

Complement Activation Levels Are Related to Disease Stage in AMD

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Received: December 3, 2018

Accepted: December 9, 2019

Published: March 16, 2020

Citation: Heesterbeek TJ, Lechanteur YTE, Lorés-Motta L, et al.

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Invest Ophthalmol Vis Sci. 2020;61(3):18.

<https://doi.org/10.1167/iovs.61.3.18>

PURPOSE. To study the levels of complement activation in different disease stages of AMD and the influence of genetic polymorphisms in complement genes.

METHODS. We included 797 patients with AMD and 945 controls from the European Genetic Database. Patients were grouped into five AMD stages: early AMD, intermediate AMD, central geographic atrophy, active choroidal neovascularization or inactive choroidal neovascularization. Differences in complement activation, as defined by the systemic C3d/C3 ratio, between AMD stages were evaluated using general linear modeling. In addition, we evaluated the influence of 18 genetic AMD polymorphisms in complement genes and their effect on complement activation. Differences in complement activation between stages were evaluated stratifying by complement associated haplotypes.

RESULTS. Complement activation levels differed significantly between AMD disease stages. As compared with controls, the C3d/C3 ratio was higher in patients with intermediate AMD ($P < 0.001$) and central geographic atrophy ($P = 0.001$). Two polymorphisms in *CFH* (rs10922109 and rs570618) and one in *CFB* (rs116503776) were significantly associated with complement activation. The association between AMD disease stage and complement activation was more pronounced in patients with haplotypes associated with the highest complement activation.

CONCLUSIONS. In general, consecutive AMD disease stages showed increasing levels of complement activation, especially in individuals with a genetic burden in complement genes. These findings contribute to the discussion on the pathogenesis of AMD in relation to complement activation and might suggest refinement in patient selection and the optimum window of treatment with complement inhibitors. Prospective studies are needed to confirm these results.

Keywords: retina, age-related macular degeneration, complement activation, complement genes

AMD is a chorioretinal disease of the posterior pole of the retina characterized by the presence of drusen and pigmentary changes in the early stages of the disease, and geographic atrophy (GA) and choroidal neovascularization (CNV) in the advanced stages.¹ The etiology of AMD is multifactorial and includes both environmental and genetic risk factors.² A major role in the pathophysiology of AMD is attributed to the alternative pathway of the complement system. The evidence for this relationship comes from several lines of research. More than two decades ago, immunohistochemical studies demonstrated the presence of complement components in drusen.^{3–5} Next, several genetic variants in genes encoding components and regulators of the complement system were associated with an

increased risk of AMD.^{6–20} In addition, serum and plasma components of the alternative pathway were shown to be elevated in AMD when compared with controls.^{21–26}

The complement system is part of our innate immunity and is important in the defense against pathogens and host homeostasis. The alternative pathway is continuously activated at a low level and this activation is tightly regulated by several inhibitory proteins.²⁷ Disturbances of these regulating mechanisms lead to an increase in complement activation and subsequently an increased inflammatory reaction, which may ultimately result in tissue damage and AMD.²⁸ Systemic levels of complement components have been associated with environmental factors such as age, sex, smoking, body mass index, triglyceride levels,^{29–31} and genetic AMD

variants in complement genes (*C3*, *CFB*, and *CFH*), indicating that at least a part of the complement activation is influenced by genetics.¹¹

Modulation of the complement system by targeting the regulating components is now the focus for the development of new treatment modalities for AMD. Several phase III clinical trials have been initiated to study the effect of complement inhibition on dry AMD, but none of them were successful in slowing down GA progression.³² Initial promising results from a phase II study on APL-2, a complement component 3 inhibitor, suggested a reduction of progression of GA at 12 months by 29% compared with sham treatment when administered monthly, although this finding needs to be validated in extensive phase III clinical trials.^{32,33}

Although it is not clear why the results from clinical trials evaluating complement inhibition in GA are not as successful as we all hoped, in part this may be due to a limited understanding of how complement activation is regulated in AMD. Currently, there is insufficient knowledge about the nature of complement activation in the different AMD stages, because all previous studies have analyzed all AMD stages together,^{24,34} focused only on advanced AMD,^{11,23,26,35} or analyzed complement activation between two AMD disease groups using small sample sizes.^{22,25,36,37} In this study, we stratified patients with AMD into their respective grading stage and investigated whether differences in systemic complement activation between AMD disease stages existed. Moreover, we investigated the influence of 18 genetic AMD polymorphisms in complement genes and their effect on complement activation differences between the AMD disease stages.

METHODS

Study Participants

All participants were recruited from the European Genetic Database (www.eugenda.org), a multicenter database for clinical and molecular analysis of AMD, including University Hospital in Cologne, Germany, and Radboud University Medical Center in Nijmegen, the Netherlands. This study adhered to the tenets of the declaration of Helsinki and was approved by the local ethics committees of both sites. All study participants provided written informed consent.

We selected all unrelated patients with AMD (≥ 50 years of age) and unrelated control individuals (≥ 65 years of age) with gradable fundus photographs and optical coherence tomograms of both eyes, as well as a recent analysis of the level of complement activation. Participants where the ophthalmic examination and date of venipuncture were separated by more than 1 month were not eligible for inclusion. Also, inclusion of patients with AMD with CNV was limited to those individuals for whom blood collection took place on the same day as ophthalmic examination, to ensure that active CNV could be distinguished from inactive CNV. We excluded all participants with other pathology possibly affecting AMD staging (such as high myopia) and patients with a history of retinal surgery.

