Plasma Complement Components and Activation Fragments: Associations with Age-Related Macular Degeneration Genotypes and Phenotypes

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Abstract

Purpose—Several genes encoding complement system components and fragments are associated with age-related macular degeneration (AMD). This study was conducted to determine whether alterations in circulating levels of these markers of complement activation and regulation are also independently associated with advanced AMD and whether they are related to AMD genotypes.

Methods—Plasma and DNA samples were selected from individuals in our AMD registry who had progressed to or developed the advanced stages of AMD, including 58 with geographic atrophy and 62 with neovascular disease. Subjects of similar age and sex, but without AMD, and who did not progress were included as controls ($n = 60$). Plasma complment components (C3, CFB, CFI, CFH, and factor D) and activation fragments (Bb, C3a, C5a, iC3b, and SC5b-9) were analyzed. DNA samples were genotyped for seven single-nucleotide polymorphisms in six genes previously shown to be associated with AMD: *CFB, CFH, C2, C3*, and *CFI* and the *LOC387715/ARMS2* gene region. The association between AMD and each complement biomarker was assessed by using logistic regression, controlling for age, sex, and proinflammatory risk factors: smoking and body mass index (BMI). Functional genomic analyses were performed to assess the relationship between the complement markers and genotypes. Concordance, or *C*, statistics were calculated to assess the effect of complement components and activation fragments in an AMD gene-environment prediction model.

Results—The highest quartiles of Bb and C5a were significantly associated with advanced AMD, when compared with the lowest quartiles. In multivariate models without genetic variants, the odds ratio (OR) for Bb was 3.3 (95% confidence interval [CI] = 1.3-8.6), and the OR for C5a was 3.6

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(95% CI = 1.2-10.3). With adjustment for genetic variants, these ORs were substantially higher. The alternative pathway regulator CFH was inversely associated with AMD in the model without genotypes (OR = 0.3; P = 0.01). Positive associations were found between BMI and plasma C3, CFB, CFH, iC3b, and C3a. There were also significant associations between C5a fragment and *LOC387715/ARMS2* and *C3* genotypes (P for trend = 0.02, 0.04), respectively. C statistics for models with behavioral and genetic factors increased to 0.94 ± 0.20 with the addition of C3a, Bb, and C5a.

Conclusions—Increased levels of activation fragments Bb and C5a are independently associated with AMD. Higher BMI is related to increased levels of complement components. C5a is associated with AMD genotypes. *C* statistics are stronger with the addition of C3a, Bb, and C5a in predictive models. Results implicate ongoing activation of the alternative complement pathway in AMD pathogenesis.

Several genes in the complement pathway are known to be associated with age-related macular degeneration (AMD), including *CFH, CFB, C2, C3*, and *CFI*.[1-9] These associations point to the involvement of inflammatory and immune mechanisms, especially the complement system, in the development of AMD. Another gene in the *LOC387715/ARMS2* region on chromosome 10 is related to AMD, although the mechanism is uncertain.[10-13] Behavioral and modifiable factors, such as smoking and body mass index (BMI), which are related to AMD,[14] influence levels of inflammatory biomarkers,[15,16] and also modify genetic susceptibility.[17] We tested levels of complement components (C3, CFB, CFI, CFH, and factor D) and activation fragments (Bb, C3a, C5a, iC3b, and SC5b-9) in plasma samples from cases with advanced AMD and controls, to further assess the role of the complement system and its association with AMD. In addition, we determined the association of these biomarkers with behavioral factors related to AMD and their association with AMD genotypes. Finally, we assessed the degree to which these components and activation fragments contribute to the algorithm previously shown to predict the prevalence and incidence of advanced AMD.[18]

