AMERICAN ACADEMY OF OPHTHALMOLOGY®

Development of a Genotype Assay for Age-Related Macular Degeneration

The EYE-RISK Consortium

Anita de Breuk, MD, MSc,[1,] Ilhan E. Acar, MSc,[1,*] Eveline Kersten, MD, PhD,[1] Mascha M.V.A.P. Schijvenaars, BASc,[2] Johanna M. Colijn, MD, MSc,[3,4] Lonneke Haer-Wigman, PhD,[5] Bjorn Bakker, BASc,[1] Sarah de Jong, MSc,[1] Magda A. Meester-Smoor, PhD,[3,4] Timo Verzijden, MSck,[3,4] Tom O.A.R. Missotten, MD,[6] Jordi Monés, MD, PhD,[7,8] Marc Biarnés, MPH, PhD,[7,8] Daniel Pauleikhoff, MD, PhD,[9] Hans W. Hense, PhD,[10] Rufino Silva, MD, PhD,[11,12,13] Sandrina Nunes, MD, PhD,[13] Joana B. Melo, PhD,[14,15] Sascha Fauser, MD, PhD,[16] Carel B. Hoyng, MD, PhD,[1] Marius Ueffing, PhD,[17] Marieke J.H. Coenen, PhD,[2] Caroline C.W. Klaver, MD, PhD,[1,3,4,18] Anneke I. den Hollander, PhD,[1] for the EYE-RISK Consortium*

Purpose: To develop a genotype assay to assess associations with common and rare age-related macular degeneration (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 = 4740) from 5 European cohorts.

Methods: We designed single-molecule molecular inversion probes for target selection and used next generation sequencing to sequence 87 single nucleotide polymorphisms (SNPs), coding and splice-site regions of 10 AMD-(related) genes (*ARMS2*, *C3*, *C9*, *CD46*, *CFB*, *CFH*, *CFI*, *HTRA1*, *TIMP3*, and *SLC16A8*), and 3 genes that cause inherited macular dystrophies (*ABCA4*, *CTNNA1*, and *PRPH2*). Genetic risk scores 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 patients and control individuals with logistic regression analysis.

Main Outcome Measures: Genetic risk score, association of genetic variants with AMD, and genotype−phenotype correlations.

Results: We observed high concordance rates between our platform and other genotyping platforms for the 69 successfully genotyped SNPs (>96%) and for the rare variants (>99%). We observed a higher GRS for patients with late AMD compared with patients with early/intermediate AMD ($P < 0.001$) and individuals without AMD ($P < 0.001$). A higher proportion of pathogenic variants in the *CFH* (odds ratio [OR] = 2.88; $P = 0.006$), *CFI* (OR = 4.45; $P = 0.005$), and *C3* (OR = 6.56; $P = 0.0003$) genes was observed in late AMD patients compared with control individuals. In 9 patients, we identified pathogenic variants in the *PRPH2*, *ABCA4*, and *CTNNA1* genes, which allowed reclassification of these patients as having inherited macular dystrophy.

Conclusions: This study reports a genotype assay for common and rare AMD genetic variants, which 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 because they harbor rare high-risk variants. Carriers of these variants could be amendable for new treatments for AMD that currently are under development. *Ophthalmology 2021;128:1604-1617*

Supplemental material available at www.aaojournal.org.

See Commentary on page 1618.

Age-related macular degeneration (AMD) is a common cause of vision loss in the aging population, with a prevalence of 0.1% in individuals 55 to 59 years of age and rising to 9.8% in individuals 85 years of age and older 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 nongenetic 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

https://doi.org/10.1016/j.ophtha.2020.07.037
ISSN 0161-6420/20

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*), and 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] Most of these variants are common genetic variants, whereas 7 variants are 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 with control individuals. In recent years, the role of rare genetic variants in AMD has gained attention because 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 of AMD developing. Considering that many genetic variants in multiple genes have been associated with AMD, only a comprehensive genotype assay including all risk variants will identify the total genetic risk accurately. Genetic testing for AMD is a contentious area, and the currently available tests mostly are limited to a low number of genetic variants and vary in their predictive ability.[17] This points to a clear need for such an assay.

Besides the limited number of genetic variants included in the tests that are available currently (Macula Risk PGx and Vita Risk [15 genetic variants], http://www.macular-risk.com; 23andMe [2 genetic variants], http://www.23and-me.com; EasyDNA [number of genetic variants unspecified], https://www.easydna.co.uk; and 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 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 (ORs) 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 disease) because 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 genetic risk score (GRS), and to differentiate between AMD and AMD-mimicking dystrophies.

Methods

Study Population

DNA samples from 5 European cohorts contributing to the EYE-RISK database were selected for genotyping: the Coimbra Eye Study (CES), the Combined Ophthalmic Research Rotterdam Biobank, the European Genetic Database (EUGENDA), the Characterization of Geographic Atrophy Progression in Patients with Age-Related Macular Degeneration, and the Muenster Aging and Retina Study. In addition, several induced pluripotent stem cells 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 1 category and used the following categories: no AMD, early or intermediate AMD, and late AMD (geographic atrophy or choroidal neovascularization). In total, 786 individuals without AMD older than 65 years, 1056 individuals with early or intermediate AMD, and 1714 individuals with late AMD were selected for analysis (Table S1, available at 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 www.aaojournal.org). Furthermore, the coding and splice-site regions of 13 genes were sequenced completely. Genes that have been described to carry rare variants in AMD (*C3*, *C9*, *CFH*, *CFI*, *TIMP3*, and *SLC16A8*),[8,10–16] candidate genes that may carry rare variants in AMD (*ARMS2*, *CD46*, *CFB*, and *HTRA1*), and genes involved in AMD-mimicking macular dystrophies (*ABCA4*, *CTNNA1*, and *PRPH2*)[20–22,28] were selected for complete sequencing. In addition, 3 intronic *ABCA4* variants affecting splicing (c.5196+1137G→A, c.5196+1216C→A, and 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 www.aaojournal.org). During the design phase of the smMIPs, 6 SNPs were covered poorly (rs11402250, rs72802342, rs61941274, rs12019136, rs67538023, and rs9708919), including 5 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 www.aaojournal.org), and accompanying smMIPs were designed. No alternative SNP was selected for rs9708919.

Data were analyzed using an in-house smMIP pipeline. We used samtools version 1.4.1 and bcftools version 1.9.20 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 alleles. For validation, we

compared the EYE-RISK smMIPs sequencing data to genotyping data of selected samples of the EUGENDA cohort that were analyzed previously on other genotyping platforms (whole exome sequencing,[4] KASP genotyping (LGC Biosearch Technologies, Middlesex, UK), and exome chip[7]). Concordance rates between the different platforms were calculated. The variants that passed these quality control steps were tested further if they were in Hardy-Weinberg equilibrium.

We compared SNP allele frequencies (AFs) of control individuals (>65 years of age) and late AMD patients in the EYE-RISK dataset with AFs of control individuals and late AMD patients in the IAMDGC dataset.[7] We assessed allelic ORs for all SNPs to test if the SNPs in our study showed the same direction and magnitude of effect compared with the 52 SNPs as reported in the IAMDGC study.[7] Further details with respect to the design of the smMIPs, the smMIP bioinformatics pipeline, and quality control steps are described in the Supplemental Methods (available at 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 described previously 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 or more *ABCA4* variants of class 3 or higher, based on the American College of Medical Genetics and Genomics classification. Retinal images of these carriers were evaluated by a retinal specialist (C.C.W.K.) 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 patients. Allele frequencies with P values of less than 7.2^{-4} (0.05/69) were considered to differ significantly between the datasets. Binary logistic regression analysis based on AF was used to assess allelic ORs for the SNPs. Weighted GRSs 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: $\mathrm{GRS} = \sum_{i=1}^{52}(G_i\,\beta_i)$. G_i 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 1 minor allele, 2 = carrier of 2 minor alleles). β_i represents the effect size of variant i (natural logarithm of the fully conditioned OR of the minor allele of variant i), based on the GWAS of the IAMDGC.[7,38] The GRS of an individual was considered to be missing if the genotype of one of the major risks 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, then we considered this variant in this individual to be missing. Differences in GRS between individuals without AMD, with early or intermediate AMD, and with late AMD were analyzed by a univariate general linear model (SPSS software version 22.0; IBM Corp., Armonk, NY). We compared the GRS distribution in individuals without AMD, with early or intermediate AMD, and with late AMD in our current study with 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 JM, Meester M, Verzijden T, et al, The EYE-RISK consortium. Genetic risk, lifestyle, and AMD in Europe: The EYE-RISK consortium; submitted 2020).