AMD Staging

We determined the AMD stage using stereo color fundus photographs (Cologne: Canon UVI fundus camera, Canon, Tokyo, Japan; and Nijmegen: Topcon TRC 50IX fundus

camera, Topcon, Tokyo, Japan) and SD-OCT (Spectralis HRA system, Heidelberg Engineering, Heidelberg, Germany). In patients suspected for neovascular AMD, we also performed fundus fluorescein angiography (Spectralis HRA system, Heidelberg Engineering). Certified graders (TS, SL, LA) assessed the images based on the protocol of the Cologne Image Reading Center and Laboratory. Because this study focused on the relationship between AMD disease stage and complement activation, we had to attribute AMD stage not only to eyes, but also to individuals. Study participants were classified as controls (no AMD) or patients with early AMD, intermediate AMD, or advanced AMD (Table 1). Advanced AMD was further specified into central GA and CNV. To select a homogeneous population for GA in advanced AMD, RPE atrophy inside the circle of the Early Treatment Diabetic Retinopathy Study grid was defined as central GA, and RPE atrophy outside the circle was defined as noncentral GA and included in the intermediate AMD group, as defined in the Cologne Image Reading Center and Laboratory grading protocol. Within the CNV group, we distinguished between active and inactive CNV. This decision was based on a previously reported finding in which lower levels of complement activation were found in patients with advanced stage AMD and no evidence of disease activity.³⁸ To take a difference in AMD stage between both eyes into account, we classified patients based on the worst eye. Patients with central GA in at least one eye without signs of CNV in the fellow eye were staged as advanced AMD with central GA. The term advanced AMD with active CNV was reserved for individuals with at least one eye with active CNV. We used advanced AMD with inactive CNV for patients with inactive CNV in at least one eye and without active CNV. Patients with central GA in one eye and CNV in the other eye were classified as advanced AMD with active or inactive CNV, depending on CNV activity. Because we investigated systemic complement levels in association with local disease activity in the worst eye, we aimed to select a homogeneous population. We therefore deemed reliable classification impossible when the AMD stage between the left and right eye differed by more than one stage and excluded these individuals from the analysis.

Complement, CRP, and Triglyceride Measurements

We conducted C3d and C3 measurements in serum samples as a measure for complement activation. The activation fragment C3d is the most prominent marker for chronic complement activation because it reflects complement turnover.^{24,39} The C3d level depends on the concentration of its parent molecule C3; therefore, the C3d/C3 ratio was calculated as a value of complement activation that is independent of individual variations in the level of C3.⁴⁰ Serum was prepared by coagulation at room temperature. After centrifugation, we stored samples at -80°C within 1 hour of collection. Complement component 3 was assessed by radial immunodiffusion using monospecific polyclonal rabbit antisera, and C3d was measured by rocket electrophoresis.^{41,42} All measurements were performed in a single assay. Triglycerides and C-reactive protein (CRP) levels were measured with the Abbott Architect C16000 system (Abbott Diagnostics, Chicago, IL). We excluded patients with CRP values of greater than 10 mg/L, because these values are suggestive for viral or bacterial inflammation or disease activity in chronic inflammatory conditions and will lead to an increase in complement activation.⁴³

TABLE 1. Classification of Eyes With AMD According to the Criteria Defined by the Cologne Image Reading Center and Laboratory Grading Protocol

AMD Stage	Criteria
Control	No drusen or only small drusen (<63 µm diameter) or only pigmentary abnormalities or <10 small drusen + pigmentary abnormalities
Early AMD	≥10 small drusen + pigmentary abnormalities or 1–14 intermediate drusen (≥63 and <125 µm)
Intermediate AMD	≥1 large drusen (≥125 µm diameter) or ≥15 intermediate drusen or GA (RPE atrophy ≥175 µm) not in the central circle of the ETDRS grid
Advanced AMD	
Central GA	GA (RPE atrophy ≥175 µm) secondary to AMD involving the central subfield of the ETDRS grid
Active CNV	CNV within the ETDRS grid secondary to AMD with signs for CNV activity (hemorrhage on CFP and/or leakage on FA and/or subretinal and/or intraretinal fluid on SD-OCT)
Inactive CNV	CNV within the ETDRS grid secondary to AMD without signs for CNV activity

CFP, color fundus photography; ETDRS, Early Treatment Diabetic Retinopathy Study; FA, fluorescein angiography.

Genetic Analysis

DNA was extracted from venous blood samples. Because we studied systemic complement activation levels in patients with AMD, DNA was analyzed for 18 AMD variants in five genes encoding components or regulators of the complement pathway, as found in the most recent genome-wide association study on AMD: *CFH* (rs10922109; rs570618; rs121913059; rs148553336; rs187328863; rs61818925; rs35292876; rs191281603), *CFB* (rs116503776; rs144629244; rs114254831; rs181705462), *C3* (rs2230199; rs147859257; rs12019136), *CFI* (rs10033900; rs141853578) and *C9* (rs62358361) (Supplementary Table S1). The AMD variants were genotyped with the HumanCoreExome array by Illumina (Illumina Inc., San Diego, CA) within the International AMD Genetics Consortium. Details regarding the design of the array, annotation, imputation, and quality control of the genotypic data have been described previously.⁷

Haplotype analysis was performed using the haplo.glm function of the R library 'haplo.stats' (version 1.7.7; The R Foundation, Vienna, Austria). We computed maximum likelihood estimates of haplotype probabilities using the haplo.em function of the same library and selected individuals with a posterior probability of pair of haplotypes of 0.80 or greater.⁴⁴ Linkage disequilibrium between the investigated variants and other AMD variants in complement genes from previous studies was calculated with the HumanCoreExome array dataset using plink2 (www.cog-genomics.org/plink/2.0/).⁴⁵

