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# Analysis of rare variants in the *CFH* gene in patients with the cuticular drusen subtype of age-related macular degeneration

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**Purpose:** Age-related macular degeneration (AMD) and cuticular drusen (CD), a clinical subtype of AMD, have been linked to genetic variants in the complement factor H (*CFH*) gene. In this study, we aimed to investigate the frequency of rare variants in the *CFH* gene in 180 cases with CD. In addition, we aimed to determine the frequency of a previously reported rare, highly penetrant *CFH* variant (p.Arg1210Cys) in a Dutch-German non-CD-type AMD case-control cohort, and to describe the phenotype of patients carrying the p.Arg1210Cys variant.

**Methods:** Study subjects were selected from the European Genetic Database (EUGENDA), a joint AMD database of the Radboud University Medical Centre and the University Hospital of Cologne, and graded at the Cologne Image Reading Centre and Laboratory (CIRCL). Additionally, two CD cases were recruited from the VU Medical Centre in Amsterdam. The *CFH* gene was analyzed in 180 CD cases with Sanger sequencing. All identified variants were analyzed for potential damaging effects with prediction software tools Sorting Intolerant from Tolerant (SIFT) and Polymorphism Phenotyping (PolyPhen). In addition, we genotyped the p.Arg1210Cys variant in 813 non-CD type AMD cases and 1175 controls.

**Results:** Sequencing identified 11 rare, heterozygous missense variants, one frameshift variant, and one splice acceptor site variant in 16 CD cases. The p.Arg1210Cys variant was identified in two CD cases but was not identified in our Dutch-German non-CD-type AMD case-control cohort.

**Conclusions:** The present study identified the presence of rare variants in the *CFH* gene in 16 (8.8%) of 180 patients with the CD subtype of AMD. The carriers of rare *CFH* variants displayed a significantly earlier age at onset than non-carriers (p=0.016). The rare missense variant p.Arg1210Cys was identified in two CD cases, but was not detected in 813 non-CD type AMD cases or in the 1,175 controls of our Dutch-German cohort. The current study suggests that the p.Arg1210Cys variant may be restricted to a subset of patients with the CD subtype of AMD. Detailed clinical phenotyping, including fluorescein angiography, of patients with AMD carrying the p.Arg1210Cys variant in other cohorts is required to confirm this finding.

Age-related macular degeneration (AMD, OMIM 603075) is the most common cause of vision loss and irreversible blindness among the elderly in the Western world [1]. A major hallmark of AMD is the appearance of drusen in the macula accompanied by loss of sharp and central vision with advancing disease [2]. Cuticular drusen (CD, OMIM 126700), also termed "basal laminar drusen" or "early adult-onset, grouped drusen," is a clinical subtype of AMD, characterized by the fundoscopic findings of innumerable, small (25–75 µm), slightly raised, round drusen, scattered throughout the central and peripheral retina [3]. CD commonly appear in early adulthood, and are readily visualized with fluorescein angiography (FA). In more advanced stages, the large number of CD present as a typical "stars-in-the-sky" appearance in the early phases of the angiogram [4]. Approximately 10% of AMD cases display the CD phenotype [5].

The early age of onset and the clustering of the CD phenotype in families implies a large genetic contribution to the development of CD [5-8]. This is supported by the observation that heterozygous mutations in the *complement factor H* (*CFH*) gene (ID 3075; OMIM 134370) segregate with the CD phenotype in multiplex families [5,8]. Common variants in and near the *CFH* gene have been associated with CD and with AMD [6,7]. In addition, a rare, highly penetrant variant (p.Arg1210Cys) in the *CFH* gene has been associated with AMD [9,10]. However, the p.Arg1210Cys variant was not detected in an Icelandic cohort or in the Han Chinese population [11,12]. This suggests that the rare p.Arg1210Cys

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variant is not consistently associated with AMD among different populations.

In this study, we aimed to investigate the frequency of rare variants in the *CFH* gene in 180 cases with CD. In addition, we aimed to determine the frequency of the rare highly penetrant p.Arg1210Cys variant in a Dutch-German non-CD type AMD case-control cohort, and to describe the phenotype of patients carrying the p.Arg1210Cys variant.

