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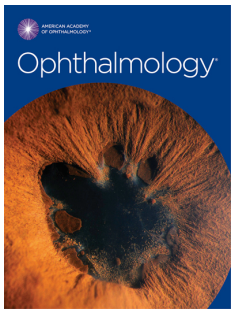

Loss-of-function mutations in the *CFH* gene affecting alternatively encoded Factor H-like 1 protein cause dominant early-onset macular drusen

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Loss-of-function mutations in the *CFH* gene affecting alternatively encoded Factor H-like 1 protein cause dominant early-onset macular drusen

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Running head: *CFH* loss-of-function mutations affecting FHL-1 cause early onset macular drusen

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This article contains additional online-only material. The following should appear online-only: Figures 1, 2, and 4, and Supplementary Data A, B, C and D.

Abstract

Purpose: To characterise the molecular mechanism underpinning early-onset macular drusen (EOMD), a phenotypically severe sub-type of age-related macular degeneration (AMD), in a sub-group of patients.

Design: Multi-centre case series, *in vitro* experimentation and retrospective analysis of previously reported variants.

Participants: Seven families with apparently autosomal dominant EOMD.

Methods: Patients underwent comprehensive ophthalmic assessment. Affected individuals from families A, B and E underwent whole exome sequencing. The probands from families C, D, F and G underwent Sanger sequencing analysis of the Complement Factor H (*CFH*) gene. Mutant recombinant Factor H Like-1 (FHL-1) proteins were expressed in HEK293 cells to assess the impact on FHL-1 expression and function. Previously reported EOMD-causing variants in *CFH* were reviewed.

Main Outcome Measures: Detailed clinical phenotypes, genomic findings, *in vitro* characterization of mutation effect on protein function, and postulation of the pathomechanism underpinning EOMD.

Results: All affected participants presented with bilateral drusen. The earliest reported age of onset was 16 years with a median of 46 years). Ultra-rare (MAF ≤0.0001) *CFH* variants were identified as the cause of disease in each family: *CFH* c.1243del, p.(Ala415ProfsTer39) het; c.350+1G>T het; c.619+1G>A het, c.380G>A, p.(Arg127His) het; c.694C>T p.(Arg232Ter)het [identified in two unrelated families in this cohort]; and c.1291T>A, p.(Cys431Ser). All mutations affect complement control protein domains (CCP) 2-7, thus are predicted to impact both FHL-1, the predominant isoform in Bruch's membrane(BrM) of the macula, and FH. *In vitro* analysis of recombinant proteins FHL-1_{R127H}, FHL-$1_{A415f/s}$ and FHL-1_{C431S} demonstrated that they are not secreted and thus are loss-of-function. Intra-cellular expression of mutant proteins was low, suggesting they may be rapidly degraded due to protein unfolding or instability. Review of 29 previously reported EOMD-causing

mutations found that 75.8% (22/29) of impact FHL-1 and FH. In total, 86.2% (25/29) EOMD-associated variants cause haploinsufficiency of FH/FHL-1.

Conclusions: EOMD is an under-recognised, phenotypically severe sub-type of AMD. We propose that haploinsufficiency of FHL-1, the main regulator of the complement pathway in BrM, where drusen develop, is an important mechanism underpinning the development of EOMD in a number of cases. Understanding the molecular basis of EOMD will shed light on AMD pathogenesis given their pathological similarities.

Key words: early onset macular drusen, age-related macular degeneration, complement factor-H, *CFH*, FHL-1

Introduction

Age-related macular degeneration (AMD) represents a leading cause of irreversible vision loss, accounting for 8.7% of global blindness [1]. The condition is characterised by inflammation and the deposition of extracellular material in the form of drusen, in Bruch's membrane (BrM). Drusen can cause metabolic disruption that leads to dysfunction and death of retinal pigment epithelium (RPE). Later stages of AMD may be characterised by geographic atrophy or choroidal neovascularisation, these are associated with severe loss of central vision. It is now widely accepted that an excessive inflammatory response driven by inadequate regulation of the complement cascade is a major contributory factor to AMD [2]. AMD is a disease of multifactorial aetiology with a strong genetic component and the role of the complement pathway in AMD pathogenesis is corroborated by the implication of genetic variants in a number of complement factors with AMD risk [3, 4]. Genetic variants associated with AMD represent a broad allelic range, from common polymorphisms (minor allele frequency (MAF) >1%) that confer a relatively low risk of disease [5-9], to relatively rare variants (MAF<1%) that demonstrate high penetrance, e.g. the p.(Arg1210Cys) substitution in *CFH* [10].

The complement system is a crucial component of host innate immunity [11]. It is a cascade system comprising three activation pathways (classical, lectin and alternative), each of which is engaged

uniquely, but which converge upon three common goals: modifying the membrane of an unwanted cell for phagocyte recognition, generation of membrane attack complexes for cell lysis, and promoting an inflammatory response[2]. Complement can activate on all surfaces, both host or foreign, so the host requires mechanisms to prevent inappropriate self-directed damage [12]. The alternative pathway (AP) is constantly active at a low level and contains a positive feedback loop to allow rapid amplification of the complement response. Tight regulation is required to maintain balanced immune homeostasis and it is increasingly recognised that defective regulation of the AP plays a central role in human disease [13, 14].

The *complement factor H* (*CFH*) gene encodes a 155 kDa plasma protein known as Factor H (FH)[15] that functions as a complement regulator by binding to host surfaces to protect them against complement activation. It mainly exerts its effect on the AP pathway, negatively regulating the positive feedback loop. FH harbours 20 complement control protein (CCP) domains, each comprising 60 amino acids, that can be grouped into three functional domains, CCP 1-4 form a binding site for co-factor activity; while both CCP 6-8 and CCP19-20 facilitate binding of FH via glycoaminoglycans (GAGs) to cell surfaces and extracellular matrices (ECM) [2, 16].

