

Systematic Functional Testing of Rare Variants: Contributions of *CFI* to Age-Related Macular Degeneration

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PURPOSE. Genome-wide association (GWAS) and sequencing studies for AMD have highlighted the importance of coding variants at loci that encode components of the complement pathway. However, assessing the contribution of such alleles to AMD, especially when they are rare, remains coarse, in part because of the persistent challenge in establishing their functional relevance. Others and we have shown previously that rare alleles in complement factor I (*CFI*) can be tested functionally using a surrogate in vivo assay of retinal vascularization in zebrafish embryos. Here, we have implemented and scaled these tools to assess the overall contribution of rare alleles in *CFI* to AMD.

METHODS. We performed targeted sequencing of *CFI* in 731 AMD patients, followed by replication in a second patient cohort of 511 older healthy individuals. Systematic functional testing of all alleles and post-hoc statistical analysis of functional variants was also performed.

RESULTS. We discovered 20 rare coding nonsynonymous variants, including the previously reported G119R allele. In vivo testing led to the identification of nine variants that alter *CFI*; six of which are associated with hypoactive complement factor I (FI). Post-hoc analysis in ethnically matched, population controls showed six of these to be present exclusively in cases.

CONCLUSIONS. Taken together, our data argue that multiple rare and ultra-rare alleles in *CFI* contribute to AMD pathogenesis; they improve the precision of the assessment of the contribution of *CFI* to AMD; and they offer a rational route to establishing both causality and direction of allele effect for genes associated with this disorder.

Keywords: in vivo testing, complement pathway, rare allele burden

Age-related macular degeneration is a complex, multifactorial ocular neurodegenerative disease and one of the leading causes of vision loss, affecting up to 8% of those over the age of 60.¹ In addition to numerous environmental susceptibility factors, including age, ethnicity, smoking, dietary fat, and antioxidant/vitamin intake,²⁻⁴ genetic risk factors are thought to confer as much as 50% of the risk.⁵

The advent of genome-wide association studies (GWAS) catalyzed the mapping of a number of AMD susceptibility loci, starting with complement factor H (*CFH*)⁶⁻¹¹ and extending progressively to single nucleotide polymorphisms (SNPs) in or near genes associated with a variety of physiological processes including angiogenesis, lipid transportation, extracellular cell matrix remodeling, and cellular stress response.¹²⁻¹⁴ Prominent among these has been the complement pathway, with six members of this cascade, *CFH*,⁶⁻¹¹ *C2/CFB*,¹⁵ *C3*,⁹ *CFI*,^{9,16-18} and *C9*⁹ now understood to contribute to the genetic burden of the disorder, accounting for as much as 60% of the genetic risk of AMD.¹⁹

The majority of our understanding of the complement pathway is derived from its roles in innate immunity, where it can be activated by antigen-antibody complexes (classical pathway); by bacterial mannose groups (lectin pathway); or is active constitutively at low levels with an amplification feedback loop (alternative pathway; AP). In the present study, we focus on a major negative regulator of the AP, *CFI*, a serine protease involved in the inactivation of C3b and the subsequent inhibition of the amplification of the AP response.^{16,20-22}

The genetic causality of *CFI* to AMD pathogenesis was suggested initially through targeted SNP analysis of (1) suggestive linkage peaks identified by a meta-analysis of whole-genome linkage studies, and (2) of complement pathway components.^{16,23} More direct proof for the gene (as opposed to the region with inferred candidacy by proxy due to a known function of the protein) was obtained by the identification of coding variants in *CFI*, with an observed increased burden of rare variants in AMD patients versus controls.^{9,16,24,25} In addition, one study reported that 40% of advanced AMD patients (i.e., patients with wet or dry AMD) have rare variants

within *CFI* and decreased hypoactive complement factor I (FI; *CFI* protein) serum levels.¹⁷ However, it is not clear what fraction of such biochemical observations is linked causally to *CFI* alleles directly, versus *CFI* cascade dysfunction (due to variants in other genes relevant to the pathway). Central to this conundrum has been the persistent challenge in interpreting the consequences of rare coding variants on protein function and the concomitant absence of power to detect statistical association with individual rare alleles. To overcome some of these obstacles and to derive a more accurate assessment of the overall burden of *CFI* to AMD, we sought to (1) sequence *CFI* in a well-phenotyped AMD cohort, (2) access functionally all discovered coding, nonsynonymous changes using a previously derived zebrafish model of retinal vasculogenesis, and (3) derive function-informed population statistics about *CFI*.