Statistical Analysis

We used the natural logarithm of the C3d/C3 ratio for the analyses because of the skewness of the data. Covariates were categorized as follows: age (continuous), sex (female/male), smoking status (never/past/current), body mass index (continuous), triglycerides (continuous), CRP (continuous), disease status (AMD/control), and the 18 AMD variants in complement genes (wild type/heterozygous/homozygous). Multivariate general linear modeling was used to analyze the association between the C3d/C3 with demographic and environmental factors, the AMD disease stages, 18 AMD variants in complement genes, and haplotypes. An additional least significant difference post hoc analysis was performed to analyze which AMD disease stages differed significantly in mean C3d/C3 ratio. To analyze the association between the 18 AMD variants and the C3d/C3 ratio, all the single

nucleotide polymorphisms (SNPs) were included in a model together with other relevant covariates that are associated with systemic complement activation. A *P* value of less than 0.002 was considered statistically significant, which corresponds with a *P* value of 0.05 with Bonferroni correction for the 24 covariates in the model. Only AMD variants that were found to be associated with systemic complement activation levels after Bonferroni correction were used to assess haplotypes. Based on the haplotypes with the highest and lowest C3d/C3 ratios, we subdivided the population into a group having haplotypes associated with the highest complement activation levels and a group having haplotypes associated with the lowest complement activation levels. Independent *t*-tests and chi square tests were used to investigate differences in demographic factors and haplotypes distributions among controls and AMD disease groups. SPSS version 22.0 (IBM Corp., Armonk, NY) was used for all these analyses.

RESULTS

A total of 2116 participants of the European Genetic Database had gradable imaging data and complement activation measurements within one month of the retinal imaging. We excluded 374 participants for the following reasons: a difference of more than one AMD stage between the left and right eye (*n* = 159); CRP levels of greater than 10 mg/L (*n* = 156); and other retinal pathology that could interfere with the diagnosis of AMD, such as myopic degeneration (*n* = 59), or previous retinal surgery (*n* = 8). This left us with 797 patients with AMD and 945 control individuals. Patients with AMD were then further stratified based on their disease stage, resulting in five stages (early AMD, intermediate AMD, central GA, CNV active, and CNV inactive).

Table 2 provides an overview of the AMD disease stages of the enrolled patients, along with extensive descriptive information of the control group and the five patient groups. A model including demographic and environmental factors showed that age, sex, smoking status, body mass index, triglycerides and disease status were significantly associated with systemic complement activation (*P* < 0.05), whereas CRP was not (*P* = 0.336) (Supplementary Table S2). Consequently, all further analyses with systemic complement activation were adjusted for these significant complement associated variables.

AMD stage was significantly associated with complement activation (*P* < 0.001). To provide a more detailed view of complement activation levels in stratified AMD disease

TABLE 2. Demographic Overview of the AMD Disease Groups

	Control (n = 945)	Early AMD (n = 270)	Intermediate AMD* (n = 144)	Central GA (n = 62)	Active CNV (n = 270)	Inactive CNV (n = 51)	P value
Age, y, mean ± SD	72.3 ± 5.9	73.1 ± 7.5	73.8 ± 8.1	78.0 ± 8.4	78.4 ± 7.3	78.8 ± 8.3	<0.001
Female sex, n (%)	529 (56.0)	157 (58.1)	103 (71.5)	36 (58.1)	173 (64.1)	30 (58.8)	0.007
Male sex, n (%)	416 (44.0)	113 (41.9)	41 (28.5)	26 (41.9)	97 (35.9)	21 (41.2)	–
Never smoked, n (%)	447 (47.3)	138 (51.1)	73 (50.7)	28 (45.2)	138 (51.1)	28 (54.9)	0.029
Smoked in the past, n (%)	449 (47.5)	116 (43.0)	60 (41.7)	30 (48.4)	106 (39.3)	18 (35.3)	–
Current smoking, n (%)	49 (5.2)	16 (5.9)	11 (7.6)	4 (6.5)	26 (9.6)	5 (9.8)	–
BMI, kg/m ² , mean ± SD	25.5 ± 3.5	25.0 ± 3.5	25.3 ± 3.7	26.4 ± 3.9	25.4 ± 3.6	26.9 ± 3.7	0.008
Triglycerides, mmol/L, mean ± SD	1.86 ± 0.90	1.76 ± 0.94	1.61 ± 0.70	1.79 ± 0.71	1.86 ± 1.02	2.06 ± 1.10	0.014
CRP, mg/mL, mean ± SD	4.3 ± 0.9	4.3 ± 0.9	4.5 ± 1.2	4.4 ± 1.1	4.5 ± 1.2	4.5 ± 1.0	0.004

Analyses are based on independent *t*-tests and chi square tests.

BMI, body mass index.

* Including noncentral GA.

TABLE 3. Association of the Three Significant AMD-Associated Variants in Genes Involved in the Complement System with Systemic Complement Activation Levels

Variant	Frequency, n (%)	Log C3d/C3 Ratio, EMM (95% CI)	P value	Bonferroni-corrected P value
<i>CFH</i> rs10922109				
CC*	634 (40.6)	1.54 (1.00-2.08)	Ref.	Ref.
CA	720 (46.1)	1.38 (0.84-1.93)	<0.001	<0.001
AA	209 (13.4)	1.25 (0.70-1.80)	<0.001	<0.001
<i>CFH</i> rs570618				
GG	514 (32.9)	1.49 (0.94-2.03)	Ref.	Ref.
GT	736 (47.1)	1.38 (0.83-1.92)	0.001	0.033
TT*	312 (20.0)	1.31 (0.76-1.86)	0.001	0.012
<i>CFB</i> rs116503776				
GG*	1159 (78.4)	1.44 (0.89-1.99)	Ref.	Ref.
GA	299 (20.2)	1.32 (0.77-1.87)	<0.001	0.001
AA	21 (1.4)	1.41 (0.86-1.97)	0.785	1.0

Analysis for every variant is based on multivariate general linear modeling and adjusted for complement associated variables, including age, sex, smoking status, body mass index, triglycerides, disease status, and the other 18 genetic AMD polymorphisms in complement genes.

