Genetic Associations with Age-related Macular Degeneration and Genetic Risk Score in the Epidemiologic Coimbra Eye Study

Associações Genéticas e *Genetic Risk Score* na Degenerescência Macular da Idade no Estudo Epidemiológico de Coimbra

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ABSTRACT

INTRODUCTION: To date several genetic variants are known to play an important role in age-related macular degeneration (AMD). Variations in the genetic pool of different populations impact the disease prevalence, incidence and risk of progression.

This report aims to determine the genetic contribution in the development of AMD in a Portuguese population from the Coimbra Eye Study (CES,NCT01298674,NCT02748824), and to determine the genetic risk score (GRS).

METHODS: Participants in the CES underwent ophthalmologic examination and imaging in baseline and 6.5-year follow-up visits. AMD staging was performed in a centralized reading center.

Two genetic analyses were performed, a case-control analysis and a progression to AMD analysis. Genomic DNA was isolated from blood samples collected in the follow-up visit. Genetic sequencing was performed using the EYERISK assay under the European Eye Epidemiology Consortium (E3). Sixty-nine single nucleotide polymorphisms (SNPs) were genotyped and tested for association under an additive model with presence/absence of AMD in the follow-up visit, and with progression/no progression in the longitudinal analysis. Logistic regression analysis was performed to assess allelic odds ratio at 95% CI for each variant, adjusted for age and sex. GRS were calculated for AMD cases/controls and progressors/non-progressors.

RESULTS: In case-control analysis samples from 237 patients and 640 controls were included. The SNPs associated to increased risk of AMD were: *ARMS2 rs10490924, ARMS2/HTRA1 rs3750846,CFH rs35292876,SLC16A8 rs8135665,TGFBR1 rs1626340.* The SNPs with protective effect were: *CFH rs10922109, CFH rs1410996,C2/CFB/SKIV2L rs429608,CETP rs5817082,CNN2 rs10422209,CFB rs641153* and *RDBP_CFB rs760070.*

In progression to AMD analysis (630 non-progressors and 137 progressors), identified risk-variants for progression were: *ARMS2 rs10490924, ARMS2/HTRA1 rs3750846,CFH rs35292876;* and protective variants were *C2_CFB_SKIV2L rs429608,CFH rs10922109,CFH rs1410996,CNN2 rs10422209,CFHR5 rs10922153,SYN3/TIMP3 rs5754227,COL10A1 rs3812111.*

The GRS for AMD cases and controls was 1.12±1.19 and 0.65±1.12 (p<0.001), and for progressors and non-progressors was 1.19±1.18 and 0.67±1.14 (p<0.001).

CONCLUSION: This is the first genetic study in AMD in a Portuguese population. Similar variants were found to be associated with the presence and progression to AMD in our epidemiological study, while others were protective. The GRS was significantly different between cases and controls showing its potential when assessing risk. Genetic characterization is important to pursue in different populations to further expand the knowledge of AMD pathophysiology.

KEYWORDS: Macular Degeneration/epidemiology; Macular Degeneration/genetics; Polymorphism, Single Nucleotide.

RESUMO

INTRODUÇÃO: Vários *single nucleotide polymorphisms* (SNPs) foram já identificados em associação à degenerescência macular da idade (DMI). Variações no *pool* genético afetam a prevalência, incidência e risco de progressão da doença em diferentes populações.

Este estudo tem como objetivo caracterizar as associações genéticas na DMI numa população portuguesa do estudo epidemiológico *Coimbra Eye Study* (CES,NCT01298674,NCT02748824) e determinar o *genetic risk score* (GRS).

MÉTODOS: Os participantes foram submetidos a exame oftalmológico e imagiologia na visita inicial do CES e na dos 6,5 anos de seguimento. O estadiamento da DMI foi realizado num centro de leitura centralizado.

Procedemos a duas análises genéticas: uma de caso-controlo e uma longitudinal de progressão para DMI. O DNA genómico foi isolado de amostras de sangue e o sequenciamento genético foi realizado usando o *EYERISK-assay* do *European Eye Epidemiology Consortium (E3)*. Sessenta e nove SNPs foram genotipados e testados para associação com presença/ausência de DMI na coorte da visita final, e com progressores/não-progressores na análise longitudinal. Usámos regressão logística para avaliar a *odds ratio* alélica com IC a 95% para cada variante, ajustada ao sexo e idade. Os GRS foram calculados para casos/controlos e progressores/não-progressores.