## METHODS

*EUGENDA Study:* The study participants were recruited from the European Genetic Database (EUGENDA), a multicenter database consisting of subjects from the Nijmegen area, the Netherlands and the Cologne area, Germany (Table 1). In addition, two CD cases were recruited at the VU Medical Centre in Amsterdam. All subjects analyzed in this study are Dutch and German and are of Caucasian descent. All subjects underwent ophthalmological examinations, and AMD staging was performed by the Cologne Image Reading Centre and Laboratory (CIRCL). CD was classified as a symmetric distributed pattern in both eyes of at least 50 scattered, uniformly-sized, small (25–75 µm), and hyperfluorescent drusen on FA in each eye, with a minimum of 20 drusen located outside the Wisconsin age-related maculopathy grading template [6,13]. AMD was classified by the presence of at least 15 intermediate (63–124 µm) drusen or at least one large (≥125 µm) druse in the Early Treatment Diabetic Retinopathy Study (ETDRS) grid (intermediate AMD) or geographic atrophy or choroidal neovascularization secondary to AMD (advanced AMD). Control subjects were ≥65 years of age and did not display AMD, which includes subjects without drusen, with only small drusen (<63 µm) or with pigmentary abnormalities alone or combined with less than 10 small drusen. Early AMD cases with ≥10 small drusen and pigmentary abnormalities or 1–14 intermediate drusen were excluded. The participants' age at onset was noted as the age at which first visual complaints were experienced.

The EUGENDA study was approved by the local research ethics committees (Commissie Mensgebonden Onderzoek Regio Arnhem-Nijmegen, the Netherlands, and the Ethics Committee of the University Hospital Cologne, Germany). Written informed consent was obtained from all participants, and the study was performed in accordance with the tenets of the Declaration of Helsinki. The study was adhered to the ARVO statement for the use of human subjects in ophthalmic and vision research.

*Sequencing:* Sanger sequencing of the *CFH* (NM_000186) gene was performed in 178 CD cases from the EUGENDA database and two CD cases from Amsterdam. Primers were designed to amplify all 22 exons and flanking intron-exon junctions with Primer3 software (Appendix 1). PCR were performed, and amplification products were sequenced using an automated sequencer (BigDye Terminator, version 3, 3730 DNA analyzer; Applied Biosystems, Waltham, MA). All sequencing chromatograms were compared to the reference sequence using ContigExpress (Vector NTI Advance, Version 11.0, Life Technologies, Waltham, MA). Each newly identified variant was confirmed with a second independent PCR and bidirectional Sanger sequencing. All identified variants were annotated based on the Human Genome Variation Society (HGVS) nomenclature. Variants with a minor allele frequency (MAF) <1% were considered rare variants. The number of carriers of rare variants discovered in the CD cohort (n=180) was compared to the general population (n=4300) using data from Exome Variant Server (EVS). Rare coding (missense, frameshift, and nonsense) and splice site variants in the *CFH* gene were used in the analysis. The average age at onset of the patients with CD were compared in carriers of rare *CFH* variants (n=16; age at onset known=13) versus non-carriers of rare *CFH* variants (n=164; age at onset known=64). The predicted effects of identified missense variants were examined using Polymorphism Phenotyping (PolyPhen) and Sorting Intolerant from Tolerant (SIFT) [14,15].

Table 1. Demographics of studied subjects of the EUGENDA cohort.

| Variables | CD | Intermediate AMD | Advanced AMD | Controls |
|---|---|---|---|---|
| Number (Total) | 180 | 207 | 606 | 1175 |
| Mean age (±SD) | 70±13.7 | 74±6.8 | 77±7.7 | 70±5.9 |
| Gender | | | | |
| Male | 59 (32.8%) | 80 (38.6%) | 251 (41.4%) | 504 (42.9%) |
| Female | 121 (67.2%) | 127 (61.4%) | 355 (58.6%) | 671 (57.1%) |

CD: Cuticular drusen; AMD: Age-related macular degeneration; SD: Standard deviation

