Soft distinct drusen (≥ 63µm) with pigmentary abnormalities
3	Soft indistinct (≥ 125µm) or reticular drusen with pigmentary abnormalities
4	Atrophic or neovascular AMD

Disease Definitions

Early AMD was defined by the presence of large (≥125 µm in diameter), soft, indistinct or reticular drusen only, or of soft distinct (≥63 µm in diameter), indistinct (≥125 µm) or reticular drusen with pigmentary abnormalities, within the macula, in the absence of signs of late AMD. This definition corresponds to stages 2 and 3 of that proposed in the Rotterdam study and is approximate to that used in the Blue Mountains and Beaver Dam Eye Study [10, 11, 15, 16].

Drusen were categorized based on their appearance, namely size, homogeneity of surface and outlines. Soft distinct drusen were ≥63 µm in diameter with uniform density and sharp edges, and soft indistinct were those ≥125 µm with decreasing density from the center outwards and fuzzy edges. Pigmentary abnormalities were classified into either hypopigmentation (without visibility of choroidal vessels) or hyperpigmentation.

Late AMD was defined by the presence of neovascular AMD (NV-AMD) or geographic atrophy (GA) within the grid (3,000 µm from the center of the fovea). NV-AMD included serous or hemorrhagic detachment of the retinal pigment epithelium or sensory retinal, subretinal or subretinal pigment epithelial hemorrhages and fibrous scar. GA was defined as an area of retinal depigmentation, 175 µm or larger, characterized by a sharp border and visualization of choroidal vessels [10, 11, 24]. When GA and NV-AMD coexisted in the same eye, it was categorized as NV-AMD. This definition of late AMD corresponds to stage 4 in the Rotterdam classification [16].

Statistical Analysis

The cumulative incidence (risk) was calculated as:

$$\frac{Number\ of\ subjects\ developing\ the\ disease\ over\ a\ time\ period}{Total\ number\ of\ subjects\ followed\ over\ that\ time\ period}$$

and reflects the probability that a subject within the population will develop AMD over the follow-up period.

Incident early AMD was defined by the appearance at follow-up of either stage 2 or 3 in either eye of participants in whom no early or late AMD was present in either eye at baseline. Participants with only distinct soft drusen or retinal pigmentary abnormalities at baseline (stage 1), not classified as early AMD at that time, who then went on to demonstrate complementary lesions that together constitute a diagnosis of early AMD were included as incident early AMD cases.

Incident late AMD (stage 4) was defined by the appearance at follow-up of NV-AMD or GA in either eye of patients in whom no late AMD lesion was present in either eye at baseline (this is, without AMD or early disease at baseline). Late disease incidence was also explored in subjects only with no AMD at baseline (this is, without early disease at baseline), and in subjects only with early disease at baseline.

Age-standardized incidence of early and late AMD was calculated for the Portuguese population according to Census 2011 of the National Institutes of Statistics (www.ine.pt).

Demographic and clinical characteristics were summarized using descriptive methods. ANOVA, Student *t* test and χ^2 tests were used to compare groups as well to evaluate the independence of early, late and no AMD, regarding age and gender.

Data were analyzed using STATA software (Stata Corp., College Station, TX, USA), version 12.1 SE. Alpha was set at 0.05 for statistical significance.

RESULTS

The mean and median times between the baseline and the follow-up examination were 6.4 and 6.5 years, respectively. Of the 4,370 eligible individuals, 3,000 participants took part in the baseline examination (68.6% response rate), and the final cohort comprised 2,975 participants after excluding 25 from grading analysis [20, 23]. In the 6.5-year follow-up examination and after excluding 413 participants who died, 123 who were bedridden and 822 who did not participate (e.g., refusal, had moved away, unreachable), 1,617 of 2,439 eligible individuals (66.3%) participated in the follow-up examinations. One participant was excluded because of ungradable photographs of both eyes. The remaining 1,616 participants were included in the analysis (Fig. 1). Demographic characterization of the population included in the follow-up analysis is presented in Table 2.

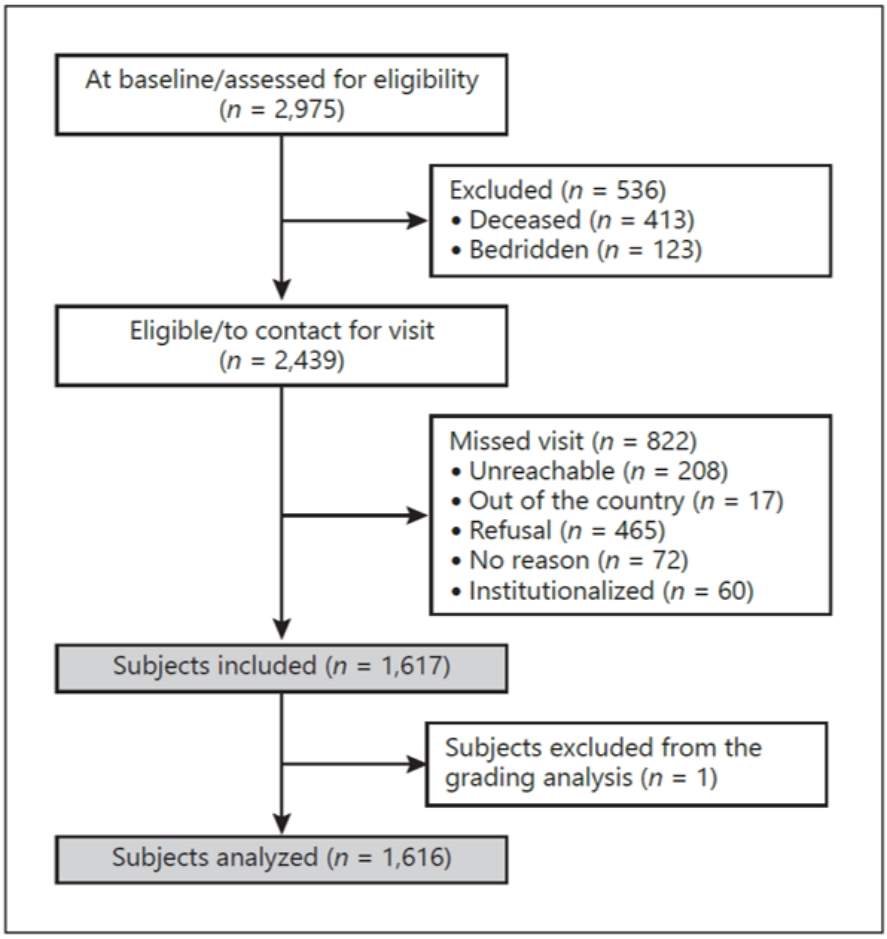

Fig. 1. Flow chart of the study participants.

Table 2. Demographic characteristics of the study population at the follow-up visit (n = 1,616).

Characteristics	Number
Gender	
Male	697 (43.1)
Female	919 (56.9)
Age	
55-64	270 (16.7)
65-74	779 (48.2)
75-84	491 (30.4)
≥ 85	76 (4.7)
Ethnicity	
Caucasian	1608 (99.6)
African	7 (0.4)
Smoking	
Smoker	33 (2.1)
Ex-smoker	214 (13.3)
Non-smoker	1362 (84.6)
Familiar history of AMD	
Yes	7 (0.4)
No	1338 (82.8)
Does not know	271 (16.8)
Diabetes	
Yes	335 (20.8)
No	1255 (77.6)
Does not know	26 (1.6)
Hypertension	
Yes	894 (55.4)
No	692 (42.8)
Does not know	30 (1.8)
Hypercholesterolemia	
Yes	750 (46.4)
No	734 (45.5)
Does not know	132 (8.1)
Hyperlipidaemia	
Yes	73 (4.5)
No	1366 (84.5)
Does not know	177 (11.0)
Menopause	
Yes	917 (99.8)
Does not know	2 (0.2)
Mean BMI ± SD (min–max)	28.1±4.5 (15.8-47.0)

Percentages in parentheses. BMI, body mass index; SD, standard deviation; min, minimum; max, maximum.

Characteristics of participants and nonparticipants at baseline are given in Table 3. Nonparticipants were divided into those who had died and those who were not observed. Compared with those followed up, subjects who had died during the follow-up were older, more likely to be smoker or ex-smoker, to have comorbidities like diabetes and hypertension, and they had a slightly superior prevalence of AMD diagnosis at baseline. Comparing the observed participants to those alive but not observed at follow-up, the latter were significantly older at baseline ($p < 0.001$), and actually there were no participants in the follow-up visit from the age category ≥85 years at baseline. Nonparticipants were also more likely to have hypertension ($p = 0.002$), but no differences were found regarding gender, history of smoking or familiar history of AMD ($p > 0.05$). Nonparticipants had inferior AMD prevalence at baseline, compared to participants ($p = 0.001$).

Table 3. Comparison of baseline characteristics between participants and nonparticipants, at 6.5 years in the incidence study of AMD in Central Portugal.

Characteristics	Observed (n=1,616)	Not observed (n=945)	*p*-value	Died (n=413)
Gender				
Male, n (%)	697 (43.1)	376 (39.8)	0.098	192 (46.5)
Female, n (%)	919 (56.9)	569 (60.2)		221 (53.5)
Age				
55-64, n (%)	764 (47.3)	309 (32.7)	**<0.001**	46 (11.1)
65-74, n (%)	672 (41.6)	338 (35.8)		101 (24.5)
75-84, n (%)	180 (11.1)	259 (27.4)		186 (45.0)
≥ 85, n (%)	0 (0.0)	39 (4.1)		80 (19.4)
Ethnicity				
Caucasian, n (%)	1611 (99.7)	941 (99.6)	0.638	413 (100.0)
African, n (%)	5 (0.3)	4 (0.4)		0 (0.0)
Smoking				
Smoker, n (%)	31 (1.9)	18 (1.9)	0.670	14 (3.4)
Ex-smoker, n (%)	81 (5.0)	40 (4.2)		21 (5.1)
Non-smoker, n (%)	1504 (93.1)	887 (93.9)		378 (91.5)
Familiar history of AMD				
Yes, n (%)	186 (11.5)	118 (12.5)	0.457	35 (8.5)
No, n (%)	1430 (88.5)	827 (87.5)		378 (91.5)
Diabetes				
Yes, n (%)	254 (15.7)	173 (18.3)	0.243	110 (26.6)

No, n (%)	1336 (82.7)	756 (80.0)		293 (70.9)
Does not know, n (%)	26 (1.6)	16 (1.7)		10 (2.4)
Hypertension				
Yes, n (%)	714 (44.2)	483 (51.1)	**0.002**	215 (52.1)
No, n (%)	862 (53.3)	436 (46.1)		174 (42.1)
Does not know, n (%)	40 (2.5)	26 (2.8)		24 (5.8)
Menopause				
Yes, n (%)	908 (99.2)	556 (98.1)	**0.010**	182 (95.8)
No, n (%)	5 (0.5)	2 (0.4)		1 (0.5)
Does not know, n (%)	2 (0.2)	9 (1.6)		7 (3.7)
Mean BMI ± SD (min–max)	28.0±4.0 (16.6-48.1)	27.9±4.4 (16.4-48.8)	0.479	27.9±4.6 (15.7-48.9)
Disease status				
No AMD, n (%)	1479 (91.5)	900 (95.2)	**0.001**	368 (89.1)
Early AMD, n (%)	128 (7.9)	38 (4.0)		42 (10.2)
Late AMD, n (%)	10 (0.6)	7 (0.7)		3 (0.7)

p value: $\chi 2$ test for the 2 groups (observed vs. not observed) for nominal variables or Student *t* test for the 2 groups. AMD, age-related macular degeneration; *n*, number; BMI, body mass index; SD, standard deviation; min, minimum; max, maximum.

At the follow-up visit 1,370 subjects were found to have no AMD, 228 were classified as having early AMD and 18 with late AMD. Demographic characteristics by group are presented in Table 4. Early AMD was more prevalent in women, but late AMD was more prevalent in men ($p = 0.014$). Prevalence of AMD increased with age, with late AMD more prevalent in older cohorts ($p < 0.001$). Subjects with late AMD had a higher proportion of smoking history compared to subjects in the early AMD or no-AMD groups ($p = 0.003$).

Table 4. Demographic characteristics of the study population classified as no AMD, early AMD or late AMD, based on the CFP in the follow-up visit.

Characteristics	No AMD (n=1,370)	Early AMD (n=228)	Late AMD (n=18)	*p*-value
Gender				
Male, n (%)	597 (43.6)	87 (38.2)	13 (72.2)	**0.014**
Female, n (%)	773 (56.4)	141 (61.8)	5 (27.8)	
Age				
55-64, n (%)	241 (17.6)	26 (11.4)	3 (16.7)	**<0.001**
65-74, n (%)	682 (49.8)	95 (41.7)	2 (11.1)	
75-84, n (%)	391 (28.5)	90 (39.5)	10 (55.6)	
≥ 85, n (%)	56 (4.1)	17 (7.5)	3 (16.7)	

Ethnicity				
Caucasian, n (%)	1368 (99.9)	223 (97.8)	18 (100.0)	**<0.001**
African, n (%)	2 (0.1)	5 (2.2)	0 (0.0)	
Smoking				
Smoker, n (%)	27 (2.0)	6 (2.6)	0 (0.0)	
Ex-smoker, n (%)	174 (12.7)	32 (14.2)	8 (44.4)	**0.003**
Non-smoker, n (%)	1164 (85.3)	188 (83.2)	10 (55.6)	
Familiar history of AMD				
Yes, n (%)	5 (0.4)	2 (0.9)	0 (0.0)	
No, n (%)	1118 (81.6)	205 (89.9)	15 (83.3)	**0.017**
Does not know, n (%)	247 (18.0)	21 (9.2)	3 (16.7)	
Diabetes				
Yes, n (%)	300 (21.9)	31 (13.6)	4 (22.2)	
No, n (%)	1047 (76.4)	194 (85.1)	14 (77.8)	0.062
Does not know, n (%)	23 (1.7)	3 (1.3)	0 (0.0)	
Hypertension				
Yes, n (%)	759 (55.4)	125 (54.8)	10 (55.6)	
No, n (%)	588 (42.9)	97 (42.5)	7 (38.9)	0.665
Does not know, n (%)	23 (1.7)	6 (2.6)	1 (5.6)	
Hypercholesterolemia				
Yes, n (%)	645 (47.1)	99 (43.4)	6 (33.3)	
No, n (%)	615 (44.9)	110 (48.2)	9 (50.0)	0.491
Does not know, n (%)	110 (8.0)	19 (8.3)	3 (16.7)	
Hyperlipidaemia				
Yes, n (%)	61 (4.5)	12 (5.3)	0 (0.0)	
No, n (%)	1163 (84.9)	189 (82.9)	14 (77.8)	0.464
Does not know, n (%)	146 (10.7)	27 (11.8)	4 (22.2)	
Menopause				
Yes, n (%)	771 (99.7)	141 (100.0)	5 (100.0)	0.828
Does not know, n (%)	2 (0.3)	0 (0.0)	0 (0.0)	
Mean BMI ± SD (min–max)	28.1±4.4 (15.8-46.1)	28.0±4.9 (17.1-47.0)	28.1± 4.1 (20.3-35.4)	0.948

p value: $\chi 2$ test for the 3 groups for nominal variables or one-way ANOVA for the 3 groups. AMD, age-related macular degeneration; *n*, number; BMI, body mass index; SD, standard deviation; min, minimum; max, maximum.

At the 6.5-year follow-up, there were 158 participants with incident early AMD and 13 participants with incident late AMD. The crude 6.5-year cumulative incidence was 10.7% for early AMD and 0.8% for late AMD, whereas the age-standardized cumulative incidence was 10.8% (95% CI, 10.74–10.80%) for early AMD and 1.0% (95% CI, 1.00–1.02%) for late AMD. Of the 13 participants with incident late AMD, 6 had GA and 7 had NV-AMD (incidence, 0.37% for GA and 0.44% for neovascular AMD). The incidence of late AMD in participants without early disease at baseline was 0.5% ($n = 7$) and in participants with early disease only at baseline it was 0.4% ($n = 6$).

Age was associated with incidence of early AMD ($p < 0.001$) and late AMD ($p = 0.003$). Regarding late forms, age was associated with incidence of GA ($p = 0.008$), but not with NV-AMD despite the incidence of the latter being superior in older cohorts. This association with increasing

age was maintained in the male population ($p = 0.011$ for late disease and $p = 0.009$ for GA). Early disease incidence increased with age in both genders ($p < 0.05$). Female gender had a trend toward having a higher incidence of early AMD (11.3%) compared with men (9.9%). However, male gender had a superior incidence of late AMD (1.4%) compared to women (0.3%), and with a higher rate of incident GA (0.9%) than NV-AMD (0.6%). All incident cases of late AMD in females were of NV-AMD (0.3%). Cumulative incidence by age and by gender and age is presented in Table 5 and Figure 2. No cases 85 years or older at baseline participated in the follow-up analysis, therefore incidence in this age group was not possible to ascertain.

Table 5. 6.5-year incidence of early and late AMD according to gender and age.

	No. at risk	**Early disease, n (%)**	**Late disease[a], n (%)**	**No. at risk**	**Late disease[b], n (%)**	**GA[b], n (%)**	**NV-AMD[b], n (%)**
Age group[c] (years)							
55-64	719	52 (7.2)	0 (0)	761	2 (0.3)	1 (0.1)	1 (0.1)
65-74	613	80 (13.1)	3 (0.5)	669	6 (0.9)	2 (0.3)	4 (0.6)
75-84	147	26 (17.7)	4 (2.7)	177	5 (2.8)	3 (1.7)	2 (1.1)
≥ 85	0	-	-	0	-	-	-
Total	1479	158 (10.7)	7 (0.5)	1607	13 (0.8)	6 (0.4)	7 (0.4)
***p*-value**		**<0.001**	**<0.001**		**0.003**	**0.008**	0.135
Females							
55-64	431	36 (8.4)	0 (0)	452	0 (0)	0 (0)	0 (0)
65-74	331	44 (13.3)	0 (0)	362	2 (0.6)	0 (0)	2 (0.6)
75-84	82	15 (18.3)	1 (1.2)	100	1 (1.0)	0 (0)	1 (1.0)
≥ 85	0	-	-	0	-	-	-
Total	844	95 (11.3)	1 (0.1)	914	3 (0.3)	0 (0)	3 (0.3)
***p*-value**		**0.010**	**0.010**		0.180	-	0.180
Males							
55-64	288	16 (5.6)	0 (0)	309	2 (0.6)	1 (0.3)	1 (0.3)
65-74	282	36 (12.8)	3 (1.1)	307	4 (1.3)	2 (0.7)	2 (0.7)
75-84	65	11 (16.9)	3 (4.6)	77	4 (5.2)	3 (3.9)	1 (1.3)
≥ 85	0	-	-	0	-	-	-
Total	635	63 (9.9)	6 (0.9)	693	10 (1.4)	6 (0.9)	4 (0.6)
***p*-value**		**0.002**	**0.002**		**0.011**	**0.009**	0.584

p value: $\chi 2$ test for comparison of the 4 groups. a Defined as the combination of no AMD at baseline and signs of late AMD at follow-up. b Defined as the combination of no AMD or early AMD at baseline and signs of late AMD at follow-up. c Age at baseline.

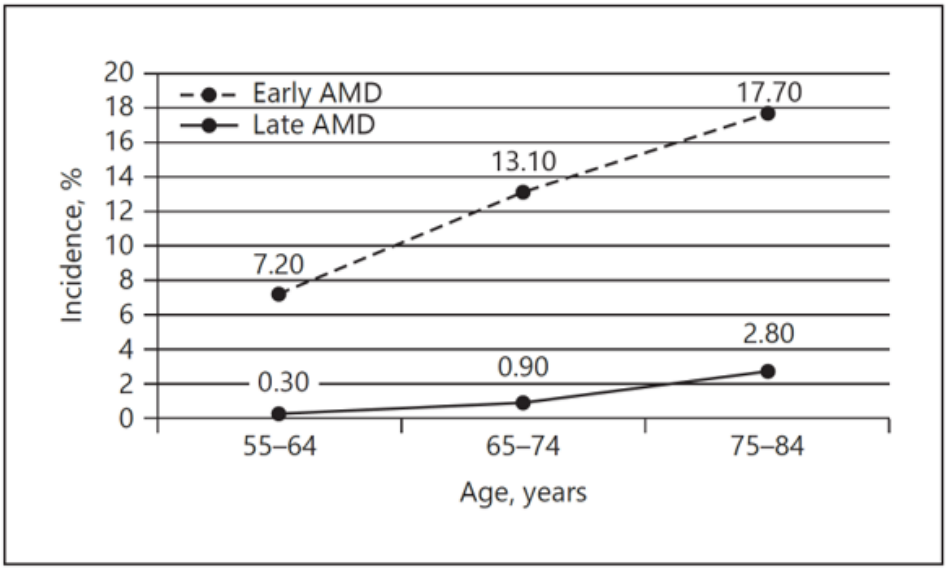

Fig. 2. Cumulative incidence of early AMD and late AMD, by age.

Progression of AMD in the included 1,616 subjects, by 1 or more levels of severity according to the Rotterdam staging system, from baseline to the follow-up visit was explored and is presented in Figure 3. A progression in AMD severity can be seen over time, and this translated to a progression rate of 17.2% ($n = 278$). Age was associated with progression of AMD ($p < 0.001$, OR = 1.04, 95% CI, 1.02–1.06%), and this was significant after adjusting for gender ($p < 0.001$, OR = 1.43, 95% CI, 1.21–1.70%). Gender alone was not associated with AMD progression ($p > 0.05$).

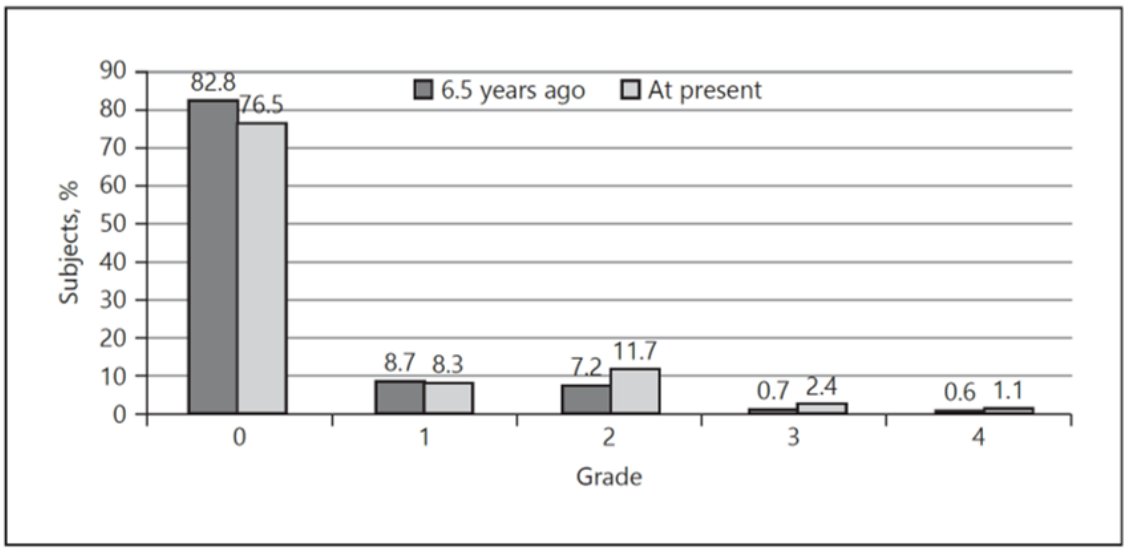

Fig. 3. Progression of AMD according to the Rotterdam staging system, from baseline to follow-up visit.

DISCUSSION

This is the first population-based cohort study to investigate the long-term incidence of AMD in Portugal, and the only one performed in a Mediterranean European country. Our findings showed that the overall, 6.5-year cumulative incidence of early AMD was 10.7%, and that of late AMD was 0.8%, while the age-standardized cumulative incidence for the Portuguese population was 10.8% (95% CI, 10.74–10.80%) for early AMD and 1.0% (95% CI, 1.00–1.02%) for late AMD. Both early and late AMD increased with advancing age. The cumulative incidence of GA was 0.37%, and that of NV-AMD was 0.44%. The progression rate in AMD severity was 17.2%.

Several long-term population-based studies reported on the incidence of AMD. However, limitations exist when comparing our results to other epidemiological studies, as there are important differences among them. For example, the minimum age for inclusion, the AMD staging system used and even the definition of early AMD. Other definitions at the level of AMD lesions are being updated, such as the multimodal-based definition of GA recently proposed [27]. Thus, no global consensus exists on AMD classification and grading, despite several attempts of uniformization by study groups [24, 28–31]. Because of this, we chose to use a grading system that was used in landmark epidemiological studies on AMD prevalence and incidence, and we also adjusted our staging system and definitions to them [5, 10, 15, 16].

Our findings are similar to most reports on AMD incidence available in the literature, namely to those originary from European countries or countries with marked
European descent, namely the USA and Australia.

In Europe, the Rotterdam eye study reports a 5-year risk of early AMD of 7.9% and of 0.9% for late AMD. The incidence was 0.4% for GA and 0.5% for NV-AMD in this population [16]. In the ALIENOR Study, from a French population, the cumulative incidence at 5 years was much superior: 32.9% for early AMD and 8.9% for late AMD, but this study comprised much older subjects (≥73 years old), and the definition of early AMD included stage 1 lesions by the Rotterdam classification, which was excluded by us [17]. The Blue Mountains Eye Study reported a cumulative incidence of 8.7% for early AMD and 1.1% for late AMD at 5 years, and 14.1% for early AMD and 3.7% for late AMD at 10 years [5, 32]. The Beaver Dam Eye Study reported a cumulative incidence of 8.2% for early AMD and 0.9% for late AMD at 5 years, and 12.1% for early AMD and 1.2% for late AMD at 10 years [6, 33].

The incidence of early AMD in our population is in line with these reports; however, our cumulative incidence of late AMD is inferior, despite a superior follow-up time, compared to the 5-year analysis in the Rotterdam Study, Blue Mountains Eye Study and Beaver Dam Eye Study. This was not totally unexpected since we previously reported that the late AMD prevalence for the coastal town was inferior to that reported in other large studies [20, 23]. Specific characteristics may explain this difference, since our population is from a coastal town and predominantly has a Mediterranean diet rich in fish, vegetables and fruits, which may have a protective effect for AMD

diagnosis [21, 22]. Genetic background may also be important, and further analysis on these factors will be explored by our group in the future. However, since the number for late disease was relatively small, conclusions must be made with caution.

In our study the incidence of NV-AMD was superior to the incidence of GA (0.44 and 0.37%, respectively), and although this difference was small, it is in line with other large-scale studies such as the Rotterdam Eye Study and the Beaver Dam Eye Study, at 5 years [6, 16]. The Blue Mountains Eye Study reports identical risk rates for GA and NV-AMD at 5 years, but a superior incidence of NV-AMD compared to GA became apparent in the 10-year and 15-year reports [5, 11, 32]. A 7-year analysis from a British population also reported on similar incidences of GA and NV-AMD [19]. In contrast the Reikjavik Eye Study reported a cumulative incidence of GA much superior to NV-AMD (in a proportion of 7: 1), and the authors hypothesize that this could be due to genetic and/or environmental factors specific to this island population [18]. The ALIENOR Study also reported a slightly superior incidence of GA over NV-AMD [17].

The incidence of early and late AMD significantly increased with advancing age. This finding was reported in several studies focusing on AMD incidence and confirms the age-related nature of the disease. Incidence of GA was also associated with age, but there was a lack of association with NV-AMD. This was probably due to the low number of the cohort, and in our previous report the NVAMD prevalence was significantly associated with age [20].

Globally, the incidence of late AMD was superior in men. This was unexpected, since most European and European- descent population-based studies report a superior incidence of late AMD in women (especially NVAMD) [4–6, 17]. On the other hand, most reports associate GA with the male gender, as was the case in our study [17]. The reason for these differences is unknown, and contradictory reports on gender as risk factor for AMD make conclusions of this subject difficult.

After staging our participants by the Rotterdam classification, we found a progression rate of 17.2% (increase in one or more stages). Similarly, Mukesh et al. [12] reported an overall 5-year progression rate of 16.8% in an Australian cohort. However, Klaver et al. [15] reported a superior cumulative progression rate of 21.5% after only 2 years of follow-up. Differences in population characteristics and in study design might be accountable for these discrepancies. Nonparticipation in the follow-up up visit from the oldest cohort in our study might have limited our results in this matter too. In spite of this, a progression from less severe to severer stages over 5 years of follow-up was clear, and the progressive and relentless course of AMD was explored and confirmed in other studies [10, 16].

This study has several limitations. First, our cohort was reduced to 54.4% of the original cohort, after accounting for death and excluding subjects who could not participate. This could have introduced a selection bias due to selective follow-up and mortality. Participants who were lost to follow-up because of nonparticipation were generally older and had a lower prevalence of AMD at baseline, and therefore they were more likely of having incident AMD, since there is a

strong relationship between age and AMD incidence. This could result in underestimation of AMD incidence in our cohort. The same was true for individuals who died during follow-up; however, one can assume that their impact on the burden of disease cannot be accounted, as only those who survive demand eye health care services. Second, we used CFP alone to grade AMD lesions in this preliminary incidence analysis. CFP has lower sensitivity in the detection of some AMD lesions compared to a multimodal approach (e.g., pseudodrusen, incipient neovascular lesions or small areas of GA). In the ALIENOR Study 34 participants developed late AMD with grading based on CFP only compared to 45 participants when the diagnosis was based on CFP plus spectral-domain OCT [17]. Thus, this is a shortcoming of the present study, and since our participants were examined not only with CFP but also with spectral-domain OCT, FAF and IR imaging, we are planning to compare AMD grading based on CFP only with AMD grading based on a multimodal approach, and to further explore differences in prevalence and incidence rates between the two methods.

The strengths of our study are the relatively long-term follow-up of a population-based cohort, the use of a validated AMD grading system used in major epidemiological studies, and we are the first study addressing the incidence of early and late AMD in a southern European country.

In summary, we documented the 6.5-year incidence of AMD in the central region of Portugal, a Mediterranean country of southern Europe. Our cumulative incidence of early AMD is similar to that reported in major epidemiological studies of European-descent populations with a close follow-up time; however, the incidence of late AMD was lower, and further analysis of risk factors will be conducted in order to explore these results.

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CHAPTER 3.

AGE-RELATED MACULAR DEGENERATION STAGING BY COLOR FUNDUS PHOTOGRAPHY VS. MULTIMODAL IMAGING: EPIDEMIOLOGICAL IMPLICATIONS *(THE COIMBRA EYE STUDY – REPORT 6)*

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In the AMD Incidence Study multimodal imaging was pursued with the objective of extensive phenotypic characterization of this population. In the first report of the Incidence Study presented in Chapter 2, only CFP data alone was analyzed to properly derive incidence data by using the same methodology both at baseline and at the follow-up visit. However, one important question emerged: if multimodal imaging is nowadays the mainstay in AMD diagnosis and management, how is it not applied in epidemiological data where the certainty of different lesions is of paramount importance to a correct classification and staging?

Not only there is no truly universal classification/ staging system in AMD, which limits comparability across major epidemiologic studies, but all of them are based on color fundus photography alone, a less reliable imaging tool in AMD. By not taking advantage of all pathophysiological information available with multimodal imaging, the grading and staging of AMD are probably less accurate in these major studies. This can be especially true for incipient late AMD for example or to detect important lesions such as subretinal drusenoid deposits. By extension, prevalence/ incidence rates might be flawed, and predictive models developed based on phenotype by CFP alone are unrefined.

Despite the importance of this topic, few studies focused on analyzing the differences in AMD grading by using the two approaches – CFP only vs Multimodal Imaging – in the same population at the same time point, and how this ultimately affects phenotypic characterization and epidemiologic data. If MMI would be found to be significantly superior, then a consensus to establish its use in a truly global, homogenous, and highly reproducible AMD classification system would be the next step. Such classification would probably have a higher predictive power regarding progression and in evaluating response to treatments.

In this report, we aimed to evaluate how the staging of AMD lesions was affected by grading with CFP only or with MMI in the Mira cohort from the Incidence Study, and the consequent changes in AMD prevalence and incidence rates in this population. Our results showed that AMD staging was more accurate with the multimodal approach, and this was especially true for late AMD. Based on our findings we proposed that multimodal imaging should be adopted in the future to better estimate and compare epidemiological data in different populations.

ABSTRACT

Epidemiology of age-related macular degeneration (AMD) is based on staging systems relying on color fundus photography (CFP). We aim to compare AMD staging using CFP to multimodal imaging with optical coherence tomography (OCT), infra-red (IR), and fundus autofluorescence (FAF), in a large cohort from the Epidemiologic AMD Coimbra Eye Study. All imaging exams from the participants of this population-based study were classified by a central reading center. CFP images were graded according to the International Classification and Grading System for AMD and staged with Rotterdam classification. Afterward, CFP images were reviewed with OCT, IR, and FAF and stage update was performed if necessary. Early and late AMD prevalence was compared in a total of 1616 included subjects. In CFP-based grading, the prevalence was 14.11% for early AMD (n = 228) and 1.05% (n = 17) for late AMD, nine cases (0.56%) had neovascular AMD (nAMD) and eight (0.50%) geographic atrophy (GA). Using multimodal grading, the prevalence increased to 14.60% for early AMD (n = 236) and 1.61% (n = 26) for late AMD, with 14 cases (0.87%) of nAMD and 12 (0.74%) of GA. AMD staging was more accurate with the multimodal approach and this was especially relevant for late AMD. We propose that multimodal imaging should be adopted in the future to better estimate and compare epidemiological data in different populations.

Key Words: age-related macular degeneration; AMD staging; multimodal imaging; early AMD; late AMD.

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of central vision loss in the elderly populations of industrialized countries [1], and this burden is expected to increase as recent estimates point to a projected number of 288 million affected individuals by 2040 [2]. Epidemiologic studies on prevalence and incidence of AMD are therefore cornerstones for planning for demand in health care systems and for establishing preventing measures [3].

To date, several population-based studies have provided important information on AMD epidemiology [4–10]. However, classification and grading in AMD, which is the basis of epidemiologic studies, differs across them and there is still no consensus. Despite several attempts by study groups, no global classification system exists, and this is especially true for classification of early/ intermediate AMD [11–15]. Furthermore, as data obtained with different imaging methods provides further insight on AMD pathophysiology, further confounding factors in this matter arise. Novel and updated definitions at the level of AMD lesions are emerging and redefined [16,17]. In fact, we now witness a change in AMD grading from the conventional color fundus based classification approach to a multimodal one, capable of more accuracy in disease diagnosis and staging [17–20]. Implementation of this multimodal approach to epidemiologic studies will probably be the necessary next step, in order to precisely stage AMD and to achieve true comparability between populations. However, the extent of the discrepancies that would arise when implementing a multimodal classification, compared to the conventional color fundus photography (CFP) based grading, is not known in epidemiologic analysis. This is especially relevant if studies using novel multimodal-based staging systems are to be compared to older studies based only on CFP.

The Coimbra Eye Study (CES) was the first population-based study providing several reports on AMD prevalence and associated risk factors in Portugal (NCT01298674) [21–24]. The study included two distinct populations in central Portugal—Mira, a coastal town, and Lousã, an inland town. Recently, we conducted a follow-up study for the coastal town of Mira and we reported on AMD incidence over six years for this population (NCT02748824) [25]. All subjects who participated in this follow-up visit were submitted to multimodal imaging, alongside conventional color fundus photography (CFP).

The purpose of the present report is to compare the staging of AMD when using color fundus photography (CFP) grading only to grading using a multimodal approach with CFP, spectral-domain optical coherence tomography (SD-OCT), infra-red (IR), and fundus autofluorescence (FAF) imaging, and to analyze the consequent epidemiologic changes in the set of patients from the coastal town of Mira that participated in the AMD Incidence Coimbra Eye Study.

EXPERIMENTAL SECTION

Study Design and Population

Information on the identification and description of the study population has been reported elsewhere [21,22,25]. Briefly, subjects who participated in the baseline analysis for the estimation of AMD prevalence in Portugal (NCT01298674) and recruited from the primary healthcare unit in Mira were identified and the surviving cohort was invited to participate in the 6.5-year follow-up analysis for the estimation of AMD incidence (NCT02748824) [25].

Signed informed consent was obtained for all participants, the study adhered to the tenets of the Declaration of Helsinki (2008) and was approved by the Association for Innovation and Biomedical Research on Light and Image (AIBILI) Ethics Committee.

Data Collection and Ophthalmic Examination

All participants underwent a detailed questionnaire-based interview on demographic, clinical, and lifestyle related information, as well as complete bilateral ophthalmological examination including best-corrected visual acuity (BCVA), tested with Early Treatment Diabetic Retinopathy Study (ETDRS) charts.

Color fundus photographs were obtained after pharmacological mydriasis. Fields 1M (centered on the optic disc), 2 (centered on the macula), and 3M (temporal to the macula) acquired at 45º for both eyes, were recorded using a digital Topcon® fundus camera (TRC-NW8; Topcon Corp., Tokyo, Japan). Fundus reflex photographs were taken to document media opacities.

Spectral-domain optical coherence tomography, fundus autofluorescence, and infrared imaging of both eyes were also acquired with Spectralis HRA+OCT (Heidelberg Engineering, Heidelberg, Germany). SD-OCT acquisitions consisted of one EDI Macular Volume Scan (20º x 20º, 49 B-scans, 16 frames per scan), 1 radial scan centered in the fovea (20º x 20º, 24 B-scans, 10 frames per scan) and 2 high resolution EDI Line Scans (30º, acquired at 0º and 90º, with ≥ 20 frames each), with signal strength ≥ 25. Both FAF (488 nm) and IR images were acquired for field 2 at 30º (High Resolution with ≥ 15 frames each).

Grading Methods

All imaging exams were sent to a centralized reading center for grading (Coimbra Ophthalmology Reading Center—CORC, AIBILI). CFP images were exported in TIFF format and OCT, FAF, and IR exams were exported as Heidelberg E2E files. All graders were ophthalmologists certified by the reading center according to the specific study protocol. All exams were analyzed and graded in a stepwise manner. Images were defined as gradable if they complied with established quality criteria by the reading center (e.g., sufficient brightness and color contrast, full macular region visible, OCT scans with good signal and well centered).

First, a general analysis of all included participants was carried out to identify major retinal pathology and to identify suspected AMD cases. Images were excluded from AMD differential grading if they had obscuring lesions (e.g., cataract) or lesions from other concomitant retinal disease hampering correct AMD grading. This analysis was independently performed by the graders, however, a second senior grader performed additional independent grading of 2% of cases. Discrepancies were analyzed and final reports were sent for the participant and his primary care physician at the healthcare unit.

After this preliminary grading, 4 senior medical retina specialists graders (C.F., M.L.C., I.P., J.P.M.) performed a differential analysis and graded the suspected AMD cases using the International Classification and Grading System (ICGS), and subsequently they staged for severity using the Rotterdam classification [12,26].

The differential grading was performed in a two-step approach as follows. First, grading was performed by grading CFP images alone. This was done to allow for comparability with our prevalence study that was based in CFP only [21,22]. CFP image quality was adjusted using color balance software to automatically standardize the brightness, contrast, and color [27]. Image grading was supported by Retmarker AMD Research software (Retmarker SA, Coimbra, Portugal), that assists manual grading of lesions according to the International Age-Related Macular Epidemiological Study Group Classification [12,28]. Afterwards, the graders classified the signs of AMD into 5 exclusive stages (stage 0–4) using the Rotterdam staging system (Table 1) [7,26]. Staging of an individual participant was based on the eye with more severe status if both eyes were gradable, and on the gradable eye if only one eye was gradable.

Table 1. Classification of mutually exclusive stages of age-related macular degeneration (AMD) according to the Rotterdam staging system.

Stage	Definition
0	
a	No signs of AMD at all.
b	Hard Drusen (< 63µm) only
1	
a	Soft distinct drusen (≥ 63µm) only
b	Pigmentary abnormalities only, no soft drusen (≥ 63µm)
2	
a	Soft indistinct drusen (≥ 125µm) or reticular drusen only
b	Soft distinct drusen (≥ 63µm) with pigmentary abnormalities
3	Soft indistinct (≥ 125µm) or reticular drusen with pigmentary abnormalities
4	Atrophic or neovascular AMD

After this first step, the graders opened for each case the correspondent SD-OCT, IR, and FAF images in the Heidelberg Eye Explorer software, and all CFP images were reviewed with

grading now being performed in a multimodal manner. A new updated stage was recorded whenever necessary.

