Common haplotypes at the $CFH$ locus and low-frequency variants in $CFHR2$ and $CFHR5$ associate with systemic FHR concentrations and age-related macular degeneration

Laura Lorés-Motta,1,2 Anna E. van Beek,3,4,5,6 Esther Willems,7,8,9 Judith Zandstra,3,4 Gerard van Mierlo,3 Alfred Einhaus,2 Jean-Luc Mary,2 Corinne Stucki,2 Bjorn Bakker,1 Carel B. Hoyng,1 Sascha Fauser,2 Simon J. Clark,10,11,12 Marien I. de Jonge,7,8 Everson Nogoceke,2 Elod Koertvely,2 Ilse Jongerius,3,4 Taco W. Kuipers,4,13 and Anneke I. den Hollander1,14,*

Summary

Age-related macular degeneration (AMD) is the principal cause of blindness in the elderly population. A strong effect on AMD risk has been reported for genetic variants at the $CFH$ locus, encompassing complement factor H ($CFH$) and the complement-factor-H-related (CFHR) genes, but the underlying mechanisms are not fully understood. We aimed to dissect the role of factor H (FH) and FH-related (FHR) proteins in AMD in a cohort of 202 controls and 216 individuals with AMD. We detected elevated systemic levels of HRR-1 ($p = 1.84 \times 10^{-6}$), FHR-2 ($p = 1.47 \times 10^{-5}$), FHR-3 ($p = 1.05 \times 10^{-5}$) and FHR-4A ($p = 1.22 \times 10^{-5}$) in AMD, whereas FH concentrations remained unchanged. Common AMD genetic variants and haplotypes at the $CFH$ locus strongly associated with HHR protein concentrations (e.g., FH p.Tyr402His and FHR-2 concentrations, $p = 3.68 \times 10^{-17}$), whereas the association with FH concentrations was limited. Furthermore, in an International AMD Genomics Consortium cohort of 17,596 controls and 15,894 individuals with AMD, we found that low-frequency and rare protein-altering $CFHR2$ and $CFHR5$ variants associated with AMD independently of all previously reported genome-wide association study (GWAS) signals ($p = 5.03 \times 10^{-10}$ and $p = 2.81 \times 10^{-10}$, respectively). Low-frequency variants in $CFHR2$ and $CFHR5$ led to reduced or absent FHR-2 and FHR-5 concentrations (e.g., p.Cys72Tyr in $CFHR2$ and FHR-2, $p = 2.46 \times 10^{-19}$). Finally, we showed localization of FHR-2 and FHR-5 in the choriocapillaris and in drusen. Our study identifies FHR proteins as key proteins in the AMD disease mechanism. Consequently, therapies that modulate FHR proteins might be effective for treating or preventing progression of AMD. Such therapies could target specific individuals with AMD on the basis of their genotypes at the $CFH$ locus.

Introduction

Age-related macular degeneration (AMD) is responsible for the majority of blindness occurring among the elderly in the Western world and is the third most common cause of severe visual impairment worldwide.1,2 The prevalence increases dramatically with age3 and, with an aging society, the number of affected individuals is expected to rise from 196 million to 288 million by 2040.4 The early stage of AMD is characterized by the appearance of drusen under the retina.5 Drusen contain inflammatory factors, suggesting a local chronic inflammatory state.6 Individuals with AMD can progress to an advanced stage of disease in which vision loss occurs.7 Advanced AMD can be classified as two types: (1) geographic atrophy, involving degeneration of the choriocapillaris, retinal pigment epithelium (RPE) cells and photoreceptors; and (2) choroidal neovascularization, in which abnormal sprouting of blood vessels from the choriocapillaris occurs.8 Therapeutic options to date are limited to the neovascular form and have variable effectiveness, leaving a vast number of individuals with AMD untreated or with a progressing disease.9

AMD is a multifactorial disease in which genetic factors play a significant role, explaining 46% to 71% of the variation in the overall severity.10 Identification of these genetic factors and understanding how they exert their effect on AMD can help disentangle the AMD disease mechanisms and lead to improved therapeutic options. The first major susceptibility locus for AMD was identified on chromosome 1 at the 1q31.3 locus, where the rs1061170

1Department of Ophthalmology, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, Nijmegen, 6525EX, the Netherlands; 2Roche Pharma Research and Early Development, Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd, Basel, 4070, Switzerland; 3Department of Immunopathology, Sanquin Research and Landsteiner Laboratory, Amsterdam University Medical Centre, University of Amsterdam, Amsterdam, 1066CX, the Netherlands; 4Department of Pediatric Immunology, Rheumatology and Infectious Diseases, Emma Children’s Hospital, Amsterdam University Medical Centre, Amsterdam, 1066CX, the Netherlands; 5Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, Basel, 4051, Switzerland; 6University of Basel, Basel, 4051, Switzerland; 7Laboratory of Medical Immunology, Department of Laboratory Medicine, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, 6525GA, the Netherlands; 8Radboud Center for Infectious Diseases, Radboud University Medical Center, Nijmegen, 6525GA, the Netherlands; 9Translational Metabolic Laboratory, Department of Laboratory Medicine, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, 6525GA, the Netherlands; 10University Eye Clinic, Department of Ophthalmology, University of Tubingen, 72076, Germany; 11Institute for Ophthalmic Research, Eberhard Karls University of Tübingen, Germany; 12Institute for Ophthalmic Research, Sanquin Research and Landsteiner Laboratory, Amsterdam University Medical Centre, University of Amsterdam, Amsterdam, 1066CX, the Netherlands; 13Department of Human Genetics, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, 6525GA, the Netherlands

*Correspondence: Anneke.denHollander@radboudumc.nl
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(p.Tyr402His) variant in complement factor H (CFH) was strongly associated with an increased risk for AMD. This finding soon implicated factor H (FH), a major regulator of the alternative pathway of the complement cascade, and its short splice variant FH-like-1 (FHL-1) in the development of AMD. More recently, the International AMD Genomics Consortium (IAMDGC) performed a large genome-wide association study (GWAS) on advanced AMD. This study included 17,832 controls and 16,144 individuals with advanced AMD and identified 52 independent genome-wide significant signals at 34 genomic loci. Out of these 52 signals, a total of eight are located at an extended CFH locus, encompassing KCNT2, CFH, CFHR1, CFHR2, CFHR3, CFHR4, and CFHR5. The lead variants of these signals include only one CFH missense variant, rs121913059 (p.Arg1210Cys), which exerts a very strong effect on AMD (odds ratio [OR] = 20.28). The remaining variants are synonymous or non-coding, and one, rs570618, is in high linkage disequilibrium (LD) with the rs1061170 (p.Tyr402His) variant in complement factor H (CFH). The underlying effects of the other AMD-associated signals at this locus are, however, not known.

Several lines of evidence suggest that genetic variants at the extended CFH locus might affect the CFHR genes. The complement FH-related (FHR) proteins are thought to compete with FH and fine tune complement regulation, but their function is not yet fully understood. A common deletion spanning CFHR3 and CFHR1 (CNP147, 86.3 kb) and a rare deletion spanning CFHR1 and CFHR4 (CNP148, 122 kb) are both associated with a protective effect for AMD. Additionally, systemic FHR-4 concentrations have been recently found to be elevated in individuals with AMD and to be associated with AMD genetic variants. These findings suggest that FHR proteins might be involved in AMD, underlying the effect of the unexplained signals at the extended CFH locus, yet a comprehensive analysis of all five FHR proteins and the eight AMD genetic variants has not been performed to date.

