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Sequence and Expression of Complement Factor H Gene Cluster Variants and Their Roles in Age-Related Macular Degeneration Risk

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PURPOSE. To investigate how potentially functional genetic variants are coinherited on each of four common complement factor H (*CFH*) and *CFH*-related gene haplotypes and to measure expression of these genes in eye and liver tissues.

METHODS. We sequenced the *CFH* region in four individuals (one homozygote for each of four common *CFH* region haplotypes) to identify all genetic variants. We studied associations between the haplotypes and AMD phenotypes in 2157 cases and 1150 controls. We examined RNA-seq profiles in macular and peripheral retina and retinal pigment epithelium/choroid/sclera (RCS) from eight eye donors and three liver samples.

RESULTS. The haplotypic coinheritance of potentially functional variants (including missense variants, novel splice sites, and the *CFHR3–CFHR1* deletion) was described for the four common haplotypes. Expression of the short and long *CFH* transcripts differed markedly between the retina and liver. We found no expression of any of the five *CFH*-related genes in the retina or RCS, in contrast to the liver, which is the main source of the circulating proteins.

CONCLUSIONS. We identified all genetic variants on common *CFH* region haplotypes and described their coinheritance. Understanding their functional effects will be key to developing and stratifying AMD therapies. The small scale of our expression study prevented us from investigating the relationships between *CFH* region haplotypes and their expression, and it will take time and collaboration to develop epidemiologic-scale studies. However, the striking difference between systemic and ocular expression of complement regulators shown in this study suggests important implications for the development of intraocular and systemic treatments.

Keywords: complement factor H, genetic variation, gene expression, genetic association, haplotype

Ten years have passed since the association between variation in the human complement factor H (*CFH*) gene and risk of age-related macular degeneration (AMD) was discovered,[1–3] yet this knowledge has not yet been translated into preventive measures for the aging population or treatments for patients with AMD. New therapeutic agents that modify the function of the pathway are in development and in trials, and stratified medicine approaches to the use of existing treatments may soon require ophthalmologists to choose treatments according to individual patients' inherited genetic variants.

Dysfunction of the complement system (a central part of the innate immune system) is key to the etiology of AMD.[4] The complement system comprises a series of proteins, C1 to C9, which are sequentially activated or inhibited by regulatory molecules, including complement factor H (FH), factor D (FD), and factor I (FI). The central component of the process, C3, forms enzymes that accelerate its own activation in a positive feedback loop. When in homeostatic balance, the system facilitates destruction of microbial pathogens, apoptosis, and the removal of cell debris without damage to healthy self-tissues.

Pharmaceuticals currently in clinical trials that inhibit the complement system include lampalizumab (used to inhibit FD, currently in phase III trials for the treatment of "dry" AMD or geographic atrophy in people with specific genotypes), compstatin (an inhibitor of C3, in phase II trials for treatment of AMD), and bikaciomab (an inhibitor of FB that is in phase II trials for treatment of AMD).[5] Numerous studies of the impact of patients' genetics on their response to intravitreal anti-VEGF for AMD have been carried out, yet they have primarily focused on the effect of only one polymorphism, Y402H, even though there are four distinct haplotypes of *CFH* with different functional variants and risk profiles. Even while potential clinical applications are being investigated there are many unanswered questions about how the complement system behaves in people with different inherited genetic variants and in the different tissues of the eye.

