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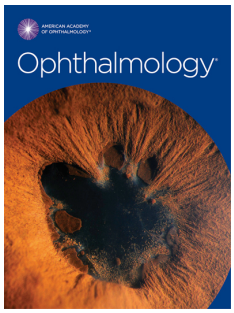

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Running head

Genotype assay for age-related macular degeneration

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Abbreviations

AMD	Age-related macular degeneration

smMIPs	Single molecule molecular inversion probes
NGS	Next generation sequencing
GRS	Genetic risk score
AF	Allele frequency
MAF	Minor allele frequency
SNP	Single nucleotide polymorphism
LD	Linkage disequilibrium
HWE	Hardy-Weinberg Equilibrium
CACD	Central areolar choroidal dystrophy
IAMDGC	International age-related macular degeneration genomics consortium
AUC	Area under the ROC curve
LoF	Loss-of-function
NA	Not applicable
N/A	Not available
ND	Not determined
iPSC	Induced pluripotent stem cells

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Online-only supplemental files

This article contains additional online-only material. The following should appear online-only: Supplemental Methods, Supplemental Dataset 1 and 2, Supplemental Tables S1-14, and Supplemental Figure S1-S2.

Abstract

Purpose: To develop a genotype assay to assess associations with common and rare AMD risk variants, to calculate an overall genetic risk score (GRS), and to identify potential misdiagnoses with inherited macular dystrophies that mimic AMD.

Design: Case-control study.

Participants: Individuals (N=4,740) from five European cohorts.

Methods: We designed single molecule molecular inversion probes (smMIPs) for target selection and used next generation sequencing (NGS) to sequence eighty-seven single nucleotide polymorphisms (SNPs), coding and splice-site regions of ten AMD-(related) genes (*ARMS2*, *C3*, *C9*, *CD46*, *CFB*, *CFH*, *CFI*, *HTRA1*, *TIMP3*, *SLC16A8*), and three genes that cause inherited macular dystrophies (*ABCA4*, *CTNNA1*, *PRPH2*). GRS for common AMD risk variants were calculated based on effect size and genotype of 52 AMD-associated variants. Frequency of rare variants was compared between late AMD cases and control individuals with logistic regression analysis.

Main Outcome Measures: GRS, association of genetic variants with AMD, genotype-phenotype correlations.

Results: We observed high concordance rates between our platform and other genotyping platforms for the 69 successfully genotyped SNPs (96.77-97.28%) and for the rare variants (99.81%). We observed a higher GRS for patients with late AMD compared to patients with early/intermediate AMD (p<0.001) and individuals without AMD (p<0.001). A higher proportion of rare loss-of-function variants and variants with a Combined Annotation Dependent Depletion score ≥20 in the *CFH* (50 [2.92%] vs 8 [1.02%], OR=2.88 [1.36-6.11], p=0.006), *CFI* (38 [2.22%] vs 4 [0.51%], OR=4.45 [1.58-12.50], p=0.005) and *C3* (56 [3.27%] vs 4 [0.51%], OR=6.56 [2.37-18.17], p=0.0003) genes was observed in late AMD cases compared to control individuals. In nine patients we identified pathogenic variants in the *PRPH2, ABCA4* and *CTNNA1* genes, which allowed reclassification of these patients as inherited macular dystrophy.

Conclusions: This study reports a high-throughput and comprehensive genotype assay for common and rare AMD genetic variants. This test can identify individuals at intermediate to high genetic risk of late AMD, and enables differential diagnosis of AMD mimicking dystrophies. Our study supports sequencing of *CFH*, *CFI* and *C3* genes as they harbor rare high-risk loss-of-function variants. Carriers of these variants could be amendable for new treatments for AMD that are currently under development.

Introduction

Age-related macular degeneration (AMD) is a common cause of vision loss in the aging population, with a prevalence of 0.1% in individuals aged 55-59 years and rising to 9.8% in individuals aged ≥ 85 years for late AMD in Europe.[1] The number of individuals affected by any form of AMD is expected to rise to 288 million worldwide by 2040.[2] Both genetic and non-genetic factors contribute to the disease pathogenesis, which makes it a complex disease.

The first evidence for a genetic contribution to AMD originates from The US Twin Study.[3] Significant progress has been achieved over the past 15 years in identifying the genetic causes of AMD. Although polymorphisms in the *CFH* and *ARMS2* genes account for an important proportion of the AMD risk, additional genetic variants in or near genes of the complement system (*CFB, CFI, C2, C3*), extracellular matrix remodeling (*COL8A1, TIMP3*), cholesterol metabolism (*ABCA1, APOE, CETP, LIPC*) and genes in other undefined pathways (e.g. *ARHGAP21, B3GALTL*) have been associated with AMD.[4-9] The largest genome-wide association study (GWAS) in AMD was published in 2016 and identified 52 independently associated genetic variants with AMD distributed across 34 loci.[7] The majority of these variants were common genetic variants, while seven variants were rare (minor allele frequency < 0.01) in the investigated population. Furthermore, a significantly higher burden of rare variants in the *CFH*, *CFI*, *TIMP3* and *SLC16A8* genes was identified in AMD patients compared to control individuals. In recent years, the role of rare genetic variants in AMD gained attention, as they can have large effect sizes. Sequencing of candidate genes in case-control studies and in AMD families resulted in the identification of rare variants in the *CFH*, *CFI*, *C3* and *C9* genes that could be linked to AMD. [8, 10-16]

Current knowledge of genetic variants contributing to the risk of AMD can be used to design genetic tests that predict the risk to develop AMD. Considering that many genetic variants in multiple genes have been associated with AMD, only a comprehensive genotype assay including all risk variants will accurately identify the total genetic risk. Genetic testing for AMD is a contentious area, and the currently available tests are mostly limited to a low number of genetic variants and vary in their predictive ability.[17] This points out a clear need for such an assay.

Besides the limited number of genetic variants included in the tests that are currently available (Macula Risk PGx and Vita Risk [15 genetic variants], http://www.maculariskcom; 23andMe [2 genetic variants], http://www.23andme.com; EasyDNA [number of genetic variants unspecified],

https://www.easydna.co.uk; RetnaGene [12 genetic variants], http://www.mynicox.com), the high costs also prevent implementation of extensive genetic testing for AMD in daily practice. Combining genomic capture using single molecule molecular inversion probes (smMIPs) and next-generation sequencing (NGS) allows for a cheap and fast way to sequence AMD-associated variants and genes.[18] Furthermore, sequencing of AMD-associated genes enables identification of potential new rare variants contributing to AMD risk. In particular rare, highly penetrant variants in the *CFH* and *CFI* genes are shown to confer high odds ratios with AMD.[19] It is also important to evaluate genes that are involved in the pathogenesis of inherited macular dystrophies (e.g. central areolar choroidal dystrophy, late-onset Stargardt's disease), since the phenotype of some of these dystrophies can mimic AMD.[20-22]

The aim of this study was to develop a comprehensive AMD genotype assay to assess associations with AMD risk variants, to calculate an overall GRS, and to differentiate between AMD and AMD-mimicking dystrophies.

Methods

Study population

DNA samples of five European cohorts contributing to the EYE-RISK database were selected for genotyping: Coimbra Eye Study (CES), Combined Ophthalmic Research Rotterdam Biobank (CORRBI), European Genetic Database (EUGENDA), Characterization of geographic atrophy progression in patients with age-related macular degeneration (GAIN), and Muenster Aging and Retina Study (MARS). In addition, several induced pluripotent stem cells (iPSC) and donor eye samples from Tübingen and Sevilla were selected for genotyping. Grading of the images was performed in each study individually by experienced graders. The final AMD stage was determined based on the worst eye. Detailed information on the included studies has been published elsewhere.[23-26] We merged early and intermediate AMD in one category and used the following categories: no AMD, early/intermediate AMD and late AMD (geographic atrophy or choroidal neovascularization). In total 786 individuals without AMD > 65 years of age, 1,056 individuals with early/intermediate AMD and 1,714 individuals with late AMD were selected for analysis (Table S1, available at http://www.aaojournal.org). In addition, 453 family members from the EUGENDA cohort were genotyped and included only for the analysis

regarding the identification of potential AMD-mimicking dystrophies. Informed consent was obtained from all individuals according to the tenets of the Declaration of Helsinki, and Ethics Committee approval was obtained.