*Genotyping:* The *CFH* p.Arg1210Cys variant was genotyped in 813 non-CD type AMD cases and 1,175 controls from the EUGENDA database using a competitive allele-specific PCR assay (KASPar SNP Genotyping System, KBiosciences, Teddington, UK). KASPar genotyping was performed according to the manufacturer's protocol in a volume of 4 µl containing 10 ng of genomic DNA, 2.5 µl of 2X reaction mix, and 0.069 µl of assay (Appendix 2). Thermal cycling conditions included a preincubation step at 94 °C for 15 min, 20 cycles of 94 °C for 10 s, 57 °C for 5 s, 72 °C for 10 s, followed by 23 cycles of 94 °C for 10 s, 57 °C for 20 s, 72 °C for 40 s. Plates were analyzed on a 7900 Fast Real-Time PCR system (Applied Biosystems).

## RESULTS

Through sequencing of the exons and flanking intron-exon boundaries of the *CFH* gene in 180 unrelated CD cases, 13 heterozygous rare variants in 16 cases (8.8%) were identified (Table 2). Of these 13 variants, 11 were missense variants, one was a frameshift variant (p.Ala301Asnfs*25), and one was a splice acceptor site variant (c.428–2A>G). Seven variants (c.428–2A>G, p.Ala161Ser, p.Ala173Gly, p.Arg175Gln, p.Ser193Leu, p.Ala301Asnfs*25, and p.Trp379Arg) were not present in public genetic variant databases (dbSNP, ESP6500/EVS) and therefore represent rare, unique variants. Six variants (p.Leu3Val / rs139254423, p.Ile216Thr/rs183474263, p.Gln400Lys/rs201671665, p.Gln950His/rs149474608, p.Thr956Met/rs145975787, and p.Arg1210Cys/rs121913059) had low reported MAFs in these databases (MAFs<0.001). The total number of carriers of rare *CFH* variants in the patients with CD (16 (8.8%) of 180) is significantly higher than the total number of carriers of rare *CFH* variants in the general population (185 (4.3%) of 4300; p=0.008, Fisher's test). The mean age at onset in carriers of rare *CFH* variants (57.2±16.8 years) is significantly earlier than non-carriers of rare *CFH* variants (66.1±10.8 years; p=0.016, Student *t* test). Using the online prediction algorithms SIFT and PolyPhen, potential damaging effects of the missense variants were assessed. Three missense variants (p.Ser193Leu, p.Trp379Arg and p.Gln950His) showed a consistent deleterious and damaging score by both PolyPhen and SIFT, while three variants were predicted deleterious or damaging by one of the prediction algorithms. Five missense variants were not predicted to be deleterious or damaging by either algorithm, including the p.Arg1210Cys variant.

The p.Arg1210Cys variant was identified in two unrelated individuals with CD. To test for a possible association of the p.Arg1210Cys variant in our Dutch-German non-CD type AMD case-control cohort, we genotyped this variant in 813 cases and 1175 controls from the EUGENDA database (Table 1). The p.Arg1210Cys variant was not found in our genotyped cohort, except in the two patients with CD in whom the p.Arg1210Cys variant was identified with sequence analysis of the *CFH* gene.

Both individuals carrying the p.Arg1210Cys variant presented with hyperfluorescent drusen on FA, typical of the CD subtype of AMD (Figure 1). In case 1, drusen were first noted at 50 years, with both eyes displaying numerous small drusen in the posterior pole and the peripheral retina (Figure 1A–D). Case 2 had onset of visual impairment at 64 years and presented with numerous small drusen in the posterior pole in both eyes. Furthermore, case 2 displayed a large fibrotic scar in the right eye (Figure 1E,G), pigmentary changes, and an occult choroidal neovascularization in the left eye (Figure 1F,H).