The major inhibitor of the system, FH, is produced in the liver and secreted into the serum, where it is highly abundant.[6] Rare *CFH* mutations are associated with early-onset AMD.[7] Relatively little is known about how variants influence pathogenesis.[8,9] Factor H protein is composed of 20 short consensus repeats (SCRs), each approximately 60 amino acids in length, which share homology at specific residues.[10,11] Full-length FH is encoded by 22 exons, and a shorter form, FHL-1, stops after splicing of an alternative 10th exon. Five homologous *CFH*-related genes (*CFHR1* to *CFHR5* encoding FHR-1 to FHR-5) exist in tandem on chromosome 1q in a region of strong linkage disequilibrium (Supplementary Fig. S1). Four common haplotypes of the *CFH* region are inherited in white Europeans, and each confers a different risk of AMD (and other diseases).[9,12,13] A common deletion of entire *CFH*-related genes (*CFHR3-CFHR1*) has been found between sites of segmental duplication,[14] and two distinct isoforms of FHR-1, one acidic and one basic, have been reported.[15] Homologous sequences cause significant technical challenges for investigators trying to genotype variants and structural rearrangements.

The research questions that we sought to answer were which of the sequence variations in the *CFH* region might be functional and therefore may be of interest as prognostic biomarkers, predictive (of therapeutic response) biomarkers, or therapeutic targets for inhibition or synthetic imitation; and in which AMD-relevant human tissues the *CFH* and *CFHR1-5* genes and their splicing variants are expressed (eye tissues for local production and liver for systemic production). In order to answer these questions, we undertook massively parallel genomic sequencing of the *CFH* region in individuals who were homozygous for each of four common haplotypes. We investigated the risk of AMD-related phenotypes from a genome-wide association study of AMD to illustrate the risk associated with each haplotype. Lastly, we studied RNA expression of *CFH* and *CFHR* gene transcripts in central and peripheral retina and retinal pigment epithelium/choroid/sclera (RCS) and in the liver. Our results will help inform the development of a personalized medicine approach to complement system therapeutics.

MATERIALS AND METHODS

Study Populations

Institutional Review Board (IRB)/Ethics Committee approval was obtained, and informed consent was obtained from all participants in the original studies. The research adhered to the tenets of the Declaration of Helsinki. DNA for the sequencing experiment was from the anonymized DNA, RNA, and Serum Bank in the Centre for Public Health at Queen's University Belfast. Patients had neovascular AMD confirmed by clinical examination, grading of digital fundal color photographs, and fluorescein angiography. No details about the clinical course or treatment were available. Research ethics approval for this bank allows analysis of DNA samples for this purpose (Office for Research Ethics Committees Northern Ireland approval reference 11/NI/0139). The in silico data analyzed for this report were from IRB-approved studies. The characteristics of participants included in these studies are summarized in Supplementary Table S1.

Investigation of *CFH* and *CFH*-Related Gene Sequence

To identify functional variants in the *CFH* gene region, we selected genomic DNA samples from individuals of white European ancestry who were homozygous for each of the four common *CFH* haplotypes, as described previously.[12,13] Full details of the preparatory, sequencing, and bioinformatics methods have been provided (Supplementary Methods). Briefly, we quantified and fragmented genomic DNA, size selected, and indexed it for Illumina sequencing using the Truseq system.[16] We used Nimblegen SeqCap EZ capture to target the entire *CFH* and *CFHR* genomic region. Sequencing was performed on a HiSequation 2000 (Illumina, San Diego, CA, USA) with 100-bp paired-end reads. We aligned genomic reads to hg19 human reference sequence with the Burroughs-Wheeler Aligner (BWA) 0.5.9 *aln* algorithm[17] and used SAMtools 0.1.14[18] for sorting, indexing, and removal of duplicate reads. We employed Genome Analysis Toolkit (GATK) to recalibrate and realign and to call polymorphisms.[19] We predicted the effect of coding polymorphisms with PolyPhen-2 (Polymorphism Phenotyping v2).[20]

Investigation of the Effect of *CFH* Region Haplotypes on AMD Phenotypes

To investigate the effect of polymorphisms on the differential effect on risk of neovascular AMD compared to drusen only (as a proxy for progression), we conducted a genome-wide case–case study and candidate gene studies using the Michigan, Mayo, AREDS, Pennsylvania (MMAP; dbGAP accession phs000182.v1.p1) AMD study, consisting of 2157 cases of neovascular AMD, geographic atrophy, or drusen and 1150 healthy controls of white European ancestry (Supplementary Table S1).[21] The candidate gene studies aimed to investigate the roles of the four *CFH* haplotypes and a representative polymorphism from each of the other major AMD loci (*CFB*, *C3*, and *HTRA1*)[1–3,22–25] on the differential risk of neovascular AMD compared to drusen. The risks of drusen and neovascular AMD compared to disease-free controls are shown to illustrate the effects associated with each haplotype.