A second senior grader performed an independent grading of all late AMD cases and of 5% the remaining cases. Consensus was achieved after discussion between the two graders, and if that could not be attained a third senior adjudicator grader oversaw the final decision.

Disease Definitions and AMD Classification

Early AMD was defined by the presence of large (≥125 μm in diameter), soft, indistinct, or reticular drusen only; or of soft distinct (≥ 63 μm in diameter), indistinct (≥ 125 μm), or reticular drusen with pigmentary abnormalities, within the macula, in the absence of signs of late AMD. This definition corresponds to stage 2 and 3 in the Rotterdam classification [7,26]. Participants with only distinct soft drusen or retinal pigmentary abnormalities (stage 1), were not defined as early AMD.

Late AMD was defined by the presence of neovascular AMD (nAMD) or geographic atrophy (GA) within the grid (3000 μm from the center of the fovea). In CFP analysis, nAMD included serous or hemorrhagic detachment of the retinal pigment epithelium (RPE) or sensory retina, intra, subretinal, or sub-RPE hemorrhages and fibrous scar. GA was defined as an area of retinal depigmentation (≥ 175 μm), characterized by sharp borders and visualization of choroidal vessels [4,5,12]. In multimodal analysis, nAMD was considered present if there were signs of any type 1, 2, and 3 neovascularization with associated features of intra/sub-retinal fluid, hemorrhage, and/or sub-retinal fibrosis. For GA to be considered present in multimodal analysis an appearance of complete RPE and outer retina atrophy (cRORA) must have been present in OCT, and a corresponding sharply demarcated area of deep hypoautofluorescence in FAF imaging was required [16,17]. When GA and nAMD coexisted in the same eye, it was categorized as nAMD. Late AMD corresponds to stage 4 in the Rotterdam classification [7].

Statistical Analysis

Descriptive statistics were used to describe the prevalence of AMD in the follow-up population in terms of the Rotterdam classification, and to explore the differences in AMD stage between the two methodologies (CFP vs. multimodal imaging). Both crude and age-standardized prevalence of early and late AMD were calculated. Age-standardization was performed for the Portuguese population according to Census 2011 of the National Institutes of Statistics (www.ine.pt). Cumulative incidence was calculated for each grading method in order to further explore for differences. Data were analyzed using STATA software (StatCorp., College Station, TX, USA), version 12.1 SE.

RESULTS

In the scope of the Coimbra Eye Study, 1617 subjects from the coastal town of Mira were included in the AMD incidence study [25]. Briefly, in the 6.5-year follow-up examination there were 2975 eligible participants, of whom 536 had died or were bedridden and 822 who did not participate. After excluding one participant because of ungradable imaging exams of both eyes, the final cohort of 1616 participants was achieved. The mean age of this population was 72.5 years old (sd. 6.83 range. 60.3–91.9).

After the general grading of the 1616 included participants, 460 were classified as suspected of having any stage of AMD and differential grading with CFP followed by multimodal grading was carried out, as stated above.

For the comparative analysis between grading methods (CFP vs. multimodal), 457 participants were included, as three participants were excluded—two participants because CFP did not have enough quality for lesion grading, and one because OCT, FAF, and IR imaging was of low quality for accurate grading.

Stage Frequency by Grading Method

The stage frequency distribution by severity and by grading modality is presented in Table 2 and Figure 1. Classification as stage 2a (soft indistinct drusen ≥ 125 μm or reticular drusen) and as stage 4 (GA and/or nAMD) was more frequent in multimodal grading compared to CFP based grading, while all other stages were less prevalent using multimodal approach.

Table 2. Frequency distribution of the mutually exclusive stages of AMD by grading methodology, according to the Rotterdam classification.

n=457	CFP only	%	Multimodal	%
0a	20	4.38	19	4.16
0b	58	12.69	57	12.47
1a	126	27.57	113	24.73
1b	8	1.75	6	1.31
2a	171	37.42	184	40.26
2b	18	3.94	15	3.28
3	39	8.53	37	8.10
4	17	3.72	26	5.69

Stage based in worst eye if both were gradable; CFP—color fundus photography.

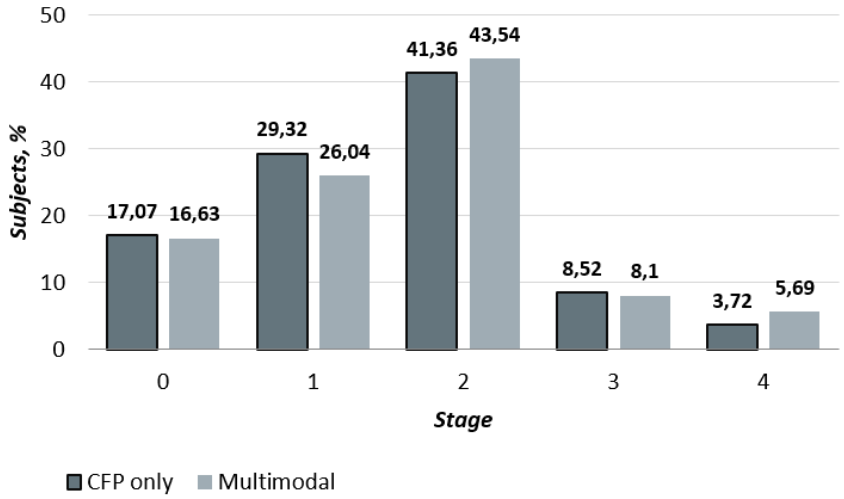

Fig. 1. Frequency distribution of differential grading cases, by stage and grading approach.

The differences and changes in the stages of all participants included in differential analysis are shown in detail in Table 3. The increase in stage 2a prevalence in multimodal grading is due to misclassification in CFP as stage 1a (n = 14) and stages 0a and 0b (n = 4). The increase in stage 4 in multimodal grading compared to CFP (n = 26 vs. n = 17) is due to misclassification in CFP as stages 3 (n = 5), 2 (n = 3) and 1b (n = 1). Participants classified as stage 3 in CFP (n = 39) maintained that stage in multimodal in 33 cases, but five were reclassified as stage 4 in multimodal evaluation and one to stage 0a (no AMD).

Table 3. Crosstab of the distribution of AMD stages based in CFP only and based in the multimodal approach.

		Multimodal								
		0a	**0b**	**1a**	**1b**	**2a**	**2b**	**3**	**4**	**Total**
CFP only	**0a**	17	0	0	1	2	0	0	0	20
	0b	0	54	1	1	2	0	0	0	58
	1a	1	2	109	0	14	0	0	0	126
	1b	0	1	1	4	0	0	1	1	8
	2a	0	0	2	0	166	0	2	1	171
	2b	0	0	0	0	0	15	1	2	18
	3	1	0	0	0	0	0	33	5	39
	4	0	0	0	0	0	0	0	17	17
	Total	19	57	113	6	184	15	37	26	**457**

Globally, CFP mostly underestimated the presence of disease, that is, early and late AMD. There were only seven cases (1.53%) misclassified as the superior stage in CFP and regraded to lower stages in the multimodal approach. In addition, only two of these (0.44%) were misclassified as stage 2a— that is, early AMD—in CFP and reclassified to 1a, or no disease, in multimodal.

Causes of stage update—CFP vs. Multimodal

The increase in stage 2a prevalence with multimodal imaging was mainly due to the more accurate detection of reticular drusen or pseudodrusen. Of the 18 cases misclassified as lower stages in CFP, we observed that in 14 of these pseudodrusen not clearly seen in CFP were detected in OCT, FAF and IR imaging, and consequently the stage was updated to 2a, this is from "no disease" to "early AMD".

Other causes identified for change in stage were drusen initially graded as present on CFP but absent on multimodal, or vice-versa. This was mainly due to: image quality interfering with accurate grading; presence of other drusen-like lesions such as vitelliform deposits or changes in the vitreoretinal interface that mimicked drusen appearance in CFP; presence of drusen-like lesions in CFP without any change visible in OCT/FAF/IR; and presence of single drusen on OCT not seen in CFP.

Concerning late AMD, we found that stage 4 was misclassified as a lower stage in nine participants when using only CFP, and this was found to be related with early cases of nAMD or GA not clearly seen in fundus images, or due to CFP image quality preventing discrimination of fine details.

Prevalence of Early and Late AMD

Considering the 1616 included participants of the Mira population in the follow-up visit, we found that using CFP grading only, the crude prevalence was 14.11% for early AMD (n = 228) and 1.05% (n = 17) for late AMD, with nine cases (0.56%) of neovascular AMD and eight (0.50%) of geographic atrophy. When using the multimodal approach, the crude prevalence increased to 14.60% for early AMD (n = 236) and 1.61% (n = 26) for late AMD. There were 14 detected cases (0.87%) of nAMD and 12 cases (0.74%) of GA. Crude and age-standardized prevalence to the Portuguese population is presented in Table 4.

Table 4. Comparison of early and late AMD prevalence based in CFP versus multimodal approach.

Prevalence %	Early AMD	Late AMD	nAMD	GA	Early AMD age-standardized	Late AMD age-standardized
CFP	14.11 (n=228)	1.05 (n=17)	0.56 (n=9)	0.50 (n=8)	13.36% (95% CI, 11.64%-15.27%)	1.23% (95% CI, 0.76%-1.91%)
Multimodal	14.60 (n=236)	1.61 (n=26)	0.87 (n=14)	0.74 (n=12)	13.84% (95% CI, 12.11%-15.80%)	1.81% (95% CI, 1.20%-2.58%)

nAMD—neovascular AMD; GA—geographic atrophy.

Globally, the difference between the prevalence of AMD (stages 2, 3, and 4) based only in CFP and based in the multimodal approach was 1.42% (95% CI, 0.90%–2.14%). When analyzing the cases with early AMD only the difference was 1.9% (95% CI, 1.3%–2.7%); and for late AMD only the difference between the two methods was 0.5% (95% CI, 0.3%–1.1%).

Incidence of Early and Late AMD

Considering those cases who performed both CFP grading and multimodal grading and our previous report on AMD prevalence in the CES by Cachulo ML et al. [22], the 6.5-year cumulative incidence was found to be 10.69% for early AMD (n = 158) and 0.75% (n = 12) for late AMD when using CFP grading only. However, if the multimodal approach is used at follow-up, the cumulative incidence changes to 11.03% for early AMD (n = 163) and 1.31% (n = 21) for late AMD.

DISCUSSION

The AMD incidence study from the Coimbra Eye Study was the first population-based study to investigate the long-term incidence of AMD in Portugal [25]. It is also one of the few that explores and compares AMD staging when accessed in a multimodal manner to that obtained with CFP only, which is to date the basis of major epidemiological studies [6,29]. Our results show that there are important differences in staging when using multimodal grading, with an increase in prevalence of both early and late AMD. These differences were mainly due to more accuracy of multimodal imaging in the detection of pseudodrusen in early AMD and incipient cases of nAMD or GA. We also found that cumulative incidence is higher, and probably overestimated, if follow-up visits in longitudinal studies are switched to multimodal grading when CFP only was used at baseline.

In our study, in which the Rotterdam classification was used, CFP grading generally underestimated the stage of AMD relative to that determined with multimodal imaging. This was observed in early AMD, with change from lower "no disease" stages to stage 2a, and in late AMD, with cases being updated mainly from stage 3 to stage 4. This is an important finding because it translated to an increase in prevalence of early AMD and of late AMD in our cohort when using a multimodal approach.

We cannot extrapolate as to how early AMD stage and prevalence would be affected in other classification systems such as the more recent Beckman classification, as for instance reticular pseudodrusen are not considered in this classification for staging purposes [14,29]. Even so, a recent preliminary report from the NICOLA study using the Beckman system revealed multimodal grading to be superior in the detection of AMD lesions compared to CFP alone, and to significantly influence staging [29]. Other classification systems do consider reticular pseudodrusen, but introduce other important differences, such as drusen area and other definitions, that makes comparisons impossible [15,30]. Even the definition of "early AMD" per se differs across classifications [14,15,26,31]. However, in cohorts classified with the Rotterdam system, our findings provide an important notion that early AMD might be underrepresented when using CFP only compared to multimodal imaging, and groups using this classification should be aware of these differences and more analysis on the subject should be conducted [8–10].

As for late AMD, the global definition is more consensual and therefore our results show an underestimation of prevalence when using conventional CFP-based classification are more readily extrapolated to other studies. Crude prevalence of late AMD using CFP only was 1.05% in our cohort, however, it increased to 1.61% with multimodal imaging (1.81% if age-standardized to the Portuguese population).

This change affected both late AMD forms—nAMD and GA—with maintenance of the nAMD/GA proportion. This means that there is a possibility that in major prevalence studies late

AMD is also underestimated, and both GA and nAMD are more prevalent than commonly thought. The ALIENOR study [6] also reported on differences in late AMD when OCT was introduced in the grading. They reported on 34 participants with incident late AMD with grading based only in CFP, but when SD-OCT was added, the number increased to 45 participants. In this regard, Colijn J et al. [9] also acknowledge in their metanalysis of AMD prevalence in Europe that an important limitation of the estimates presented is that classification of AMD used in the included studies was based in CFP only, and as multimodal imaging better visualizes edema and subtle changes resulting from choroidal neovascularization (CNV), their data might have underestimated the true prevalence of CNV. As for GA, it is now increasingly recognized that both FAF and OCT are recommended for diagnosis and detection of atrophy progression, and therefore the higher detection rate of GA when using multimodal imaging in our study was not unexpected [16,17].

The accuracy of CFP in late AMD diagnosis seems therefore below optimal, and we demonstrated an important difference of stage 4 diagnosis between methods. Multimodal imaging shows superior accuracy in this particular setting and we believe that this approach should be considered the gold standard in the future in epidemiologic studies. Greater diagnostic accuracy might even mitigate significant discrepancies on prevalence and incidence between populations from whom CFPs were graded and analyzed in different conditions.

Based on the more complete information provided by multimodal imaging, Spaide [19] recently proposed a multimodal classification system for AMD that aims to incorporate the diverse spectrum of AMD presentations in order to better predict outcomes. This approach considers soft drusen, but also pachydrusen and pseudodrusen as well as choroidal features and associates them with progression towards specific phenotypes of late AMD. For example, thinner choroids are more likely to present with pseudodrusen and type 3 neovascularization, while AMD eyes with thicker choroids are at greater risk of type 1 CNV, including polypoidal choroidal vasculopathy. Therefore, the understanding that AMD is better characterized by multimodal imaging is essential, and it must be extended to classification systems and epidemiology studies.

Another finding in our study was that with multimodal grading the incidence rate of early and late AMD was also different. For early AMD, the 6.5-year cumulative incidence was 11.03% (n = 163) with multimodal grading and 10.69% (n = 158) with CFP. As for late AMD, the incidence was 1.31% (n = 21) with multimodal and 0.75% (n = 12) with CFP. Although there is a chance that the incidence is truly higher when using multimodal imaging, this probably represents an overestimation for using a more disease and stage-sensitive method in the follow-up analysis. Disease burden at baseline would also be expected to change in the same proportion if OCT was used at this time-point. Therefore, if multimodal imaging is to be used in older stablished cohorts analyzed only with CFP at baseline, this overestimation bias in incidence must be kept in mind.

This study has several limitations. First, our follow-up visit cohort was reduced to 54.4% of the original one and this might have introduced a selection bias due to selective follow-up and

mortality. However, the final population in analysis for differences in grading methodology on prevalence is still large, and important differences were readily seen. Another bias could be that we did not a corrected prevalence for participants with only one gradable eye. These could be misclassified towards a lower stage if the ungraded eye is worse than the observed one. However, the main purpose of the study was to compare between different grading methods and to analyze their impact in staging.

The strengths of our study are the relatively long-term follow-up of a population-based cohort, the use of a validated AMD grading system used in major epidemiologic studies, and we are one of the first studies extensively addressing prevalence and incidence of early and late AMD with different grading methodologies—CFP vs. multimodal. The utilization of multimodal grading allowed us to better detect AMD lesions such as pseudodrusen, incipient neovascular lesions, or small areas of GA. Therefore, we provide a more accurate report on early and late AMD prevalence in a large cohort from a Portuguese population, as well as of the impact that introduction of multimodal imaging carries in the epidemiologic context of AMD.

In summary, we present the first report in a populational study truly comparing AMD staging with Rotterdam classification using CFP versus multimodal imaging grading. AMD staging proved to be more accurate using the multimodal approach in our study and this was especially true for the correct identification of late AMD, where the difference between the two methods is critical. We believe that multimodal imaging should be adopted in future AMD classification systems and epidemiologic studies, in order to better estimate and compare AMD prevalence and incidence in different populations.

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CHAPTER 4.
RETINAL LAYER THICKNESSES AND NEURODEGENERATION IN EARLY AGE-RELATED MACULAR DEGENERATION: INSIGHTS FROM THE COIMBRA EYE STUDY

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As previously presented, the AMD Incidence Study was designed to include an ophthalmological evaluation with multimodal imaging with the objective of extensive phenotypic characterization of this population. This approach is currently advised for AMD diagnosis and management in both clinical practice and research. Furthermore, given the results described in the previous chapter, a choice was made to consider the staging of AMD obtained with the multimodal approach the most accurate in the Incidence Study, and it will be used through the next chapters.

Multimodal imaging not only provides the means to increase diagnostic certainty in AMD but also sets the possibility of identifying new imaging biomarkers of progression. By using phenotypic data obtained with different but complementary imaging techniques, these biomarkers could be used to increase the predictive power of progression models and in evaluating response to treatments.

In this chapter, we aimed to explore quantitative and qualitative biomarkers obtained with MMI and their association with AMD severity in a cohort from the Incidence Study. Despite the extensive focus on the external retinal layers and choroid as biomarkers of AMD progression, there is still little knowledge on how the inner retinal layers change over time or by stage of progression. In fact, degenerative changes in the inner retina seem to be present in the early stages of AMD eyes.

Our results show that several inner and outer neuroretinal layers are thinner with a higher stage in early AMD. These findings support the notion that there is early and progressive neurodegeneration in AMD affecting almost all retinal layers. In addition, subretinal drusenoid deposits seem to be biomarkers of a phenotype characterized by more prominent and faster neurodegeneration, as their presence was associated with even thinner retinal layers and choroids.

Based on our findings we suggest that neuronal retinal layer thicknesses obtained with OCT might be used as quantitative biomarkers of disease progression in AMD and can be included in predictive models. Fully automatization of the segmentation process in the era of Artificial Intelligence will possibly facilitate this task in the future.

ABSTRACT

Purpose: This study aims to analyze the retinal layers and choroidal thickness in a large set of eyes with early age-related macular degeneration (AMD), in order to detect differences by stage suggestive of early neurodegeneration, and to explore biomarkers of different phenotypes.

Methods: This study is a population-based, cross-sectional study. Patients from the incidence AMD study (NCT02748824) with early AMD (Rotterdam 2a, 2b, 3) were included. All performed spectral-domain optical coherence tomography (SD-OCT) (Spectralis, Heidelberg Engineering, Germany) and automatic segmentation of all retinal layers was obtained with built-in software. Manual correction was performed whenever necessary. The mean thicknesses (ETDRS grid) and volume of each layer were recorded. Subfoveal choroidal thickness was manually measured. Estimates for each layer thickness were calculated with linear mixed models and tested for pairwise differences between stages. Associations between layer thickness and microstructural findings were assessed by multivariate regression analysis.

Results: The final cohort comprised 346 eyes (233 patients): 82.66% (n = 286) in stage 2a, 5.49% (n = 19) in stage 2b, and 11.85% (n = 41) in stage 3. A global tendency for lower/inferior thickness of the neuroretinal layers was found comparing stage 3 to 2a: retinal nerve fiber layer (RNFL), ganglion cell layer (GCL), and inner plexiform layer (IPL) were inferior in the inner/outer ETDRS circles and the outer nuclear layer (ONL) and photoreceptors' segments layer in the central circle ($p \leq 0.002$). The retinal pigment epithelium–Bruch's membrane (RPE/BrM) layer was thicker in stage 3 ($p \leq 0.001$). Subretinal drusenoid deposits (SDD) were associated with thinner neuroretinal layers and choroid ($p < 0.05$).

Conclusions: Our results showed in a large population-based dataset that several inner and outer neuroretinal layers were thinner with a higher stage in early AMD. These findings support the existence of early and progressive neurodegeneration. Neuronal retinal layer thicknesses might thus be used as quantitative biomarkers of disease progression in AMD. The presence of SDD is possibly associated to more prominent and faster neurodegeneration.

Keywords: Early age-related macular degeneration; Microstructural spectral-domain optical coherence tomography analysis; Biomarkers of AMD progression; Retinal layers and choroidal thicknesses; Subretinal drusenoid deposits

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss and blindness in older adults in industrialized countries [1–3]. The global tendency of population aging anticipates an increase in the burden of disease, with estimations in Europe of 15% more affected individuals by 2050 and doubling of late AMD cases by 2040 [1, 2]. Unfortunately, treatment is still limited in late AMD, and thus, comprehension on the pathophysiology of disease progression is of paramount importance, as it will hopefully lead to the development and implementation of preventive measures in early stages [4, 5]. The understanding of the pathophysiological mechanisms underlying disease development and progression is, however, a difficult challenge, since several interacting genetic and environmental factors are known to play a role. Besides, with the development of new imaging techniques, imagiological features are being updated and recognized as phenotypic biomarkers of disease progression [4, 6–8].

The diagnosis and classification of AMD were conventionally based on clinical examination and assessment of color fundus photographs (CFP), which is to date the basis of staging systems used in epidemiological data. However, the introduction of spectral-domain optical coherence tomography (SD-OCT), fundus autofluorescence (FAF), infrared (IR) imaging, and more recently OCT angiography (OCTA) provided improved ability to detect pathologic changes earlier and to more accurately stage AMD in a multimodal basis [9–12].

The SD-OCT allows in vivo precise and reproducible quantitative and qualitative analysis of the individual layers of the retina and choroid, allowing to detect subtle longitudinal changes before the development of late-stage disease [13]. Recently, automatic "layer-by-layer" segmentation algorithms were developed and are commercially available in SD-OCT devices. They provide layer segmentation in close correlation to retinal histology; however, manual correction is often necessary, especially when the quality of the exam is insufficient, or the retinal architecture is compromised by pathology. Semiautomatic segmentation in this type of quantitative layer-by-layer analysis is therefore a more precise term [14].

AMD is a disease that predominantly affects the outer retina and choroid and already extensively characterized by multimodal imaging. For example, studies using in-built or customized SD-OCT segmentation algorithms reported that drusen volume is an important predictor for the development of late AMD, that is, a biomarker to identify eyes at the highest risk for progression [15]. However, and despite the extensive focus in the external retinal layers and choroid as biomarkers of disease progression and functional prognosis, there is still little knowledge on how the inner retinal layers change over time or by stage of progression in AMD. In fact, it is recently being recognized that progressive degenerative changes in the inner retina might be present as well in early stages of the disease, and studies using histopathological analyses or SD-OCT have shown differences between AMD patients to aged-matched controls. However, most

had small samples and did not focus on all inner retinal layers [14, 16–21]. Plus, the correlation on this apparent degenerative process and specific AMD features or phenotypes has yet not been explored.

The Coimbra Eye Study (CES) is an epidemiologic population-based study on the prevalence and incidence of AMD in a Portuguese population (NCT01298674, NCT02748824). All participants in the follow-up visit for the estimation of AMD incidence performed color fundus photography, SD-OCT, FAF, and IR imaging. AMD stage in the CES was defined by the Rotterdam classification and based in a multimodal imaging grading [12, 22–25].

The purpose of this report is to perform a detailed quantitative analysis of all retinal layers by using semiautomatic segmentation with SD-OCT and of the subfoveal choroid, in the set of eyes staged with early AMD from the incidence CES, in order to detect differences by stage suggestive of early progressive neurodegeneration, and to explore specific phenotypes.

MATERIAL AND METHODS

Study design and selection of participants

Information on the identification and description of the study population has appeared in previous reports [12, 22–24]. Briefly, the CES is a single-center population-based study whose cohort included two geographically distinct populations aged ≥ 55 years for the estimation of AMD prevalence: one from a coastal town (Mira) and the second from an inland town (Lousã) [22, 23]. The subsequent AMD incidence study (NCT01298674) conducted 6.5 years later only included the subjects recruited from the primary health-care unit in Mira. This latter population was extensively characterized in this follow-up visit from a demographic and clinical perspective, including multimodal imaging (MMI) [12, 24]. For inclusion in the present analysis, patients from the AMD incidence study staged with early AMD by MMI, with stages 2a, 2b, and 3 by Rotterdam classification, were identified as eligible. The Rotterdam staging system, developed in the Rotterdam Study, stratifies AMD signs into five mutually exclusive stages and is the classification system adopted in the CES (Table 1) [12, 25]. Exclusion criteria were defined: refractive error over +5.0 or –6.0 diopters, significant media opacities, history of vitreoretinal surgery, other comorbidities, and/ or significant vitreoretinal interface abnormalities preventing accurate segmentation or potentially compromising reliable outcomes.

Signed informed consent was obtained for all participants in the scope of the AMD incidence study. The study adhered to the tenets of the Declaration of Helsinki (2008) and was approved by the Association for Innovation and Biomedical Research on Light and Image (AIBILI) Ethics Committee.

Data collection and AMD staging

All CES participants underwent a detailed questionnaire-based interview on demographic, clinical, and lifestyle-related information, as well as complete bilateral ophthalmological examination including BCVA, tested with early treatment diabetic retinopathy study (ETDRS) charts.

In respect to AMD grading, in the CES, the Rotterdam staging system was used: early AMD was defined as stages 2a, 2b, and 3 and late AMD as stage 4 (Table 1). As reported elsewhere, a multimodal approach including CFP, SD-OCT, FAF, and IR imaging was used for this purpose [12]. Color fundus photographs (fields 1M, 2, and 3M) were acquired at 45° using a digital Topcon® fundus camera (TRC-NW8; Topcon Corp., Tokyo, Japan). SD-OCT, FAF, and IR of both eyes were acquired with Spectralis HRA+OCT (Heidelberg Engineering, Heidelberg, Germany). SD-OCT acquisitions consisted of one EDI macular volume scan (20° × 20°, 49 or 97 B-scans, 16 frames per scan), 1 radial scan centered in the fovea (20° ×20°, 24 B-scans, 10 frames per scan), and 2 high-resolution EDI line scans (30°, acquired at 0° and 90°, with ≥ 20 frames

each), with signal strength ≥ 25. Both FAF (488 nm) and IR images were acquired for field 2 at 30° (high-resolution with ≥ 15 frames each) [12, 24].

CFP image grading was supported by the RetmarkerAMD Research software (Retmarker SA, Coimbra, Portugal), and staging of all included eyes was performed while simultaneously analyzing the correspondent SD-OCT, IR, and FAF exams with the Heidelberg Eye Explorer software (version 1.10.4.0). This assessment was performed at a centralized reading center (Coimbra Ophthalmology Reading Center, AIBILI, Portugal), by 4 senior medical retina specialist graders trained according to the study protocol (CF, MLC, IP, JPM).

Table 1. Classification of mutually exclusive stages of age-related macular degeneration (AMD) according to the Rotterdam staging system. Early AMD corresponds to stages 2a, 2b, and 3.

Stage	Definition
0	
a	No signs of AMD at all.
b	Hard Drusen (< 63µm) only
1	
a	Soft distinct drusen (≥ 63µm) only
b	Pigmentary abnormalities only, no soft drusen (≥ 63µm)
2	
a	Soft indistinct drusen (≥ 125µm) or reticular drusen only
b	Soft distinct drusen (≥ 63µm) with pigmentary abnormalities
3	Soft indistinct (≥ 125µm) or reticular drusen with pigmentary abnormalities
4	Atrophic or neovascular AMD

SD-OCT qualitative assessment and FAF/IR analysis in the CES

Besides AMD staging, an extensive qualitative analysis of the SD-OCT scans was performed in all patients included in the follow-up visit of the CES, by the same senior graders at the reading center. This analysis was performed according to the recently proposed "European Eye Epidemiology spectral-domain optical coherence tomography classification of macular diseases for epidemiological studies" [26]. Several features were graded: (a) assessment of the neuroretina and retinal pigment epithelium (RPE) layer, including soft drusen, subretinal drusenoid deposits (SDD), hyperreflective foci (HRF), intraretinal/subretinal fluid, outer retinal tubulations, subretinal hyperreflective material, and RPE atrophy, and (b) assessment of the vitreomacular interface, including vitreomacular adhesion/ traction and epiretinal membrane.

In FAF and IR grading, the presence of SDD was confirmed, and the area of the affected retina was measured with the Heidelberg Eye Explorer software in-built tools.

For this study, the presence of SDD and hyperreflective foci was considered relevant to detect associations with retinal layer and choroidal thicknesses. These features were selected on

the basis that they could be biomarkers of different AMD phenotypes or pathways of disease progression. In fact, both are known as predictors of progression to late stages [13, 27].

SD-OCT segmentation and quantitative assessment

For the retinal layer thickness analysis, the EDI macular volume scan was used, and segmentation of all retinal layers was performed for each eye as follows: first, automatic segmentation was applied to the complete volume scan by using the in-built feature for automated segmentation in the Heidelberg Eye Explorer software (version 1.10.4.0); after this, all retinal layers were reviewed for segmentation in all scans of the volume, and manual correction was performed whenever necessary, using appropriate tools of the software. If after maximum effort a layer could not be correctly segmented, it was automatically subtracted from the map. When manual correction was impossible because of the scan's low quality and poor reflectivity for properly distinguishing retinal layers, the entire scan was excluded. Decentration of the macular volume was also manually rectified if necessary. Eyes with concomitant retinal disease distorting the macular layers or compromising accurate thickness assessment were also excluded. Both automatic segmentation and the necessary manual corrections were conducted by a trained grader (ALS), certified by the reading center, and supervised by a senior grader (CF).

The Heidelberg segmentation software displays seven distinct retinal layers: retinal nerve fiber layer (RNFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and RPE/Bruch's membrane layer (RPE/BrM). The software also provides information on three additional "combination" layers: (1) the inner retinal layers (IRL), extending from the inner limiting membrane (ILM) to the external limiting membrane (ELM); (2) the outer retinal layers (ORL), extending from ELM to Bruch's membrane (BrM); and (3) the total retinal thickness, from ILM to the BrM (Fig. 1). Since the software does not provide the retinal thickness of inner and outer segments of the photoreceptors, and as these are of paramount interest in AMD pathophysiology, we have calculated the thickness and volume of an additional layer, the "photoreceptors' segments" layer (PRL), by subtracting the RPE/BrM layer from the ORL, as described by Brandl et al. [14].

After verification of each individual B-scan and correction of segmentation errors, the thickness from the nine macular fields, as determined by the ETDRS grid available in the software, and the macular volume for each of the segmented retinal layers were recorded (Fig. 1). The mean retinal thicknesses in the inner and outer circles (3- and 6-mm diameter, respectively) were calculated by averaging the thickness measurements of the inner and outer superior, inferior, nasal, and temporal fields of the grid, respectively [14, 28].

Besides the retinal thickness, the sub-foveal choroidal thickness was manually measured using the caliper tool available in the software, using the 1:1 μm viewing mode, in the horizontal EDI scan passing through the center of the fovea. Choroidal thickness was defined as the vertical

distance from the hyperreflective line of the BrM to the innermost hyperreflective line of the chorioscleral interface. If the hyperreflective line of the BrM was not separated from the RPE, the choroid was measured from beneath the outermost hyperreflective line of the RPE/BrM layer to the innermost hyperreflective line of the chorioscleral interface [29].

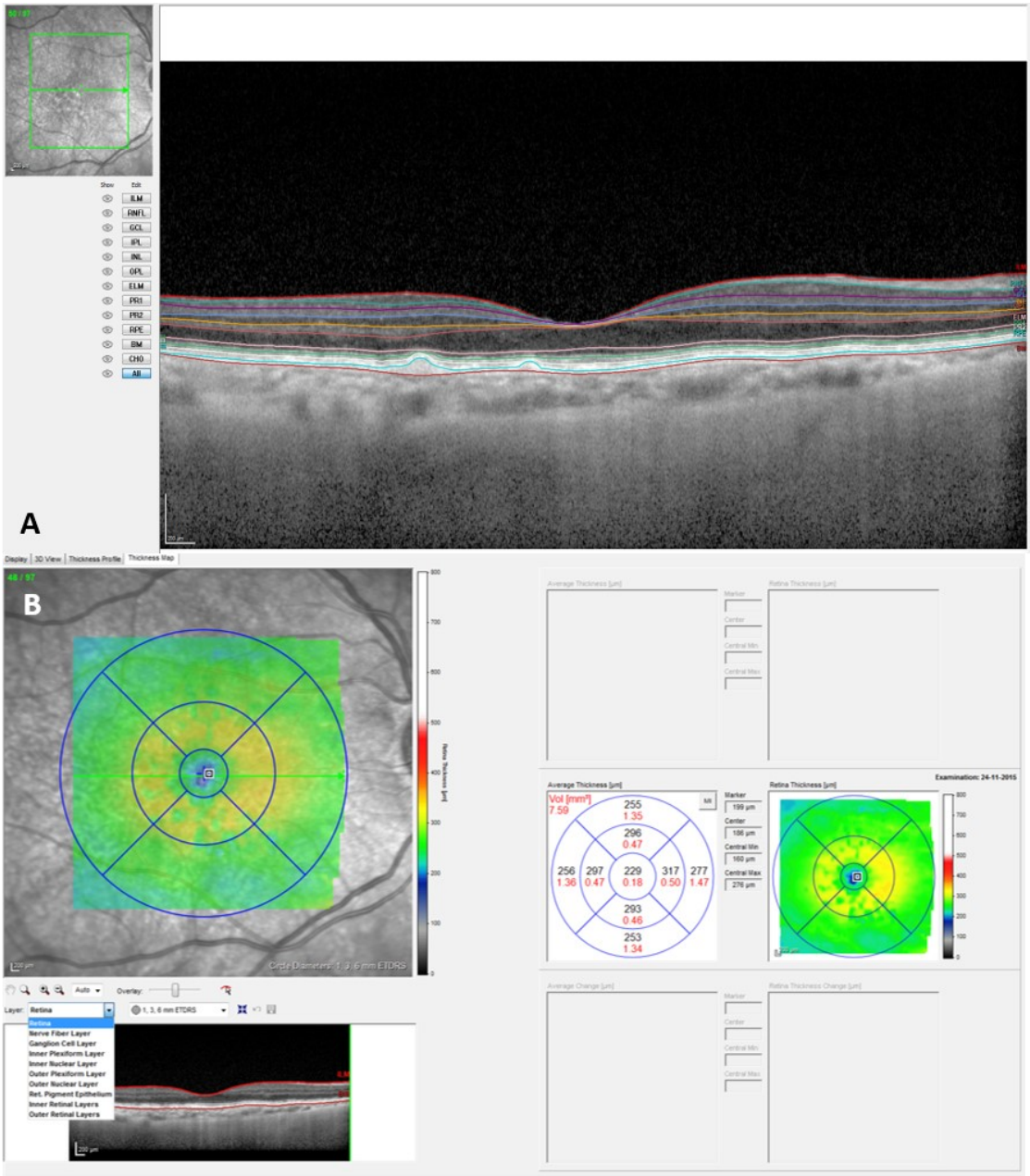

Fig. 1 a) Representation of the automatic segmentation of retinal layers provided by the Heidelberg software segmentation tool, in a foveal B-scan. **b)** Retinal layers output, with corresponding thickness display according to the ETDRS grid, plus macular volume.

Statistical analyses

Data were analyzed using STATA software (StataCorp. 2019. Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC). Categorical variables were summarized with

frequencies and percentages, and numerical variables were presented as median and interquartile range (IQR), due to the non-normal distribution of the data, assessed by Shapiro–Wilk test.

Two major analyses were performed.

In the first analysis, to test for differences in retinal layer thickness within eyes with early AMD stages, and in order to include eyes from the same patient in the analysis, linear mixed model were used for each layer separately (RNFL, GCL, IPL, INL, OPL, ONL, PRL, and RPE/BrM). For each layer, the dataset consists of multiple measurements per study participant, eyes, and macular circle. This correlation structure was taken into account by including a participant-specific random intercept and a nested random intercept for each eye per participant into each model. The dependent variable of each model was the natural logarithm of manually corrected retinal layer thickness by circle (central, inner, and outer) and by eyes. The independent variables were age, gender, ETDRS circle, and AMD stage (2a, 2b, 3). Age and gender were integrated as potential cofounders, due to significant variation in the thickness of retinal layers in normal eyes depending on these variables [14, 28]. An interaction effect of the AMD stage with the macular circle was included to account for differing retinal layer thickness by circle and to estimate potentially differing effects of the disease stages on retinal layer thickness in the different circles. Model-based expected retinal layer thicknesses by early AMD stage, for central, inner, and outer circles, were produced. To test for pairwise differences, we used approximate *F*-tests based on the Kenward–Rogers approach, where *p*-values were adjusted by the Bonferroni method for 3 comparisons. To take into account multiple comparisons for the 3 macular circles in the 8 retinal layers, the significance level was set as 0.05/24 = 0.002. The ORL and IRL were excluded from the analysis, as they are collinear with other retinal layers.

For the second analysis, to explore and characterize the different retinal layers, only the worst AMD staged eye per patient was considered, to prevent exclusion of more severe stages that were less represented in the total sample. The associations between the different retinal layers and the RPE/ BrM layer, regarding thickness and volume, were tested using Spearman correlation due to the non-normal distribution of the variables, followed by a stepwise backward multivariate regression analysis to adjust for possible confounders (age, gender, and AMD stage). Variables with *p*-value > 0.1 were removed from the model. This same statistical methodology was applied to test the association between the retinal layers' thicknesses and other morphologic AMD features assessed by SD-OCT, FAF, and IR imaging. To statistical significance, *p*-value was established at 0.05.

RESULTS

Study population characteristics and AMD stage

In the scope of the Coimbra Eye Study, 1616 subjects comprised the final cohort of the AMD incidence study. Early AMD was found in 370 eyes from 248 patients. From these, 346 eyes from 233 patients (93.51%) were eligible for segmentation analysis and included in the study. Eyes with the following criteria were excluded (n = 24): poor image quality for accurate segmentation, absence of SD-OCT scans in the macular volume, concomitant retinal disease distorting the retinal layers preventing reliable manual segmentation correction, and concomitant optic atrophy (Fig. 2).

The complete demographic and clinical characteristics of the patients with early AMD included in the present analysis are provided in Supplementary Table 1. The median (IQR) age was 74.9 (70.1–80.5) years-old, and the majority were women (64.0%). The median (IQR) spherical equivalent was +0.625 (−3.75 to +1.75) diopters.

Of the 346 eyes with early AMD included, 82.66% (n = 286) were in stage 2a, 5.49% (n = 19) in stage 2b, and 11.85% (n = 41) in stage 3.

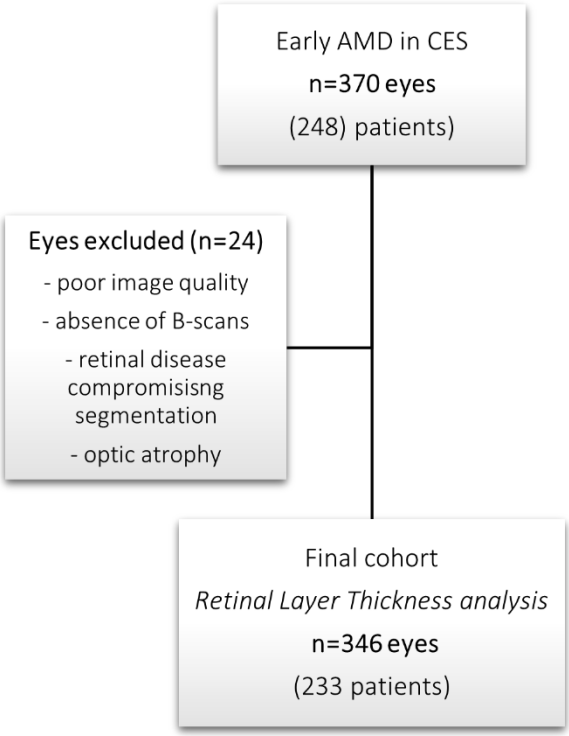

Fig. 2 Flow chart of eyes selected and included in the retinal layer thickness (RLT) analysis.

Retinal layer thicknesses analysis: differences between stages

The median retinal thicknesses by ETDRS circle and macular volumes of each retinal layer in all eyes are presented in Table 2.