Design of the genotype assay, bioinformatics pipeline and quality control

The EYE-RISK genotype assay was designed to genotype 87 single-nucleotide polymorphisms (SNPs), including the 52 independently associated SNPs identified by the International AMD Genomics Consortium (IAMDGC),[7] SNPs previously associated with AMD,[27] and several candidate SNPs (Table S2, available at http://www.aaojournal.org). Furthermore, the coding and splice-site regions of thirteen genes were completely sequenced. Genes that have been described to carry rare variants in AMD (*C3, C9, CFH, CFI, TIMP3, SLC16A8),*[8, 10-16] candidate genes that might carry rare variants in AMD (*ARMS2, CD46, CFB, HTRA1*), and genes involved in AMD-mimicking macular dystrophies (*ABCA4, CTNNA1, PRPH2*)[20-22, 28] were selected for complete sequencing. In addition, three intronic *ABCA4* variants affecting splicing (c.5196+1137G>A, c.5196+1216C>A, c.5196+1056A>G) were targeted.[29]

All smMIPs were designed using the MIPGEN pipeline,[30] and the GrCh37/hg19 was used as the reference genome build. Each smMIP covered a 110-bp genomic region with a maximum overlap of 40 bp with the adjacent smMIP (Supplemental Dataset 1, available at http://www.aaojournal.org). During the design phase of the smMIPs six SNPs were poorly covered (rs11402250, rs72802342, rs61941274, rs12019136, rs67538023, rs9708919), including five SNPs of the 52 top hits from the latest GWAS. For those SNPs the second best hit from the GWAS[7] was selected (Table S3, available at http://www.aaojournal.org), and accompanying smMIPs were designed. No alternative SNP was selected for rs9708919.

Data was analyzed using an in-house smMIP-pipeline. We used samtools (v1.4.1) and bcftools (v1.9.20) for genotype calling. We applied a minimum of 40 reads coverage for the SNPs, and a more stringent filtering for the rare variants of 40 reads coverage on both reference and alternate allele. For validation, we compared the EYE-RISK smMIPs sequencing data to genotyping data of selected samples of the EUGENDA cohort that were previously analyzed on other genotyping platforms (whole exome sequencing,[4] KASP genotyping, exome chip[7]). Concordance rates between the different platforms were calculated. The variants that passed these quality control steps were further tested if they were in Hardy-Weinberg Equilibrium (HWE).

We compared SNP allele frequencies (AFs) of control individuals (>65 years of age) and late AMD cases in the EYE-RISK dataset with AFs of control individuals and late AMD cases in the IAMDGC dataset.[7] We

assessed allelic odds ratios (ORs) for all SNPs to test if the SNPs in our study showed the same direction and magnitude of effect compared to the 52 SNPs as reported in the IAMDGC study.[7] Further details with respect to the design of the smMIPs, the smMIPs bioinformatics pipeline and quality control steps are described in the Supplemental Methods (available at http://www.aaojournal.org).

Phenotypes of *ABCA4*, *CTNNA1* and *PRPH2* rare variant carriers

Genetic variants identified in the *ABCA4*, *CTNNA1, PRPH2* and *TIMP3* genes were filtered for rare and low-frequency protein-altering and splice-site variants. Based on literature we selected rare variants that were previously described to cause inherited macular dystrophies (Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php), and an in-house database of the department of Human Genetics, Nijmegen, The Netherlands).[21, 28, 31-37] For the *ABCA4* gene we filtered for carriers of ≥ 2 *ABCA4* variants of class 3 or higher, based on the American College of Medical Genetics and Genomics (ACMG) classification. Retinal images of these carriers were evaluated by a retinal specialist (CCWK) to identify patients with potential misdiagnoses of inherited macular dystrophies.

Statistical analysis

We used chi-square tests to compare AFs between control individuals and late AMD cases. AFs with p-values below 7.2^{-4} (0.05/69) were considered to differ significantly between the datasets. Binary logistic regression analysis based on AF was used to asses allelic ORs for the SNPs. Weighted genetic risk scores (GRS) were calculated based on the 52 independently associated variants from the IAMDGC GWAS.[7] For each individual we generated a GRS according to the formula: $GRS = \sum_{i=1}^{52}(G_i\,\beta_i)$. G_1 represents the genotype of variant i, where genotypes were coded as 0, 1 or 2 based on the number of minor alleles (0 = carrier of 0 minor alleles, 1 = carrier of one minor allele, 2 = carrier of two minor alleles). β_1 represents the effect size of variant i (natural logarithm of the fully conditioned odds ratio [OR] of the minor allele of variant i), based on the GWAS of the IAMDGC.[7, 38] The GRS of an individual was considered as missing if the genotype of one of the major risk or protecting variants (*CFH* rs570618, *CFH* rs10922109, *C2/CFB/SKIV2L* rs429608, *ARMS2* rs3750846 or *C3* rs2230199) was not available. If the genotype of one of the other variants was missing we considered this variant in this individual as missing. Differences in GRS between individuals without AMD, early/intermediate AMD and late AMD were analyzed by a univariate general linear model (SPSS version 22.0 [IBM Corp., Armonk,

NY]). We compared the GRS distribution in individuals without AMD, early/intermediate AMD and late AMD in our current study to the GRS distribution in the study of Colijn *et al*, which included both population-based studies and clinic-based studies, and used the same method for GRS calculation (*Colijn et al., submitted*).

For the rare variant analysis we first performed a single variant association test with RAREMETALWORKER (version 4.13.8) [https://genome.sph.umich.edu/wiki/RAREMETALWORKER] to test if any of the single variants were associated with late AMD. We adjusted for age, gender and institute within this analysis. Variants with a P-value < 1.89^{-5} (0.05/2642) were considered statistically significant (Bonferroni correction). The number of 2642 was based on the number of tested variants, which included all genetic variants with a minor allele frequency (MAF) < 0.05.

Subsequently, we performed logistic regression analyses to assess the cumulative effect of rare variants with AMD. ANNOVAR was used to annotate the variants.[39] Rare (MAF < 0.01) protein-altering and splice-site variants were stratified into the following categories: (1) CADD < 20 and (2) CADD ≥ 20 or loss-of-function, according to the Combined Annotation Dependent Depletion (CADD) score, which is an algorithm predicting the functional effect of genetic variants. Loss-of-function variants were defined as nonsense, splice-site and frameshift variants, and missense variants with a described functional effect based on functional studies (Table S4, available at http://www.aaojournal.org). Another way of categorizing rare variants is according to the Polyphen2 prediction score, where we used the following categories: (1) benign, (2) possibly damaging, (3) probably damaging and (4) loss-of-function. We used binary logistic regression analysis to assess association of the different categories of variants with late AMD. P-values < 0.05 were considered statistically significant. Noncarriers were used as the reference category. In case of same event status we applied Firth correction (Statistical Analysis System Institute, V9.4).

Results

Performance of the genotype assay

Out of the 87 SNPs, 69 SNPs were genotyped successfully, while 11 SNPs were excluded due to low coverage (Figure S1 and Table S5, available at http://www.aaojournal.org), five SNPs were removed due to deviation of HWE, and two SNPs were removed due to low genotype concordance with other genotyping platforms (Table S6, available at http://www.aaojournal.org). The concordance rates between SNPs genotyped

with the EYERISK smMIPs sequencing platform compared to the whole exome sequencing, KASP genotyping and exome chip datasets were 96.77%, 97.28% and 96.96%, respectively (Table S7, available at http://www.aaojournal.org). To ensure a complete dataset of the 52 AMD-associated variants we genotyped ten SNPs by KASP genotype assays. Genotyping and validation of the assays was carried out by LGC Genomics (Table S8, available at http://www.aaojournal.org).