RESULTADOS: Na análise caso-controlo foram incluídas amostras de 237 doentes e 640 controlos. Os SNPs associados a risco aumentado de DMI foram: *ARMS2 rs10490924,ARMS2/HTRA1 rs3750846,CFH rs35292876,SLC16A8 rs8135665,TGFBR1 rs1626340.* Os SNPs com efeito protetor foram: *CFH rs10922109,CFH rs1410996,C2 / CFB / SKIV2L rs429608,CETP rs5817082,CNN2 rs10422209,CFB rs641153* e *RDBP_CFB rs760070.*

Na análise de progressão para DMI (630 não-progressores e 137 progressores), as variantes de risco identificadas foram: *ARMS2 rs10490924,ARMS2/HTRA1 rs3750846,CFH rs35292876;* e as variantes protetoras foram *C2_CFB_SKIV2L rs429608,CFH rs10922109,CFH rs1410996,CNN2 rs10422209,CFHR5 rs10922153,SYN3/TIMP3 rs5754227, COL10A1 rs3812111.*

O GRS de casos e controlos foi 1,12±1,19 e 0,65±1,12 (p <0,001), e de progressores e não-progressores foi 1,19±1,18 and 0.67±1.14(p <0,001).

CONCLUSÃO: Este é o primeiro estudo genético na DMI numa população portuguesa. Vários SNPs foram identificados, sendo semelhantes aqueles associados à presença e à progressão para DMI, enquanto outros têm um efeito protetor. O GRS foi significativamente superior nos casos de DMI demonstrando o seu potencial na avaliação de risco. É importante caracterizar geneticamente diferentes populações para melhor compreensão da fisiopatologia da DMI.

PALAVRAS-CHAVE: Degenerescência Macular/epidemiologia; Degenerescência Macular/genética; Polimorfismo de Nucleotídeo Único.

INTRODUCTION

Age-related macular degeneration (AMD) is a major leading cause of irreversible blindness in industrialized countries, and the burden of disease is expected to increase in the next decades.[1–3] Understanding the pathophysiology of AMD and its genetic basis is important, not only to develop new therapeutic strategies, but also to provide advice to patients on their individual risk.

The heritable component in AMD is estimated to be as high as 45% to 70%, and research groups already identified several common and rare genetic variants associated with risk of disease development and progression.[4–7] 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 7 were rare variants (minor allele frequency <0.01). Susceptibility genes were found to be grouped into four main pathways: 1) complement system, 2) high density lipoprotein metabolism, 3) angiogenesis, and 4) extracellular matrix remodeling. 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).[5] Since different variants with important functional effects can be differently distributed among populations, influencing phenotype and progression of disease, case-control studies are important to be carried out in different populations.[5,8,9]

The genetic risk score (GRS) represents a way to express the risk of AMD based on the genotype of the individual, by cumulatively identifying the variants known to be associated with disease. Calculating the GRS can be useful for example when integrating the genetic information with information from interacting environmental and demographic risk factors, to more accurately predict disease development and progression.[8,10–13]

The Coimbra Eye Study (CES) is a 2-visit epidemiologic study on the prevalence and incidence of AMD in a Portuguese population (NCT01298674, NCT02748824).[14–17] The environmental and nutritional risk-factors associated to AMD prevalence were previously explored and reported.[15,18,19] However, subjects who participated in the 6.5-year follow-up visit for the estimation of AMD incidence also had blood samples collected for further genetic characterization.[17]

The purpose of this study is to determine the contribution of genetic variants, known to be associated with AMD, in the development of the disease in a Portuguese population, and to determine differences between the GRS of AMD patients compared to non-AMD participants in our cohort.

MATERIAL AND METHODS

STUDY DESIGN AND POPULATION

The Epidemiological Study (NCT01298674) is a population-based study which 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).[14,15] The AMD Incidence Study (NCT027048824) was conducted 6.5 years later and included only the subjects recruited from the primary health care unit in Mira. Information on the identification, recruitment and complete description of the study population have been published elsewhere.[14–16]

Signed informed consent was obtained for all participants. The study adhered to the tenets of the Declaration of Helsinki (2013) 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 favorable opinion for the conduction of the study.