C3, complement component 3; C3d, complement component 3d; *CFB*, complement factor B; *CFH*, complement factor H; EMM, estimated marginal mean.

*AMD risk-increasing allele.

stages, we performed a least significant difference post hoc test in the model. Figure 1A shows the mean complement activation levels between the AMD disease stages of the total population with the corresponding *P* values of the least significant difference post hoc test. The mean complement activation level in participants with intermediate AMD was higher when compared with control individuals (*P* < 0.001), participants with early AMD (*P* = 0.003), and participants with active CNV (*P* = 0.024). Participants with central GA had higher complement activation levels when compared with controls (difference of 0.17 log C3d/C3 ratio; *P* = 0.001) and participants with early AMD (difference of 0.10 log C3d/C3 ratio; *P* = 0.025). Participants with active CNV had also higher complement activation when compared with controls (*P* = 0.017).

Next, we evaluated the influence of 18 AMD variants in five complement genes on the level of complement activation. Supplementary Table S3 shows the association of all 18 AMD-associated variants with systemic complement activation. After Bonferroni correction, three SNPs were significantly associated with altered levels of complement

activation: rs10922109 and rs570618 in the *CFH* gene and rs116503776 in the *CFB* gene (Table 3). Supplementary Table S4 describes the linkage disequilibrium of these three SNPs with other AMD variants in complement genes from the current study and previous studies.^{6–12}

To assess the effect of the combination of variants at the *CFH* locus in the same allele on systemic complement activation, we evaluated the effect of distinct haplotypes across the *CFH* locus on systemic complement activation levels using the two *CFH* SNPs in combination with the *CFB* SNP. As seen in Table 4, the *CFH* haplotype CG, including the combination of allele C in *CFH* rs10922109 and allele G in *CFH* rs570618, with the combination of allele G in *CFB* rs116503776, had the highest complement activation level (Log C3d/C3 ratio 1.61; Table 4) and was taken as the reference haplotype. Other *CFH* haplotypes with *CFB* variant combinations had significantly lower systemic complement activation levels compared with the reference haplotype (*P* ≤ 0.001), except for haplotypes with a frequency of less than 2% in the cohort study. Because the minor allele A of *CFB* rs116503776 was not very common in the study cohort, we only used the