In this study we aimed to dissect the role of FH and all five FHR proteins encoded by genes at the extended CFH locus in AMD. We measured serum concentrations of FH and the FHR proteins in individuals with advanced AMD and controls by using highly specific ELISAs and assessed associations with AMD disease and with common variants and haplotypes at the extended CFH locus. In addition, we assessed the association of low-frequency and rare protein-altering variants in the CFH and CFHR genes with AMD and with systemic protein concentrations. Finally, we examined the localization of the FHR proteins at the local AMD disease site in the eye.

Material and methods

Study cohort

Individuals included in this study were retrieved from the European Genetic Database (EUGENDA), a database created for the study of AMD. EUGENDA encompasses clinical and molecular information collected at the Radboud University Medical Center, Nijmegen, the Netherlands, and at the University Hospital of Cologne, Cologne, Germany. Certified graders determined the AMD and control status of each individual included in the study by using multimodal image grading according to the standard protocol of the Cologneman Image Reading Center (as described in Heisterbeek et al., 2020; Table S1). Controls included in this study were older than 65 years, and individuals with AMD were older than 60 years. All participants were ascertained to be of European descent via genome-wide array genotyping (see below). Information on age and sex were obtained from standardized interviewer-assisted questionnaires, and information on sex was confirmed with genetic data (see below).

Written informed consent was obtained from all study participants regarding clinical examination, epidemiological data collection, and blood measurements, as well as genetic analyses. This study adhered to the tenets of the Declaration of Helsinki (7th revision), and ethical approval was obtained from the local ethics committees (Arnhem-Nijmegen Commissie Mensgebonden Onderzoek [CMO] and Ethics Commission of Cologne University’s Faculty of Medicine).

Protein measurements

Serum concentrations of FH, the FHR proteins, and the homo- and heterodimers of FHR-1 and FHR-2 were measured in serum samples of the EUGENDA AMD case-control cohort. In total, 418 serum samples of 202 controls and 216 individuals with advanced AMD were included in the study. Serum samples were obtained via a standard coagulation and centrifugation protocol and were stored at –80°C within 1 h. Samples were not thawed before being used for this study. Serum concentrations of FH and FHR proteins were determined in two batches of 130 and 288 samples, respectively (Table S1), by in-house developed ELISAs. For specific measurements of FH, an in-house generated, monospecific mouse monoclonal antibody (mAb) directed against CCP domains 16/17 of FH (anti-FH.16, Sanquin Research, Amsterdam, the Netherlands) was used as capture mAb, and polyclonal goat anti-human FH (Quidel, San Diego, CA, USA), conjugated with HRP in house, was used as detection Ab. FHR-1/1 homodimers and FHR-1/2 heterodimers were captured by anti-FH.02 (Sanquin Research, directed against CCP20 of FH and CCP5 of FHR-1) and detected with biotinylated anti-FH.02 or anti-FHR-2 (monospecific for FHR-2, clone MAB5484, R&D Systems), respectively. FHR-2/2 homodimer concentrations and total concentrations of both FHR-1 and FHR-2 were calculated on the basis of the observed concentrations of FHR-1/1 and FHR-1/2 dimers and in consideration of the molecular weights of 39.5 kDa for FHR-1 (average of 37 and 42 kDa) and 26.5 kDa for FHR-2 (average of 24 and 29 kDa), respectively. Hence, in the 14 cases where FHR-1 protein was lacking in serum, FHR-2/2 and total FHR-2 concentrations could not be determined. For measurements of FHR-3, the in-house mAb anti-FHR-3.1 (directed against FHR-3 and FHR-4A, Sanquin Research) was used as a capture mAb, and biotinylated mAb anti-FHR-3.4 (directed against FHR-3 and FH, Sanquin Research) was used as a detecting mAb. FHR-4A was captured by monospecific mAb directed against FHR-3 and FH, Sanquin Research) was used as a capture mAb, and biotinylated mAb anti-FHR-3.4 (directed against FHR-3 and FH, Sanquin Research) was used as a detecting mAb.
FHR-4A.04 (directed against CCP5) and detected by biotinylated rabbit anti-human FHR-3 antiserum (Sanquin Research). Lastly, FHR-5 was measured with the monospecific anti-FHR-5.1 as a capture mAb and the monospecific biotinylated anti-FHR-5.4 as a detection mAb (both from Sanquin Research). All assays included a standard curve of normal human serum pool (>400 donors) and two control sera to ensure limited inter-assay variation. Sera were tested as two independent duplicates (on separate plates) and measured in two dilutions.

Genotypic data
Except for one individual with advanced AMD and one control individual, all individuals with measured FH and FHR serum concentrations were part of the IAMDGC GWAS and had been genotyped with a custom-modified Illumina HumanCoreExome array at the Centre for Inherited Disease Research (CIDR). Genotype calling, ancestry, and sex assessment, as well as genotype imputation via the 1000 Genomes Project reference panel, were performed by the IAMDGC as previously described.15

Statistical analysis
Association of FH and FHR concentrations with advanced AMD
A total of 202 controls and 216 individuals with advanced AMD and ELISA-based FH and FHR measurements were included in the analysis. A log \( \pi \) transformation and a standardization were applied to the concentrations of FH and FHR proteins. Association analyses of FH and FHR protein concentrations with advanced AMD were performed by means of a logistic regression using the ‘glm’ function of R; adjustments were made for age, sex, measuring batch, sampling cohort, AMD status, and ten ancestry principal components. Imputation quality control was performed as previously described, and genotype dosages were used for analysis of imputed variants. For the eight AMD-associated variants, we additionally performed comparable analyses without adjusting for AMD status and by including only controls. Comparable effect estimates were found for the associated variants, suggesting that adding AMD status as a covariate did not bias the estimation of the genetic effects on FH and FHR concentrations (Table S2).

Manhattan and quantile-quantile plots were generated with the “qqman” R package (version 0.1.4). In order to assess whether more than one genome-wide-significant signal was present at the extended CFH locus in each of the GWASs, we repeated the analysis conditioning on the lead variant(s) at this locus by adding the index variant(s) as covariates to the model until we found no additional genome-wide associations.

Low-frequency and rare-variant analysis of CFH and CFHR genes via the IAMDGC dataset
For the association analysis of low-frequency and rare variants with AMD, genotypes at the extended CFH locus were extracted from the IAMDGC dataset, which included 17,596 controls and 15,894 individuals with AMD (a total of 33,488 individuals). Gene-based tests were performed with SKAT-O as implemented in EPACTS, and the ENCODE v14 annotation was used. Protein-altering variants defined as missense, nonsense, and frameshift and as affecting the canonical splice sites with a MAF < 0.05 were included in the analyses. A threshold on imputation quality of ≥0.8 was set for the low-frequency and rare variants. The SKAT-O gene-based tests of low-frequency and rare variants were adjusted for the first two ancestry principal components and source of DNA (whole-blood or whole-genome amplified DNA). Furthermore, and in order to confirm that the associations were not driven by known AMD-associated variants in the same locus, we performed the same analysis by adding as covariates the index variants of the signals that have been previously reported to be associated with AMD at this locus (rs187328863, rs148553336, rs570618, rs10922109, rs35292876, rs121913059, rs61818925, and rs191281603), that is, a locus-wide conditioned analysis. We performed this analysis in two steps: first we adjusted only for the common index variants (rs570618, rs10922109, rs61818925), and then we adjusted for all eight index variants. We also performed sensitivity analyses by adjusting for seven additional principal components and including only variants with a PIHRED-CADD score ≥10. We applied multiple testing correction to the gene-based tests by using the Bonferroni procedure.

Association of low-frequency and rare variants in CFH, CFHR2, and CFHR5 with systemic FH, FHR-2, and FHR-5 protein concentrations
The association of low-frequency and rare variants identified in the gene-based test (see above) with (log \( \pi \) transformed and standardized) FH and FHR concentrations was modeled with adjustment for age, sex, measuring batch, sampling cohort, and AMD status, as well as for the eight variants that had previously been associated with AMD (locus-wide conditioning). All the association analyses were performed with the “glm” R function.