Methods

Study Population

Patients with and without AMD were enrolled in our genetic epidemiologic studies, using standardized clinical examinations, questionnaires, and fundus photography, as previously described.[19-21] Grades were based on clinical and fundus photographic data using the Clinical Age-Related Maculopathy Grading System (CARMS).[22] From our AMD registry, we selected 180 unrelated Caucasian individuals with DNA and plasma samples. The 120 patients (60 men and 60 women), included 58 individuals with geographic atrophy (GA) and 62 with neovascular disease (NV). Among the patients, 108 had a baseline CARMS grade of 1, 2, or 3 and progressed to either grade 4 with central or noncentral GA (n = 48) or grade 5 with NV (n = 62) in one or both eyes. Controls (n = 60) had a CARMS grade of 1 in both eyes, did not progress, and consisted of 30 men and 30 women. The mean ± SD of ages of the case and control groups was 82 ± 6.9 and 79 ± 4.4 years, respectively. The research complied with the tenets of the Declaration of Helsinki and was approved by the institutional review board. Informed consent was obtained from all subjects.

Genotyping

DNA samples were genotyped for seven single-nucleotide polymorphisms (SNPs) in genes previously demonstrated to be related to AMD: (1) complement factor H *CFHY402H rs1061170* in exon 9 of the *CFH* gene on chromosome 1, region q32, a change 1277T>C, resulting in a substitution of histidine for tyrosine at codon 402 of the CFH protein; (2) *CFH rs1410996*, an independently associated SNP variant within intron 14 of *CFH*; (3) *LOC387715*/A69S *ARMS2* (rs10490924 in the LOC387715/ARMS2 region of chromosome 10, a nonsynonymous coding SNP variant in exon 1 of *LOC387715*), resulting in a substitution

of the amino acid serine for alanine at codon 69; (4) complement component 2 or C2BE318D (rs9332739), the nonsynonymous coding SNP variant in exon 7 of *C2* resulting in a substitution of aspartic acid for glutamic acid at codon 318; (5) complement factor B or CFBR32Q (rs641153), the nonsynonymous coding SNP variant in exon 2 of *CFB* resulting in the substitution of the amino acid glutamine for arginine at codon 32; (6) complement component 3 or C3R102G (rs2230199), the nonsynonymous coding SNP variant in exon 3 of *C3* resulting in the substitution of the amino acid glycine for arginine at codon 102; and (7) complement factor I *CFIrs10033900*, an independently associated SNP located in the linkage peak region of chromosome 4, 2781 bp upstream of the 3′ untranslated region of *CFI.*

For the genetic variant on chromosome 10, *LOC387715A69S*, it remains a subject of debate whether the gene *HTRA1* adjacent to it may in fact be the AMD susceptibility gene on 10q26[10-13]; however, the relevant SNPs in these two genes have been reported to be in near-perfect correlation. Thus, although the other SNP is a promising candidate variant, rs10490924, used in this study, can be considered a surrogate for the causal variant that resides in this region. For the *C2/CFB* genes, there are two independent associations to the *C2/CFB* locus, but because of linkage disequilibrium, we do not know whether one of the two genes or both are functionally affected.[1,2] Genotyping was performed using primer mass extension and MALDI-TOF MS analysis (MassEXTEND methodology; Sequenom, San Diego, CA) at the Broad Institute Center for Genotyping and Analysis (Cambridge, MA).

Plasma Samples and Complement Components and Activation Fragments

Fasting plasma samples were drawn into tubes (BD Biosciences, Bedford, MA) containing K_2 EDTA, primarily at time-of-baseline grade. The blood was centrifuged and plasma separated within 30 minutes of collection. The samples were frozen and stored in liquid nitrogen until testing was performed. The following complement activation fragments and complement system proteins were analyzed in the Complement Laboratory of PCG: Bb, C3a, C5a, iC3b, SC5b-9, C3, factor D, CFB, CFH, and CFI.

C3 was measured by standard clinical laboratory nephelometric methods. CFB, CFH, and CFI were measured by radial immunodiffusion (RID) with anti-human antibodies specific for the individual proteins in 1% agarose gels.[23] The CFH antiserum (Quidel, San Diego, CA) was produced against purified human factor H. It is specific for CFH but has not been evaluated for cross-reactivity to the CFH-related proteins. The complement laboratory reference range for CFH is 160 to 412 μg/mL, in agreement with ranges used by other complement reference laboratories in the United States and Europe. The antibodies used for CFB and CFI are also goat polyclonal antisera specific for the given proteins (Quidel). Purified CFB, CFH, and CFI were used to standardize the assays (Quidel, and CompTech, Tyler TX). Appropriate controls were included with each batch of patient samples, and the results were calculated from the area under the precipitin rings.