## DISCUSSION

The present study identified the presence of rare variants (MAF<1%) in the *CFH* gene in 16 (8.8%) of 180 patients diagnosed with the CD subtype of AMD. This number is significantly higher than the number of carriers of rare *CFH* variants in the general population (4.3%, p=0.008). This study showed that carriers of rare *CFH* variants display an earlier age at onset than non-carriers of rare *CFH* variants (p=0.016). The rare missense variant p.Arg1210Cys was identified in two CD cases, but was not detected in the 813 non-CD-type AMD cases and 1,175 controls of our Dutch-German cohort. The p.Arg1210Cys variant was previously found to be highly associated with AMD in North American cohorts, with a frequency in patients with AMD of 40/2,423 (1.65%) [10] and 23/2,335 (0.99%) [9], respectively. However, the p.Arg1210Cys variant was not detected in an Icelandic cohort (consisting of 1,143 patients with AMD) or in a Han Chinese cohort (consisting of 258 patients with AMD) [11,12].

The current study suggests that the p.Arg1210Cys variant may be restricted to a subset of patients with AMD with CD. Since the cohort of patients with CD analyzed in our study was too small to reliably test for an association, this finding must be confirmed in additional cohorts of patients with CD, and/or by detailed clinical phenotyping of patients with AMD carrying the p.Arg1210Cys variant using FA. An under- or overrepresentation of patients with AMD with CD-like characteristics between cohorts might explain the discrepancy in the p.Arg1210Cys association observed among the North American [10], Icelandic [11], Han Chinese [12], and Dutch-German cohorts. However, the distribution of low-frequency alleles vary among populations, since they tend to be the result of recent mutations and are expected to geographically

cluster around the location at which the mutation first arose [16]. The frequency of disease-causing variants can differ significantly among populations [17] or can even be restricted to a geographic region [18].

The CFH protein is an important regulator of the alternative pathway of the complement cascade that plays a key role in the clearance of pathogens and immune complexes, and modulates adaptive immunity [19]. CFH is composed of 20 sequential complement control protein (CCP) domains (Figure 2). Five of the identified rare variants (p.Ala161Ser, p.Ala173Gly, p.Arg175Gln, p.Ser193Leu, and p.Ile216Thr) are clustered within the N-terminal domains CCPs 1–4. This region has been demonstrated to be involved in cofactor activity [20], suggesting that these variants potentially have an impact on CFH cofactor activity. Two variants, p.Trp379Arg and p.Gln400Lys, are located within the CCP6 and CCP7 domains, respectively, close to the common p.Tyr402His [21] AMD risk variant, which causes defective heparin-binding properties [20]. This raises the possibility that these variants may exert a similar effect. Three variants (p.Gln950His, p.Thr956Met, and p.Arg1210Cys) are clustered in the C-terminal CCP domains 16–20. Functional studies have demonstrated that these four C-terminal CCP domains are necessary for the host cell recognition or discrimination properties of CFH [22]. In addition, we identified one frameshift (p.Ala301Asnfs*25) variant and one splice-acceptor site (428–2A>G) variant, which are predicted to abolish CFH function.

Notably, not all missense variants identified in this study were predicted by SIFT and PolyPhen to be pathogenic. In particular, this is the case for the p.Arg1210Cys variant, which was not predicted to be deleterious or damaging by either algorithm. However, functional studies have demonstrated that the p.Arg1210Cys variant compromises CFH function, as the mutant protein exhibits defective binding to C3d, C3b, heparin, and endothelial cells, and forms a covalent interaction with human serum albumin [10,23-26]. Therefore, although prediction software tools such as SIFT and PolyPhen