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 of less than 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) of less than 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) combined annotation-dependent depletion (CADD) score of less than 20 and (2) CADD score of 20 or more or loss-of-function (LoF), according to the 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 as missense variants with a described functional effect based on functional studies (Table S4, available at www.aaojournal.org). Another way of categorizing rare variants is according to the Polyphen2 prediction score, for which we used the following categories: (1) benign, (2) possibly damaging, (3) probably damaging, and (4) LoF. We used binary logistic regression analysis to assess association of the different categories of variants with late AMD. P values of less than 0.05 were considered statistically significant. Noncarriers were used as the reference category. In cases of same event status, we applied Firth correction (Statistical Analysis System Institute version 9.4, Cary, NC).

Results

Performance of the Genotype Assay

Of the 87 SNPs, 69 SNPs were genotyped successfully, whereas 11 SNPs were excluded because of low coverage (Fig S1 and Table S5, available at www.aaojournal.org), 5 SNPs were removed because of deviation of Hardy-Weinberg equilibrium, and 2 SNPs were removed because of low genotype concordance with other genotyping platforms (Table S6, available at www.aaojournal.org). The concordance rates between SNPs genotyped with the EYE-RISK smMIPs sequencing platform compared with the whole exome sequencing, KASP genotyping, and exome chip datasets were 96.77%, 97.28%, and 96.96%, respectively (Table S7, available at www.aaojournal.org). To ensure a complete dataset of the 52 AMD-associated variants, we genotyped 10 SNPs by KASP genotype assays. Genotyping and validation of the assays were carried out by LGC Biosearch Technologies (Middlesex, UK) (Table S8, available at www.aaojournal.org).

Ten genes (*ABCA4*, *C3*, *C9*, *CD46*, *CFH*, *CFI*, *CTNNA1*, *PRPH2*, and *TIMP3*) were well covered because at least 95% of the base pairs in these genes were covered at least ×40. For 3 genes (*ARMS2*, *HTRA1*, and *SCL16A8*), a lower percentage (between 70.6% and 83.6%) of the base pairs were covered at least ×40. The lower coverage in these genes was attributed mainly to specific exonic regions in these genes (Tables S9 and S10, available at www.aaojournal.org). The concordance rates of rare variants identified in the EYE-RISK smMIPs dataset compared with the whole exome sequencing dataset was more than 99% (Table S7).

We observed similar AFs for 61 of the 69 SNPs in control individuals as in the previous IAMDGC GWAS study. For late AMD patients, we observed similar AFs for 66 of 69 SNPs (Table S11, available at www.aaojournal.org). Regarding differences in patients, we observed a lower AF in late AMD patients from the EYE-RISK study for *MIR* rs4351242, *C3* (*NRTN*/*FUT6*) rs17855739, and *MMP9* rs142450006 compared with late AMD patients from 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 www.aaojournal.org). The differences in AF in patients were not assigned to a specific cohort. However, for 6 of 8 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 EYE-RISK 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 www.aaojournal.org). The effects of the significantly associated SNPs were all in the same direction compared with those from the IAMDGC study.

Genetic Risk Scores

The GRS for AMD was calculated for 786 individuals without AMD older than 65 years, 1056 early or intermediate AMD patients, and 1714 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; standard deviation, 1.29) compared with patients with early or intermediate AMD (mean, 0.86; standard deviation, 1.27; $P < 0.001$) and individuals without AMD (mean, 0.30; standard deviation, 1.06; $P < 0.001$). We compared the GRS distribution in early or intermediate patients, late AMD patients, and control individuals in our current study with the GRS distribution in the study of Colijn et al and observed a similar distribution of the GRS among the different groups (Colijn JM, Meester M, Verzijden T, et al, The EYE-RISK consortium. Genetic risk, lifestyle, and AMD in Europe: The EYE-RISK consortium; submitted 2020).

Figure 2 demonstrates 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 JM, Meester M, Verzijden T, et al, The EYE-RISK consortium. Genetic risk, lifestyle, and AMD in Europe: The EYE-RISK consortium; submitted 2020). The proband (age, 65 years) was affected by late-stage AMD and demonstrated a GRS of 3.86. Sixty-four percent of the individuals in GRS category 3 to 4 were affected by late-stage AMD. Her 1-year-younger brother demonstrated 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 demonstrated a GRS of 1.02. Thirty-one percent of the individuals within GRS category 1 to 2 were affected by late-stage AMD, whereas 69% were affected by early or 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 an MAF of less than 0.01 and 11 protein-altering variants with an MAF of between 0.01 and 0.05 were identified in 13 genes (Supplemental Dataset 2, available at www.aaojournal.org), based on AF data of European (non-Finnish) individuals (http://gnomad.broadinstitute.org/). In addition, 1 variant (*ABCA4* p.Asn1868Ile) with an MAF of 0.07 was present in the dataset. Most of the variants included missense variants, representing 412 unique variants. Furthermore, we identified several splice-site, nonsense, frameshift, and nonframeshift variants (number of unique variants: 9, 18, 16, and 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 13 genes selected for this project. A higher number of rare LoF variants or variants with a CADD score of 20 or more 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 patients compared with 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 patients compared with control individuals (OR, 1.77; $P = 0.04$). Another interesting finding included the observation of more probably damaging rare variants in late AMD patients compared with control individuals in the *ABCA4* gene (OR, 1.78; $P = 0.03$; Table S14, available at 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 no single variants were statistically significantly associated with late AMD in the single variant analysis, we observed a higher MAF in late AMD patients compared with 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 www.aaojournal.org). All 3 variants represented variants of unknown clinical significance (American College of Medical Genetics and Genomics 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 patients (early or intermediate and late AMD combined) is depicted in Table S14.

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 patients (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