Factor D was measured by ELISA (Quantikine kit; R&D Systems, Minneapolis, MN), with a plate precoated with specific monoclonal antibody for factor D. The standard provided with the kit is 40 ng of recombinant human complement factor D (CFD). The enzyme conjugate is polyclonal anti-CFD linked to horseradish peroxidase. Three controls: high, medium, and low, are run with each set of samples. The reference range given by the manufacturer is 1468 to 3657 ng/mL for EDTA plasma. The reference range established by the complement laboratory is 1688 to 3076 ng/mL for EDTA plasma, which was converted to μg/mL: 1.69 to 3.08 (mean $\pm$ 2 SD) for reporting in this study.

Complement activation markers were measured by commercially available ELISA kits with extra in-house controls. Bb, iC3b, and SC5b-9 ELISA kits were from Quidel. The tests for C3a and C5a were performed with kits (OptEIA) from BD-Pharmingen (San Diego, CA). Reference

ranges for all the complement fragments were established in the complement laboratory under stringent validation protocols. Reference ranges for the assays are as follows: C3a (305-1239 ng/mL), C5a (5.0-25.4 ng/mL), Bb (0.41-1.49 μg/mL), iC3b (0-17.4 ng/mL), and SC5b-9 (72-244 ng/mL). The complement laboratory is accredited by the College of American Pathologists and the Clinical Laboratory of 1988 to perform tests of high complexity.

Covariates

Data on smoking were collected from a standardized risk factor questionnaire. Smokers were defined as having smoked at least one cigarette per day for 6 months or longer. Pack years were calculated by multiplying the number of cigarettes smoked per day by the number of years smoked divided by 20. Height and weight were measured at the time of the baseline grade to calculate BMI (weight in pounds, multiplied by 703, divided by height in inches squared), or in a few instances by self-report.

Statistical Analysis

Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for covariates, and genotypes by using logistic regression (controlling for age [60-79, 80+] and sex) to evaluate their association with each maculopathy group (GA and NV), and total AMD (GA and NV combined), with controls. *t*-Tests were used to calculate probabilities values for age between cases and controls. $P \leq 0.05$ was considered statistically significant for all analyses.

The Wilcoxon rank sum test was used to calculate probabilities to assess the relationship between the median plasma level of complement components and activation fragments and maculopathy group.

ORs and 95% CIs for total AMD were computed to compare the fourth quartile with the first quartile of the component and activation fragments using logistic regression. In model A, we controlled for age (60-79, 80+), sex, BMI (<25, 25-29.9, 30+), and smoking (ever, never). In model B, we controlled for the same factors as in model A, plus all the genotypes: *CFB* (CC and CT/TT), *CFH:Y402H* (TT, CT, and CC), *CFH:rs1410996* (TT, CT, and CC), *C2* (GG and CG/CC), *LOC387715/ARMS2* (GG and GT/TT), *C3* (CC, CG, and GG), and *CFI* (CC, CT, and TT).

ORs and 95% CIs for AMD were calculated for each genotype separately with one component or activation fragment at a time to assess whether the effect of genotype was mediated by that complement component or activation fragment. We used log component and activation fragment values, because the distribution was slightly skewed, to assess associations with genotype among controls using linear regression. In addition, linear regression was used to test the relationship between each complement component or activation fragment and smoking and BMI, both among the controls and among the cases and controls combined. To see whether the effects of genotype and complement components and activation fragments were dependent on one another, we used logistic regression to test for interaction effects on risk of AMD.

General linear model analysis was used to calculate the least-squares (LS) mean, to assess the relationship between the mean level of components or fragments and genotype among the cases and controls combined. In this model we controlled for age, sex, AMD status, and all the genotypes.