To assess the differences in retinal layer thicknesses within early AMD stages, all 346 eyes were considered as described above. The expected retinal layer thicknesses derived from linear mixed models were calculated by early AMD stage, regarding central, inner, and outer circles, and adjusted for age and gender and nested random effects for within-person and within-eye correlations. They are presented in detail in Fig. 3. We could observe that globally, the retinal layers were reduced in thickness in stage 3, compared to stage 2a. The RNFL, GCL, IPL, and INL were thinner in the inner and outer ETDRS circles, while the ONL and PRL presented with more marked thinning in the central circle. In contrast, the RPE/ BrM layer showed increased thickness in stage 3 in the central and inner circles.

After descriptive comparison analysis, we tested for pairwise differences in retinal layer thickness measurements between AMD stages and separately for each macular circle. Significant differences between stages were found and are presented in Table 3. For completeness, we also provide information on all layers and respective pairwise comparisons in Supplementary Table 2. Again, differences were found when comparing stage 3 to stage 2a: the RNFL, GCL, and IPL thicknesses were inferior in the inner/outer circles and the ONL and PRL in the central circle (p = 0.002 to 0.006). The RPE/BrM layer was significantly increased in the central circle in stage 3, compared to both stage 2a and stage 2b ($p \leq 0.001$).

Table 2. Retinal layers thicknesses in the central, inner, and outer circles of the ETDRS grid, plus volume, in a population aged 55+ with early AMD from the CES.

Retinal layers	Central circle	Inner circle	Outer circle	Volume
RNFL	12.00 (11.00–14.00)	20.50 (19.25–22.25)	34.50 (31.50–37.75)	0.87 (0.80–0.96)
GCL	14.00 (12.00–16.00)	48.00 (43.75–51.00)	33.125 (30.50–36.00)	1.02 (0.94–1.09)
IPL	20.00 (18.00–22.00)	40.0 (37.75–42.50)	28.50 (26.25–30.25)	0.87 (0.81–0.92)
INL	20.00 (17.00–24.00)	40.75 (38.75–43.50)	32.50 (31.00–34.25)	0.96 (0.91–1.01)
OPL	25.00 (22.00–29.00)	31.50 (29.25–34.25)	26.25 (25.25–27.75)	0.78 (0.74–0.82)
ONL	95.00 (87.00–102.00)	70.25 (65.00–77.25)	55.25 (50.25–60.00)	1.69 (1.54–1.83)
PRL	69.00 (67.00–72.00)	65.25 (64.00–66.25)	65.00 (63.50–65.75)	1.84 (1.80–1.86)
RPE	16.00 (14.00–17.00)	14.75 (13.50–15.75)	13.0 (12.00–14.00)	0.38 (0.35–0.41)
IRL	186.00 (174.00–201.00)	252.5 (240.75–264.00)	210.375 (200.75–220.25)	6.21 (5.90–6.49)
ORL	86.00 (83.00–89.00)	80.375 (78.25–82.00)	77.75 (76.00–79.25)	2.23 (2.17–2.26)
Overall Retina	272.00 (260.00–288.00)	331.75 (320.50–344.75)	287.75 (277.50–298.5)	8.41 (8.11–8.72)

Median thickness (μm) and volume values (mm^3) with IQR (25th to 75th quartile) of the eight distinct retinal layers plus three combinations (IRL, ORL, and overall retina) in all 346 eyes analyzed, after semiautomatic segmentation. IQR, interquartile range.

Fig. 3 Model-based expected retinal layer thicknesses (µm) by early AMD stage, regarding central, inner, and outer circles. Estimates are derived from linear mixed models of 346 eyes with stage 2a (n = 286), stage 2b (n = 19), and stage 3 (n = 41) of early AMD, adjusted for linear age and gender and nested random effects for within-person and within-eye correlations.

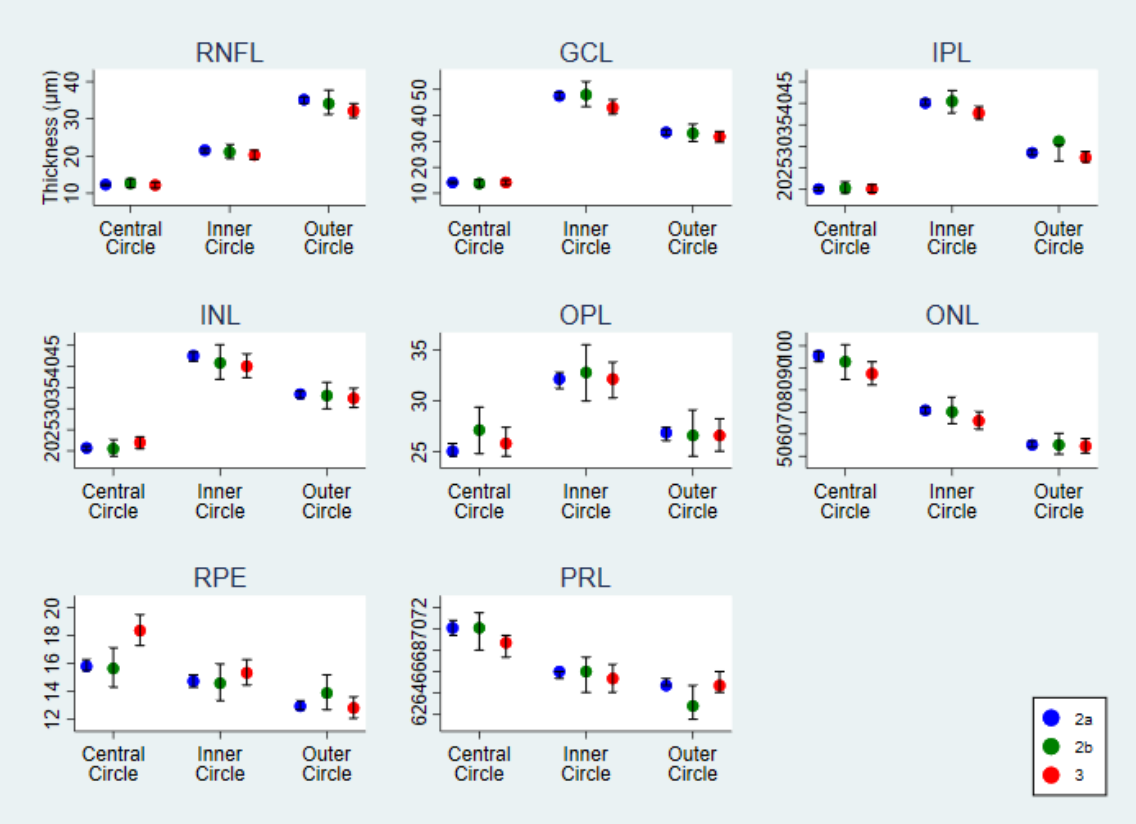

Table 3. Expected retinal layer thicknesses by ETDRS circle with significant differences between early AMD stages.

	EXPECTED RETINAL LAYER THICKNESSES, µM			EFFECT ESTIMATES / PAIRWISE TEST *P*-VALUE		
Retinal layers	**2a (n=286)**	**2b (n=19)**	**3 (n=41)**	**2a vs. 2b**	**2a vs. 3**	**2b vs. 3**
RNFL, outer circle	35.2 (34.1 – 35.9)	34.1 (31.2 – 37.7)	32.1 (30.3 – 34.1)	0.021/1.000	**0.086/0.005**	0.065/0.471
GCL, inner circle	47.5 (46.5-48.9)	47.9 (43.4 – 53.0)	42.9 (40.4 – 46.1)	-0.002/1.000	**0.099/0.002**	0.101/0.120
IPL, inner circle	40.0 (39.6 – 40.9)	40.4 (37.7 – 42.9)	37.7 (36.2 – 39.3)	-0.002/1.000	**0.065/0.002**	0.068/0.118
ONL, central circle	95.6 (92.8 – 97.5)	92.8 (84.8 – 100.5)	87.4 (82.3 – 92.8)	0.029/1.000	**0.088/0.002**	0.059/0.489
PRL, central circle	70.1 (69.4 – 70.8)	70.1 (68.0 – 71.5)	68.7 (67.4 - 69.4)	0.003/1.000	**0.023/0.006**	0.020/0.335

RPE, central circle	15.8 (15.5 – 16.3)	15.6 (14.3 – 17.1)	18.4 (17.3 – 19.5)	0.016/1.000	**-0.143/<0.0001**	**- 0.160/0.001**

Expected retinal layer thicknesses (μm), effect estimates (on log scale), and *p*-values of pairwise tests, obtained from layer-specific linear mixed models. The analysis includes 346 eyes with early AMD (stages 2a, 2b, and 3), obtained from the original cohort of AMD incidence study. *p*-values were judge at Bonferroni-corrected significance level, $p < 0.05/24 = 0.002$. Significant and strong near-significant results are highlight with bold text.

Association between neuroretinal layers and RPE/BrM layer

To explore the possible associations between the thickness and volume of the segmented neuroretinal layers and the RPE/BrM layer, where drusen are located, but also where degenerative changes take place as AMD progresses, a stepwise backward multivariate linear regression analysis was performed. Only the worst staged eye of each subject was included for this purpose: 233 eyes, with 78.11% (n = 182) in stage 2a, 6.87% (n = 16) in stage 2b, and 15.02% (n = 35) in stage 3.

Regarding thickness and volume, only weak correlations were found between the RPE/BrM and the external neuronal retinal layers (OPL, ONL, and ORL), while no associations were detected to the inner neuronal layers ($p < 0.05$) (Supplementary Table 3). In stepwise backward multivariate linear regression analysis, after adjusting for age, sex, and AMD stage, only associations of the RPE/Bruch layer to ONL and PRL were significant, but these were also weak and thus not clinically relevant (Supplementary Table 4).

Choroid in early AMD

Considering again only one eye (worst staged eye) per subject, to discard inter-eye interactions, the global median subfoveal choroidal thickness (CT) was 249.0 μm (IQR, 191.0 to 296.0 μm). Regarding stage, we found that the choroidal thickness was lower in stage 3 compared to stage 2a and 2b, but the difference did not reach statistical significance ($p > 0.05$). As expected, older individuals (> 74.7 years old, median age for this population) had an inferior median CT compared to younger patients, and women also had thinner choroids compared to men (Table 4).

Table 4. Choroidal thickness descriptive analysis (only worst eye per subject is considered).

		N	Median CT (IQR) (μM)
Stage	2a	182	249 (190-295)
	2b	16	260.5 (228 - 317)
	3	35	239 (168 - 322)
Gender	Male	84	262.5 (209.5 – 303.5)
	Female	149	235 (117- 295)
Age	≤74.7	117	260 (208-318)
	>74.7	116	230 (156-281.5)

Qualitative SD-OCT analysis and association to retinal layer and choroidal thickness

The presence of specific retinal features obtained in the SD-OCT, FAF, and IR grading from the AMD Incidence study, such as the presence of SDD and hyperreflective foci, was also analyzed in order to detect the associations with retinal layer thickness. For this purpose, only the worst staged eye was selected.

The SDD were found to be definitely present in 20.69% of the eyes (n = 48) and questionable in 4 cases (1.72%). Comparing to eyes without SDD, eyes having SDD were found to have significantly thinner neuroretinal layers (IPL, INL, OPL, ONL, and PRL) and thinner RPE/BrM layer and choroid, compared to eyes without SDD ($p < 0.05$) (Table 5). The definite presence of HRF was found in 22 eyes (9.48%), being questionable in another 4 (1.72%).

To assess the association to each retinal layer thickness, by macular circle and choroidal thickness, stepwise multivariate linear regression analysis was performed. Statistically significant results, corrected for age, gender, and stage, are presented in Table 6.

Eyes with SDD were significantly associated and predictive of a thinner OPL, ONL, PRL, and RPE/BrM layer and a thinner subfoveal choroid (Fig. 4). Eyes with HRF were associated to thicker ONL and RPE/BrM layer ($p < 0.05$). In the model, we also found that age was associated to a decrease in the ONL, PRL, and choroidal thickness, and stage 3 was associated to thinner ONL and PRL and to a thicker RPE/BrM.

Table 5. Comparison between eyes with SDD and without, regarding the retinal/choroidal layers thicknesses (only worst eye per subject is considered).

Layers	Eyes with SDD	Eyes without SDD	*p*-value
IPL, inner circle	38.875 (36.875–41.00)	40.50 (38.00–42.75)	**0.026**
INL, inner circle	40 (38 – 42.75)	41.5 (39.25 - 44)	**0.044**
INL, outer circle	31.75 (30–33)	32.75 (31.25–35)	**0.009**
OPL, outer circle	25.25 (24.5–27.0)	26.5 (25.75–28.0)	**<0.001**
ONL, central circle	89 (84.5 - 100)	96 (88.5 - 102)	**0.029**
ONL, inner circle	67.5 (60.75–72.375)	71.75 (65.75–78.25)	**0.002**
ONL, outer circle	52.375 (47.75–58)	55.5 (51.75–61.00)	**0.005**
PRL, central circle	67 (65.5–70.0)	70 (68.0–73.0)	**< 0.001**
PRL, inner circle	64.75 (63.00–65.75)	65.5 (64.25–67.0)	**< 0.001**
PRL, outer circle	64.25 (62.75–65.25)	65.00 (63.75–65.75)	**0.003**
RPE/BrM, central circle	15.0 (13.0–16.0)	16.0 (15.0–17.0)	**0.002**
RPE/BrM, inner circle	13.75 (12.5–15.125)	14.75 (13.75–15.875)	**<0.001**
Choroidal Layer (subfoveal)	188.1 ± 12.4	258.2 ± 5.76	**<0.001**

Median retinal thickness values (μm) and IQR (25th to 75th quartile), by circle (central, inner, and outer) with significant differences between eyes with and without SDD. Mean choroidal thickness (μm) with standard deviation is also provided. To statistical significance, *p*-value was established at 0.05.

Table 6. Statistically significant results from the association between retinal layers and choroidal thicknesses with the presence of SDD and HRF.

	SDD present (*β*/*P*-VALUE)	**HRF present** (*β*/*P*-VALUE)	**Age** (*β*/*P*-VALUE)	**Sex - Female** (*β*/*P*-VALUE)	**AMD stage 3**
GCL, central	0.093 [0.015,0.171]/**0.019**	–	–	-0.119 [-0.185,-0.052]/**0.001**	–
OPL, outer	-0.048 [-0.071, 0.025]/**<0.001**	–	–	–	–
ONL, inner	-0.066 [-0.126,-0.005]/**0.035**	0.082 [0.003,0.161]/**0.042**	-0.004 [-0.007,-0.000]/**0.039**		-0.078 [-0.142,-0.013]/**0.018**
PRL, central	-0.021 [-0.054,-0.018]/**0.031**		-0.002 [-0.004/-0.001]/**<0.001**		-0.031 [-0.050,-0.011]/**0.002**
RPE/BrM, central	-0.079 [-0.150,-0.008]/**0.029**	0.135 [0.035,0.236]/**0.009**			0.159 [0.077,0.241]/**<0.001**
RPE/BrM, inner	-0.086 [-0.129,0.042]/**<0.001**	0.113 [0.052,0.0175]/**<0.001**			
Choroid, subfoveal	-51.9 [-79.0,-24.8]/**<0.001**	–	-3.0 [-4.6,-1.4]/**<0.001**		

Regression coefficient *β* with 95% CI, and the *p*-value, derived from stepwise backward multivariate linear regression analysis separately for each macular layer. Only significant results are presented, based on the set of 233 eyes—worst eye per subject.

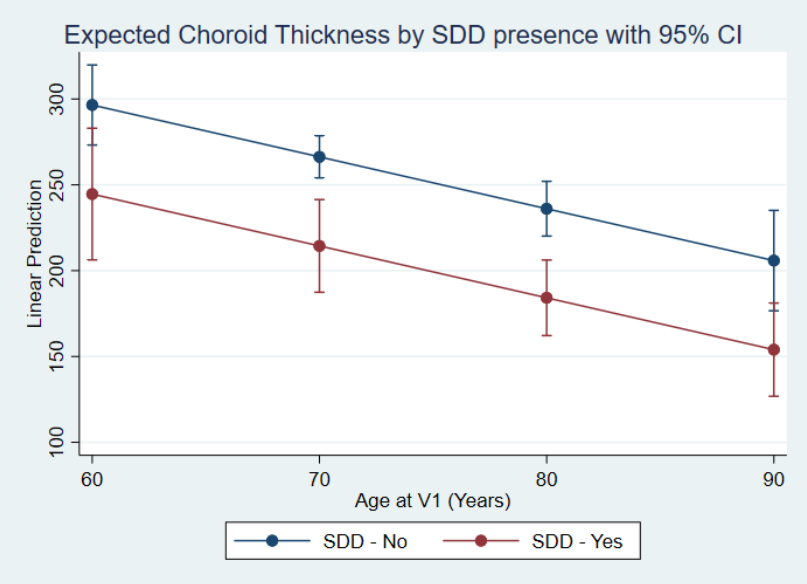

Fig. 4 Model-based expected subfoveal choroidal thicknesses (µm), by the presence of SDD, adjusted for progressive age.

DISCUSSION

In this study, we present a detailed quantitative morphological evaluation of all retinal layers obtained with SD-OCT through semiautomatic segmentation and of the choroid, in eyes with early AMD, in a cohort of patients from the AMD Coimbra Eye Study. We have demonstrated that changes in all retinal layers are observed early in the disease process, with stage 3 being associated with thinner inner and outer neuronal retinal layers, compared to stage 2a. These outcomes in a large cohort seem to support the existence of progressive neurodegenerative changes in early AMD, a finding not consistently reported so far. The association between known biomarkers of disease progression and retinal and choroidal layers was also highlighted, with SDD being predictive of thinner outer neuronal retinal layers, RPE/Bruch layer, and choroid. Interestingly the presence of HRF was associated to thicker ONL and RPE/BrM, consistent with their known association to drusen volume increase, RPE disruption, and migration to the ONL.

We first provide data on the retinal thickness and volume of all retinal layers individually, by ETDRS circle (central, inner, and outer) and by early AMD stage (2a, 2b, and 3), acquired after semiautomatic segmentation. We found that the inner retinal layers, namely, the RNFL, GCL, IPL, and INL, tendentially decrease in thickness when comparing stage 3 to stage 2a but not to stage 2b. This change took place mainly in the inner and outer circles of the EPDRS grid, and pairwise comparison between stages revealed significant or near-significant differences in all except for the INL. Despite some layers reaching only near-significant difference, considering the global thinning tendencies graphically depicted across stage, they appear to be nonetheless clinically relevant. Interestingly, both the ONL and PRL were thinner too in stage 3 but at the central circle. We speculate that this pattern might represent some form of contiguous process of atrophy or degeneration, being more prominent at the photoreceptor level in the fovea and then extending to the inner layers of the parafovea and perifovea. This suggestive pattern of neuronal anterograde degeneration in almost all neuroretinal layers at the macula of early AMD patients stratified by stage has not yet been consistently described. In fact, some studies provide insight in differences in retinal layer thickness between AMD and non-AMD eyes; however, progression within early stages suggesting an early and continuous neurodegeneration was rarely addressed in the literature [18–21].

The German AugUR study is the only epidemiologic study, besides ours, that provides an extensive report on retinal layers thickness in AMD through similar methodology, allowing for comparability. In their study Brandl et al. [14] found that the photoreceptor layers (ONL and PRL) were thinner between AMD and non-AMD eyes. Although not specifically tested, the analysis of their results shows a tendency in decrease in thickness in mild, moderate, and severe early AMD. Regarding the inner layers, they also found differences between AMD and non-AMD eyes; however, the results were not reproducible in their confirmation cohort, and only ONL and PRL

remained significant. When analyzing thickness by stage progression within early AMD, our results seem similar regarding both the outer and inner neuronal layers; however, they did not test between early AMD stages, and thus, comparability across stages is limited. Another study by Lamin et al. [17] analyzed the volume of the INL and GCL+ IPL between AMD and non-AMD eyes and found that volume was inferior in the first group. Muftuoglu et al. [19] found that in intermediate AMD patients, the RNFL and GCL were well preserved, but as macular degeneration progressed, the IPL became thinned, suggesting that a transsynaptic degeneration of ganglion cell dendrites might be present with photoreceptor loss. In both studies, the authors conclude that some form of neuronal degeneration due to photoreceptors loss, resulting in reduced transneuronal input to the inner retina causing its degeneration, is present in early or intermediate AMD. These findings are in accordance with our own results.

Regarding the RPE/BrM layer, we showed increased thickness in stage 3 in the central circle, which is consistent with the expected drusen volume increase as the disease progresses and is in accordance with the results from the AugUR study [14]. In this respect, Lamin et al. [17] showed that the volume of the outer retinal layers (photoreceptors and RPE/BrM) was greater in AMD eyes compared to controls, as expected due to the presence of drusen, but in addition, they found that in AMD eyes, some retinal layers underwent volume change over a 2-year follow-up: a reduction in ONL volume and an increase in RPE/BrM.

Our findings that the RPE/BrM complex was thicker, and the neuronal retinal layers were tendentially thinner for eyes with higher early AMD stage highlight that quantitative assessment by automatic segmentation of the latter has potential to be used as quantitative biomarkers of progression. Improvement in segmentation algorithms in the future together with the development and inclusion of these parameters in artificial intelligence models could be valuable in identifying individuals at risk of developing late AMD. Besides, understanding disease pathophysiology per se also contributes to the development of preventive strategies or treatments capable of delaying progression to late stages [4].

The created model of linear association between the neuronal layers and the RPE-BrM layer thickness and volume (an indirect representation of drusen load in the macula) identified only weak associations, significant for the ONL and PRL. This led us to conclude that they not fit into a linear model that can fully explain the behavior observed. In this respect, Lee et al. [18] found that the average GCL+ IPL combined layer thickness was negatively correlated with the drusen area automatically given by the SD-OCT software used in their study. We could not find such association. But our model took into account all segmented retinal layers, so further interactions might explain the different results. Borreli et al. [30] results were more in accordance to ours as they found no association between GCL thickness and drusen area or volume. Therefore, a mechanist cause of simple compression of the inner layers by the elevation of the RPE and outer retina over drusen leading to secondary inner cell damage does not seem probable and corroborates

the postreceptoral hypothesis as a better explanation for the observed neuronal retinal damage, progressive over the course of disease.

Regarding the association to specific qualitative features, we found SDD to be present in eyes with not only thinner choroids but also thinner RPE-BrM layer and several neuroretinal layers, including the IPL, INL, OPL, ONL, and PRL. Besides, eyes with SDD were significantly predictive of a thinner OPL, ONL, PRL, and RPE/BrM layer and a thinner subfoveal choroid in multivariate linear regression. The association to thinner choroids is well described in the literature, and some authors propose that choroidal fibrosis could be a causative mechanism, leading to the disorganization of the RPE and consequently SDD [31]. Garg et al. [31] also found that the retina was thinner in SDD eyes, suggesting that atrophy of the choroid could be related with atrophy of the retina in early AMD. Nittala et al. [13] examined longitudinally the behavior of the external retina in eyes with SDD and found that the rate of PRL outer segment thinning was higher in these eyes compared to eyes without SDD, and most importantly, the rates of thickness change in the neurosensory retina, RPE + drusen complex, and choroid were higher in eyes with SDD although not statistically significant. This enhances not only our assumptions that neurodegeneration might happen early and progress in initial stages in AMD but also that specific biomarkers such as SDD can represent a more rapidly progressive phenotype or pathway of neurodegeneration. In fact, it is well-known that SDD are a risk factor to advanced AMD, so it should not be unexpected that faster inner retinal compromise can be associated to their presence too.

Hyperreflective foci are defined as well-circumscribed lesions within the neurosensory retina with a reflectivity similar to the RPE. They correspond in non-neovascular AMD to migrating RPE and are seen as hyperpigmentation in CFP. Their presence in longitudinal studies was associated to progression to advanced AMD, and they can be a potential structural endpoint for studies focusing on preventing conversion to late stages [27, 32]. In our analysis, we found that HRF were associated to thicker ONL and RPE/BrM layer. This confirms that they are associated to increased drusen load and thus are biomarkers of progressing disease. Their association in the model to thicker inner circle ONL might be explained by lateral displacement of the ONL in the central circle by lager drusen in the RPE-BrM layer. Another hypothesis is that in the model, SDD were considered, and while SDD are predictive of a thinner retinal phenotype, HRF were associated to larger soft drusen and thus predictive of another phenotype with higher ONL thickness.

Study strengths are the multimodal-based staging approach in the context of a large epidemiologic study and the systematic and reliable segmentation of all retinal layers by a supervised trained grader in the setting of a reading center; we provide significant insight on neurodegeneration in all retinal layers in early AMD by stage of severity, and we also explore its association to specific biomarkers of progression to late AMD.

The main limitation of our study is its cross-sectional nature and relatively small samples of higher early AMD stages. The lower representation of higher stages is due to the underlying

design of a population-based epidemiologic study such as the AMD Incidence study; however, we still found important differences in almost all retinal layers by using robust statistical analysis with correction for multiple confounders. We also did not include a control non-AMD group, and this can be a shortcoming when comparing our results to other studies; however, the primary objective of our work was to detect differences in all retinal layers within early AMD stages to infer a progressive neurodegenerative process with disease severity and to find associations with biomarkers of progression. Prospective longitudinal evaluation with semiautomated or fully automated layer segmentation in early AMD eyes should be conducted in order to validate our findings.

In conclusion, we present one of the few population-based studies reporting with great detail the effect of progressing stage on all retinal layers and in the choroid of eyes with early AMD, and we demonstrate that neurodegeneration is present in all retinal layers and progresses before late stages are reached. SDD seem to be biomarkers of a different phenotype characterized by more prominent and faster neurodegeneration and probably associated to different genetic susceptibilities.

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Demographic and clinical characteristics of the study population.

Characteristics	n (%)
Gender	
Male, n (%)	84 (36.0)
Female, n (%)	149 (64.0)
Age	
55-64 years, n (%)	26 (11.2)
65-74 years, n (%)	94 (40.3)
75-84 years, n (%)	93 (39.9)
≥ 85 years, n (%)	20 (8.6)
Smoking	
Smoker	5 (2.2)
Ex-smoker	26 (11.3)
Non-smoker	200 (86.6)
Diabetes	
Yes	29 (12.5)
No	200 (85.8)
Does not know	4 (1.7)
Hypertension	
Yes	132 (56.7)
No	95 (40.8)
Does not know	6 (2.6)
Hypercholesterolemia	
Yes	102 (43.8)
No	114 (48.9)
Does not know	17 (7.3)
BMI, median (IQR)	27.4 (24.1 – 30.2) Kg/m^2
BCVA, median (IQR)	79 (73 – 83) letters
Spherical equivalent, median (IQR)	+0.625 (-3.75 – +1.75) diopters

BMI, body mass index; BCVA, best-corrected visual acuity; IQR, interquartile range.

Supplementary Table 2. Expected retinal layer thicknesses by ETDRS circle of all segmented retinal layers, and pairwise comparisons between early AMD stages.

	Expected Retinal Layer Thicknesses, µm			**Effect Estimates / Pairwise Test *p*-value**		
Retinal layers	**2a (n=286)**	**2b (n=19)**	**3 (n=41)**	**2a vs. 2b**	**2a vs. 3**	**2b vs. 3**
RNFL, central circle	2.51 (2.48-2.53)	2.54 (2.44 – 2.63)	2.50 (2.43 – 2.56)	- 0.028/1.000	0.013/1.000	0.041/1.000
RNFL, inner circle	3.07 (3.04 – 3.09)	3.05 (2.96 – 3.14)	3.01 (2.95 – 3.07)	0.017/1.000	0.057/0.111	0.040/1.000
RNFL, outer circle	3.56 (3.53 – 3.58)	3.53 (3.44 – 3.63)	3.47 (3.41 – 3.53)	0.021/1.000	**0.086/0.005**	0.065/0.471
GCL, central circle	2.65 (2.62-2.67)	2.62 (2.52-2.72)	2.65 (2.58-2.71)	0.028/1.000	-0.003/1.000	- 0.031/1.000
GCL, inner circle	3.86 (3.84-3.89)	3.87 (3.77 – 3.97)	3.76 (3.70 – 3.83)	- 0.002/1.000	**0.099/0.002**	0.101/0.120
GCL, outer circle	3.51 (3.48 – 3.54)	3.50 (3.40-3.60)	3.46 (3.39 – 3.52)	0.012/1.000	0.055/0.193	0.042/1.000
IPL, central circle	3.00 (2.98 – 3.02)	3.01 (2.95 – 3.08)	3.00 (2.96 – 3.05)	- 0.015/1.000	-0.005/1.000	0.010/1.000
IPL, inner circle	3.69 (3.68 – 3.71)	3.70 (3.63 – 3.76)	3.63 (3.59 – 3.67)	- 0.002/1.000	**0.065/0.002**	0.068/0.118
IPL, outer circle	3.35 (3.33 -3.37)	3.44 (3.28 – 3.41)	3.31 (3.27 -3.36)	0.008/1.000	0.040/0.124	0.032/0.994
INL, central circle	3.03 (3.00-3.05)	3.02 (2.93 -3.12)	3.09 (3.02-3.15)	0.005/1.000	-0.060/0.132	- 0.064/0.568
INL, inner circle	3.75 (3.72 – 3.77)	3.71 (3.61 -3.81)	3.69 (3.62 – 3.76)	0.036/1.000	0.057/0.172	0.021/1.000
INL, outer circle	3.51 (3.48 – 3.53)	3.50 (3.40 – 3.59)	3.48 (3.41 – 3.55)	0.011/1.000	0.028/1.000	0.017/1.000
OPL, central circle	3.22 (3.20 – 3.25)	3.30 (3.21 – 3.38)	3.25 (3.20-3.31)	- 0.072/0.154	-0.028/0.782	0.043/0.940
OPL, inner circle	3.47 (3.44 – 3.49)	3.49 (3.40 – 3.57)	3.47 (3.41 – 3.52)	- 0.020/1.000	0.001/1.000	0.021/1.000
OPL, outer circle	3.29 (3.26 – 3.31)	3.28 (3.20 – 3.37)	3.28 (3.22 – 3.34)	0.004/1.000	0.010/1.000	0.006/1.000
ONL, central circle	4.56 (4.53 – 4.58)	4.53 (4.44 – 4.61)	4.47 (4.41 – 4.53)	0.029/1.000	**0.088/0.002**	0.059/0.489
ONL, inner circle	4.26 (4.24 – 4.28)	4.25 (4.17 -4.34)	4.19 (4.13 – 4.25)	0.009/1.000	0.068/0.020	0.060/0.463
ONL, outer circle	4.01 (3.99 -4.04)	4.01 (3.93-4.10)	4.00 (3.94 – 4.06)	0.002/1.000	0.014/1.000	0.013/1.000
PRL, central circle	4.25 (4.24-4.26)	4.25 (4.22-4.27)	4.23 (4.21-4.24)	0.003/1.000	**0.023/0.006**	0.020/0.335
PRL, inner circle	4.19 (4.18-4.19)	4.19 (4.16-4.21)	4.18 (4.16-4.20)	- 0.001/1.000	0.004/1.000	0.005/1.000
PRL, outer circle	4.17 (4.17 – 4.18)	4.14 (4.12 – 4.17)	4.17 (4.16 -4.19)	0.033/0.009	0.002/1.000	- 0.031/0.046
RPE, central circle	2.76 (2.74-2.79)	2.75 (2.66-2.84)	2.91 (2.85-2.97)	0.016/1.000	**- 0.143/<0.0001**	**- 0.160/0.001**
RPE, inner circle	2.69 (2.66 – 2.72)	2.68 (2.59 – 2.77)	2.73 (2.67 -2.79)	0.012/1.000	-0.037/0.459	- 0.049/0.818
RPE, outer circle	2.56 (2.54-2.59)	2.63 (2.54-2.72)	2.55 (2.49-2.61)	- 0.063/0.338	0.016/1.000	0.078/0.249

Expected Retinal Layer Thicknesses (µm), with 95% CI in parenthesis, effect estimates (on log scale), and *p*-values of pairwise tests, obtained from layer-specific linear mixed models. The analysis includes 346 eyes with early AMD (stages 2a, 2b, and 3), obtained from the original cohort of AMD Incidence study. *P*-values were judge at Bonferroni-corrected significance level, $p < 0.05/24=0.002$. Significant and strong near significant results are highlight with bold text.

Supplementary Table 3. Correlation between the RPE/BrM layer thickness and volume to the neuroretinal layers, age, gender, spherical equivalent, and AMD stage.

	RPE/BRM THICKNESS			RPE/BRM
	Central circle	**Inner circle**	**Outer circle**	**VOLUME**
RNFL	-0.005 (0.942)	-0.113 (0.086)	-0.060 (0.362)	-0.065 (0.323)
GCL	-0.009 (0.889)	0.015 (0.826)	0.016 (0.805)	0.025 (0.707)
IPL	-0.010 (0.876)	0.004 (0.949)	0.053 (0.425)	0.031 (0.642)
INL	-0.107 (0.105)	0.008 (0.905)	0.047 (0.476)	0.062 (0.347)
OPL	**-0.156 (0.017)**	-0.032 (0.625)	-0.021 (0.754)	0.032 (0.629)
ONL	0.029 (0.663)	**0.208 (0.001)**	0.003 (0.970)	0.083 (0.206)
PRL	-0.046 (0.481)	**0.188 (0.004)**	**0.240 (<0.001)**	**0.229 (<0.001)**
Age	-0.064 (0.328)	**-0.180 (0.006)**	-0.023 (0.732)	-0.067 (0.309)
Gender	-0.043 (0.512)	0.003 (0.970)	0.025 (0.702)	0.048 (0.471)
Spherical equivalent	0.104 (0.171)	0.073 (0.339)	-0.076 (0.322)	-0.045 (0.550)
Stage	**0.219 (<0.001)**	**0.160 (0.014)**	0.122 (0.064)	**0.160 (0.014)**

The table above provides the pairwise Spearman correlation coefficient and, in parenthesis, the *p*-value. These results are based on 233 eyes - worst eye per subject. Statistically significant correlations are highlighted with bold text ($p<0.05$).

Supplementary Table 4. Multivariate linear regression analysis of thickness and volume between RPE/BrM layer and neuroretinal layers.

	RPE/BRM THICKNESS (central)			RPE/BRM VOLUME
	Central circle (β/*p*-value)	**Inner circle** (β/*p*-value)	**Outer circle** (β/*p*-value)	(β/*p*-value)
ONL	0.196 (0.035-0.358) /0.018	0.198 (0.095-0.302)/ <0.001)	–	–
PRL	–	–	1.139(0.647,1.630) /<0.001	0.828 (0.475 – 1.82) / <0.001
Stage 2b			0.05(0.002–0.113) /0.041	0.069(0.013-0.125) /0.015
Stage 3	0.174 (0.099-0.248)/<0.001)	0.077 (0.027-0.127)/0.003		0.053 (0.0140.092) /0.008

Regression coefficient β with 95% CI, in parenthesis, and the *p*-value, derived from stepwise backward multivariate linear regression analysis separately for each macular layer. Only significant results are presented, based on the set of 233 eyes - worst eye per subject. All retinal layer thicknesses were log-transformed and adjusted for age, gender, spherical equivalent, and AMD stage. Variables with *p*-value>0.1 were removed from the model.

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CHAPTER 5.
COMMON AND RARE GENETIC RISK VARIANTS IN AGE-RELATED MACULAR DEGENERATION AND GENETIC RISK SCORE IN THE COIMBRA EYE STUDY

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In the last two decades, several research groups provided important information on AMD genetics with the identification of several common and rare variants associated with the risk of disease development and progression. Pursuing genetic analysis of AMD in different populations is important since it will allow us to uncover phenotype-genotype-environmental associations and contributes to expanding the global knowledge of the disease's pathophysiology. Besides, exploring strategies such as calculating the genetic risk score, this is, the cumulative risk of developing AMD based on the genotype of variants known to be associated with disease in a given population, can also be useful when integrating the genetic information with other interacting environmental and demographic factors to better predict disease risk. Despite this, to date, no data on the genetics of AMD was available in any Portuguese population.

As previously presented the AMD Incidence Study included extensive multimodal phenotypic characterization of the participants and blood samples were collected from those who consented to further genetic analysis.

After exploring the effects of multimodal imaging in AMD classification and staging and on new imaging biomarkers obtained with this comprehensive approach, we set out to characterize the genetics of this cohort regarding AMD risk.

In this Chapter we present the contribution of common and rare genetic variants in the development of AMD in this Portuguese population, we will explore the effect of the burden of pathogenic rare variants in disease development, and calculate differences between the genetic risk score of AMD patients compared to non-AMD participants. The effect of rare variants is of major interest because they are known to have a strong impact due to high penetrance and may predispose to more severe disease.

Our results showed that both common and rare variants were associated with AMD, but interestingly, it was a CFH rare variant that conferred the highest risk of disease while three major risk variants had a lower-than-expected allele frequency in this population originating from a geographic region with lower rates of AMD. Still, the GRS was significantly higher in AMD patients. Finally, damaging CFH rare variants were cumulatively more common in AMD cases.

ABSTRACT

Purpose: To determine the contribution of common and rare genetic variants in age-related macular degeneration (AMD) in a Portuguese population from the Coimbra Eye Study (CES), and the genetic risk score (GRS).

Methods: Participants underwent ophthalmologic examination and imaging. A centralized reading centre performed AMD staging. Genetic sequencing was carried out with the EYE-RISK assay. Sixty-nine single nucleotide polymorphisms (SNPs) were genotyped and tested for association with AMD. Case‑control and progression-to-AMD analyses were performed using logistic regression to assess allelic odds ratio (OR) at a 95% confidence interval (CI) for each variant. GRS was calculated for cases/controls and progressors/non-progressors. Cumulative impact of rare variants was compared between cases/controls using logistic regression.

Results: In case‑control analysis (237 cases/640 controls) variants associated with risk of disease were: *ARMS2* rs10490924, *ARMS2_HTRA1* rs3750846, *CFH* rs35292876, *SLC16A8* rs8135665, *TGFBR1* rs1626340. Major risk variants *ARMS2/ HTRA1* rs3750846, *CFH* rs570618 and *C3* rs2230199 had unexpected lower allele frequency (AF), and the highest risk-conferring variant was a rare variant, *CFH* rs35292876 (OR, 2.668; *p*-value = 0.021). In progression-to-AMD analysis (137 progressors/ 630 non-progressors), variants associated with risk of progression were *ARMS2* rs10490924, *ARMS2_HTRA1* rs3750846, *CFH* rs35292876. GRS of cases/ controls was 1.124 ± 1.187 and 0.645 ± 1.124 (*p*-value < 0.001), and of progressors/non-progressors was 1.190 ± 1.178 and 0.669 ± 1.141 (*p*-value < 0.001). Higher proportion of pathogenic rare *CFH* variants was observed in cases (OR, 9.661; *p*-value < 0.001).

Conclusions: Both common and rare variants were associated with AMD, but a *CFH* rare variant conferred the highest risk of disease while three major risk variants had a lower-than-expected AF in our population originary from a geographic region with lower prevalence of AMD. GRS was still significantly higher in AMD patients. Damaging *CFH* rare variants were cumulatively more common in AMD cases.

Keywords: age-related macular degeneration, Coimbra eye study, common genetic variants, genetic risk score, rare genetic variants, single nucleotide polymorphism

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in the older population of industrialized countries (Colijn et al., 2017; Li et al., 2020; Wong et al., 2014). As the burden of disease is expected to increase in the next decades (Colijn et al., 2017; Li et al., 2020), further understanding on the pathophysiology of disease is of utmost importance, not only in order to develop therapeutic strategies capable of halting disease progression but also to provide the best advice to patients on their individual risk.

In the last two decades several research groups provided important information on AMD genetics with identification of several common and rare variants associated to risk of disease development and progression. In fact, the heritable component in AMD is estimated to be as high as 45%‑70% (Fritsche et al., 2013, 2016; Geerlings, de Jong, et al., 2017; Jordan-Yu et al., 2021). Recently, a landmark genome-wide association study (GWAS) identified 52 variants at 34 genomic regions to be independently associated with AMD. Forty-five were common variants while seven were rare variants (minor allele frequency [MAF] <0.01). Susceptibility genes were grouped into four main pathways: (1) complement system, (2) high density lipoprotein metabolism, (3) angiogenesis and (4) extracellular matrix remodelling. Most of the identified variants were in or near a gene of the complement system: complement factor H (*CFH*), complement factor I (*CFI*), complement component 3 (*C3*), complement component 2 (*C2*), complement component 9 (*C9*), complement factor B (*CFB*) and vitronectin (*VTN*). Furthermore, a significant burden of rare variants was observed in the *CFH* and *CFI* genes (in addition to TIMP metallopeptidase Inhibitor 3 (*TIMP3*) and solute carrier family 16 member 8 (*SLC16A8*)) (Fritsche et al., 2016). In fact, the interest in rare variants in AMD is significantly growing since they can have strong impact due to high penetrance and may predispose to more severe disease in a given cluster of subjects or population. Several other rare and low-frequency variants (MAF 0.010‑0.050) were already identified and might explain the missing heritability in AMD (Geerlings, de Jong, et al., 2017). Since population-specific rare variants tend to have a strong functional effect, case‑control studies are, therefore, of most importance to be carried out in different populations (de Breuk et al., 2020; Fritsche et al., 2016; Gibson, 2012).