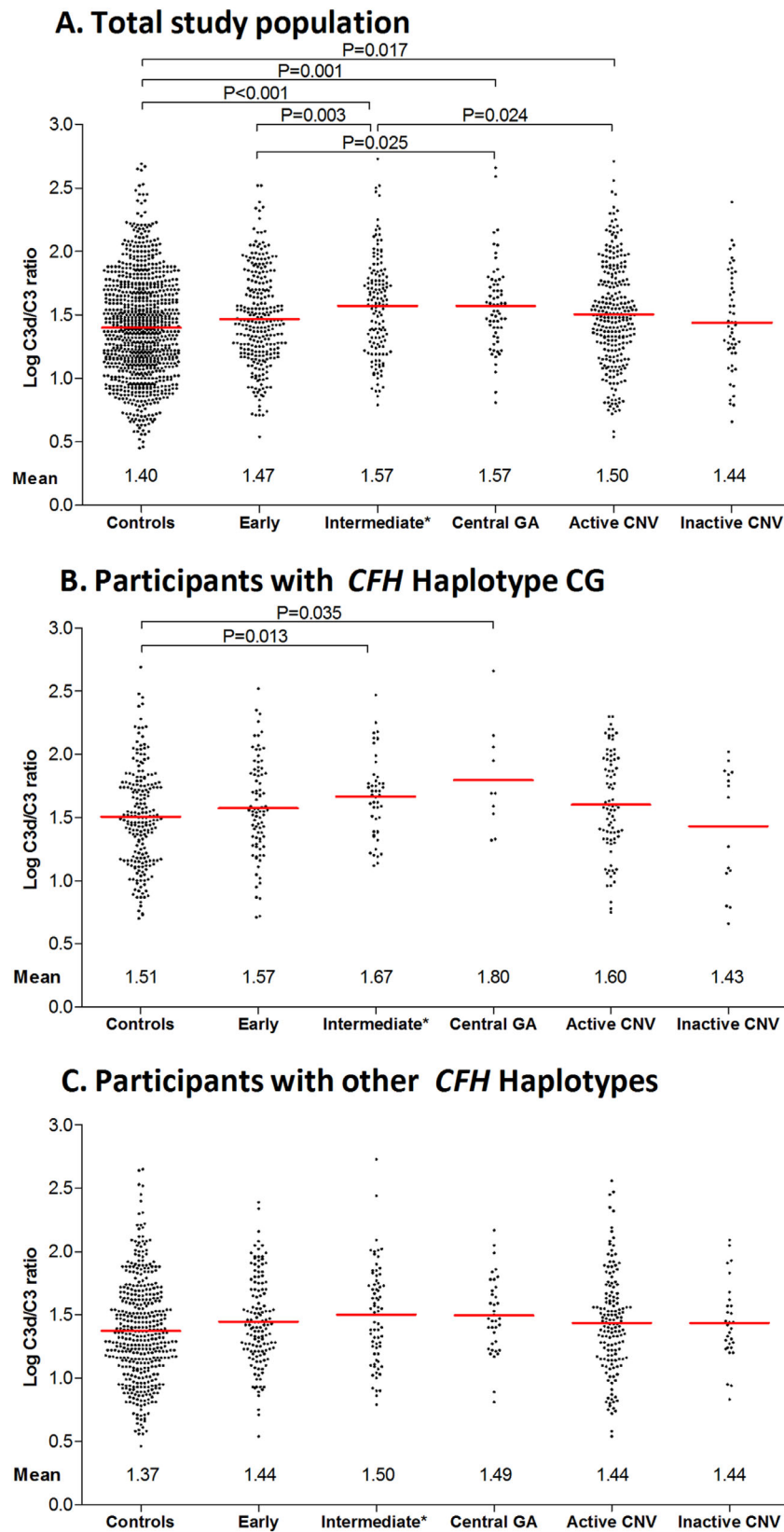


FIGURE 1. Association between systemic complement activation and AMD disease stage in (A) total study population, (B) participants with *CFH* haplotype CG, and (C) participants with other *CFH* haplotypes. Analysis is based on multivariate general linear modeling. *P* values are based on a least significant difference post hoc test between the patient groups and adjusted for the following covariates: age, sex, smoking status, BMI, and triglycerides. Overall *P* value of the association between AMD disease stage and systemic complement activation is $P < 0.001$. Red lines represent mean levels of log C3d/C3 ratio per subgroup. *Including noncentral GA. BMI, body mass index; C3, complement component 3; C3d, complement component 3d; *CFH*, complement factor H.

TABLE 4. Association of the *CFH* Haplotypes and *CFB* Variant with Systemic Complement Activation Levels

<i>CFH</i> rs10922109	<i>CFH</i> rs570618	<i>CFB</i> rs116503776	Frequency, <i>n</i> (%)	Log C3d/C3 ratio, EMM (95% CI)	<i>P</i> value
C [*] ,†	G [†]	G [*] ,†	477 (18.5)	1.61 (1.56–1.67)	Ref.
C [*] ,†	G [†]	A [†]	29 (1.1)	1.58 (1.42–1.75)	0.751
C [*]	T [*]	G [*]	1081 (41.9)	1.49 (1.44–1.55)	0.001
C [*]	T [*]	A	65 (2.5)	1.42 (1.32–1.53)	0.001
A	G	G [*]	865 (33.5)	1.47 (1.43–1.51)	<0.001
A	G	A	58 (2.2)	1.30 (1.20–1.40)	<0.001
A	T [*]	G [*]	5 (0.2)	1.37 (1.02–1.73)	0.188
A	T [*]	A	0 (0)	–	–

Analysis is based on multivariate general linear modeling and adjusted for the following complement associated variables: age, sex, smoking status, body mass index, triglycerides, and disease status.

C3, complement component 3; C3d, complement component 3d; *CFB*, complement factor B; *CFH*, complement factor H; EMM, estimated marginal mean.

^{*}AMD risk-increasing allele.

[†]*CFH* haplotype CG included the combination of allele C in *CFH* rs10922109 and allele G in *CFH* rs570618.

two *CFH* variants to divide the patients and controls in two groups: individuals carrying the *CFH* haplotype CG, with the two complement raising alleles, and individuals carrying other *CFH* haplotypes. Then, we looked again for differences in complement activation between the AMD disease stages separately for each haplotype group. In participants with the *CFH* haplotype CG (Fig. 1B), the differences in complement activation levels between the control group and the AMD disease stages became more pronounced as compared with participants with other *CFH* haplotypes that are associated with lower complement activation levels (Fig. 1C). In participants with the *CFH* haplotype CG, patients with intermediate AMD had a significant higher complement activation level when compared with control individuals ($P = 0.013$) and patients with central GA had the highest complement activation levels when compared with controls (difference of 0.29 Log C3d/C3 ratio; $P = 0.035$). These alterations in complement activation levels were not caused by differences in the distribution of genetic variants over the AMD disease stages, since there was no significant difference in the distribution of *CFH* haplotype CG among the AMD disease groups (Supplementary Table S5). In participants with other *CFH* haplotypes that are associated with lower complement activation levels, the level of complement activation did not differ between the disease stages (Fig. 1C).

DISCUSSION

Complement activation levels have been previously described to be elevated in patients with AMD compared with controls.^{11,22–26,34–37} In line with these findings, patients with intermediate AMD, central GA, and active CNV had higher complement activation levels compared with controls in our study. Between AMD disease stages, there is a trend of relatively higher complement activation levels in patients with intermediate AMD and central GA when compared with the other AMD stages. This variation of complement activation levels over the AMD disease stages seemed to be more pronounced in individuals with the *CFH* haplotype CG with complement raising alleles from SNPs in *CFH* (rs10922109, rs570618) and less evident in individuals with other *CFH* haplotypes. Although statistically significant differences in complement activation levels in participants with *CFH* haplotype CG were only observed between controls and patients with intermediate

AMD or central GA, this was probably due to a lack of power.

The relation between the AMD variants in complement genes, complement activation, and the onset and progression of the disease is very complex and not well understood. In Figure 2, we propose a hypothetical overview of complement activation in AMD that speculates on this relationship. Complement activation is thought to contribute to the local disease process in AMD.⁴⁶ Certain polymorphisms in complement genes increase the risk for onset and progression of AMD, and these genetic risk factors drive systemic complement activation.^{21–23,30} This leads to the hypothesis of a chronic low-grade systemic complement activation in patients with genetic risk variants, ultimately resulting in retinal pathology, with the macula as the main locus, presumably because of its high metabolic properties.⁴⁷ In this scenario, the continuous low-grade complement activation contributes to early AMD changes and increases promoting progression of the disease (Fig. 2, arrow 1). In line with this hypothesis, our findings suggest that, especially in participants with haplotypes associated with the highest complement activation levels, complement activation increases up to intermediate AMD and even later stage of central GA.