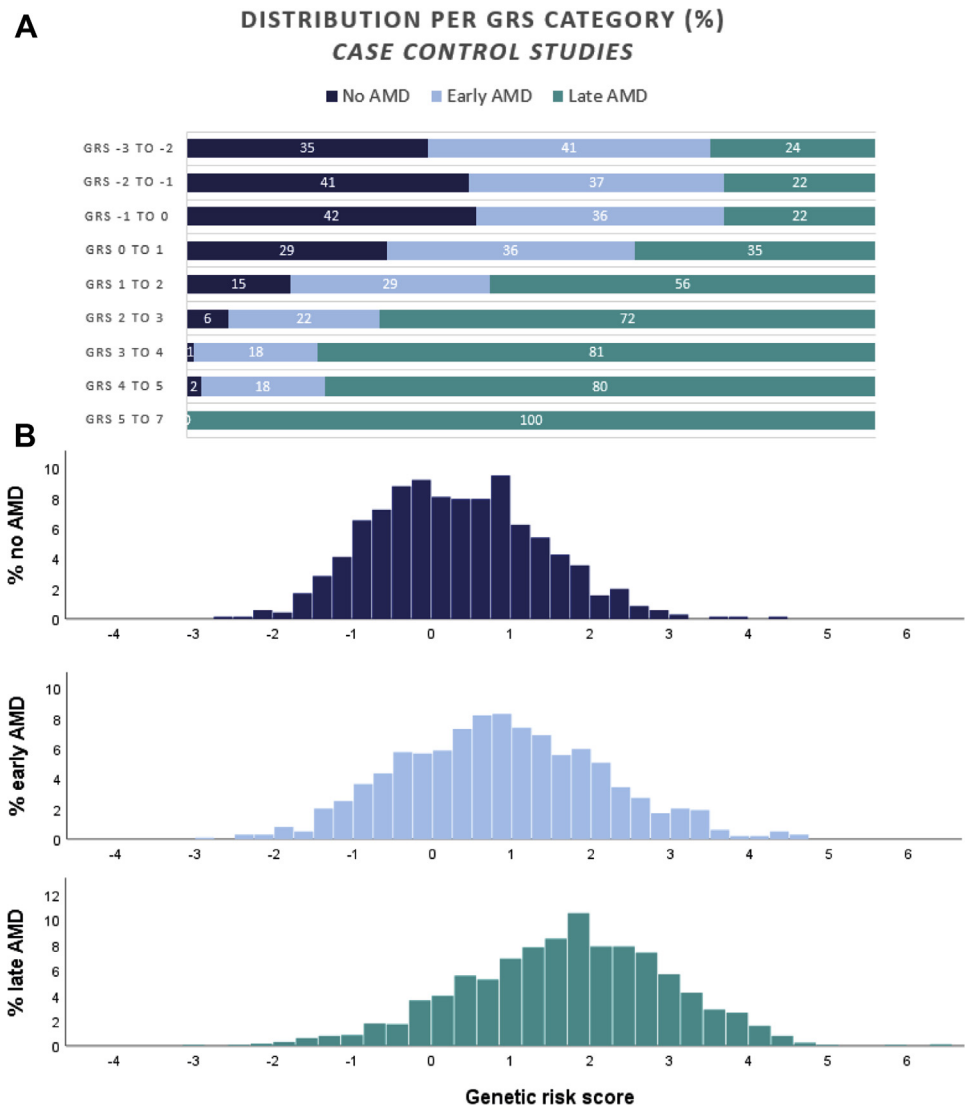

Figure 1. Bar graphs showing the distribution of the genetic risk score (GRS) in case-control studies. **A**, Stratification of the GRS in the different GRS categories. **B**, Distribution of the GRS in individuals without age-related macular degeneration (AMD), early or intermediate AMD, and late AMD.

to cause autosomal-dominant central areolar choroidal dystrophy (CACD),[40] was found in 1 early AMD patient (GRS, −0.89) and 1 late AMD patient (GRS, 2.19) and also in 2 family members (both graded as AMD; GRS, 0.45 and 0.95). The phenotypes of all 4 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, and 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 dystrophy, except for the *PRPH2* p.Trp246Arg carrier. Figure 3 shows the images of the 4 patients diagnosed with AMD primarily with a *PRPH2* p.Arg142Trp variant. The color fundus photographs (CFPs) of the patient in Fig 3A showed an increased parafoveal reflectivity, without clear drusen (Fig 3A1) No abnormalities were observed outside the parafoveal area. In the patient in Fig 3B, a large area of chorioretinal atrophy in both eyes was visible on CFPs (Fig 3B1). The right eye of the patient in Fig 3C was characterized by central hyperpigmentation on CFP (Fig 3C1) and parafoveal photoreceptor loss on OCT (Fig 3C2). The CFP of the left eye showed yellow deposits in the macula (Fig 3C1). The CFPs of the patient in Fig 3D show an increased parafoveal reflectivity (Fig 3D1). Hyperfluorescent parafoveal changes were visible on the corresponding fluorescein angiography images of this patient (Fig 3D3).

Rare Variants in the ABCA4 Gene. Sequencing of the *ABCA4* gene revealed 121 unique, rare protein-altering and splice-site variants in 383 AMD patients (383/5540 alleles [6.91%]) and 101 control individuals (101/1572 alleles [6.42%]; Supplemental Dataset 2). In addition, 3 deep intronic *ABCA4* variants affecting splicing were genotyped. Only 1 of these deep intronic variants (*ABCA4* c.5196+1137G→A) was identified in 3 control individuals younger than 65 years. No second low-frequency variant in the coding or splice-site regions of the *ABCA4* gene was identified in these 3 individuals within the smMIPs dataset. We further analyzed the phenotypes of 18 individuals carrying 2 or more heterozygous *ABCA4* variants that were classified as class 3 or