Strategies such as calculating the genetic risk score (GRS), the cumulative risk of developing AMD based on the genotype of variants known to be associated with disease, can also be useful. This is especially true when integrating the genetic information with other interacting environmental and demographic factors to better predict disease risk (Colijn et al., 2021; Cooke Bailey et al., 2016; de Breuk et al., 2020; Lambert et al., 2016; Wang et al., 2014). Furthermore, the GRS is important to explore in different cohorts as its calculation depends on the presence of risk variants that may be differently distributed across populations.

The Coimbra Eye Study (CES) is a 2-visit epidemiologic population-based study on the prevalence and incidence of AMD in a Portuguese population (NCT01298674, NCT02748824) (Cachulo et al., 2015, 2016; Farinha et al., 2019, 2020). The environmental and nutritional risk factors associated with AMD prevalence were previously explored and reported (Cachulo et al., 2016; Raimundo et al., 2018). Subjects who participated in the 6.5-year follow-up visit for the estimation of incidence also had blood samples collected for further genetic characterization (Farinha et al., 2020).

The purpose of this study is to determine the contribution of common and rare genetic variants in the development of AMD in a Portuguese population, to explore the burden of pathogenic rare variants, and to determine differences between the GRS of AMD patients compared to non-AMD participants.

MATERIALS AND METHODS

Study design and population

The Epidemiological Study (NCT01298674) is a single-centre population-based study whose cohort included two geographically distinct populations aged ≥55 years for the estimation of AMD prevalence: one from a coastal town (Mira), and the second from an inland town (Lousã) (Cachulo et al., 2016, 2015).

The AMD Incidence Study (NCT027048824) was conducted 6.5 years later and included only the subjects from the coastal town Mira, which had been recruited in the primary health care unit. This population was extensively characterized in this follow-up visit from a demographic and clinical perspective, including multimodal imaging (MMI). Complete information on the identification and description of the study population, as well on the patients' recruitment details, have been published elsewhere (Cachulo et al., 2016, 2015; Farinha et al., 2019).

Signed informed consent was obtained for all participants. The study adhered to the tenets of the Declaration of Helsinki (2008) and of the International Conference on Harmonization ‑ Good Clinical Practice Guideline. The Association for Innovation and Biomedical Research on Light and Image (AIBILI) Ethics Committee issued a favourable opinion for the conduction of the study.

Data collection and AMD staging

Briefly, all participants from the follow-up incidence study underwent a detailed questionnaire-based interview on demographic, clinical and lifestyle related information by a trained nurse from the primary health care centre, and blood samples were collected from the participants who consented for further genetic and laboratorial analysis. Afterwards, all participants underwent bilateral ophthalmological assessment, including best-corrected visual acuity (BCVA) tested with Early Treatment Diabetic Retinopathy Study (ETDRS) charts and MMI. This multimodal approach included Colour Fundus Photography (CFP) (Topcon® fundus camera, TRC-NW8; Topcon Corp., Tokyo, Japan), Spectral Domain Optical Coherence Tomography (SD-OCT), Fundus Autofluorescence (FAF) and Infrared (IR) imaging with Spectralis HRA + OCT (Heidelberg Engineering, Heidelberg, Germany) (Farinha et al., 2020, 2019).

In respect to AMD grading the Rotterdam staging system was used: early AMD was defined as stages 2a, 2b and 3 (this is, presence of large (≥125 μm), soft, indistinct or reticular drusen only; or of soft distinct (≥63 μm), indistinct (≥125 μm) or reticular drusen with pigmentary abnormalities), and late AMD as stage 4 (neovascular AMD (nAMD), and/or geographic atrophy (GA)) (Klaver et al., 2001; Vingerling et al., 1995). Staging of an individual participant was based on the eye with more severe status if both eyes were gradable, and on the gradable eye if only one

eye was gradable. AMD staging was performed at a centralized reading centre (Coimbra Ophthalmology Reading Center, AIBILI, Portugal), by senior medical retina specialist graders.

Genetic sequencing procedures and selection of cases/controls

Genomic DNA samples of the CES participants were genotyped according to standard procedures in the context of collaboration with The European Eye Epidemiology Consortium (E3). As reported elsewhere, our cohort genetic data was obtained through the recently published EYE-RISK genotype assay, which was designed to genotype 87 single nucleotide polymorphisms (SNPs), including the 52 independently associated SNPs identified by the International AMD Genomics Consortium (IAMDGC) (de Breuk et al., 2020; Fritsche et al., 2016). The assay also includes genes that have been described to carry rare variants in AMD (*C3, C9, CFH, CFI, TIMP3, SLC16A8*), candidate genes possibly carrying rare variants in AMD ((*age-related maculopathy susceptibility 2 (ARMS2), CD46 molecule (CD46), CFB, htrA serine peptidase 1 (HTRA1)),* and genes involved in AMD-mimicking macular dystrophies ((*ATP binding cassette subfamily a member 4 (ABCA4), catenin alpha1 (CTNNA1), peripherin2 (PRPH2*)). Sequencing was performed by combining genomic capture using single-molecule molecular inversion probes (smMIPs) and next-generation sequencing, as described by de Breuk et al. (2020). After quality control, 69 SNPs were successfully genotyped in our cohort. To ensure a complete dataset of the 52 AMD-associated variants 10 SNPs were genotyped by KASP genotyping assays.

Cases were defined as participants from the AMD Incidence Study with early or late AMD, this is stages 2, 3 and 4. Controls were participants that in the Incidence Study were staged as 0 (no signs of AMD or only hard drusen) if their age was above 60 years old, or stage 1 (only soft distinct drusen (≥63 μm) or pigmentary changes) if their age was above 70 years old. This was done to avoid including controls that could develop AMD. All cases that consented to the genetic analysis and with viable DNA samples were genotyped by the EYE-RISK consortium, as well as age and sex-matched controls.

Genetic analysis –Association to disease/ no disease and genetic risk score

The successfully genotyped samples and 69 SNPs were tested for association under an additive model, using the presence of AMD as a binary outcome. A logistic regression analysis was performed to assess allelic odds ratio (OR) at 95% confidence interval (CI) for each variant, adjusted for age and sex, with a significance level set to 0.05. We compared SNP allele frequencies (AFs) of control individuals and AMD patients in the CES cohort to those of the EYE-RISK and IAMDGC datasets, and we explored if the allelic ORs for all SNPs in our study showed the same direction and magnitude of effect compared with those reported in the EYE-RISK study and IAMDGC primary analysis (de Breuk et al., 2020; Fritsche et al., 2016). The GRS was also computed in our population. Fifty-two independent variants identified by Fritsche et al. (2016)

were selected and the OR from the IAMDGC GWAS fully conditioned analysis was used to compute the GRS. For each participant the GRS was generated according to the formula: $GRS = \sum_{i=1}^{52}(G_i\beta_i)$, where G_i represents the genotype of variant i coded as 0, 1 or 2 based on the number of minor alleles and β_i represents the effect size of variant i (natural logarithm of the odds ratio of the minor allele variant i, based on the GWAS of the IAMDGC fully conditioned analysis. No data imputation was performed. The GRS was considered as missing if the genotype of one of the major risk variants (*CFH* rs570618, *CFH* rs10922109, *C2/CFB/* ski2 like RNA helicase [*SKIV2L*] rs429608, *ARMS2/HTRA1* rs3750846 and *C3* rs2230199) was not available.

Progression to AMD – Genetic associations and GRS

Since the CES is a longitudinal study, it was possible to also explore genetic associations with progression to AMD in the 6.5-year follow-up. For this analysis we compared progressors to non-progressors. Progressors were participants that progressed from no AMD at baseline (stages 0 or 1) to having AMD at the follow-up visit in the Incidence study (this is, stages 2,3 or 4). Non-progressors were those participants that were classified as not having AMD (stages 0 or 1) in both baseline and follow-up visits. Genetic associations were performed using the same methodology described in the previous section, as well as the calculation of the GRS for progressors versus non-progressors.

Rare variants analysis

For the rare variant analysis, we performed logistic regression analyses to assess the cumulative effect of rare variants with AMD for the *CFH*, *CFI* and *ARSM2* genes. All genetic variants with a MAF < 0.01 were included in the analysis.

Filtering of variants to ensure quality of the data was carried out by the EYE-RISK Consortium. Variants with less than 40 reads coverage on reference allele were changed to missing values. For homozygous reference samples, genotype was kept unchanged, even if it did not have 40 reads coverage in alternate alleles. Following the EYE-RISK quality control steps regarding rare variants, samples with more than 10% missing calls were removed from our dataset.

To predict the functional effect of the rare variants found in our population, two algorithms were explored: the PolyPhen 2 prediction score and the combined annotation-dependent depletion (CADD) score. According to the PolyPhen 2 prediction score the variants included were stratified into: benign (b), possibly damaging (P) and probably damaging (D). Variants with a described loss-of-function (LoF) effect based on functional studies were included as a separate category. According to the CADD score the functional effect of genetic variants was stratified in: score of less than 20, of 20 or more, or LoF. Loss-of-function variants were defined as nonsense, splice-site and frameshift variants and as missense variants with a described functional effect based on functional studies (de Breuk et al., 2020).

Macular dystrophies mimicking AMD in the CES

For *ABCA4*, *CTNNA1* and *PHPR2* genes, sequenced with the EYE-RISK assay, we filtered for carriers of variants of class 3 or higher, based on the American College of Medical Genetics and Genomics classification (de Breuk et al., 2020). Retinal images of carriers were re-evaluated by a retinal specialist (C.F.) to identify patients with potential misdiagnose of AMD caused by mimicking inherited macular dystrophies.

RESULTS

From the original cohort of 1.617 participants in the AMD incidence study, where 237 (14.7%) were early AMD cases and 28 (1.73%) were late AMD cases, a total of 922 samples were successfully genotyped for a total of 69 SNPs, in association with the EYE-RISK/ E3. In addition, to include only controls respecting the above-mentioned age criteria, 45 samples from controls were excluded. The final cohort in analysis comprised 877 genotyped samples from 237 cases and 640 controls (Figure 1). Regarding AMD cases, 24.3% (*n* = 213) were early AMD (stages 2 and 3) and 2.73% (*n* = 24) were late AMD (stage 4). The global mean age of the cohort was 72.6 ± 6.8 years and 57.8% were female. The mean age was 71.9 ± 6.4 years in controls versus 74.7 ± 7.3 in cases, and 56.2% of controls versus 62.0% of cases were female. Characterization of the analysed genotyped population is presented in Table S1.

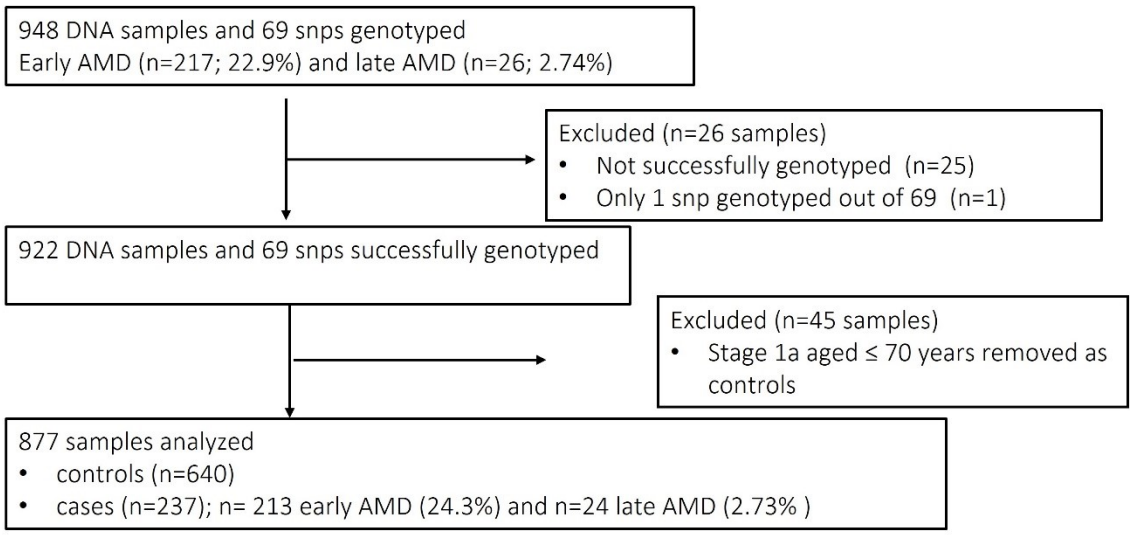

FIGURE 1. Flowchart of genotyped samples from the CES.

The AFs of the tested SNPs in AMD cases and controls are presented in Table 1 and Figure 2. Comparing the AFs from the CES cohort to the AFs of the EYE-RISK and the IAMDGC datasets, the following inverse trends in MAF distribution between cases and controls in our study were found: the MAF was higher in controls for acyl-CoA dehydrogenase family member 10/BRCA1 associated protein (*ACAD10/ BRAP*) rs61941272, *C3* rs2230199, *C9* rs62358361, collagen type VIII alpha 1 chain (*COL8A1*) rs13081855 and rs140647181 and NPL4 homologue, ubiquitin recognition factor/tetraspanin 10 (*NPLOC4/TSPAN10*) rs656559; and the MAF was higher in AMD cases for ATP binding cassette subfamily A member 1 (*ABCA1)* rs1883025 and rs2740488, apolipoprotein E (exocyst complex component 3 like 2/microtubule affinity regulating kinase 4) *APOE (EXOC3L2/MARK4)* rs73036519, *CFH* rs3753394, collagen type IV alpha 3 chain (*COL4A3)* rs11884770, transforming growth factor beta 1 (*TGBR1)* rs334353, transforming growth factor beta receptor 1 (*TGFBR1)* rs1590, rs1626340 and rs334349, and vascular endothelial

growth factor A (*VEGFA)* rs943080. Another interesting finding was that for *ARMS2/HTRA1* rs3750846, a major risk variant for AMD, the allele frequency in cases was much lower than expected when comparing our cohort to EYE-RISK and IAMDGC reports (AF: 0.197 CES versus 0.432 EYE-RISK/ 0.436 IAMDGC). The same was true for *CFH* rs570618 and *C3* rs2230199, other major risk variants, albeit to a lesser extent.

TABLE 1. Allele frequencies (AFs) of the SNPs from AMD cases and controls in the CES and comparison to the EYE-RISK and the IAMDGC datasets

Gene	SNP	Major/ Minor allele	MAF controls CES	MAF cases CES	MAF controls EYE-RISK	MAF cases EYE-RISK	MAF controls IAMDGC	MAF cases IAMDGC
ABCA1	**rs1883025**	**C / T**	**0.264**	**0.288**	**0.266**	**0.239**	**0.261**	**0.243**
ABCA1	**rs2740488**	**A / C**	**0.292**	**0.297**	**0.285**	**0.244**	**0.275**	**0.255**
ACAD10/BRAP	**rs61941272**	**C / A**	**0.009**	**0.006**	**0.010**	**0.015**	**0.018**	**0.024**
ADAMTS9	rs6795735	C / T	0.521	0.530	0.458	0.450	0.433	0.465
ADAMTS9-AS2	rs62247658	T / C	0.525	0.537	0.472	0.457	0.433	0.466
APOE	rs429358	T / C	0.106	0.072	0.114	0.108	0.135	0.099
APOE (EXOC3L2/MARK4)	**rs73036519**	**G / C**	**0.216**	**0.257**	**0.286**	**0.281**	**0.302**	**0.284**
ARHGAP21	rs12357257	G / A	0.317	0.297	0.270	0.230	0.223	0.243
ARMS2	rs10490924	G / T	0.142	0.201	0.181	0.437	0.208	0.436
ARMS2/HTRA1	rs3750846	T / C	0.140	0.197	0.181	0.432	0.208	0.436
B3GALTL	rs9542236	T / C	0.461	0.483	0.466	0.474	0.437	0.452
B3GALTL	rs9564692	C / T	0.329	0.319	0.302	0.260	0.299	0.277
C2	rs4151667	T / A	0.017	0.004	0.029	0.028	0.046	0.025
C2/CFB/SKIV2L	rs2746394	G / A	0.012	0.008	0.008	0.009	0.012	0.016
C2/CFB/SKIV2L	rs429608	G / A	0.142	0.078	0.134	0.087	0.148	0.090
C2/CFB/SKIV2L (PBX2)	rs204993	A / G	0.182	0.191	0.201	0.259	0.260	0.284
C3	rs147859257	T / G	*0.000*	*0.000*	0.001	0.014	0.004	0.012
C3	**rs2230199**	**G / C**	**0.183**	**0.168**	**0.182**	**0.249**	**0.208**	**0.266**
C3 (NRTN/FUT6)	rs17855739	C / T	0.001	0.000	0.001	0.001	0.049	0.038
C9	rs34882957	G / A	0.013	0.011	0.009	0.017	0.009	0.016
C9	**rs62358361**	**G / T**	***0.013***	***0.011***	**0.009**	**0.017**	**0.009**	**0.016**
CFB	rs641153	G / A	0.125	0.085	0.102	0.050	0.090	0.048
CETP	rs17231506	C / T	0.292	0.301	0.313	0.335	0.315	0.348
CETP	rs3764261	C / A	0.303	0.311	0.320	0.341	0.317	0.350
CETP	rs5817082	C / CA	0.290	0.236	0.260	0.221	0.264	0.232
CFB	rs4151672	C / T	0.015	0.004	0.029	0.029	0.045	0.025
CFH	rs10922109	C / A	0.443	0.361	0.461	0.243	0.426	0.223
CFH	rs121913059	C / T	0.000	0.000	0.000	0.001	0.000	0.003
CFH	rs1410996	G / A	0.443	0.360	0.460	0.242	0.426	0.223
CFH	rs148553336	T / C	*0.002*	*0.002*	0.005	0.003	0.009	0.003
CFH	rs191281603	C / G	*0.005*	*0.002*	0.008	0.007	0.006	0.007
CFH	rs35292876	C / T	*0.010*	*0.023*	0.011	0.018	0.009	0.021
CFH	**rs3753394**	**C / T**	**0.304**	**0.308**	**0.281**	**0.262**	**0.291**	**0.266**
CFH	rs570618	G / T	0.310	0.340	0.347	0.578	0.364	0.580
CFHR5	rs10922153	G / T	0.550	0.506	0.531	0.360	0.499	0.342
CFI	rs10033900	C / T	0.304	0.341	0.421	0.510	0.477	0.511
CFI	rs141853578	C / T	*0.000*	*0.000*	0.001	0.004	0.001	0.003
CNN2	rs10422209	C / G	0.228	0.165	0.165	0.136	0.123	0.142
COL10A1	rs3812111	T / A	0.451	0.415	0.426	0.355	0.387	0.372
COL4A3	**rs11884770**	**C / T**	**0.355**	**0.374**	**0.330**	**0.267**	**0.278**	**0.258**

COL8A1	**rs13081855**	**G / T**	**0.083**	**0.080**	**0.088**	**0.113**	**0.092**	**0.104**
COL8A1	**rs140647181**	**T / C**	**0.028**	**0.024**	**0.020**	**0.022**	**0.016**	**0.023**
COL8A1	rs55975637	G / A	0.114	0.118	0.114	0.141	0.117	0.132
CSK_MIR4513	rs2168518	A / G	0.339	0.327	0.335	0.328	0.345	0.328
CTRB2/CTRB1	rs55993634	C / G	0.127	0.105	0.105	0.069	0.089	0.075
HTRA1	rs11200638	G / A	0.131	0.164	0.177	0.424	0.207	0.431
LIPC	rs2043085	C / T	0.402	0.382	0.390	0.370	0.384	0.354
LIPC	rs2070895	G / A	0.209	0.209	0.203	0.195	0.217	0.195
LIPC	rs493258	C / T	0.526	0.500	0.494	0.444	0.465	0.442
LPL	rs12678919	A / G	0.102	0.078	0.118	0.100	0.099	0.100
MIR	rs4351242	C / T	0.094	0.086	0.075	0.038	0.067	0.063
MIR6130/RORB	rs10781182	G / T	0.327	0.335	0.300	0.314	0.306	0.328
MMP9	rs142450006	TTTTC / T	0.100	0.118	0.098	0.080	0.141	0.124
NPLOC4/TSPAN10	**rs6565597**	**C / T**	**0.318**	**0.287**	**0.339**	**0.380**	**0.381**	**0.400**
PILRB/PILRA	rs7803454	C / T	0.200	0.205	0.199	0.210	0.190	0.209
PRLR/SPEF2	rs74767144	C / G	0.010	0.006	0.021	0.021	0.022	0.017
RAD51B	rs2842339	A / G	0.140	0.144	0.110	0.092	0.094	0.107
RAD51B	rs8017304	A / G	0.527	0.489	0.425	0.348	0.372	0.349
RDBP_CFB	rs760070	T / C	0.124	0.086	0.106	0.050	0.091	0.049
SLC16A8	rs8135665	C / T	0.150	0.203	0.181	0.228	0.195	0.217
SYN3/TIMP3	rs5754227	T / C	0.116	0.096	0.133	0.109	0.137	0.109
TGFBR1	**rs334353**	**T / G**	**0.231**	**0.249**	**0.257**	**0.227**	**0.248**	**0.231**
TGFBR1	**rs1590**	**T / G**	**0.236**	**0.256**	**0.268**	**0.236**	**0.260**	**0.242**
TGFBR1	**rs1626340**	**G / A**	**0.181**	**0.219**	**0.211**	**0.182**	**0.209**	**0.189**
TGFBR1	rs334348	A / G	0.238	0.257	0.265	0.235	0.260	0.242
TGFBR1	**rs334349**	**G / A**	**0.224**	**0.248**	**0.259**	**0.236**	**0.261**	**0.242**
TMEM97/VTN	rs11080055	C / A	0.481	0.478	0.498	0.485	0.486	0.463
VEGFA	**rs943080**	**T / C**	**0.465**	**0.467**	**0.484**	**0.460**	**0.497**	**0.465**
ZBTB41	rs12724106	A / G	0.088	0.105	0.088	0.151	0.105	0.168

Note: To compare allele frequencies in cases and controls in CES with EYE-RISK and IAMDGC datasets, major and minor alleles were selected to match the ones from Fritsche et al., 2016 (table S11).

Abbreviations: *ABCA1*, ATP binding cassette subfamily A member 1; *ACAD10/BRAP*, acyl-CoA dehydrogenase family member 10/BRCA1 associated protein; *ADAMTS9*, ADAM metallopeptidase with thrombospondin type 1 motif 9; *ADAMTS9-AS2*, ADAMTS9 antisense RNA 2; AFs, allele frequencies; AMD, age-related macular degeneration; *APOE (EXOC3L2/MARK4)*, apolipoprotein E (exocyst complex component 3 like 2/microtubule affinity regulating kinase 4); *APOE*, apolipoprotein E; *ARHGAP21*, rho GTPase activating protein 21; *ARMS2/HTRA1*, age-related maculopathy susceptibility 2/htrA serine peptidase 1; *B3GALTL*, beta 3-glucosyltransferase; *C2/CFB/SKIV2L*, complement component 2/complement factor B/ski2 like RNA helicase; *C3*, complement component 3; *C9*, complement component 9; CES, Coimbra Eye Study; *CETP*, cholesteryl ester transfer protein; *CFB*, complement factor B; *CFH*, complement factor H; *CFHR5*, complement factor h related 5; *CFI*, complement factor I; *CNN2*, calponin 2; *COL10A1*, collagen type X alpha 1 chain; *COL4A3*, collagen type IV alpha 3 chain; *COL8A1*, collagen type VIII alpha 1 chain; *CSK_MIR4513*, c-terminal src kinase/microRNA 4513; *CTRB2/CTRB1*, chymotrypsinogen B2/chymotrypsinogen B; *HTRA1*, htrA serine peptidase 1; IAMDGC, International AMD Genomics Consortium; *LIPC*, lipase c, hepatic type; *LPL*, lipoprotein lipase; MAF, minor allele frequency; *MIR6130/RORB*, microRNA 6130/RAR related orphan receptor b; *MMP9*, matrix metallopeptidase 9; *NPLOC4/TSPAN10*, NPL4 homologue, ubiquitin recognition factor/tetraspanin 10; *NRTN/ FUT6*, neurturin /fucosyltransferase 6*; PBX2*, PBX homeobox 2; *PILRB/PILRA*, paired immunoglobulin like type 2 receptor beta/ paired immunoglobulin like type 2 receptor alpha; *PRLR/SPEF2*, prolactin receptor/sperm flagellar 2; *RAD51B*, RAD51 paralog b; *SLC16A8*, solute carrier family 16 member 8; SNPs, single nucleotide polymorphisms; *SYN3/TIMP3*, synapsin III/TIMP metallopeptidase inhibitor 3*; TGFBR1*, transforming growth factor beta receptor 1; *TMEM97/VTN*, transmembrane protein 97/vitronectin; *VEGFA*, vascular endothelial growth factor A; *ZBTB41*, zinc finger and BTB domain containing 41.

Single nucleotide polymorphisms with an inverse trend in AF between cases and controls in the CES in comparison to the EYE-RISK and IAMDGC datasets are presented in bold. Rare variants in our cohort are presented in grey.

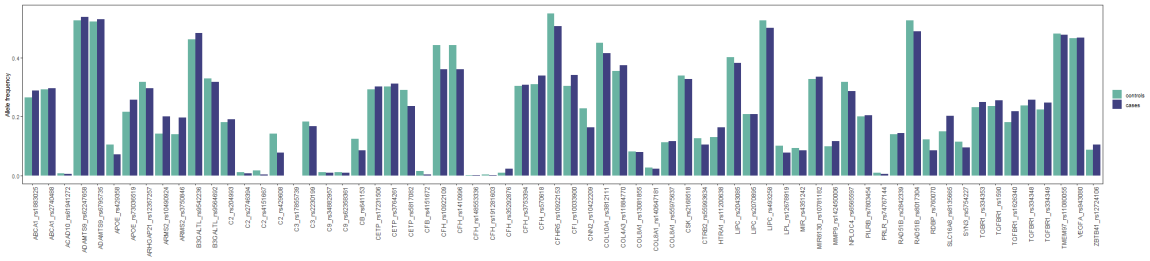

FIGURE 2. Minor allele frequencies in the CES incidence cohort. Comparison between cases and controls.

Associations with AMD risk

To test for variants associated with AMD cases, the total of 877 samples and 69 SNPs were tested for association under an additive model, using the presence of AMD as a binary outcome, in a univariate logistic regression analysis adjusted for age and sex.

Five risk variants were associated to increased risk of AMD: *ARMS2* rs10490924 (OR 1.474; CI 95% 1.121–1.933, *p*-value = 0.005), *ARMS2/HTRA1* rs3750846 (OR 1.462; CI 95% 1.106–1.924, *p*-value = 0.007), *CFH* rs35292876 (OR 2.668; CI 95% 1.136–6.171, *p*-value = 0.021), *SLC16A8* rs8135665 (OR 1.436; CI 95% 1.052–1.951, *p*-value = 0.021) and *TGFBR1* rs1626340 (OR 1.321; CI 95% 1.014–1.713, *p*-value = 0.037). Moreover, we identified seven variants with protective effect: *CFH* rs10922109, *CFH* rs1410996, *C2/CFB/SKIV2L* rs429608, *CETP* rs5817082, calponin 2 (*CNN2)* rs10422209, *CFB* rs641153 and *RDBP/CFB* rs760070. Significant associations are depicted in Table 2, as well as comparisons to the EYE-RISK and the IAMDGC datasets (de Breuk et al., 2020; Fritsche et al., 2016). For purpose of completeness all risk associations tested are depicted in Table S2.

TABLE 2. Variants significantly associated with risk of AMD in the CES, and comparison to EYE-RISK and IAMDGC reports.

Gene	SNP	REF	ALT	Major/ Minor allele	MAF controls CES	MAF cases CES	OR CES (95% CI)	*P*-value CES	OR EYE-RISK[a]	*P*-value EYE-RISK[a]	OR IAMDGC primary analysis[a]	*P*-value IAMDGC primary analysis[a]
C2/CFB/SKIV2L	rs429608	G	A	G/A	0.142	0.078	0.507 [0.338 - 0.741]	0.001	0.62	1.00^{-6}	0.57	1.2^{-103}
CFH	rs1410996	G	A	G/A	0.443	0.360	0.713 [0.571 -0.888]	0.003	0.37	1.64^{-47}	0.38	0
CFH	rs10922109	C	A	C/A	0.443	0.361	0.717 [0.574-0.893]	0.003	0.37	3.93^{-47}	0.38	9.6^{-618}
ARMS2	**rs10490924**	G	T	G/T	0.142	0.201	**1.474 [1.121 -1.933]**	0.005	3.29	9.04^{-55}	2.81	0
ARMS2/HTRA1	**rs3750846**	T	C	T/C	0.140	0.197	**1.462 [1.106 - 1.924]**	0.007	3.18	5.26^{-52}	2.81	6.5^{-735}
CNN2	rs10422209	C	G	C/G	0.228	0.165	0.655 [0.464 - 0.913]	0.014	0.80	0.02	1.15	2.7^{-8}
CFB	rs641153	G	A	G/A	0.125	0.085	0.629 [0.424 - 0.915]	0.018	0.44	9.00^{-12}	0.51	1.1^{-89}
CETP	rs5817082	C	CA	C/CA	0.290	0.236	0.731 [0.562 - 0.945]	0.018	0.81	0.003	0.84	3.6^{-19}
SLC16A8	**rs8135665**	C	T	C/T	0.150	0.203	**1.436 [1.052 - 1.951]**	0.021	1.33	0.001	1.14	5.5^{-11}
CFH	**rs35292876**	C	T	C/T	0.010	0.023	**2.668 [1.136 - 6.171]**	0.021	1.62	0.09	2.42	8.2^{-37}
RDBP_CFB	rs760070	T	C	T/C	0.124	0.086	0.648 [0.438 - 0.939]	0.025	0.44	3.56^{-12}	0.51	9.5^{-91}
TGFBR1	**rs1626340**	G	A	G/A	0.181	0.219	**1.321 [1.014 - 1.713]**	0.037	0.83	0.02	0.88	3.8^{-10}

Abbreviations: ALT, alternative allele; AMD, age-related macular degeneration; *ARMS2*, age-related maculopathy susceptibility 2; *ARMS2/HTRA1*, age-related maculopathy susceptibility 2/htrA serine peptidase 1; *C2/CFB/SKIV2L*,
complement component 2/complement factor B/ski2 like RNA helicase; CES -Coimbra Eye Study; *CETP*, cholesteryl ester transfer protein; *CFB*, complement factor B; *CFH*, complement factor H; CI, confidence interval; *CNN2*, calponin 2; IAMDGC, International AMD Genomics Consortium; MAF, minor allele frequency; OR, odds ratio; REF, reference allele; *SLC16A8*, solute carrier family 16 member 8; SNP, single nucleotide polymorphisms; *TGFBR1*, transforming growth factor beta receptor 1.
Variants in bold are associated with an increased risk of having AMD; the remaining variants are associated with a protective effect towards AMD.
[a] Data from de Breuk et al., 2020

Genetic risk score

To assess individual genetic risk, the GRS was calculated. The SNPs from the IAMDGC fully conditioned analysis used in the GRS calculation in the CES study are presented in Table S3. However, if the genotype of one of the major risk variants (*CFH* rs570618, *CFH* rs10922109, *C2/CFB/SKIV2L* rs429608, *ARMS2* rs3750846 and *C3* rs2230199) was not available, the GRS was considered as missing. For this reason, the analysed cohort to compute the GRS comprised 829 subjects: 607 controls and 222 cases.

Significant differences between the GRS from controls and AMD cases were found in our population: 0.645 ± 1.124 versus 1.124 ± 1.187, respectively ($p < 0.001$). The GRS varied from −2.905 to 5.526, and there was a clear shift towards a higher GRS in AMD cases comparing to controls. It was, however, not possible to completely distinguish between cases and controls based on the GRS alone, as there is substantial overlap (Figure 3). We further explored on the GRS from early ($n = 213$) and late ($n = 24$) AMD cases, but no significant differences were found between them.

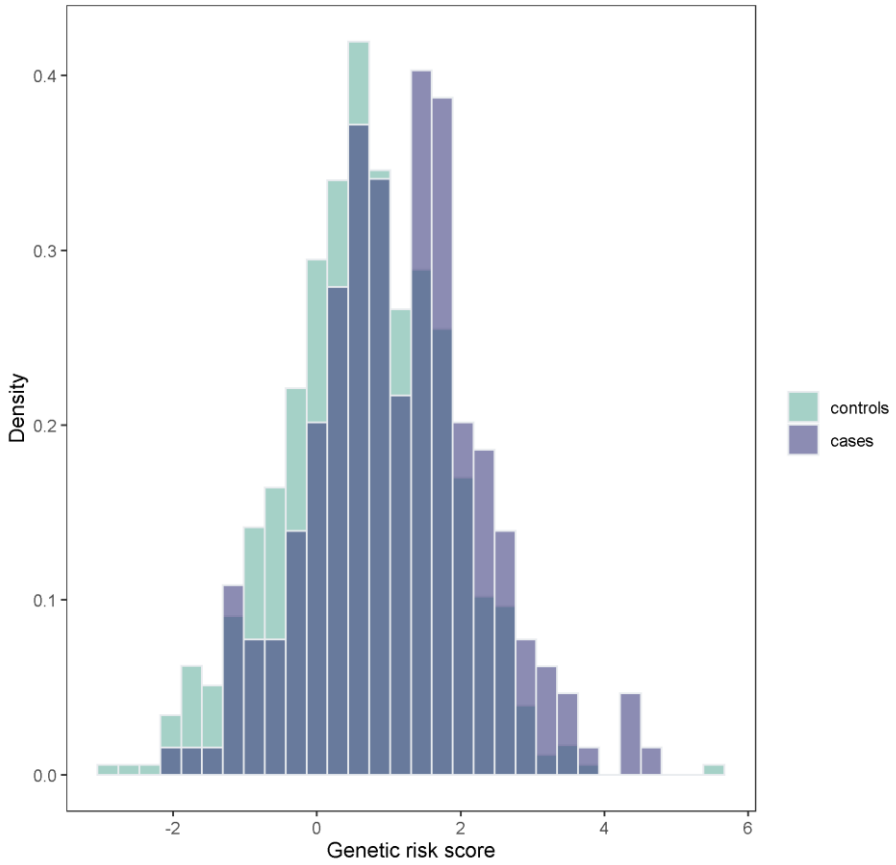

FIGURE 3. GRS of cases and controls.

Progression to AMD – Genetic associations and GRS

We obtained 137 samples from progressors and 630 samples from non-progressors. Variants associated to risk of progression were: *ARMS2* rs10490924, *ARMS2/HTRA1* rs3750846, *CFH* rs35292876; and variants protective for progression were again *C2/CFB/SKIV2L* rs429608, *CFH* rs10922109, *CFH* rs1410996, *CNN2* rs10422209 but also complement factor h related 5 (*CFHR5)* rs10922153, synapsin III (*SYN3)/TIMP3* rs5754227 and collagen type X alpha 1 chain (*COL10A1)* rs3812111 (Table 3).

Non-progressors and progressors also had a significantly different GRS: 0.669 ± 1.141 and 1.190 ± 1.178, respectively ($p < 0.001$). Again, and despite the substantial overlap, there was a shift towards a higher GRS in those who progressed to AMD (Figure 4).

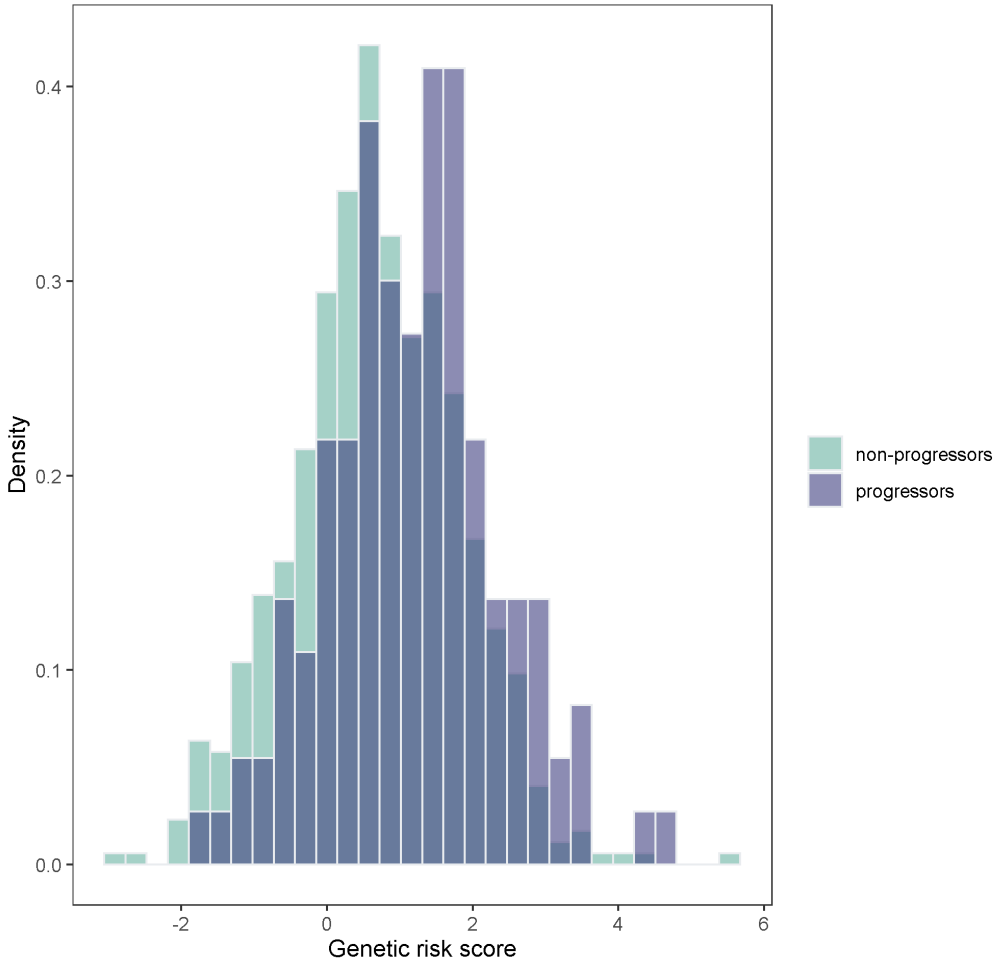

FIGURE 4. GRS of progressors and non-progressors.

TABLE 3. Variants significantly associated with risk of progression to AMD in the 6.5-year follow-up from the CES.