PARTICIPANTS' DATA COLLECTION AND AMD STAGING

All participants from the follow-up incidence study underwent a detailed questionnaire-based interview on demographic, clinical and lifestyle information, and were submitted to bilateral ophthalmological assessment, including best-corrected visual acuity (BCVA) tested with Early Treatment Diabetic Retinopathy Study (ETDRS) charts and multimodal imaging (MMI). This included Color Fundus Photography (CFP) (Topcon® fundus camera, TRC-NW8; Topcon Corp., Tokyo, Japan), Spectral Domain Optical Coherence Tomography (SD-OCT), fundus autofluorescence (FAF), and Infra-Red (IR) imaging with Spectralis HRA+OCT (Heidelberg Engineering, Heidelberg, Germany). Finally, blood samples were collected from the participants who consented for further genetic and laboratorial analysis.[16,17]

In respect to AMD grading the Rotterdam staging system was used through the CES: early AMD was defined as stages 2a, 2b and 3 (presence of large (≥125 μm), soft, indistinct drusen 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 and/or geographic atrophy).[20,21] 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 center (Coimbra Ophthalmology Reading Center, AIBILI, Portugal) at baseline and follow-up visits, by senior medical retina specialist graders trained according to study protocol.[15,17]

GENETIC SEQUENCING PROCEDURES AND SELECTION OF CASES/CONTROLS

Genomic DNA samples from the participants were genotyped according to standard procedures through the recently published EYERISK genotype assay, in collaboration with the E3 - The European Eye Epidemiology Consortium.[8] This assay was designed to genotype 87 single nucleotide polymorphisms (SNPs), including the 52 SNPs independently associated with AMD identified in the GWAS study

conducted by the International AMD Genomics Consortium (IAMDGC).[5,8] 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 (*ARMS2, CD46, CFB, HTRA1*), and genes involved in AMD-mimicking macular dystrophies (*ABCA4, CTNNA1, 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*.[8] After quality control, 69 SNPs were successfully genotyped. To ensure a complete dataset of the 52 AMD-associated variants 10 SNPs were genotyped by KASP genotyping assays.

Cases were defined as the participants from the AMD Incidence Study having 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 aged > 60 years old, or stage 1 (soft distinct drusen (≥63 µm) or pigmentary changes) if aged > 70 years old. This was to avoid including controls that could progress to AMD.

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 (ORs) at 95% CI for each variant, adjusted for age and sex, with a significance level set to 0.05.

We compared the SNP allele frequencies (AFs) of controls and AMD patients in the CES cohort to those of the GWAS IAMDGC dataset, and we explored if the allelic ORs showed the same direction and magnitude of effect.[5]

The genetic risk score was also computed in our population. Fifty-two independent variants identified by Fritsche *et al* [5] were selected and the OR from the IAMDGC GWAS 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). 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/SKIV2L* rs429608, *ARMS2* rs3750846 and *C3* rs2230199) was not available.

PROGRESSION TO AMD IN THE CES – 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 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.

RESULTS

A total of 877 samples from 237 cases and 640 controls were successfully genotyped for a total of 69 SNPs in the cohort of the follow-up Incidence CES. The global mean age was 72.6 ±6.8 years (71.9 ±6.4 in controls and 74.7 ±7.3 in cases), and 57.8 % were female (56.2% of controls and 62.0% of cases).

The allele frequencies (AFs) of the tested SNPs from AMD cases and controls are presented in Table 1. Comparing to the IAMDGC dataset, we found the following inverse trends in MAF distribution between cases and controls: our MAF was higher in controls for *ACAD10/BRAP* rs61941272, *C3* rs2230199, *C9* rs62358361, *COL8A1* rs13081855 and rs140647181 and *NPLOC4/TSPAN10* rs656559; and the MAF in our cases was higher for *ABCA1* rs1883025 and rs2740488, *APOE (EXOC3L2/MARK4)* rs73036519, *CFH* rs3753394, *COL4A3* rs11884770, *TGBR1* rs334353, *TGFBR1* rs1590 and rs1626340, *TGFBR1* rs334349 and *VEGFA* rs943080.

ASSOCIATIONS WITH AMD RISK IN CASE-CONTROL ANALYSIS

Five risk-variants were associated to increased risk of AMD: *ARMS2* rs10490924 (OR 1.47; CI 95% 1.12 -1.93, *p*=0.005), *ARMS2/HTRA1* rs3750846 (OR 1.46; CI 95% 1.11 -1.92, *p*=0.007), *CFH* rs35292876 (OR 2.67; CI 95% 1.14 -6.17, *p*=0.021), *SLC16A8* rs8135665 (OR 1.44; CI 95% 1.05 -1.95, *p*=0.021), and *TGFBR1* rs1626340 (OR 1.32; CI 95% 1.01 -1.71, *p*=0.037).