Interestingly, the relatively low level of complement activity in patients with CNV, both active and inactive, does not fit this scenario and leads to a cause-and-effect paradigm in which the level of systemic complement activation not only drives the disease, but, in turn, may also be influenced by local disease activity. One might speculate that in the setting of a genetically driven increased activation of the complement system, a small, local stimulus is sufficient for further amplification of the complement response (Fig. 2, arrow 2). In this case, the active macular disease process elicits a systemic inflammatory signal that results in amplification and further systemic activation of complement as measured by elevated systemic C3d/C3 levels, especially in those with AMD SNPs in complement genes. The relatively low systemic complement activation levels in the CNV groups may reflect a shift from a proinflammatory environment to a VEGF-driven environment in the retina (Fig. 2, arrow 3), implying that, next to complement activation, other alternative mechanisms may also be involved in CNV. This finding was also advocated in preclinical studies.⁴⁸

In recent years, the importance of local complement activation in AMD is becoming more evident. A recent genome-

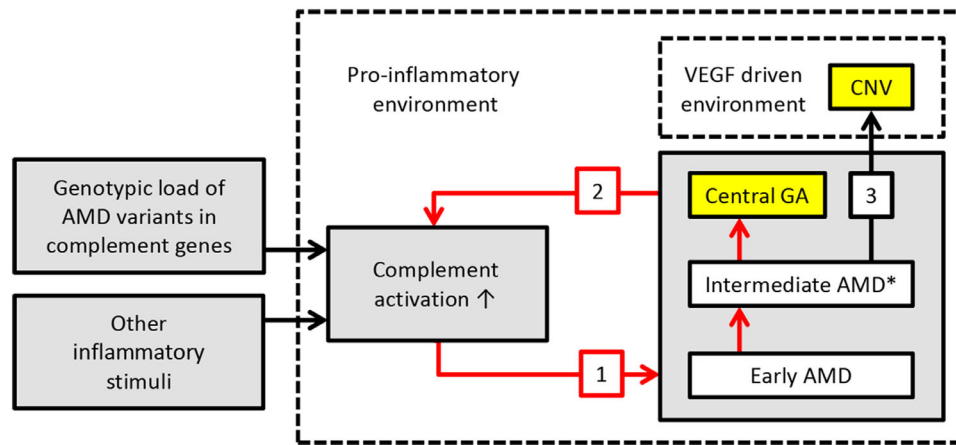


FIGURE 2. Hypothetical overview of potential underlying mechanisms between genetics, complement activation and AMD. We hypothesize that, in the presence of a genetic burden and other inflammatory stimuli, complement activation levels increase. The increased complement activation may contribute directly to the onset and progression of AMD, eventually toward advanced stage central GA (arrow 1). One might speculate that the active macular disease process also elicits an inflammatory signal, especially in those with a high genetic burden, resulting in a self-perpetuating amplification loop of increasing complement activation in a proinflammatory environment (arrow 2). Progression to CNV may also involve alternative mechanisms in a VEGF-driven environment, explaining the relatively lower levels of complement activation in this patient group (arrow 3). *Including noncentral GA.

wide association study on complement activation showed that, despite multiple variants affect systemic complement activation, only some variants were associated with AMD, suggesting tissue-specific effects of these variants, and that systemic complement activation may not always reflect the local disease activity.¹¹ Multiple immunohistochemical studies have found evidence for local complement activity in AMD. Bruch's membrane, a sheet of extracellular matrix between the choroid capillary and the RPE, has a protective function in local complement activation. Disruptions of the Bruch's membrane may lead to a reduced capacity for factor H like protein-1, a complement protect regulator within the Bruch's membrane, to deal with C3b leading to increased local complement activation and ultimately the development of AMD.⁴⁹ In addition, local complement levels in aqueous and vitreous humor are also shown to be elevated in patients with AMD, providing more evidence for local complement dysregulation in AMD.^{34,50} The relation between systemic complement activation and local complement activity and by extension the development of AMD are still incompletely understood.

These trends of complement activation levels over the AMD disease stages might have implications for the rationale and timing of complement-inhibiting treatment in AMD, if confirmed in prospective studies. Patients with intermediate AMD show relatively high levels of complement activation. For this reason, treatment of this group with a complement inhibitor is theoretically sound and might prove beneficial. A downside of treating these patients with expensive complement inhibitors is that only a minority of these patients will progress to central GA.^{1,51}

Current clinical trials, however, tend to select patients with advanced AMD, in most cases GA. The rationale for choosing GA patients as the primary target for complement-inhibitory therapy is supported by the finding that the central GA group has the highest complement activation of all groups. However, it could be argued that intervention at that stage may be too late.³² In a very proinflammatory environment, with irreversible loss of tissue, inhibiting complement may not be very effective anymore. In addition to this

optimum window of treatment, not every patient might be even suitable for complement-inhibiting therapy and selection by means of genotyping may help to identify patients who might benefit most of complement inhibiting therapy.

Strengths and Limitations

The robust datasets from the European Genetic Database are a major strength of this study. The large set of control individuals and patients with AMD allows for a detailed study between systemic complement activation and disease stages. Ideally, we would have like to monitor complement activity in individual patients as their disease progresses. The current approach, however, is much more feasible and suggests that complement activation is a dynamic process that varies with the AMD stage. To our knowledge, there are no studies that evaluate the association between complement activation and AMD stage over time and prospective studies would be needed to further strengthen our findings. Second, the number of participants with central GA and inactive CNV was relatively small, because we collected our patients from a tertiary referral center. This factor has reduced our power to detect any differences in complement activation between these groups. Larger studies are needed to confirm whether complement activation levels are indeed higher in patients with central GA, especially in those having haplotypes associated with higher complement activation levels.

Of all 18 selected AMD associated variants in *C3*, *C9*, *CFB*, *CFH*, and *CFI*, only three variants were significantly associated with systemic complement activation in our study. However, the other 15 AMD-associated variants could still be associated with complement activation, but were not found to be associated in our study. This finding was possibly due to a less strong effect of these variants on systemic complement activation, a small minor allele frequency for some of these variants that decreased the power to detect associations with complement activation, and a possible stronger effect on local complement activation. Contrary to the expectation, allele G of *CFH* rs570618 was associated with higher complement activation, while being a protective

allele for AMD. It could be that this variant has an opposite effect on local complement activation.