Gene	SNP	REF	ALT	Major/ Minor allele	MAF Progressors CES	MAF Non-progressors CES	OR CES (95% CI)	*P*-value CES	OR EYE-RISK[a]	*P*-value EYE-RISK[a]	OR IAMDGC primary analysis[a]	*P*-value IAMDGC primary analysis[a]
C2/CFB/SKIV2L	rs429608	G	A	G/A	0.066	0.136	0.428 (0.243 - 0.707)	0.002	0.62	1.00-6	0.57	1.2-103
CFH	rs1410996	G	A	G/A	0.335	0.437	0.655 (0.493-0.862)	0.003	0.37	1.64-47	0.38	0
CFH	rs10922109	C	A	C/A	0.344	0.437	0.682 (0.514 - 0.897)	0.007	0.37	3.93-47	0.38	9.6-618
CFHR5	rs10922153	T	G	G/T	0.471	0.556	0.704 (0.539 - 0.916)	0.009	0.5	3.77-27	0.52	0
ARMS2/HTRA1	**rs3750846**	T	C	T/C	0.203	0.141	**1.519 (1.078-2.120)**	0.015	3.18	5.26-52	2.81	6.5-735
CFH	**rs35292876**	C	T	C/T	0.029	0.012	**3.060 (1.183 - 7.412)**	0.015	1.62	0.09	2.42	8.2-37
ARMS2	**rs10490924**	G	T	G/T	0.204	0.144	**1.508 (1.073 - 2.098)**	0.016	3.29	9.04-55	2.81	0
SYN3/TIMP3	rs5754227	T	C	T/C	0.070	0.115	0.560 (0.326 -0.912)	0.026	0.8	0.02	0.77	1.1-24
CNN2	rs10422209	C	G	C/G	0.154	0.223	0.631 (0.403 - 0.958)	0.036	0.8	0.02	1.15	2.7-8
COL10A1	rs3812111	T	A	T/A	0.408	0.463	0.761 (0.581 - 0.992)	0.045	0.75	4.00-6	0.94	1.2-4

Note: Variants in bold are associated with an increased risk of progression to AMD; the remaining variants are associated with a protective effect towards progression. Abbreviations: ALT, alternative allele; AMD, age-related macular degeneration; *ARMS2*, age-related maculopathy susceptibility 2; *ARMS2/HTRA1*, age-related maculopathy susceptibility 2 /htrA serine peptidase 1; *C2/CFB/SKIV2L*, complement component 2/complement factor B/ski2 like RNA helicase; CES -Coimbra Eye Study; *CFH*, complement factor H; *CFHR5*, complement factor h related 5; CI, confidence interval; CNN2, calponin 2; *COL10A1*, collagen type X alpha 1 chain; IAMDGC, International AMD Genomics Consortium; MAF, minor allele frequency; OR, odds ratio; REF, reference allele; SNP, single nucleotide polymorphisms; *SYN3/TIMP3*, synapsin III/TIMP metallopeptidase inhibitor 3.

[a] Data from de Breuk et al., 2020

Rare and low-frequency variants analysis

A total of 859 samples and 1031 rare variants were successfully genotyped in our cohort. After filtering, 973 SNPs and 804 samples from 591 controls and 213 AMD cases were analysed. We investigated the presence of rare variants and their association with disease for the *CFH*, *CFI* and *ARMS2* genes.

For the *CFH* gene, a total of 90 rare variants were included (Table S4). The cumulative analysis revealed that AMD patients had more rare variants with a CADD score ≥ 20 or LoF variants compared to controls (OR, 9.661; *p*-value < 0.001) (Tables 4 and 5).

As for the *CFI* gene the rare variants found are reported in Table S5. Controls had more benign variants according to PolyPhen-2 score and higher frequency of a CADD score < 20; however, the cumulative difference did not reach statistical significance when comparing controls with cases (Table S6).

For the *ARSM2* gene the only two rare variants assessed were 10:124214262:G:C (Gly7Arg) and 10:124214475:C:G (Pro78Ala), and none was found in our population.

Macular dystrophies mimicking AMD

No AMD cases in the CES had two class 3 or higher variants previously reported as pathogenic in the *ABCA4* gene. Two controls were homozygotes for class 3 variant Asn1868Iln (rs1801466) in *ABCA4* gene, but the fundus imaging did not show features compatible with macular dystrophy after revising the exams. Furthermore, the cumulative analysis of variants for the *ABCA4* gene did not reveal more rare variants in AMD cases compared to controls (Table S7). No pathogenic variants were found for genes *CTNNA1* and *PRPH2*.

TABLE 4. *CFH* rare variants identified in the CES cohort.

Gene	Position GRCh37 (hg19)	REF	ALT	Function	SNP	Nucleotide Change	Protein change	SIFT[a]	Polyphen2_HDIV[a]	CADD[a]	Variants (N)	MAC cases	MAC controls	MAF cases	MAF controls
CFH	196642206	C	T	nonsynonymous_SNV	rs757785149	C157T	R53C [#*]	D	D	29.8	1	1	0	0.002	0.000
CFH	196646659	G	T	nonsynonymous_SNV	rs777300338	G481T	A161S [§]	T	P	2.863	1	1	0	0.002	0.000
CFH	196648794	A	G	nonsynonymous_SNV	rs774239374	A661G	I221V [#]	T	B	0.001	1	0	1	0.000	0.001
CFH	196658607	G	A	nonsynonymous_SNV	rs371192606	G1022A	R341H [§]	NA	NA	7.702	1	1	0	0.002	0.000
CFH	196658733	T	C	nonsynonymous_SNV	rs762389370	T1148C	V383A	T	P	0.006	1	0	1	0.000	0.001
CFH	196684751	T	A	nonsynonymous_SNV	rs147403664	T1548A	N516K [§]	T	D	16.62	1	1	0	0.002	0.000
CFH	196684825	A	G	nonsynonymous_SNV	1:196684825:A:G	A1622G	E541G	T	D	21.3	1	1	0	0.002	0.000
CFH	196695985	C	A	nonsynonymous_SNV	rs763441589	C2151A	F717L [§]	D	B	12.93	1	1	0	0.002	0.000
CFH	196706659	C	A	nonsynonymous_SNV	rs114743644	C2651A	S884 [§]	T	P	10.94	1	0	1	0.000	0.001
CFH	196711052	G	C	nonsynonymous_SNV	rs201816520	G3004C	G1002R [§]	T	D	7.236	1	1	0	0.002	0.000
CFH	196716415	T	A	nonsynonymous_SNV	1:196716415:T:A	T3668A	L1223Q	D	D	24.1	1	0	1	0.000	0.001
CFH	196694418	A	G	nonsynonymous_SNV	1:196694418:A:G	A1864G	I622V	T	B	0.194	2	1	1	0.002	0.001

CFH	196648906	C	T	nonsynonymous_SNV	rs768526062	C773T	P258L §	NA	NA	21.1	17	13	4	0.033	0.004
CFH	196706677	G	T	nonsynonymous_SNV	rs515299	G2669T	S890I §	T	B	0.463	25	9	16	0.021	0.014
CFH	196712596	A	T	nonsynonymous_SNV	rs35274867	A3148T	N1050Y #*	T	B	3.424	32	3	29	0.007	0.025

Abbreviations*: CFH* -complement factor H; CES -Coimbra Eye Study; GRCh37(hg19) - genome reference consortium human reference 37 ; REF – reference allele; ALT – alternative allele; SIFT – Sorting intolerant from tolerant; CADD - combined annotation-dependent depletion; MAC – Minor allele counts; D: probably damaging; P - Possible damaging; B – Benign; AMD- Age-related macular degeneration.

Variants reported as significantly associated with AMD in one or more AMD case-control cohorts.

§ Variants found in one or more studies.

***** Variants with a functional effect on the protein or change systemic levels (Geerlings *et al.* 2017).

[a] Data from de Breuk *et al.* 2020

TABLE 5. *CFH* rare variants score in AMD cases versus controls

***CFH* rare variant carriers by Polyphen2 score**	**Controls, N (%) (n=591)**	**Cases, N (%) (n=213)**	**OR (95%CI)**	***p*-value**
Noncarrier	545	195	1 reference	
Carrier-B	44	13	0.826 (0.419 -1.524)	0.558
Carrier-P	1	1	2.795 (0.110 – 70.904)	0.468
Carrier-D	0	2	NA	0.981
Carrier-LoF	0	1	NA	0.986
***CFH* rare variant carriers by CADD score**				
Noncarrier	541	182		
Carrier-CADD<20	45	17	1.123 (0.612-1.975)	0.695
Carrier-CADD≥20 or LoF	4	13	**9.661** (3.371-34.636)	**< 0.001**

Abbreviations: AMD, Age-related macular degeneration, N, number of subjects; B, Benign; CADD, combined annotation-dependent depletion; *CFH*, complement factor H; CI, confidence interval; D, probably damaging; LoF, loss-of-function; OR, odds ratio; P, Possible damaging.

DISCUSSION

Several variants were found to be associated with the presence of AMD and its progression in our epidemiological longitudinal study, while others had a protective role. These genes act in different pathophysiologic pathways sustaining the multifactorial aetiology of AMD. Their effects in our population agree with major reports, including large GWAS studies, although some risk variants considered major were found in lower frequency than expected. Despite this, the GRS was still significantly different between AMD and non-AMD cases and between progressors and non-progressors, supporting its role when assessing individual risk. Furthermore, we also found that rare and low-frequency variants in the *CFH* gene with damaging effects were more common in our AMD patients.

Genome-wide association studies have identified several genetic risk variants that are strongly associated with AMD: 52 variants at 34 genomic regions, of which 45 were common variants while 7 were rare variants (Fritsche et al., 2016). In our study 12 variants sequenced by the genotype assay developed by the EYE-RISK consortium were found to be associated with AMD. Eleven are common variants while one in the *CFH* gene (rs35292876) is a rare variant that increases the risk of AMD.

The analysis of the MAF of all sequenced SNPs in AMD cases versus controls revealed that some variants had an inverse trend in our cohort compared to what was found in the larger databases of the EYE-RISK and IAMDGC. These differences can be due to the relatively low number of our sample or most probably due to real specificities of our study population, which originates from a small populational area in central Portugal. These discrepancies were found in different pathways, such as the complement system (*CFH*, *C3* and *C9*), extracellular matrix (*COL4A3*, *COL8A1*, matrix metallopeptidase 9 [*MMP9]*), cholesterol metabolism (*ABCA1*, *ACAD10/BRAP*, *APOE*) and the *TGFBR1* gene (de Breuk et al., 2020; Fritsche et al., 2016).

Another interesting finding when analysing the MAF distribution was that regarding the major risk variants for AMD, we observed that for both *ARMS2/HTRA1* rs3750846 and *CFH* rs570618, the allele frequency in our cases was much lower compared to the AFs of cases from the EYE-RISK and IAMDGC datasets (de Breuk et al., 2020, Fritsche et al., 2016). In addition, not only the same was true for *C3* rs2230199, another major risk variant, but even an inverse distribution between cases and controls was found in our cohort for this variant. This lower-than-expected AF in major risk variants in AMD cases translates into lower odds ratios with implications in AMD risk in our cohort. We previously reported in our epidemiologic study that this coastal population had significantly lower prevalence of both early and late AMD compared to the inland cohort. Furthermore, in the subsequent incidence study of the coastal population we found that incidence of late AMD was lower than expected compared to other European cohorts (Cachulo et al., 2016; Farinha et al., 2019). These differences could be due to different habits and

lifestyle profiles, as well as for different genetic patterns such as we now describe in this report. Furthermore, we also previously reported that a higher adherence to the Mediterranean diet was significantly protective for AMD, and that the coastal population had a significantly higher adherence to it (Nunes et al., 2018). The interplay between these lifestyle and genetic background differences could be the cause of our previous epidemiologic findings for this population and are in accordance to the findings on genetic and lifestyle interaction by Colijn et al. (2021).

The variants significantly associated to AMD in our population were fewer than expected but as discussed above, specific genetic differences in our population cannot be excluded, as for instance there were sequenced variants in the complement pathway totally absent in our cohort. Furthermore, genes associated to having the disease were just in part the same as those associated with conversion to AMD in the longitudinal analysis and located only in *CFH* and *AMRS2/HTRA1* genes. This discrepancy might be related to the more prominent role of these genes in disease progression. The variant *ARMS2* rs10490924, is known to be associated with incidence of early AMD and progression to both neovascular AMD and geographic atrophy (Seddon et al., 2015; Heesterbeek et al., 2020).

The development of AMD is influenced not only by common variants but also by rare genetic variants, and the impact of such rare variants can be quite significant. The *CFH* rs121913059 (Arg1210Cys) for instance is associated to a 47 times higher risk of developing AMD but was not found in our cohort (Geerlings, de Jong, et al., 2017). The *CFH* rare variant rs35292876 was identified in our population as a *low-frequency* variant and in addition conferred the highest risk of AMD (OR, 2.67), even when compared to major common risk variants. Moreover, it was associated to the highest risk of progression to AMD in follow-up analysis (OR, 3.06). Interestingly, for this variant the EYE-RISK did not find association to AMD risk while the IAMDGC reported an OR of 2.42 (de Breuk et al., 2020; Fritsche et al., 2016). Geographic variations might explain the discrepancies, and this specific variant was found to be more common in western Europe compared to other globe regions, justifying its superior prevalence and effect in our population (Geerlings et al., 2018). This variant was not found to be associated to FH or FHR concentrations in serum, but other rare variants have, and their burden analysis is important to pursue in different cohorts. The *CFH* rs757785149 (Arg53Cys), has been previously identified in AMD families with high disease burden and was identified in our study only in cases. It is reported to possibly affect the local conformation of Factor H, slightly reducing the binding affinity to C3b (Geerlings, de Jong, et al., 2017; Lores-Motta et al., 2021). Our cumulative analysis of rare variants in the *CFH* gene revealed that they had impact in disease development in our cohort, as damaging rare variants were more frequent in AMD patients compared to non-AMD controls. More functional studies are necessary to determine their pathophysiological effect.

Rare variants in the *CFI* gene have also been associated with a four-fold increased risk of AMD, younger age at AMD onset and with late AMD (de Breuk et al., 2020; de Jong et al., 2020; Seddon et al., 2013). In our population controls had more benign variants, while none of the reported high risk rare variants. The *CFI* variant Pro553Ser was observed more in controls and is reported to be benign in respect to Factor I levels measured in the plasma of carriers (de Jong et al., 2020; Geerlings, de Jong, et al., 2017; Geerlings, Kremlitzka, et al., 2017). Our lack of significance when cumulatively comparing between cases and controls is probably related to the small number of carriers. No *ARMS2* rare variants were found, as observed in the EYE-RISK report, which is interesting since it is a gene known to play a fundamental role in AMD progression. Despite altering the protein structure or splicing, the sequenced variants have a low CADD score (<10) and were absent from our population. Other rare variants known to be associated to increased risk of AMD such as *C3* rs147859257 or *C9* rs62358361 were not found in our cohort or were not associated to AMD, and this is probably related to the different distribution of rare alleles across populations.

Addressing the cumulative risk of damaging rare variants may be more useful when analysing differences between cohorts than focusing only on a few variants with low effect in larger study populations. This approach might even have a role in the near future in the identification of those who would benefit more of targeted therapies. Rare variants in the *CFH* and *CFI* genes were already found to cause higher levels of complement activation, thus the carriers might respond more to complement-inhibiting therapies. Phase I/II clinical trials for subretinal gene therapy in AMD are currently underway, and others targeting the complement inhibition are already in Phase III (Cabral De Guimaraes et al., 2021; de Jong et al., 2021; Jaffe et al., 2020; Liao et al., 2020).

Regarding the Genetic Risk Score, it was found to be significantly different between AMD cases and controls, and between progressors and non-progressors. This confirms that the conjoined heritable component in a given individual is important for developing the disease and should be taken into account, if personalized medicine is to be pursued in the future. However, since there was a substantial overlap, it was not possible to completely distinguish between AMD patients and controls based on the GRS alone. This is not unexpected and is in line with what was found in previous publications, since the complex aetiology of AMD depends not only of the genetic background, but is greatly impacted and modified by environmental factors (Colijn et al., 2021; de Breuk et al., 2020). Thus, a score that comprehensively assesses genetic and lifestyle risk factors, such as smoking, body mass index, nutrition and even concomitant medication, together with phenotypic characteristics of the disease, might be more informative of the risk of disease than the GRS alone (Heesterbeek et al., 2019; Seddon et al., 2015). This is more relevant as one must remember that the GRS is calculated on the basis of pre-defined risk variants, and as we found, not even major risk variants are evenly distributed across populations, compromising

the generalization of such tool if used alone in risk calculation. Awareness of this is especially important if the GRS is to be implemented in settings such as clinical trials and clinical practice to assess the individual risk of a patient.

Genetic studies on AMD are based on the principle that diagnosis is correct. However, it is sometimes challenging to differentiate from mimicking inherited macular dystrophies, especially in late atrophic stages. Moreover, it is crucial to correctly identify AMD patients before their inclusion in clinical trials (de Breuk et al., 2020; Kersten et al., 2018). We evaluated the occurrence of rare genetic variants associated with AMD-mimicking dystrophies and no pathogenic variants were found in our patients, thus excluding this possible bias from our analysis, and further strengthening the genetic characterization of our cohort.

This study has some limitations that should be addressed. Despite being originally an epidemiological population-based study, for the purpose of genetic analysis it is a relatively small cohort, and the population is from a single location. As some genetic variants are geographically and regionally heterogeneous there is the risk of bias, and the analysis cannot be fully extended to the entire Portuguese population. However, this is the first and only genetic study in AMD in a Portuguese cohort, and we provide extensive characterization regarding common and rare variants. We also found differences in AFs that might explain previous findings in both prevalence and incidence in the CES, further contributing to the disease genetic knowledge in Europe and differences towards other regions. Another limitation was that in progression analysis a comprehensive understanding of the genetic risk of progression to late AMD and of fast progressors, which would be of most interest to explore, was not possible due to small sample size available for these analyses. However, we still derived important information on those who progressed to develop AMD during follow-up. Finally, as part of the EYE-RISK project our results are based in a comprehensive genotype assay recently validated in European populations.

In summary, several variants were identified in association to AMD in our cohort, and the *CFH* rare variant rs35292876 conferred the highest risk of disease, while three major AMD risk variants in *ARMS2/HTRA1*, *CFH* and *C3* had a lower-than-expected AF. Damaging rare variants in the *CFH* gene were significantly more frequent in AMD patients when cumulatively analysed. The GRS was significantly higher in AMD cases, but it was insufficient to discriminate from controls and non-progressors, reinforcing the need to include lifestyle and other risk factors when personalizing risk. Our study adds new information regarding the common and rare variants associated to AMD in a European population, which can be used for comparison with other populational cohorts and further expanding the knowledge of AMD pathophysiology.

SUPPLEMENTARY MATERIAL

Supplementary Table S1. Demographic and clinical characteristics of the genotyped population from the CES.

	Controls, N = 640	Early AMD, N = 213	Late AMD, N = 24	*p*-value[1]
Gender, N (%)				0.003
Female	360.0 (56.2%)	139.0 (65.3%)	8.0 (33.3%)	
Male	280.0 (43.8%)	74.0 (34.7%)	16.0 (66.7%)	
Age, mean (SD)	71.9 (6.4)	74.3 (7.1)	78.6 (8.2)	<0.001
Smoking				<0.001
Ex-Smoker	79.0 (12.4%)	25.0 (11.8%)	11.0 (45.8%)	
Non-smoker	547.0 (86.1%)	182.0 (86.3%)	12.0 (50.0%)	
Smoker	9.0 (1.4%)	4.0 (1.9%)	1.0 (4.2%)	
Familiar history of AMD, N (%)				0.025
Doesn't know	107.0 (16.7%)	18.0 (8.5%)	3.0 (12.5%)	
No	530.0 (82.8%)	193.0 (90.6%)	21.0 (87.5%)	
Yes	3.0 (0.5%)	2.0 (0.9%)	0.0 (0.0%)	
Diabetes, N (%)				0.019
Doesn't know	12.0 (1.9%)	4.0 (1.9%)	0.0 (0.0%)	
No	496.0 (77.5%)	186.0 (87.3%)	19.0 (79.2%)	
Yes	132.0 (20.6%)	23.0 (10.8%)	5.0 (20.8%)	
Hypertension, N (%)				0.300
Doesn't know	10.0 (1.6%)	6.0 (2.8%)	1.0 (4.2%)	
No	290.0 (45.3%)	85.0 (39.9%)	12.0 (50.0%)	
Yes	340.0 (53.1%)	122.0 (57.3%)	11.0 (45.8%)	
BMI, mean (SD)	28.2 (4.3)	27.8 (4.9)	27.6 (3.8)	0.200

[1]Pearson's Chi-squared test (or Fisher's exact test when appropriate) for categorical variables; Kruskal-Wallis rank sum test for continuous variables;

Abbreviations: CES- Coimbra Eye Study; SD – standard deviation; AMD- age-related macular degeneration; BMI – body mass index.

Supplementary Table S2. Variants tested for association with AMD in the CES, and comparison to the EYE-RISK and IAMDGC reports.

Gene	SNP	REF	ALT	Major/ Minor allele	MAF controls CES	MAF cases CES	OR CES (95% CI)	*P*-value CES	OR EYE-RISK[1]	*P*-value EYE-RISK[1]	OR IAMDGC primary analysis[1]	*P*-value IAMDGC primary analysis[1]
ABCA1	rs1883025	C	T	C/T	0.264	0.288	1.125 [0.877 - 1.440]	0.351	0.86	0.04	0.91	1.5^{-7}
ABCA1	rs2740488	A	C	A/C	0.292	0.297	1.016 [0.802 - 1.283]	0.894	0.81	0.003	0.90	1.2^{-8}
ACAD10/BRAP	rs61941272	C	A	C/A	0.009	0.006	0.662 [0.146 - 2.218]	0.539	1.51	0.16	1.51	1.1^{-9}
ADAMTS9	rs6795735	C	T	C/T	0.521	0.530	1.074 [0.869 - 1.328]	0.508	0.97	0.61	1.13	4.1^{-14}
ADAMTS9-AS2	rs62247658	C	T	T/C	0.525	0.537	1.085 [0.876 - 1.344]	0.457	0.95	0.36	1.14	1.8^{-14}
APOE	rs429358	T	C	T/C	0.106	0.072	0.685 [0.410 - 1.103]	0.132	0.94	0.55	0.70	2.4^{-42}
APOE(EXOC3L2/MARK4)	rs73036519	G	C	G/C	0.216	0.257	1.314 [0.977 - 1.764]	0.069	0.98	0.78	0.91	3.1^{-7}
ARHGAP21	rs12357257	G	A	G/A	0.317	0.297	0.892 [0.701 - 1.132]	0.351	0.81	0,003	1.11	4.4^{-8}
ARMS2	rs10490924	G	T	G/T	0.142	0.201	1.474 [1.121 - 1.933]	0.005	3.29	9.04^{-55}	2.81	0
ARMS2/HTRA1	rs3750846	T	C	T/C	0.140	0.197	1.462 [1.106 - 1.924	0.007	3.18	5.26^{-52}	2.81	6.5^{-735}
B3GALTL	rs9542236	T	C	T/C	0.461	0.483	1.086 [0.874 - 1.350]	0.458	1.03	0.63	1.06	1.8^{-4}
B3GALTL	rs9564692	C	T	C/T	0.329	0.319	0.953 [0.752 - 1.205]	0.690	0.80	0.002	0.89	3.3^{-10}
C2	rs4151667	T	A	T/A	0.017	0.004	0.301 [0.048 - 1.009]	0.103	0.95	0.78	0.54	7.8^{-41}
C2/CFB/SKIV2L	rs2746394	G	A	G/A	0.012	0.008	0.576 [0.129 - 1.862]	0.403	1.10	0.87	1.39	2.6^{-6}

C2/CFB/SKIV2L	rs429608	G	A	G/A	0.142	0.078	0.507 [0.338 - 0.741]	0.001	0.62	1.00^{-6}	0.57	1.2^{-103}
C2/CFB/SKIV2L (PBX2)	rs204993	A	G	A/G	0.182	0.191	1.079 [0.816 - 1.420]	0.588	1.39	1.70^{-5}	1.13	9.4^{-12}
C3	rs147859257	T	G	T/G	0.000	0.000	NA	NA	9.34	0.002	2.86	3.1^{-28}
C3	rs2230199	G	C	G/C	0.183	0.168	0.909 [0.684 - 1.196]	0.501	1.49	4.08^{-7}	1.43	3.8^{-69}
C3 (NRTN/FUT6)	rs17855739	C	T	C/T	0.001	0.000	NA	0.981	0.73	0.68	0.72	8.4^{-15}
C9	rs34882957	G	A	G/A	0.013	0.011	0.935 [0.297 - 2.480]	0.899	1.87	0.04	1.79	1.6^{-14}
C9	rs62358361	G	T	G/T	0.013	0.011	0.932 [0.296 - 2.473]	0.894	1.92	0.03	1.80	1.3^{-14}
CFB	rs641153	G	A	G/A	0.125	0.085	0.629 [0.424 - 0.915]	0.018	0.44	9.00^{-12}	0.51	1.1^{-89}
CETP	rs17231506	C	T	C/T	0.292	0.301	1.038 [0.818 - 1.311]	0.758	1.10	0.13	1.16	2.2^{-18}
CETP	rs3764261	C	A	C/A	0.303	0.311	1.029 [0.816 -1.295]	0.807	1.10	0.14	1.16	9.7^{-18}
CETP	rs5817082	C	CA	C/CA	0.290	0.236	0.731 [0.562 - 0.945]	0.018	0.81	0.003	0.84	3.6^{-19}
CFB	rs4151672	C	T	C/T	0.015	0.004	0.330 [0.052 - 1.168]	0.142	1.00	0.99	0.54	8.9^{-41}
CFH	rs10922109	C	A	C/A	0.443	0.361	0.717[0.574 - 0.893]	0.003	0.37	3.93^{-47}	0.38	9.6^{-618}
CFH	rs121913059	C	T	C/T	0.000	0.000	NA	NA	4.09	0.40	20.28	8.9^{-24}
CFH	rs1410996	G	A	G/A	0.443	0.360	0.713 [0.571 - 0.888]	0.003	0.37	1.64^{-47}	0.38	0
CFH	rs148553336	T	C	T/C	0.002	0.002	1.099 [0.053 - 8.946]	0.936	0.70	0.45	0.29	8.6^{-26}

CFH	rs191281603	C	G	C/G	0.005	0.002	0.414 [0.021 - 2.610]	0.426	0.85	0.64	1.07	0.68
CFH	rs35292876	C	T	C/T	0.010	0.023	2.668 [1.136 - 6.171]	0.021	1.62	0.09	2.42	8.2^{-37}
CFH	rs3753394	C	T	C/T	0.304	0.308	1.010 [0.804 - 1.265]	0.928	0.91	0.17	0.88	1.7^{-11}
CFH	rs570618	T	G	G/T	0.310	0.340	1.114 [0.891 - 1.389]	0.342	2.49	8.98^{-44}	2.38	2.0^{-590}
CFHR5	rs10922153	T	G	G/T	0.550	0.506	0.844 [0.683 - 1.042]	0.115	0.50	3.77^{-27}	0.52	0
CFI	rs10033900	T	C	C/T	0.304	0.341	1.239 [0.972 - 1.578]	0.082	1.44	8.76^{-9}	1.15	5.4^{-17}
CFI	rs141853578	C	T	C/T	0.000	0.000	NA	NA	5.44	0.10	3.64	6.3^{-10}
CNN2	rs10422209	C	G	C/G	0.228	0.165	0.655 [0.464 -0.913]	0.014	0.80	0.02	1.15	2.7^{-8}
COL10A1	rs3812111	T	A	T/A	0.451	0.415	0.834 [0.672 - 1.032]	0.097	0.75	4.00^{-6}	0.94	1.2^{-4}
COL4A3	rs11884770	T	C	C/T	0.355	0.374	1.115 [0.892 - 1.392]	0.339	0.75	1.20^{-5}	0.90	2.9^{-8}
COL8A1	rs13081855	G	T	G/T	0.083	0.080	1.024 [0.680 - 1.515]	0.906	1.33	0.01	1.15	5.2^{-7}
COL8A1	rs140647181	T	C	T/C	0.028	0.024	0.886 [0.417 - 1.746]	0.737	1.10	0.68	1.59	1.4^{-11}
COL8A1	rs55975637	G	A	G/A	0.114	0.118	1.086 [0.768 - 1.519]	0.634	1.29	0.01	1.15	1.3^{-8}
CSK_MIR4513	rs2168518	G	A	A/G	0.339	0.327	0.962 [0.760 - 1.214]	0.744	0.97	0.63	0.92	3.3^{-6}
CTRB2/CTRB1	rs55993634	C	G	C/G	0.127	0.105	0.802 [0.554 - 1.142]	0.229	0.62	1.80^{-5}	0.81	5.1^{-12}
HTRA1	rs11200638	G	A	G/A	0.131	0.164	1.309 [0.898 - 1.891]	0.154	3.11	2.59^{-39}	2.76	0

LIPC	rs2043085	T	C	C/T	0.402	0.382	0.896 [0.718 - 1.117]	0.332	0.92	0.17	0.87	4.3^{-15}
LIPC	rs2070895	G	A	G/A	0.209	0.209	1.009 [0.775 - 1.307]	0.945	0.95	0.49	0.87	2.4^{-11}
LIPC	rs493258	T	C	C/T	0.526	0.500	0.883 [0.708 - 1.101]	0.269	0.82	0.002	0.91	1.2^{-8}
LPL	rs12678919	A	G	A/G	0.102	0.078	0.740 [0.493 - 1.085]	0.133	0.82	0.047	1.00	0.9
MIR	rs4351242	C	T	C/T	0.094	0.086	0.892 [0.601 - 1.299]	0.560	0.50	1.31^{-7}	0.94	0.04
MIR6130/RORB	rs10781182	T	G	G/T	0.327	0.335	1.045 [0.829 - 1.314]	0.707	1.07	0.31	1.11	2.6^{-9}
MMP9	rs142450006	TTTTC	T	TTTTC/T	0.100	0.118	1.161 [0.821 - 1.620]	0.389	0.80	0.04	0.85	2.4^{-10}
NPLOC4/TSPAN10	rs6565597	C	T	C/T	0.318	0.287	0.852 [0.636 - 1.133]	0.275	1.19	0.02	1.13	1.5^{-11}
PILRB/PILRA	rs7803454	C	T	C/T	0.200	0.205	1.036 [0.780 - 1.369]	0.804	1.07	0.41	1.13	4.8^{-9}
PRLR/SPEF2	rs74767144	C	G	C/G	0.010	0.006	0.579 [0.129 - 1.870]	0.406	1.00	0.99	0.74	8.7^{-7}
RAD51B	rs2842339	G	A	A/G	0.140	0.144	1.018 [0.744 - 1.377]	0.912	0.82	0.06	1.14	1.4^{-6}
RAD51B	rs8017304	G	A	A/G	0.527	0.489	0.870 [0.701 - 1.077]	0.202	0.72	2.34^{-7}	0.90	3.0^{-10}
RDBP_CFB	rs760070	T	C	T/C	0.124	0.086	0.648 [0.438 - 0.939]	0.025	0.44	3.56^{-12}	0.51	9.5^{-91}
SLC16A8	rs8135665	C	T	C/T	0.150	0.203	1.436 [1.052 - 1.951]	0.021	1.33	0.001	1.14	5.5^{-11}
SYN3/TIMP3	rs5754227	T	C	T/C	0.116	0.096	0.791 [0.544 - 1.130]	0.208	0.80	0.02	0.77	1.1^{-24}
TGFBR1	rs334353	T	G	T/G	0.231	0.249	1.139 [0.885 - 1.461]	0.308	0.85	0.02	0.91	1.8^{-6}

TGFBR1	rs1590	T	G	T/G	0.236	0.256	1.161 [0.904 - 1.488]	0.239	0.84	0.01	0.91	3.0^{-7}
TGFBR1	rs1626340	G	A	G/A	0.181	0.219	1.321 [1.014 - 1.713]	0.037	0.83	0.02	0.88	3.8^{-10}
TGFBR1	rs334348	A	G	A/G	0.238	0.257	1.153 [0.898 - 1.476]	0.260	0.85	0.03	0.91	2.9^{-7}
TGFBR1	rs334349	G	A	G/A	0.224	0.248	1.181 [0.915 - 1.520]	0.198	0.88	0.07	0.91	3.4^{-7}
TMEM97/VTN	rs11080055	A	C	C/A	0.481	0.478	0.973 [0.775 - 1.221]	0.813	0.95	0.41	0.91	1.0^{-8}
VEGFA	rs943080	C	T	T/C	0.465	0.467	1.004 [0.810 - 1.245]	0.969	0.91	0.12	0.88	1.1^{-14}
ZBTB41	rs12724106	A	G	A/G	0.088	0.105	1.239 [0.856 - 1.775]	0.248	1.84	2.71^{-9}	1.73	1.6^{-111}

[1] Data from de Breuk *et al*. 2020

Abbreviations: AMD- age-related macular degeneration; CES -Coimbra Eye Study; IAMDGC - International AMD Genomics Consortium; SNP - single nucleotide polymorphism; REF – reference allele; ALT – alternative allele; MAF - minor allele frequency; OR - odds ratio; CI - confidence interval; *ABCA1* - ATP binding cassette subfamily A member 1*; ACAD10/BRAP* - acyl-CoA dehydrogenase family member 10/BRCA1 associated protein*; ADAMTS9* -ADAM metallopeptidase with thrombospondin type 1 motif 9; *ADAMTS9-AS2* - ADAMTS9 antisense RNA 2*; APOE* - apolipoprotein E; *APOE(EXOC3L2/MARK4)* - apolipoprotein E(exocyst complex component 3 like 2/microtubule affinity regulating kinase 4); *ARHGAP21* - rho GTPase activating protein 21; *ARMS2/HTRA1* - age-related maculopathy susceptibility 2 /htrA serine peptidase 1; B3GALTL - beta 3-glucosyltransferase; *C2* - complement component 2; *C2/CFB/SKIV2L* - complement component 2/complement factor B/ski2 like RNA helicase*; PBX2* - PBX homeobox 2; *C3* - complement component 3; *NRTN/FUT6* - neurturin/fucosyltransferase 6; *C9* - complement component 9; *CFB* - complement factor B; *CETP* - cholesteryl ester transfer protein; *CFH* - complement factor H*; CFHR5* - complement factor h related 5; *CFI* - complement factor I; *CNN2* - calponin 2; *COL10A1* - collagen type X alpha 1 chain; *COL4A3* - collagen type IV alpha 3 chain; *COL8A1* - collagen type VIII alpha 1 chain; *CSK_MIR4513* - c-terminal src kinase/microRNA 4513*; CTRB2/CTRB1* - chymotrypsinogen B2/chymotrypsinogen B; *HTRA1* - htrA serine peptidase 1; *LIPC* - lipase c, hepatic type; *LPL* - lipoprotein lipase*; MIR6130/RORB* - microRNA 6130/RAR related orphan receptor b; *MMP9* - matrix metallopeptidase 9; *NPLOC4/TSPAN10* - NPL4 homolog, ubiquitin recognition factor/tetraspanin 10; *PILRB/PILRA* - paired immunoglobin like type 2 receptor beta/ paired immunoglobin like type 2 receptor alpha; *PRLR/SPEF2* - prolactin receptor/sperm flagellar 2; *RAD51B* - RAD51 paralog b; SLC16A8 - solute carrier family 16 member 8; *SYN3/TIMP3* - synapsin III/TIMP metallopeptidase inhibitor 3; *TGFBR1* - transforming growth factor beta receptor 1; *TMEM97/VTN* - transmembrane protein 97/vitronectin; *VEGFA* - vascular endothelial growth factor A; *ZBTB41* - zinc finger and BTB domain containing 41.

Supplementary Table S3. SNPs from the IAMDGC[1] used in the GRS calculation in the CES study.

Gene	SNP	Alternative SNP	Chr:Position	Major/ Minor allele	OR Fully Conditioned Analysis [1]	*P*-value Fully Conditioned Analysis [1]	EYE-RISK Method[b]
CFH	rs10922109		1:196,704,632	C/A	0.51	1.0×10^{-131}	smMIP
CFH	rs570618		1:196,657,064	G/T	1.74	9.2×10^{-76}	smMIP
CFH	rs121913059		1:196,716,375	C/T	47.63	2.2×10^{-35}	smMIP
CFH	rs148553336		1:196,613,173	T/C	0.31	8.8×10^{-17}	smMIP
CFH	rs187328863	rs79436252 (R^2=1.0)	1:196,358,288	A/G	1.47	2.8×10^{-12}	KASPar
CFH (CFHR3/CFHR1)	rs61818925	rs61818924 (R^2=0.80)	1:196,815,374	A/T	1.18	6.3×10^{-9}	KASPar
CFH	rs35292876		1:196,706,642	C/T	1.54	9.5×10^{-8}	smMIP
CFH	rs191281603		1:196,958,651	C/G	0.41	7.7×10^{-7}	smMIP
COL4A3	rs11884770		2:228,086,920	C/T	0.92	2.6×10^{-4}	smMIP
ADAMTS9-AS2	rs62247658		3:64,715,155	T/C	1.14	7.8×10^{-11}	smMIP
COL8A1	rs140647181		3:99,180,668	T/C	1.85	1.6×10^{-14}	smMIP
COL8A1	rs55975637		3:99,419,853	G/A	1.16	3.8×10^{-7}	smMIP
CFI	rs10033900		4:110,659,067	C/T	1.15	1.2×10^{-13}	smMIP
CFI	rs141853578		4:110,685,820	C/T	5.12	7.4×10^{-12}	smMIP
C9	rs62358361		5:39,327,888	G/T	1.67	7.2×10^{-9}	smMIP

PRLR/SPEF2	rs114092250	rs74767144 (R^2=0.77)	5:35,588,257	C/G	0.71	9.5×10^{-6}	smMIP
C2/CFB/SKIV2L	rs116503776	rs429608 [a]	6:31,930,462	G/A	0.51	5.0×10^{-96}	smMIP
C2/CFB/SKIV2L	rs144639244	rs2746394 [a]	6:31,946,792	G/A	2.79	1.0×10^{-32}	smMIP
C2/CFB/SKIV2L (PBX2)	rs114254831	rs204993[a]	6:32,155,581	A/G	1.13	8.8×10^{-9}	smMIP
C2/CFB/SKIV2L	rs181705462	rs114212545 (R^2=0.84)	6:31,932,368	G/A	1.56	2.8×10^{-8}	KASPar
VEGFA	rs943080		6:43,826,627	T/C	0.87	5.8×10^{-13}	smMIP
KMT2E/SRPK2	rs1142		7:104,756,326	C/T	1.14	1.3×10^{-10}	KASPar
PILRB/PILRA	rs7803454		7:99,991,548	C/T	1.15	2.8×10^{-9}	smMIP
TNFRSF10A	rs79037040		8:23,082,971	T/G	0.89	5.1×10^{-9}	KASPar
MIR6130/RORB	rs10781182		9:76,617,720	G/T	1.12	1.5×10^{-6}	smMIP
TRPM3	rs71507014		9:73,438,605	GC/G	1.11	2.3×10^{-8}	KASPar
TGFBR1	rs1626340		9:101,923,372	G/A	0.88	4.0×10^{-7}	smMIP
ABCA1	rs2740488		9:107,661,742	A/C	0.89	6.0×10^{-7}	smMIP
ARHGAP21	rs12357257		10:24,999,593	G/A	1.12	1.8×10^{-6}	smMIP
ARMS2/HTRA1	rs3750846		10:124,215,565	T/C	2.93	6.0×10^{-645}	smMIP
RDH5/CD63	rs3138141		12:56,115,778	C/A	1.18	4.7×10^{-8}	KASPar
ACAD10	rs61941274	rs61941272 (R^2=1.0)	12:112,116,776	C/A	1.60	3.2×10^{-9}	smMIP
B3GALTL	rs9564692		13:31,821,240	C/T	0.90	1.0×10^{-6}	smMIP

RAD51B	rs61985136		14:68,769,199	T/C	0.88	8.2×10^{-10}	KASPar
RAD51B	rs2842339		14:68,986,999	A/G	1.18	3.3×10^{-7}	smMIP
LIPC	rs2043085		15:58,680,954	T/C	1.15	7.7×10^{-13}	smMIP
LIPC	rs2070895		15:58,723,939	G/A	0.86	1.8×10^{-10}	smMIP
CETP	rs5817082		16:56,997,349	C/CA	0.87	2.7×10^{-8}	smMIP
CETP	rs17231506		16:56,994,528	C/T	1.11	1.2×10^{-6}	smMIP
CTRB2/CTRB1	rs72802342	rs55993634 (R^2=0.89)	16:75,236,763	C/G	0.79	8.0×10^{-9}	smMIP
TMEM97/VTN	rs11080055		17:26,649,724	C/A	0.92	1.5×10^{-5}	smMIP
NPLOC4/TSPAN10	rs6565597		17:79,526,821	C/T	1.12	2.1×10^{-7}	smMIP
C3	rs2230199		19:6,718,387	C/G	1.47	1.6×10^{-60}	smMIP
C3	rs147859257		19:6,718,146	T/G	3.22	4.1×10^{-26}	smMIP
C3 (NRTN/FUT6)	rs12019136	rs17855739 (R^2=0.95)	19:5,831,840	C/T	0.74	4.0×10^{-9}	smMIP
CNN2	rs67538026	rs113772652 (R^2=0.996)	19:1,031,550	C/T	0.90	1.4×10^{-6}	KASPar
APOE	rs429358		19:45,411,941	T/C	0.67	3.9×10^{-39}	smMIP
APOE(EXOC3L2/MARK4)	rs73036519		19:45,748,362	G/C	0.91	2.4×10^{-5}	smMIP
MMP9	rs142450006		20:44,614,991	TTTTC/T	0.84	5.3×10^{-9}	smMIP
C20orf85	rs201459901	rs117420707 (R^2=0.99)	20:56,663,846	C/A	0.76	3.8×10^{-12}	KASPar
SYN3/TIMP3	rs5754227		22:33,105,817	T/C	0.79	5.7×10^{-16}	smMIP

SLC16A8	rs8135665	22:38,476,276	C/T	1.14	1.4×10^{-8}	smMIP

[1] Data from Fritsche *et al*. 2016

[a] The SNP database has been updated and some rs numbers has been updated accordingly.