Disease-causing variants in *CFH* result in three distinct pathological syndromes: atypical haemolytic uraemic syndrome (aHUS), C3 glomerulopathy (C3G; a clinical entity that encompasses C3 glomerulonephritis and dense deposit disease (DDD) -formerly membranoproliferative glomerulonephronophthitis type II or MPGN II), and AMD. An apparent genotype-phenotype correlation exists whereby the majority of aHUS-causing variants affect CCP19 and CCP20 [17], whilst variants associated with AMD predominantly affect CCPs 1-4, and 6-8 [18, 19]. In 2008, Boon et al., and then van de Ven et al. in 2012, showed that an early-onset sub-type of AMD was caused by monogenic inheritance of ultra-rare variants in *CFH* [20, 21]. This AMD sub-type (which for clarity we term 'early-onset macular drusen' (EOMD)) demonstrates much earlier age of onset (mean age = 50 years), causing many more years of substantial visual loss than AMD [20, 21]. There have since been few EOMD cases reported [22-25].

Alternative splicing of *CFH* exon 9 produces a variant of FH known as factor H-like 1 (FHL-1) that is identical to CCPs 1-7 of FH, has a unique carboxy-terminal tail of four amino acids, and is significantly smaller (49kDa). Recent work has suggested that FHL-1 retains the same regulatory functions as FH and is able to bind to surfaces via its single GAG-interaction domain at CCPs 6-7 to regulate the complement cascade [26]. Impaired FH and FHL-1 function leads to disease as a result of inflammation and cellular debris mishandling due to excessive AP activation driven by defective regulation of the complement cascade [2, 26]. Genetic studies of AMD fail to distinguish between FH and FHL-1. FHL-1 is the major isoform present within BrM of the retina [26, 27], a major site of AMD pathogenesis and where drusen, the characteristic lesions of AMD, form. It has been suggested that it is this isoform that protects BrM against complement activation [26].

Herein, we perform functional and variant localization analysis of the EOMD-causing variants that lie within FHL-1 and define the mechanism responsible for one subgroup of patients affected by this phenotypically severe condition.

Methods

Ethics and Patient Recruitment

Ethics committee approval was obtained from the North West Regional Ethics Committee for all aspects of this study (15/YH/0365) and the protocol observed the tenets of the Declaration of Helsinki. Written informed consent was obtained from each study participant as an essential pre-requisite for study inclusion. Patients with early-onset macular drusen (EOMD) were included in our analysis. Given the heterogeneous nature of drusen and to limit bias, we did not select patients based on the type/size of drusen they presented with.

Clinical Assessment

Each patient underwent full ophthalmic assessment including visual acuity and dilated fundus examination. Fundus photographs were obtained using Ultra-Widefield Optos™ colour fundus imaging (Optos plc, Dunfermlin, UK). Fundus autofluorescence (FAF) imaging was conducted using either the Spectralis (Heidelberg Engineering Ltd., Heidelberg, Germany) or ultra-widefield confocal scanning laser imaging (Optos™ plc, Dunfermlin, UK). Optical coherence tomography (OCT) was

performed using the Spectralis OCT platform (Heidelberg Engineering). Electroretinography (ERG) was performed to standards specified by the International Society for Clinical Electrophysiology of Vision (ISCEV). Clinical history was also obtained to discern the presence of additional health problems.

Whole-exome sequencing

Whole-exome sequencing (WES), performed as previously published[28], was carried out as part of an ongoing study on inherited retinal disease (UK Inherited Retinal Dystrophy Consortium, UKIRDC) in families without a molecular diagnosis following NGS screening for a panel of 105 or 176 genes known to cause inherited retinal dystrophy. Detailed methodology can be found in Supplementary Data A (available at www.aaojournal.org). WES data from affected relatives (A:II.1 and A:II.3; E:III.11 and E:II.8) was analysed for shared rare variants. Variants in genes known to be involved in IRD were examined as a priority. Identified variants were interpreted according to the Association for Clinical Genetic Science Best Practice Guidelines for Variant Classification 2018.

Sanger sequencing of CFH

Seventy-five patients diagnosed with dominant/early drusen who tested negative for the *EFEMP1* c.1033C>T; p.(Arg345Trp) variant were retrospectively identified from a referrals database and were subjected to screening for variants in the *CFH* gene by Sanger sequencing. The coding exons and flanking intronic sequences of *CFH* (NM_000186) plus an additional four amino acids unique to the alternative transcript, FHL-1 (exon 10, NM_0010149975) were amplified by PCR and subject to bi-directional Sanger sequencing (see supplementary data A, available at www.aaojournal.org,for details). Variants were interpreted as before (see 'whole exome sequencing').

Functional Characterization of Genetic Variants

N-terminal His-tagged, DNA sequences encoding human FHL-1 or mutant versions of FHL-1, were synthesized and inserted in to pcDNA3.1 by GeneArt (Invitrogen). Plasmid DNA was stably transfected in to HEK293 cells using polyethylenimine (PEI) and culture media was harvested from transfected cells at 24, 48, 72, and 144 hours. His-tagged recombinant proteins were purified from the harvested media using Amintra Ni-NTA affinity resin (Expedeon, CA, USA) by gravity flow

chromatography. Cell lysates were made from transfected HEK293 cells and quantified using the Pierce BCA protein assay. Western blotting was conducted as previously described[26]. Detailed methodologies for plasmid preparation, transformation and transfection, purification of recombinant proteins and Western blotting can be found in Supplementary Data A (available at www.aaojournal.org).