METHODS

Cohort Description

Recruitment was performed at Johns Hopkins Hospital in Baltimore, MD of unrelated subjects.²⁶ The study was approved by the institutional review board at Johns Hopkins University and informed consent was attained for all subjects. All subjects were treated in accordance with the Declaration of Helsinki. All subjects included in the study were of self-described Northern European descent and underwent standard clinical examinations, assessed for visual acuity, stereoscopic color fundus photography, and dilated slit-lamp biomicroscopy. Grading was performed following the Age-Related Eye Disease Study (AREDS) classification system and diagnosis of AMD was equivalent to AREDS category 4, presenting with either geographic atrophy or choroidal neovascularization. The AMD cohort consisted of 415 wet AMD samples and 316 dry AMD samples. Our control subjects were those individuals who were at least 60-years old with no signs of AMD (no drusen or RPE abnormalities).

Sequencing and Analysis

Sequencing of *CFI* was performed on 731 AMD cases and 161 age- and ethnically matched controls using an Ion Torrent platform. The Torrent Suite Software version 4.2 (Thermo Fisher Scientific, Waltham, MA, USA) was used to generate FASTQ files from bcl (base-calling) files. Alignment to hg19 reference genome was performed using the Burrows-Wheeler Aligner²⁷ and to assess mapping quality and duplication rates we used the BamUtil package. For removal of unmapped reads and PCR duplicates, we used Picard and SAMtools. Genotyping calls were made according to the Genome Analysis Toolkit²⁸ multisample Haplotype Caller and annotation with ANNOVAR.²⁹ Variant files were converted to PLINK binary files through PLINK/SEQ for downstream sample and variant assessment.³⁰ All variants were confirmed by Sanger sequencing (Supplementary Table S2).

Zebrafish Injection and Analysis

All animal work performed was under approved protocols of the Duke Institutional Animal Care and Use Committee and in adherence to the ARVO Animal Statement. The *CFI* homolog in zebrafish (ENSDART00000162353.1) encodes for the protein that is 40% identical to humans, therefore we used the human encoding mRNA in our studies to determine the effect of the alleles in the context in which they are found. Overexpression studies were performed by injecting 100 pg of wild-type or mutant encoding human mRNA into 1- to 4-cell staged

fli1:EGFP zebrafish embryos ($n > 30$ –50 embryos/condition/experiment). All experiments were performed in triplicate. In vitro transcription of the capped-mRNA used for injections was done using the SP6 mMessage mMachine kit (Ambion, Foster City, CA, USA). For the in vivo analysis, 5 days post fertilization (dpf) embryos injected with either wild-type or mutant *CFI* were imaged at 5 dpf using the Nikon AZ100 microscope (Nikon Corporation, Yokohama, Japan) with a $\times 2$ objective and a 5.0-megapixel DS-Fi1 digital camera with the $\times 8$ zoom (Nikon Corporation). Samples were masked and measurements of hyaloid vessel diameter were taken using ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

We compared the frequency of the variants identified in our AMD affected samples with our controls and to ethnically matched population controls from ExAC or the Welllderly cohort, and we assessed differences in frequency using one-tailed Fisher's exact test. To compare our AMD cohort and the ExAC cohorts, we made two assumptions: (1) there was minimal missingness in the sequencing data to determine total allele count, and (2) that each individual had only one rare variant. Comparisons between wild-type- and mutant-injected conditions were analyzed using Student's *t*-test. *P* values less than 0.05 were considered nominally statistically significant.