[b] Ten SNPs from the 52 AMD-associated variants genotyped by the smMIP method did not pass quality controls. These variants were then genotyped by KASP genotyping assays.

Abbreviations: SNP - single nucleotide polymorphism; IAMDGC - International AMD Genomics Consortium; GRS – genetic risk score; CES -Coimbra Eye Study; Chr – chromosome; OR - odds ratio; CI - confidence interval; *ABCA1* - ATP binding cassette subfamily A member 1; *ACAD10* - acyl-CoA dehydrogenase family member 10*; ADAMTS9-AS2* - ADAMTS9 antisense RNA 2; *APOE* - apolipoprotein E *; APOE (EXOC3L2/MARK4)* - apolipoprotein E(exocyst complex component 3 like 2/microtubule affinity regulating kinase 4); *ARHGAP21* - rho GTPase activating protein 21; *ARMS2/HTRA1* - age-related maculopathy susceptibility 2 /htrA serine peptidase 1; *B3GALTL* - beta 3-glucosyltransferase; *C2/CFB/SKIV2L* - complement component 2/complement factor B/ski2 like RNA helicase; *PBX2* - PBX homeobox 2; *C3* - complement component 3; *NRTN/FUT6* - neurturin/fucosyltransferase 6; *C9* - complement component 9; *CETP* - cholesteryl ester transfer protein; *CFH* - complement factor H; *CFHR1* - complement factor h related 1*; CFHR3* - complement factor h related 3; *CFI* - complement factor I; *CNN2* - calponin 2; *COL4A3* - collagen type IV alpha 3 chain; *COL8A1* - collagen type VIII alpha 1 chain; *CTRB2/CTRB1* - chymotrypsinogen B2/chymotrypsinogen B; *LIPC* - lipase c, hepatic type; *KMT2E/SRPK2* -Lysine Methyltransferase 2E (Inactive)/ SRSF Protein Kinase 2 ; *MIR6130/RORB* - microRNA 6130/RAR related orphan receptor b; *MMP9* - matrix metallopeptidase 9; *NPLOC4/TSPAN10* - NPL4 homolog, ubiquitin recognition factor/tetraspanin 10; *PILRB/PILRA* - paired immunoglobin like type 2 receptor beta/ paired immunoglobin like type 2 receptor alpha; *PRLR/SPEF2* - prolactin receptor/sperm flagellar 2; *RAD51B* - RAD51 paralog b; *RDH5/CD63* - Retinol Dehydrogenase 5/CD63 Molecule ; *SLC16A8* - solute carrier family 16 member 8; *SYN3/TIMP3* - synapsin III/TIMP metallopeptidase inhibitor 3*; TGFBR1* - transforming growth factor beta 1; TGFBR1 - transforming growth factor beta receptor 1; *TMEM97/VTN* - transmembrane protein 97/vitronectin; *TNFRSF10A* - TNF Receptor Superfamily Member 10a; *VEGFA* - vascular endothelial growth factor A; *SYN3/TIMP3* - synapsin III/ TIMP metallopeptidase inhibitor 3; *TRPM3* - Transient Receptor Potential Cation Channel Subfamily M Member 3 ; *C20orf85* - chromosome 20 open reading frame 85.

Supplementary Table S4. Genotyped *CFH* rare variants in the CES population.

Gene	Position GRCh37 (hg19)	REF	ALT	Function	SNP	Nucleotide Change	Protein change	SIFT[1]	Polyphen2_HDIV[1]	CADD[1]	Variants (N)	MAC cases	MAC controls	MAF cases	MAF controls
CFH	196621254	C	G	nonsynonymous_SNV	rs139254423	C7G	L3V	T	P	14.24	0	0	0	0,000	0,000
CFH	196642173	T	G	nonsynonymous_SNV	1:196642173:T:G	T124G	Y42D	D	D	23.7	0	0	0	0,000	0,000
CFH	196642194	A	G	nonsynonymous_SNV	rs747546121	A145G	I49V	T	B	0.003	0	0	0	0,000	0,000
CFH	196642206	C	T	nonsynonymous_SNV	rs757785149	C157T	R53C	D	D	29.8	1	1	0	0,002	0,000
CFH	196642221	T	G	nonsynonymous_SNV	rs141336681	T172G	S58A	T	B	0.811	0	0	0	0,000	0,000
CFH	196642260	T	A	nonsynonymous_SNV	1:196642260:T:A	T211A	W71R	T	D	24.2	0	0	0	0,000	0,000
CFH	196643064	G	A	nonsynonymous_SNV	rs868394050	G322A	V108I	D	B	23.6	0	0	0	0,000	0,000
CFH	196645133	G	A	nonsynonymous_SNV	1:196645133:G:A	G365A	G122D	D	D	25.8	0	0	0	0,000	0,000
CFH	196645145	A	T	nonsynonymous_SNV	1:196645145:A:T	A377T	Y126F	T	B	1.414	0	0	0	0,000	0,000
CFH	196645148	G	A	nonsynonymous_SNV	rs121913058	G380A	R127H	D	D	33.0	0	0	0	0,000	0,000
CFH	196645156	G	A	nonsynonymous_SNV	rs147002633	G388A	D130N	T	P	23.3	0	0	0	0,000	0,000
CFH	196645188	A	G	nonsynonymous_SNV	1:196645188:A:G	A420G	I140M	T	P	23.3	0	0	0	0,000	0,000
CFH	196645195	G	A	nonsynonymous_SNV	1:196645195:G:A	G427A	V143I	T	D	26.6	0	0	0	0,000	0,000
CFH	196646659	G	T	nonsynonymous_SNV	rs777300338	G481T	A161S	T	P	2.863	1	1	0	0,002	0,000
CFH	196646674	C	T	nonsynonymous_SNV	1:196646674:C:T	C496T	R166W	D	D	25.2	0	0	0	0,000	0,000
CFH	196646682	C	A	stopgain	1:196646682:C:A	C504A	Y168X			24.1	0	0	0	0,000	0,000
CFH	196646684	A	G	nonsynonymous_SNV	rs768647508	A506G	H169R	T	B	0.001	0	0	0	0,000	0,000
CFH	196646701	C	T	nonsynonymous_SNV	1:196646701:C:T	C523T	R175W				0	0	0	0,000	0,000
CFH	196646702	G	A	nonsynonymous_SNV	1:196646702:G:A	G524A	R175Q	T	B	0.015	0	0	0	0,000	0,000
CFH	196646727	GA	G	frameshift_deletion	1:196646727:GA:G	550delA	I184fs				0	0	0	0,000	0,000
CFH	196646750	A	G	nonsynonymous_SNV	1:196646750:A:G	A572G	H191R	T	D	18.63	0	0	0	0,000	0,000
CFH	196646755	T	C	nonsynonymous_SNV	1:196646755:T:C	T577C	S193P	D	D	24.0	0	0	0	0,000	0,000
CFH	196646756	C	T	nonsynonymous_SNV	1:196646756:C:T	C578T	S193L	T	D	23.4	0	0	0	0,000	0,000

CFH	196646760	C	A	nonsynonymous_SNV	1:196646760:C:A	C582A	D194E	T	B	0.001	0	0	0	0,000	0,000
CFH	196648780	T	C	nonsynonymous_SNV	rs183474263	T647C	I216T	T	B	0.001	0	0	0	0,000	0,000
CFH	196648794	A	G	nonsynonymous_SNV	rs774239374	A661G	I221V	T	B	0.001	1	0	1	0,000	0,001
CFH	196648906	C	T	nonsynonymous_SNV	rs768526062	C773T	P258L			21.1	17	13	4	0,033	0,004
CFH	196654203	G	A	nonsynonymous_SNV	1:196654203:G:A	G800A	C267Y	D	D	23.8	0	0	0	0,000	0,000
CFH	196654290	A	G	nonsynonymous_SNV	1:196654290:A:G	A887G	N296S	T	P	0.001	0	0	0	0,000	0,000
CFH	196654303	TG	T	frameshift_deletion	1:196654303:TG:T	901delG	A301fs				0	0	0	0,000	0,000
CFH	196654311	G	A	nonsynonymous_SNV	rs766408580	G908A	R303Q	T	B	0.217	0	0	0	0,000	0,000
CFH	196654313	G	A	nonsynonymous_SNV	1:196654313:G:A	G910A	G304R	D	D	4.395	0	0	0	0,000	0,000
CFH	196654350	C	T	nonsynonymous_SNV	1:196654350:C:T	C947T	P316L	D	D	25.5	0	0	0	0,000	0,000
CFH	196658582	G	A	nonsynonymous_SNV	1:196658582:G:A	G997A	G333R	D	D	26.1	0	0	0	0,000	0,000
CFH	196658607	G	A	nonsynonymous_SNV	rs371192606	G1022A	R341H			7.702	1	1	0	0,002	0,000
CFH	196658658	A	G	nonsynonymous_SNV	rs576059537	A1073G	D358G	T	D	13.36	0	0	0	0,000	0,000
CFH	196658660	G	T	stopgain	1:196658660:G:T	G1075T	E359X			23	0	0	0	0,000	0,000
CFH	196658733	T	C	nonsynonymous_SNV	rs762389370	T1148C	V383A	T	P	0.006	1	0	1	0,000	0,001
CFH	196658738	T	C	nonsynonymous_SNV	1:196658738:T:C	T1153C	C385R	D	D	23.9	0	0	0	0,000	0,000
CFH	196659231	C	A	nonsynonymous_SNV	rs201671665	C1198A	Q400K	T	B	0.001	0	0	0	0,000	0,000
CFH	196659255	C	T	stopgain	rs121913061	C1222T	Q408X			32	0	0	0	0,000	0,000
CFH	196659309	C	T	stopgain	1:196659309:C:T	C1276T	Q426X			34	0	0	0	0,000	0,000
CFH	196682946	C	T	nonsynonymous_SNV	rs371053403	C1418T	A473V	T	B	8.370	0	0	0	0,000	0,000
CFH	196683035	C	G	nonsynonymous_SNV	rs570523689	C1507G	P503A	T	D	22.7	0	0	0	0,000	0,000
CFH	196684751	T	A	nonsynonymous_SNV	rs147403664	T1548A	N516K	T	D	16.62	1	1	0	0,002	0,000
CFH	196684798	T	C	nonsynonymous_SNV	rs201193547	T1595C	L532S	D	D	24.5	0	0	0	0,000	0,000
CFH	196684807	A	G	nonsynonymous_SNV	1:196684807:A:G	A1604G	E535G	D	D	24.5	0	0	0	0,000	0,000
CFH	196684814	T	A	nonsynonymous_SNV	1:196684814:T:A	T1611A	H537Q	T	B	0.009	0	0	0	0,000	0,000
CFH	196684825	A	G	nonsynonymous_SNV	1:196684825:A:G	A1622G	E541G	T	D	21.3	1	1	0	0,002	0,000
CFH	196694253	A	G	nonsynonymous_SNV	rs757756991	A1699G	R567G	T	D	23	0	0	0	0,000	0,000
CFH	196694262	G	A	nonsynonymous_SNV	rs76405615	G1708A	E570K		B	0.235	0	0	0	0,000	0,000

CFH	196694332	T	A	stopgain	1:196694332:T:A	T1778A	L593X			36	0	0	0	0,000	0,000
CFH	196694418	A	G	nonsynonymous_SNV	1:196694418:A:G	A1864G	I622V	T	B	0.194	2	1	1	0,002	0,001
CFH	196695609	A	G	nonsynonymous_SNV	1:196695609:A:G	A1883G	Q628R	T	B	0.001	0	0	0	0,000	0,000
CFH	196695648	T	C	nonsynonymous_SNV	rs371768180	T1922C	V641A	T	B	0.258	0	0	0	0,000	0,000
CFH	196695675	G	T	nonsynonymous_SNV	rs143237092	G1949T	G650V	T	B	0.034	0	0	0	0,000	0,000
CFH	196695721	G	A	nonsynonymous_SNV	1:196695721:G:A	G1995A	M665I	D	B	23.3	0	0	0	0,000	0,000
CFH	196695756	A	G	nonsynonymous_SNV	1:196695756:A:G	A2030G	E677G	T	B	0.849	0	0	0	0,000	0,000
CFH	196695927	A	C	nonsynonymous_SNV	1:196695927:A:C	A2093C	E698A	T	B	7.448	0	0	0	0,000	0,000
CFH	196695975	C	G	stopgain	1:196695975:C:G	C2141G	S714X			35	0	0	0	0,000	0,000
CFH	196695985	C	A	nonsynonymous_SNV	rs763441589	C2151A	F717L	D	B	12.93	1	1	0	0,002	0,000
CFH	196696029	C	T	nonsynonymous_SNV	rs201360629	C2195T	T732M	T	B	11.39	0	0	0	0,000	0,000
CFH	196697494	A	G	nonsynonymous_SNV	1:196697494:A:G	A2255G	K752R	T	B	8.420	0	0	0	0,000	0,000
CFH	196697498	C	A	stopgain	1:196697498:C:A	C2259A	C753X			36	0	0	0	0,000	0,000
CFH	196697519	A	G	nonsynonymous_SNV	1:196697519:A:G	A2280G	I760M	T	B	0.131	0	0	0	0,000	0,000
CFH	196697552	C	A	nonsynonymous_SNV	1:196697552:C:A	C2313A	F771L	D	B	13.01	0	0	0	0,000	0,000
CFH	196697568	A	G	nonsynonymous_SNV	rs761904009	A2329G	I777V	T	B	0.001	0	0	0	0,000	0,000
CFH	196705989	A	T	nonsynonymous_SNV	1:196705989:A:T	A2449T	I817F	D	P	25.7	0	0	0	0,000	0,000
CFH	196706001	C	T	nonsynonymous_SNV	rs367687415	C2461T	H821Y	T	B	0.001	0	0	0	0,000	0,000
CFH	196706028	C	T	nonsynonymous_SNV	rs62641696	C2488T	R830W		P	22.2	0	0	0	0,000	0,000
CFH	196706112	T	A	nonsynonymous_SNV	1:196706112:T:A	T2572A	W858R	D	D	25.5	0	0	0	0,000	0,000
CFH	196706633	G	C	nonsynonymous_SNV	1:196706633:G:C	G2625C	Q875H	T	B	0.001	0	0	0	0,000	0,000
CFH	196706659	C	A	nonsynonymous_SNV	rs114743644	C2651A	S884Y	T	P	10.94	1	0	1	0,000	0,001
CFH	196706677	G	T	nonsynonymous_SNV	rs515299	G2669T	S890I	T	B	0.463	25	9	16	0,021	0,014
CFH	196709816	G	T	nonsynonymous_SNV	rs149474608	G2850T	Q950H	D	P	21.4	0	0	0	0,000	0,000
CFH	196709833	C	T	nonsynonymous_SNV	rs145975787	C2867T	T956M	T	D	15.09	0	0	0	0,000	0,000
CFH	196709874	A	G	nonsynonymous_SNV	rs759625279	A2908G	I970V	T	B	0.01	0	0	0	0,000	0,000
CFH	196709875	T	C	nonsynonymous_SNV	1:196709875:T:C	T2909C	I970T	T	B	0.004	0	0	0	0,000	0,000
CFH	196711052	G	C	nonsynonymous_SNV	rs201816520	G3004C	G1002R	T	D	7.236	1	1	0	0,002	0,000

CFH	196711098	C	T	nonsynonymous_SNV	rs34362004	C3050T	T1017I	D	P	12.48	0	0	0	0,000	0,000
CFH	196711125	G	A	nonsynonymous_SNV	1:196711125:G:A	G3077A	G1026E	D	D	24.7	0	0	0	0,000	0,000
CFH	196712596	A	T	nonsynonymous_SNV	rs35274867	A3148T	N1050Y	T	B	3.424	32	3	29	0,007	0,025
CFH	196712624	T	C	nonsynonymous_SNV	rs35343172	T3176C	I1059T	D	B	12.25	0	0	0	0,000	0,000
CFH	196712674	C	G	nonsynonymous_SNV	rs62625015	C3226G	Q1076E	T	B	0.003	0	0	0	0,000	0,000
CFH	196712682	G	T	nonsynonymous_SNV	rs121913062	G3234T	R1078S	T	B	0.637	0	0	0	0,000	0,000
CFH	196716253	T	C	nonsynonymous_SNV	1:196716253:T:C	T3506C	I1169T	D	B	23.8	0	0	0	0,000	0,000
CFH	196716261	G	T	stopgain	rs121913060	G3514T	E1172X			39	0	0	0	0,000	0,000
CFH	196716375	C	T	nonsynonymous_SNV	rs121913059	C3628T	R1210C	T	B	11.77	0	0	0	0,000	0,000
CFH	196716399	T	G	nonsynonymous_SNV	1:196716399:T:G	T3652G	C1218G	D	D	23.5	0	0	0	0,000	0,000
CFH	196716415	T	A	nonsynonymous_SNV	1:196716415:T:A	T3668A	L1223Q	D	D	24.1	1	0	1	0,000	0,001

[1] Data from de Breuk *et al*. 2020

Abbreviations: *CFH* – complement factor H; CES -Coimbra Eye Study; GRCh37(hg19) - genome reference consortium human reference 37; REF – reference allele; ALT – alternative allele; D: probably damaging; P - Possible damaging; B – Benign; T – Tolerated; SIFT – Sorting intolerant from tolerant; CADD - combined annotation-dependent depletion ; MAC – Minor allele counts; MAF – minor allele frequency; SNV – singe nucleotide variant; SNP – single nucleotide polymorphism.

Supplementary Table S5. Rare variants in *CFI* gene.

Gene	Position GRCh37 (hg19)	REF	ALT	Function	SNP	Nucleotide Change	Protein change	SIFT	Polyphen2_HDIV	CADD	Variants (n)	MAC cases	MAC controls	MAF cases	MAF controls
CFI	110662144	G	A	nonsynonymous_SNV	rs113460688	C1657T	P553S	T	B	15.35	4	1	3	0.002	0.003
CFI	110663656	C	T	nonsynonymous_SNV	4:110663656:C:T	G1525A	E509K	T	P	23.6	1	0	1	0.000	0.001
CFI	110667402	C	T	nonsynonymous_SNV	rs763276049	G1405A	V469I	T	B	0.002	1	1	0	0.002	0.000
CFI	110667657	C	T	nonsynonymous_SNV	rs762315947	G1150A	A384T	T	P	3.034	1	0	1	0.000	0.001
CFI	110678925	T	C	nonsynonymous_SNV	rs11098044	A898G	T300A	T	B	0.002	3	1	2	0.002	0.002
CFI	110682723	G	A	nonsynonymous_SNV	rs138346388	C608T	T203I	T	B	0.957	12	1	11	0.002	0.009

Abbreviations: CFI - complement factor I; SIFT – Sorting intolerant from tolerant; GRCh37(hg19) - genome reference consortium human reference 37 ; SNV – single nucleotide variant; REF – reference allele; ALT – alternative allele; SIFT – Sorting intolerant from tolerant; CADD - combined annotation-dependent depletion ; MAC – Minor allele counts; MAF – minor allele frequency; D - probably damaging; P - Possible damaging; B – Benign; T – Tolerated.

Supplementary Table S6. *CFI* rare variants score in AMD cases vs Controls.

***CFI* rare variant carriers by Polyphen2 score**	**Controls, N (%) (N=591)**	**Cases, N (%) (N=213)**	**OR (95%CI)**	***P*-value**
Noncarrier	574	209	1 reference	
Carrier-B	12	3	0.687 (0.155 -2.187)	0.563
Carrier-P	2	0	NA	0.974
Carrier-D	0	0	NA	NA
Carrier-LoF	2	1	1.373 (0.064-1.441)	0.796
***CFI* rare variant carriers by CADD score**				
Noncarrier	574	209	1 reference	
Carrier-CADD<20	13	3	0.634 (0.144-1.990)	0.480
Carrier-CADD≥20 or LoF	3	1	0.915 (0.045-7.195)	0.939

Abbreviations*: CFI* – complement factor I; AMD – age-related macular degeneration; CADD- combined annotation-dependent depletion; OR - odds ratio; CI -confidence interval; B - benign; P- possibly damaging; D- probably damaging; LoF- loss-of-function.

Supplementary Table S7. *ABCA4* rare variants score in AMD cases vs Controls.

***ABCA4* rare variant carriers by Polyphen2 score**	**Controls, N (%) (n=591)**	**Cases,N (%) (n=213)**	**OR (95%CI)**	***p*-value**
Noncarrier	469	163	1 reference	
Carrier-B	38	14	1.060 (0.543 -1.963)	0.858
Carrier-P	67	25	1.074 (0.646 – 1.737)	0.777
Carrier-D	13	9	1.992 (0.808 - 4.704)	0.120
Carrier-LoF	0	0	NA	NA
***ABCA4* rare variant carriers by CADD score**				
Noncarrier	442	1579	1 reference	
Carrier-CADD<20	7	2	0.804 (0.119-3.369)	0.787
Carrier-CADD≥20 or LoF	141	52	1.038 (0.716-1.491)	0.841

Abbreviations: ABCA4 - ATP binding cassette subfamily A member 4; AMD – age-related macular degeneration; CADD- combined annotation-dependent depletion; OR - odds ratio; CI -confidence interval; B - benign; P- possibly damaging; D- probably damaging; LoF- loss-of-function

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CHAPTER 6.
PHENOTYPIC EXPRESSION OF *CFH* RARE VARIANTS IN AGE-RELATED MACULAR DEGENERATION PATIENTS IN THE COIMBRA EYE STUDY

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As previously presented, the AMD Incidence Study included extensive phenotypic characterization of the participants through multimodal imaging which provided insight into biomarkers of AMD progression, and genetic analysis regarding AMD risk was reported for the first time in a Portuguese population.

The subsequent logical step is to explore the associations between phenotype and genotype in our cohort. Genotype-phenotype associations are important to pursue in different populations, in order to fully grasp the variability of pathophysiology in AMD. Specific biomarkers obtained with MMI can be linked to specific genotypes. The identification of associations with rare variants, in particular, is of major interest because they can have a strong impact due to high penetrance and may predispose to more severe disease. Despite this, very few studies addressed the phenotypic characteristics associated with rare variants in AMD, and most of them were based on color fundus photography alone.

A better understanding of phenotype-genotype associations with respect to rare variants and based on multimodal imaging could contribute to improving the identification of patients at greater risk of progression to late-stage disease. This might be of special interest to detect patients who can benefit more from genetic screening, especially when complement and genetic-targeted therapies are rapidly becoming a reality.

The CFH gene encodes the factor H, which is an inhibitor of the alternative complement pathway. Compromise of this pathway leads to a pro-inflammatory state and rare variants located in the CFH gene are among those which confer the highest risk for AMD. Thus, our objective in this Chapter was to explore the presence of rare variants in the CFH gene and their relationship with the phenotypic features of our AMD patients from the Mira Cohort. Our results showed that there are indeed phenotypic differences between carriers and noncarriers of rare CFH variants in AMD patients. Carriers presented with more severe disease, and they might be at increased risk of progression.

ABSTRACT

Purpose: To determine the association between rare genetic variants in complement factor H (*CFH*) and phenotypic features in age-related macular degeneration (AMD) patients from the Coimbra Eye Study (CES).

Methods: AMD patients from the Incidence CES (NCT02748824) underwent ophthalmologic examination and color fundus photography, spectral-domain optical coherence tomography (SD-OCT), fundus autofluorescence, and near-infrared imaging. Multimodal phenotypic characterization was carried out in a centralized reading center. The coding and splice-site regions of the *CFH* gene were sequenced through single-molecule molecular inversion probe‐based next-generation sequencing in association with the EYE-RISK consortium. Variants with minor allele frequency <0.05 resulting in splice-site or protein change were selected. Differences in phenotypic features between carriers and noncarriers were analyzed using generalized estimated equations logistic regression models, considering intereye correlations.

Results: We included 39 eyes of 23 patients carrying rare *CFH* variants and 284 eyes of 188 noncarriers. Carrier status was associated with having higher drusen burden in the macula in the inner Early Treatment Diabetic Retinopathy Study circle (odds ratio [OR], 5.44 [95% confidence interval {CI}, 1.61‐18.37]; $P = 0.006$), outer circle (OR, 4.37 [95% CI, 1.07‐17.77]; $P = 0.04$), and full grid (OR, 4.82 [95% CI, 1.13‐20.52]; $P = 0.033$). In SD-OCT, a lower total macular volume and lower inner retinal layers' volume (OR, 0.449 [95% CI, 0.226‐0.894]; $P = 0.023$; OR, 0.496 [95% CI, 0.252‐0.979]; $P = 0.043$) and pigment epithelial detachments (PEDs) (OR, 5.24 [95% CI, 1.08‐25.44]; $P = 0.04$) were associated with carrying a rare *CFH* variant. Carriers with subretinal drusenoid deposits (SDD) had the rare variant P258L in all cases except one.

Conclusions: We identified in our cohort phenotypic differences between carriers and noncarriers of rare variants in the *CFH* gene. Carriers had more severe disease, namely superior drusen burden, PEDs, and thinner retinas. The rare variant P258L may be associated with SDD. Carriers are probably at increased risk of progression.

Keywords: age-related macular degeneration, Coimbra Eye Study, rare *CFH* variants, AMD phenotype, genotype-phenotype associations

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness in the older population in industrialized countries, and its prevalence is expected to significantly increase in the future.[1,2] Although in the early stages of disease visual loss is commonly not perceived by patients, in late-stage AMD the visual compromise can be profound and irreversible. Thus significant effort is being made to develop strategies capable of halting disease progression and predicting individual risk. Further understanding of the pathophysiology is essential to achieve these goals because AMD is a complex multifactorial disease, influenced by demographic, environmental, and genetic factors.[3-7]

Several genetic risk variants have been identified in recent years, and a landmark genome-wide association study (GWAS) identified 52 variants at 34 genomic regions as independently associated with AMD (45 common variants and seven rare variants [minor allele frequency <1%]).[5] A large risk effect has been reported for common genetic variants located at the *CFH* and *ARMS2/HTRA1* loci.[6,8,9] However, Fritsche et al.[5] also noted that a significant burden of rare variants was observed in the *CFH* and *CFI*, whereas other groups confirmed that rare genetic variants located in these genes conferred a high risk of disease.[10-12] To date, more than 100 rare variants are described to be associated with AMD.[5,11]

The identification of rare variants is important because they can have a strong impact due to high penetrance and may predispose to more severe disease. The *CFH* gene encodes factor H, which is an inhibitor of the alternative complement pathway, leading to decreased activity and preventing complement overactivation. Compromise of this regulatory function leads to a proinflammatory state that is associated with both AMD development and progression.[13,14] Additionally, Triebwasser et al.[11] showed that rare variants act in an autosomal dominant manner, in that haploinsufficiency of the cofactor protein (FH) or the necessary protease (Factor I) to inactivate C3b is sufficient to allow this proinflammatory state.

Despite this, there is not much information on the phenotypic characteristics associated with rare variants in AMD.[15,16] Furthermore, most of these reports address features based on color fundus photography alone. A better understanding of phenotype-genotype associations with respect to rare variants and based on multimodal imaging could contribute to improving the identification of patients at greater risk of progression to late-stage disease. It would also help in selecting those who could benefit more from targeted therapies inhibiting specific pathways in the complement system or even gene therapy.[17-19]

The Coimbra Eye Study (CES) is a two-visit epidemiologic population-based study on the prevalence and 6.5-year incidence of AMD in a Portuguese population (NCT01298674, NCT02748824). Subjects who participated in the Incidence study also had blood samples collected for genetic analysis.[20-23] We have previously reported on the genetic characterization of this cohort,

and we found that rare and low-frequency variants in the *CFH* gene with damaging effects were more common in AMD cases (Farinha C, et al., unpublished data, 2021). The purpose of this study is to determine the association between the carrier status of rare genetic variants in the *CFH* gene and the phenotypic features in AMD patients who participated in the Incidence study.

MATERIAL AND METHODS

Study Population and Data Collection

The AMD Incidence Study (NCT027048824) is a single-center population-based study that was conducted in the context of the Coimbra Eye Study, an epidemiologic project for the estimation of AMD prevalence and incidence in a Portuguese population. The Incidence Study was conducted 6.5 years after the Epidemiological Study (NCT01298674), which reported on AMD prevalence. A detailed description of the global study population and recruitment details have been published elsewhere.[20-22]

In the Incidence Study, the population was extensively characterized, including demographic, clinical, and lifestyle/nutritional information, and blood samples were collected from the participants who consented to further genetic analysis.[22,24] Bilateral ophthalmological assessment was performed including multimodal imaging. This multimodal approach included color fundus photography (CFP) of fields 1M, 2, and 3M acquired at 45° (Topcon fundus camera, TRC-NW8; Topcon Corp., Tokyo, Japan), spectral-domain optical coherence tomography (SD-OCT), fundus autofluorescence (FAF), and infrared (IR) imaging with Spectralis HRA+OCT (Heidelberg Engineering, Heidelberg, Germany). SD-OCT acquisitions consisted of one EDI Macular Volume Scan (20° × 20°, 49 or 97 B-scans, 16 frames per scan), one radial scan centered in the fovea (20° × 20°, 24 B-scans, 10 frames per scan), and two high-resolution EDI Line Scans (30°, acquired at 0° and 90°, with ≥20 frames each), with signal strength ≥25. Both FAF (488 nm) and IR images were acquired for field 2 at 30° (high resolution with ≥15 frames each).[23]

Signed informed consent was obtained for all participants. The study adhered to the tenets of the Declaration of Helsinki (2008) and the International Conference on Harmonization‐Good Clinical Practice Guideline. The Association for Innovation and Biomedical Research on Light and Image Ethics Committee issued a favorable opinion for the conduction of the study.

AMD Definitions and Staging

The participants' imaging examination results were sent to a centralized reading center for grading (Coimbra Ophthalmology Reading Center (CORC), AIBILI, Coimbra, Portugal). All graders were senior medical retina specialists certified by the reading center. CFP image grading was supported by Retmarker AMD Research software (Retmarker SA, Coimbra, Portugal) according to the International Age-Related Macular Epidemiological Study Group Classification, while simultaneously analyzing the corresponding SD-OCT, IR, and FAF images in the Heidelberg Eye Explorer software (version 1.10.4.0) as previously reported.[21,23,25,26]

The Rotterdam staging system was used to assess the AMD severity status of all included eyes.[27] Early AMD was defined by the presence of large (≥125 μm in diameter), soft, indistinct, or

reticular drusen only; or of soft distinct (≥63 μm in diameter), indistinct (≥125 μm), or reticular drusen with pigmentary abnormalities, within the macula (3000 μm radius Early Treatment Diabetic Retinopathy Study [ETDRS] grid, centered in the fovea), which corresponds to Rotterdam stages 2a, 2b and 3. Late AMD was defined by the presence of neovascular AMD (nAMD) or geographic atrophy (GA). Neovascular AMD was defined by the presence of any type 1, 2, or 3 macular neovascularization (MNV), associated with features such as intraretinal/subretinal fluid, hemorrhage, fibrosis, or subretinal hyperreflective material. Geographic atrophy was defined as a sharply demarcated area of retinal depigmentation, with a corresponding appearance of complete RPE and outer retina atrophy in OCT, and deep hypoautofluorescence in FAF imaging.[28,29] When GA and nAMD coexisted in the same eye, it was categorized as nAMD. Late AMD corresponds to stage 4 in the Rotterdam classification. Both eyes were graded and staged in this manner, but the stage of an individual participant was based on the eye with a more severe status.

AMD Multimodal Grading of Phenotypic Features

The following fundus features were assessed and quantified directly in CFP with Retmarker AMD Software in the total 6 mm ETDRS grid centered in the fovea, and in the central, inner, and outer circles: (1) number, type, and size of drusen; (2) predominant type of drusen and the confluence of drusen; (3) total area occupied by drusen (<10%, 10%‑50%, ≥50%) and the cumulative real drusen area – total and in each ETDRS circle; (4) presence of hyperpigmentation and hypopigmentation; (5) presence of GA and neovascular AMD. Grading of these features was confirmed by visualizing the corresponding SD-OCT, FAF, and IR images.

Analysis of the SD-OCT scans concerning the vitreomacular interface, neuroretina, and RPE was performed according to the "European Eye Epidemiology spectral-domain optical coherence tomography classification of macular diseases for epidemiological studies." [30] Several features were graded including the presence of soft drusen (size, location, confluence, internal core reflectivity), subretinal drusenoid deposits (SDD)/pseudodrusen, hyperreflective foci, intraretinal/subretinal fluid, subretinal hyperreflective material, pigment epithelial detachments (PED), RPE atrophy, and presence of MNV. Quantification in the macula of the total retinal thickness and volume, and layer-by-layer including the RPE/Bruch's membrane layer (which is an indirect measurement of the drusen load), was performed in patients with early AMD (stages 2 and 3) through semiautomatic segmentation as reported previously.[31] Eyes in stage 4 were excluded from retinal thickness and volume measurements because of the inherent retinal layer distortion caused by GA or MNV. In brief, the Heidelberg segmentation software displays seven distinct retinal layers (retinal nerve fiber layer, ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, and RPE/Bruch's membrane layer), and also provides information on two additional "combination" layers: (1) the inner retinal layers, extending

from the inner limiting membrane to the external limiting membrane; and (2) the outer retinal layers, from external limiting membrane to Bruch's membrane. The subfoveal choroidal thickness was measured manually in both early and late AMD patients with the inbuilt caliper tool available in the software.[31]

In FAF and IR imaging the presence of SDD and geographic atrophy were confirmed, and the corresponding areas were measured with the Heidelberg Eye Explorer software in-built tools. The SDD total area was measured by using the manual Heidelberg Eye Explorer region overlay tool to draw the borders of the area of interest, and GA was measured by using the semi-automated RegionFinder software.[31,32]

Genetic Sequencing and Selection of Carriers/Non-Carriers

Genomic DNA samples obtained from the AMD Incidence study participants were genotyped according to standard procedures in the context of a collaboration with the E3‐The European Eye Epidemiology Consortium and the EYE-RISK Consortium. The EYE-RISK genotype assay is designed to genotype 87 single nucleotide polymorphisms, including the 52 independently associated single nucleotide polymorphisms identified by the International AMD Genomics Consortium.[5,9] The sequencing of rare variants in the *CFH* gene was obtained with the EYE-RISK single-molecule molecular inversion probes‐based next-generation sequencing (NextSeq500; Illumina, San Diego, CA, USA), as described in detail by de Breuk et al.[9] All coding and splice-site regions of the *CFH* gene were sequenced, and the EYE-RISK carried out the selection and filtering of variants to ensure the quality of the data. Variants with fewer than 40 reads coverage on reference allele and variants with less than 40 reads coverage on alternate allele were changed to

missing values. For homozygous reference samples genotype was kept unchanged, even if it did not have 40 reads coverage in alternate alleles. Variants with a minor allele frequency (MAF) > 0.05 were removed from the dataset to retain only rare and low-frequency variants.[9]

For this analysis AMD patients were eligible for inclusion, whereas participants without AMD were excluded, to compare only between carriers and noncarriers of rare variants in the *CFH* gene who had the disease. Carriers were AMD patients carrying a rare variant in the *CFH* gene that results in a splice-site or protein change (nonsynonymous), because these variants are more likely to be pathogenic.[9] Patients carrying rare *CFH* variants with a described protective effect in case-control analyses (Q950H) and with a likely benign effect in functional studies (S890I, T956M, and V1007L) were excluded.[15] Noncarriers were the remaining patients not having a rare *CFH* variant.

Statistical Analysis

General (demographic, environmental, clinical, and genetic) characteristics between carriers and noncarriers were compared, using Mann-Whitney U Test and Pearson's $\chi 2$ test for continuous and categorical variables, respectively (significance level was set to 0.05). The genotyped samples and selected rare variants were tested regarding the presence of the above-mentioned phenotypic features obtained with multimodal imaging-based grading. For this purpose, odds ratios (ORs) at 95% confidence interval (CI) was computed for the presence of any *CFH* rare variant according to the presence of the selected phenotypic features of interest using binary logistic regression models, while adjusting for age, sex, AMD stage, and history of smoking. Generalized estimated equations were used to account for intereye correlations. A nominal significance level was set to 0.05, as correction for multiple comparisons was hampered by small sample size. All statistical analyses were performed using R Statistical Software (v4.0.2; R Core Team 2020).

RESULTS

From the original cohort of 1617 participants in the AMD incidence study, of which 237 (14.7%) were early AMD cases and 28 (1.73%) were late AMD cases, a total of 859 samples, including 218 from AMD patients, were genotyped under the association with the EYE-RISK/E3 - The European Eye Epidemiology Consortium. Eyes with spherical equivalent >3.00 diopters or poor image quality hampering grading were excluded. The final cohort in the analysis comprised 323 eyes from 211 AMD patients. Of these, 256 eyes (79.3%) were in stage 2, 41 eyes (12.7%) were in stage 3, and 26 eyes (8%) were in stage 4.

A total of 90 unique splice-site or protein change rare *CFH* variants were genotyped in the 859 samples from AMD cases and controls. Of these, and after excluding one patient with a variant with described benign effect (c.2669G>T p.Ser890Ile), 15 rare variants were present in our total population, and 11 were found in AMD patients and included in the analysis.[15]

Our final cohort in analysis thus included 39 eyes of 23 carriers (AMD patients having at least one of these 11 rare variants [mean ± SD age, 73.1 ± 6.5 years; 60.9% female]) and 284 eyes of 188 noncarriers (AMD patients not having at least one of these variants [mean ± SD age, 75.0 ± 7.5 years; 61.2% female]). Demographic and environmental characteristics were well balanced between carriers and noncarriers (Table 1).

Regarding common major risk variants for AMD, we found slight differences in MAF distribution between carriers and noncarriers, although these were non-significant (Table 1).[9] The major risk variants *ARMS2* rs10490924, *ARMS2/HTRA1* rs3750846, and *CFH* rs570618 had a lower MAF in carriers compared to non-carriers, while the *C3* rs2230199 risk variant was well balanced between groups. Moreover, in non-carriers all 4 major risk variants had a lower MAF than that reported in AMD patients from larger populations such as from the EYE-RISK and International AMD Genomics Consortium (Supplementary Table S1).[5,9] The MAF of the protective variant *CFH* rs10922109 was lower in carriers compared to non-carriers, but similar between the latter and AMD patients from these larger cohorts.[5,9] The *C2/CFB/SKIV2L* rs429608 protective variant had a higher MAF in carriers.

The rare *CFH* variants found in both AMD cases and non-AMD cases from the CES are presented in Table 2, and for completeness, all sequenced variants are presented in Supplementary Table S2. The most frequent rare variant found in AMD patients was *CFH* rs768526062 (Pro258Leu). Also, variants known to have a functional effect such as *CFH* rs757785149 (Arg53Cys) were present.