Full details of quality control and analyses are shown in the Supplementary Methods. Briefly, genotyping was conducted on Illumina Human370 microarray chips. Final analyses included 867 individuals with neovascular AMD, 519 with drusen, and 1115 healthy controls. A Q-Q plot is shown in Supplementary Figure S2. We used additive model univariate binary logistic regression in PLINK v1.07.[26,27] Statistical significance was accepted at two-sided $P < 0.05$ after Bonferroni correction for multiple testing in the genome-wide association studies. We did not apply correction to the candidate polymorphisms because their selection was based on prior hypotheses due to their known association with the AMD phenotype. We phased haplotypes of *CFH* in PLINK and used SNP Annotation and Proxy Search (Broad Institute)[28] to identify proxy single nucleotide polymorphisms (SNPs).

Investigation of FH, FHL-1, and FHR-1 to FHR-5 Expression in the Retinal Pigment Epithelium, Choroid, and Sclera and in the Retina

To investigate the relative expression of FH, FHL-1, and FHR-1 to FHR-5 in eye tissues and to investigate the expression of alternatively sliced variants, we aligned RNA-seq reads from our (ML, CAC, DS) previous studies[29] to the human reference hg19 genome using GSNAP.[30,31] Eight participants of white European ancestry from the United States were included. Full details are described in Supplementary Methods. We used SAMtools (version 0.1.19)[18] to sort reads; Picard (version 1.121)[32] to mark duplicate reads; and SAMtools[18] depth to obtain read depths. Relative read depths were calculated for FH and FHL-1 relative to FH and FHL-1 combined. An estimate of the proportion of FH relative to both FH and FHL-1 combined for

TABLE 1. Putatively Functional Polymorphisms Identified on Common *CFH* Region Haplotypes in Sequenced Homozygotes (*n* = 1 Homozygous Participant per Haplotype)

		Haplotype A	Haplotype B	Haplotype C	Haplotype D
CFH rs800292	V62I	V	V	I	V
CFH rs1061170	Y402H	H	Y	Y	Y
CFH rs1065489	D936E	E	D	E	E
CFH rs203685	Possible splice variant CCG|>CAG|	Predict reduced FH	–	–	–
CFHR3	Present/deleted	Present	Present	Present	Deleted
CFHR3 rs379370	P241S	P	S	P	–
CFHR1	Isoform/deleted	*CFHR1*A*	*CFHR1*A*	*CFHR1*B*	Deleted
CFHR1 rs145545132	H157Y	H	H	Y	–
CFHR1 rs410232	L159V	L	L	V	–
CFHR1 rs388862	E175Q	E	E	Q	–
CFHR4 rs7417769	S209N	S	S	S	N/S
CFHR4 rs181498339	Q388L	Q	Q	Q>L	Q
CFHR4 rs10494745	G552E	E/G	G	G	G
CFHR2 rs79351096	C72Y	C	Y	C	C
CFHR2 rs4085749	Splice variant	CRSTI	CRSTI	F	CRSTI
CFHR5 rs141321678	M514R	M	M	M	M>R

N/S and E/G represent heterozygosity for rare SNPs.

all samples was calculated by random effects meta-analysis using metaprop in meta (v4.0-3) in R 3.2.1.[33] This method was chosen as it allowed calculation of an appropriately weighted average proportion (taking into account the total number of reads from each individual) with an overall estimate of uncertainty (confidence interval [CI]) for each tissue.