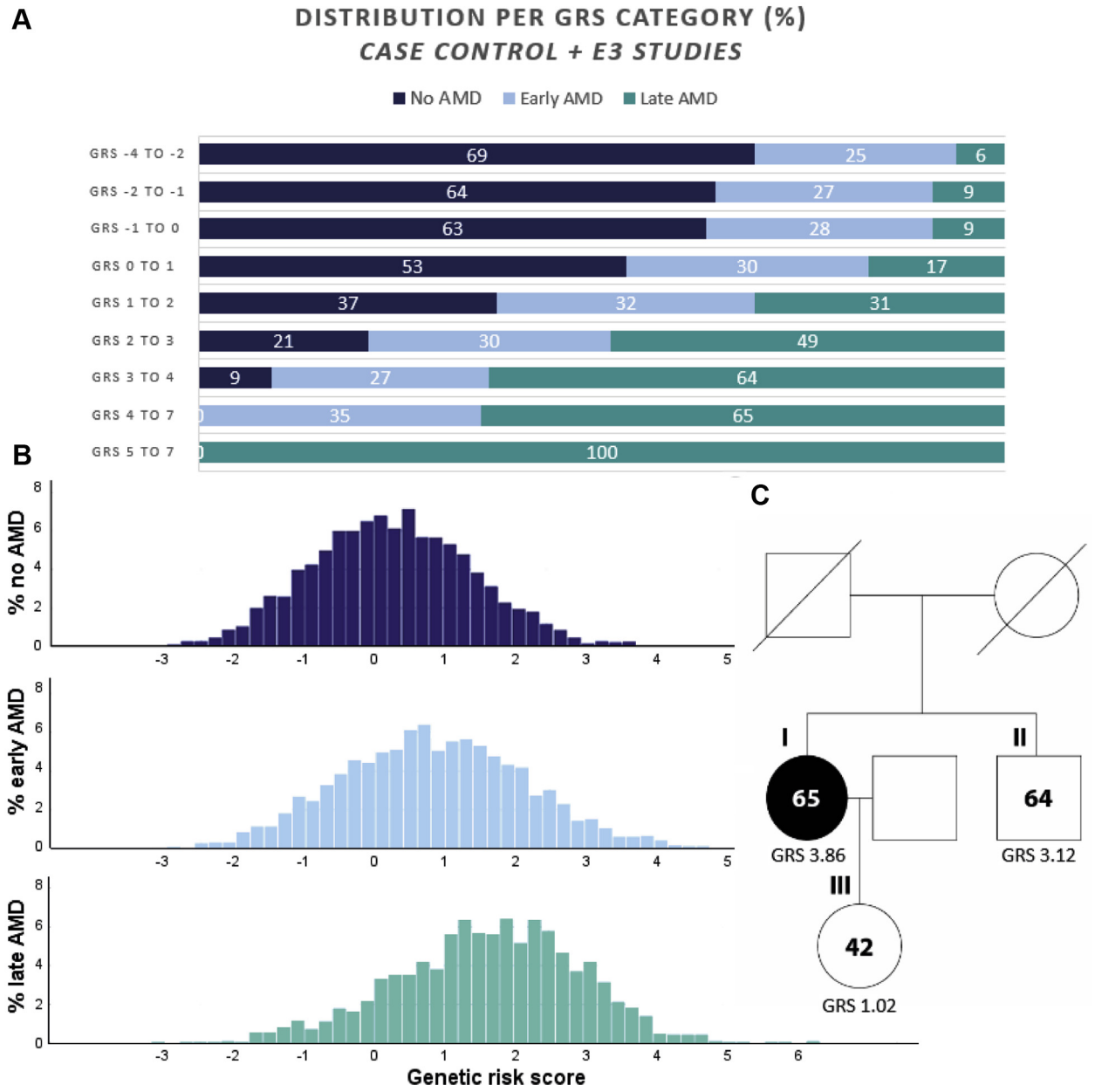

Figure 2. Genetic risk report detailing the distribution of the genetic risk score (GRS) in case-control and population studies combined, including a demonstration of a GRS report based on an example of a small family. **A**, Stratification of the GRS into the different GRS categories. **B**, Genetic risk score distribution among early and intermediate age-related macular degeneration (AMD) patients, late AMD patients, and control individuals based on case-control studies and population studies. **C**, Pedigree in which individual I, 65-year-old woman affected by late-stage AMD, confers a high GRS of 3.86 and individual II, 64-year-old man without signs of AMD, confers a high GRS of 3.12, and individual III, 42-year-old woman without signs of AMD, confers an intermediate GRS of 1.02.

higher based on the American College of Medical Genetics and Genomics classification, although it cannot be deduced from the current genotyping data whether the variants are located on different alleles. In 4 patients, both the genotype and the phenotype suggested (late-onset) Stargardt disease (Fig 3E–H; Table 2). The overall GRS in these patients was low to intermediate (−1.47, 0.19, 1.80, and 2.39).

Rare Variants in the *CTNNA1* Gene. Screening of the *CTNNA1* gene revealed 20 unique rare missense variants in 51 AMD patients (51/5540 alleles [0.92%]) and 12 control individuals (12/1572 alleles [0.76%]). Rare variants that were described previously to cause a butterfly-shaped pigment dystrophy (p.Leu318Ser, p.Ile431Met, and p.Glu307Lys) were not identified in any of the individuals in this study.[28] For 1 variant (p.Arg54Cys), the pathogenicity remains unclear.[28] We identified 1 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

Table 1. Association of Rare Variants with Age-Related Macular Degeneration

Rare Variant Carriers Categorized by Combined Annotation-Dependent Depletion Score	Controls (n = 786), No. (%)	Late Age-Related Macular Degeneration Patients (n = 1714), No. (%)	Odds Ratio (95% Confidence Interval)	P Value
C3				
Noncarrier	761 (96.82)	1623 (96.82)	1 (Reference)	
Carrier: CADD score, <20)	21 (2.67)	35 (2.04)	0.781 (0.452–1.352)	0.378
Carrier: CADD score, ≥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 score, <20	22 (2.80)	37 (2.16)	0.775 (0.454–1.323)	0.351
Carrier: CADD score, ≥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 score, <20	9 (1.15)	23 (1.34)	1.199 (0.552–2.604)	0.646
Carrier: CADD score, ≥20 or loss of function	4 (0.51)	38 (2.22)	4.450 (1.584–12.503)	0.005

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

dystrophy, we did observe an egg-yolk lesion in 1 eye, which is also observed in patients with Best vitelliform macular dystrophy (Fig 3I; 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 the literature that specific mutations in the *TIMP3* gene can cause Sorsby's fundus dystrophy (SFD).[41] Caution is always required in AMD patients with choroidal neovascularization because phenotypic characteristics of SFD and AMD can show overlap. We identified 2 individuals in this study carrying a rare variant in the *TIMP3* gene (p.Pr077Ser). This mutation is not among 1 of the 16 mutations that have been associated with SFD previously.[41] Both patients (age, >70 years) were graded as having neovascular AMD. One of the patients demonstrated choroidal neovascularization in both eyes without any drusen, which phenotypically raised suspicion for SFD (Fig S2, available at 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 genotype successfully 69 SNPs and the coding and splice-site regions of 10 AMD-related genes and 3 dystrophy genes. We computed GRSs for AMD patients and control individuals and observed high GRSs predominantly in patients with late AMD, whereas low GRSs were observed more commonly in control individuals. With regard to the role of rare genetic variants, we observed a higher occurrence of rare LoF variants or variants with a CADD score of 20 or more in the *CFH*, *CFI*, and *C3* genes in late AMD patients compared with control individuals. Furthermore, we highlighted the importance of sequencing the *PRPH2* and *ABCA4* genes by revealing that in 9 patients, both genotype and phenotype pointed toward inherited macular dystrophy, rather than AMD.

Population Differences in Allele Frequencies

Allele frequencies of most of the SNPs in patients (66/69) and control individuals (61/69) included in our study were comparable with AFs in patients 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 6 of these 8 SNPs. For example, we observed an MAF of 0.500, 0.503, 0.512, and 0.311 for *CFI* rs10033900 within control individuals of the Combined Ophthalmic Research Rotterdam Biobank, EUGENDA, Muenster Aging and Retina Study, and CES cohorts, respectively. An MAF of 0.477 was reported for this particular SNP within the IAMDGC study. Because the different distribution in the CES cohort was limited to only these 6 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 with other European populations.

Genetic Risk Score

Within our data, we observed a significantly higher GRS in individuals with late AMD compared with both patients with early or intermediate AMD and control individuals. Genetic risk profiling allowed us to identify individuals who carried an intermediate and high genetic risk for AMD. Despite the substantial differences in GRS among control individuals, early and intermediate AMD patients, and late AMD patients, an overlap remained between the groups, and therefore, one cannot completely distinguish the 3 groups based on GRS only. Furthermore, we reported genetic risk based on prevalence data of a large group of patients and control individuals. Unfortunately, follow-up data

