Phenotypic Expression of *CFH* Rare Variants in Age-Related Macular Degeneration Patients in the Coimbra Eye Study

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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

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

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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

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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 semi-automatic 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 in-built 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 χ^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]

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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 χ^2 test; Fisher's exact test.
‡ No. of minor alleles/total No. of alleles (%).

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.

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

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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
	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.

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.

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

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

Phenotypic Characteristics	Noncarriers (n = 284 Eyes)	Carriers (n = 39 Eyes)	OR (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				
Overall Retina (OCT), mm^3	8.40 ± 0.50	8.29 ± 0.32	0.449 [0.226–0.894]	**0.023**
IRL (OCT), mm^3	6.17 ± 0.51	6.05 ± 0.36	0.496 [0.252–0.979]	**0.043**
ORL (OCT), mm^3	2.22 ± 0.09	2.20 ± 0.10	0.033 [0.0005–2.26]	0.114
RPE-Bruch layer (OCT), mm^3	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).

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.

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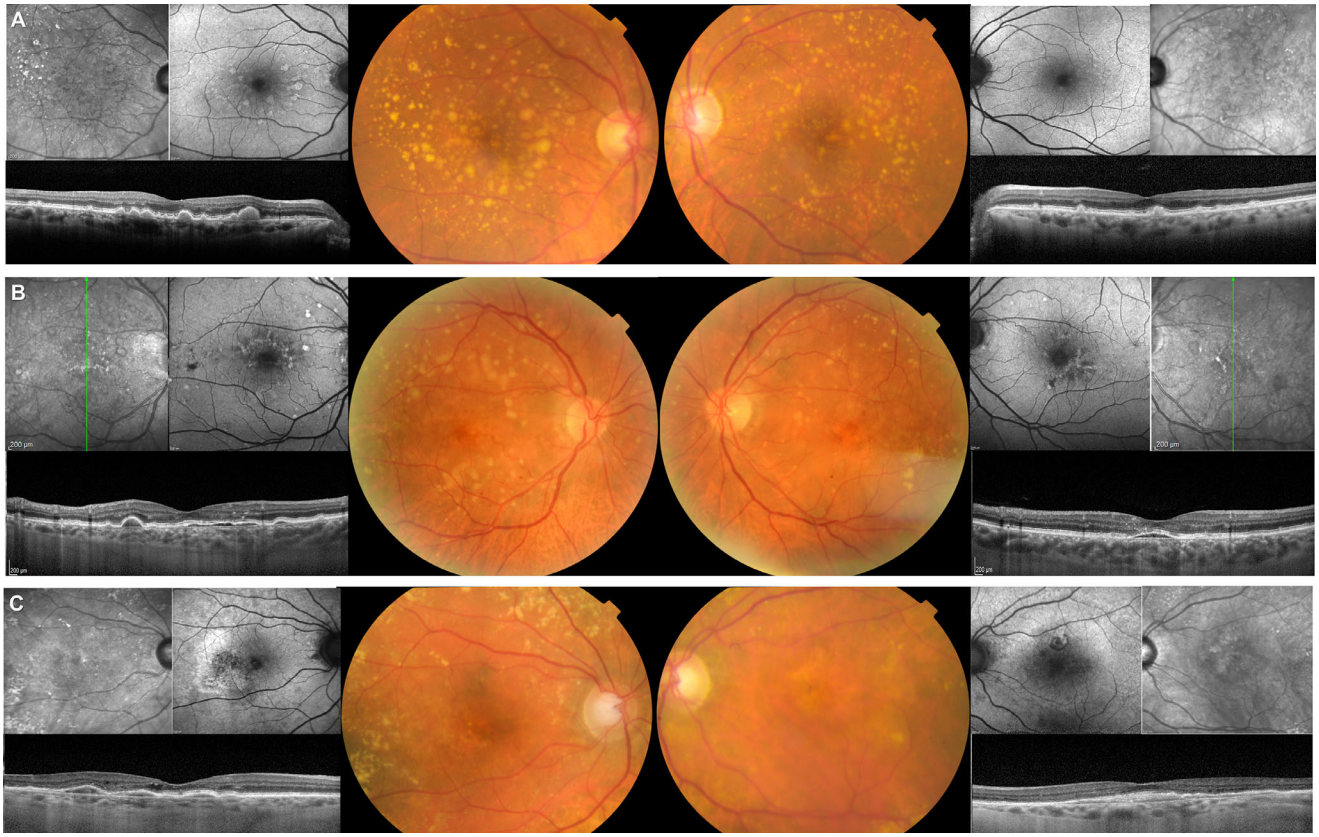