Immunocytochemistry of human retinas
Human donor eyes were obtained from Discovery Life Sciences. Eyes were collected within 12 h of death and transected behind
the limbus so that the anterior part including the lens was removed. The posterior eyecup and the globe were immediately fixed in 4% paraformaldehyde solution for 24 h. Next, the tissue was transferred into PBS for 48 h. The vitreous was carefully removed, and the eyecup was cryopreserved in 30% sucrose overnight. The tissue was embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, the Netherlands). 8 μm cryosections were cut with a CryoStar NX70 Cryostat (Thermo Scientific).

The immunostaining was performed with the VENTANA DISCOVERY ULTRA automated staining system (Ventana Medical Systems). Sections from three different donors were incubated with the primary antibodies (anti-FHR-2.1, Sanquin (2.0 mg/ml), diluted [dil.] 1:250; anti-FHR-5.4, Sanquin (2.7 mg/ml), dil. 1:800) for 30 min at 37°C. The antibody anti-FHR-2.1 was generated against FHR-2 but also recognizes FHR-1. Anti-FHR-5.4 is monospecific for FHR-5. After thorough washing, sections were incubated with anti-mouse secondary antibodies (OmniMap monospecific antibody clone detecting FHR-2 (MAB5484, R&D Systems) and frozen sections from three donors as described against FHR-2 but also recognizes FHR-1. Anti-FHR-5.4 is monospecific for FHR-5. After thorough washing, sections were incubated with anti-mouse secondary antibodies (OmniMap anti-mouse HRP detection system, Roche Diagnostics) for 15 min at 37°C, followed by incubation with the DISCOVERY Purple chromogen for another 15 min. Sections were counterstained with VENTANA HE 600 Blueing in conjunction with VENTANA HE 600 Hematoxylin for 4 min and mounted with Kaiser’s glycerol gelatin (Merck). Images were captured by a VS120 Olympus slide scanner and processed by VS-ASW L100 imaging software (version 2.9., Olympus). The results for FHR-2 were confirmed with a monospecific antibody clone detecting FHR-2 (MAB5484, R&D Systems) and frozen sections from three donors as described above. All tissues were acquired with consent of the donor or donor family in accordance with the principles outlined in the Declaration of Helsinki.

Results

Systemic concentrations of FHR-1, FHR-2, FHR-3, and FHR-4A are higher in individuals with advanced AMD than in controls

The five CFHR genes, located downstream of CFH, encode FHR-1, FHR-2, FHR-3, FHR-4A, FHR-4B, and FHR-5; FHR-4A is the only splice variant of CFHR4 found in circulation.14 FHR-1, FHR-2, and FHR-5 are present as homodimers, and FHR-1 and FHR-2 are also able to form heterodimers.33,46 First, we aimed to determine systemic concentrations of all five FHR proteins in serum samples of individuals with AMD and of control individuals. Until recently, the high degree of homology between members of the FH protein family and the presence of homo- and heterodimers presented a major challenge for their accurate measurement in serum. However, we used recently validated in-house-developed ELISAs to measure each protein and dimer specifically.32–34 Here, we measured serum concentrations of FH, FHR-1, FHR-2, FHR-3, and FHR-4, FHR-5, and the homo- and heterodimers of FHR-1 and FHR-2 in a cohort of 418 individuals, including 202 control individuals and 216 individuals with advanced AMD. Details of the study cohort are described in Table S1.

We performed an association analysis of FHR and FH serum concentrations with advanced AMD. After correction for multiple testing, total FHR-1, total FHR-2, FHR-1/2 dimers, FHR-1/2 dimers, FHR-2/2 dimers, FHR-3, and FHR-4A concentrations were found to be associated with advanced AMD, and for all FHR proteins, an increase in systemic concentrations was associated with an increased risk for advanced AMD (Table S3, Figure 1). The strongest effect on AMD risk was observed for total FHR-1 concentrations (OR per SD increase = 2.14, 95% CI = 1.56–2.92, pFDR = 5.52 × 10−6), followed by FHR-1/2 dimer concentrations (OR = 2.04, CI = 1.53–2.73, pFDR = 5.52 × 10−6) and FHR-1/1 dimer concentrations (OR = 1.99, CI = 1.50–2.64, pFDR = 5.52 × 10−6). Additional significant associations were found for FHR-3 concentrations (OR = 1.69, CI = 1.34–2.14, pFDR = 2.36 × 10−5), total FHR-2 concentrations (OR = 1.65, CI = 1.28–2.15, pFDR = 2.64 × 10−4), FHR-2/2 dimer concentrations (OR = 1.33, CI = 1.04–1.70, pFDR = 0.03), and FHR-4A concentrations (OR = 1.33, CI = 1.06–1.65, pFDR = 0.02). No significant differences were found between advanced AMD and controls for FHR-5 and FH concentrations (pFDR = 0.058 and pFDR = 0.847, respectively).

AMD variants and haplotypes at the extended CFH locus are strongly associated with FHR-1, FHR-2, FHR-3, FHR-4A, and FHR-5 concentrations

Next, we set out to determine the effect of genetic variants at the CFH locus on AMD concentrations. The largest GWAS carried out to date on advanced AMD included 17,832 controls and 16,144 individuals with advanced AMD of European descent and identified 52 independent genome-wide-significant signals.15 Out of these 52 signals, eight were located at an extended CFH locus encompassing CFH and CFHR. The lead variants of these signals were either common in the population (rs570618, rs10922109, and rs61818925), present at a low frequency (minor-allele frequency [MAF] 1%–5%; rs187328863) or rare (MAF < 1%; rs148553336, rs35292876, rs121913059, and rs191281603). Out of the eight lead variants, one is a missense variant in CFH (rs121913059, FH p.Arg1210Cys), one is a synonymous variant in CFH (rs35292876, FH p.His878His), and the remaining variants are non-coding: intronic in CFH (rs570618 and rs10922109), intronic in CFHRS (rs191281603), intronic in KCNT2 (rs187328863), and intergenic (rs148553336 upstream of CFH and rs61818925 downstream of CFH1 and upstream of CFHR4) (Figure 2). The intronic rs570618 variant is in high LD with the p.Tyr402His variant. We performed a comprehensive association analysis of these AMD variants with serum concentrations of FH and FHR.

Strong associations with FHR concentrations in serum were found for the three common variants rs10922109, rs570618, and rs61818925 (Figure 2, Table S4). The top variant of the first signal of the AMD GWAS, rs10922109 (1.1), is a protective variant that was associated with decreased serum concentrations of total FHR-1 and FHR-2, FHR-1/1 dimers, FHR-2/2 dimers, FHR-2/2 dimers, FHR-3, and FHR-4A. The strongest association was found with FHR-1/2 dimers (β = −0.749, SE = 0.064,
Although to a lesser degree, rs10922109 also showed an association with increased concentrations of FH. The second-most common variant of the AMD GWAS, rs570618 (1.2, in high LD with rs1061170 [p.Tyr402His]), is an AMD-risk-conferring variant and was strongly associated with

\[ pFDR = 5.27 \times 10^{-26}, \text{ Table S4, Figure S2} \].
increased serum concentrations of total FHR-1 and FHR-2, FHR-1/1 dimers, FHR-2/2 dimers, FHR-4A, and to a lesser degree, FHR-5. Similar to findings for rs10922109, the strongest association was found with FHR-1/2 dimers ($b = 0.587$, SE = 0.064, $p_{\text{FDR}} = 2.21 \times 10^{-17}$, Table S4, Figure S2). rs570618 was associated with increased FH concentrations as well, but to a much lesser degree. The third-most common variant is rs61818925, the sixth signal of the AMD GWAS (1.6). rs61818925 was identified as protective in the single-variant analysis but was reported to be risk-conferring in the locus-wide conditioned analysis. The variant rs61818925 was associated with decreased serum concentrations of FH, total FHR-2, FHR-2/2 dimers, FHR-4A, and FHR-5 and with increased serum concentrations of FHR-1/1 dimers and FHR-3. The strongest association of this variant was found with decreased serum FHR-4A concentrations ($b = -0.756$, SE = 0.066, $p_{\text{FDR}} = 3.16 \times 10^{-17}$, Table S4, Figure S2). The rare variant rs121913059 (1.3) was not present in our cohort, and the variant rs14855336 (1.4) was present in only one individual. Therefore, no analyses were performed for these two variants (gray).