Results

Retinal Findings

We report ten individuals from seven unrelated families with a monogenic form of early-onset macular drusen (Figure 1 available at www.aaojournal.org). Retinal phenotypes are summarised below and in Table 1. Detailed ophthalmic histories for each case can be found in Supplementary data A (available at www.aaojournal.org). None of the individuals included in this study had evidence of renal disease. The median age of drusen onset was 46 years The average age at onset in our cohort is skewed by late identification of disease in individual B:II.2 who did not receive a diagnosis until 80 years of age. The earliest documented age at which drusen were identified was 16 years in individual A:II.1, younger than has previously been reported in EOMD. In all affected study participants, drusen were bilateral and broadly symmetrical, and visible on fundus examination/colour photos (Figure 2, available at www.aaojournal.org), fundus autofluorescence (FAF) (Figure 3) and/or optical coherence tomography (OCT) (Figure 4, available at www.aaojournal.org). The impact on visual acuity was varied, from no apparent impact (B:I.1), to mild (A:II.3, A:II.1, C:I.2, D:I.7, E:III.11, F:II.2, G:III.7), moderate (D:II:2, E:II.2), and severe visual loss (B:II.2). As shown in Figure 2-4, varying phenotypes and degrees of disease severity were present in study participants. This is possibly reflective of the fact that individuals in our cohort are a range of ages and thus represent various disease stages. However, in younger participants, we cannot rule out non-progressive disease, at this time.

In family A, both affected siblings presented with small drusen, scattered throughout the retina in an appearance typical of 'basal laminar drusen' (i.e.- 'stars in the sky' appearance). Ophthalmic

examination of their mother found that she was unaffected. Proband B:I.1 from family B, was found to have multiple small drusen at the macula and nasal to the optic discs. His mother, B:II.2, was more advanced, showing drusen as well as atrophy at both the macula and the nasal retina. C:I.1, presented with central scotomata, and drusen surrounding a region of central atrophy that extended to the vascular arcades. She had patchy atrophy with reticular and drusenoid features in the retinal periphery with mid-peripheral sparing. Her mother and sister had also received a diagnosis of macular drusen. The proband from family D (D:I.7) presented with multiple large drusen bilaterally, associated with atrophy of the right macula. Her affected father (D:II.2) presented with advanced neovascular macular degeneration. Family history revealed the proband's paternal aunt (D:II.1) and great grandfather (D.IV.6) had experienced visual deterioration in their forties. It was also noted that her paternal great aunt (D.IV.2) and great uncle (D:IV.5) were affected in their fifties, although no further information was available. Her paternal grandmother (D:III.3) was deceased aged 56 years and it is not known whether she was also affected. The proband from family E (E:III.11) presented with central vision problems. FAF revealed hypoautofluorescence centrally due to geographic atrophy with a surrounding ring of hyperautofluorescence and drusen nasal to the optic disc. Pigmentary changes and drusen were also seen in the peripheral retina. Her affected son (E:II.8) was found to have small, sparse macular drusen at the age of 40 years. Family history revealed multiple affected members in family E. Fundoscopy of the proband from family F (F:II.2) revealed isolated sparse drusen within the temporal macula. At the time of her diagnosis, her mother (F:III.2) was being treated for choroidal neovascularisation. Proband G:III.7 from family G presented with clustered drusen spread throughout the macula, with early non-foveal geographic atrophy in the left eye. Her sister (G:III.3) and two brothers (G:III.1 and G.III.4) were also affected. Her mother died aged 30 years and her father died aged 80 with no known visual problems.

Genetic Findings

The proband from families A, B, and E underwent testing for variants in the coding and flanking intronic regions of 105 or 176 known retinal dystrophy-causing genes, including a number of genes

associated with macular drusen (*ABCA4*: NM_000350; *CA4*: NM_000717; *CNGB3*: NM_019098; *EFEMP1*: NM_001039348; *PROM1*: NM_006017; *TIMP3*: NM_000362). No putative disease-causing or carrier variants were identified. Subsequently, each was recruited to the UKIRDC study and WES was conducted on DNA from the affected sibling pair of family A (A:II.1 and A:II.3), the proband of family B (B:II.2), and the affected mother (E.II.2) and son (E.I.1) of family E. In each family, an ultra-rare (MAF<0.01%) or novel heterozygous *CFH* variant was identified as the probable cause of disease (Table 1). NGS analysis and interpretation of variants can be found in Supplementary Data F (available at www.aaojournal.org).

A cohort of 75 patients diagnosed with macular drusen, and negative for the *EFEMP1* c.1033C>T p.(Arg345Trp) variant, were screened for variants in the coding and flanking intron regions of the *CFH* gene (NM_000186) including an alternatively encoded exon from the transcript NM_0010149975. Four patients (the probands of families C,D, F, and G: C:I.2, D:I.7, F:II.2, G:III.7) were found to harbour novel or ultra-rare (MAF<0.01%) heterozygous variants in the *CFH* gene (Table 1).

In total, six different *CFH* variants were identified in seven EOMD families (Table 1). Segregation analysis was performed where possible and the respective variant segregated with disease in each case (family members tested, their disease status and their genetic status are indicated in Supplementary Data B, available at www.aaojournal.org). Three mutations, which have not previously been reported in association with disease, represent novel EOMD-causing mutations: *CFH* c.1243del, p.(Ala415ProfsTer39) het; *CFH* c.350+1G>T het; and *CFH* c.619+1G>A het. The three remaining variants have previously been reported as disease-causing: *CFH* c.380G>A, p.(Arg127His)[25, 29]; *CFH* c.694C>T, p.(Arg232Ter)[30] identified in two unrelated families in this cohort; and *CFH* c.1291T>A, p.(Cys431Ser), which has been identified in the homozygous state underlying MPGN type I[31] and DDD[30].