TABLE 1. Demographic and Clinical Characteristics of AMD Patients − Carriers Versus Noncarriers

Characteristic	Non-carriers, N = 188* (284 Eyes)	Carriers, N = 23* (39 Eyes)	*P*-Value †
Age	75.0 (7.5)	73.1 (6.5)	0.26
Gender			0.98
Male	73.0 / 188.0 (38.8%)	9.0 / 23.0 (39.1%)	
Female	115.0 / 188.0 (61.2%)	14.0 / 23.0 (60.9%)	
Smoking			0.41
Non-smoker	151.0 / 186.0 (81.2%)	20.0 / 23.0 (87.0%)	
Ex-Smoker	31.0 / 186.0 (16.7%)	2.0 / 23.0 (8.7%)	
Smoker	4.0 / 186.0 (2.2%)	1.0 / 23.0 (4.3%)	
Familiar history of AMD			0.30
No	172.0 / 188.0 (91.5%)	19.0 / 23.0 (82.6%)	
Doesn't know	14.0 / 188.0 (7.4%)	4.0 / 23.0 (17.4%)	
Yes	2.0 / 188.0 (1.1%)	0.0 / 23.0 (0.0%)	
Diabetes			0.25
No	165.0 / 188.0 (87.8%)	18.0 / 23.0 (78.3%)	
Doesn't know	4.0 / 188.0 (2.1%)	0.0 / 23.0 (0.0%)	
Yes	19.0 / 188.0 (10.1%)	5.0 / 23.0 (21.7%)	
Arterial hypertension			0.36
No	75.0 / 188.0 (39.9%)	11.0 / 23.0 (47.8%)	
Doesn't know	4.0 / 188.0 (2.1%)	1.0 / 23.0 (4.3%)	
Yes	109.0 / 188.0 (58.0%)	11.0 / 23.0 (47.8%)	
Dyslipidemia			>0.99
No	151.0 / 188.0 (80.3%)	19.0 / 23.0 (82.6%)	
Doesn't know	27.0 / 188.0 (14.4%)	3.0 / 23.0 (13.0%)	
Yes	10.0 / 188.0 (5.3%)	1.0 / 23.0 (4.3%)	
BMI	27.8 (4.9)	28.3 (4.1)	0.47
AMD stage - worst eye			0.83
2	142.0 / 188.0 (75.6%)	18.0 / 23.0 (78.2%)	
3	29.0 / 188.0 (15.4%)	2.0 / 23.0 (8.7%)	
4	17.0 / 188.0 (9.0%)	3.0 / 23.0 (13.0%)	
Major common risk variants MAF‡			
ARMS2 rs10490924, T	76/376 (20.2)	8/46 (17.4)	0.56
ARMS2/HTRA1 rs3750846, C	75/376 (19.9)	7/46 (15.2)	0.29

CFH rs570618, T	130/374 (34.8)	11/46 (23.9)	0.34
CFH rs10922109, A	134/374 (35.8)	14/46 (30.4)	0.77
C2/CFB/SKIV2L rs429608, A	28/370 (7.6)	5/46 (10.9)	0.21
C3 rs2230199, C	64/376 (17.0)	8/46 (17.4)	0.36

* Mean (SD); n/N (%).
† Wilcoxon rank sum test; Pearson's $\chi 2$ test; Fisher's exact test.
‡ No. of minor alleles/total No. of alleles (%).

TABLE 2. *CFH* Rare Variants Identified in the CES Cohort

Gene	Position GRCh37 (hg19)	REF	ALT	ID	Nucleotide Change	Protein change	Maf CES	Variants (n)	MAC cases	MAC controls	Maf cases	Maf controls
CFH	196642206	C	T	rs757785149	C157T	R53C * § ‡	0.000612	1	1	0	0.002	0.000
CFH	196646659	G	T	rs777300338	G481T	A161S †	0.000612	1	1	0	0.002	0.000
CFH	196648794	A	G	rs774239374	A661G	I221V * §	0.000612	1	0	1	0.000	0.001
CFH	196648906	C	T	rs768526062	C773T	P258L†	0.011409	17	13	4	0.033	0.004
CFH	196658607	G	A	rs371192606	G1022A	R341H †	0.000612	1	1	0	0.002	0.000
CFH	196658733	T	C	rs762389370	T1148C	V383A	0.000612	1	0	1	0.000	0.001
CFH	196684751	T	A	rs147403664	T1548A	N516K †	0.000614	1	1	0	0.002	0.000
CFH	196684825	A	G	1:196684825:A:G	A1622G	E541G	0.000612	1	1	0	0.002	0.000
CFH	196694418	A	G	1:196694418:A:G	A1864G	I622V †	0.001224	2	1	1	0.002	0.001
CFH	196695985	C	A	rs763441589	C2151A	F717L†	0.000612	1	1	0	0.002	0.000
CFH	196706659	C	A	rs114743644	C2651A	S884Y †	0.000612	1	0	1	0.000	0.001
CFH	196706677	G	T	rs515299	G2669T	S890I †‖	0.0153	25	9	16	0.021	0.013
CFH	196711052	G	C	rs201816520	G3004C	G1002R †	0.000612	1	1	0	0.002	0.000
CFH	196712596	A	T	rs35274867	A3148T	N1050Y *‡	0.019584	32	3	29	0.007	0.024
CFH	196712624	T	C	rs35343172	T3176C	I1059T †	0.000612	1	1	0	0.002	0.000
CFH	196716415	T	A	1:196716415:T:A	T3668A	L1223Q	0.000612	1	0	1	0.000	0.001

* Variants reported to be significantly associated with AMD in one or more AMD case-control cohorts.
† Variants found in one or more studies.
‡ Variants with a functional effect on the protein or change in systemic levels.[10]
§ Risk-conferring variants in GWAS.[5]
‖ Variant removed from the analyzed dataset due to having a described protective effect in case-control analyses or a likely begin effect in functional studies.

Associations With Phenotypic Features

Regarding the phenotypic features analyzed with multimodal imaging in AMD patients, associations were found with carrying rare variants in the *CFH* gene.

The risk of carrying at least one of these variants increased with a larger drusen area in the inner ETDRS circle (OR, 3.22 [95% CI, 1.18‑8.78]; $P = 0.022$) and higher percentual coverage of the ETDRS grid by drusen in color fundus photography. Specifically, having a 10% to 50% area of the ETDRS grid occupied by drusen in the inner circle (OR, 5.44 [95%CI, 1.61‑18.37]; $P = 0.006$), the outer ETDRS circle (OR, 4.37 [95%CI, 1.07‑17.77]; $P = 0.04$), and in the full ETDRS grid (OR, 4.82 [95%CI, 1.13‑20.52]; $P = 0.033$), but not in the central fovea.

In SD-OCT phenotypic analysis we found that having a higher macular retinal volume appears to decrease the risk of having a rare variant (OR, 0.449 [95% CI, 0.226‑0.894]; $P = 0.023$), and the same was true for having a higher volume of all combined inner retinal layers (OR, 0.496 [95% CI, 0.252‑0.979]; $P = 0.043$). The presence of pigment epithelial detachments in OCT was predictive of having a rare variant (OR, 5.24 [95% CI, 1.08‑25.44]; $P = 0.04$). A trend in the same direction was found regarding the presence of hyperreflective foci (OR, 2.61 [95% CI, 0.88‑7.71]; $P = 0.08$).

Despite not reaching statistical significance, hard drusen were more common in noncarriers, and intermediate and large drusen were more common in carriers. Plus, carriers had on average thinner choroids (208.7 ± 83.8 μm vs. 228.3 ± 87.7 μm, $P = 0.15$) and larger retinal areas affected by SDD (7.89 ± 16.8 mm^2 vs. 4.64 ± 10.10 mm^2, $P = 0.13$). An interesting finding was that in carriers of the most frequent rare variant (*CFH* rs768526062, Pro258Leu), 46.2% (n = 6) had SDD in both eyes, and in most cases affecting an extensive retinal area. In fact, and except for only one case, all carriers with SDD shared this rare variant in our cohort.

Regarding late AMD, both GA and MNV were more common in carriers, but this was more striking for MNV (10.26% in carriers vs. 2.82% in noncarriers); however, it was not statistically significant. The associations between all assessed phenotypic features and carrier status are shown in Table 3. Exemplificative multimodal images of the main phenotypic characteristics of carriers are presented in Figures 1 and 2.

TABLE 3. Phenotypic Characterization of Carriers Versus Noncarriers of Rare *CFH* Variants.

Phenotypic Characteristics	Non-carriers (n=284 Eyes)	Carriers (n=39 Eyes)	Odds Ratio (95% CI)	*P*-Value
Area covered by drusen (in ETDRS grid), mm^2				
Central subfield	0.028 (0.063)	0.048 (0.084)	na	0.16
Inner circle	0.15 (0.30)	0.36 (0.56)	3.22 [1.18 – 8.78]	**0.022**
Outer circle	0.54 (0.98)	0.96 (1.25)	1.34 [0.93 – 1.94]	0.11

% Area occupied by drusen in ETDRS grid (all subfields)				
< 10 %	275 (96.8)	33 (84.6)	1.0	ref
10 - 50%	9 (3.2)	6 (15.4)	4.82 [1.13 – 20.52]	**0.033**
≥ 50 %	0	0	na	na
% Area occupied by drusen – Central field				
0 – 10 %	266 (93.66)	33 (84.62)		ref
10 - 50%	18(6.34)	6 (15.38)	3.28 [0.83-12.97]	**0.091**
≥ 50 %	0	0	na	na
% Area occupied by drusen – Inner circle				
0 – 10 %	273 (96.1)	32 (82.1)	1.0	ref
10 - 50%	11 (3.9)	7 (17.8)	5.44 [1.61 – 18.37]	**0.006**
≥ 50 %	0	0	na	na
% Area occupied by drusen – Outer circle				
0 – 10 %	274 (96.5)	33 (84.6)	1.0	Ref.
10 - 50%	10 (3.5)	6 (15.4)	4.37 [1.07 – 17.77]	**0.040**
≥ 50 %	0	0	Na	na
Predominant drusen type within ETDRS grid				
Absent	28 (10.18)	4 (10.81)		ref
Hard drusen	114 (52.36)	15 (40.54)	0.60 [0.11-3.22]	0.55
Intermediate drusen	83 (30.18)	15 (40.54)	1.15 [0.21-6.22]	0.87
Large drusen	20 (7.27)	3 (8.11)	0.98 [0.13 – 7.88]	0.99
Hyperpigmentation (CFP)				
No	232 (81.7)	32 (82.0)		ref
Yes	52 (18.3)	7 (17.9)	1.20 [0.16 – 9.19]	0.86
Hypopigmentation (CFP)				
No	253 (89.1)	34 (87.2)		ref
Yes	31 (10.9)	5 (12.8)	1.32 [0.25 – 6.86]	0.74
Presence of SDD (FAF+IR+OCT)				
No	203 (71.48)	27 (69.23)		ref
Yes	81 (28.52)	12 (30.77)	1.43 [0.48 – 4.27]	0.52
Total area of SDD (FAF), mm^2	4.64 ± 10.1	7.89 ± 16.8	1.03 [0.99 – 1.08]	0.128
Retinal thickness in central subfield (OCT), µm	278.4 ± 34.9	273.0 ± 26.8	0.996 [0.98-1.01]	0.58
Volume (OCT), mm^3				
Overall Retina	8.40 ± 0.50	8.29 ± 0.32	0.449 [0.226-0.894]	**0.023**
IRL	6.17 ± 0.51	6.05 ± 0.36	0.496 [0.252– 0.979]	**0.043**
ORL	2.22 ± 0.09	2.20 ± 0.10	0.033 [0.0005– 2.26]	0.114
RPE-Bruch layer	0.39 ± 0.05	0.38 ± 0.05	na	0.88
Subfoveal Choroidal Thickness (OCT), µm	228.3 ± 87.7	208.7 ± 83.8	0.995 [0.99-1.00]	0.145
Pigment epithelial detachment (OCT)				
No	275 (96.83)	34 (87.18)		**ref**
Yes	9 (3.17)	5 (12.82)	**5.24 [1.08-25.44]**	**0.04**
Hyperreflective foci (OCT)				
No	239 (84.15)	28 (71.79)		**ref**
Yes	45 (15.85)	11 (28.21)	**2.61 [0.88-7.71]**	**0.083**
MNV (OCT)				
No	276 (97.18)	35 (89.74)		ref
Yes	8 (2.82)	4 (10.26)	6.08 [0.48 – 76.82]	0.16

Geographic atrophy/ (CFP+FAF+IR+OCT)				
No	251 (93.66)	35 (92.11)		ref
Yes	17 (6.34)	3 (7.89)	0.57 [0.07-1.08]	0.52

ORL, outer retinal layers; IRL, inner retinal layers.
Generalized estimated equation logistic regression analysis, adjusted by AMD stage, age, sex, and smoking (non-smokers vs smokers/ex-smokers).

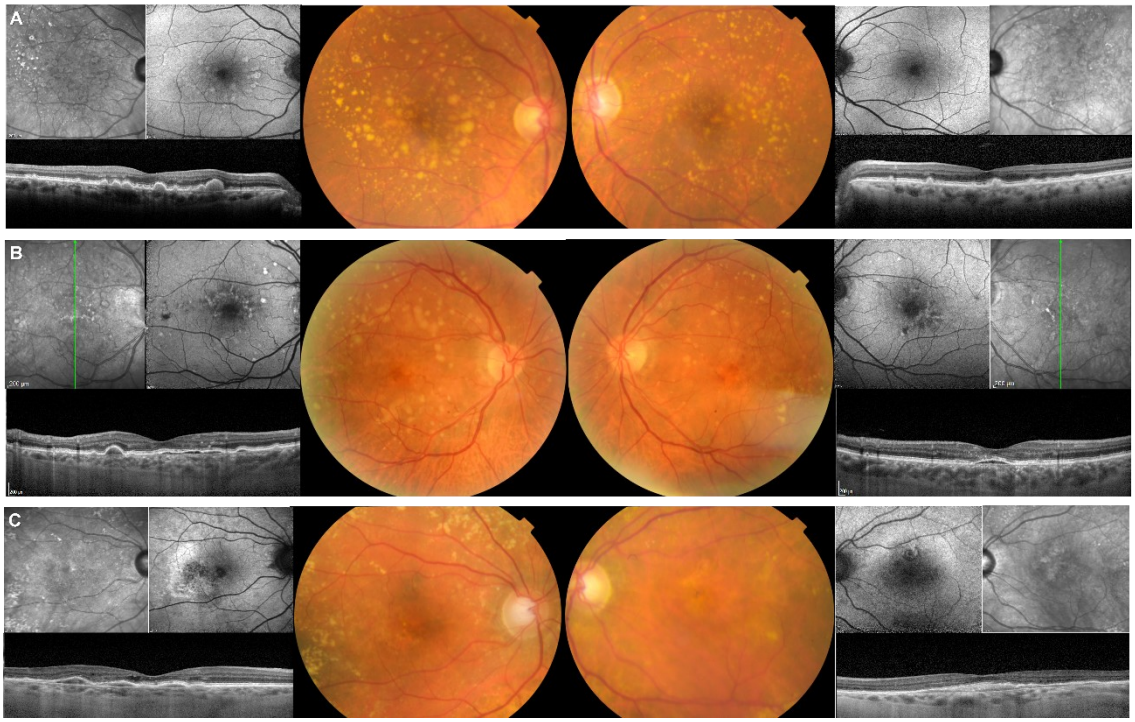

FIGURE 1. Exemplificative images of fundus features of carriers.
(A) Female, 68 years old (yo) (*CFH* rs757785149; Arg53Cys) with extensive soft drusen both inside the ETDRS grid and outside extending beyond the vascular arcades, and crystalline drusen temporal to the macula. There is a high degree of phenotypic symmetry between both eyes.
(B) Male, 68yo (*CFH* rs371192606; Arg341His) with large, soft, confluent drusen mainly located in the outer ETDRS grid circle and extending to the vascular arcades and nasal peripapillary area, along with hypo and hyperpigmentation in the central macula. The OCT reveals shallow and heterogeneously hyporreflective PEDs under the fovea in both
eyes, but no intraretinal or subretinal fluid. The symmetry of all pathologic changes in multimodal imaging is striking.
(C) Female, 80yo (*CFH* 1:196694418:A:G; Ile622Leu) with large soft, confluent drusen mainly located in the outer ETDRS grid circle and temporal to the macula. They extend outside the vascular arcades and to the nasal peripapillary area. There is hypopigmentation and hyperpigmentation in the central macula in both eyes. The OCT shows PED under the fovea in both eyes, in the right eye with intraretinal fluid (type 1 MNV), and the left eye without fluid (probably quiescent MNV).

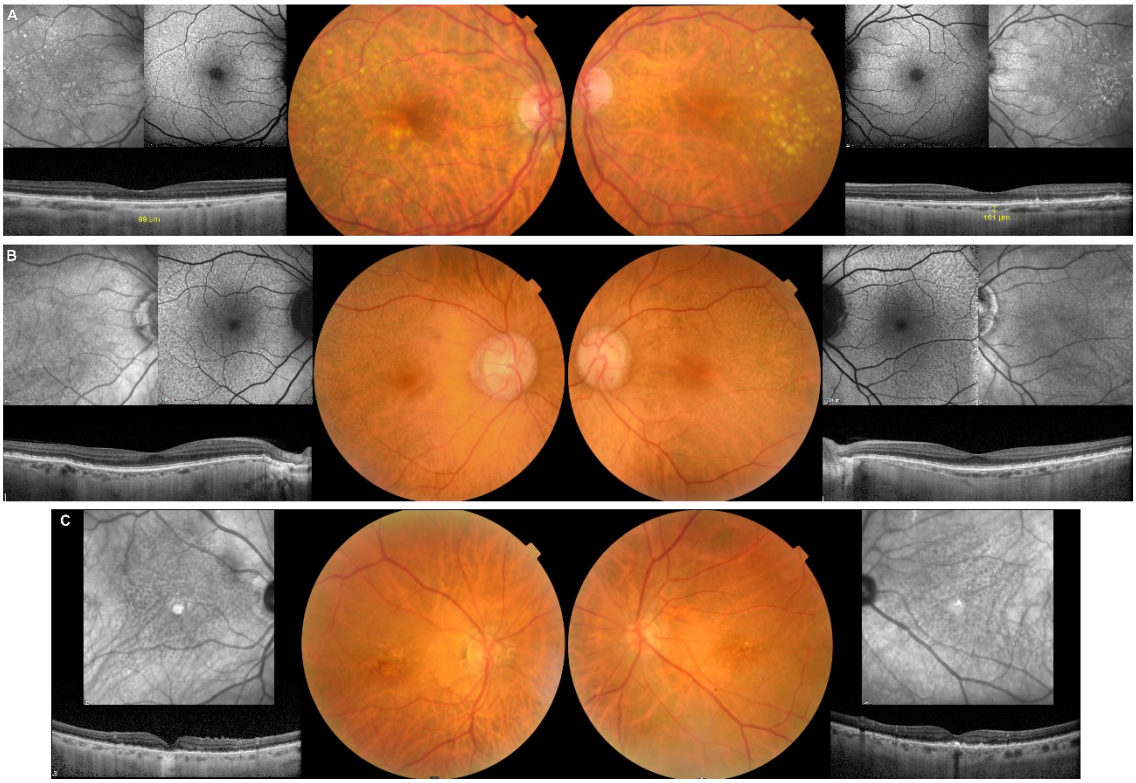

FIGURE 2. Exemplificative multimodal images of *CFH* rs768526062 (Pro258Leu) carriers.
(A) Female, 72yo with soft drusen mainly clustering in the temporal macula and SDD in parafoveal, nasal, and superior distribution in both eyes. The choroid is very thin (99 micra in right eye and 101 micra in left eye, subfoveal).
(B) Female, 68yo with extensive SDD in both eyes affecting the posterior pole, except for the fovea, and extending to the vascular arcades.
(C) Male, 81yo with SDD in both eyes affecting the posterior pole. There is foveal geographic atrophy in the right eye and soft drusen with hyperpigmentary changes in the fovea of the left eye.

DISCUSSION

In our study, we identified phenotypic differences between carriers and noncarriers of rare *CFH* variants in AMD patients through multimodal imaging. Carriers presented with more severe disease, including superior drusen burden in the macula, more PEDs of any cause, and thinner retinas, especially at the level of the inner retinal layers, independently of AMD stage. Our findings agree with previous studies reporting a significant association between having rare variants in the *CFH* gene and increased drusen load. However, the finding of carriers also having thinner retinas, namely thinner inner retinal layers as quantified by SD-OCT, is described here for the first time to the best of our knowledge.[13,15,16] Our results also suggest that the AMD phenotype characterized by thinner choroid and SDD seems to be more common in carriers of rare *CFH* variants, namely the association of SDD with the P258L variant, as well as having MNV in late stages, although these differences did not reach statistical significance in our analyzed population.

The first genetic studies in AMD mainly focused on common variants in the population through GWAS. A major GWAS has established 52 genetic risk variants to be strongly associated with AMD: 45 common plus 7 rare variants.[5] Genetic causality in a disease can be further explored by the identification of protein-altering variants in coding regions. These variants might be rare in the population, and several studies thus focused on their discovery by sequencing genes in AMD loci. In these studies, rare variants were found to be individually associated with AMD. These variants have mainly been identified in genes involved in the complement pathway: *CFH*, *CFI*, *C3*, and *C9*.[11,12,33] In the CES we previously described 12 variants to be associated with AMD. Eleven of these were common variants, whereas one noncoding variant in the *CFH* gene (rs35292876) was a rare variant. Furthermore, we also found that rare or low-frequency variants in the *CFH* gene with a predicted damaging effect were more common in AMD cases (Farinha C, et al., unpublished data, 2021).

Rare genetic variants located in the *CFH* gene are among the variants that confer the highest risk for AMD.[10] Because of their high penetrance and strong effect size, these variants may account for familial clustering of AMD and lead to more severe disease.[14] Despite this, few studies exploring genotype-phenotype associations considering only rare variants are available.[15,16] Thus our objective in this report was to explore the presence of rare variants in the *CFH* gene and their relationship with the phenotypic features of our AMD patients.

Ferrara et al.[13] focused on the rare *CFH* variant rs121913059 (Arg1210Cys), the strongest genetic risk variant of AMD in North-American populations and showed that the typical phenotype was characterized by extensive, voluminous, and confluent soft-drusen accumulation in the macula but also throughout the fundus. There was also a higher risk of developing late AMD, namely geographic atrophy. Wagner et al.[16] further reported that four highly penetrant rare *CFH* variants were strongly associated with advanced AMD, a higher frequency of drusen, earlier age of disease

onset, and phenotypic symmetry. Kersten et al.[15] evaluated the phenotypic effect of rare *CFH* variants cumulatively, and they reported in their work that patients with an extensive drusen area, drusen with crystalline appearance, and drusen nasal to the optic disc were more likely to have at least one rare variant in the *CFH* gene. In our study, we too identified phenotypic differences between carriers and noncarriers of rare *CFH* variants in a cumulative analysis. In the same way as these previous reports, AMD patients who were carriers presented with more severe disease in our study, including superior drusen burden in the macula, both in the perifovea and parafovea. This apparent difference was found by quantifying the real drusen area derived from the sum of each druse, as well as by measuring the percentage of the ETDRS grid and respective rings covered by drusen.

Regarding common variants, several studies addressed the clinical and phenotypic implications of individual variants. Dietzel et al.[34] showed that common variants in the *CFH*, *ABCA1*, and *ARMS2* genes were related to the presence and progression of drusen burden in early AMD. Another study by Seddon et al.[35] measured drusen burden through SD-OCT quantification and found that variants in *CFH* and *ARMS2/HTRA1* were independently associated with an increase in both drusen volume and area in eyes with early and intermediate AMD. Thee et al.[36] recently found in a large cohort from the E3/EYE-RISK consortium that the *ARMS2/HTRA1* locus was highly associated with intermediate AMD features, including a five times higher risk of a large macular area covered by drusen, and a six times higher risk of SDD, and with late AMD at a younger age. The same group reported, however, that phenotypic risk differences between *ARMS2/HTRA1* and complement genes were found only for MNV and not for other AMD lesions, because they overlapped significantly.[36] In our study, we found no significant difference in the MAF distribution of major common AMD risk variants between the carrier and non-carrier groups. However, there were some relevant findings because the MAF of these risk variants (*ARMS2* rs10490924, *ARMS2/HTRA1* rs3750846, *CFH* rs570618, *C3* rs2230199) were not only inferior in carriers compared to noncarriers, but also inferior or similar in noncarriers when compared to AMD patients from larger populations.[5,9] Kersten et al.[15] reported similar findings as the frequency of common genetic variants in *CFH* and *ARMS2* were inferior in carriers compared with noncarriers in their study. They suggested that carriers of rare *CFH* variants are less burdened by common AMD risk variants and that their AMD risk and associated phenotypic features are thus attributable to the rare variants. This relevant finding seems to be supported by our study. Regarding the inferior MAF of major risk variants in AMD patients from the CES compared to other cohorts, this was already described in our previous report and is probably related to populational differences (Farinha C, et al., unpublished data, 2021). Plus, our sample is relatively small and originally from a limited geographic area. Our AMD cases seem not only less burdened by common genetic major risk variants, but carriers of *CFH* rare variants are even less.

When further exploring other phenotypic features in multimodal imaging, we found that in SD-OCT analysis a lower macular volume was associated with carrier status, and this was also true when considering only the inner retinal layers but not the outer retinal layers where drusen are located. Quantitative SD-OCT-derived information on all segmented retinal layers is here presented for the first time in association with rare *CFH* variants and suggests that carriers present with thinning of the retina and mainly of the inner neuronal retina. This could be due to neurodegeneration in the context of more severe disease and caused or enhanced by genetic factors, independently of AMD stage. The association of PEDs and hyperreflective foci graded in OCT with the carrier status also points towards a more severe phenotype and perhaps increased risk of disease progression, related to more pathogenic *CFH* rare variants.

Our group previously reported that in early AMD patients from the CES the presence of SDD was associated with both thinner neuroretinal layers and thinner choroids.[31] Subretinal drusenoid deposits are a distinct feature of AMD and a known risk factor for advanced disease. However, their pathophysiological mechanism remains unclear.[37] Spaide[38] demonstrated in this respect that one difference between drusen and SDD was the significantly thinner underlying choroid of the latter, but despite the phenotypic differences, genetic data yielded conflicting results, and the total genetic risk score for SDD did not differ significantly from that seen for drusen in AMD. Other studies analyzed possible genetic associations, and a post hoc analysis of the Comparison of AMD Treatment Trials observed that common risk variants *ARMS2* rs10490924 and *HTRA1* rs11200638 were associated with an increased risk of SDD, while *CFH* rs1061170 was associated with a lower risk.[37] Bonyadi et al.[39] also found a stronger contribution of *ARMS2* rs10490924 in comparison with *CFH* genotypes in AMD with SDD versus without. Dutheil et al.,[40] however, found that in participants of the ALIENOR study the risk variants *ARMS2* rs10490924, *LIPC* rs10468017, and *CFH* rs1061170 were all associated with incident SDD. When analyzing our data, the distribution of common variants was not different between groups, including the *ARMS2* rs10490924. Regarding rare variants, to the best of our knowledge, there isn't yet any report addressing the associations between the presence and extension of SDD and the presence of rare variants in the *CFH* gene in AMD patients. Saksens et al.[14] observed a higher familial occurrence of AMD and an earlier age at onset in the carriers of the rare genetic variants *CFI* rs141853578 (Gly119Arg), *C3* rs147859257 (Lys155Gln), and *C9* rs34882957 (Pro167Ser), but no association to the presence of SDD. In our study, we found that carriers of at least one rare *CFH* variant had larger areas of retinal involvement by SDD, and carriers also had on average thinner choroids, besides thinner retinas as discussed above. We acknowledge that associations with SDD and choroidal thickness did not reach statistical significance, but this can be attributed to the relatively small sample size of our study. Interestingly, we also found that for the rare variant *CFH* rs768526062 (Pro258Leu), which was the most common in our carriers' group, almost half of the carriers had an SDD phenotype, alone or in combination with drusen, and most strikingly, virtually

all eyes from carriers who also had SDD shared this rare variant. We speculate that there could be a role for rare and more pathogenic *CFH* variants in the development of SDD and associated increased risk of disease progression, and we propose that this finding should be further explored in larger studies on genotype-phenotype correlation.

Some limitations should be addressed in our study. First, despite being originally an epidemiological population-based study, for genetic analysis, it has a small cohort, and the population is originally from a single location in Portugal. Second, we focused on rare variants in the *CFH* gene alone, but both common and rare variants in other genes of the complement pathway and other biological pathways also influence phenotype in AMD (Fig. 3). In addition, the *CFH* variants were assessed cumulatively, making phenotypic associations to all individual variants not possible. However, given that they are rare, single associations would not be feasible to establish in our cohort. Assessing their conjoined effect still revealed an association to more severe disease status, so they seem to share or overlap phenotypic characteristics. Pursuing a similar cumulative-based approach in other genes for rare variants would be of interest in future studies. Rare variants in other complement genes were, however, too few to analyze in our study. Another important limitation is that an analysis of the identified rare variants in family members from carriers would be most relevant to pursue to better characterize their pathogenic role and to better establish genotype-phenotype correlations. However, this was not possible due to the design of the epidemiological study on which this report is based. Still, we found that patients carrying these rare *CFH* variants were phenotypically different when compared to noncarriers, and because the phenotype was more severe, the overall effect of these variants is probably pathogenic. It would also be of interest to quantify the serum levels of FH in carriers, and functional studies are important to pursue to confirm our findings. We also recognize that nominal significance levels are provided in phenotypic analysis, as corrected significance was not possible to achieve, likely due to the small sample size. Finally, we only assessed drusen burden in the macula, but studies evaluating extramacular and peripheral retina would be important to further expand the phenotype-genotype correlation in AMD.[41] Nevertheless, our study is one of the few genetic studies addressing the effect of rare variants in the complement pathway in AMD phenotype through extensive multimodal imaging characterization, and the associations to the carrier status here documented for the first time strengthen our results. Furthermore, as part of the EYE-RISK project, our results are based on a comprehensive genotype assay recently validated in European populations.

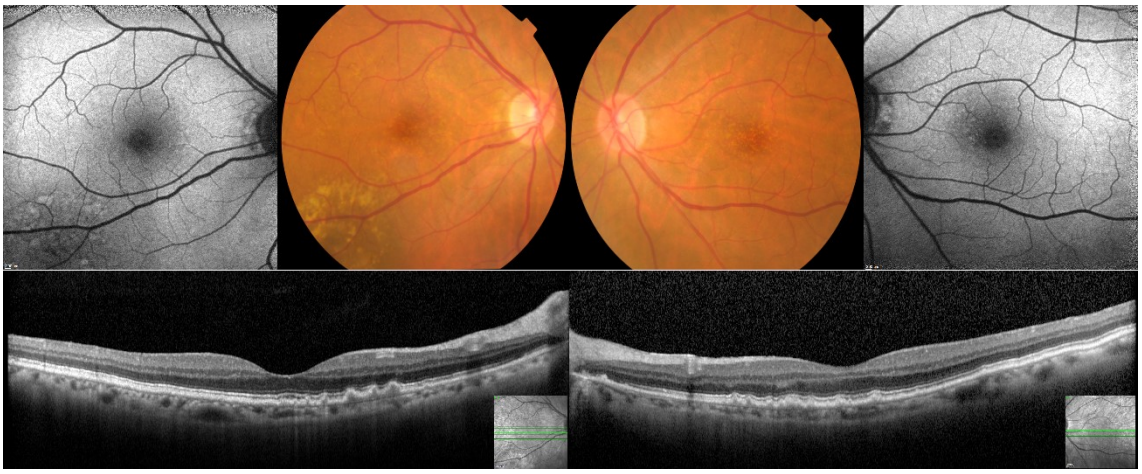

FIGURE 3. Female, 74yo (rare variant *CFH* rs35274867; Asn1050Tyr) with cuticular drusen in the fovea and nasal parafovea. Besides the rare variant, this patient harbors multiple common variants, including three other *CFH* variants (rs10922109, rs1410996, rs3753394) and three risk-conferring variants in *ARHGAP21* (rs12357257), *NPLOC4_TSPAN10* (rs6565597) and *SLC16A8* (rs8135665).

In summary, we identified phenotypic differences between carriers and noncarriers of rare *CFH* variants in AMD patients. Carriers presented with more severe disease, including superior drusen burden in the macula, PEDs, hyperreflective foci, and thinner retinas, mainly at the level of the inner retinal layers. Our results also suggest that exudative late AMD and the phenotype of SDD with thin choroid seem to be more prevalent in carriers, which was especially true for those carrying the variant P258L. These patients might be at increased risk of progression, and identification of such features can help in the selection of those who could benefit from genetic investigation. This can be especially relevant if complement-targeted therapies and genetic-based therapies are to be pursued in the future.

SUPPLEMENTARY MATERIAL

Supplementary Table S1. Allele frequencies (AFs) of major risk variants in AMD in the CES and comparison to the EYE-RISK [9] and the IAMDGC [5] datasets.

Gene	SNP	REF	ALT	Major/ Minor allele	MAF controls CES	MAF cases CES	OR CES (95% CI)	P-value CES	MAF controls EYE-RISK	MAF cases EYE-RISK	OR EYE-RISK	P-value EYE-RISK	MAF controls IAMDGC	MAF cases IAMDGC	OR IAMDGC primary analysis	P-value IAMDGC primary analysis
ARMS2	rs10490924	G	T	G/T	0.142	0.201	1.47 [1.12 - 1.93]	0.005	0.181	0.437	3.29	9.04-55	0.208	0.436	2.81	0
ARMS2/HTRA1	rs3750846	T	C	T/C	0.140	0.197	1.46 [1.11 - 1.92]	0.007	0.181	0.432	3.18	5.26-52	0.208	0.436	2.81	6.5^{-735}
C2/CFB/SKIV2L	rs429608	G	A	G/A	0.142	0.078	0.51 [0.34 - 0.74]	0.001	0.134	0.087	0.62	1.00-6	0.148	0.090	0.57	1.2^{-103}
C3	rs2230199	G	C	G/C	0.183	0.168	0.91 [0.68 - 1.20]	0.501	0.182	0.249	1.49	4.08-7	0.208	0.266	1.43	3.8^{-69}
CFH	rs10922109	C	A	C/A	0.443	0.361	0.72 [0.57 -0.89]	0.003	0.461	0.243	0.37	3.93-47	0.426	0.223	0.38	9.6^{-618}
CFH	rs570618	G	T	G/T	0.310	0.340	1.11 [0.89 - 1.39]	0.342	0.347	0.578	2.49	8.98-44	0.364	0.580	2.38	2.0^{-590}

Supplementary Table S2. Genotyped *CFH* rare variants in the CES population.

Gene	Position GRCh37 (hg19)	REF	ALT	Function	ID	Nucleotide Change	Protein change	SIFT	Polyphen2_HDIV	CADD	Variants (n)	MAC cases	MAC controls	maf cases	maf controls
CFH	196621254	C	G	nonsynonymous_SNV	rs139254423	C7G	L3V	T	P	14.24	0	0	0	0.000	0.000
CFH	196642173	T	G	nonsynonymous_SNV	1:196642173:T:G	T124G	Y42D	D	D	23.7	0	0	0	0.000	0.000
CFH	196642194	A	G	nonsynonymous_SNV	rs747546121	A145G	I49V	T	B	0.003	0	0	0	0.000	0.000
CFH	196642206	C	T	nonsynonymous_SNV	rs757785149	C157T	R53C	D	D	29.8	1	1	0	0.002	0.000
CFH	196642221	T	G	nonsynonymous_SNV	rs141336681	T172G	S58A	T	B	0.811	0	0	0	0.000	0.000
CFH	196642260	T	A	nonsynonymous_SNV	1:196642260:T:A	T211A	W71R	T	D	24.2	0	0	0	0.000	0.000
CFH	196643064	G	A	nonsynonymous_SNV	rs868394050	G322A	V108I	D	B	23.6	0	0	0	0.000	0.000
CFH	196645133	G	A	nonsynonymous_SNV	1:196645133:G:A	G365A	G122D	D	D	25.8	0	0	0	0.000	0.000
CFH	196645145	A	T	nonsynonymous_SNV	1:196645145:A:T	A377T	Y126F	T	B	1.414	0	0	0	0.000	0.000
CFH	196645148	G	A	nonsynonymous_SNV	rs121913058	G380A	R127H	D	D	33.0	0	0	0	0.000	0.000
CFH	196645156	G	A	nonsynonymous_SNV	rs147002633	G388A	D130N	T	P	23.3	0	0	0	0.000	0.000
CFH	196645188	A	G	nonsynonymous_SNV	1:196645188:A:G	A420G	I140M	T	P	23.3	0	0	0	0.000	0.000
CFH	196645195	G	A	nonsynonymous_SNV	1:196645195:G:A	G427A	V143I	T	D	26.6	0	0	0	0.000	0.000
CFH	196646659	G	T	nonsynonymous_SNV	rs777300338	G481T	A161S	T	P	2.863	1	1	0	0.002	0.000
CFH	196646674	C	T	nonsynonymous_SNV	1:196646674:C:T	C496T	R166W	D	D	25.2	0	0	0	0.000	0.000
CFH	196646682	C	A	stopgain	1:196646682:C:A	C504A	Y168X			24.1	0	0	0	0.000	0.000
CFH	196646684	A	G	nonsynonymous_SNV	rs768647508	A506G	H169R	T	B	0.001	0	0	0	0.000	0.000
CFH	196646701	C	T	nonsynonymous_SNV	1:196646701:C:T	C523T	R175W				0	0	0	0.000	0.000
CFH	196646702	G	A	nonsynonymous_SNV	1:196646702:G:A	G524A	R175Q	T	B	0.015	0	0	0	0.000	0.000
CFH	196646727	GA	G	frameshift_deletion	1:196646727:GA:G	550delA	I184fs				0	0	0	0.000	0.000
CFH	196646750	A	G	nonsynonymous_SNV	1:196646750:A:G	A572G	H191R	T	D	18.63	0	0	0	0.000	0.000

CFH	196646755	T	C	nonsynonymous_SNV	1:196646755:T:C	T577C	S193P	D	D	24.0	0	0	0	0.000	0.000
CFH	196646756	C	T	nonsynonymous_SNV	1:196646756:C:T	C578T	S193L	T	D	23.4	0	0	0	0.000	0.000
CFH	196646760	C	A	nonsynonymous_SNV	1:196646760:C:A	C582A	D194E	T	B	0.001	0	0	0	0.000	0.000
CFH	196648780	T	C	nonsynonymous_SNV	rs183474263	T647C	I216T	T	B	0.001	0	0	0	0.000	0.000
CFH	196648794	A	G	nonsynonymous_SNV	rs774239374	A661G	I221V	T	B	0.001	1	0	1	0.000	0.001
CFH	196648906	C	T	nonsynonymous_SNV	rs768526062	C773T	P258L			21.1	17	13	4	0.033	0.004
CFH	196654203	G	A	nonsynonymous_SNV	1:196654203:G:A	G800A	C267Y	D	D	23.8	0	0	0	0.000	0.000
CFH	196654290	A	G	nonsynonymous_SNV	1:196654290:A:G	A887G	N296S	T	P	0.001	0	0	0	0.000	0.000
CFH	196654303	TG	T	frameshift_deletion	1:196654303:TG:T	901delG	A301fs				0	0	0	0.000	0.000
CFH	196654311	G	A	nonsynonymous_SNV	rs766408580	G908A	R303Q	T	B	0.217	0	0	0	0.000	0.000
CFH	196654313	G	A	nonsynonymous_SNV	1:196654313:G:A	G910A	G304R	D	D	4.395	0	0	0	0.000	0.000
CFH	196654350	C	T	nonsynonymous_SNV	1:196654350:C:T	C947T	P316L	D	D	25.5	0	0	0	0.000	0.000
CFH	196658582	G	A	nonsynonymous_SNV	1:196658582:G:A	G997A	G333R	D	D	26.1	0	0	0	0.000	0.000
CFH	196658607	G	A	nonsynonymous_SNV	rs371192606	G1022A	R341H			7.702	1	1	0	0.002	0.000
CFH	196658658	A	G	nonsynonymous_SNV	rs576059537	A1073G	D358G	T	D	13.36	0	0	0	0.000	0.000
CFH	196658660	G	T	stopgain	1:196658660:G:T	G1075T	E359X			23	0	0	0	0.000	0.000
CFH	196658733	T	C	nonsynonymous_SNV	rs762389370	T1148C	V383A	T	P	0.006	1	0	1	0.000	0.001
CFH	196658738	T	C	nonsynonymous_SNV	1:196658738:T:C	T1153C	C385R	D	D	23.9	0	0	0	0.000	0.000
CFH	196659231	C	A	nonsynonymous_SNV	rs201671665	C1198A	Q400K	T	B	0.001	0	0	0	0.000	0.000
CFH	196659255	C	T	stopgain	rs121913061	C1222T	Q408X			32	0	0	0	0.000	0.000
CFH	196659309	C	T	stopgain	1:196659309:C:T	C1276T	Q426X			34	0	0	0	0.000	0.000
CFH	196682946	C	T	nonsynonymous_SNV	rs371053403	C1418T	A473V	T	B	8.370	0	0	0	0.000	0.000
CFH	196683035	C	G	nonsynonymous_SNV	rs570523689	C1507G	P503A	T	D	22.7	0	0	0	0.000	0.000
CFH	196684751	T	A	nonsynonymous_SNV	rs147403664	T1548A	N516K	T	D	16.62	1	1	0	0.002	0.000
CFH	196684798	T	C	nonsynonymous_SNV	rs201193547	T1595C	L532S	D	D	24.5	0	0	0	0.000	0.000
CFH	196684807	A	G	nonsynonymous_SNV	1:196684807:A:G	A1604G	E535G	D	D	24.5	0	0	0	0.000	0.000
CFH	196684814	T	A	nonsynonymous_SNV	1:196684814:T:A	T1611A	H537Q	T	B	0.009	0	0	0	0.000	0.000
CFH	196684825	A	G	nonsynonymous_SNV	1:196684825:A:G	A1622G	E541G	T	D	21.3	1	1	0	0.002	0.000