NS = non-significant; NA = not applicable (variant not analyzed)

Figure 2. Associations of systemic FH, FHR-1, FHR-2, FHR-3, FHR-4A, and FHR-5 with AMD variants at the CFH locus

The variants depicted are the lead variants identified in the International AMD Genomics Consortium AMD GWAS at the extended CFH locus in chromosome 1. Variants in blue are protective for advanced AMD and variants in red are risk-conferring in the primary analysis. The number refers to the signal identification order. If a $p$ value is lower than 0.05, it is represented with one star (*), if it is lower than 0.01, with two (**), and if it is lower than 0.001, with three stars (***)). A dash (-) indicates a non-significant $p$ value. The rare variant rs121913059 (1.3) was not present in our cohort, and the variant rs14855336 (1.4) was present in only one individual. Therefore, no analyses were performed for these two variants (gray).
### Table 1. AMD haplotypes at the extended CFH locus are strongly associated with FHR protein concentrations: FH, total FHR-1, total FHR-2, and FHR-1

| H haplotype frequency | OR association with AMD | FH | Total FHR-1 | Total FHR-2 | FHR1-1 |
|-----------------------|------------------------|----------------|-------------|-------------|--------|
|                       |                        | B | Reference | SE | p value | p FDR | B | Reference | SE | p value | p FDR | B | Reference | SE | p value | p FDR |
| H1 0.375              |                         | 0.36 | -0.180 | 0.098 | 0.066 | 0.149 | 0.096 | 0.073 | 0.187 | 0.299 | -1.042 | 0.087 | <0.001 | -0.023 | 0.074 | 0.756 |
| H2 0.162              |                         | 0.30 | 0.252 | 0.105 | 0.017 | 0.045 | -1.247 | 0.078 | <0.001 | <0.001 | -0.496 | 0.105 | 3.45 × 10⁻⁶ | 1.46 × 10⁻⁵ | -1.273 | 0.081 | <0.001 |
| H3 0.148              |                         | 0.68 | -0.772 | 0.102 | 2.07 × 10⁻¹³ | 1.35 × 10⁻¹² | 0.051 | 0.075 | 0.493 | 0.623 | -0.224 | 0.091 | 0.014 | 0.039 | 0.089 | 0.075 | 0.238 |
| H4 0.059              |                         | 0.59 | -0.245 | 0.144 | 0.091 | 0.182 | -0.212 | 0.104 | 0.043 | 0.103 | -0.546 | 0.131 | 3.64 × 10⁻⁵ | 1.38 × 10⁻⁴ | -0.148 | 0.106 | 0.162 |
| H5 0.045              |                         | 1.39 | -0.299 | 0.162 | 0.065 | 0.149 | -0.043 | 0.119 | 0.720 | 0.836 | -0.329 | 0.144 | 0.023 | 0.057 | 0.000 | 0.119 | 0.997 |
| H6 0.019              |                         | 0.44 | 0.274 | 0.248 | 0.270 | 0.395 | -2.389 | 0.284 | 8.88 × 10⁻¹⁶ | 6.39 × 10⁻¹⁰ | 0.445 | 0.291 | 0.128 | 0.236 | -1.980 | 0.361 | 7.47 × 10⁻⁸ | 3.84 × 10⁻⁷ |
| H7 0.010              |                         | 1.50 | -0.192 | 0.204 | 0.346 | 0.488 | 0.121 | 0.151 | 0.422 | 0.573 | 0.066 | 0.183 | 0.719 | 0.836 | 0.011 | 0.152 | 0.436 |
| H8 0.005              |                         | 1.54 | -0.192 | 0.204 | 0.346 | 0.488 | 0.121 | 0.151 | 0.422 | 0.573 | 0.066 | 0.183 | 0.719 | 0.836 | 0.011 | 0.152 | 0.436 |

H = haplotype; p value < 0.001 is indicated when the obtained p value was 0.

*The variants in the haplotype are as follows: rs187328863, rs148553336, rs570618, rs6677604, rs10922109, rs35292876, rs121913059, rs61818925, and rs191281603. H1 = CTGAGGCCG, H2 = CTGACCGTC, H3 = CTGAACCTG, H4 = CTGCGCCCT, H5 = CTGCGCCGC, H6 = TTGGCCCGT, H7 = CTGAAACCTC, H8 = CTGGCCCTC, and H9 = CTTGCTGC.

### Table 2. AMD haplotypes at the extended CFH locus are strongly associated with FHR protein concentrations: FHR1-2, FHR2-2, FHR-3, FHR4A, and FHR5

| H haplotype frequency | OR association with AMD | FHR1-2 | FHR2-2 | FHR-3 | FHR4A | FHR5 |
|-----------------------|------------------------|--------|--------|-------|-------|------|
|                       |                        | B | Reference | SE | p value | p FDR | B | Reference | SE | p value | p FDR | B | Reference | SE | p value | p FDR |
| H1 0.375              |                         | 0.36 | -0.626 | 0.084 | 7.48 × 10⁻¹³ | 4.49 × 10⁻¹² | -0.920 | 0.093 | <0.001 | <0.001 | -0.019 | 0.073 | 0.793 | 0.865 | -1.012 | 0.089 | <0.001 | <0.001 | -0.363 | 0.100 | 3.10 × 10⁻⁴ | 0.001 |
| H2 0.162              |                         | 0.30 | -1.013 | 0.092 | 0.001 | 0.001 | -0.047 | 0.112 | 0.676 | 0.811 | -0.740 | 0.078 | <0.001 | <0.001 | -0.140 | 0.096 | 0.146 | 0.263 | 0.143 | 0.108 | 0.187 | 0.299 |
| H3 0.148              |                         | 0.68 | -0.125 | 0.087 | 0.153 | 0.269 | -0.310 | 0.097 | 0.002 | 0.006 | 0.993 | 0.075 | <0.001 | <0.001 | -0.533 | 0.093 | 1.83 × 10⁻⁸ | 1.01 × 10⁻⁷ | -0.356 | 0.104 | 0.001 | 0.003 |
| H4 0.059              |                         | 0.59 | -0.519 | 0.123 | 2.95 × 10⁻³ | 1.18 × 10⁻⁴ | -0.482 | 0.138 | 0.001 | 0.003 | 0.367 | 0.108 | 0.001 | 0.003 | 0.227 | 0.130 | 0.082 | 0.174 | -0.369 | 0.145 | 0.012 | 0.035 |
| H5 0.045              |                         | 1.39 | -0.254 | 0.139 | 0.069 | 0.151 | -0.358 | 0.155 | 0.022 | 0.057 | -0.132 | 0.120 | 0.274 | 0.395 | 0.206 | 0.149 | 0.168 | 0.281 | -0.106 | 0.167 | 0.524 | 0.650 |
| H6 0.019              |                         | 0.44 | -1.628 | 0.304 | 1.46 × 10⁻⁷ | 7.01 × 10⁻⁷ | -0.069 | 0.308 | 0.823 | 0.884 | -0.907 | 0.182 | 9.44 × 10⁻⁷ | 4.25 × 10⁻⁶ | -0.394 | 0.232 | 0.090 | 0.182 | 0.339 | 0.261 | 0.194 | 0.304 |
| H7 0.010              |                         | 0.50 | -0.164 | 0.275 | 0.550 | 0.671 | -0.008 | 0.378 | 0.983 | 0.997 | 0.775 | 0.292 | 0.008 | 0.024 | -0.537 | 0.340 | 0.114 | 0.216 | -0.006 | 0.387 | 0.987 | 0.997 |
| H8 0.025              |                         | 1.54 | 0.134 | 0.176 | 0.447 | 0.585 | -0.023 | 0.196 | 0.909 | 0.949 | -0.043 | 0.151 | 0.776 | 0.860 | -0.164 | 0.188 | 0.383 | 0.530 | 0.029 | 0.210 | 0.891 | 0.943 |