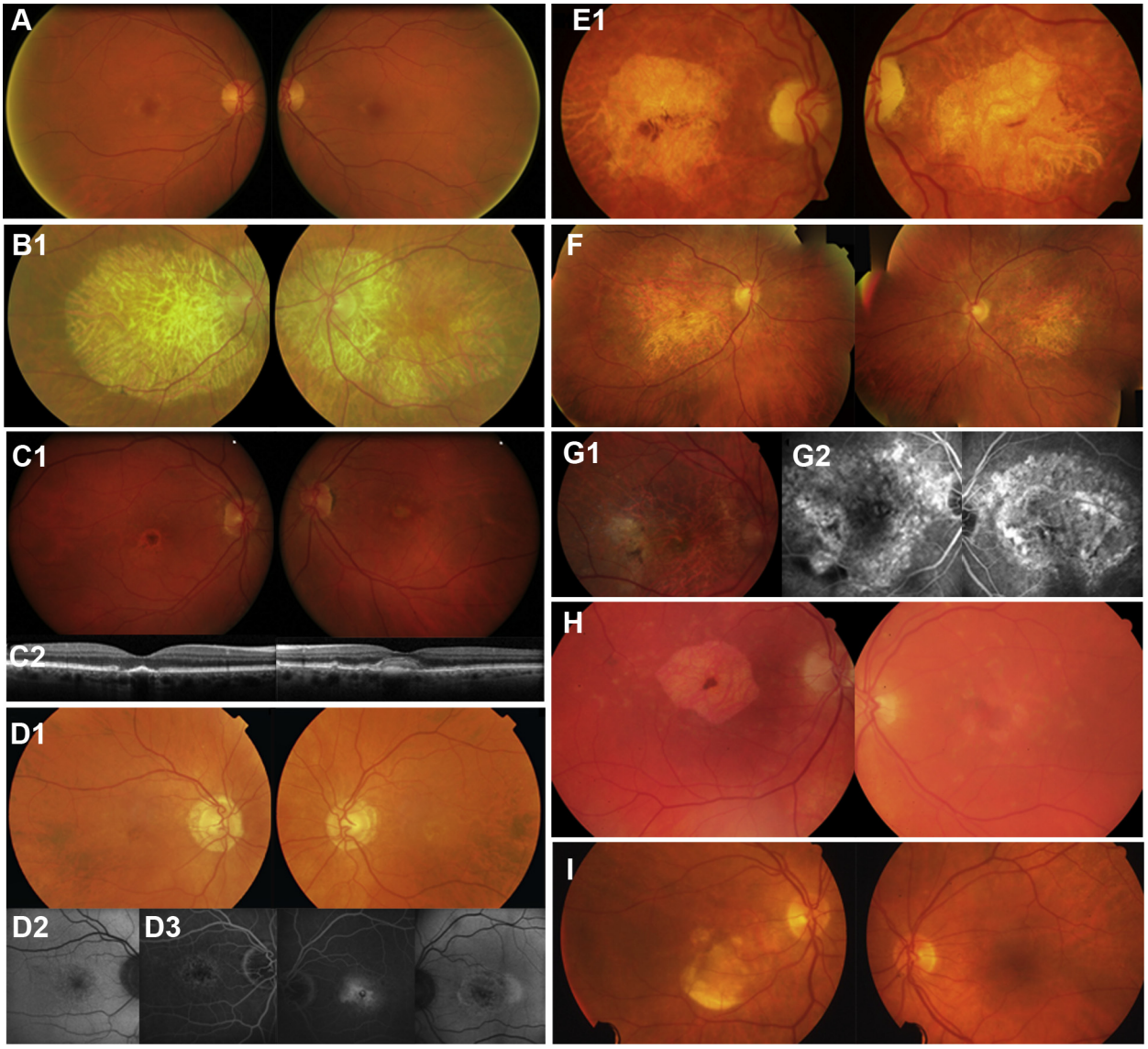

Figure 3. Retinal images showing phenotypic characteristics of carriers harboring 1 or more rare and low-frequency variants in the *ABCA4*, *CTNNA1*, and *PRPH2* genes: (**A**–**D**) individuals carrying the *PRPH2* p.Arg142Trp variant heterozygous, (**E**–**H**) individuals carrying 2 or more *ABCA4* variants, and (**I**) individuals carrying the *CTNNA1* p.Arg54Cys variant heterozygous.

were 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 LoF variants and variants with a CADD score of 20 or more in patients compared with control individuals for most of the complement genes tested within this study. Our study underlined the important role of the complement system, but its crucial role also was demonstrated in the study of Colijn et al, whose results showed that the complement system was the main driving pathway in AMD (Colijn JM, Meester M, Verzijden T, et al, The EYE-RISK consortium. Genetic risk, lifestyle, and AMD in Europe: The EYE-RISK consortium; submitted 2020). 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 most rare variants, the functional effect currently remains 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

Table 2. Rare and Low-Frequency Variants in Inherited Macular Dystrophy Genes

Individual	Variant	Minor Allele Frequency			Variant Classification (American College of Medical Genetics[41])	Gender	Age (yrs)	Phenotypic Characteristics on Retinal Imaging
		gnomAD non-Finnish European (%)	*Patients (%; n = 2770)*	*Control Participants (%; n = 786)*				
A	*PRPH2* p.Arg142Trp	0.002	0.04	0.00	Class 5	F	72	Parafoveal hypopigmentation
B						M	76	Extensive central GA and PPA
C						M	74	RE central hyperpigmentation with atrophy, LE central hypopigmentation
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

CFP = color fundus photograph; F = female; FA = fluorescein angiography; FAF = fundus autofluorescence; GA = geographic atrophy; LE = left eye; M = male; N/A = not available; NFE = Non-Finnish European; PPA = peripapillary atrophy; RE = right eye.

recombinant adeno-associated virus targeting complement factor I (clinicaltrialsregister.eu identifier, 2019-003421-22) and GEM103, a recombinant factor H protein (ClinicalTrials.gov identifier, 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 potentially could be attributed to the smaller sample size compared with the GWAS of the IAMDGC study. Furthermore, 2 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 9 potential misdiagnoses of inherited macular dystrophies. All 9 individuals were diagnosed primarily with AMD (both early and late stages). However, after critical evaluation of the retinal images of these individuals, 4 individuals were most likely affected by CACD, 4 individuals were most likely affected by (late-onset) Stargardt disease, and 1 individual demonstrated a phenotype similar to Best vitelliform macular dystrophy. It is also worth noting that none of these 9 individuals demonstrated a very high GRS (range, −1.47 to 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 of the 86 variants included variants with a CADD score of 20 or more, which indicates that they potentially could 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 patients compared with control individuals in the *ABCA4* gene (69 [4.03%] vs. 18 [2.29%]; OR, 1.78 [95% confidence interval, 1.05−3.02]; $P = 0.03$). A potential link between AMD and Stargardt disease was 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 found only when categorizing the rare variants according to the Polyphen2 prediction score, and because the other categories (LoF and possibly damaging variants) did not show the same effect, not enough evidence exists in our data that supports this potential link. Sequence analysis in larger AMD cohorts is required to investigate further 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 also is important for future clinical trials. Because of 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 (e.g., *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 be easily confused, not only in the late stages, but also in the early stages of the disease.[22] Furthermore, in 4 individuals with a large area of atrophy and in some patients with yellow deposits in the macula, 2 or more *ABCA4* variants of class 3 or higher were identified, which in conclusion match with the diagnosis of (late-onset) Stargardt disease. Results of this study demonstrate that in some patients, 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 is 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 cause of AMD and how that affects the subsequent advice to the patient and family members. The lack of treatment options also was 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 reconsidered.

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, for example, 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. Regardless of this argument, identification of rare variant carriers and calculation of a GRS also is 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 that 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 a high risk of AMD, whereas other rare variants do not influence the protein or are even protective for AMD.[19] For most rare variants, the functional effect currently is unknown. When rare variants in the *CFH* or *CFI* genes are identified, we recommend performing an enzyme-linked immunosorbent assay to determine Factor H (FH) or Factor I (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 patients, functional assays such as a C3b degradation assay can be performed (Fig 4). Patients carrying rare variants with either decreased protein levels or reduced functionality are eligible for clinical trials.

The importance of a healthy lifestyle, cessation of smoking, and the use of antioxidant supplements has been demonstrated already[61,62] and should be advised to all AMD patients, regardless of their genetic profile. Whether patients with a high genetic risk benefit more from such lifestyle modifications needs to be investigated further. The study of Colijn et al provided interesting findings. The authors observed that an unhealthy lifestyle resulted in a 2-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 JM, Meester M, Verzijden T, et al, The EYE-RISK consortium. Genetic risk, lifestyle, and AMD in Europe: The EYE-RISK consortium; submitted 2020).

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 lifetime 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 recommend computing an overall GRS based on the 52 AMD-associated SNPs and in addition sequencing 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, because in some (familial) patients, a high suspicion that rare variants are involved already exists. Furthermore, one may consider including the *PRPH2* p.Arg142Trp variant in the genetic test and sequencing the coding and splice-site regions of the *ABCA4* gene. Despite critical evaluation of the patients' phenotypes, 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 patients (Fig 4). Considering the complexity of AMD, it is essential to obtain an accurate genetic testing report, and therefore, we recommend performing genetic testing in a Clinical Laboratories Improvement Amendments- or IS015189-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

Because 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 it in individuals of non-European descent. Another limitation is the relatively small number of control individuals compared with the patients who were included in this study. Although ideally, the number of control individuals should be higher, we decided to exclude individuals without AMD younger than 65 years because a reasonable chance exists that AMD still could develop in those individuals. To maintain a substantial control group, we set the threshold at 65 years of age. Finally, the design of some smMIPs failed, and the coverage of some regions was low; therefore, the smMIPs assay will need to be optimized before implementation of the genetic test into the clinic.