Moreover, we have also identified 7 variants with a potential protective effect towards AMD: *CFH* rs10922109 (OR 0.72; CI 95% 0.57 -0.89, *p*=0.003), *CFH* rs1410996 (OR 0.71; CI 95% 0.57 -0.89, *p*=0.003), *C2/CFB/SKIV2L* rs429608 (OR 0.51; CI 95% 0.34 -0.74, *p*=0.001), *CETP* rs5817082 (OR 0.73; CI 95% 0.56 -0.95, *p*=0.018), *CNN2* rs10422209 (OR 0.66; CI 95% 0.46 -0.91, *p*=0.014), *CFB* rs641153 (OR 0.63; CI 95% 0.42 -0.91, *p*=0.018) and *RDBP_CFB* rs760070 (OR 0.65; CI 95% 0.44 -0.94, *p*=0.025).

All significant associations are depicted in Table 2, plus comparison to the IAMDGC dataset. All SNPs were associated with AMD in the same direction of effect (protective *vs* increased risk), except for the *TGFBR1* rs1626340.

GENETIC RISK SCORE:

The cohort to compute the GRS comprised 829 subjects: 607 controls and 222 cases. This was because the GRS was considered as missing from the analysis if the genotype of

Table 1. Allele frequencies (AFs) of the SNPs from AMD cases and controls in the CES and comparison to the IAMDGC dataset.

Gene	Rs number	Major/ Minor allele	MAF controls CES	MAF cases CES	MAF controls IAMDGC	MAF cases IAMDGC
ABCA1	**rs1883025**	**C / T**	**0.264**	**0.288**	**0.261**	**0.243**
ABCA1	**rs2740488**	**A / C**	**0.292**	**0.297**	**0.275**	**0.255**
ACAD10/BRAP	**rs61941272**	**C / A**	**0.009**	**0.006**	**0.018**	**0.024**
ADAMTS9	rs6795735	C / T	0.521	0.530	0.433	0.465
ADAMTS9-AS2	rs62247658	T / C	0.525	0.537	0.433	0.466
APOE	rs429358	T / C	0.106	0.072	0.135	0.099
APOE(EXOC3L2/MARK4)	**rs73036519**	**G / C**	**0.216**	**0.257**	**0.302**	**0.284**
ARHGAP21	rs12357257	G / A	0.317	0.297	0.223	0.243
ARMS2	rs10490924	G / T	0.142	0.201	0.208	0.436
ARMS2/HTRA1	rs3750846	T / C	0.140	0.197	0.208	0.436
B3GALTL	rs9542236	T / C	0.461	0.483	0.437	0.452
B3GALTL	rs9564692	C / T	0.329	0.319	0.299	0.277
C2	rs4151667	T / A	0.017	0.004	0.046	0.025
C2/CFB/SKIV2L	rs2746394	G / A	0.012	0.008	0.012	0.016
C2/CFB/SKIV2L	rs429608	G / A	0.142	0.078	0.148	0.090
C2/CFB/SKIV2L (PBX2)	rs204993	A / G	0.182	0.191	0.260	0.284
C3	rs147859257	T / G	0.000	0.000	0.004	0.012
C3	**rs2230199**	**G / C**	**0.183**	**0.168**	**0.208**	**0.266**
C3 (NRTN/FUT6)	rs17855739	C / T	0.001	0.000	0.049	0.038
C9	rs34882957	G / A	0.013	0.011	0.009	0.016
C9	**rs62358361**	**G / T**	**0.013**	**0.011**	**0.009**	**0.016**
CFB	rs641153	G / A	0.125	0.085	0.090	0.048
CETP	rs17231506	C / T	0.292	0.301	0.315	0.348
CETP	rs3764261	C / A	0.303	0.311	0.317	0.350
CETP	rs5817082	C / CA	0.290	0.236	0.264	0.232
CFB	rs4151672	C / T	0.015	0.004	0.045	0.025
CFH	rs10922109	C / A	0.443	0.361	0.426	0.223
CFH	rs121913059	C / T	0.000	0.000	0.000	0.003
CFH	rs1410996	G / A	0.443	0.360	0.426	0.223
CFH	rs148553336	T / C	0.002	0.002	0.009	0.003
CFH	rs191281603	C / G	0.005	0.002	0.006	0.007
CFH	rs35292876	C / T	0.010	0.023	0.009	0.021
CFH	**rs3753394**	**C / T**	**0.304**	**0.308**	**0.291**	**0.266**
CFH	rs570618	G / T	0.310	0.340	0.364	0.580
CFHR5	rs10922153	G / T	0.550	0.506	0.499	0.342
CFI	rs10033900	C / T	0.304	0.341	0.477	0.511
CFI	rs141853578	C / T	0.000	0.000	0.001	0.003
CNN2	rs10422209	C / G	0.228	0.165	0.123	0.142
COL10A1	rs3812111	T / A	0.451	0.415	0.387	0.372
COL4A3	**rs11884770**	**C / T**	**0.355**	**0.374**	**0.278**	**0.258**
COL8A1	**rs13081855**	**G / T**	**0.083**	**0.080**	**0.092**	**0.104**
COL8A1	**rs140647181**	**T / C**	**0.028**	**0.024**	**0.016**	**0.023**
COL8A1	rs55975637	G / A	0.114	0.118	0.117	0.132

Note: SNPs with an inverse trend in MAF between cases and controls in the CES in comparison to the IAMDGC dataset are presented in bold.