Concordance, or *C*, statistics were calculated to assess whether activation fragments contribute to the predictability of developing advanced AMD. Similar to our previous studies,[16,18,24] the area under the receiver operating characteristic (ROC) curve was obtained, and an age-adjusted *C* statistic based on the ROC curve was calculated. *C* statistics were calculated for six models with various combinations of covariates, genotypes, and activation fragments, to assess

the probability that the risk score from a random case was higher than the corresponding risk score from a random control, based on the group of risk factors in each model, such that a perfect score would be 1.0, or 100%, predictability. We obtained standard errors of estimated C statistics and compared C statistics from alternative risk prediction models by using correlated ROC curve methods.

Results

Table 1 shows associations between increasing age and all AMD groups. The NV-only and total AMD groups were more likely to be smokers or heavy smokers and had higher BMIs than did the control subjects, although these associations were not statistically significant. All genotypes were associated with both types of advanced AMD. There was a significant protective effect of genotype *CFB* (*CT/TT*) for GA and a trend for reduced risk of NV, compared with *CC* genotype. There were strong positive associations between the *CFH Y402H* and *CFH*: *rs1410996* CC genotypes (vs. TT) and both forms of advanced AMD, and a protective association of *C2* CG/CC (vs. GG) with overall AMD and GA. *LOC387715/ARMS2* GT/TT was associated with AMD compared with GG. The *C3* GG genotype was positively associated with GA, and a similar trend was seen for NV. For *CFI*, ORs were in the direction of increased risk for the T allele for both GA and NV (*P*-trend = 0.08 for total AMD).

Table 2 shows that the median levels of the activation fragments, Bb, C3a, and C5a, were significantly higher in the GA group than in the control group. CFH component was significantly lower in the GA group than in the control group (median of 289 vs. 312 μg/mL, *P* = 0.008). Results for the total AMD group were similar to those for the GA group.

Table 3 shows a positive association between activation fragment Bb and the total AMD group, both with and without adjustment for genotype (OR = 3.3, 95% CI = 1.3-8.6, and OR = 5.4, 95% CI = 1.3-22.9, respectively). Similar results were found for C5a (OR = 3.6, 95% CI = 1.2-10.3; OR = 25.2, 95% CI = 3.7-171.7), with and without adjustment for genotypes, respectively. CFH component had a significant inverse association with AMD in the model that did not control for genotype (OR = 0.3, 95% CI = 0.09-0.75, *P* = 0.01), and which became nonsignificant after adjustment for genotype.

Table 4 shows the change in the association between genotype and AMD, with addition of complement fragments Bb, C5a, and component CFH to the models. The ORs for risk of AMD increased for both *CFH* loci, *LOC*, and *CFI* genotypes, with the addition of activation fragment C5a into the statistical models. There was also an increase in the OR for the *C3* gene when the CFH component was added to the model. The associations of the other genetic loci with AMD (data not shown) were not materially changed after inclusion of complement components and activation fragments one at a time.

We found significant associations between BMI $\geq$ 25 and increased levels of fragments C3a and iC3b, and component CFH among the control subjects (Table 5). When the entire study population was included to further assess these relationships, controlling for AMD status, CFH remained significant, CFB and C3 became significant (*P* = 0.001 and 0.005, respectively), and C3a and iC3b became nonsignificant. We found no significant association between complement components or fragments and smoking.

Interactions between genotypes and components and fragments were also assessed (data not shown). We found a significant interaction between component CFH and the protective *CFB* genotype (*P* interaction = 0.04). This finding indicates that the component CFH is inversely associated with risk of AMD among individuals with the *CFB* genotype CC, but is unrelated to risk of AMD in the presence of a protective allele *CFB* genotype of CT/TT.

When controlling for all genotypes, we found significant associations between the LS mean for fragment C5a and the *LOC387715/ARMS2* and *C3* genotypes, although the differences were small (Table 6). For the *LOC387715/ARMS2* genotype, the LS mean for C5a was lower with the addition of the risk allele T. For the *C3* genotype the trend for increasing C5a is significant with the addition of each risk allele. The Bb LS means are negative because they are on a log scale and have values less than 1 in some instances.