Ten genes (*ABCA4*, *C3*, *C9, CD46, CFH, CFI, CTNNA1, PRPH2* and *TIMP3*) were well covered, as at least 95% of the base pairs in these genes were covered at least 40x. For three genes (*ARMS2*, *HTRA1*, *SCL16A8*) a lower percentage (between 70.6-83.6 %) of the base pairs were covered at least 40x. The lower coverage in these genes was mainly attributed to specific exonic regions in these genes (Tables S9 and S10, available at http://www.aaojournal.org). The concordance rates of rare variants identified in the EYERISK smMIPs dataset compared to the whole exome sequencing dataset was >99% (Table S7, available at http://www.aaojournal.org).

We observed similar AFs for 61 of the 69 SNPs in control individuals as in the previous IAMDGC GWAS study. For late AMD cases we observed similar AFs for 66 of 69 SNPs (Table S11, available at http://www.aaojournal.org). Regarding differences in cases, we observed a lower AF in late AMD cases of the EYE-RISK study for *MIR* rs4351242, *C3 (NRTN/FUT6)* rs17855739 and *MMP9* rs142450006 compared to late AMD cases of the IAMDGC study. Differences in AF in control individuals were observed for *COL4A3* rs11884770, *CFI* rs10033900, *C2/CFB/SKIV2L* rs204993, *ARHGAP21* rs12357257, *RAD51B* rs8017304, *CNN2* rs10422209, *C3 (NRTN/FUT6)* rs17855739 and *MMP9* rs142450006. Next, we evaluated the different cohorts in more detail to determine whether the differences were caused by a specific cohort (Table S12, available at http://www.aaojournal.org). The differences in AF in cases were not assigned to a specific cohort. However, for six out of eight SNPs the difference in AF in control individuals was attributed to a different AF distribution in the CES cohort.

Association analysis of 69 SNPs with late AMD in the EYERISK smMIPs genotyping dataset identified 40 SNPs that were associated with late AMD ($p < 0.05$). For 29 SNPs we observed no association. After correction for multiple comparisons, 19 of 40 SNPs showed a significant association with late AMD ($p < 7.2^{-4}$) (Table S13, available at http://www.aaojournal.org). The effects of the significantly associated SNPs were all in the same direction compared to the IAMDGC study.

Genetic risk scores

The GRS for AMD was calculated for 786 individuals without AMD > 65 years of age, 1,056 early/intermediate AMD patients, and 1,714 late AMD patients, based on 52 AMD-associated SNPs. Figure 1 shows the distribution of the GRS in this study. We observed a higher GRS in patients with late AMD (mean 1.71, SD 1.29) compared to patients with early/intermediate AMD (mean 0.86, SD 1.27, $p < 0.001$) and individuals without AMD (mean 0.30, SD 1.06, $p < 0.001$). We compared the GRS distribution in early/intermediate cases, late AMD cases and control individuals in our current study to the GRS distribution in the study of Colijn *et al*, and observed a similar distribution of the GRS among the different groups (*Colijn et al., submitted*).

In Figure 2 we demonstrated how the GRS can be used to report the AMD risk to individuals, using a small family as an example. For this purpose we combined the data of the case-control studies with the data of population based studies, as presented in the study of Colijn *et al* (*Colijn et al., submitted*). The proband (age 65) was affected by late stage AMD and presented with a GRS of 3.86. Sixty-four percent of the individuals in GRS category 3-4 were affected by late stage AMD. Her one year younger brother presented with a GRS of 3.12, and consequently belonged to the same GRS category. Both individuals were reported to belong to a high genetic risk category, whereas the 42-year-old daughter of the proband presented with a GRS of 1.02. Thirty-one percent of the individuals within GRS category 1-2 were affected by late stage AMD, whereas 69 percent was affected by early/intermediate AMD or no AMD. This individual was reported to belong to the intermediate genetic risk category.

Rare variants

In total 446 unique protein-altering and splice-site variants with a MAF < 0.01 and 11 protein-altering variants with a MAF between 0.01 and 0.05 were identified in 13 genes (Supplemental Dataset 2, available at http://www.aaojournal.org), based on AF data of European (non-Finnish) individuals (http://gnomad.broadinstitute.org/). In addition, one variant (*ABCA4* p.Asn1868Ile) with a MAF of 0.07 was present in the dataset. The majority of the variants included missense variants, representing 412 unique variants. Furthermore, we identified several splice-site, nonsense, frameshift and non-frameshift variants (number of unique variants: 9, 18, 16, 3, respectively).

Rare variant association tests

First, we performed a single-variant association test to determine associations of single variants (MAF < 0.05) with late AMD. No statistically significant associations were observed ($p > 1.89^{-5}$). Next, we categorized the rare (MAF < 0.01) protein-altering and splice-site variants according to their predicted functional effect and performed logistic regression analyses to test the cumulative effect of rare protein-altering and splice-site variants for each of the thirteen genes selected for this project. A higher number of rare loss-of-function variants or variants with a CADD score ≥ 20 were observed in the *CFI* (OR 4.45, $p = 0.005$), *C3* (OR 6.56, $p = 0.0003$) and *CFH* (OR 2.88, $p = 0.006$) genes in late AMD cases compared to control individuals (Table 1).

In addition, we categorized rare variants according to the Polyphen2 prediction score. Besides the association with late AMD for the *CFI* and *C3* genes, we also observed a higher number of rare variants in the *C9* gene in late AMD cases compared to control individuals (OR 1.77, $p = 0.04$). Another interesting finding included the observation of more probably damaging rare variants in late AMD cases compared to control individuals in the *ABCA4* gene (OR 1.78, $p = 0.03$) (Table S14, available at http://www.aaojournal.org). With regard to the association of the probably damaging variants with AMD in the *ABCA4* gene, we focused on the individual variants included in this category. Although there were no single variants that were statistically significant associated with late AMD in the single variant analysis, we observed a higher MAF in late AMD cases compared to control individuals for the missense variants p.Leu1970Phe, p.Thr901Ala and p.Thr897Ile (0.25 % vs 0.06 %, 0.09 % vs 0.06 % and 0.13 % vs 0.06 %, respectively) (Supplemental Dataset 2, available at http://www.aaojournal.org). All three variants represented variants of unknown clinical significance (ACMG classification). No significant associations were observed for rare variants in the *ARMS2*, *CFB*, *CTNNA1*, *HTRA1*, *PRPH2*, *SLC16A8* and *TIMP3* genes. An overview of the results of all tested genes, including logistic regression analyses for all AMD cases (early/intermediate and late AMD combined) is depicted in Table S14 (available at http://www.aaojournal.org).

Rare variants in inherited macular dystrophy genes

Rare variants in the *PRPH2* gene

Sequence analysis of the *PRPH2* gene revealed 20 unique rare protein-altering variants in 64 AMD cases (64/5540 alleles [1.16 %]) and 15 control individuals (15/1572 alleles [0.95 %]) (Supplemental Dataset 2, available at http://www.aaojournal.org). The rare pathogenic missense variant *PRPH2* p.Arg142Trp, which has been described to cause autosomal dominant central areolar choroidal dystrophy (CACD),[40] was found in one

early (GRS -0.89) and one late AMD case (GRS 2.19), and in addition in two family members (both graded as AMD) (GRS 0.45 and 0.95). The phenotypes of all four individuals carrying the pathogenic *PRPH2* p.Arg142Trp variant were suspect for CACD. Five of the identified *PRPH2* variants (p.Ile32Val, p.Arg142Trp, p.Gly208Asp, p.Ser289Leu, p.Trp246Arg) identified in this cohort were described previously in *PRPH2*-associated macular dystrophies or autosomal dominant retinitis pigmentosa.[32, 34, 40] The phenotypes of the individuals carrying these variants were not suspect for a dystrophy, except for the *PRPH2* p.Trp246Arg carrier. Figure 3 shows the images of the four patients primarily diagnosed with AMD with a *PRPH2* p.Arg142Trp variant. The color fundus photographs (CFP) of patient A showed an increased parafoveal reflectivity, without clear drusen (A1). No abnormalities were observed outside the parafoveal area. In patient B a large area of chorioretinal atrophy in both eyes was visible on CFPs (B1). The right eye of patient C was characterized by central hyperpigmentation on CFP (C1) and parafoveal photoreceptor loss on optical coherence tomography (OCT) (C2). The CFP of the left eye showed yellow deposits in de macula (C1). CFPs of patient D showed an increased parafoveal reflectivity (D1). Hyperfluorescent parafoveal changes were visible on the corresponding fluorescein angiography (FA) images of this patient (D3).