RESULTS

Identification of *CFI* Coding Variants

During the past 20 years, using the AREDS severity scale grading system,³¹ we have collected and phenotyped an AMD cohort through the Wilmer Eye Center clinic (Johns Hopkins Hospital, Baltimore, MD, USA), while at the same time obtaining DNA samples from clinically assessed healthy retinas (individuals > 60 -years old, with no RPE abnormalities and fewer than five small [< 63 - μ m] drusen), which we have contributed to a host of GWAS studies for AMD.^{32–35} To identify *CFI* variants in this cohort, we performed targeted sequencing of 731 AMD and 161 controls across the coding region and splice sites of the canonical *CFI* transcript (NM_000204). Subsequent to filtering and Sanger-sequence verification, we obtained 18 missense and two nonsense alleles (Table 1), all of which were heterozygous in the patients. All detected variants were rare (minor allele frequency [MAF] < 0.01) and 15/20 were found exclusively in cases as observed by comparison to ethnically matched population samples (from ExAC). While several of these alleles have been reported in AMD cohorts, three were novel (M532V, E305X, and D249E) and ultrarare (MAF < 0.001). Overall, variants were distributed across the entire protein (Fig. 1) with no apparent enrichment in *CFI* domains.

Functional Assessment of Discovered *CFI* Alleles

The presence of benign rare (or ultrarare) alleles can cause a loss of power in locus burden tests and additionally, their rarity typically precludes single-variant association testing. One solution relies on the establishment of the relevance of discovered variants to pathology, followed by revised burden testing of only functionally relevant alleles. This has helped improve the assessment of risk, as well offer an estimation of penetrance in some complex traits.^{36–38} In the context of *CFI* and AMD, we and others have reported previously the development and implementation of a vascular assay in the zebrafish retina as a functional surrogate for some AMD loci.^{18,39} Specifically, use of the *fli1:EGFP* (enhanced green

TABLE 1. Comparison of Frequency of CFI Coding Variants Identified in Duke Cohort With Population Controls (ExAC)

Nucleotide	Amino Acid	Duke			Relative Risk	ExAC EUR-Non-Finnish Allele Count/Total Alleles (%MAF)	Duke AMD vs. ExAC One-Tailed Fisher's Exact χ^2 P Value
		AMD Alleles/Total Alleles (%MAF)	Unaffected Alleles/ Total Alleles (%MAF)				
C191T	P64L	1/1266 (0.079)	0/306 (0)	1.2421	1/66716 (0.0015)	0.0424	
G355A	G119R	3/1462 (0.2052)	0/322 (0)	1.2212	63/66532 (0.0947)	0.1685	
G373A	G125R	2/1462 (0.1368)	1/322 (0.3106)	0.813		NA	
A452G	N151S	1/1462 (0.0684)	0/322 (0)	1.2205	1/66740 (0.0001)	0.0424	
G454A	V152M	1/1462 (0.0684)	0/322 (0)	1.2205	3/66740 (0.0045)	0.083	
G560A	R187Q	1/1462 (0.0684)	0/322 (0)	1.2205	9/66736 (0.0135)	0.1948	
C608T	T203I	1/1462 (0.0684)	0/322 (0)	1.2205	29/66738 (0.0435)	0.4781	
C747A	D249E	1/1458 (0.0686)	0/322 (0)	1.2212		NA	
G782A	G261D	2/1454 (0.1376)	2/320 (0.625)	0.609	131/66730 (0.1963)	0.7811	
G859A	G287R	3/1456 (0.206)	1/320 (0.3125)	0.9145	3/66738 (0.0096)	0.0002	
A870C	E290D	1/1456 (0.0687)	0/320 (0)	1.2201	2/66738 (0.0030)	0.0629	
G907A	E303K	2/1446 (0.1383)	0/322 (0)	1.2233	0/66328 (0)	0.0005	
G913T	E305X	2/1448 (0.1381)	0/322 (0)	1.223		NA	
C930G	D310E	2/1444 (0.1385)	0/322 (0)	1.2236		NA	
G1217A	R406H	0/1374 (0)	1/308 (0.3247)	0	111/66550 (0.1668)	0.9999	
A1322G	K441R	14/1456 (0.9615)	1/322 (0.3106)	1.1425	329/66716 (0.4931)	0.0172	
C1342T	R448C	2/1456 (0.1374)	0/322 (0)	1.2218	2/66710 (0.0030)	0.0027	
C1420T	R474X	2/1454 (0.1376)	0/322 (0)	1.2221	5/66592 (0.0075)	0.009	
A1594G	M532V	1/1458 (0.0686)	0/320 (0)	1.2198		NA	
C1657T	P553S	1/1458 (0.0686)	0/320 (0)	1.2198	144/66708 (0.2159)	0.957	