C statistics for all cases versus controls were calculated for various models to assess the predictability of advanced AMD (Table 7): model 1 (age, sex, AMD status, smoking, and BMI); model 2 (all variables in model 1 plus genetic variants CFB, CFH:Y402H, CFH:rs1410996, C2, LOC387715/ARMS2, C3, and *CFI*); and models 3, 4, 5, and 6 (model 2 plus fragments C3a, Bb, and C5a, and all three markers, respectively). There were significant increases in *C* statistics on adding Bb (0.870) and C5a (0.895) in model 2 (*P* = 0.029, 0.043, respectively). Combining C3a, Bb, and C5a in the model resulted in a *C* statistic of 0.944 and a *P* < 0.001 compared with model 2. We plotted the frequency distribution of risk scores separately for cases and controls for model 6, selecting a cutoff of 4 (risk score ≥ 0), which yields a sensitivity of 88% and a specificity of 73% (Fig. 1). In general, cases had higher risk scores than the controls.

Discussion

In our analyses, we found an independent relationship between activation fragments Bb and C5a and advanced AMD. Our study provides new information regarding the association between the complement system and AMD in several ways. We evaluated each complement component or fragment, both with and without seven AMD genotypes, controlling for other covariates including age, sex, smoking, and BMI. We found a significant association between median levels of fragments Bb and C5a and advanced AMD as well as the GA group separately. There was a significant inverse association between the median level of CFH component and the total AMD group and the GA group separately. Median C3a level was significantly higher among GA cases, but this association was not significant after adjustment for other genetic and nongenetic factors. Some associations were stronger in GA cases than NV cases, compared with controls. Reasons for this are unclear, and additional studies may clarify the biologic mechanisms responsible for these differences in advanced AMD.

Bb is a catalytic domain of the alternative pathway C3 convertase, C3bBb. Upon decay or disassociation of this two-part protein complex by complement regulators such as the membrane protein decay accelerating factor (DAF, CD55) or the plasma protein factor H, Bb is liberated into the surrounding milieu. Thus, it is a marker of alternative pathway activation and increased levels provide strong evidence for engagement of the alternative pathway in AMD patients. Of note, elevated levels of Bb were also recently reported in preeclampsia, another condition in which excessive alternative pathway activation has been implicated in etiopathogenesis.[25] Preeclampsia is characterized by small vessel angiopathy and rare mutations in the complement regulatory proteins of the alternative pathway have also been identified in this condition and in the hemolytic uremic syndromes, another thrombomicroangiopathy.[25,26]

We also found associations between higher BMI and activation fragments C3a, iC3b, and component CFH among the control subjects, as well as associations between BMI and components C3, CFB, and CFH among the entire study population, when controlling for AMD. Higher BMI is a known risk factor for the development of AMD,[14,17,24] it has been shown to be related to higher CRP,[15,16] and the independent association between BMI and complement components and fragments found in this study is also noteworthy. We found a significant inverse association between C5a fragment and *LOC387715/ARMS2* genotypes, and significant

positive trend between *C3* genotypes and higher C5a fragment. We calculated ROC curves and *C* statistics for C3a, Bb, and C5a which included demographic and environmental covariates and seven SNPs. Significant increases in *C* statistics were observed when these fragments were added to the prediction model, especially when they were considered together, with discrimination between advanced AMD and a control increasing to as high as 94%. These findings add to the evidence that the complement system, in particular the alternative pathway, is activated and involved in the development of advanced AMD, such as through chronic low-level activation of complement in the retina.