Investigation of FH, FHL-1, and FHR-1 to FHR-5 Expression in Liver

To investigate the relative expression of FH, FHL-1, and FHR-1 to FHR-5 in liver, we accessed liver RNA-seq reads from three individuals (no details of ancestry were available) from the EBI Illumina body map[34] and EBI Expression Atlas[35] and aligned them to the human reference hg19 chromosome 1 using STAR aligner (version 2.4.0f1).[36] We sorted the alignments with SAMtools and viewed sequence in IGV.[18,37]

RESULTS

Sequencing of *CFH* and *CFH*-Related Genes From Genomic DNA

We identified nine missense coding SNPs (cSNPs) within *CFH* and the *CFH*-related genes, each of which had a minor allele that was homozygous and restricted to one of the four common haplotypes (Table 1). In addition, four heterozygous missense cSNPs (interpreted as rare SNPs against specific haplotype backgrounds; Table 1) and numerous synonymous cSNPs were also identified (Supplementary Table S2). Haplotype A carried a SNP that was predicted to create a novel splice site that would result in nonsense-mediated decay (rs203685, A) of the transcript (creating CAG\|). An ancestral gene conversion event has caused the replacement of part of the *CFHR1* gene with part of *CFH* on haplotype C only, resulting in the FHR-1 157Y, 159V, and 175Q polymorphisms in SCR 3. This means that on haplotype C, SCR 3 of FHR-1 is identical to SCR 18 of FH. These variants correlate with the previously reported[15] FHR-1 acidic and basic isoforms (CFHR1*A and CFHR1*B): Our haplotypes A and B produced the acidic isoform, and haplotype C produced the basic isoform. Haplotype D carried the deletion of *CFHR3* and *CFHR1* that we identified previously, and therefore neither isoform of FHR-1 is produced.[14] Haplotype C also carried *CFHR2* rs4085749 T,

which created a premature splice donor site GCAGG>GTAGG, resulting in replacement of residues 140 to 144 by a single phenylalanine residue, removing one of the key cysteine residues responsible for the SCR structure.

Investigation of Risk of AMD Phenotypes

The risks of drusen and neovascular AMD associated with each *CFH* haplotype were calculated to illustrate the effects of each *CFH* haplotype (Table 2). We compared genome-wide variation in individuals with neovascular AMD to those with drusen and found that two SNPs were significantly associated with differential risk (a proxy for a "progression" phenotype) after Bonferroni correction for multiple testing: rs932275 and rs2248799, both in *HTRA1* (Supplementary Table S3). We identified several SNPs with a suggestive *P* value < 5×10^{-5}, many of which were in biologically plausible genes (Supplementary Table S3). We also compared individuals with neovascular AMD to those with drusen for previously reported major AMD loci (Supplementary Table S4), but the only association identified from among these candidates was the same one that was identified at the *HTRA1* locus in the genome-wide analysis. The complement system gene variants were not significantly different between people with drusen and people with neovascular AMD in this cohort.

Retinal Expression of *CFH* and *CFH*-Related Genes

We found no expression of any *CFH*-related genes (*CFHR1-5*) in any of the eye tissues studied. Expression of both long and short *CFH* transcripts was 30- to 50-fold higher in the RCS than in peripheral or macular retina (Table 3). Within the retina, the full-length FH transcripts were considerably more abundant than FHL-1 transcripts in the macula compared to the periphery (Table 3). The RCS showed increased ratio of FH to FHL-1 transcripts in the periphery compared to the macula in seven of eight eyes (Supplementary Table S5).