To keep a moderate sample size and a homogeneous population, we have decided to group patients together if the left and right eye differed by less than two disease stages. However, in an ideal setting, only patients with the same disease stage in both eyes would be included. Finally, because the Cologne Image Reading Center and Laboratory grading protocol defines having RPE atrophy outside the central circle of the Early Treatment Diabetic Retinopathy Study grid as intermediate AMD, the results of higher complement activation levels in this group could still be caused by patients who have, similar to the central GA group.

CONCLUSION

Systemic complement activation levels differ between AMD disease stages, especially in individuals with genetic variants associated with high complement activation levels. In general, patients with intermediate AMD, central GA, or active CNV have higher systemic complement activation levels when compared with controls. Patients with intermediate AMD or central GA have relatively higher complement activation levels when compared with patients with early AMD or active CNV. The association between AMD disease stage and complement activation was more pronounced in patients with haplotypes that were associated with higher complement activation levels. These findings contribute to the discussion on the pathogenesis of AMD in relation to complement activation and might suggest refinement in patient selection and the optimum window of treatment with complement inhibitors. Prospective studies are needed to confirm these results.

Acknowledgments

We thank J.M.M. Groenewoud, MSc (Department for Health Evidence, Radboud University Medical Center, Nijmegen, the Netherlands), for his advice on the statistical analyses.

Supported by MD-fonds, Oogfonds, Algemene Nederlandse Vereniging ter Voorkoming van Blindheid.

Disclosure: **T.J. Heesterbeek**, None; **Y.T.E. Lechanteur**, None; **L. Lorés-Motta**, None; **T. Schick**, None; **M.R. Daha**, None; **L. Altay**, None; **S. Liakopoulos**, None; **D. Smailhodzic**, None; **A.I. den Hollander**, None; **C.B. Hoyng**, None; **E.K. de Jong**, None; **B. J. Klevering**, None

References

1. Joachim N, Mitchell P, Burlutsky G, Kifley A, Wang JJ. The incidence and progression of age-related macular degeneration over 15 years: the Blue Mountains Eye Study. *Ophthalmology*. 2015;122:2482–2489.
2. de Jong PT. Age-related macular degeneration. *N Engl J Med*. 2006;355:1474–1485.
3. Anderson DH, Mullins RF, Hageman GS, Johnson LV. A role for local inflammation in the formation of drusen in the aging eye. *Am J Ophthalmol*. 2002;134:411–431.
4. Johnson LV, Ozaki S, Staples MK, Erickson PA, Anderson DH. A potential role for immune complex pathogenesis in drusen formation. *Exp Eye Res*. 2000;70:441–449.
5. van der Schaft TL, Mooy CM, de Bruijn WC, de Jong PT. Early stages of age-related macular degeneration: an immunofluorescence and electron microscopy study. *Br J Ophthalmol*. 1993;77:657–661.
6. Fritsche LG, Chen W, Schu M, et al. Seven new loci associated with age-related macular degeneration. *Nat Genet*. 2013;45:433–439, 439e1–2.
7. Fritsche LG, Igl W, Bailey JN, et al. A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. *Nat Genet*. 2016;48:134–143.
8. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci USA*. 2005;102:7227–7232.
9. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science*. 2005;308:385–389.
10. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet*. 2006;38:458–462.
11. Lores-Motta L, Paun CC, Corominas J, et al. Genome-wide association study reveals variants in CFH and CFHR4 associated with systemic complement activation: implications in age-related macular degeneration. *Ophthalmology*. 2018;125:1064–1074.
12. Li M, Atmaca-Sonmez P, Othman M, et al. CFH haplotypes without the Y402H coding variant show strong association with susceptibility to age-related macular degeneration. *Nat Genet*. 2006;38:1049–1054.
13. Fagerness JA, Maller JB, Neale BM, Reynolds RC, Daly MJ, Seddon JM. Variation near complement factor I is associated with risk of advanced AMD. *Eur J Hum Genet*. 2009;17:100–104.
14. Yates JR, Sepp T, Matharu BK, et al. Complement C3 variant and the risk of age-related macular degeneration. *N Engl J Med*. 2007;357:553–561.
15. Raychaudhuri S, Iartchouk O, Chin K, et al. A rare penetrant mutation in CFH confers high risk of age-related macular degeneration. *Nat Genet*. 2011;43:1232–1236.
16. Helgason H, Sulem P, Duvvari MR, et al. A rare nonsynonymous sequence variant in C3 is associated with high risk of age-related macular degeneration. *Nat Genet*. 2013;45:1371–1374.
17. van de Ven JP, Nilsson SC, Tan PL, et al. A functional variant in the CFI gene confers a high risk of age-related macular degeneration. *Nat Genet*. 2013;45:813–817.
18. Seddon JM, Yu Y, Miller EC, et al. Rare variants in CFI, C3 and C9 are associated with high risk of advanced age-related macular degeneration. *Nat Genet*. 2013;45:1366–1370.
19. van de Ven JP, Boon CJ, Fauser S, et al. Clinical evaluation of 3 families with basal laminar Drusen caused by novel mutations in the complement factor H gene. *Arch Ophthalmol*. 2012;130:1038–1047.
20. Triebwasser MP, Roberson ED, Yu Y, et al. Rare variants in the functional domains of complement factor H are associated with age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2015;56:6873–6878.
21. Hecker LA, Edwards AO, Ryu E, et al. Genetic control of the alternative pathway of complement in humans and age-related macular degeneration. *Hum Mol Genet*. 2010;19:209–215.
22. Reynolds R, Hartnett ME, Atkinson JP, Giclas PC, Rosner B, Seddon JM. Plasma complement components and activation fragments: associations with age-related macular degeneration genotypes and phenotypes. *Invest Ophthalmol Vis Sci*. 2009;50:5818–5827.