In AMD, there is increased complement deposition in Bruch's membrane and in drusen.[27] Photo-oxidation of bisretinoid lipofuscin in cultured RPE cells has been shown to lead to complement activation and release of fragments iC3b and C3a in vitro.[28] This finding supports the thinking that increased complement activation in AMD may occur as a result of photo-oxidation within RPE. Having insufficient functional CFH to dampen the complement-induced injury in the outer retina may then lead to pathologic features in advanced AMD. This line of thinking also is supported by the inverse relationship we found between CFH and AMD for analyses that were not controlled by genotype. The major predisposing effect is thus one of decreased regulation of activation of the complement cascade in the retina, such as through dysfunctional CFH gene protein or from insufficient production of protein. Whether there is systemic activation of the complement system or the elevated levels reflect systemically circulating fragments from local activation in the eye (as is observed in rheumatoid arthritis with complement activation in a joint) is not clear.[29] These are not mutually exclusive possibilities, in that both systemic activation and local (i.e., retinal) complement activation could play a role. The latter is established based on findings of complement deposition in drusen.[27] Biologically, the same outcome would occur if CFH were present in lower than normal amounts or were not fully functional. Complement activation, although probably not the initiating cause of the injury in AMD, can nevertheless substantially contribute to subsequent tissue damage. Animal models of laser induced damage to Bruch's membrane provide evidence of the importance of C5a and other components of alternative pathway in the development of choroidal neovascularization.[30,31] Elevated plasma activation fragments Bb, C3a, and C5a are consistent with continuous, low-level alternative pathway activation in patients with advanced AMD. In sum, we hypothesize that subtle alterations in the efficiency of activation and/or in regulatory capacity negatively influence a pathologic process that plays out over the years.

Activation of the complement system may lead to the formation the membrane attack complex (MAC or C5b-9). This terminal pathway begins with the cleavage of C5 to C5a and C5b. SC5b-9 is the fluid phase terminal complex that usually circulates bound to the inhibitor vitronectin. The quantity of SC5b-9 in the circulation is in part a reflection of the MAC that is generated locally. Thus, a fraction is deposited at the site and part is in the fluid phase. Our results and those of others indicate increased activation of C5 based on the elevated C5a levels in the blood and deposition of C5b-9 in the retina in AMD patients. C5a can bind to its receptor, C5aR, and has been shown to be important in models of AMD.[32] C5b can be generated by activation of the alternative pathway or by direct cleavage by proteases including trypsin and thrombin. A recent report provided considerable evidence to indicate C5 activation by thrombin in a lung model of immune complex activation.[33] In our study, the SC5b-9 was not increased in AMD, although it was in a similar study by Scholl et al.[34] That study had few GA patients, and we speculate that the efficiency of C5b-9 deposition and retention in the two forms or the difference in the proportion of the types of advanced disease compared with our sample may account for the differences in results regarding MAC and other differences between the two reports. Another possibility relates to the quantity of local versus systemic activation which could alter the ratio of blood to tissue MAC. We still have much to learn about how the complement system is triggered and regulated, especially in niche locations such as the retina.

BMI is a known risk factor for the development of AMD.[14],[17],[24] The association described in this report between high BMI and elevated C3, CFB, and CFH as well as increased concentrations of complement activation fragments derived from several components of the alternative pathway point to a role of the complement system in obesity. Adipose tissue is the source of acylation-stimulating protein (ASP), which is also C3a desArg. C3a desArg is a C3-derived protein formed by removal of an arginine from the carboxyl terminus of C3a and is the major form of circulating C3a. Before the relationship to C3 was discovered, the lipogenic function of ASP/C3a desArg was discovered.[35] Sivaprasad et al.[36] studied C3a desArg as an indicator of C3 systemic complement activation among 84 cases of advanced AMD (42 GA, 42 NV), as well as in association with the *CFH:rs1061170* genotype. They found no significant association between genotype and C3a desArg, but did find an increase in C3a desArg concentration among cases, which suggests systemic activation. Largely consistent with these data, we found associations with C3a and AMD, and additionally found a new association between a second C3 split product, iC3b and BMI. Adipsin, an adipose-specific factor linked to adipocyte differentiation, was shown to be factor D of the alternative pathway of the complement system.[37] We did not find a relationship with plasma factor D, whereas a previous study showed factor D was increased among patients with AMD compared with controls with a different proportion of geographic atrophy to neovascular AMD cases.[34] Other noteworthy adipose-complement alterations include altered lipid clearance in $C3^{-/-}$ mice and partial lipodystrophy in children with an autoantibody that stabilizes the alternative pathway C3 convertase.[38],[39] Also, there are a few reports of elevated C3 concentrations in individuals with a high BMI.[40],[41] Our data support increased turnover and activation of the alternative pathway in individuals with high BMI, evidenced by increased activation fragments, Bb, C3a, and iC3b. Enhanced alternative pathway activation in obesity and in AMD may jointly accelerate tissue damage.