In conclusion, within the EYE-RISK project, we developed a comprehensive genotype assay that 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 suggested that the *CFH*, *CFI*, *C3*, and *C9* genes also should be sequenced because rare LoF variants and variants with a CADD score of 20 or more in these genes can confer a high risk for AMD, and carriers of these variants could be amendable for new (targeted) treatments that currently are being developed for AMD. Furthermore, this study emphasized that

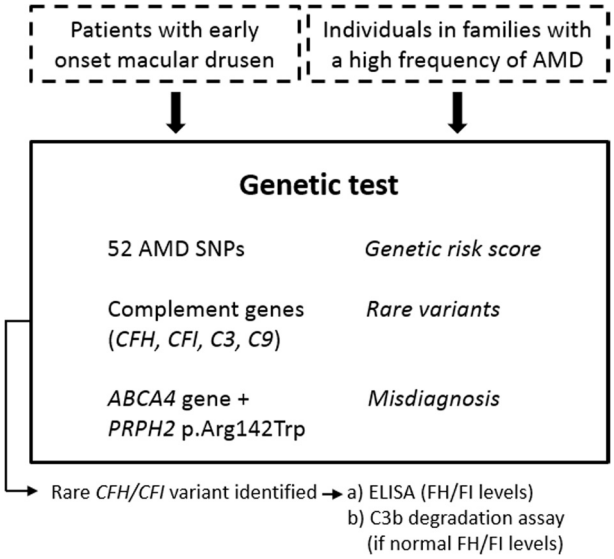

Figure 4. Flow chart showing genetic testing for specific subgroups that might benefit from genetic testing for age-related macular degeneration (AMD). ELISA = enzyme-linked immunosorbent assay; FH = Factor H; FI = Factor I; SNP = single nucleotide polymorphism.

sequencing inherited macular dystrophy genes confers the potential benefit of avoiding serious misdiagnoses.

Acknowledgments

The authors thank the International Age-Related Macular Degeneration Genomics Consortium for making the allele frequencies and odds ratios of the 52 age-related macular degeneration variants and several additional variants available for comparing allele frequencies and computing genetic risk scores, Johanne Groothuismink for technical assistance, Johannes Groenewoud for assisting in statistical analyses, and Birte Claes for her involvement in the data management of the Muenster Aging and Retina Study cohort.

Footnotes and Disclosures

Originally received: March 21, 2020.
Final revision: June 30, 2020.
Accepted: July 16, 2020.
Available online: July 25, 2020. Manuscript no. D-20-00604.

[1] Department of Ophthalmology, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, Nijmegen, The Netherlands.

[2] Department of Human Genetics, Radboud University Medical Center, Radboud Institute for Health Sciences, Nijmegen, The Netherlands.

[3] Department of Ophthalmology, Erasmus Medical Center, Rotterdam, The Netherlands.

[4] Department of Epidemiology, Erasmus Medical Center, Rotterdam, The Netherlands.

[5] Department of Human Genetics, Donders Centre for Neuroscience, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.

[6] The Rotterdam Eye Hospital, Rotterdam, The Netherlands.

[7] Barcelona Macula Foundation, Barcelona, Spain.

[8] Institut de la Màcula, Barcelona, Spain.

[9] Department of Ophthalmology, St. Franziskus Hospital, Münster, Germany.

[10] Institute of Epidemiology and Social Medicine, Westfälische Wilhelms University, Münster, Germany.

[11] Department of Ophthalmology, Centro Hospitalar e Universitário de Coimbra (CHUC), Coimbra, Portugal.

[12] Coimbra Institute for Clinical and Biomedical Research, Faculty of Medicine, University of Coimbra (iCBR-FMUC), Coimbra, Portugal.

[13] Association for Innovation and Biomedical Research on Light and Image (AIBILI), Coimbra, Portugal.

[14] Cytogenetics and Genomics Laboratory, Faculty of Medicine, University of Coimbra, Coimbra, Portugal.

[15] iCBR-CIMAGO, Center of Investigation on Environment, Genetics and Oncobiology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal.

[16] Department of Ophthalmology, University Hospital of Cologne, Cologne, Germany.

[17] Centre for Ophthalmology, Institute for Ophthalmic Research, University of Tübingen, Tübingen, Germany.

[18] Institute of Molecular and Clinical Ophthalmology, Basel, Switzerland.

*Both authors contributed equally as first authors.

Disclosure(s):

All authors have completed and submitted the ICMJE disclosures form.

The author(s) have made the following disclosure(s): J.M.: Consultant — Novartis, Alcon, Bayer, Iveric Bio, Notal Vision, Roche, Cellcure, Genentech; Financial support — Novartis, Alcon, Allergan, Bayer, Iveric Bio, Ophthotech, Notal Vision, Roche, Apellis, Cellcure, Kodiak Sciences, Lineage Cell Therapeutics, Maculogix, Reneuron, Genentech; Lecturer — Novartis, Iveric Bio, Roche, Apellis, Genentech, M.B.: Financial support — Roche, Bayer, R.S.: Advisory board — Allergan, Alimera, Bayer, Novartis, Roche, THEA, NovoNordisk, S.F.: Employee — Roche, A.I.dH.: Consultant — Ionis Pharmaceuticals, Gyroscope Therapeutics, Gemini Therapeutics, Roche; Financial support — F. Hoffmann-La Roche Ltd.

Supported by the Dutch Organization for Scientific Research (grant no.: 016.Vici.170.024 [A.I.d.H.]); the European Union Horizon 2020 Research and Innovation Programme (grant no.: 634479 [EYE-RISK]); F. Hoffmann-La Roche, Ltd., Basel, Switzerland. The sponsor or funding organizations had no role in the design or conduct of this research.

The EYE-RISK Consortium: Université Bordeaux, Inserm, Bordeaux Population Health Research Center, Bordeaux, France: Soufiane Ajana, Audrey Cougnard-Grégoire, Cécile Delcourt, Bénédicte M. J. Merle; Institute for Ophthalmic Research, Eberhard Karls University Tübingen, University Clinic Tübingen, Tübingen, Germany: Blanca Arango-Gonzalez, Sascha Dammeier, Sigrid Diether, Sabina Honisch, Ellen Kilger, Marius Ueffing; AYOXXA Biosystems GmbH, Cologne, Germany: Tanja Endermann, Markus Zumbansen; Pro-Retina Deutschland, Aachen, Germany: Franz Badura; Department of Regeneration and Cell Therapy, Andalusian Molecular Biology and Regenerative Medicine Centre (CABIMER), Seville, Spain: Berta De la Cerda; Barcelona Macula Foundation, Barcelona, Spain: Marc Biarnés, Anna Borrell, Lucia L. Ferraro, Míriam Garcia, Jordi Monés, Eduardo Rodríguez; Department of Epidemiology, Erasmus Medical Center, Rotterdam, The Netherlands: Johanna M. Colijn, A. Ikram, Caroline C. W. Klaver, Magda Meester-Smoor, Timo Verzijden, Johannes Vingerling; Radboud University Medical Center, Nijmegen, The Netherlands: Anneke I. den Hollander, Thomas J. Heesterbeek, Caroline C. W. Klaver, Eveline Kersten, Eiko K. de Jong, I. Erkin Acar, Anita de Breuk; Centre for Experimental Medicine, Queen's University Belfast, Belfast, United Kingdom: Eszter Emri, Imre Lengyel; Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd., Basel, Switzerland: Hanno Langen, Everson Nogoceke; Centre for Public Health, Queen's University Belfast, Belfast, United Kingdom: Tunde Peto; Institute of Ophthalmology, University College London, London, United Kingdom: Phil Luthert, Frances M. Pool.