Rare variants in the *ABCA4* gene

Sequencing of the *ABCA4* gene revealed 121 unique rare protein-altering and splice-site variants in 383 AMD cases (383/5540 alleles [6.91 %]) and 101 control individuals (101/1572 alleles [6.42 %]) (Supplemental Dataset 2, available at http://www.aaojournal.org). In addition, three deep intronic *ABCA4* variants affecting splicing were genotyped. Only one of these deep intronic variants (*ABCA4* c.5196+1137G>A) was identified in three young control individuals < 65 years of age. No second low-frequency variant in the coding or splice-site regions of the *ABCA4* gene was identified in these three individuals within the smMIPs dataset. We further analyzed the phenotypes of 18 individuals carrying ≥ 2 heterozygous *ABCA4* variants that were classified as class 3 or higher based on the ACMG classification, although it cannot be deduced from the current genotyping data whether the variants are located on different alleles. In four patients both the genotype and the phenotype suggested a (late-onset) Stargardt's disease (Figure 3E-H and Table 2). The overall GRS in these patients was low to intermediate (-1.47, 0.19, 1.80, 2.39).

Rare variants in the *CTNNA1* gene

Screening of the *CTNNA1* gene revealed 20 unique rare missense variants in 51 AMD cases (51/5540 alleles [0.92 %]) and 12 control individuals (12/1572 alleles [0.76 %]). Rare variants that were previously described to cause a butterfly-shaped pigment dystrophy (p.Leu318Ser, p.Ile431Met, p.Glu307Lys) were not identified in any of the individuals in this study.[28] For one variant (p.Arg54Cys) the pathogenicity remains unclear.[28] We identified one individual carrying this particular variant. The overall GRS of this individual was 1.39. Although the phenotype of this individual did not match with a butterfly-shaped pigment dystrophy, we did observe an egg-yolk lesion in one eye, which is also observed in patients with Best vitelliform macular dystrophy (Figure 3I and Table 2).

Rare variants in the *TIMP3* gene

In addition, we evaluated the rare variants identified in the *TIMP3* gene. Although rare variants in this gene have been associated with a higher risk for AMD previously,[7] it is also known from literature that specific mutations in the *TIMP3* gene can cause Sorsby's fundus dystrophy (SFD).[41] Caution is always required in AMD patients presenting with a choroidal neovascularization (CNV), since phenotypic characteristics of SFD and AMD can show overlap. We identified two individuals in this study carrying a rare variant in the *TIMP3* gene (p.Pro77Ser). This mutation is not among one of the sixteen mutations that have been associated with SFD previously.[41] Both patients (age > 70 years) were graded as neovascular AMD. One of the patients presented with a CNV in both eyes without any drusen, which phenotypically raised suspicion for SFD (Figure S2, available at http://www.aaojournal.org). The overall GRS of this patient was 0.74.

Discussion

In the EYE-RISK consortium we developed a comprehensive genotype assay for AMD and demonstrated the added value of extensive genetic testing for AMD. When comparing the EYE-RISK smMIPs genotype assay with other genotyping platforms we observed high genotype concordance rates for both the SNPs (>96%) and the rare variants (>99%). Although several SNPs need to be redesigned, we were able to successfully genotype 69 SNPs and the coding and splice-site regions of 10 AMD-related and 3 dystrophy genes. We computed GRS for AMD patients and control individuals and observed high GRS predominantly in patients with late AMD, whereas low GRS were more commonly observed in control individuals. With regard to the role

of rare genetic variants, we observed a higher occurrence of rare loss-of-function variants or variants with a CADD score ≥ 20 in the *CFH*, *CFI* and *C3* genes in late AMD cases compared to control individuals. Furthermore, we highlighted the importance of sequencing the *PRPH2* and *ABCA4* genes by revealing that in nine cases both genotype and phenotype pointed towards an inherited macular dystrophy rather than AMD.

Population differences in allele frequencies

AFs of the majority of the SNPs in cases (66/69) and control individuals (61/69) included in our study were comparable with AFs in cases and control individuals from the IAMDGC study.[7] Eight SNPs in control individuals showed a different distribution. It is striking that the different distribution was attributed to the CES cohort for six of these eight SNPs. For example, we observed a MAF of 0.500, 0.503, 0.512 and 0.311 for *CFI* rs10033900 within control individuals of the CORRBI, EUGENDA, MARS and CES cohort, respectively. A MAF of 0.477 was reported for this particular SNP within the IAMDGC study. Since the different distribution in the CES cohort was limited to only these six SNPs, and the SNPs passed all the quality control steps, we consider that these differences may be attributed to AF differences in the Portuguese population compared to other European populations.

Genetic risk score

Within our data we observed a significantly higher GRS in individuals with late AMD compared to both individuals with early/intermediate AMD and control individuals. Genetic risk profiling allowed identifying individuals who carried an intermediate and high genetic risk for AMD. Despite the substantial differences in GRS between control individuals, early/intermediate AMD cases and late AMD cases, there is still an overlap between the groups, and therefore one cannot completely distinguish the three groups based on GRS only. Furthermore, we reported genetic risk based on prevalence data of a large group of cases and control individuals. Unfortunately, follow-up data was not available, and therefore could not be used for risk prediction in this study.

Rare variants in complement genes

Results of our study showed a higher occurrence of rare loss-of-function variants and variants with a CADD score ≥ 20 in cases compared to control individuals for the majority of the complement genes tested

within this study. Our study underlined the important role of the complement system, but its crucial role was also demonstrated in the study of Colijn *et al*; results showed that the complement system was the main driving pathway in AMD (*Colijn et al., submitted*). It is important to note that the rare variants in our study are categorized according to both the CADD score and the Polyphen2 prediction score. Ideally, rare variants should be categorized based on functional effect using functional studies. To date, the functional effect of several rare variants has been studied,[8, 11, 14, 15, 42-50] but for the majority of rare variants the functional effect is currently still unknown. A more comprehensive analysis of the functional effect of rare variants in the complement genes is needed to determine the clinical relevance of these variants in individual patients.

In the framework of upcoming complement inhibiting therapies and gene therapies targeting the complement system, sequencing of the complement genes and functional analysis of rare variants becomes more important. Clinical trials investigating the safety and effectivity of GT005, a recombinant adeno-associated virus (rAAV) targeting complement factor I (https://www.clinicaltrialsregister.eu/ctr-search/trial/2019-003421-22/GB) and GEM103, a recombinant factor H protein (https://clinicaltrials.gov/ct2/show/study/NCT04246866) are ongoing. If trials show conclusively that such treatments are effective, carriers of rare variants in the *CFI, CFH* or other genes could be eligible for precise and individualized therapies.

In the GWAS of the IAMDGC study the authors identified a burden of rare variants for the *CFH, CFI, SLC16A8* and *TIMP3* genes.[7] In our study we did not observe a higher occurrence of rare variants in the *SLC16A8* and *TIMP3* genes. This could potentially be attributed to the smaller sample size compared to the GWAS of the IAMDGC study. Furthermore, two exons of the *SCL16A8* gene showed a lower coverage on our genotype platform, therefore, we potentially could have missed rare variants in these regions.