Values in bold are those that show significant *P*-values <0.05 when comparing Duke AMD cohort vs. ExAC.

fluorescent protein) transgenic reporter, which allows the visualization of the vascular endothelium in live zebrafish, has enabled the detection and quantitative scoring of vascular or angiogenic phenotypes,^{40,41} including suppression of two AMD risk loci: the *VEGFA*, and phospholipase D1 (*PLD1*).^{42,43} Extending this paradigm to *CFI*, we have shown that the rare, penetrant mutation *G119R*, has reduced activity compared with wild-type,¹⁸ as measured by comparing the effects of wild-type versus mutant message on the vascular architecture of the hyaloid vessels in the retinas of 5-dpf zebrafish larvae. Importantly, reduced FI plasma levels in the sera of 119R heterozygotes and the mutant protein's decreased ability to degrade C3b supported this effect of *G119R*.¹⁸

We took advantage of this paradigm to study the contribution of functional coding variation of *CFI* to AMD, wherein we assayed 17 missense variants in the hyaloid vessel paradigm (the 18th discovered allele was the previously assayed *G119R*). The nonsense mutation *E305X* was also included as a positive loss of function control, while two alleles *K441R* and *P553S* that are of high frequency in ExAC (in the public domain, <http://exac.broadinstitute.org/>)⁴⁴ yet show no association with AMD were

used as negative (specificity) controls. We injected 1- to 4-cell stage *flil*:EGFP zebrafish embryos with 100 pg of wild-type or mutant human mRNA (*n* = 30–50 embryos per injection in triplicate) and imaged the hyaloid vessels of 5-dpf larvae, by measuring the diameter of the vessels at three different positions in the imaged field of each embryo (each field was anchored with the optic nerve at the bottom center of the field to ensure similar placement within eye cup).¹⁸ We reproduced previously published observations in which *CFI* is dosage sensitive (either too much or too little results in abnormal hyaloid architecture); in triplicate experiments, injection of 100 pg of wild-type mRNA induced a decreased diameter of the hyaloid vessels when compared with uninjected controls.¹⁸ We therefore expressed all coding variants identified in our cohort. Embryos injected with 100 pg of human mRNA encoding *N151S*, *V152M*, *R187Q*, *T203I*, *G261D*, *G287R*, *R406H*, *K441R*, *M532V*, or *P553S* presented a hyaloid vessel morphology and diameter that were indistinguishable from zebrafish injected with 100 pg of human wild-type *CFI* mRNA, suggesting that these variants are likely benign (or at least are so mild that they cannot induce pathology within the dynamic range of our assay). In contrast, we

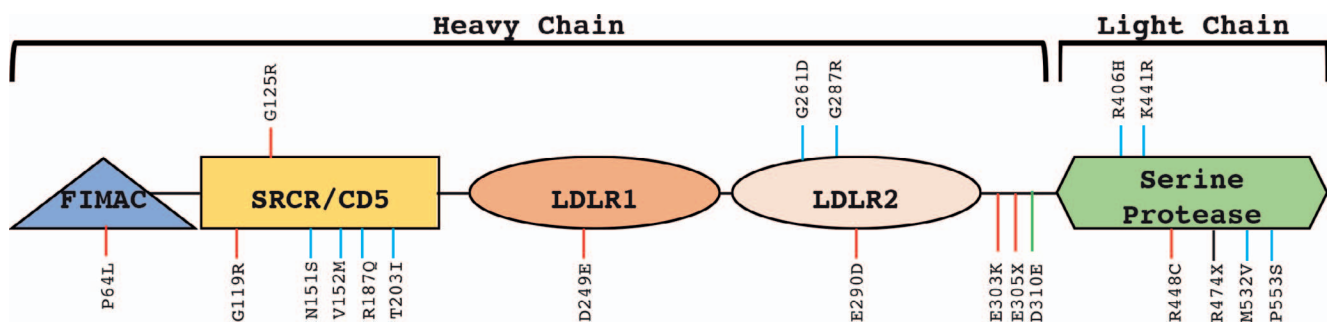


FIGURE 1. Domain structure of *CFI*. Variants identified in our AMD cohort are positioned along the domains of *CFI*. Those above the domains were present in cases and controls, whereas those beneath the domains were exclusively in cases (red lines, hypoactive alleles; blue lines, benign; green line, hyperactive; black line, not tested). Complement factor I has a FI membrane-attack-complex domain (FIMAC), a CD5-like/scavenger receptor cysteine-rich (SRCR) domain, two low-density lipoprotein receptor (LDLR) domains, and a serine protease domain.