Expression of *CFH* and *CFH*-Related Genes in Liver and Confirmation of the Effect of rs4085749 T on *CFHR2* mRNA

Relative to total *CFH* transcription in the liver, *CFHR1* was expressed approximately 2-fold more strongly, *CFHR2* at a

TABLE 2. *CFH* Region Haplotype Frequencies and Associated Risk of Neovascular AMD and Drusen (Additive Model Univariate Logistic Regression With Each Haplotype Compared to All Others); the Reported Risk of Invasive Meningococcal Disease and Atypical Hemolytic Uremic Syndrome Shown to Provide Context

	Haplotype A	Haplotype B	Haplotype C	Haplotype D
Frequency in normal-eye controls, $n = 1115$	0.36	0.17	0.23	0.20
Frequency in individuals with drusen, $n = 519$	0.59	0.15	0.11	0.10
Frequency in individuals with NV AMD, $n = 867$	0.60	0.15	0.11	0.09
Odds ratio for drusen vs. normal-eye controls (95% CI)	2.59 (2.21–3.04)	0.89 (0.73–1.08)	0.44 (0.35–0.55)	0.47 (0.37–0.59)
Odds ratio for NV AMD vs. normal-eye controls (95% CI)	2.75 (2.39–3.16)	0.90 (0.75–1.07)	0.41 (0.34–0.49)	0.41 (0.34–0.50)
Odds ratio for AMD progression, NV AMD vs. drusen (95% CI)	1.05 (0.89–1.24)	1.02 (0.82–1.26)	0.95 (0.74–1.21)	0.88 (0.68–1.14)
Meningococcal disease risk[13]	Neutral	Reduced	Neutral	Neutral
Atypical hemolytic uremic syndrome risk[8,15]	–	–	Increased in homozygosity	Increased in homozygosity

NV, neovascular.

similar level, and *CFHR3* approximately 2-fold less strongly (Supplementary Table S6). *CFHR4* and *CFHR5* showed lower expression, between 5% and 17% of *CFH* levels. Relative expression of *CFH* long (FH) and short (FHL-1) transcripts varied widely, with reduction of FH transcripts in proportion to the number of copies haplotype C carried (Supplementary Table S7).

CFHR2 reads from the haplotype AC heterozygote showed that the early splice donor site created by the T allele of rs4085749 carried on haplotype C was used for 66% of exon-spanning reads, while the original site 12 bases downstream was used for the remaining 34% of reads from this haplotype. Reads from the homozygous haplotype CC sample showed 68% reduction of read depth across the site, and 60% of exon-spanning reads used the novel splice site (Supplementary Fig. S3).

The liver RNA-seq reads also revealed several novel exons that were occasionally found in other transcripts. Of note was an exon within intron 4 of *CFHR3* (196,757,875–196,758,074) that was included in about half of all *CFHR3* transcripts, despite carrying an in-frame stop codon.

DISCUSSION

The central role of factor H in innate immunity and the effects of its genetic variation on risk of AMD, atypical hemolytic uremic syndrome (aHUS),[8] systemic lupus erythematosus,[38] rheumatoid arthritis,[39] and invasive meningococcal infection[9] make it intriguing, and it has yielded many surprises. Our study is the first to investigate the extended *CFH* region in individuals who are homozygous for the common European haplotypes. The results of this study should provide a clear foundation for future studies of FH function and aid the development of personalized approaches based on patients' own complement system genetics.

Haplotype A confers greatest risk of AMD and carries the 402H risk variant.[12] Codon 402 is found in both FH and FHL-1, so both products of the *CFH* gene are affected.[40] This haplotype also carries a possible new splice site (rs203685), which could reduce expression of *CFH* through nonsense-mediated decay. Haplotype A produces the acidic isoform of FHR-1.

Haplotype B has no effect on AMD risk,[12] but confers reduced risk of meningococcal disease, which may be due to altered binding of meningococci to FH or FH-related proteins.[9] We showed that the minor alleles of virtually all variants within the *CFHR3* region were found on haplotype B. *CFH* 936D and *CFHR3* 241S, two variants in the 3′untranslated region (UTR) of *CFHR3* (rs402372 and rs390837), and *CFHR2* 72Y are carried on haplotype B. This haplotype also produces the acidic isoform of FHR-1.