Table 1. Allele frequencies (AFs) of the SNPs from AMD cases and controls in the CES and comparison to the IAMDGC dataset (Cont.).

Gene	Rs number	Major/ Minor allele	MAF controls CES	MAF cases CES	MAF controls IAMDGC	MAF cases IAMDGC
CSK_MIR4513	rs2168518	A / G	0.339	0.327	0.345	0.328
CTRB2/CTRB1	rs55993634	C / G	0.127	0.105	0.089	0.075
HTRA1	rs11200638	G / A	0.131	0.164	0.207	0.431
LIPC	rs2043085	C / T	0.402	0.382	0.384	0.354
LIPC	rs2070895	G / A	0.209	0.209	0.217	0.195
LIPC	rs493258	C / T	0.526	0.500	0.465	0.442
LPL	rs12678919	A / G	0.102	0.078	0.099	0.100
MIR	rs4351242	C / T	0.094	0.086	0.067	0.063
MIR6130/RORB	rs10781182	G / T	0.327	0.335	0.306	0.328
MMP9	rs142450006	TTTTC / T	0.100	0.118	0.141	0.124
NPLOC4/TSPAN10	**rs6565597**	**C / T**	**0.318**	**0.287**	**0.381**	**0.400**
PILRB/PILRA	rs7803454	C / T	0.200	0.205	0.190	0.209
PRLR/SPEF2	rs74767144	C / G	0.010	0.006	0.022	0.017
RAD51B	rs2842339	A / G	0.140	0.144	0.094	0.107
RAD51B	rs8017304	A / G	0.527	0.489	0.372	0.349
RDBP_CFB	rs760070	T / C	0.124	0.086	0.091	0.049
SLC16A8	rs8135665	C / T	0.150	0.203	0.195	0.217
SYN3/TIMP3	rs5754227	T / C	0.116	0.096	0.137	0.109
TGBR1	**rs334353**	**T / G**	**0.231**	**0.249**	**0.248**	**0.231**
TGFBR1	**rs1590**	**T / G**	**0.236**	**0.256**	**0.260**	**0.242**
TGFBR1	**rs1626340**	**G / A**	**0.181**	**0.219**	**0.209**	**0.189**
TGFBR1	rs334348	A / G	0.238	0.257	0.260	0.242
TGFBR1	**rs334349**	**G / A**	**0.224**	**0.248**	**0.261**	**0.242**
TMEM97/VTN	rs11080055	C / A	0.481	0.478	0.486	0.463
VEGFA	**rs943080**	**T / C**	**0.465**	**0.467**	**0.497**	**0.465**
ZBTB41	rs12724106	A / G	0.088	0.105	0.105	0.168

Note: SNPs with an inverse trend in MAF between cases and controls in the CES in comparison to the IAMDGC dataset are presented in bold.

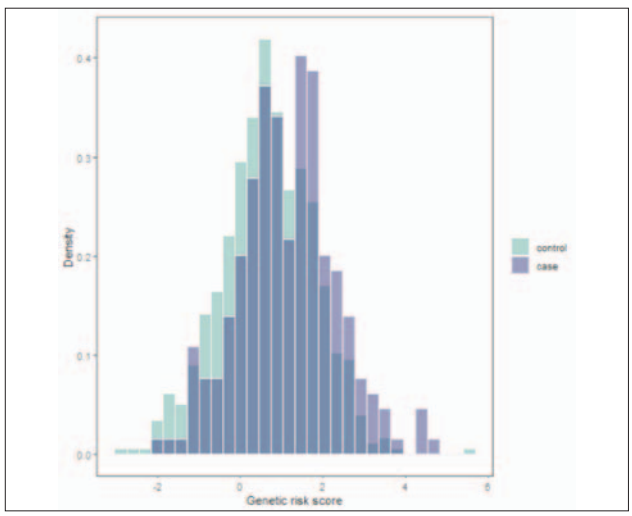

Figure 1. GRS in AMD cases and in controls in the CES.

one of the major risk variants was not available in a given participant, as explained in the methods section.