All six mutations identified in this study affect complement control protein (CCP) domains 2-7 (Table 1), and are therefore predicted to impact both FH and FHL-1 (Figure 5).

Functional characterization of CFH mutations in Recombinant FHL-1

Previous studies have shown that rare *CFH* variants underlying aHUS and EOMD can prevent or delay secretion of FH [32-35], or severely impair protein function causing reduced FH activity [22, 25, 33, 36], leading to dysregulation of the complement pathway. However, the impact of rare *CFH* variants on the function of the alternative isoform FHL-1 has not previously been investigated. Given the probable importance of FHL-1 in the EOMD phenotype [26], we investigated the expression and secretion of mutant forms of FHL-1 containing the respective mutations identified in EOMD families A (FHL-$1_{A415f/s}$), E (FHL-1_{C431S}), and F (FHL-1_{R127H}) compared with full-length wild-type FHL-1 (FHL-1_{402Y}). Secreted His-tagged recombinant protein was purified from media by affinity chromatography, and lysates were made from transfected cells for analysis of intracellular expression of recombinant wildtype or mutant FHL-1.

Western blotting for both the N-terminal His-tag of the recombinant proteins revealed that FHL-1_{402Y} (wildtype) transfected cells secreted a protein product of expected size (51kDa, slightly larger than native FHL-1 due to its N-terminal His-tag modification). However, cells transfected with mutant plasmids did not secrete a 51kDa protein product at detectable levels within 144 hours of transfection (Figure 6). Mock (i.e. - no vector) transfected cells did not secrete any detectable His-tagged protein product, as expected. Next we investigated whether the mutant proteins were being expressed but retained intracellularly by examining lysates made from the transfected cells. Western blot for the His-tag of recombinant mutant and wildtype proteins indicated mutant proteins were not detectable when analysed using OX23.In comparison, FHL-1_{402Y} (wildtype) was present in abundance as indicated by the intense ~51kDa band despite equal protein loading as indicated by SOD2 (~26kDa). These findings suggest that although wildtype recombinant FHL-1 is expressed and secreted, mutant forms of FHL-1 are not. The absence of mutant proteins intracellularly suggests they are rapidly degraded by the cell soon after synthesis, possibly because they are unfolded or unstable, rather than synthesized and accumulated within the cell because they cannot be secreted.

Our findings indicate that the c.1243del, p.(Ala415ProfsTer39) het, c.1291T>A, p.(Cys431Ser) het and c.380G>A, p.(Arg127His) het variants found to underlie disease in families A, E and F respectively, could consequently be considered to result from loss-of-function of FHL-1, since the mutant proteins may be rapidly degraded upon synthesis and/or not secreted by the cell. T. Although beyond the scope of this study, it may be possible to assess this via the application of inhibitors of degradation pathways. The remaining putative EOMD-causing variants: c.350+1G>T het (family B), c.694C>T p.(Arg232Ter) het (families C and D), and c.619+1G>A het (family G), are also predicted to be loss-of-function variants. This leads us to suggest that haploinsufficiency of FHL-1 is the pathological mechanism underlying this severe, dominant, early-onset macular drusen phenotype in a subset of cases(i.e. the identified variants result in reduction in the amount of functional FHL-1 protein produced which is not sufficient to support the normal function of the retinal pigment epithelium, leading to disease).

Review of previously reported EOMD mutations

Following on from this hypothesis, we reviewed previously reported cases of EOMD to further define their mutational mechanisms. For this analysis we considered literature reports of mutations found to cause 'basal laminar drusen' [20, 21], 'cuticular drusen' [23, 37] and 'early-onset AMD' [22, 24, 25] in the absence of kidney disease. A total of 25 different variants in 29 families have been reported (Supplementary Data D, available at www.aaojournal.org). Of these, 19 are non-synonymous missense variants. Three variants - c.1198C>A; p.(Gln400Lys) het, c.2850G>C; p.(Gln950His) het, and c.3628C>T; p.(Arg1210Cys) het - have each been identified in 2 unrelated families [23]. Six different loss-of-function (i.e. - splice-altering, frameshift or nonsense) variants have also been reported. One nonsense variant (c.1222C>T, p.Gln408Ter het) has been identified in two unrelated families [20]. Of the 29 reported EOMD cases, 75.8% (n=22) are due to variants affecting CCP1-7 and impact both FH and FHL-1. Overall, including the cases reported here, 80.5% (n=29) of reported EOMD-causing variants (n=36) affect CCP1-7 and thus impact FHL-1 and FH. Furthermore, 100% of previously reported putative loss-of-function variants (i.e. - nonsense (n=2), frameshift (n=2), splice-altering

(n=3)) causing EOMD affect CCP1-7. Of the 15 missense variants affecting CCP1-7, 71.3% (n=11) have evidence that they prevent secretion or severely reduce protein function. The remaining four variants have not been assessed. In total, 81.8% (n=18) of previously reported EOMD variants affecting CCP1-7, likely result in haploinsufficiency of FHL-1/FH. Taking into account the variants reported herein, this figure increases to 86.2% (n= 25). Thus, there is considerable evidence in support of the hypothesis that haploinsufficiency of FHL-1 is the molecular mechanism underpinning the development of this severe, early-onset phenotype in a proportion of cases.

Two of the other reported missense variants (p.(Pro139Ala) and p.(Arg175Gln)) have supporting evidence for their pathogenicity in that a different amino acid change at the same position of the peptide has been reported to cause *CFH*-related disease with a demonstrated effect on FH serum levels, suggesting the mutant protein is not secreted or is degraded upon synthesis/secretion [25, 38].