Haplotype C protects against AMD.[12] *CFHR1* 157Y, 159V, and 175Q, which have arisen by conversion from *CFH*, code for the basic isoform of FHR-1 on this haplotype. This may result in altered competition between FHR-1 and FH for binding sites, or alteration of the regulatory roles of FHR-1. On haplotype C, *CFH* 62I encodes FH with increased binding activity for C3b, and increased cofactor activity for complement factor I–mediated cleavage of fluid phase and cell-bound C3b.[41] The preferential use of an alternative splice donor site created by *CFHR2* rs4085749 on haplotype C may be functionally important.[42] This site is predicted to result in omission of one of the four cysteine residues that form the SCR structure and would have a major effect on the protein structure. Haplotype D is missing the entire *CFHR3* and *CFHR1* genes.[14] There are no cSNPs in *CFH* on this haplotype. Homozygotes for haplotypes C and D are at elevated risk of aHUS.[8,15,43,44] Absence of FHR-1 is thought to be critical, resulting in lack of immune tolerance to an epitope of FHR-1 that mimics a neoepitope of FH that may be formed during interaction with bacteria and development of antibodies that cross-react against FH.[15,43]

Although *CFH* was expressed in the retina and in the RCS of normal aged eyes, we found no evidence of transcription of any of the *CFH*-related genes (*CFHR1-5*) in these tissues. This finding contrasts with reports by Bennis et al.[45] and Booij et al.[46,47] of a microarray experiment that measured transcriptome expression of six human eyes, which reported *CFHR1* transcription at approximately one-fourth the level of *CFH* (and lower levels of *CFHR4*, *CFHR5*, and *CFHR2*, but not *CFHR3*). They found no *CFHR1* expression in mouse eye tissues, which is consistent with an earlier report by Luo et al.[48] in which RT-PCR indicated no expression of *CFHR1* in mouse retina or RPE/choroidal tissue. The conflicting results indicate either that our method did not detect *CFHR* mRNA where it was present, or that the Bennis et al.[45] and Booij et al.[46,47] experiments falsely detected *CFHR* mRNA. If *CFHR* genes are expressed in these tissues, the most likely explanation for their apparent absence in our experiment would be differences in the tissue processing between the two studies. Alternatively, the Bennis et al.[45] and Booij et al.[46,47] custom microarrays may have

TABLE 3. The Mean Combined Expression of FH and FHL-1 Measured by RNA-seq Read Depth in Four Eye Tissues From Eight Individuals and the Percentage of the Total That Is FH

	Mean FH + FHL-1 Read Depth (95% CI)	% FH (95% CI)
Peripheral retina, $n = 7$	13.6 (7.5–19.8)	45.3 (34.6–56.5)
Macular retina, $n = 7$	16.0 (8.0–24.0)	75.8 (60.6–87.0)
Peripheral RPE/choroid/ sclera, $n = 8$	412.6 (241.1–584.1)	48.5 (42.8–54.3)
Macular RPE/choroid/ sclera, $n = 8$	287.1 (133.1–441.1)	32.1 (22.9–42.9)

suffered cross-reactivity from FH and FHL-1 transcripts (Supplementary Fig. S1), leading to the apparent presence of *CFHR* genes. Absent expression would not necessarily indicate that *CFHR* genes have no role in these tissues, as it is likely that they could diffuse across Bruch's membrane, as FHL-1 does (though FH does not).[49] Further studies are needed to assess *CFHR* expression (if any) in AMD eyes and the effect of inflammation. *CFH* transcripts for FH and FHL-1 showed relatively low expression in the macular and peripheral retina and much greater expression in the RCS, suggesting reduced protection in the retina and greater protection in at least one of the RCS tissues. Our results suggested a greater proportion of FH compared to FHL-1 in the macular retina compared to the peripheral retina. This may be due to regulation by microRNAs. Both transcripts appear to be heavily regulated by microRNAs; however, target sites for miR146a and miR155 are adjacent on the 3′ UTR of *CFH* and absent from the 3′ UTR of the short (FHL-1) transcript. These are among the microRNAs with greatest influence on immune signalling pathways and immune homeostasis.[50]