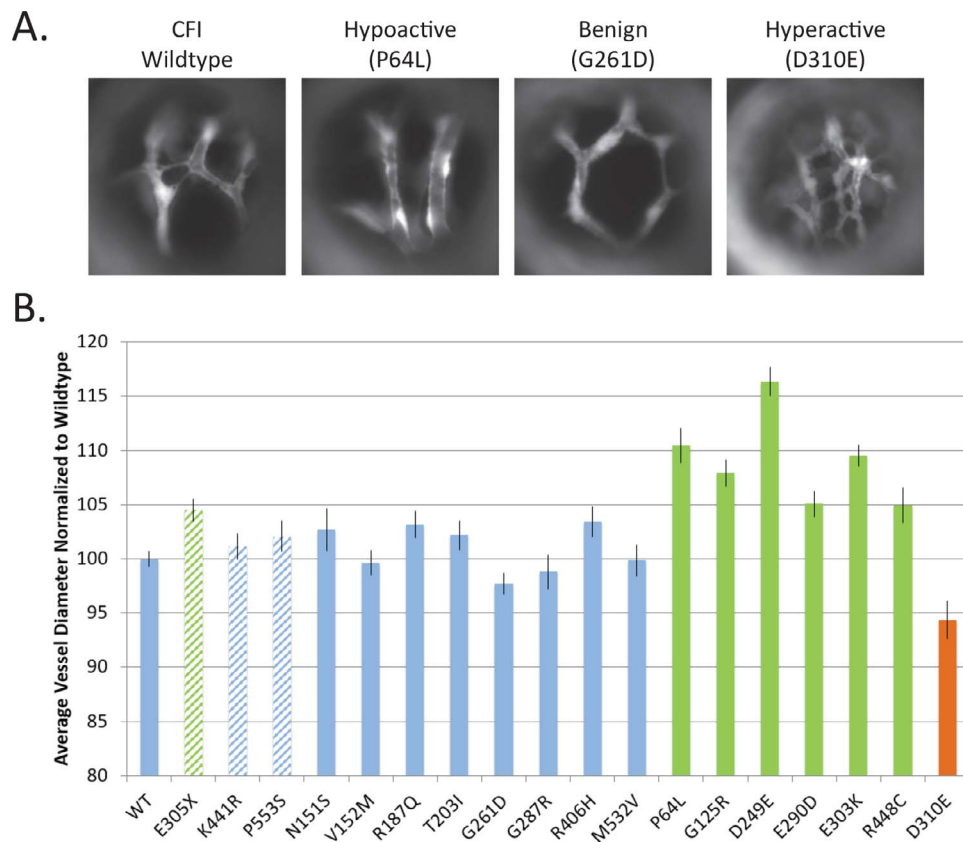


FIGURE 2. Zebrafish in vivo analysis of variants. (A) Representative images of the hyaloid vessels in 5dpf *fli1:EGFP* zebrafish. (B) Comparison of the overexpression of wild-type and mutant encoding human mRNA hyaloid vessel diameter normalized to wild-type. * $P < 0.01$ for green and orange bars (bars with stripes, positive and negative control; blue bars, benign; green bars, hypoactive allele; orange bar, hyperactive allele). Error bars represent standard error.

identified one hyperactive variant, D310E, (expression of mutant mRNA induced a significantly smaller hyaloid vessel diameter), and seven hypoactive variants, P64L, G125R, D249E, E290D, E303K, E305X, and R448C in which we saw a larger diameter of hyaloid vessels when compared with wild-type (Fig. 2, $n = 79$ –448 embryos, normalized to wild-type; $P < 0.01$, two-tailed Student's *t*-test; Supplementary Table S1). Similar to our previous study, we did not appreciate any abnormalities in the truncal vasculature, nor did we observe gross dysmorphologies of the injected larvae, abating possible toxicity concerns.