Discussion

This study identified unrelated families with a history of EOMD associated with deleterious ultra-rare (MAF≤0.0001) heterozygous *CFH* pathogenic variants. It provides evidence that clinical signs begin significantly earlier than seen in typical age-related macular degeneration cases; the earliest reported onset of retinal changes in our cohort is 16 years of age. The six different variants identified (Table 1) all localized within CCP2-7 and consequently impact FHL-1 and FH. FHL-1 is a short form of FH that retains all of the regulatory functions of the full-length protein that has been suggested to be the predominant regulator of complement in BrM, the extracellular matrix (ECM) layer that lies beneath the human retina, and the intercapillary septa [26]. We propose that lost or reduced function of FHL-1 leads to dysregulation of complement turnover in the ECM of Bruch's membrane and choriocapillaris, and may be the mechanism driving development of EOMD in these cases; complement over-activation is thought to be an important driver of pathogenesis in early AMD [39].

The identified putative splice-altering and nonsense variants are likely to result in haploinsufficiency of both FH and FHL-1. By contrast, the c.1243del mutation in exon 9 of *CFH* is predicted to have

differing effects on the two isoforms. In FH (NM_000186), this frameshift is predicted to undergo NMD while in FHL-1 (NM_001014975), this same single base pair deletion is predicted to result in a frameshift within the penultimate exon that is likely to escape NMD, creating a stop codon 47 amino acids downstream [p.(Ala415ProfsTer47)] and resulting in an extension of the normal 449 amino acid product by 13 amino acids, as well as loss of its unique four amino acid C-terminal tail which is known to have a role in the proteins binding to pro-inflammatory monomeric C-reactive protein [40]. Predicting the impact of missense variants on protein function is challenging, particularly for the *CFH* gene [33]. Both of the missense variants, p.(Cys431Ser) and p.(Arg127His), as well as the p.(Ala415ProfsTer47) frameshift variant, were investigated *in vitro*. By expressing these recombinant mutant FHL-1 proteins we have demonstrated that none of the three allowed the production of a secreted FHL-1 product. We suggest that all three variants may also result in loss of protein function. The *CFH* p.(Cys431Ser) variant replaces the highly conserved, third cysteine residue of the 7th CCP domain. One of the defining characteristics of a CCP domain is the presence of two disulphide bonds with the cysteine-cysteine pattern of C_1-C_3 and C_2-C_4, therefore this amino acid substitution likely disrupts the structure and function of CCP7, and results in a free cysteine that may covalently interact with surrounding residues. Functional characterization of a different amino acid substitution at the same position of the peptide, p.(Cys431Tyr), showed that it decreased stability of the recombinant FH [41]. Moreover, mass spectrometry analysis of plasma from the affected patient found that the protein product of the mutated allele was not present, suggesting that the change prevents secretion and/or the mutated protein is rapidly degraded [41]The *CFH* p.(Arg127His) substitution alters CCP2, in a region of FH/FHL-1 previously shown to be important for C3b binding and co-factor activity [35]. *In vivo* investigations have shown that p.(Arg127His) results in retention of mutant full-length FH in the endoplasmic reticulum (ER) of cultured patient fibroblasts [35].Review of 29 *CFH* mutations previously reported as the underlying cause of EOMD ('basal laminar drusen, 'cuticular drusen', and 'early-onset AMD'), found that 75.8% impact FHL-1 as well as FH. This figure increases to 80.5% upon inclusion of the variants reported by this study. Importantly all 12

reported nonsense, splice-altering and frameshift variants (5 by this study, 7 reported previously in the literature) fall within CCP1-7. Furthermore, all missense substitutions (n =10) affecting CCP1-7, which have been evaluated *in vitro,* have been found to prevent secretion or result in loss of protein function. Consequently, at least 81% of reported EOMD variants result in loss of protein function, strongly supporting the hypothesis that haploinsufficiency of FHL-1 is one important mechanism underpinning the development of drusen in early adulthood. The contribution of AMD-related loci to EOMD if any, remains elusive but is an important area for future research. Moreover, identification of higher numbers of affected individuals or families would likely be required to associate demographics such as age at onset and severity with AMD risk alleles in this subgroup of patients.

Strict control of innate immunity at BrM is critical for maintaining normal homeostasis and health of the retina. It is becoming increasingly apparent that one of the main pathological characteristics of AMD is inflammation of the central retina and consequential particulate accumulation (drusen development), cellular damage and subsequent loss of vision as a result of complement dysregulation [42]. Factor H, and almost certainly FHL-1, are the only components of the complement system known to downregulate alternative pathway activation on host extracellular matrix and self-surfaces via interaction with binding partners and co-factors [12, 39]. There is increasing evidence that the eye synthesises complement pathway components locally [5, 43, 44], and the importance of functional FHL-1 at the retina is becoming ever more apparent [26, 27, 40].