HUMAN SUBJECTS: Human subjects were included in this study. The institutional review boards of the Radboud University Medical Center (Nijmegen, the Netherlands), the Erasmus Medical Center (Rotterdam, the Netherlands), the Barcelona Macula Foundation (Barcelona, Spain), the St. Franziskus Hospital (Muenster, Germany), the University of Coimbra (Coimbra, Portugal), and the University of Cologne (Cologne, Germany) approved the study. Informed consent was obtained from all individuals according to the tenets of the Declaration of Helsinki.

No animal subjects were included in this study.

Author Contributions:

Conception and design: Kersten, Schijvenaars, Coenen, den Hollander

Analysis and interpretation: de Breuk, Acar, Colijn, Haer-Wigman, de Jong, Meester-Smoor, Coenen, Klaver, den Hollander

Data collection: de Breuk, Acar, Kersten, Schijvenaars, Bakker, Meester-Smoor, Verzijden, Missotten, Monés, Biarnés, Pauleikhoff, Hense, Silva, Nunes, Melo, Fauser, Hoyng, Ueffing, Coenen, den Hollander

Obtained funding: Ueffing, Klaver, den Hollander; Study was performed as a part of the authors' regular employment duties.

Overall responsibility: de Breuk, Acar, Colijn, Klaver, den Hollander

Abbreviations and Acronyms:
AF = allele frequency; **AMD** = age-related macular degeneration; **CADD** = combined annotation-dependent depletion; **CACD** = central areolar choroidal dystrophy; **CES** = Coimbra Eye Study; **CFP** = color fundus photograph; **EUGENDA** = European Genetic Database; **GRS** = genetic risk score; **GWAS** = genome-wide association study; **IAMDGC** = International Age-Related Macular Degeneration Genomics Consortium; **LoF** = loss-of-function; **MAF** = minor allele frequency; **NA** = not applicable; **N/A** = not available; **ND** = not determined; **OR** = odds ratio; **SFD** = Sorsby's fundus dystrophy; **smMIP** = single-molecule molecular inversion probe; **SNP** = single nucleotide polymorphism.

Keywords:
Age-related macular degeneration, Genetics, Genetic counseling, Genetic testing.

Correspondence:
Anneke I. den Hollander, PhD, Department of Ophthalmology (409), Radboud University Medical Center, Philips van Leydenlaan 15, 6525EX Nijmegen, The Netherlands. E-mail: Anneke.denHollander@radboudumc.nl.

References

1. Colijn JM, Buitendijk GHS, Prokofyeva E, et al. Prevalence of age-related macular degeneration in Europe: the past and the future. *Ophthalmology*. 2017;124(12):1753−1763.
2. Wong WL, Su X, Li X, et al. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. *Lancet Glob Health*. 2014;2(2):e106−e116.
3. Seddon JM, Cote J, Page WF, et al. The US Twin Study of age-related macular degeneration: relative roles of genetic and environmental influences. *Arch Ophthalmol*. 2005;123(3):321−327.
4. Corominas J, Colijn JM, Geerlings MJ, et al. Whole-exome sequencing in age-related macular degeneration identifies rare variants in COL8A1, a component of Bruch's membrane. *Ophthalmology*. 2018;125(9):1433−1443.
5. Fritsche LG, Chen W, Schu M, et al. Seven new loci associated with age-related macular degeneration. *Nat Genet*. 2013;45(4):433−439, 439e1−439e2.
6. Fritsche LG, Fariss RN, Stambolian D, et al. Age-related macular degeneration: genetics and biology coming together. *Annu Rev Genomics Hum Genet*. 2014;15:151−171.
7. Fritsche LG, Igl W, Bailey JN, et al. A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. *Nat Genet*. 2016;48(2):134−143.
8. Seddon JM, Yu Y, Miller EC, et al. Rare variants in CFI, C3 and C9 are associated with high risk of advanced age-related macular degeneration. *Nat Genet*. 2013;45(11):1366−1370.
9. Yu Y, Reynolds R, Fagerness J, et al. Association of variants in the LIPC and ABCA1 genes with intermediate and large drusen and advanced age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2011;52(7):4663−4670.
10. Duvvari MR, Paun CC, Buitendijk GH, et al. Analysis of rare variants in the C3 gene in patients with age-related macular degeneration. *PLoS One*. 2014;9(4):e94165.
11. van de Ven JP, Nilsson SC, Tan PL, et al. A functional variant in the CFI gene confers a high risk of age-related macular degeneration. *Nat Genet*. 2013;45(7):813−817.
12. Boon CJ, Klevering BJ, Hoyng CB, et al. Basal laminar drusen caused by compound heterozygous variants in the CFH gene. *Am J Hum Genet*. 2008;82(2):516−523.
13. van de Ven JP, Boon CJ, Fauser S, et al. Clinical evaluation of 3 families with basal laminar drusen caused by novel mutations in the complement factor H gene. *Arch Ophthalmol*. 2012;130(8):1038−1047.
14. Yu Y, Triebwasser MP, Wong EK, et al. Whole-exome sequencing identifies rare, functional CFH variants in families with macular degeneration. *Hum Mol Genet*. 2014;23(19):5283−5293.
15. Wagner EK, Raychaudhuri S, Villalonga MB, et al. Mapping rare, deleterious mutations in factor H: association with early onset, drusen burden, and lower antigenic levels in familial AMD. *Sci Rep*. 2016;6:31531.
16. Zhan X, Larson DE, Wang C, et al. Identification of a rare coding variant in complement 3 associated with age-related macular degeneration. *Nat Genet*. 2013;45(11):1375−1379.
17. Buitendijk GH, Amin N, Hofman A, et al. Direct-to-consumer personal genome testing for age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2014;55(10):6167−6174.
18. Neveling K, Mensenkamp AR, Derks R, et al. BRCA testing by single-molecule molecular inversion probes. *Clin Chem*. 2017;63(2):503−512.
19. Geerlings MJ, de Jong EK, den Hollander AI. The complement system in age-related macular degeneration: a review of rare genetic variants and implications for personalized treatment. *Mol Immunol*. 2017;84:65−76.
20. Saksens NT, Fleckenstein M, Schmitz-Valckenberg S, et al. Macular dystrophies mimicking age-related macular degeneration. *Prog Retin Eye Res*. 2014;39:23−57.
21. Kersten E, Geerlings MJ, Pauper M, et al. Genetic screening for macular dystrophies in patients clinically diagnosed with dry age-related macular degeneration. *Clin Genet*. 2018;94(6):569−574.
22. Smailhodzic D, Fleckenstein M, Theelen T, et al. Central areolar choroidal dystrophy (CACD) and age-related macular degeneration (AMD): differentiating characteristics in multimodal imaging. *Invest Ophthalmol Vis Sci*. 2011;52(12):8908−8918.
23. Cachulo M da L, Lobo C, Figueira J, et al. Prevalence of age-related macular degeneration in Portugal: the Coimbra Eye Study—report 1. *Ophthalmologica*. 2015;233(3−4):119−127.
24. Ristau T, Ersoy L, Lechanteur Y, et al. Allergy is a protective factor against age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2014;55(1):210−214.
25. Biarnes M, Arias L, Alonso J, et al. Increased fundus autofluorescence and progression of geographic atrophy secondary to age-related macular degeneration: the GAIN study. *Am J Ophthalmol*. 2015;160(2):345−353.e5.
26. Heesterbeek TJ, de Jong EK, Acar IE, et al. Genetic risk score has added value over initial clinical grading stage in predicting disease progression in age-related macular degeneration. *Sci Rep*. 2019;9(1):6611.
27. Buitendijk GHS, Rochtchina E, Myers C, et al. Prediction of age-related macular degeneration in the general population: the Three Continent AMD Consortium. *Ophthalmology*. 2013;120(12):2644−2655.
28. Saksens NT, Krebs MP, Schoenmaker-Koller FE, et al. Mutations in CTNNA1 cause butterfly-shaped pigment dystrophy and perturbed retinal pigment epithelium integrity. *Nat Genet*. 2016;48(2):144−151.
29. Braun TA, Mullins RF, Wagner AH, et al. Non-exomic and synonymous variants in ABCA4 are an important cause of Stargardt disease. *Hum Mol Genet*. 2013;22(25):5136−5145.