Figure 1. Exemplificative images of fundus features of carriers. (**A**) Female, 68 years old (yo) (*CFH* rs757785149; Arg53Cys) with extensive soft drusen both inside the ETDRS grid and outside extending beyond the vascular arcades, and crystalline drusen temporal to the macula. There is a high degree 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).

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

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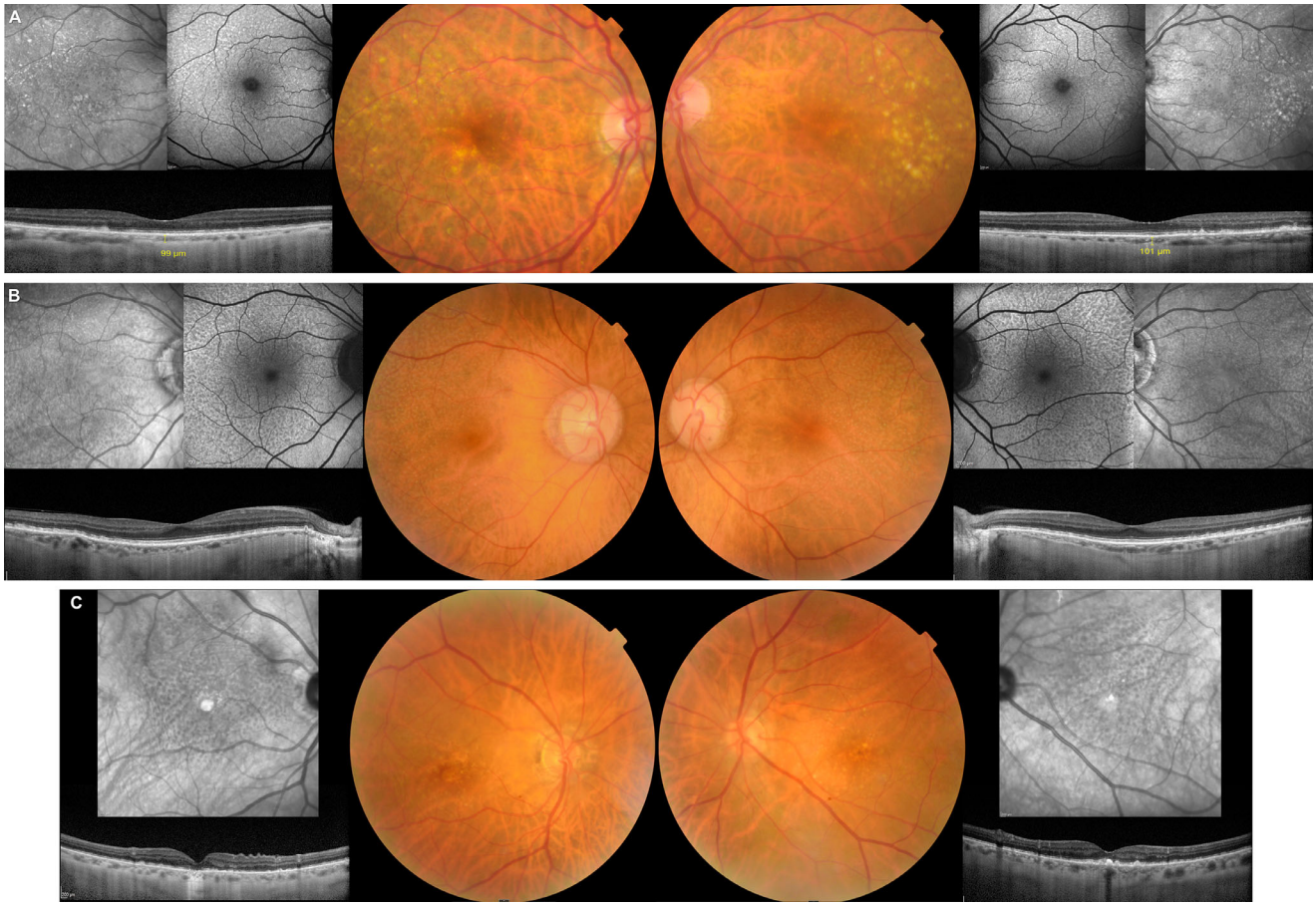