See legend for Table 1.
GWASs on FH and FHR reveal strong signals at the extended CFH locus and a tight link to AMD risk

We additionally performed GWASs on FH, total FHR-1, total FHR-2, FHR-3, FHR4A, and FHR-5 concentrations to examine their genetic determinants. All proteins showed a genome-wide signal located at the extended CFH locus (Figure 3: Manhattan plots, Figure S4: Q-Q plots, Table 3: main results, Tables S6–S11: genome-wide-associated variants for each GWAS). After consecutive conditioning on the lead variants, the GWAS on total FHR-2 concentrations showed three additional genome-wide-significant signals at this locus, the GWAS on FHR-3 concentrations showed one additional signal, and the GWAS on FHR4-A concentrations showed two additional signals.

The top variant in the total FHR-1 GWAS, rs148235292, was associated with decreased FHR-1 concentrations (B = −1.376, SE = 0.073, p = 1.39 × 10^{-57}), and it is also a protective variant for AMD at a genome-wide significant level (OR = 0.41, p = 1.23 × 10^{-284}). The same scenario was found for the first signal of the total FHR-2 GWAS (FHR-2.1), rs3790414, which was associated with decreased total FHR-2 concentrations (B = −0.979, SE = 0.080, p = 1.59 × 10^{-25}), and it is also associated with a protective effect for AMD at a genome-wide significant level (OR = 0.54, p = 3.16 × 10^{-187}). The top variant of the first signal of the FHR-4A GWAS (FHR-4A.1), rs56994749, was associated with increased FHR-4A levels (B = 0.770, SE = 0.068, p = 6.11 × 10^{-26}), and with a higher risk for AMD at a genome-wide significant level (OR = 1.58, p = 1.12 × 10^{-135}). The top variant for the FHR-5 GWAS, rs6695321, was associated with decreased FHR-5 concentrations (B = −0.398, SE = 0.071, p = 4.03 × 10^{-8}), and it is protective for AMD at a genome-wide significant level (OR = 0.62, p = 2.24 × 10^{-170}; Table 3). Notably, the r² between rs56994749 and rs6695321 (FHR-4A.1 and FHR-5.1) is 0.76, whereas the
Table 3. GWAS on FH and FHR proteins concentrations identify independent genome-wide-significant signals at the extended CFH locus overlapping with AMD risk

| Protein | Signal number | Top variant | Chromosomal position<sup>a</sup> | Major/ minor allele<sup>b</sup> | Location (aa change) | MAF | B | SE | p value | OR association with AMD<sup>c</sup> | p value association with AMD<sup>d</sup> | Linkage disequilibrium of R<sup>2</sup> > 0.3 with index AMD GWAS signals |
|---------|---------------|-------------|----------------------------------|-------------------------------|----------------------|-----|---|----|----------|--------------------------|---------------------|-------------------------------------------------|
| FH      | FH.1          | rs70620<sup>e</sup> | 1:196704997                      | G/A                           | intronic CFH          | 0.170 | -0.754 | 0.087 | 8.73 × 10<sup>-17</sup> | 0.935 | 0.003 |
|         | total FHR-1   | rs148235292 | 1:196814850                      | T/A                           | intergenic of CFH1, upstream of CFHR4 | 0.167 | -1.376 | 0.073 | 1.39 × 10<sup>-57</sup> | 0.410 | 1.23 × 10<sup>-284</sup> |
| FHR-2   | FHR-2.1       | rs3790414   | 1:196920299                      | T/A                           | Intron CFHR2          | 0.177 | -0.979 | 0.080 | 1.59 × 10<sup>-29</sup> | 0.537 | 3.16 × 10<sup>-197</sup> |
|         | FHR-2.2       | rs79351096  | 1:196918741                      | G/A                           | exon CFHR2            | 0.012 | -2.268 | 0.241 | 5.07 × 10<sup>-19</sup> | 0.634 | 1.01 × 10<sup>-11</sup> |
|         | FHR-2.3       | rs41310132  | 1:19628188                       | A/G                           | exon CFHR2            | 0.009 | -1.833 | 0.279 | 1.65 × 10<sup>-10</sup> | 0.408 | 1.54 × 10<sup>-28</sup> |
|         | FHR-2.4       | rs114645367 | 1:196716998                      | G/A                           | intergenic of CFH     | 0.012 | -1.507 | 0.228 | 1.32 × 10<sup>-10</sup> | 1.500 | 1.14 × 10<sup>-7</sup> |
| FHR-3   | FHR-3.1       | rs529541    | 1:196719716                      | A/G                           | intergenic of CFH     | 0.178 | 1.161  | 0.070 | 1.92 × 10<sup>-47</sup> | 0.949 | 0.018 |
|         | FHR-3.2       | rs138396963 | 1:196724984                      | AC/A                          | intergenic of CFH, upstream of CFHR3 | 0.343 | -0.772 | 0.066 | 1.01 × 10<sup>-27</sup> | 0.578 | 1.82 × 10<sup>-202</sup> |
|         | FHR-4A        | FHR-4A.1    | rs56994749<sup>e</sup>           | T/A                           | intergenic of CFH1, upstream of CFHR4 | 0.329 | 0.770  | 0.068 | 6.11 × 10<sup>-26</sup> | 1.577 | 1.12 × 10<sup>-135</sup> |
|         | FHR-4A.2      | rs10494745  | 1:196887457                      | G/A                           | exon CFHR4            | 0.099 | -1.302 | 0.089 | 6.35 × 10<sup>-39</sup> | 1.763 | 2.24 × 10<sup>-121</sup> |
|         | FHR-4A.3      | rs10922108  | 1:19701473                       | A/T                           | intronic CFH           | 0.335 | -0.449 | 0.053 | 2.78 × 10<sup>-16</sup> | 0.382 | 1.48 × 10<sup>-612</sup> |
| FHR-5   | FHR-5.1       | rs6695321<sup>e</sup> | 1:196675861                      | A/G                           | intronic CFH           | 0.337 | -0.398 | 0.071 | 4.03 × 10<sup>-8</sup> | 0.616 | 2.24 × 10<sup>-170</sup> |

<sup>a</sup>Chromosomal position is based on NCBI RefSeq hg19.<br><sup>b</sup>Effect allele.<br><sup>c</sup>Extracted from the single-variant unconditioned results of the GWAS on AMD from the IAMDGC.<br><sup>d</sup>Linkage disequilibrium with index variants from the eight independent signals of the GWAS on AMD from the IAMDGC, also described in Figure 2. The matrix of pairwise linkage disequilibrium statistics was generated with LDlink (accessed on February 2021) and is displayed in Table S12. All European reference populations from the reference haplotypes from phase 3 (version 5) of the 1000 Genomes Project were used for the linkage-disequilibrium calculations.<br><sup>e</sup>R<sup>2</sup> for these two variants is 0.987 ("LDlink," accessed on May 2020 and including all European reference populations from the reference haplotypes from phase 3 (Version 5) of the 1000 Genomes Project). The model was adjusted for age, sex, measuring batch, sampling cohort, and AMD status, as well as ten ancestry principal components. The resulting betas reflect the allele effect on log-transformed +1 and standardized protein concentrations. Second, third, and fourth signals were identified after adjustment for the lead variant(s) of the previously identified signal.
other above-mentioned lead variants have an $r^2 < 0.5$, (Table 3 and Table S12).