Rare variants in genes associated with inherited macular dystrophies

The *ABCA4*, *CTNNA1* and *PRPH2* genes were included in this study to identify potential misdiagnoses. Genotype and phenotype data of our study revealed nine potential misdiagnoses of inherited macular dystrophies. All nine individuals were primarily diagnosed with AMD (both early and late stages). However, after critical evaluation of the retinal images of these individuals, four individuals were most likely affected by CACD, four individuals by (late-onset) Stargardt and one individual presented with a phenotype similar to Best vitelliform macular dystrophy. It is also worth noting that none of these nine individuals presented with a very

high GRS (range -1.47 - 2.39) based on the 52 AMD-associated variants. Although the number of potential misdiagnoses is limited, it is important to note that not all images of patients carrying variants in the *PRPH2*, *ABCA4* and *CTNNA1* genes were re-evaluated. We focused on variants previously described in patients with inherited macular dystrophies, and subsequently evaluated the retinal images of those patients. In our dataset we also identified 86 variants in the *PRPH2*, *ABCA4* and *CTNNA1* genes that were not reported previously in individuals with inherited macular dystrophies, and therefore represent variants of unknown clinical significance. Fifty-three out of the 86 variants included variants with a CADD score ≥ 20, which could potentially be damaging variants.

An interesting finding in this study is the observation of a higher proportion of rare variants predicted to be probably damaging in late AMD cases compared to control individuals in the *ABCA4* gene (69 [4.03 %] vs 18 [2.29 %], OR 1.78 [1.05 - 3.02], $p = 0.03$). A potential link between AMD and Stargardt's disease has been proposed previously.[51, 52] However, some other studies did not support this proposed link between AMD and the *ABCA4* gene.[53, 54] This observation was only found when categorizing the rare variants according to the Polyphen2 prediction score, and since the other categories (loss-of-function and possibly damaging variants) did not show the same effect, there is not enough evidence in our data that supports this potential link. Sequence analysis in larger AMD cohorts is required to further investigate the potential link between the *ABCA4* gene and AMD.

Screening of specific inherited macular dystrophy genes that can mimic AMD is important for genetic counseling of patients and their family members, but is also important for future clinical trials. Due to the different underlying disease mechanisms it is not desired to include, unintentionally, inherited macular dystrophies into clinical trials for AMD. Therefore, one might consider screening for specific genes (*ABCA4*) or specific genetic variants (e.g. *PRPH2* p.Arg142Trp) before inclusion of patients in clinical trials. As demonstrated in this study, phenotypic characteristics of CACD and AMD show significant overlap and can easily be confused, not only in the late stages, but also in early stages of the disease.[22] Furthermore, in four individuals presenting with a large area of atrophy and in some cases with yellow deposits in the macula, two or more *ABCA4* variants of class 3 or higher were identified, which in conclusion match with the diagnosis of (late-onset) Stargardt's disease. Results of this study demonstrate that in some cases genetic testing combined with detailed image analysis is needed to avoid misdiagnoses.

Translation to the clinic

Currently, routine genetic testing for AMD is a contentious area and not yet recommended by professional organizations, such as the American Academy of Ophthalmology.[55, 56] Major concerns include the lack of knowledge regarding the complex etiology of AMD and how that affects the subsequent advice to the patient and family members. The lack of treatment options was also an argument against routine genetic testing for AMD, as were incidental findings and cost-effectiveness. The field of AMD is evolving rapidly, and we believe that the opinion about genetic testing needs to be re-considered.

Individuals with an early onset of AMD (< 55 years of age) and individuals in families with a high frequency of AMD are likely to carry a high genetic risk. Previous reports have shown that highly penetrant rare variants in complement genes confer a high risk for AMD, can cluster in AMD families, and can be present in individuals with early onset macular drusen.[8, 11, 14, 16, 57-60] Sequencing of the complement genes (*CFH, CFI, CFB, C3* and C9) can identify rare variant carriers, who may be eligible for specific treatment trials, e.g. the GT005 and GEM103 trials mentioned above, in which patient inclusion is based on genotype. Genetic testing for inherited eye disorders has been recommended with the argument that patients can enter gene-specific clinical trials,[55] which is now also the case for AMD patients carrying specific genotypes. Irrespective of this argument, identification of rare variant carriers and calculation of a GRS is also relevant in terms of family counseling (e.g. patients with early-onset AMD, families with a high frequency of AMD).

When one or more rare variants are identified in a patient we believe it is important to take into account the functional effect of the rare variant. For some variants the functional effect has been tested previously and it has been reported that some rare variants confer high risk of AMD, whereas other rare variants do not influence the protein or are even protective for AMD.[19] For the majority of the rare variants the functional effect is currently unknown. When rare variants in the *CFH* or *CFI* genes are identified we would recommend to perform an ELISA assay to determine FH levels or FI levels, respectively. Not all rare variants cause lower protein levels. Some rare variants present with normal protein levels, whereas the functionality has been reduced.[44] In these cases functional assays such as a C3b degradation assay can be performed (Figure 4). Patients carrying rare variants with either decreased protein levels or a reduced functionality are eligible for clinical trials.

The importance of a healthy lifestyle, cessation of smoking and the usage of antioxidant supplements has already been demonstrated,[61, 62] and should be advised to all AMD patients, irrespective of their genetic

profile. Whether patients with a high genetic risk benefit more from such lifestyle modifications needs to be further investigated. The study of Colijn *et al* provided interesting findings. The authors observed that an unhealthy lifestyle resulted in a two-fold increase in AMD risk. In individuals at high genetic risk the OR for late AMD even increased from 15 in patients with a favorable lifestyle to 30 in patients with an unfavorable lifestyle (*Colijn et al., submitted*).

The demand for genetic testing is growing,[63] however the currently commercially available genetic tests for AMD include only a small number of variants and are limited in their predictive ability. The reported predictive ability ranges from 1.4 % to 16.1 % for life-time risk assessment.[17] In this study we developed a comprehensive genetic test for AMD including all 52 AMD-associated variants. In terms of genetic risk profiling we would recommend to compute an overall GRS based on the 52 AMD associated SNPs and in addition sequence the coding and splice-site regions of the complement genes (*CFH, CFI, C3* and *C9*) to identify rare genetic variants that might contribute to AMD risk, since in some (familial) cases there is already a high suspicion that rare variants are involved. Furthermore, one may consider to include the *PRPH2* p.Arg142Trp variant in the genetic test and sequence the coding and splice-site regions of the *ABCA4* gene. Despite critical evaluation of the patients' phenotype, geographic atrophy in AMD can mimic geographic atrophy in inherited macular dystrophies, which at times leads to misdiagnoses, and therefore genetic testing can be valuable in some cases (Figure 4). Considering the complexity of AMD it is essential to obtain an accurate genetic testing report, and therefore we would recommend to perform genetic testing in a Clinical Laboratories Improvement Amendments (CLIA)– or ISO15189-approved laboratory. In addition, education for ophthalmologists needs to be upgraded regarding AMD genetics and the interpretation and clinical follow-up of genetic test reports for AMD.[64]

Study limitations

Since the EYE-RISK consortium is a European initiative, only European cohorts were included in this study. Therefore, the genetic test developed within this study would be less accurate when applying in individuals of non-European descent. Another limitation is the relatively small number of control individuals compared to the cases that were included in this study. Although ideally the number of control individuals should be higher, we decided to exclude individuals without AMD < 65 years of age, since there is a reasonable chance that those individuals could still develop AMD. To maintain a substantial control group we set the

threshold at 65 years of age. Last, the design of some smMIPs failed and the coverage of some regions was low, therefore, the smMIPs assay will need to be optimized prior to implementation of the genetic test into the clinic.