Significant differences between the GRS from controls and AMD cases were found in our population: 0.645 ±1.124 *vs* 1.124 ±1.187, respectively (p<0.001). The GRS varied from -2.9 to 6, and there was a clear shift towards a higher GRS in AMD cases. It was, however, not possible to completely distinguish between cases and controls based on the GRS alone, as there was substantial overlap (Fig. 1).

PROGRESSION TO AMD – GENETIC ASSOCIATIONS AND GRS

In respect to the genetic associations with progression to AMD in the 6.5-year follow-up longitudinal analysis we compared progressors (progression to stages 2,3 or 4) to non-progressors (stage 0 or 1 in both visits). We

Table 2. Variants significantly associated with risk of AMD in the CES, and comparison to the IAMDGC report.

Gene	SNP	REF	ALT	Major/ Minor allele	MAF controls CES	MAF cases CES	OR CES (95% CI)	*p*-value CES	OR IAMDGC	*p*-value IAMDGC
C2/CFB/SKIV2L	rs429608	G	A	G/A	0.142	0.078	0.51 [0.34 - 0.74]	0.001	0.57	1.2-103
CFH	rs1410996	G	A	G/A	0.443	0.360	0.71 [0.57 -0.89]	0.003	0.38	0
CFH	rs10922109	C	A	C/A	0.443	0.361	0.72 [0.57-0.89]	0.003	0.38	9.6-618
ARMS2	**rs10490924**	G	T	G/T	0.142	0.201	**1.47 [1.12 -1.93]**	0.005	2.81	0
ARMS2/HTRA1	**rs3750846**	T	C	T/C	0.140	0.197	**1.46 [1.11 - 1.92]**	0.007	2.81	6.5-735
CNN2	rs10422209	C	G	C/G	0.228	0.165	0.66 [0.46 - 0.91]	0.014	1.15	2.7-8
CFB	rs641153	G	A	G/A	0.125	0.085	0.63 [0.42 - 0.91]	0.018	0.51	1.1-89
CETP	rs5817082	C	CA	C/CA	0.290	0.236	0.73 [0.56 - 0.95]	0.018	0.84	3.6-19
SLC16A8	**rs8135665**	C	T	C/T	0.150	0.203	**1.44 [1.05 - 1.95]**	0.021	1.14	5.5-11
CFH	**rs35292876**	C	T	C/T	0.010	0.023	**2.67 [1.14 - 6.17]**	0.021	2.42	8.2-37
RDBP_CFB	rs760070	T	C	T/C	0.124	0.086	0.65 [0.44 - 0.94]	0.025	0.51	9.5-91
TGFBR1	**rs1626340**	G	A	G/A	0.181	0.219	**1.32 [1.01 - 1.71]**	0.037	0.88	3.8-10

Variants in bold are associated with increased risk of having AMD; The remaining variants are associated with protective effect towards AMD.

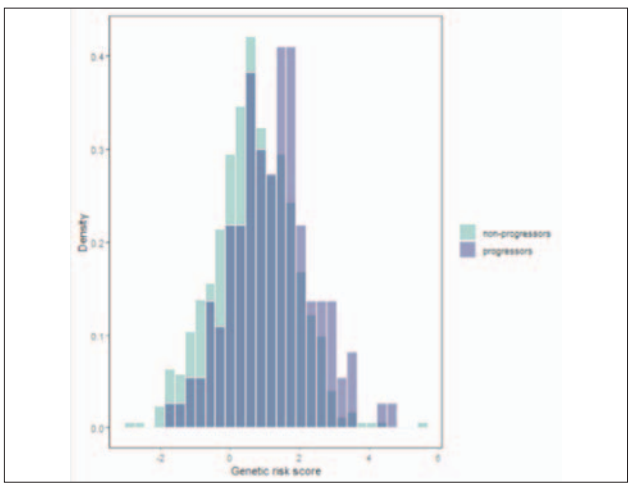

Figure 2. GRS in progressors and in non-progressors in the CES.

obtained 137 samples from progressors and 630 samples from non-progressors. Variants associated to increased risk of progression to AMD were: *ARMS2* rs10490924, *ARMS2/HTRA1* rs3750846, and *CFH* rs35292876. The variants found to have a protective effect regarding progression to AMD were again *C2_CFB_SKIV2L* rs429608, *CFH* rs10922109, *CFH* rs1410996 and *CNN2* rs10422209, but also the variants *CFHR5* rs10922153, *SYN3/TIMP3* rs5754227 and *COL10A1* rs3812111 (Table 3).

Non-progressors and progressors had significantly different GRS: 0.67±1.14 and 1.19±1.18, respectively (p<0.001). Again, and despite the substantial overlap, there was a shift towards a higher GRS in those who progressed to AMD (Fig. 2).