The top variant of the only signal in the FH GWAS, rs70620, was associated with decreased FH concentrations and with a lower risk for AMD, but with a much lower degree of significance ($OR = 0.94, p = 0.003$). The top variant of the first signal of the FHR-3 GWAS (FHR-3.1), rs529541, was associated with increased FHR-3 concentrations but with a protective effect for AMD at a $p$ value just below the 0.05 threshold ($OR = 0.95, p = 0.018$). These two variants, rs70620 and rs529541 (FH.1 and FHR-3.1), have an $r^2$ of 0.98 (Table S12). The second signal of the FHR-3 GWAS, rs138396963 (FHR-3.2), was associated with decreased FHR-3 concentrations ($B = -0.772, SE = 0.066, p = 1.01 \times 10^{-27}$) and with a protective effect for AMD at a genome-wide-significant level ($OR = 0.58, p = 1.82 \times 10^{-202}$; Table 3).

The total FHR-2 GWAS revealed four genome-wide-significant signals, of which the FHR-2.2, FHR-2.3, and FHR-2.4 signal were low-frequency variants. The top associated variants of the FHR-2.2 and FHR-2.3 signals, rs79351096 and rs41310132, are missense variants in FHR-2, leading to p.Cys72Tyr and p.Tyr264Cys. Both were associated with decreased concentrations of FHR-2 and a protective effect for AMD at genome-wide-significant level. The top variant of the FHR-2.4 signal, rs114645367, was associated with decreased FHR-2 concentrations but a higher risk for AMD. The FHR-4A GWAS revealed three genome-wide-significant signals. The top variant of the FHR-4A.2 signal, rs10494745, is a missense $CFHR4$ variant that was associated with decreased FHR-4A concentrations but with an increased risk for AMD at a genome-wide-significant level. Finally, the top variant of the FHR-4A.3 signal, rs10922108, was associated with decreased FHR-4A concentrations and with a protective effect for AMD at a genome-wide-significant level (Table 3).

Interestingly, rs148235292 (FH.1.1) is in LD ($r^2 = 0.94$) with rs6677604, the tag variant for the $CFHR3-CFHR1$ deletion, and rs10922108 (FHR-4A.3) is in LD ($r^2 = 1$) with the AMD GWAS 1.1 top variant rs10922109 (Table 3, Table S12). Several index variants of these FHR GWAS signals are in some degree of LD with known index variants of AMD GWAS signals (Table 3 and Table S12).

### Low-frequency and rare variants in $CFHR2$ and $CFHR5$ are associated with advanced AMD

The largest GWAS on advanced AMD was performed on an exome-chip platform that included not only common variants but also low-frequency (MAF = 1%–5%) and rare (MAF < 1%) variants. The evaluation of the effect of rare protein-altering variants in $CFH$ revealed a burden associated with advanced AMD independently of the eight signals identified in the GWAS. Nonetheless, the effect of low-frequency variants on AMD has not been evaluated yet. We identified p.Cys72Tyr and p.Tyr264Cys (low-frequency variants) as lead variants of the total FHR-2 GWAS. We identified an association of systemic FHR-2 concentrations with advanced AMD and, therefore, reasoned that low-frequency and rare variants in $CFHR2$ and other $CFHR$ might be associated with AMD risk. Because such protein-altering variants most likely impact protein function, they can help pinpoint causal genes for AMD. Using the IAMDGC dataset, we performed gene-based tests for protein-altering low-frequency and rare variants in $CFHR1$, $CFHR2$, $CFHR3$, $CFHR4$, and $CFHR5$ (and $CFH$ for consistency). A total of 17,596 controls and 15,894 individuals who had advanced AMD in the IAMDGC dataset and were of European descent were included in this analysis. We also performed the association tests after adjusting for the eight previously reported AMD signals at the extended $CFH$ locus in order to rule out the possibility that the associations were driven by previously described signals.

Associations of protein-altering low-frequency and rare variants with advanced AMD in the locus-wide conditioned analysis were found for the $CFH$, $CFHR2$, and $CFHR5$ (SKAT-O $p$ values of $7.52 \times 10^{-6}$, $5.03 \times 10^{-3}$, and $2.81 \times 10^{-6}$, respectively; Table 4). Sensitivity analysis with adjustment for seven additional ancestry principal components confirmed lack of confounding due to population stratification (Table S13). The gene-based test for $CFH$ included 55 variants with 13 singletons, for $CFHR2$ included 11 variants with three singletons, and for $CFHR5$ included 30 variants with 13 singletons (Table 4 and Table S14; details of all the variants included in each test are provided).

We additionally performed single-variant association analysis for the variants included in the gene-based tests of $CFH$, $CFHR2$ and $CFHR5$ in order to assess whether the direction of the effect of these variants could be determined. This analysis was also adjusted for the eight previously reported AMD signals. A total of 11 variants had $p < 0.05$; these included two variants in FHR-5 (p.Arg356His [$OR = 0.75, p = 7.88 \times 10^{-7}$] and p.Tyr468IlefsTer16 [$OR = 7.19, p = 0.020$]) and one variant in FHR-2 (p.Tyr264Cys [$OR = 0.75, p = 6.60 \times 10^{-6}$] (Table S14, Figure 4A).

Several tools can help with predicting the deleteriousness of genetic variants. The tool “combined annotation-dependent depletion” (CADD) integrates multiple annotations into one metric. Variants with PhRED-scaled CADD scores higher than 10 are predicted to be the 10% most deleterious substitutions in the human genome. We performed an additional sensitivity analysis including only variants with a PhRED-scaled CADD score > 10. We found stronger associations for all three genes ($CFH$, $CFHR2$, and $CFHR5$) when we included only variants with a PhRED-scaled CADD score > 10 (SKAT-O p = $2.77 \times 10^{-6}$, $3.26 \times 10^{-3}$ and $8.62 \times 10^{-7}$, respectively; Table S15, Figure 4A).

### Low-frequency variants in $CFHR2$ and $CFHR5$ are associated with decreased systemic concentrations of FHR-2 and FHR-5

Finally, we evaluated whether carriers of variants included in the gene-based tests of $CFH$, $CFHR2$, and $CFHR5$ have
Table 4. Gene-based tests identify low-frequency and rare variants in CFH, CFHR2, and CFHR5 associated with age-related macular degeneration

| Gene   | Chromosomal location | Number of variants included | Number of singletons included | Number of individuals carrying low-frequency and rare variants | AC in AMD | AC in controls | Unconditioned p value* | p value conditioned on common variants* | Locus-wide conditioned p value* | SKAT-O p* |
|--------|----------------------|-----------------------------|-------------------------------|---------------------------------------------------------------|----------|----------------|-----------------------|----------------------------------------|-------------------------------|-----------|
| CFH    | 1:196621254–196716353| 55                          | 13                            | 2,077                                                          | 922      | 1,364          | 8.63 × 10−60           | 4.50 × 10−13                          | 7.52 × 10−6                   | 0         |
| CFHR1  | 1:196794703–196797262| 4                           | 2                             | 11                                                             | 6        | 5              | 0.669                 | 0.557                                 | 0.601                         | 1         |
| CFHR2  | 1:196918615–196928188| 11                          | 3                             | 3,177                                                          | 1,262    | 2,026          | 2.52 × 10−53           | 5.87 × 10−10                          | 0.005                         | 0.3       |
| CFHR3  | 1:196748349–196748468| 4                           | 2                             | 31                                                             | 15       | 16             | 0.179                 | 0.545                                 | 0.578                         | 0         |
| CFHR4  | 1:196871563–196887530| 20                          | 1                             | 555                                                            | 191      | 378            | 1.47 × 10−11           | 0.363                                 | 0.408                         | 0         |
| CFHR5  | 1:196952046–196977807| 30                          | 10                            | 2,771                                                          | 1,247    | 1,708          | 4.78 × 10−29           | 9.57 × 10−18                          | 2.81 × 10−6                   | 0         |