Our functional data were intimating that there might be a marked overrepresentation of functional alleles in AMD patients, because all but one of the functional variants we discovered were present exclusively in our patient cohort. However, our clinically assessed control cohort is too small to offer confidence of this assessment. Therefore, we turned to ExAC as a cross-sectional population control set and we asked how frequent were all eight alleles, deemed to be pathogenic in our assay, compared with approximately 67,000 population control chromosomes (ExAC, European, non-Finnish).⁴⁴ The rarity of each individual allele impedes the generation of a signal. Even the G119R variant, which has shown robust association to AMD and functional relevance to CFI¹⁸ is found in 63 ExAC chromosomes and fails to reach significance ($P = 0.169$; Table 1), likely due to the fact that ExAC population controls were not assessed for AMD. Nonetheless, when all functionally derived pathogenic alleles are treated together as a group, their total incidence was significantly higher in AMD versus ExAC ($P = 4.067 \times 10^{-5}$, odds ratio [OR] = 6.363, 95% confidence interval [CI] = 3.015–11.944). In contrast, grouping

and testing all benign variants in a similar fashion was marginal ($P = 0.054$), supportive of the idea that there is a significant enrichment of functionally pathogenic variants in CFI in AMD patients. Notably, given that a subset of ExAC “controls” will eventually become AMD patients, we anticipate that the observed enrichment is a conservative estimate.

Because of the lack of late-onset phenotypic data in ExAC, we sought out a healthy aging cohort and identified the Wellderly cohort, composed of “healthy elderly,” individuals older than 85 years of age with no history of chronic disease. We assessed all 20 variants identified within our cohort in 511 Wellderly exomes. Among the 511 individuals of European descent who did not have AMD, we detected four variants, G261D (4/1022 alleles), R406H (1/1022 alleles), K441R (1/1022 alleles), and P553S (1/1022 alleles). All four of these alleles were predicted to be benign from population genetics and tested benign in our zebrafish assay). Overall, there was no increase in incidence in the four benign variants between AMD versus Wellderly ($P = 0.1613$, OR = 1.64, 95% CI = 0.719–4.122). These data recapitulate the expected enrichment of pathogenic variants in CFI in AMD patients but also provide an assessment of the total burden of this locus in AMD that cannot be captured by allele counting alone.

Plasma FI Levels as a Biomarker for CFI Dysfunction

Nine of 20 variants tested in our assays have been reported in a previous AMD study, with concomitant measurements of plasma FI levels,¹⁷ a phenotype not available in our cohort.

TABLE 2. Concordance of *CFI* Variants Identified in Our AMD Cohort for Zebrafish In Vivo Assay and Patient Serum Reports From Kavanagh et al.¹⁷

<i>CFI</i> Coding Variant	Kavanagh et al. # Samples & FI Serum Level	Domain	Zebrafish Functional Assay	Zebrafish & Patient Serum Functional Concordant
P64L	1 low	FIMAC	Hypoactive	Yes
G119R	4 low	CD5/SRCR	Hypoactive	Yes
V152M	2 low	CD5/SRCR	Benign	No
G261D	12 normal	LDLR 2	Benign	Yes
G287R	3 low	LDLR 2	Benign	No
D310E	1 low, 1 normal	Connecting Region	Hyperactive	Inconclusive
R406H	3 normal	SP	Benign	Yes
K441R	2 low, 16 normal	SP	Benign	Yes
P553S	10 normal	SP	Benign	Yes

We reasoned that a direct comparison of *CFI* functionality and FI levels might inform the utility of this surrogate test and provide initial evidence of its utility as a biomarker. We therefore compared the in vivo–derived allele pathogenicity with the direction of the reported FI serum levels from Kavanagh et al.¹⁷ (Table 2). Encouragingly, four of nine variants tested benign in our zebrafish model (G261D, R406H, K441R, and P553S) were reported to have normal FI serum levels, whereas two partial loss of function variants, G119R and P64L, tracked with low FI serum levels in human patients. A seventh variant, D310E, which we report as hyperactive, was reported with inconsistent patient data. Finally, two variants that scored benign in the in vivo assay, V152M and G287R, have been found in patients with low FI serum levels. This discordance may be due to either a limitation in the dynamic range and/or sensitivity of the zebrafish assay or to the possibility that these variants themselves do not alter FI functional in a direct manner, but rather exert their effect by disrupting *CFI* interactions with factors such as IL-6, which has been reported to regulate FI synthesis in the liver.⁴⁵