Table 3. Variants significantly associated with risk of progression to AMD.

Gene	SNP	REF	ALT	Major/Minor allele	MAF progressors CES	MAF non-progressors CES	OR CES (95% CI)	*p*-value CES
C2/CFB/SKIV2L	rs429608	G	A	G/A	0.066	0.136	0.43 (0.24 - 0.71)	0.002
CFH	rs1410996	G	A	G/A	0.335	0.437	0.65 (0.49-0.86)	0.003
CFH	rs10922109	C	A	C/A	0.344	0.437	0.68 (0.51 - 0.90)	0.007
CFHR5	rs10922153	T	G	G/T	0.471	0.556	0.70 (0.54 - 0.92)	0.009
ARMS2/HTRA1	**rs3750846**	T	C	T/C	0.203	0.141	**1.52 (1.08-2.12)**	0.015
CFH	**rs35292876**	C	T	C/T	0.029	0.012	**3.06 (1.18 - 7.41)**	0.015
ARMS2	**rs10490924**	G	T	G/T	0.204	0.144	**1.51 (1.07 - 2.10)**	0.016
SYN3/TIMP3	rs5754227	T	C	T/C	0.070	0.115	0.56 (0.33 -0.91)	0.026
CNN2	rs10422209	C	G	C/G	0.154	0.223	0.63 (0.40 - 0.96)	0.036
COL10A1	rs3812111	T	A	T/A	0.408	0.463	0.76 (0.58 - 0.99)	0.045

Variants in bold are associated with increased risk of progression to AMD; The remaining variants are associated with protective effect towards progression to AMD.

DISCUSSION

We identified in our epidemiological longitudinal study several SNPs associated with the risk of having AMD and with progression towards this diagnosis, as well as others which demonstrated a protective role. These SNPs are located in genes associated with AMD that act in different pathophysiologic pathways, sustaining its multifactorial etiology. Their effects in our population agree with major reports, including large GWAS. The GRS was significantly different between AMD and non-AMD cases and between progressors and non-progressors, supporting its role when assessing individual risk, especially when performing a multifactorial analysis including other clinical and environmental risk-factors.

In the past decade a large genome-wide association study by Fritsche *et al*[5] identified several genetic risk variants strongly associated with AMD: 52 variants at 34 genomic regions, of which 45 were common variants while 7 were found to be rare variants for having a MAF < 0.01%. In our study 12 variants sequenced by the genotype assay developed by the EYERISK consortium were found to be associated with AMD, by either increasing the risk of having disease or by having a protective effect towards the diagnosis. Eleven of these variants are described in the literature as common variants while one in the *CFH* gene (rs35292876) is a rare variant known to be associated to increased risk of AMD.

The analysis of the MAF of all sequenced SNPs in AMD cases *vs* controls revealed that some variants from different pathways had an inverse trend in the CES compared to what was found in the larger database of the IAMDGC. These differences can be due to the relatively low number of our sample or due to true genetic differences of our cohort, which originates from a relatively small populational area in central Portugal. Genes with discrepancies were found in different pathways, such as the complement system (*CFH, C3* and *C9*), extracellular matrix (*COL4A3, COL8A1, MMP9*), cholesterol metabolism (*ABCA1, ACAD10/BRAP* and *APOE*), and the *TGFBR1* gene. The latter variant rs1626340 was the only one associated to AMD risk in our population while being considered protective in the IAMDGC GWAS dataset and in the complete EYERISK study cohort.[5,8]

The variants found to be significantly associated to AMD in our population were fewer than expected and this was probably due to the relatively small sample of our study. However, specific genetic differences in our population cannot be completely 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 to those associated with conversion to AMD after 6.5 years of follow-up. The variants associated with progression to AMD were fewer and located only in *CFH* and *AMRS2/HTRA1* genes. The discrepancy might be related to a more prominent role of these genes in initiating AMD and promoting its progression.