AC = allele count. SKAT-O gene-based tests were carried out for comparing protein-altering variants with a minor-allele frequency < 0.05 in 17,596 controls and 15,894 individuals with advanced AMD (a total of 33,488 individuals). Bonferroni correction for multiple testing was applied, and p values > 0.008 were considered statistically significant. Details about the variants included in the test are displayed in Table S14.

*Chromosomal position according to the NCBI RefSeq hg19 human genome reference assembly.

SKAT-O tests were adjusted for the first two ancestry principal components and source of DNA (whole-blood or whole-genome amplified DNA).

SKAT-O test was additionally adjusted for the common index variants in the locus that have been previously reported to be associated with AMD (rs570618, rs109221099 and rs61818925).

SKAT-O test was additionally adjusted for all the index variants in the locus that have been previously reported to be associated with AMD (rs187328863, rs148553336, rs570618, rs10922109, rs35292876, rs121913059, rs61818925, and rs191281603).

As computed in the locus-wide conditioned analysis.
altered protein concentrations in their serum. A total of 12 variants were present in more than one individual within our cohort; therefore, serum measurements of FH, FHR-2, and FHR-5 were available for these individuals (Table S16). Low-frequency and rare variants in CFHR2 were associated with decreased serum FHR-2 concentrations in individuals with the p.Cys72Tyr (β = −2.334, SE = 0.272, pFDR = 2.95 \times 10^{-3}), p.Glu199Ter (β = −1.491, SE = 0.266, pFDR = 2.42 \times 10^{-3}), and p.Tyr264Cys (β = −1.524, SE = 0.327, pFDR = 1.72 \times 10^{-3}) variants (Figures 4A and 4B). The p.Cys208Arg variant in CFHR5 was associated with decreased FHR-5 concentrations (β = −1.869, SE = 0.679, pFDR = 0.018; Table S16, Figures 4A and 4C). Carriers of rare variants in CFH did not have altered FH concentrations (Table S16).

FHR-2 and FHR-5 localize in the interface between the retinal pigment epithelium and the choroid
In this study, we describe an association of FHR-2 and FHR-5 with AMD. We additionally assessed whether they can also be detected at sites where the pathological changes manifest in the eye. We investigated the localization of these proteins in human ocular tissue sections by using anti-FHR-2 and anti-FHR-5 antibodies. Strikingly, both FHR-2 and FHR-5 were detected in the intercapillary septa and the extracellular matrix surrounding the choriocapillaris (Figure 5 and Figure S5). A signal in the deeper layers of the choroid was also present, but it was fainter than in the intercapillary septa. The signal intensities varied significantly within the same layer but in different areas of a single section, where regions with strong deposit accumulation exhibited the strongest staining. Sub-RPE drusen showed strong immunolabeling for both FHR-2 and FHR-5, whereas no immunostaining was detected in the neuroretina. Importantly, no intracellular staining was detected for either FHR-2 or FHR-5 in the choroid-RPE interface, indicating an extracellular (hepatic) origin (Figure 5 and Figure S5).

Discussion
In the present study, we measured FH and FHR proteins in serum of individuals with advanced AMD and controls by
and D, for FHR-2 and FHR-5, respectively; the scale bar represents 20 μm). Sections were incubated with the primary antibodies anti-FHR-2.1 from Sanquin for FHR-2 and anti-FHR-5.4 from Sanquin for FHR-5. The antibody anti-FHR-2.1 was generated against FHR-2 but also recognizes FHR-1. Anti-FHR-5.4 is monospecific for FHR-5; therefore, staining with a monoclonal antibody found to recognize FHR-2 exclusively (R&D Systems, MAB5484) was also performed (Figure S5).

using ELISAs that allowed for specific quantification, despite a high level of homology between these proteins. We identified elevated systemic concentrations of total FHR-1, total FHR-2, FHR-1/1 dimers, FHR-1/2 dimers, FHR-2/2 dimers, FHR-3, and FHR-4A in individuals with AMD, and we found associations of all FHR concentrations with common AMD genetic variants and haplotypes at the extended CFH locus. In addition, we identified low-frequency and rare CFHR2 and CFHR5 variants associated with AMD independently of the previously reported GWAS signals. Low-frequency variants in CFHR2 and CFHR5 also led to decreased or absent FHR-2 and FHR-5 concentrations. Finally, we observed that FHR-2 and FHR-5 localize in the choriocapillaris, specifically in the intercapillary septa, as well as in drusen, the hallmark lesions of AMD.

Our study reports elevated concentrations of total FHR-1, total FHR-2, FHR-1/1 dimers, FHR-1/2 dimers, FHR-2/2 dimers, FHR-3, and FHR-4A in AMD. The association between increased FHR-1 and FHR-3 concentrations and advanced AMD is in line with the previously reported protective effect of the CFHR3-CFHR1 deletion.22,29 Our findings suggest that the deletion exerts its protective effect on AMD by decreased or absent FHR-1 and FHR-3 in the carriers. However, this finding contrasts with a previous study that described decreased FHR-1 concentrations in individuals with AMD.45 The authors claimed that the effect of the CFHR3-CFHR1 deletion could therefore not be mediated by reduced concentrations of FHR-1 and would be mediated by increased FH concentrations instead. However, the ELISA used in that study showed protein concentrations of FHR-1 in CFHR1-deficient donors, suggesting that the assay is not specific for FHR-1. A recent study reported elevated FHR-4A concentrations in advanced AMD in a meta-analysis of two independent cohorts,30 measured with a different anti-FHR-4A antibody, which is confirmed in our current study. FHR-5 was the only FHR that was not associated with AMD in our analysis; however, the p value was close to significance (p = 0.052). Therefore, an extended analysis with a larger sample size might confirm elevated FHR-5 concentrations in AMD.

Importantly, we did not observe any differences in systemic FH concentrations between individuals with advanced AMD and controls. Previous studies have reported conflicting results on this regard.30,49–54 Potential explanations might be differences in size and ethnicity of the cohorts; cross-reactivity of the assays with FHL-1,30,49–54 CFH,51 or both FHL-1 and FHR-1;50,52,55 or unclear cross-reactivity.53,54,56 In the current study, we used an antibody that targets CCP16/17 in FH but does not detect any other member of the FH protein family, allowing us to specifically measure systemic FH only.55 We conclude that systemic FHR concentrations and not FH concentrations are associated with advanced AMD.

We also find that, out of the eight AMD signals reported in the large AMD IAMDGCC GWAS at the extended CFH locus,15 all three common variants were strongly associated with FHR protein concentrations. An association of these variants with total FHR-4A has been recently described,30 but in the current study we demonstrate that this association extends to all five FHR proteins.