Figure 2. Exemplificative multimodal images of *CFH* rs768526062 (Pro258Leu) carriers. (**A**) Female, 72yo with soft drusen mainly clustering in the temporal macula and SDD in parafoveal, nasal, and superior distribution in both eyes. The choroid is very thin (99 micra in right eye and 101 micra in 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.

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

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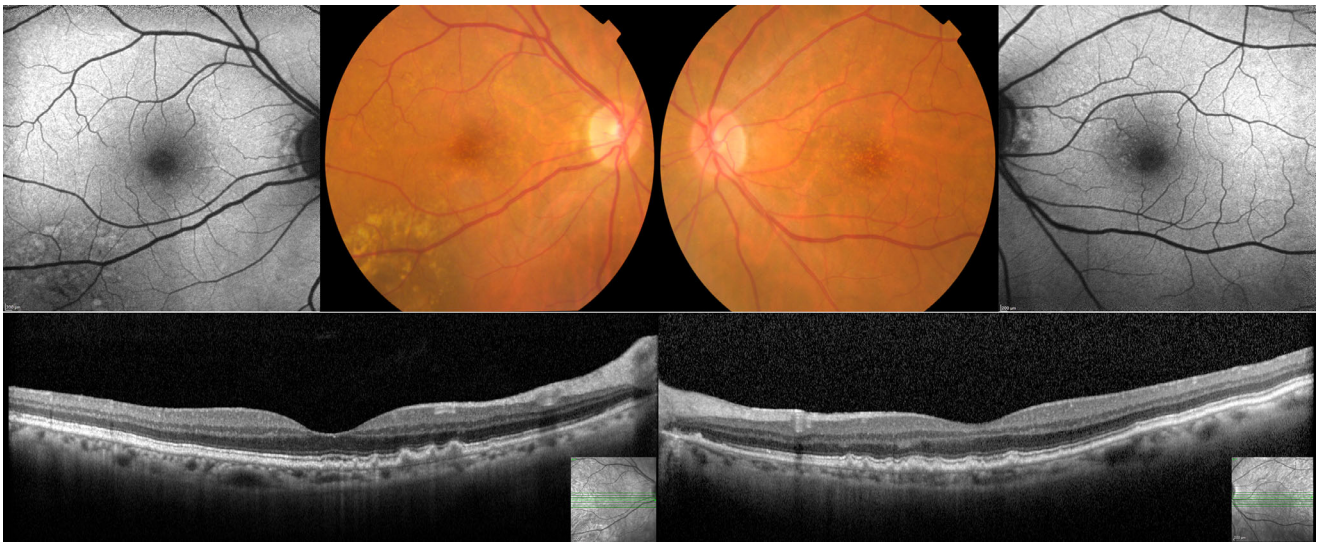

Figure 3. Female, 74yo (rare variant *CFH* rs35274867; Asn1050Tyr) with cuticular drusen in the fovea and nasal parafovea. Besides the rare variant, this patient harbors multiple common variants, including three other *CFH* variants (rs10922109, rs1410996, rs3753394) and three risk-conferring variants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

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

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project, our results are based on a comprehensive genotype assay recently validated in European populations.

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.

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