It is known that FHL-1 is the only isoform that can passively diffuse across Bruch's membrane; FH cannot because of its large size [26]. FHL-1 is immobilized in Bruch's membrane and the ECM of the choriocapillaris by interaction with heparan sulfate *via* its glycosaminoglycan (GAG)-binding site in CCP7. In this way, FHL-1 functions to protect the ECM from complement activation[26]. The Y402H polymorphism has been shown to affect the function of FHL-1; the 402H variant reduces FHL-1 binding to heperan sulphate[26]. According to the GnomAD database, 44.08% of individuals from all populations (ALL) (42.6% in the European (non-Finnish) population (EUR)) are heterozygous for the 402H allele, whereas 32.54% ALL (38.4% EUR) are homozygous. Individuals heterozygous for the

402H variant are at a two-fold increased risk of AMD, whereas those that are homozygous have a greater than five-fold increased risk[4]. Studies have shown that while the AMD-associated Y402H allele does not alter FH protein conformation, nor does it alter FH levels in blood, it does result in decreased ability of FHL-1 to bind heparan sulfate[26]: any changes to FH binding to GAGs appears minimal due to this larger protein's second GAG binding site. Furthermore, recent work has shown that the Y402H polymorphism has a more pronounced effect on FHL-1 binding of Pentraxin-3 (PTX3) - an inflammation-associated protein that binds FH at CCP7 and CCP19-20 and serves to increase interaction of FH with apoptotic cells for iC3b opsonisation – than it does on FH binding of PTX3, most likely because it affects the only PTX3 binding site within FHL-1[40]. Taken together, this evidence suggests that loss of FHL-1 expression or function would have a detrimental impact on regulation of the complement system in the retina.

Previous publications that have identified rare, highly penetrant *CFH* mutations in association with EOMD have focussed on the location of mutations within the FH protein with respect to known functions of domains[23, 37]. However, it is now recognised that FHL-1 is the predominant regulator of the complement pathway within BrM and the intercapillary septa[26]. FH and FHL-1 both negatively regulate the alternative complement pathway by competing for binding to C3b with factor B (FB) to govern the removal of immune complexes and pathogens, and modulate adaptive immunity. They also serve as co-factors for factor-I (FI) cleavage of C3b into its haemolytically inactive state, iC3b. We suggest that pathogenic variations in FHL-1 resulting in loss / impairment of function are an important cause of EOMD in the vast majority of cases.

There exists a well-recognised genotype-phenotype correlation with respect to *CFH* variants and disease; with variants affecting CCP1-4 and CCP6-8 mainly causing eye disease, whereas those affecting CCP19-20 cause kidney disease[17]. This is supported by research that has shown the GAG-binding site in CCP7 demonstrates selectivity towards heparan sulfates in Bruch's membrane and the choriocapillaris, whereas the CCP19-20 region preferentially binds heparan sulfates in the glomerular basement membrane of the kidneys[12, 47]. However, a degree of allelic overlap exists that cannot be

explained by current understanding of the function of FH and FHL-1, representing an important area for future research.

In conclusion, rare, deleterious mutations in *CFH* resulting in haploinsufficiency of FH/FHL-1 are an important and under-recognised cause of dominant EOMD. Identification of *CFH* variants underlying EOMD has important consequences for clinical care, allowing genetic testing of other family members and counselling where appropriate; the identification of the underlying molecular cause can allow the provision of more accurate prognostic information, particularly where variants are known to increase the risk progressing to a severe phenotype resulting in significant visual loss[10, 48]. Furthermore, with complement modulating therapeutics being under development for AMD such genetic analyses may identify subsets of patients who may benefit from these new treatments. The impact of variants on the expression and/or function of FHL-1 have not previously been considered for EOMD, and we propose that this truncated form of FH plays a crucial role in EOMD and potentially AMD. Identification of further mutations causing the rare, genetically heterogeneous EOMD phenotype will lead to a better understanding of disease pathogenesis.

Figure Legends

Figure 1: Fundus autofluorescence imaging in patients with EOMD with rare *CFH* variants. a-b) Patient A:II.3 aged 51 years showing drusen at the macula and extending beyond the vascular arcades; c-d) Patient A:II.1 aged 49 years with drusen extending outside the macular region (c); e-f) Patient B:II.2 aged 89 showing drusen and retinal atrophy at the macula and in the nasal retina in both eyes; g-h) Patient B:I.1 aged 61 years with bilateral macular drusen and drusen nasal to the optic discs; i-j) Patient C:I.2 aged 64 years showing loss of central signal consistent atrophy, drusen are present around the atrophy and optic nerve; k-l) Patient D:I.7 aged 26 years showing geographic atrophy and large colloidal macular drusen; m-n) Patient E:II.2 aged 53 years showing hypoautofluorescence centrally due to geographic atrophy with a surrounding ring of hyperautofluorescence and drusen nasal to the optic disc; o-p) Patient F:II.2 aged 54 years showing

sparse temporal drusen; q-r) Patient G:III.7 aged 66 showing scattered macular drusen and patches of geographic atrophy.

Figure 2: Protein schematic of FH and FHL-1. Schematic diagram of FH and FHL-1 detailing protein domains and corresponding amino acid positions with locations of mutations identified in our EOMD cohort. Factor H (FH) contains 20 CCP domains (top), whereas FHL-1 encodes 7 CCP domains (bottom) FH CPP 1-7. Regions associated with C3b binding are indicated by adjacent red bars; co-factor activity by the green bar; heparin binding sites by blue bars; and sialic acid binding site by the purple bar. The locations of mutations identified by this study are indicated by grey-dashed lines. Bracketed letter following mutation nomenclature indicates the study ID of the family in which the mutation was identified.

Figure 3 Expression of wild type and mutant FHL-1 in transfected HEK293 cells. HEK293 cells were stably transfected with His-tagged wildtype pcDNA3.1-FHL-1 (FHL-1_{Y402}) or one of three mutant constructs (FHL-1_{C431S}, FHL-1_{R127H}, FHL-$1_{A415f/s}$) as indicated at the top of each gel image, to assess the effect of the identified variants on protein expression and secretion over 144hours. Recombinant proteins purified from culture media (secreted) and cell lysates (intracellular) were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Figure shows Western blot results from analysis of experimental samples for OX23. Presence of a band indicates presence of recombinant FHL-1. Cells transfected with mutants did not secrete a detectable FHL-1 product (~51kDa) in comparison to wildtype FHL-1. Mock (no DNA) transfected cells did not produce an FHL-1 product, as expected. Analysis of cell lysates found no accumulation of mutant recombinant proteins intracellularly but wildtype recombinant FHL-1 was found to be present. SOD2 (~26kDa) indicates equal sample loading of cell lysates.