**Table 2. Rare sequence variants identified in the *CFH* gene in 180 CD cases.**

| Number of cases | Nucleotide change | Protein change | SNP Id | MAF (%) CD cases | EVS/ dbSNP | Previous disease associations | Prediction algorithms | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | SIFT | PolyPhen2 |
| 1 | c.7C>G | p.Leu3Val | rs139254423 | 0.27 | 0.02 | Novel | Tolerated (0.06) | Damaging (0.91) |
| 1 | c.428–2A>G | Splice-acceptor site | NA | 0.27 | 0 | Novel | NA | NA |
| 1 | c.481G>T | p.Ala161Ser | NA | 0.27 | 0 | Novel | Tolerated (0.17) | Benign (0.09) |
| 1 | c.518C>G | p.Ala173Gly | NA | 0.27 | 0 | Novel | Deleterious (0.03) | Benign (0.08) |
| 1 | c.524G>A | p.Arg175Gln | NA | 0.27 | 0 | Novel | Tolerated (0.17) | Benign (0.00) |
| 1 | c.578C>T | p.Ser193Leu | NA | 0.27 | 0 | Novel | Deleterious (0.0) | Damaging (0.99) |
| 1 | c.647T>C | p.Ile216Thr | rs183474263 | 0.27 | 0.001 | Novel | Tolerated (0.19) | Benign (0.003) |
| 1 | c.901_902del | p.Ala301Asnfs*25 | NA | 0.27 | 0 | Novel | NA | NA |
| 1 | c.1135T>C | p.Trp379Arg | NA | 0.27 | 0 | Novel | Deleterious (0.0) | Damaging (1.0) |
| 2 | c.1198C>A | p.Gln400Lys | rs201671665 | 0.55 | 0.01 | aHUS [29] | Tolerated (0.94) | Benign (0.01) |
| 2 | c.2850G>C | p.Gln950His | rs149474608 | 0.55 | 0.61 | aHUS [28] | Deleterious (0.0) | Damaging (0.80) |
| 1 | c.2867C>T | p.Thr956Met | rs145975787 | 0.27 | 0.16 | aHUS [30] | Tolerated (0.38) | Damaging (0.96) |
| 2 | c.3628C>T | p.Arg1210Cys | rs121913059 | 0.55 | 0.02 | aHUS/ AMD [10,24] | Tolerated (0.05) | Benign (0.02) |

MAF: Minor Allele Frequency SIFT: Sorting Intolerant from Tolerant (Intolerance ≤0.05) PolyPhen2: Polymorphism Phenotyping (score 0→1).

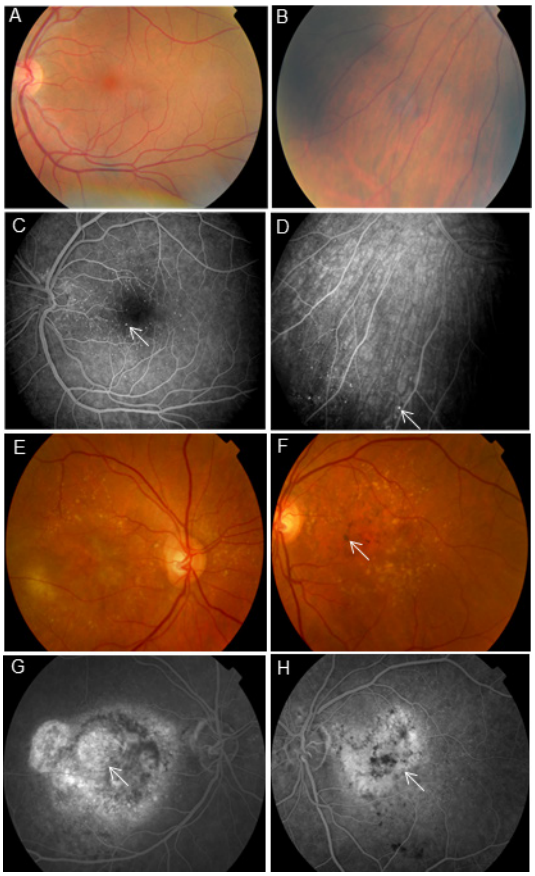


Figure 1. Fundus photographs and fluorescein angiographs of two cases carrying the Arg1210Cys variation. Case 1 displayed numerous small drusen (arrow) in the posterior pole and in the peripheral retina in both eyes. **A**, **B**: Color fundus photographs of the posterior pole and the periphery of the left eye, respectively, of case 1. **C**, **D**: Fluorescein angiographs of the posterior pole and the periphery of the left eye, respectively, of case 1. Case 2 showed small drusen of the posterior pole in both eyes. **E**, **F**: Color fundus photographs of the right and left eyes, respectively, of case 2. **G**, **H**: Fluorescein angiographs of the right and left eyes, respectively, of case 2. In addition, case 2 displayed a large fibrotic scar (**G**, arrow) in the right eye. In the left eye, pigmentary changes (**F**, arrow) and an occult choroidal neovascularization (**H**, arrow) were observed.

may assist in assessing potential damaging rare variants, functional validation of CFH mutant proteins is needed to properly assess the functional consequence of these genetic variations.