Conclusion

In conclusion, within the EYE-RISK project we developed a comprehensive genotype assay, which enables genotyping of all currently known AMD-associated SNPs and the coding and splice-site regions of AMD(-related) genes and genes that can mimic AMD. Genotyping of AMD-associated SNPs can identify individuals carrying an intermediate to high risk of AMD. Our study suggests that the *CFH*, *CFI*, *C3* and *C9* genes should also be sequenced as rare loss-of-function variants and variants with a CADD score ≥ 20 in these genes can confer a high risk for AMD, and carriers of these variants could be amendable for new (targeted) treatments that are currently being developed for AMD. Furthermore, this study emphasizes that sequencing inherited macular dystrophy genes confers the potential benefit of avoiding serious misdiagnoses.

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The EYE-RISK Consortium

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Legends

Figure 1 Genetic risk score. Figure detailing the distribution of the GRS in case-control studies. GRS = genetic risk score. (**A**) Stratification of the GRS in the different GRS categories. (**B**) Distribution of the GRS in individuals without AMD, early/intermediate AMD and late AMD.

Figure 2 Genetic risk report. Figure detailing the distribution of the GRS in case-control and population studies combined, including a demonstration of a genetic risk score report based on an example of a small family. GRS = genetic risk score. (**A**) Stratification of the GRS into the different GRS categories. (**B**) GRS distribution among early/intermediate AMD cases, late AMD cases and control individuals based on case-control studies and population studies. (**C**) I 65-year-old female affected by late stage AMD, conferring a high GRS of 3.86. II 64 year-old male without signs of AMD, but conferring a high GRS (3.12) as well, III 42-year-old female without signs of AMD, conferring an intermediate GRS (1.02).

Figure 3 Phenotypic characteristics of *ABCA4*, *CTNNA1* and *PRPH2* variant carriers. Figure detailing the phenotypic characteristics of individuals carrying one or more rare and low-frequency variants in the *ABCA4, CTNNA1* and *PRPH2* genes. A-D: retinal images of individuals carrying the *PRPH2* p.Arg142Trp variant heterozygous, E-H: retinal images of individuals carrying ≥ two *ABCA4* variants, I: retinal images of an individual carrying the *CTNNA1* p.Arg54Cys variant heterozygous.

Figure 4 Flow chart genetic testing. Figure detailing the proposed flow chart for specific subgroups that might benefit from genetic testing for AMD.

Table 1. Association of rare variants with AMD

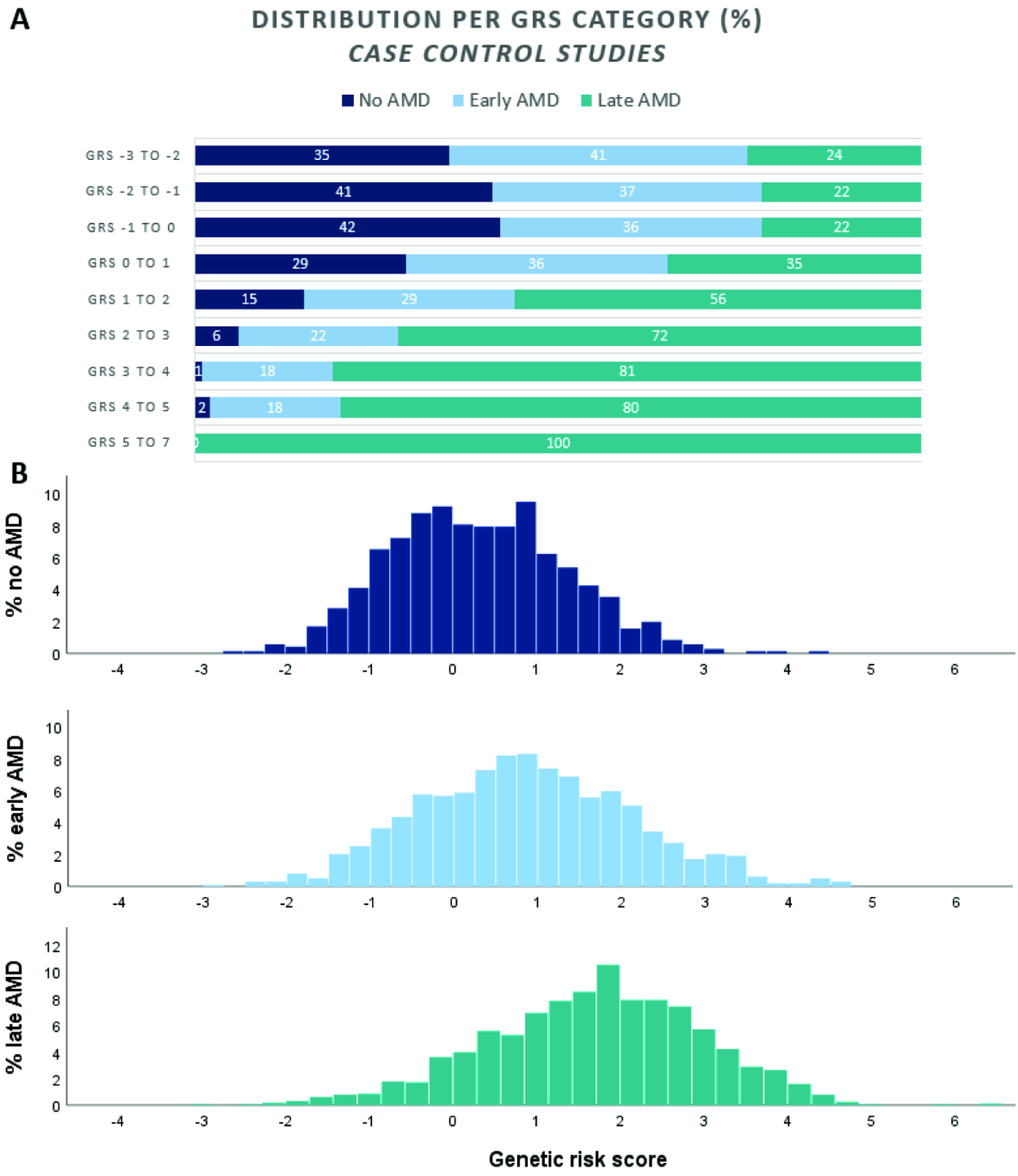
Rare variant carriers categorized by CADD score	Controls, n (%) (n=786)	Cases (late AMD), n (%) (n=1714)	OR [95% CI]	P-value
C3				
Noncarrier	761 (96.82)	1623 (96.82)	1 [reference]	
Carrier - CADD < 20	21 (2.67)	35 (2.04)	0.781 [0.452 - 1.352]	0.378
Carrier - CADD ≥ 20 or loss of function	4 (0.51)	56 (3.27)	6.564 [2.372 - 18.167]	0.0003
CFH				
Noncarrier	749 (95.29)	1625 (94.81)	1 [reference]	
Carrier - CADD < 20	22 (2.80)	37 (2.16)	0.775 [0.454 - 1.323]	0.351
Carrier - CADD ≥ 20 or loss of function	8 (1.02)	50 (2.92)	2.880 [1.359 - 6.106]	0.006
CFI				
Noncarrier	773 (98.35)	1647 (96.09)	1 [reference]	
Carrier - CADD < 20	9 (1.15)	23 (1.34)	1.199 [0.552 - 2.604]	0.646
Carrier - CADD ≥ 20 or loss of function	4 (0.51)	38 (2.22)	4.450 [1.584 - 12.503]	0.005