In contrast to the eye, we found that all *CFH*-related transcripts were present in liver RNA. Both *CFH* transcripts, *CFHR1*, *CFHR2*, and *CFHR3* were much more highly expressed than *CFHR4* or *CFHR5*. Our analysis of liver RNA-seq data suggested that haplotype C may be associated with either considerable reduction in FH or increase in FHL-1 mRNA transcript levels compared to the other haplotypes. Unexpectedly, there seems to be poor correlation between levels of transcripts in the liver and the reported concentrations of their translated products in the blood plasma. FHL-1 is reported at only 2% to 8% of FH levels in plasma, despite having transcript levels in the liver greater than that of *CFH* mRNA.[6] It may be important to understand the stability of FH, FHL-1, and their degradation products. Measurement of FH and related proteins is difficult because of the potential for related proteins to cross-react with antibodies used in immunoassays. Reported plasma concentrations of FH also include FHL-1, which reacts with antibodies specific for the N-terminal SCRs common to both proteins, and there is also considerable scope for cross-reactivity with FH-related proteins. Scholl et al.[51] reported increased concentrations of most complement proteins and activation products in AMD compared to normal controls, compatible with systemic complement activation and low-grade inflammation Complement activation products were highest in those carrying *CFH* risk haplotypes for AMD.[51] In a study that used an immunoassay with antibodies highly specific for either FH 402H or 402Y, Hakobyan et al.[52] showed that plasma FH levels of normal adults were not significantly different in those with FH 402YY, 402YH, and 402HH genotypes. The largest study of genetic influences on plasma FH and AMD was reported by Ansari et al. in 2013.[53] They showed a relationship between *CFH* genotypes and concentrations of FH and of FHR-1, though they reported plasma FHR-1 concentrations in people with no copies of the *CFHR1* gene

to be >50% of those with two copies of the gene, suggesting that their assay cross-reacted with other proteins.[53] It is possible, though difficult, to create antibodies against specific variants, and gene conversion adds another layer of complexity to the measurement and assessment of function in this region.

We conducted a secondary analysis of a genome-wide association study of AMD[21] to investigate whether variation was associated with progression from drusen to neovascular AMD. Only SNPs in *HTRA1* (near *ARMS2*) showed an association when comparing individuals with neovascular AMD to those with drusen. Variants in *CFH*, *C3*, and *CFB* increased risk of drusen and AMD equally, but added no extra risk effect to differentiate those with drusen and those with neovascular AMD, suggesting the possibility that complement dysfunction is necessary but not sufficient for progression to neovascular AMD. Interestingly, earlier case–control studies reported similar findings for *CFH* and *ARMS2/HTRA1*,[54,55] whereas a longitudinal study showed a role for *CFH* on progression from early to late disease over a period of 6 years.[56] It is possible that in the case–control studies, age, sex, smoking, and/or other factors might confound or interact with genetic risk factors in a manner not seen in the cohort study.

The most important limitation of our study is that the number of donated eyes is small, and availability of a greater number of eye samples would improve our ability to draw firm conclusions about the levels of FH and FHL-1 expression in people with different *CFH* haplotypes. Collaborative studies may be required in the future to bring together enough expression data to fully explore the effects of haplotypes on the levels of expression in different eye tissues.

We have comprehensively characterized the variants that may be the underlying causes of the AMD risk conferred by the four common haplotypes. Taking into account the real-world biological complexity of the region may allow some clearer thought about the mechanisms of disease and protection, as well as the potential for future development of personalized treatments based on these mechanisms.

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