23. Scholl HP, Charbel Issa P, Walier M, et al. Systemic complement activation in age-related macular degeneration. *PLoS One*. 2008;3:e2593.
24. Smailhodzic D, Klaver CC, Klevering BJ, et al. Risk alleles in CFH and ARMS2 are independently associated with systemic complement activation in age-related macular degeneration. *Ophthalmology*. 2012;119:339–346.
25. Sivaprasad S, Adewoyin T, Bailey TA, et al. Estimation of systemic complement C3 activity in age-related macular degeneration. *Arch Ophthalmol*. 2007;125:515–519.
26. Stanton CM, Yates JR, den Hollander AI, et al. Complement factor D in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2011;52:8828–8834.
27. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement system part I - molecular mechanisms of activation and regulation. *Front Immunol*. 2015;6:262.
28. Bora NS, Matta B, Lyzogubov VV, Bora PS. Relationship between the complement system, risk factors and prediction models in age-related macular degeneration. *Mol Immunol*. 2015;63:176–183.
29. Paun CC, Ersoy L, Schick T, et al. Genetic Variants and systemic complement activation levels are associated with serum lipoprotein levels in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2015;56:7766–7773.
30. Ristau T, Paun C, Ersoy L, et al. Impact of the common genetic associations of age-related macular degeneration upon systemic complement component C3d levels. *PLoS One*. 2014;9:e93459.
31. Gaya da Costa M, Poppelaars F, van Kooten C, et al. Age and sex-associated changes of complement activity and complement levels in a healthy Caucasian population. *Front Immunol*. 2018;9:264.
32. Dolgin E. Age-related macular degeneration foils drug makers. *Nature Biotechnology*. 2017;35:1000–1001.
33. Apellis. Appellis Pharmaceuticals announces that APL-2 met its primary endpoint in a phase 2 study in patients with geographic atrophy, an advanced form of age-related macular degeneration. *Statistically Significant Slowing of Disease Progression Seen at 12 Months*. Louisville: Apellis Pharmaceuticals, Inc.; 2017:1–2.
34. Kirschfink M, Schick T, Steinhauer M, et al. Local complement activation in aqueous humor in patients with age-related macular degeneration. *Mol Immunol*. 2017;89:163–163.
35. Silva AS, Teixeira AG, Bavia L, et al. Plasma levels of complement proteins from the alternative pathway in patients with age-related macular degeneration are independent of complement factor H Tyr(4)(0)(2)His polymorphism. *Mol Vis*. 2012;18:2288–2299.
36. Machalinska A, Dziedziejko V, Mozolewska-Piotrowska K, Karczewicz D, Wiszniewska B, Machalinski B. Elevated plasma levels of C3a complement compound in the exudative form of age-related macular degeneration. *Ophthalmic Res*. 2009;42:54–59.
37. Hecker LA, Edwards AO, Ryu E, et al. Genetic control of the alternative pathway of complement in humans and age-related macular degeneration. *Hum Mol Genet*. 2010;19:209–215.
38. Smailhodzic D, Fv Asten, Blom AM, et al. Zinc supplementation inhibits complement activation in age-related macular degeneration. *PLoS One*. 2014;vol. 9:e112682.
39. Troldborg A, Jensen L, Deleuran B, Stengaard-Pedersen K, Thiel S, Jensenius JC. The C3dg fragment of complement is superior to conventional C3 as a diagnostic biomarker in systemic lupus erythematosus. *Front Immunol*. 2018;9:581.
40. Rother E, Lang B, Coldewey R, Hartung K, Peter HH. Complement split product C3d as an indicator of disease activity in systemic lupus erythematosus. *Clin Rheumatol*. 1993;12:31–35.
41. Reddingius RE, Schroder CH, Daha MR, Monnens LA. The serum complement system in children on continuous ambulatory peritoneal dialysis. *Perit Dial Int*. 1993;13:214–218.
42. Siezenga MA, Chandie Shaw PK, van der Geest RN, et al. Enhanced complement activation is part of the unfavourable cardiovascular risk profile in South Asians. *Clin Exp Immunol*. 2009;157:98–103.
43. Clyne B, Olshaker JS. The C-reactive protein. *J Emerg Med*. 1999;17:1019–1025.
44. Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA. Score tests for association between traits and haplotypes when linkage phase is ambiguous. *Am J Hum Genet*. 2002;70:425–434.
45. Chang CC, Chow CC, Tellier LC, Vattikuti S, Purcell SM, Lee JJ. Second-generation PLINK: rising to the challenge of larger and richer datasets. *Gigascience*. 2015;4:7.
46. Hageman GS, Luthert PJ, Victor Chong NH, Johnson LV, Anderson DH, Mullins RF. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. *Prog Retin Eye Res*. 2001;20:705–732.
47. Bora NS, Matta B, Lyzogubov VV, Bora PS. Relationship between the complement system, risk factors and prediction models in age-related macular degeneration. *Mol Immunol*. 2015;63:176–183.
48. Yang Y, Liu F, Tang M, et al. Macrophage polarization in experimental and clinical choroidal neovascularization. *Sci Rep*. 2016;6:30933.
49. Clark SJ, Schmidt CQ, White AM, Hakobyan S, Morgan BP, Bishop PN. Identification of factor H-like protein 1 as the predominant complement regulator in Bruch's membrane: implications for age-related macular degeneration. *J Immunol*. 2014;193:4962–4970.
50. Loyet KM, Deforge LE, Katschke KJ, Jr, et al. Activation of the alternative complement pathway in vitreous is controlled by genetics in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2012;53:6628–6637.
51. Klein R, Klein BE, Knudtson MD, Meuer SM, Swift M, Gangnon RE. Fifteen-year cumulative incidence of age-related macular degeneration: the Beaver Dam Eye Study. *Ophthalmology*. 2007;114:253–262.