The development of AMD can be influenced not only by common variants, but also by low-frequency and rare 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 *CFH* (rs148553336, rs35292876, rs191281603). One of these, the *CFH* rare variant rs35292876 was identified in our population as low frequency, and interestingly it conferred the highest risk of having AMD in our cohort (OR, 2.67), when compared to the remaining common risk variants. Furthermore, this variant was associated to the highest risk of progression to AMD in the 6.5-year follow-up analysis (OR, 3.06). The OR in our patients was even superior to that reported in the GWAS from the IAMDGC (OR, 2.42).[5] This variant was also previously found at a higher frequency in Western Europe, compared to other globe regions, which might explain its superior effect in our population.[22]

In respect to their functional impact, our main risk-conferring rare variant *CFH* rs35292876 was not found in other studies to be associated to FH or FHR concentrations in serum, thus its functional impact is not obvious, nor the mechanism by which it confers increased risk of AMD.[6,23] Regarding the identified protective common variant *CFH* rs10922109, it is described to decrease *CFHR1*, *CFHR3*, *CFHR4* and *CFH* gene expression in liver, decreased systemic C3d/C3 ratio and decreased systemic FHR-4 levels.[24] More functional studies are therefore increasingly necessary to determine the pathophysiological effect of all identified common and rare variants, and to correctly estimate their true impact at the physiologic level besides simple association to disease/no-disease. Furthermore, the cumulative effect of these variants when located in the same haplotype must also be addressed to fully assess the mechanisms behind their role in AMD development and progression.

Our findings thus support pursuing case-control analysis of rare variants in AMD in different populations, as different rare variants with potential significant functional impact may be associated to AMD risk in different populations. However, instead of focusing only on isolated rare variants that are underpowered to detect association to AMD when we consider the global population study, adopting a strategy of addressing the total cumulative risk of several damaging rare variants could be more informative about risk when analysing differences between cohorts. This type of approach might have a role in the near future when trying to identify patients who would benefit of targeted therapies, for example by inhibiting the complement cascade. The carriers of more of these rare variants might benefit more of such complement-inhibiting therapies. Phase I/II clinical trials for subretinal gene supplementation in AMD are currently underway, and several others targeting the complement will probably follow.[24,25]

Regarding the Genetic Risk Score, we found it to be significantly different between AMD cases and controls and between progressors and non-progressors. This confirms that the conjoined hereditable 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 the cases 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 etiology of AMD is greatly impacted and modified by other clinical and environmental factors.[8,13] A global score that comprehensively assesses genetic and lifestyle risk factors, such as smoking, body mass index, nutrition, and even concomitant medication, will be more informative of the risk of the disease than the GRS alone.

This study has some limitations that should be addressed. Despite being originally an epidemiological population-based study, for the purposes of genetic analysis it has a small cohort. Furthermore, the population subjected to genetic characterization originates from a single location in Portugal. As some genetic variants are geographically and regionally heterogenous, or with more expression in some families, 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 population, and we provide extensive characterization regarding the variants associated to AMD further contributing to the disease genetic knowledge in Europe and the differences towards other regions. Furthermore, as part of the EYERISK project our results are based in a comprehensive genotype assay recently validated in European populations.

CONCLUSION

In summary, the same variants in *CFH* and *ARMS2*/*HTRA1* genes were both associated to having the disease and with conversion to disease, while others had a protective effect. The *CFH* rare variant rs35292876 conferred the highest risk of both having the disease and of progression to it. Regarding the Genetic Risk Score, it was found to be significantly higher in AMD cases and progressors, but it was unable to fully discriminate them from controls. Our study adds new information regarding the variants associated to AMD in a Portuguese population, which can be used for comparison with other populational cohorts and further expanding the knowledge of AMD pathophysiology.

PRESENTATIONS AND AWARDS

This work was presented at the ARVO 2021 meeting and at EURETINA 2021.

CONTRIBUTORSHIP STATEMENT / DECLARAÇÃO DE CONTRIBUIÇÃO:

All authors contributed to the final manuscript.
CF, PB, RC, RS: Conception or design of the work.
CF, MLC: Data collection.
CF, PB, RC, RS: Data analysis and interpretation.
CF, PB, RC: Drafting the article.
CBH, JCV, JM, RS: Critical revision of the article.
CBH, RS: Final approval of the version to be published.

RESPONSABILIDADES ÉTICAS

Conflitos de Interesse: Os autores declaram a inexistência de conflitos de interesse na realização do presente trabalho.

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Confidencialidade dos Dados: Os autores declaram ter seguido os protocolos da sua instituição acerca da publicação dos dados de doentes.

Proteção de Pessoas e Animais: Os autores declaram que os procedimentos seguidos estavam de acordo com os regulamentos estabelecidos pelos responsáveis da Comissão de Investigação Clínica e Ética e de acordo com a Declaração de Helsínquia revista em 2013 e da Associação Médica Mundial.

Proveniência e Revisão por Pares: Não comissionado; revisão externa por pares.

ETHICAL DISCLOSURES

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Confidentiality of Data: The authors declare that they have followed the protocols of their work center on the publication of data from patients.

Protection of Human and Animal Subjects: The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki as revised in 2013).

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