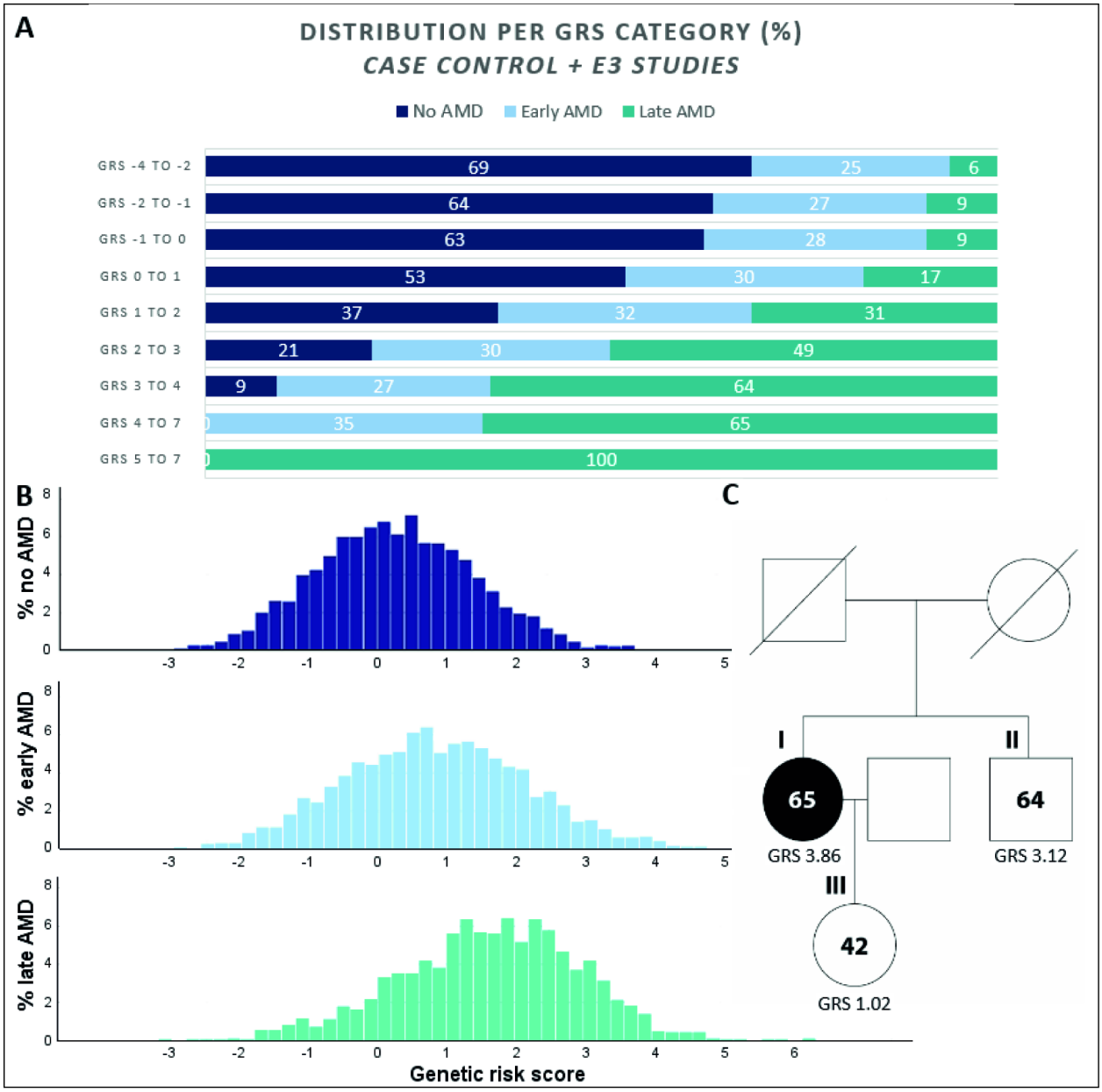
Table detailing the association of rare variants with late AMD. Logistic regression analysis was performed to assess association of the different rare variant categories with late AMD. Reference category: noncarriers. CADD = combined annotation dependent depletion.

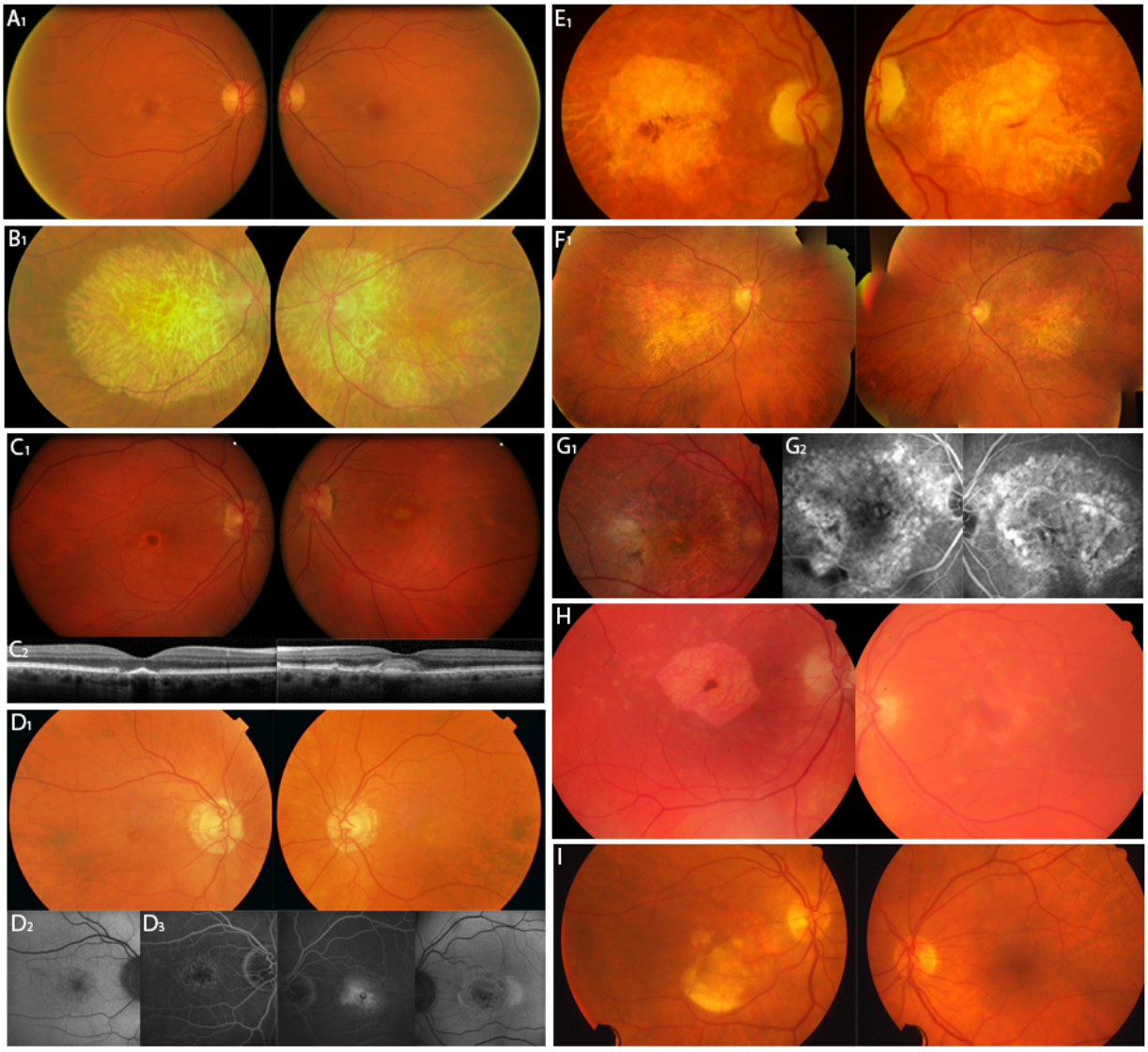
Table 2. Rare and low-frequency variants in inherited macular dystrophy genes