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Table 1:- Phenotypic and genetic findings in early-onset macular drusen patients with *CFH* mutations. Table details study ID, age at most recent exam, retinal and extra-ocular phenotype (if any), visual acuity, findings from electrophysiology investigations, mutation identified, CCP domain affected, allele frequency according to the GnomAD database.

Study ID/gender (M/F)/age at onset† (Y)	Age at most recent exam	Family history*	Phenotype	Visual Acuity (Snellen (LogMar))		Electrophysiology Findings	Mutation	CCP domain	GnomAD Allele Count (allele frequency)
				OD	OS				
A:II.3/M/18	51	Yes	Bilateral, scattered/widespread early-onset drusen, RPE mottling at the fovea, reduced VA	+5.75 (0.22; 6/9-1)	+3.75 (0.0; 6/6)	Normal and grossly symmetrical light adapted response. Slightly reduced dark adapted response in RE suggesting asymmetrical rod involvement. EOG normal	c.1243del, p.(Ala415Profs*39) het	7	-
A:II.1/M/16	49		Bilateral widespread drusen, concentrated temporal to and within the macular	6/3.8-1	6/3.8-1	Normal and grossly symmetrical light adapted response. Slightly reduced dark adapted response in LE suggesting asymmetrical rod involvement. EOG normal			
B:II.2/F/80	89	Yes	Bilateral, symmetrical, outer retinal atrophy, multiple drusen, severe visual loss	-	-	NA	c.350+1G>T het	2	1/245972 (0.000004066)
B:I.1/M/61	61		Multiple drusen bilaterally, pattern similar to that seen in affected mother, II.1	6/6	6/6	NA			
C:I.2/F/54	64	Yes	Bilateral drusen surrounding central atrophy and extending into the arcades. Patchy atrophy in the peripheral retina with reticular and drusenoid features.	6/6	6/15	NA	c.694C>T p.(Arg232Ter) het**	4	2/244650 (0.000008175)

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D:I.7/F/26	50	Yes	Large 'colloid' drusen	6/5	6/5	NA	c.694C>T p.(Arg232Ter) het**	4	2/244650 (0.000008175)
D:II.2/M/50	64		Bilateral retinal drusenoid dystrophy with CNV and significant scarring	1/60	6/24	NA			
E:II.2/F/50	52	Yes	Early onset macula dystrophy, macular and mid-peripheral drusen	6/120 (1.34)	6/96 (1.24)	Extinguished PERGs, normal EOG, normal ERG; Ishihara: 1/17 OD, 2/17 OS	c.1291T>A, p.(Cys431Ser) het**	6	1/245702 (0.000004070)
E:III.2/M/40	53		Bilateral small, sparse drusen at the maculae.	6/4	6/7.5	NA			
F:II.2/F/46	54	Yes	Isolated sparse drusen within the macula and temporal raphes.	6/6 (-4/-1.00 x 180)	6/4.8 (-4.50/-1.00 x 170)	NA	c.380G>A, p.(Arg127His) het**	2	2/121206 (0.0000165)
G:III.7/F/45	66	Yes	Bilateral large, sparse white/yellow drusen at the maculae, nasal to the disc and the surrounding arcades. Patchy geographic atrophy in the LE.	6/6 (+2.75/-1.00 x 17)	6/6 (+2.75/-1.50 x 165)	NA	c.619+1G>A het	3-4	-

†Age at onset is defined as age at which retinal changes were first detected; Y:Years; M: Male; F:Female; VA: visual acuity; NA: information not available; PED: pigment epithelial detachment; LE: left eye; RP: retinitis pigmentosa; EDT: Electrodiagnostic Testing; PERGs: Pattern electroretinograms; EOG: Electrooculogram; ERG: Electroretinogram; CNV: Choroidal neovascularisation *A positive family history is defined as another blood relative reported to be affected by macular disease/drusen; **mutation previously reported as disease causing.