We report that the AMD GWAS top variant 1.1, rs10922109, associates with altered FHR-1, FHR-2, FHR-3, and FHR-4A concentrations. This variant has recently been described to be an eQTL for CFHR1, CFHR3, and CFHR4 in liver.57 The top variant of the 1.2 AMD GWAS
In our GWAS this variant had a p value of 0.300 and was not associated with FH concentrations. The index variant in our FH GWAS, rs70620, was associated with decreased FH concentrations but was only nominally associated with a lower risk for AMD, and we found a relatively small effect size and a p value that did not reach genome-wide significance in the AMD GWAS (OR = 0.94, p = 0.003). After conditioning for rs70620, we did not find associations for any other variants with FH concentrations at a genome-wide significant level, confirming that there is not an evident association between systemic FH concentrations and AMD. A GWAS on FHR-4A had been previously performed in a study using a different antibody and had shown comparable results. Several index variants of these FHR GWAS signals were also strongly associated with AMD and in LD with reported index variants of AMD GWAS signals (Table 3 and Table S12). Therefore, FHR-associated variants could be underlying the known AMD associations at the extended CFH locus. Genetic variations in CFHR genes have also been reported to be involved in other complement diseases. Consequently, the signals that we have identified could be further explored in future studies in these contexts.

Protein-altering variants most likely impact protein function and can help pinpoint causal genes. We analyzed the effect of low-frequency and rare variants in CFHR genes on AMD, independently of all previously reported signals, in 17,596 controls and 15,894 individuals who had advanced AMD and were included in the JAMDG WGS. Low-frequency and rare functional variants in CFHR2 and CFHR5 were associated with advanced AMD, which directly implicates these genes in the disease and also shows that additional genetic associations are present at this locus.

Of the variants included in the gene-based test, 68 variants had a PHRED-CADD score > 10, demonstrated an individual association with AMD (p < 0.05) independently of previously reported GWAS signals, or had an effect on protein concentrations (p < 0.05), and 11 variants met two or more of these criteria (Figure 4A). In FH, p.Arg53Cys, p.Asn517Lys, p.Pro562His, p.Gln950His, p.Thr956Met, and p.Asn1050Tyr had a PHRED-CADD score > 10 and showed an association with AMD. These variants had been previously described in AMD, but we also report p < 0.05 for p.Asni517Lys, p.Pro562His, and p.Thr956Met.

In FHR-2, p.Cys72Tyr, p.Glu199Ter, and p.Tyr264Cys had a PHRED-CADD score > 10 and a lowering effect on total FHR-2 protein concentrations. p.Cys72Tyr and p.Glu199Ter had been previously reported to result in a complete absence of FHR-2, in the case of the p.Cys72Tyr variant because of a disulfide bridge that cannot be formed. The p.Tyr264Cys variant introduces a cysteine residue and is likely to affect folding of FHR-2. The p.Tyr264Cys variant additionally shows a protective effect on AMD independently of previously reported signals (OR = 0.75, p = 6.60 × 10⁻⁴). The p.Tyr264Cys variant in CFHR2 had been recently highlighted in the context of AMD. In that study, the extended CFH locus was sequenced with molecular inversion probes. Single-variant analysis was performed and was followed by a meta-analysis that included 1,574 AMD cases and 855 controls.
from three cohorts.29 The p.Tyr264Cys variant was found to be associated with AMD in an analysis adjusted for age, sex, and the p.Tyr402His variant (OR = 0.37, p = 1.45 × 10^{-3}). Interestingly, p.Cys72Tyr is in some degree of LD (r^2 = 0.421, Table S12) with rs148553336, the 1.4 signal of the AMD GWAS. The rs148553336 variant is a low-frequency, non-coding variant located upstream of CFH; therefore, the protective effect of the 1.4 signal in the AMD GWAS could be partially reflecting a protective effect of p.Cys72Tyr on AMD.

Finally, in FHR-5, p.Cys208Arg had a PHRED-CADD score >10 and a lowering effect on total FHR-5 protein concentrations. The p.Cys208Arg variant most likely interferes with the formation of a disulfide bridge, leading to retention of the aberrantly folded FHR-5 protein in the cell. The p.Arg356His and p.Tyr468IlefsTer16 variants had a PHRED-CADD score >10 and showed an association with AMD (OR = 0.75, p = 7.88 × 10^{-7} and OR = 7.19, p = 0.02, respectively). A protective effect would have been expected for the frameshift variant and therefore requires further research. Gene-based tests often arbitrarily analyze rare variants with a MAF < 1%. The FHR-2 variants p.Cys72Tyr, p.Glu199Ter, and p.Tyr264Cys are all low-frequency, which highlights the importance of analyzing variants with a MAF < 5%. Low-frequency variants may therefore be also analyzed in other AMD loci or throughout the genome, as they might play a relevant role in AMD.

The effects that we report are at a systemic level, and the local effects at the AMD disease site remain to be elucidated. We describe localization of FHR-2 and FHR-5 to the intercapillary septa of the choriocapillaris and in drusen. The lack of the immunopositive cells in the choroid and RPE suggests that these FHR proteins derive from the systemic circulation entering the choroidal stroma through the fenestration of the choriocapillaris and diffusion through the Bruch’s membrane. This has been previously described as well for FHR-A.30 Alterations in systemic FHR proteins abundance may therefore impact pathogenesis locally at the AMD disease site.

Rare protein-altering variants impairing the function of FH or FHL-160,61 and common variants at the extended CFH locus that increase FHR concentrations could shift the delicate balance between FH and FHR in the choriocapillaris and Bruch’s membrane and thus increase the risk for AMD. On the other hand, low-frequency variants leading to low or absent concentrations of FHR-2 and FHR-5 are protective for AMD. Our findings highlight the FHR proteins as potential drug targets that could be inhibited for the treatment or prevention of disease progression in AMD. Selection of individuals with AMD for FHR-inhibiting therapy would need to involve consideration of both common haplotypes in the extended CFH locus, as well as low-frequency and rare variants in CFHR2 and CFHR5.

In conclusion, our results deepen the understanding of the effects of genetic variation at the extended CFH locus and pinpoint a relevant role of FHR proteins in AMD; these results are also supported by a recent study by Unwin et al.62 We describe the involvement of FHR-2 and FHR-5 in AMD and identify low-frequency CFHR2 and CFHR5 variants with a protective effect on AMD. Our study could set a precedent for the analysis of other GWAS loci and pinpoints FHR proteins as potential targets for developing new AMD treatments where individuals with AMD could be selected on the basis of their genetic profile.

Data and code availability
GWAS results will be accessible at the GWAS catalog repository (https://www.ebi.ac.uk/gwas/).

Serum FH concentrations GWAS GCST90019041
Serum FHR-1 concentrations GWAS GCST90019042
Serum FHR-2 concentrations GWAS GCST90019043
Serum FHR-3 concentrations GWAS GCST90019044
Serum FHR-4A concentrations GWAS GCST90019045
Serum FHR-5 concentrations GWAS GCST90019046

Supplemental information
Supplemental information can be found online at https://doi.org/10.1016/j.ajhg.2021.06.002.

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Declaration of interests
A.E., J.L.M., C.S., E.N., E.K., and S.F. are employees of F. Hoffmann-La Roche. S.C. is an inventor on patent applications that describe the use of complement inhibitors for therapeutic purposes and is a co-founder and director of Complement Therapeutics. T.W.K. is coinventor on a patent (PCT/NL2015/050584) describing the use of complement inhibitors for therapeutic purposes and is a co-founder and director of Complement Therapeutics. A.E., J.L.M., C.S., E.N., E.K., and S.F. are employees of F. Hoffmann-La Roche.
Web resources
CADD, https://cadd.gs.washington.edu
EPACTS, http://genome.sph.umich.edu/wiki/EPACTS
EUCLIDS, www.euclids-project.eu
LDlink, https://ldlink.nci.nih.gov

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