CFH	196694253	A	G	nonsynonymous_SNV	rs757756991	A1699G	R567G	T	D	23	0	0	0	0.000	0.000
CFH	196694262	G	A	nonsynonymous_SNV	rs76405615	G1708A	E570K		B	0.235	0	0	0	0.000	0.000
CFH	196694332	T	A	stopgain	1:196694332:T:A	T1778A	L593X			36	0	0	0	0.000	0.000
CFH	196694418	A	G	nonsynonymous_SNV	1:196694418:A:G	A1864G	I622V	T	B	0.194	2	1	1	0.002	0.001
CFH	196695609	A	G	nonsynonymous_SNV	1:196695609:A:G	A1883G	Q628R	T	B	0.001	0	0	0	0.000	0.000
CFH	196695648	T	C	nonsynonymous_SNV	rs371768180	T1922C	V641A	T	B	0.258	0	0	0	0.000	0.000
CFH	196695675	G	T	nonsynonymous_SNV	rs143237092	G1949T	G650V	T	B	0.034	0	0	0	0.000	0.000
CFH	196695721	G	A	nonsynonymous_SNV	1:196695721:G:A	G1995A	M665I	D	B	23.3	0	0	0	0.000	0.000
CFH	196695756	A	G	nonsynonymous_SNV	1:196695756:A:G	A2030G	E677G	T	B	0.849	0	0	0	0.000	0.000
CFH	196695927	A	C	nonsynonymous_SNV	1:196695927:A:C	A2093C	E698A	T	B	7.448	0	0	0	0.000	0.000
CFH	196695975	C	G	stopgain	1:196695975:C:G	C2141G	S714X			35	0	0	0	0.000	0.000
CFH	196695985	C	A	nonsynonymous_SNV	rs763441589	C2151A	F717L	D	B	12.93	1	1	0	0.002	0.000
CFH	196696029	C	T	nonsynonymous_SNV	rs201360629	C2195T	T732M	T	B	11.39	0	0	0	0.000	0.000
CFH	196697494	A	G	nonsynonymous_SNV	1:196697494:A:G	A2255G	K752R	T	B	8.420	0	0	0	0.000	0.000
CFH	196697498	C	A	stopgain	1:196697498:C:A	C2259A	C753X			36	0	0	0	0.000	0.000
CFH	196697519	A	G	nonsynonymous_SNV	1:196697519:A:G	A2280G	I760M	T	B	0.131	0	0	0	0.000	0.000
CFH	196697552	C	A	nonsynonymous_SNV	1:196697552:C:A	C2313A	F771L	D	B	13.01	0	0	0	0.000	0.000
CFH	196697568	A	G	nonsynonymous_SNV	rs761904009	A2329G	I777V	T	B	0.001	0	0	0	0.000	0.000
CFH	196705989	A	T	nonsynonymous_SNV	1:196705989:A:T	A2449T	I817F	D	P	25.7	0	0	0	0.000	0.000
CFH	196706001	C	T	nonsynonymous_SNV	rs367687415	C2461T	H821Y	T	B	0.001	0	0	0	0.000	0.000
CFH	196706028	C	T	nonsynonymous_SNV	rs62641696	C2488T	R830W		P	22.2	0	0	0	0.000	0.000
CFH	196706112	T	A	nonsynonymous_SNV	1:196706112:T:A	T2572A	W858R	D	D	25.5	0	0	0	0.000	0.000
CFH	196706633	G	C	nonsynonymous_SNV	1:196706633:G:C	G2625C	Q875H	T	B	0.001	0	0	0	0.000	0.000
CFH	196706659	C	A	nonsynonymous_SNV	rs114743644	C2651A	S884Y	T	P	10.94	1	0	1	0.000	0.001
CFH	196706677	G	T	nonsynonymous_SNV	rs515299	G2669T	S890I	T	B	0.463	25	9	16	0.021	0.014
CFH	196709816	G	T	nonsynonymous_SNV	rs149474608	G2850T	Q950H	D	P	21.4	0	0	0	0.000	0.000
CFH	196709833	C	T	nonsynonymous_SNV	rs145975787	C2867T	T956M	T	D	15.09	0	0	0	0.000	0.000
CFH	196709874	A	G	nonsynonymous_SNV	rs759625279	A2908G	I970V	T	B	0.01	0	0	0	0.000	0.000

CFH	196709875	T	C	nonsynonymous_SNV	1:196709875:T:C	T2909C	I970T	T	B	0.004	0	0	0	0.000	0.000
CFH	196711052	G	C	nonsynonymous_SNV	rs201816520	G3004C	G1002R	T	D	7.236	1	1	0	0.002	0.000
CFH	196711098	C	T	nonsynonymous_SNV	rs34362004	C3050T	T1017I	D	P	12.48	0	0	0	0.000	0.000
CFH	196711125	G	A	nonsynonymous_SNV	1:196711125:G:A	G3077A	G1026E	D	D	24.7	0	0	0	0.000	0.000
CFH	196712596	A	T	nonsynonymous_SNV	rs35274867	A3148T	N1050Y	T	B	3.424	32	3	29	0.007	0.025
CFH	196712624	T	C	nonsynonymous_SNV	rs35343172	T3176C	I1059T	D	B	12.25	0	0	0	0.000	0.000
CFH	196712674	C	G	nonsynonymous_SNV	rs62625015	C3226G	Q1076E	T	B	0.003	0	0	0	0.000	0.000
CFH	196712682	G	T	nonsynonymous_SNV	rs121913062	G3234T	R1078S	T	B	0.637	0	0	0	0.000	0.000
CFH	196716253	T	C	nonsynonymous_SNV	1:196716253:T:C	T3506C	I1169T	D	B	23.8	0	0	0	0.000	0.000
CFH	196716261	G	T	stopgain	rs121913060	G3514T	E1172X			39	0	0	0	0.000	0.000
CFH	196716375	C	T	nonsynonymous_SNV	rs121913059	C3628T	R1210C	T	B	11.77	0	0	0	0.000	0.000
CFH	196716399	T	G	nonsynonymous_SNV	1:196716399:T:G	T3652G	C1218G	D	D	23.5	0	0	0	0.000	0.000
CFH	196716415	T	A	nonsynonymous_SNV	1:196716415:T:A	T3668A	L1223Q	D	D	24.1	1	0	1	0.000	0.001

D Probably damaging/ deleterious; P Possible damaging; B Benign; T Tolerated.

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CHAPTER 7.
DISCUSSION AND FUTURE DIRECTIONS

Age-related macular degeneration is the leading cause of central vision loss in the elderly populations of industrialized countries and accounts for 8.7% of legal blindness globally.(1) This burden is expected to increase as population aging becomes a global phenomenon, although much more pronounced in industrialized countries.(1, 2) Epidemiologic studies on the prevalence and incidence of AMD are therefore cornerstones for planning for demand in health care systems and for establishing prevention measures.

A comprehensive meta-analysis of population-based studies conducted by Wong *et al* (1) estimated the global prevalence of early and late AMD to be, respectively, 8.01% (95% CrI 3.98–15.49%) and 0.37% (95% CrI 0.18–0.77%). In addition, their projections for the future were alarming: 288 million affected individuals by 2040, and a doubling of late AMD cases, corresponding to an increase of 9 million more individuals.(1)

AMD is broadly classified into early, intermediate, and late forms. Early and intermediate forms are characterized by the presence of drusen and pigmentary changes in the macula. At this stage patients usually are asymptomatic or only refer to mild visual symptoms. Late AMD is however responsible for severe visual loss. Atrophic or dry late AMD is currently a non-treatable condition and in neovascular AMD the significant initial gains obtained with antiangiogenic treatments may be lost in the long term because of atrophy and/or fibrosis.(3, 4) In fact, nvAMD accounts for approximately 10–20% of total cases of AMD but is responsible for 80% of cases of severe vision loss.(5) Since the introduction of intravitreal therapy with anti-VEGF agents visual impairment due to nvAMD was significantly reduced, by around 50%.(6) Nonetheless, the treatment burden is quite significant, and sub-optimal outcomes are not uncommon in clinical practice.(4, 7) The need for long-term and even life-long treatments and follow-up appointments to maintain visual gains causes a significant burden upon patients and their relatives, as well as in health institutions.

Medicine is now moving from a reactive approach, which is to treat diseases and complications as they occur, to a more preventive, personalized-based approach, in which preventive strategies and treatments are to be implemented before significant damage occurs. Thus, much effort is currently being made to develop treatments capable of halting AMD progression before vision loss occurs and in developing monitoring strategies that can accurately predict an individual's risk and/or rate of such progression. To achieve these goals a profound understanding of the pathophysiology of AMD is essential.

AMD is a multifactorial disease, influenced by demographic, environmental, clinical, and genetic risk factors. The most consistent modifiable risk factors are smoking and diet, while variants in *CFH* and *ARMS2/HTRA1* genes confer the highest genetic risk of AMD.(8-12) The landmark GWAS on advanced AMD by the International AMD Genomics Consortium definitively identified 52 independent genome-wide significant signals at 34 genomic loci.(10) But more variants, including rare variants, are continuously being associated with AMD risk and severity. The interplay of all these interacting factors is however complex and to date, not fully understood.

The phenotypic characterization of AMD is also continuously evolving, and today Multimodal Imaging is the standard approach in AMD diagnosis and management.(13-15) Different imaging technologies provide complementary information, thereby improving the understanding of its pathophysiology. Imaging biomarkers obtained through a multimodal approach can further help in predicting progression and in guiding treatment decisions. As technology improves, and especially with the recent introduction of artificial intelligence algorithms in retinal imaging data, new biomarkers are being uncovered and their role in AMD management explored.(16-18) The identification of biomarkers predictive of disease progression is another step to improving functional and anatomical outcomes in the clinical setting and tailoring preventive measures and treatments in the context of personalized medicine. Adapting staging systems of AMD to this new reality, to accompanying imaging technology developments, is a necessary step to link phenotype to risk factors and their interplay.

Large population-based studies provide extensive information, not only on epidemiology, but also on the associated demographic, environmental, and lifestyle risk factors linked to the disease onset and progression, and in addition, they are a remarkable opportunity to extensively characterize different populations from both genetic and phenotypic perspectives.

Until recently there were no studies on AMD prevalence and incidence in Portugal. The Coimbra Eye Study aimed to address this gap and comprises two main studies, so far: the Epidemiological Study (NCT01298674) and the AMD Incidence Study (NCT027048824). Together, they provide unique information on AMD in Portuguese populations.

In the Epidemiologic Study, an important finding was that the subjects from the inland town Lousã had a significantly higher risk of early and late AMD compared to the subjects from the coastal town Mira. In addition, when analyzing the Mira cohort alone, the authors found that late AMD prevalence was inferior in this subpopulation to that reported in large epidemiologic studies.(19, 20) The cause for this discrepancy led to further investigation regarding risk factors that could have influenced this outcome. In fact, the impact of demographic, environmental, and lifestyle-related risk factors (such as diet and physical activity), was reported by our group in recent years, and for example, the Mira cohort was found to have higher adherence to the Mediterranean diet, which was globally found to be protective for AMD in the CES.(19-24)

In this Thesis, the results of the subsequent AMD Incidence Study are now presented from an epidemiological perspective and the impact of transitioning to multimodal imaging in epidemiologic data is explored. Imaging biomarkers obtained with this MMI approach and their association with disease severity were identified in our analyzed population. The next step was to extensively characterize the genetics of AMD in this population including common and rare variants analysis, the genetic risk score applied to a Portuguese population, and genetic differential regarding mimicking macular dystrophies. Finally, new genotypic-phenotypic associations were

uncovered, namely regarding the effect of rare *CFH* variants on the phenotype of our AMD patients.

The main findings of this Thesis are the following:

1. **Early AMD incidence in a coastal town of central Portugal was found to be similar to that of major epidemiological studies of European-descent populations; however, the incidence of late AMD was lower than expected.**

In our first paper (Chapter 2) we reported the results from the first population-based cohort study to investigate the long-term incidence of AMD in a Portuguese population. We could confirm that there are indeed differences in the epidemiology of the Mira population. Not only the Mira cohort had a lower prevalence of late AMD compared to large epidemiologic international studies, but the incidence of late AMD was again found to be lower than expected in this new study.(19-21)
In the Incidence study subjects who participated in the previous prevalence study and who were recruited from the primary health care unit in Mira were invited to participate again. All participants underwent ophthalmological examination including multimodal imaging (CFP, SD-OCT, FAF, NIR). A general analysis of imaging exams was carried out to identify suspected AMD cases, which were then extensively graded according to the International Age-Related Macular Epidemiological Study Group Classification and staged using the Rotterdam staging system.(25, 26) In the Incidence study primary report only CFP images were used for grading and staging in order to maintain comparability to the previous prevalence study.(19, 20) Early AMD was again defined as stages 2 and 3 of the Rotterdam staging system and late AMD was stage 4.
The final analyzed cohort comprised 1,616 participants of whom 246 had AMD. Subjects with late AMD were significantly older and had a smoking history more frequently compared to subjects with early AMD and subjects without AMD.
The 6.5-year crude cumulative incidence of early AMD was 10.7%, and late AMD was 0.8%. The age-standardized incidence calculated for the Portuguese population was 10.8% for early AMD and 1.0% for late AMD. The incidence of both neovascular AMD and geographic atrophy was 0.4%. Interestingly, the incidence of late AMD in participants without early disease at baseline was 0.5% and in participants with early disease at baseline was 0.4%. Meaning that nearly half of the incident cases of late AMD were relatively fast progressors, transitioning from no-AMD (stages 0 and 1) to late AMD during the 6.5-year follow-up. Of note, only CFP was used in the baseline Epidemiologic Study, thus there is the possibility that some cases with SDD were missed at baseline, and this might explain, in part, why some of these cases were fast in progressing to late AMD. Without surprise, age was once again significantly associated with the incidence of early and late AMD. As for progression, 17.2% progressed by one or more stages during follow-up.

Several population-based studies have provided important information on the incidence of AMD.(26-31) However, in Europe most reports are from northern countries.(26, 32, 33) Southern European countries, however, are known to have distinct sociocultural characteristics that may impact the risk for AMD causing true epidemiologic differences.

Practical limitations exist when comparing our results as there is great heterogeneity between epidemiologic study designs (e.g., minimum age for inclusion, non-uniform AMD staging systems, the definition of early AMD). On top of that staging in epidemiological data is based on CFP only, in an era where multimodal-based definitions in AMD are now the rule. Despite this, the definition of late AMD is relatively unanimous and comparable.

Our findings were similar to most reports on AMD incidence available in the literature, namely from European countries or countries with marked European descent such as the U.S.A. and Australia.(26, 30, 34) The similarities, however, were more visible for the incidence of early AMD while the cumulative incidence of late AMD was inferior when comparing to the 5-year analysis of the Rotterdam Study, Blue Mountains Eye Study and Beaver Dam Eye Study, despite the superior follow-up time of the Mira cohort.(26, 30, 34) This was not unexpected since our group previously reported that the late AMD prevalence for the coastal town was inferior to that reported in these large studies.(19)

When comparing the progression rate to the recent Gutenberg Health Study (GHS) with 5 years of follow-up, ours was also inferior (17.2% vs 18.1 %, respectively).(32) Klaver *et al* (35) also reported a superior progression rate of 21.5% after only 2 years of follow-up in the Rotterdam Study. In these two studies, the definition of progressors was the same as ours (progression by one or more levels of severity by the Rotterdam staging system).

Specific risk factors might be in the genesis of these differences. The Mira population is a coastal town and predominantly has a Mediterranean diet, which appears to have a protective effect on AMD diagnosis. The Mediterranean diet is a factor under intensive investigation in recent years regarding AMD risk, and we already showed in the CES that a higher adherence to this type of diet is protective for AMD, and most importantly, we found that in the Mira cohort the adherence was superior compared to the Lousã cohort, which had a higher prevalence of both early and late AMD.(20, 23, 24) Other factors, namely genetic could also contribute to this apparent decreased risk of late AMD in Mira and this hypothesis propelled further investigation by our group.

In conclusion, we present the 6.5-year incidence of AMD in the coastal town of Mira, located in central Portugal, and this is the first report of AMD incidence in a Portuguese population. Our cumulative incidence of early AMD was similar to that reported in major epidemiological studies of European-descent populations, however, the incidence of late AMD was lower, and further analysis of risk factors was pursued based on these findings.

2. AMD grading and staging is more accurate with a multimodal imaging approach, and this is especially relevant for late AMD, significantly impacting our epidemiological data.

In Chapter 3 we present our second paper in which we explored the impact in epidemiologic data of introducing a multimodal imaging approach in AMD grading and staging. We compared grading with CFP only to grading with MMI in the Mira cohort from the Incidence Study and evaluated the consequent changes in AMD stage, prevalence and incidence rates in this population.

All imaging exams from the participants were classified by the central reading center: first, only the CFP images were graded according to the International Classification and Grading System for AMD and staged with Rotterdam classification; afterward, CFP images were reviewed together with SD-OCT, NIR, and FAF and classification was updated if necessary. Definitions of late AMD were also updated for this purpose to incorporate newer terminologies.(14, 25, 26, 36)

When analyzing stage differences between CFP and MMI grading in AMD patients, an increase in the rates of AMD cases staged as 2a and 4 were readily apparent when MMI was used, with a corresponding decrease in stages 0, 1, and 3. The increase of stage 2a with MMI was mainly due to superior detection of subretinal drusenoid deposits. This fact is interesting for two reasons. First, detection of SDD is important in AMD pathophysiology since they are a known risk factor for progression to late-stage disease. Second, this difference existed because we used the Rotterdam staging system. In the more recent Clinical Classification from the Beckman Initiative SDD are not even considered but, as the authors acknowledge, classification based on CFP alone ignores changes relevant to the disorder, such as the development of SDD. Therefore, this feature should be reconsidered, and instead the development of AMD staging based on MMI should be attained.(37-39) Concerning late AMD, we found that stage 4 was misclassified as a lower stage when using only CFP. These were early cases of nvAMD or GA not seen in fundus images, which suggests a systemic underreporting of late AMD, probably also affecting larger landmark epidemiologic studies.

Early and late AMD prevalence was then compared considering all included subjects. In CFP-based grading, the prevalence was 14.11% for early AMD and 1.05% for late AMD (0.56% with nvAMD, 0.50% with GA). Using multimodal grading, the prevalence increased to 14.60% for early AMD and 1.61% for late AMD (0.87% with nvAMD and 0.74% with GA). In summary, CFP alone mostly underestimated the presence of disease, that is, of both early and late AMD. We objectively quantified these differences which are especially relevant if new studies using multimodal-based staging systems are to be compared to older studies based only on CFP.

When re-evaluating the 6.5-year cumulative incidence with MMI we found that it increased to 11.03% for early AMD and 1.31% for late AMD. However, MMI was not used at baseline in our study. So epidemiologic conclusions are limited but we demonstrated this possible overestimation

bias, which should be considered if follow-up visits in longitudinal studies are switched to multimodal grading when CFP only was used at baseline.

Our results confirmed our hypothesis that AMD staging is more accurate with a multimodal approach, and this was especially true for late AMD. Multimodal imaging is currently the standard in AMD diagnosis and management, and in identifying predictive biomarkers of treatment response and prognosis. Thus, is logical that if epidemiologic data is to be accurate not only in reporting real data of AMD prevalence and incidence but in its contribution to risk factors and biomarkers analysis in AMD, it must be based on MMI.

In summary, we present the first report in a populational study truly comparing AMD staging using CFP versus multimodal imaging grading. AMD staging proved to be more accurate using the multimodal approach and this was especially true for the correct identification of late AMD, where the difference between the two methods was critical. Based on our findings here reported we propose that MMI should be adopted in the future to better estimate and compare epidemiological data in different populations, and we decided to use MMI as the basis for AMD classification in the next chapters of this Thesis.

3. **A higher stage in early AMD is associated with thinning of several inner and outer neuroretinal layers, supporting the existence of progressive neurodegeneration. Neuronal retinal layer thicknesses might be used as quantitative biomarkers of disease progression in AMD. The presence of SDD is possibly associated with more prominent and faster neurodegeneration.**

Our third paper presented in Chapter 4 explores the potential of Multimodal Imaging in unraveling new biomarkers of disease severity in our cohort from Mira. Despite the extensive focus on the features located in the external retinal layers and choroid as biomarkers of AMD progression in the literature, degenerative changes in the inner retina seem to be present in the early stages of AMD.

In this study, we performed a detailed quantitative analysis of all retinal layers in the macula by using semiautomatic segmentation in SD-OCT scans and of the subfoveal choroid, in the set of eyes staged with early AMD (stages 2a, 2b, and 3) with MMI in the Incidence Study. Our purpose was to detect differences by stage suggestive of progressive neurodegeneration and to explore associations with recognized biomarkers of progression such as SDD and HRF into specific phenotypes.

The final cohort comprised 346 eyes from 233 patients, 82.66% in stage 2a, 5.49% in stage 2b, and 11.85% in stage 3. We could observe that globally, the retinal layers were reduced in thickness in stage 3, compared to stage 2a. The retinal nerve fiber layer (RNFL), ganglion cell layer (GCL), and inner plexiform layer (IPL) were thinner in the inner and the outer ETDRS grid circles, and the outer nuclear layer (ONL) and photoreceptors' segments layer (PRL) were thinner in the central

ETDRS circle. This pattern might represent a contiguous process of neurodegeneration, more prominent at the level of the photoreceptors in the fovea and extending to the inner layers of the parafovea and perifovea. This is suggestive of neuronal anterograde degeneration on almost all neuroretinal layers at the macula in early AMD patients, progressive with stage, and is an important contribution of our study to the comprehension of AMD pathophysiology. On the contrary, the retinal pigment epithelium–Bruch's membrane (RPE/BrM) layer, which is an indirect measure of the drusen load, was thicker in stage 3 in the central and inner circles as expected. The choroidal thickness was lower in stage 3 compared to stages 2a/2b, but the difference did not reach statistical significance.

Eyes having SDD were found to have significantly thinner neuroretinal layers (IPL, Inner Nuclear Layer, Outer Plexiform Layer, ONL, and PRL) and thinner RPE/BrM layer, and choroid, compared to eyes without SDD. In addition, SDD were associated in multivariate analysis with thinner OPL, ONL, PRL, RPE/BrM layer, and choroid. On the contrary, eyes with HRF were associated with thicker ONL and RPE/BrM layer, consistent with their known association to drusen volume increase, RPE disruption, and migration to the ONL.

In summary, with this work, we demonstrated that several inner and outer neuroretinal layers are thinner with a higher stage in early AMD. These findings support the notion that there is early and progressive neurodegeneration in AMD affecting almost all retinal layers. We suggest that neuronal retinal layer thicknesses obtained with OCT may be used as quantitative biomarkers of disease progression and that SDD appear to be biomarkers of a phenotype characterized by more prominent and faster neurodegeneration and probably associated with different genetic susceptibilities.

4. **Both common and rare variants were associated with AMD, but a *CFH* rare variant conferred the highest risk of disease while three major risk variants had a lower-than-expected allele frequency in our population originary from a geographic region with lower prevalence/incidence of AMD. GRS was still significantly higher in AMD patients. Damaging *CFH* rare variants were cumulatively more common in AMD cases.**

In our fourth article (Chapter 5) we present the results of the first genetic analysis in a Portuguese population on genetic risk for AMD. We determined the contribution of common and rare genetic variants in the development of disease, explored the burden of pathogenic rare variants, and calculated differences between the genetic risk score of AMD patients compared to non-AMD participants. Rare variants were explored since they are known to have a strong impact due to high penetrance and may predispose to more severe disease.(40-42)

A set of the Incidence study had genetic sequencing carried out in association with the EYE-RISK/ E3 consortium with the EYE-RISK genotype assay.(43) Sixty-nine SNPs were genotyped and

tested for association with AMD in case-control and progression-to-AMD analyses. Besides common variants associated with AMD, this assay includes genes described to carry rare variants and genes from AMD-mimicking macular dystrophies.

In our case-control analysis (877 genotyped samples from 237 cases and 640 controls) variants associated with risk of disease were: *ARMS2* rs10490924, *ARMS2_HTRA1* rs3750846, *CFH* rs35292876, *SLC16A8* rs8135665, *TGFBR1* rs1626340. The major risk variants *ARMS2/ HTRA1* rs3750846, *CFH* rs570618, and *C3* rs2230199 had unexpected lower allele frequency in our cohort compared to the large databases from the IAMDGC and EYE-RISK, while the highest risk-conferring variant in our population was the rare variant, *CFH* rs35292876 (OR, 2.67).(10, 43) This finding came across as important since as we described previously, our population is from a geographic region with a lower prevalence of AMD and with a lower incidence of late AMD (Chapter 2). More surprisingly, it was a *CFH* rare variant that conferred the highest risk of disease, leading us to assume that the Mira population appears to have specificities in its genetic background explaining in part the epidemiologic findings. Furthermore, we also previously reported that higher adherence to the Mediterranean diet was significantly protective for AMD and that the coastal population Mira had significantly higher adherence to it.(24) The interplay between these lifestyle and genetic background differences could be the cause of our epidemiologic findings for this population and are in accordance with the findings on genetic and lifestyle interaction reported by large study groups.(11, 44)

In progression-to-AMD analysis, this is eyes that progressed from no-AMD to AMD (137 progressors/ 630 non-progressors), variants associated with risk of progression were again the rare variant *CFH* rs35292876 (OR, 3.06) and the *ARMS2* rs10490924 and *ARMS2_HTRA1* rs3750846. As for the GRS, we found that it was significantly higher for AMD cases and progressors, but the overlap with the GRS from controls and non-progressors was significant which led us to the important conclusion that this method does not adequately individualize risk for AMD if used alone. In addition, the GRS is calculated based on the effect size of variants determined by the IAMDGC GWAS, and as we found the effect size of some of these variants is lower in our cohort raising concerns on real applicability to populations with different genetic backgrounds as ours. Therefore, the GRS should be used with a critical sense if it is to be implemented in settings such as clinical trials and clinical practice to assess the individual risk of a patient. Still, since GRS was significantly higher in our AMD patients, we suggest that genetic risk scores can be included for example in multivariate analyses integrating genetic-and non-genetic risk factors.

Regarding rare variant analysis, 973 SNPs and 804 samples from 591 controls and 213 AMD cases were analyzed. We investigated the presence of rare variants and their association with AMD for the *CFH*, *CFI,* and *ARMS2* genes. A higher proportion of pathogenic rare *CFH* variants was observed in cases (OR, 9.66). These results from our study thus support the notion that pathogenic rare variants in the complement pathway significantly increase the risk of AMD and should be

further explored.(40-42) They probably also contribute to a worse phenotype, which led us to the next research question, presented in Chapter 6. Addressing the cumulative risk of damaging rare variants causing higher levels of complement activation might be useful in the near future in the identification of those who could benefit more from complement-inhibiting therapies in the pipeline.

No pathogenic variants from AMD-mimicking macular dystrophies (*ABCA4, CTNNA1,* and *PRPH2)* were found in our cohort, strengthening the genetic characterization of AMD risk.

In conclusion with this study, we added new information regarding the common and rare variants associated with AMD in a European population, which can be used for comparison with other populational cohorts and further expanding the knowledge of AMD pathophysiology.

5. **Phenotypic differences were found between carriers and noncarriers of rare variants in the *CFH* gene in AMD patients. Carriers had more severe disease, namely superior drusen burden, PEDs, and thinner retinas. The rare variant P258L may be associated with SDD. Carriers are probably at increased risk of progression.**

In our last paper presented in this thesis (Chapter 6) we explored the presence of rare variants in the *CFH* gene and their association with the phenotypic features obtained with MMI from our AMD patients of the Mira cohort.

We decided to pursue this analysis since a better understanding of phenotype-genotype correlations with respect to rare variants based on multimodal imaging contributes to improving the identification of patients at greater risk of progression to late-stage disease, and since there is limited knowledge on this topic in the literature.(40, 41, 45) Our work is also relevant by helping recognize which patients could benefit more from genetic screening, especially when genetic and complement-inhibiting targeted therapies are becoming a reality.(46-49)

The *CFH* gene encodes the factor H, which is an inhibitor of the alternative complement pathway. Compromise of this pathway leads to a pro-inflammatory state and rare variants located in the *CFH* gene are among those which confer the highest risk for AMD.(40, 41, 45, 50) Rare variant analysis in the *CFH* gene is therefore of relevance.

As presented in the previous chapters, participants from the Incidence study underwent MMI examination including CFP, SD-OCT, FAF, and NIR. Phenotypic characterization of AMD patients in all imaging data available was carried out in a centralized reading center. In SD-OCT analysis this included the quantification in the macula of the total and layer-by-layer retinal thicknesses and volumes (Chapter 4). The coding and splice-site regions of the *CFH* gene were sequenced in association with the E3/ EYE-RISK consortium. Variants with a minor allele frequency <0.05 resulting in splice-site or protein change (more likely to be pathogenic) were selected and rare *CFH* variants with a described protective/benign effect were excluded. The

phenotypic features of AMD patients with and without these probably pathogenic variants were analyzed.

In this study, we included 39 eyes of 23 patients carrying rare *CFH* variants and 284 eyes of 188 noncarriers. Association analysis revealed that the carrier status was associated with having a higher drusen burden in the macula in the inner ETDRS circle (OR, 5.44), outer circle (OR, 4.37), and full ETDRS grid (OR, 4.82). In SD-OCT, a lower total macular volume and lower inner retinal layers' volume (OR, 0.449; OR, 0.496, respectively), and pigment epithelial detachments (OR, 5.24) were associated with carrying a rare *CFH* variant. A trend in the same direction was found regarding the presence of HRF (OR, 2.61). Plus, carriers had on average thinner choroids and larger retinal areas affected by SDD, although this was not significant. Remarkably, carriers with SDD had the same rare variant (P258L) in all cases except one. Based on this we propose that there could be a role for pathogenic rare *CFH* variants in the development of SDD and associated increased risk of disease progression, and we believe that this finding should be explored in future studies.

We also explored the distribution of common major risk variants for AMD between carriers and non-carriers of rare *CFH* variants, as they could influence phenotype. There were some relevant findings because the MAF of these major risk variants were tendentially inferior in carriers compared to noncarriers, but also inferior or similar in noncarriers compared to AMD patients from larger populations.(10, 43) This means that our AMD cases are not only less burdened by common genetic major risk variants (a finding also reported in Chapter 5), but carriers of *CFH* rare variants are even less. Other authors found similar results, and we agree that in carriers of rare variants the AMD risk and associated phenotypic features are mainly attributable to these and not to common major risk variants.(41)

In summary, with our study, one of the few available in the literature, we confirmed previous findings documenting phenotypic differences between carriers and noncarriers of rare *CFH* variants in AMD patients, and we documented new ones by using MMI.(40, 41, 45) Like these studies, carriers presented with more severe disease in our cohort, and they are probably at increased risk of progression. Functional and family-based association studies would be of utmost importance to confirm the pathogenic role of these variants and should be pursued in the future to confirm our findings.

Conclusions

The 6.5-year cumulative incidence of early AMD in a coastal town of central Portugal was found to be similar to that reported in major epidemiological studies of European-descent populations; however, the incidence of late AMD was lower than expected.

In this populational study AMD staging was more accurate using a multimodal approach and this was especially striking in the correct identification of late AMD, where the difference between the two methods was critical. Late AMD is underreported if CFP alone is used.

Multimodal imaging should be adopted in future AMD classification systems and epidemiologic studies, to better estimate and compare AMD prevalence and incidence in different populations.

Regarding imaging biomarkers obtained with MMI, several inner and outer neuroretinal layers were found to be thinner with a higher stage in early AMD, which supports the existence of early and progressive neurodegeneration.

Neuronal retinal layer thicknesses may be used as quantitative biomarkers of disease progression in AMD.

The presence of SDD appears to be associated with more prominent and faster neurodegeneration and probably with different genetic susceptibilities.

Several genetic variants were identified in association with AMD in our cohort, and the *CFH* rare variant rs35292876 conferred the highest risk of disease, while three major AMD risk variants in *ARMS2/HTRA1*, *CFH,* and *C3* had a lower-than-expected allele frequency.

The GRS was significantly higher in AMD cases, but it was insufficient to discriminate them from controls and non-progressors, reinforcing the need to include lifestyle and other risk factors in personalized medicine.

Damaging rare variants in the *CFH* gene were more frequent in AMD patients when cumulatively analyzed in our cohort, confirming their importance in AMD heritability.

AMD patients from the Mira study are not only less burdened by common major risk variants but carriers of *CFH* rare variants are even less, thus AMD risk and associated phenotypic features in these patients are mainly attributable to the rare variants and not to common major risk variants.

Carriers of *CFH* rare variants presented with more severe disease: superior drusen burden, PEDs, HRF, and thinner retinas. The rare variant P258L seems to be associated with SDD.

Carriers of *CFH* rare variants are probably at increased risk of progression.

Our findings reported through this Thesis have the potential to contribute to AMD pathophysiology knowledge not only in Portugal but also in a global setting. Both imaging biomarkers and genetic biomarkers are the next revolutions in AMD, where personalized medicine strategies as well as targeted treatments at different pathways in the earlier stages of disease are more than ever needed.

Future Directions

Genetic testing is not currently recommended in patients with AMD. The primary reasons for this are the inexistence of strategies capable to decrease risk in those without disease but with higher genetic risk**;** for now, there are no approved treatments that are dependent on risk genotypes; and phenotype alone still has high accuracy in predicting disease progression, with some studies showing only slightly improved accuracy from adding genotype. Because of this, AMD genetic screening is not currently advisable in the clinical setting and testing is important mostly in the investigational setting.(51)

However, this concept may rapidly change in the near future. Targeting treatments according to the patients' major contributing pathways or according to specific susceptibility genotypes is now regarded as a possibility to achieve better results in complement inhibition and genetic therapies in new clinical trials.(42, 47, 52) This may imply, for example, selecting patients with abnormal systemic complement overactivation or who have specific risk genotypes that promote complement overactivation.(42, 52) In addition, directing treatments to the pathways most affected in early/intermediate AMD, in a personalized medicine approach, could be another direction to treat AMD before it reaches late stages. Functional studies correlating genotype with systemic levels of complement components and studies focusing on the genetic characterization of large populations from different backgrounds are thus, important.

Comprehension of the interplay of all interacting genetic and non-genetic risk factors in AMD is complex and to date the full picture of pathophysiologic mechanisms is still not completely understood. This is necessary not only to develop new and more targeted preventive strategies but also to develop new treatments that halt progression before irreversible vision loss associated with late-stage disease occurs.

The interplay between lifestyle (smoking, diet, physical exercise), clinical factors (including chronic medications), and genetic background are probably at the root of our epidemiologic findings for the Mira population, and differences detected compared to other cohorts, which will merit further study. In fact, other groups already reported significant modulation of genetic risk according to lifestyle choices.(11, 44)

Regarding phenotype, we only assessed drusen burden and other phenotypic features in the macula and posterior pole, but studies evaluating extramacular, and peripheral retina with UWF imaging would be important to further expand the phenotype-genotype correlation in AMD.(53-55) Data obtained with newer imaging technologies such as OCTA will also be of interest to explore new biomarkers of disease progression and response to new treatments.

Future steps in AMD imaging and biomarkers will probably benefit from the introduction in the last decade of artificial intelligence-based techniques such as machine learning and deep learning. The primary objective of such a revolution was to automatically compute a large amount of imaging data helping in clinical decisions and exploring new biomarkers of disease progression, activity, and prognosis. Automatic identification and quantification of different features are now actively explored by different groups.(16, 56) This will add power to predictive models and further strengthen phenotype-genotype-lifestyle interaction models.

Considering these unmet needs, the future developments envisioned by our AMD Research Group are:

1. Extramacular and peripheral retinal drusen and reticular pigmentary changes have been observed and described in subjects with and without AMD. Preliminary analyses revealed an association between these peripheral retinal phenotypes and a family history of AMD.(54) Furthermore, extramacular and peripheral changes may also be present in around 10%–31% of eyes with an otherwise completely normal macula. It is still unclear whether these extramacular or peripheral changes represent risk factors for the development of AMD or if they are features of AMD itself as a disease involving the entire retina and not only located in the macular area.(53)

   We are starting a new study that aims to explore genotype-phenotype associations between the sequenced genetic variants found in the AMD Incidence study and the presence of extramacular drusen and extramacular pigment irregularities. In this way, we will determine whether these extramacular features could represent an expression of genetic susceptibility among individuals with or without AMD and if a common genetic basis exists with AMD.

   The main objectives are:

   - To evaluate the relationship between extramacular drusen and pigmentary changes and the presence or absence of AMD.
   - To evaluate the relationship between extramacular drusen and pigmentary changes with genotypes associated with AMD.
   - Associations will be tested between the AMD risk variants sequenced in the context of the EYE-RISK collaboration and the graded retinal phenotypes.

2. In our first Epidemiologic Study on AMD prevalence and risk factors, one of the main findings was the surprisingly low frequency of early and late AMD in the Mira cohort compared to Lousã.(19-21) The two populations are separated by only 80 km, but they appear to have some important differences. One is a smaller town close to the sea (Mira), while the other is closer to large cities in a more urban setting (Lousã). So not only demographic but also lifestyle-related

and even genetic factors are at play. When comparing the two cohorts we found that in the CES a higher adherence to the Mediterranean diet was protective for AMD and the coastal population (Mira) had significantly higher adherence to this diet compared to the inland population (Lousã).(23, 24) Plus, in the Incidence study in Mira, the genetic analysis was performed, and we came across interesting findings such as a lower allele frequency of AMD major risk variants in this population and the presence of rare *CFH* variants associated with the cases with a more severe phenotype (Chapters 5 and 6 of this Thesis). Is this interplay of factors the cause of our epidemiologic findings?

New work on the interplay between genetic and non-genetic risk factors in this cohort from Mira is now under development and publication. The preliminary results show interesting data (Barreto P, *et al*, unpublished data, 2022):

- Higher adherence to the Mediterranean diet (assessed with mediSCORE) showed a global protective effect regarding AMD diagnosis, but this effect was more pronounced and significant in patients with high GRS.
- Physical exercise was a protective factor in high GRS subjects but not in low GRS subjects (despite a trend in protective effect).
- In subjects with a high GRS, age increased the risk of having AMD by a 2- to 3-time-fold in the older subjects. The same was observed for smoking though it was only marginally significant.

The benefits of a healthier lifestyle are undeniable, but the protective or risk-conferring effects of non-genetic factors appear to significantly rely on the underlying genetic risk of the subject. Other groups found similar results.(11, 44, 57) Preventive medicine is the goal for all doctors, and healthy lifestyles are recommended not only in AMD but in cardiovascular and cerebrovascular diseases, and as common good clinical practice. Assessing the individual genetic risk for disease may be useful to predict the benefit of non-pharmacological personalized strategies, especially when they rely mainly upon modifiable risk factors. Objectively knowing of this interplay effect opens again the discussion on when and to whom to perform genetic testing in AMD, and for which purpose.

3. Last but not least, our research group recently received the EURETINA Clinical Research Award 2022 by the European Society of Retina Specialists (EURETINA) to develop a new project that follows the steps of the work presented in this Thesis. With this new project, we will now focus on the inland population of Lousã that hasn´t been assessed in terms of genetics and incidence/ progression of AMD. Our primary objective is to explore how the interplay between genetic, environmental and lifestyle factors (e.g., Mediterranean diet), and systemic comorbidities/ chronic medications modulates the risk for AMD onset and progression in the global population of the CES and in each cohort (Mira vs Lousã). We will also characterize the

genetics of the inland population of Lousã, including rare variants analysis; explore genotypic-phenotypic associations; and determine the 10-year incidence of AMD.

The major contributions of this new project will be:

- Expansion of our population-based study, which will allow us to extensively analyze multimodal images (including UWF fundus images and OCTA) and different genetic and non-genetic risk/protective factors for AMD, and in a longitudinal way.
- Creation of a large database in AMD from a Portuguese population that will contribute to the disease genetic knowledge in Europe.
- Deepen the comprehension of the interplay between genetic and non-genetic risk factors, including analysis of synergistic effects.
- Creation of a predictive model of AMD development and progression including genetic, non-genetic risk factors, and phenotypic data for our global population of the CES.

Finally, we hope that our added knowledge will contribute to the development of personalized medicine strategies, which will be the necessary basis for AMD management in the future.

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