Another noteworthy finding was the OR for the relationship between AMD and *LOC387715/ ARMS2* increased with the addition of C5a into the model. Since the function of ARMS2 is unknown, further research is needed. However, one possibility is that a tissue metalloproteinase directly activates C5 leading to formation of C5a and C5b, with the former binding to its receptor and the latter beginning the MAC.

Conclusion

Regardless of known genotype, both Bb and C5a are strongly associated with increased risk of advanced AMD in this study. We found a new association between AMD and Bb and a new significant inverse association with median level of CFH. Our case population consisted solely of advanced AMD cases, most of which were documented progressors to GA and NV disease. The GA and NV groups are in similar proportion and median levels are displayed for each subtype. We expand on the relationships between AMD and complement components and fragments by considering six genes (seven genetic loci) and covariates known to be related to AMD, and we evaluated the effect of these genetic factors on function or levels of the complement proteins. We also provide evidence of increased BMI in association with complement activation fragments, suggesting that BMI itself increases the chronic activation of the complement system. Our AMD prediction model with genetic, demographic, and environmental factors was improved with the addition of plasma complement markers. Results provide new evidence that the complement system, and particularly the alternative pathway, is chronically activated in AMD. These findings contribute to our understanding of the pathophysiology of advanced AMD.

Acknowledgments

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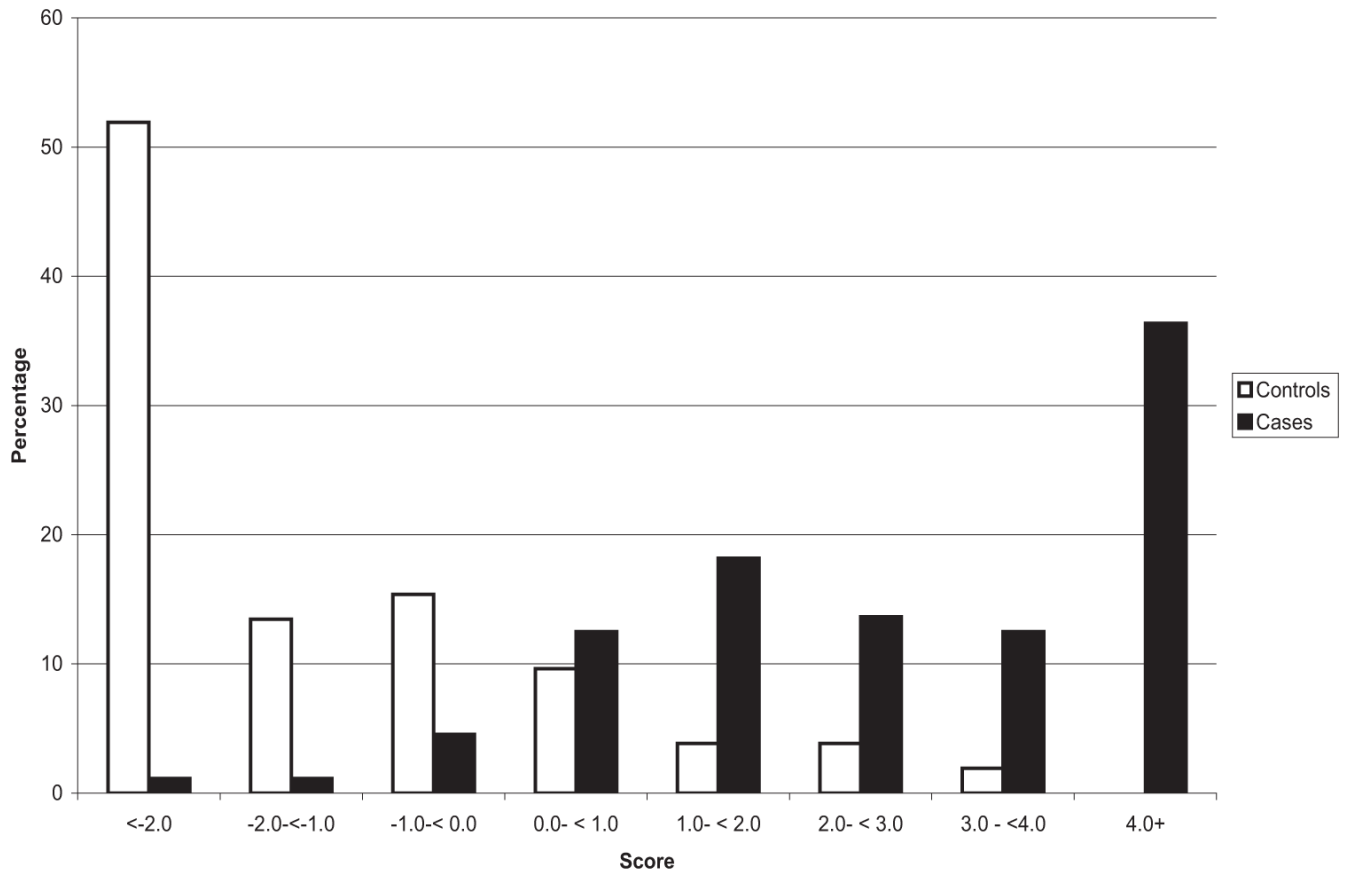
Figure 1.
Risk scores for cases and controls based on age, sex, smoking, BMI, seven genetic variants, and C3a, Bb, and C5a fragments.