The majority of the identified variants were not present in public databases and have not been previously linked to other diseases, demonstrating that many *CFH* variants are novel and unique for individual patients. The number of carriers of rare variants is significantly higher in patients with CD than the number of carriers in the general population (EVS; p=0.008). The carriers of rare *CFH* variants displayed an earlier age at onset than non-carriers (p=0.016). This emphasizes the importance of a sequence analysis of the entire *CFH* coding region and the splice junction of the *CFH* gene to identify the causative allele, in particular in individuals with the CD subtype of AMD. The p.Arg1210Cys variant has previously been demonstrated to confer a high risk of developing AMD [10], underscoring its pathogenicity. However,

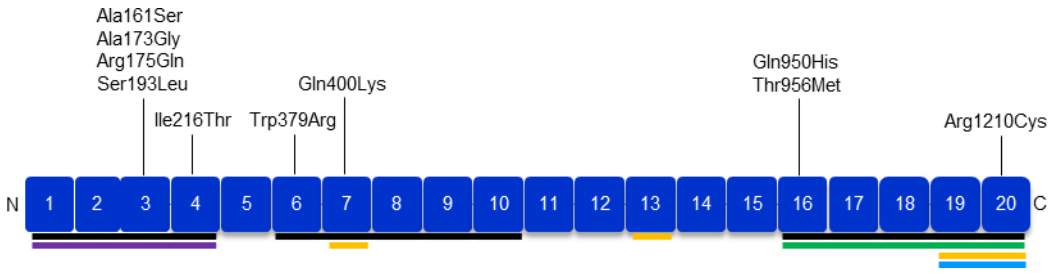


Figure 2. Schematic representation of factor H and its functional domains. Factor H is composed of 20 complement control protein (CCP) domains, and the approximate locations of missense variations are indicated at the top of the diagram. The location of the binding sites for C3b (black), cofactor activity (purple), heparin (orange), sialic acid (green), and self-surface recognition (blue bars) are mentioned below the diagram.

two *CFH* variants were recently identified in a large scale sequencing study, and were found not to be associated with the disease: p.Glu950His was identified in 9/3,343 patients with AMD and 10/1,480 controls (p=0.98), p.Thr956Met was identified in 4/3,348 patients with AMD and 6/1,484 controls (p=0.99) [27]. This implies that these alleles may not be causative for AMD. Four of the identified variants (p.Gln400Lys, p.Gln950His, p.Thr956Met, and p.Arg1210Cys) were previously reported in patients with atypical hemolytic uremic syndrome (aHUS), a devastating renal disease, supporting a previously proposed theory that an allelic overlap exists between two distinct pathologies, AMD and aHUS [28-30]. Patients with CD carrying aHUS mutations did not have renal complaints at the time of recruitment (Appendix 3). This suggests that additional genetic variants and/or external triggers determine the disease outcome in individuals carrying these alleles.

In conclusion, the present study identified the presence of rare variants in the *CFH* gene in 16 (8.8%) of 180 patients with the CD subtype of AMD. The carriers of rare *CFH* variants displayed an earlier age at onset than non-carriers (p=0.016). A previously reported rare missense variant, p.Arg1210Cys, was identified in two CD cases, but was not detected in the 813 non-CD type AMD cases and 1,175 controls of our Dutch-German cohort. The current study suggests that the p.Arg1210Cys variant may be restricted to a subset of patients with the CD subtype of AMD. Detailed clinical phenotyping, including fluorescein angiography, of patients with AMD carrying the p.Arg1210Cys variant in other cohorts is required to confirm this finding.

## APPENDIX 1: LIST OF *CFH* GENE SEQUENCING PRIMERS.

To access the data, click or select the words "Appendix 1."

## APPENDIX 2: ARG1210CYS VARIANT KASPAR PRIMERS.

To access the data, click or select the words "Appendix 2."

## APPENDIX 3: CLINICAL DESCRIPTION OF CD PATIENTS WITH RARE VARIANTS IN THE *CFH* GENE.

To access the data, click or select the words "Appendix 3."

## ACKNOWLEDGMENTS

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