Figure 3

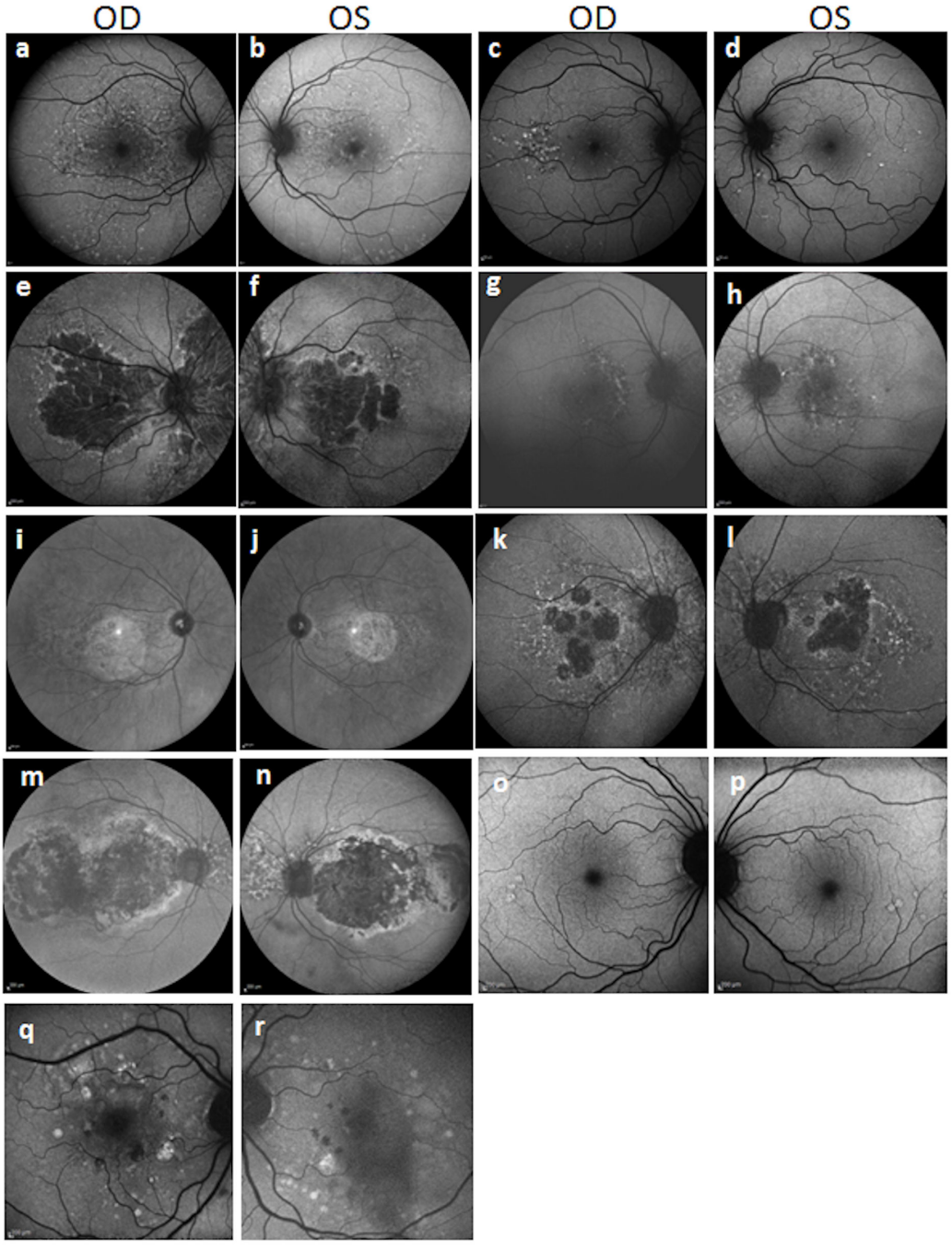

Figure 5

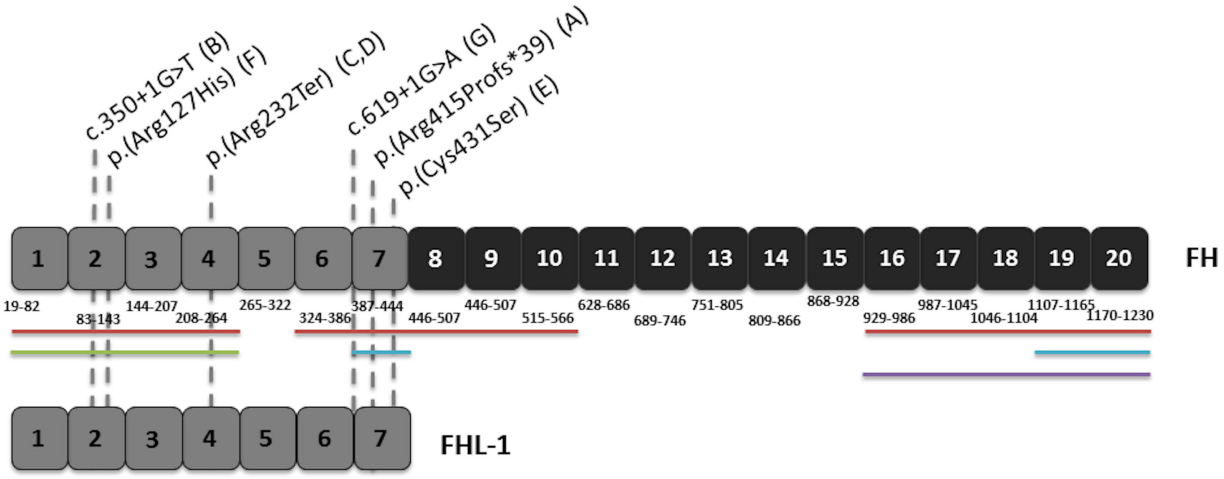

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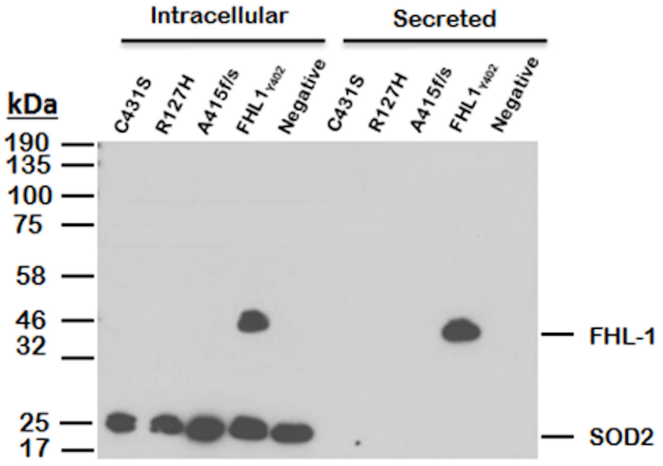
Figure 6



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Precis

Loss-of-function mutations in *CFH* that impact FHL-1, the main regulator of the complement pathway in Bruch's membrane, cause early-onset macular drusen providing a novel mechanistic insight into macular disease that could inform AMD pathogenesis.

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