30. Boyle EA, O'Roak BJ, Martin BK, et al. MIPgen: optimized modeling and design of molecular inversion probes for targeted resequencing. *Bioinformatics*. 2014;30(18):2670–2672.
31. Cornelis SS, Bax NM, Zernant J, et al. In silico functional meta-analysis of 5,962 ABCA4 variants in 3,928 retinal dystrophy cases. *Hum Mutat*. 2017;38(4):400–408.
32. Renner AB, Fiebig BS, Weber BH, et al. Phenotypic variability and long-term follow-up of patients with known and novel PRPH2/RDS gene mutations. *Am J Ophthalmol*. 2009;147(3):518–530.e1.
33. Jacobson SG, Cideciyan AV, Kemp CM, et al. Photoreceptor function in heterozygotes with insertion or deletion mutations in the RDS gene. *Invest Ophthalmol Vis Sci*. 1996;37(8):1662–1674.
34. Kohl S, Christ-Adler M, Apfelstedt-Sylla E, et al. RDS/peripherin gene mutations are frequent causes of central retinal dystrophies. *J Med Genet*. 1997;34(8):620–626.
35. Boon CJ, den Hollander AI, Hoyng CB, et al. The spectrum of retinal dystrophies caused by mutations in the peripherin/RDS gene. *Prog Retin Eye Res*. 2008;27(2):213–235.
36. Boon CJ, van Schooneveld MJ, den Hollander AI, et al. Mutations in the peripherin/RDS gene are an important cause of multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus. *Br J Ophthalmol*. 2007;91(11):1504–1511.
37. Stone EM, Andorf JL, Whitmore SS, et al. Clinically focused molecular investigation of 1000 consecutive families with inherited retinal disease. *Ophthalmology*. 2017;124(9):1314–1331.
38. Grassmann F, Kiel C, Zimmermann ME, et al. Genetic pleiotropy between age-related macular degeneration and 16 complex diseases and traits. *Genome Med*. 2017;9(1):29.
39. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res*. 2010;38(16):e164.
40. Hoyng CB, Heutink P, Testers L, et al. Autosomal dominant central areolar choroidal dystrophy caused by a mutation in codon 142 in the peripherin/RDS gene. *Am J Ophthalmol*. 1996;121(6):623–629.
41. Anand-Apte B, Chao JR, Singh R, Stohr H. Sorsby fundus dystrophy: insights from the past and looking to the future. *J Neurosci Res*. 2019;97(1):88–97.
42. Bienaime F, Dragon-Durey MA, Regnier CH, et al. Mutations in components of complement influence the outcome of factor I-associated atypical hemolytic uremic syndrome. *Kidney Int*. 2010;77(4):339–349.
43. Dragon-Durey MA, Fremeaux-Bacchi V, Loirat C, et al. Heterozygous and homozygous factor H deficiencies associated with hemolytic uremic syndrome or membranoproliferative glomerulonephritis: report and genetic analysis of 16 cases. *J Am Soc Nephrol*. 2004;15(3):787–795.
44. Geerlings MJ, Kremlitzka M, Bakker B, et al. The functional effect of rare variants in complement genes on C3b degradation in patients with age-related macular degeneration. *JAMA Ophthalmol*. 2017;135(1):39–46.
45. Kavanagh D, Richards A, Noris M, et al. Characterization of mutations in complement factor I (CFI) associated with hemolytic uremic syndrome. *Mol Immunol*. 2008;45(1):95–105.
46. Kavanagh D, Yu Y, Schramm EC, et al. Rare genetic variants in the CFI gene are associated with advanced age-related macular degeneration and commonly result in reduced serum factor I levels. *Hum Mol Genet*. 2015;24(13):3861–3870.
47. Liszewski MK, Atkinson JP. Complement regulator CD46: genetic variants and disease associations. *Hum Genomics*. 2015;9:7.
48. Recalde S, Tortajada A, Subias M, et al. Molecular basis of factor H R1210C association with ocular and renal diseases. *J Am Soc Nephrol*. 2016;27(5):1305–1311.
49. Richards A, Kemp EJ, Liszewski MK, et al. Mutations in human complement regulator, membrane cofactor protein (CD46), predispose to development of familial hemolytic uremic syndrome. *Proc Natl Acad Sci U S A*. 2003;100(22):12966–12971.
50. Volokhina E, Westra D, Xue X, et al. Novel C3 mutation p.Lys65Gln in aHUS affects complement factor H binding. *Pediatr Nephrol*. 2012;27(9):1519–1524.
51. Allikmets R. Further evidence for an association of ABCR alleles with age-related macular degeneration. The International ABCR Screening Consortium. *Am J Hum Genet*. 2000;67(2):487–491.
52. Allikmets R, Shroyer NF, Singh N, et al. Mutation of the Stargardt disease gene (ABCR) in age-related macular degeneration. *Science*. 1997;277(5333):1805–1807.
53. De La Paz MA, Guy VK, Abou-Donia S, et al. Analysis of the Stargardt disease gene (ABCR) in age-related macular degeneration. *Ophthalmology*. 1999;106(8):1531–1536.
54. Stone EM, Webster AR, Vandenburgh K, et al. Allelic variation in ABCR associated with Stargardt disease but not age-related macular degeneration. *Nat Genet*. 1998;20(4):328–329.
55. Stone EM, Aldave AJ, Drack AV, et al. Recommendations for genetic testing of inherited eye diseases: report of the American Academy of Ophthalmology Task Force on Genetic Testing. *Ophthalmology*. 2012;119(11):2408–2410.
56. Stone EM. Genetic testing for age-related macular degeneration: not indicated now. *JAMA Ophthalmol*. 2015;133(5):598–600.
57. Raychaudhuri S, Iartchouk O, Chin K, et al. A rare penetrant mutation in CFH confers high risk of age-related macular degeneration. *Nat Genet*. 2011;43(12):1232–1236.
58. Helgason H, Sulem P, Duvvari MR, et al. A rare nonsynonymous sequence variant in C3 is associated with high risk of age-related macular degeneration. *Nat Genet*. 2013;45(11):1371–1374.
59. Saksens NT, Geerlings MJ, Bakker B, et al. Rare genetic variants associated with development of age-related macular degeneration. *JAMA Ophthalmol*. 2016;134(3):287–293.
60. Taylor RL, Poulter JA, Downes SM, et al. Loss-of-function mutations in the CFH gene affecting alternatively encoded factor H-like 1 protein cause dominant early-onset macular drusen. *Ophthalmology*. 2019;126(10):1410–1421.
61. Lutein + zeaxanthin and omega-3 fatty acids for age-related macular degeneration: the Age-Related Eye Disease Study 2 (AREDS2) randomized clinical trial. *JAMA*. 2013;309(19):2005–2015.
62. Merle BMJ, Colijn JM, Cougnard-Gregoire A, et al. Mediterranean diet and incidence of advanced age-related macular degeneration: the EYE-RISK Consortium. *Ophthalmology*. 2019;126(3):381–390.
63. McCarty CA, Fuchs MJ, Lamb A, Conway P. How do patients respond to genetic testing for age-related macular degeneration? *Optom Vis Sci*. 2018;95(3):166–170.
64. Loss J, Muller D, Weigl J, et al. Views of ophthalmologists on the genetics of age-related macular degeneration: results of a qualitative study. *PLoS One*. 2018;13(12):e0209328.