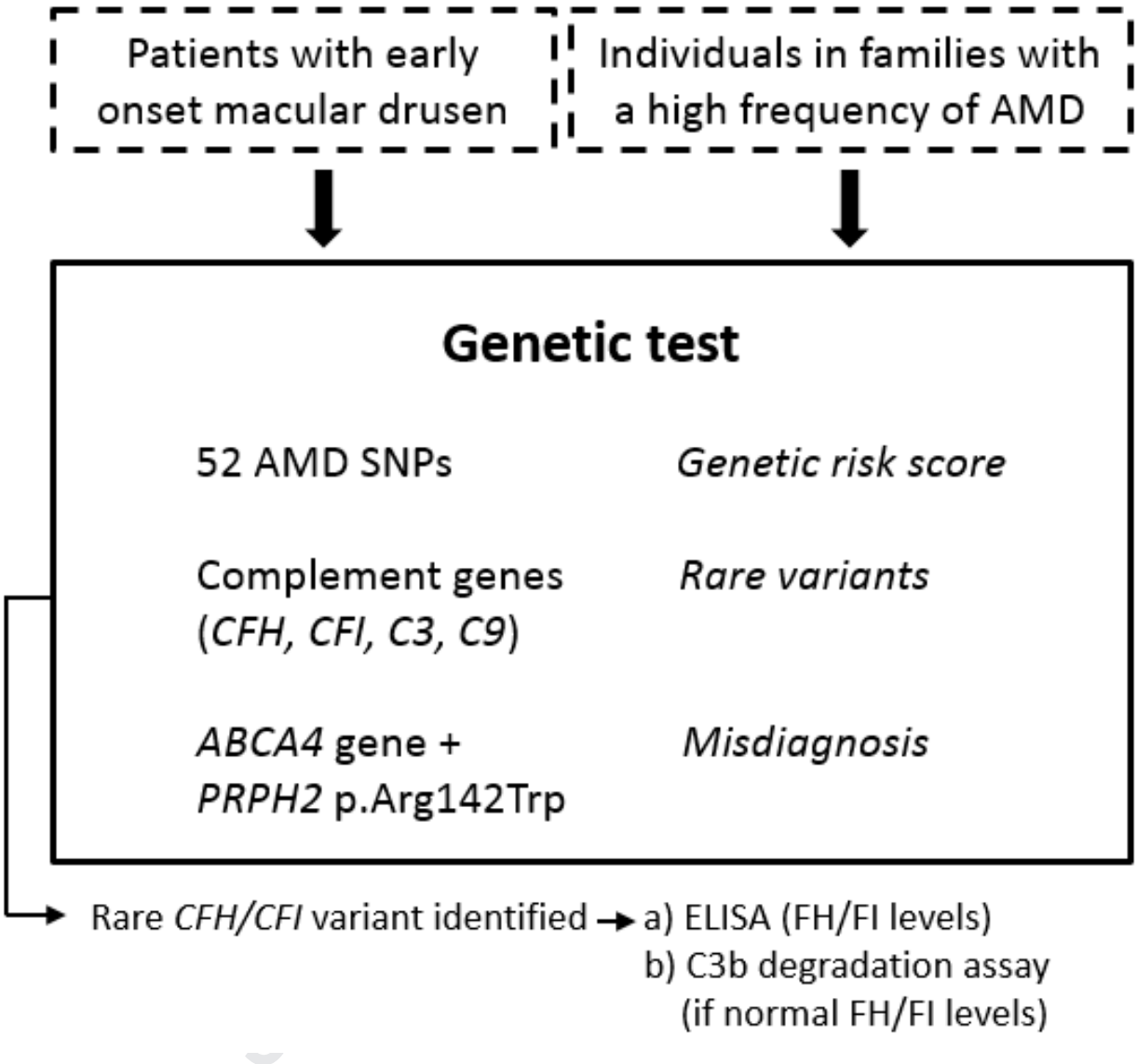
	Variant	MAF gnomAD NFE, %	MAF cases, % n=2770	MAF controls, % n=786	Variant classification (ACMG[41])	Gender	Age	Phenotypic characteristics on retinal imaging
A						F	72	Parafoveal hypopigmentation
B						M	76	Extensive central GA and PPA
C						M	74	RE central hyperpigmentation with atrophy, LE central hypopigmentation
	PRPH2 p.Arg142Trp	0.002	0.04	0.00	Class 5			
D						M	86	Central hypopigmentation on CFP, hyperautofluorescence on FAF and hyperfluorescent signal on FA
E	*ABCA4* p.Ser2255Ile	3.96	3.36	4.20	Class 1	M	67	Extensive central GA with some small yellow deposits at the border of the GA
	ABCA4 p.Asn1868Ile	6.65	6.62	5.03	Class 3			
	ABCA4 p.Cys1488Arg	0.002	0.02	0.00	Class 5			
F	*ABCA4* p.Ala1038Val	0.23	0.32	0.45	Class 4	F	69	Central GA and flecks
	ABCA4 p.Phe608Ile	0.003	0.02	0.00	Class 4			
G	*ABCA4* p.Asn1868Ile	6.65	6.62	5.03	Class 3	F	79	Large central GA in a bull's-eye configuration
	ABCA4 p.Thr901Ala	0.31	0.20	0.06	Class 3			
	ABCA4 p.Arg212His	3.60	2.38	2.10	Class 1			
H	*ABCA4* p.Ser2235*	N/A	0.00	0.06	Class 5	M	80	RE central GA surrounded by yellow deposits, LE paracentral GA with foveal sparing
	ABCA4 p.Asn1868Ile	3.60	2.38	2.10	Class 3			
I	*CTNNA1* p.Arg54Cys	0.00	0.02	0.00	N/A	F	83	Yellow, egg yolk-like lesion inferior in the macula of the RE, with a pseudohypopyon appearance. LE no abnormalities

Table detailing the rare and low-frequency variants in the *ABCA4, CTNNA1* and *PRPH2* genes that were identified in an AMD cohort.
MAF = minor allele frequency, NFE = Non-Finnish European, ACMG = American College of Medical Genetics, N/A = not available, GA = geographic atrophy, RE = right eye, LE = left eye, PPA = peripapillary atrophy, FAF = fundus autofluorescence, FA = fluorescein angiography, CFP = color fundus photograph, [41] Richards et al, 2015.


A
DISTRIBUTION PER GRS CATEGORY (%)
CASE CONTROL STUDIES
No AMD
Early AMD
Late AMD
GRS -3 TO -2
35
41
24
GRS -2 TO -1
41
37
22
GRS -1 TO 0
42
36
22
GRS 0 TO 1
29
36
35
GRS 1 TO 2
15
29
56
GRS 2 TO 3
6
22
72
GRS 3 TO 4
1
18
81
GRS 4 TO 5
2
18
80
GRS 5 TO 7
100
B
% no AMD
% early AMD
% late AMD
Genetic risk score


A
DISTRIBUTION PER GRS CATEGORY (%)
CASE CONTROL + E3 STUDIES
No AMD
Early AMD
Late AMD
GRS -4 TO -2
69
25
6
GRS -2 TO -1
64
27
9
GRS -1 TO 0
63
28
9
GRS 0 TO 1
53
30
17
GRS 1 TO 2
37
32
31
GRS 2 TO 3
21
30
49
GRS 3 TO 4
9
27
64
GRS 4 TO 7
35
65
GRS 5 TO 7
100
B
% no AMD
% early AMD
% late AMD
Genetic risk score
C
I
II
III
65
GRS 3.86
64
GRS 3.12
42
GRS 1.02


A1
E1
B1
F1
C1
G1
G2
C2
H
D1
I
D2
D3


Patients with early onset macular drusen
Individuals in families with a high frequency of AMD
Genetic test
52 AMD SNPs
Genetic risk score
Complement genes (CFH, CFI, C3, C9)
Rare variants
ABCA4 gene + PRPH2 p.Arg142Trp
Misdiagnosis
Rare CFH/CFI variant identified
a) ELISA (FH/FI levels)
b) C3b degradation assay (if normal FH/FI levels)

Précis

This study reports a genetic test for age-related macular degeneration, which can identify individuals at high risk for late age-related macular degeneration, carriers of rare high-risk variants, and potential misdiagnoses with inherited macular dystrophies.