DISCUSSION

Here, we report a sequencing and functional testing study of coding, nonsynonymous variants in *CFI*, one of the loci associated with AMD, for which the majority of the variants due to their rarity will never generate genome-wide statistical significance. We found a number of rare and ultrarare alleles that likely contribute to disease, improving the estimate of the overall genetic burden of this locus to AMD. Moreover, comparison of annotated allele data (and direction of effect) with FI plasma serum levels suggest that functional *CFI* variants correlated with reduced FI plasma serum levels, but the reciprocal predictor (FI plasma serum levels being diagnostic of AMD status¹⁷) is not as informative. To our knowledge, this is the first systematic in vivo functionalization of an allelic series in AMD and might be a useful approach for the detection and assessment of rare alleles in other AMD-associated loci for which sequencing data are either sparse or challenging to interpret.

To our knowledge there are no known AMD genes that can predict which advanced form of AMD (either dry or wet) a patient will manifest or what additional differentiating factors dichotomously contribute to AMD disease progression. However, we know that an individual allele is capable of manifesting either wet or dry AMD or even another disease further convoluting our understanding of allele function on disease outcome. Our data thus highlights a challenge and an opportunity in complex disease genetics, in which both common and rare variants can contribute to pathogenesis but the latter are essentially invisible due to lack of statistical

power. This architecture can confound rare-allele association studies and distort calculations of true burden. For some loci, such as *CFI*, the genetic burden to disease appears to be derived almost exclusively from rare and ultrarare coding alleles.^{9,17,46} Functional studies such as ours can inform these approaches.

At the same time, caution is warranted. Functional assays are subject to false-positive and false-negative rates. In the present study, we recognize that a developmental surrogate is used for a late-onset disorder. Our utilization of human genetics to evaluate our assay suggests that this surrogate phenotype can contribute to our understanding of the effects of alleles in the context they are identified in (through the use of human encoding mRNA) on protein function. Additionally, the effect of alleles on protein function remains true irrespective of time, while the potential impact of false positive/negatives of such assays must be balanced with the absence of an alternative practical surrogate. We are encouraged by two observations: (1) that the alleles deemed to be functional by our in vivo assay were found to be more common in cases compared with controls, and (2) the four alleles that have elevated frequency in the general population behave as benign. Additionally, the fact that in total, all coding variants identified (with 15/20 in ExAC solely) were observed significantly more in our AMD cohort compared with population controls (ExAC: $P = 0.0012$, $OR = 1.865$, 95% $CI = 1.29-2.60$) supports further the idea that there is an increased mutational burden in *CFI* in relation to AMD. Further, only four of 20 variants were identified in the Welllderly cohort (all benign in our zebrafish in vivo assay) and these variants, in total, were observed at a similar rate to those in our AMD cohort ($P = 0.1613$, $OR = 1.64$, 95% $CI = 0.719-4.122$). Moreover, for the limited set of pathogenic variants for which we have FI serum levels, we see general concordance, also providing a measure of reassurance. Although the allele counts are modest, these data are consistent with the notion that the false-positive rate of these assays is likely to be low. At the same time, it remains challenging to estimate the false-negative rate. Two of the alleles that scored benign in our assays were found in patients with low FI serum levels. We cannot resolve between the possibilities that (1) these alleles are benign and another *CFI* variant drives the serum phenotype, (2) these alleles are benign and mutations at another locus drives the serum phenotype, or (3) that these alleles are pathogenic but below the dynamic range of our assays.

Finally, we note that several additional AMD-associated genes can potentially benefit from this approach. Credible in vivo models for *VEGFA* and *PLDI* have previously been reported^{42,43} and we reason that utilization of the same retinal vascular paradigm could be applied to the majority of other AMD-associated loci to attain functional relevance. Further

studies can also be done to fully quantify the effects of variants on vascular branching across the entire eyecup of the zebrafish. Ultimately, the expansion of such assays across multiple genes and the concomitant increase of the number of alleles tested will provide both a credible measure of the specificity/sensitivity of such assays and of the true contribution of rare coding variants to this disorder.

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