Table 1

Baseline Demographic, Behavioral, and Genetic Factors in the Control and Maculopathy Groups

	Control (n = 60)	GA (n = 58)			NV (n = 62)			Total Advanced AMD (n = 120)		
	n (%)	n (%)	OR (95% CI)*	P	n (%)	OR (95% CI)*	P	n (%)	OR (95% CI)*	P
Characteristics										
Female	30 (50)	30 (52)	1.0		30 (48)	1.0		60 (50)	1.0	
Male	30 (50)	28 (48)	0.9 (0.5-19)	0.85	32 (52)	0.9 (0.4-2.0)	0.86	60 (50)	1.0 (0.5-1.9)	0.91
Smoking status										
Never	29 (48)	31 (52)	1.0		22 (35)	1.0		53 (44)	1.0	
Ever	31 (52)	27 (47)	0.8 (0.4-1.7)	0.54	40 (85)	1.6 (0.8-3.5)	0.20	67 (56)	1.2 (0.6-2.2)	0.66
Pack years										
0	29 (48)	31 (54)	1.0		22 (36)	1.0		53 (45)	1.0	
0.1-14.4	12 (20)	11 (19)	0.8 (0.3-2.2)		9 (15)	0.9 (0.3-2.8)		20 (17)	0.8 (0.3-2.0)	
14.5-33	10 (17)	7 (12)	0.6 (0.2-1.9)		15 (25)	1.9 (0.7-5.4)		22 (19)	1.3 (0.5-3.1)	
34+	9 (15)	8 (14)	0.9 (0.3-2.7)	0.58[†]	15 (25)	2.1 (0.7-6.1)	0.10[†]	23 (59)	1.3 (0.5-3.4)	0.48[†]
Body mass index										
<25	35 (58)	25 (43)	1.0		24 (39)	1.0		49 (40)	1.0	
25-29.9	14 (23)	19 (33)	1.6 (0.7-3.6)		25 (40)	2.3 (1.0-5.4)		44 (37)	1.8 (0.9-3.8)	
≥30	11 (18)	14 (24)	1.5 (0.6-3.7)	0.15[†]	13 (21)	1.1 (0.4-2.8)	0.17[†]	27 (23)	1.3 (0.6-2.8)	0.09[†]
Genotypes										
CFB:rs641153 (R32Q)										
CC	46 (80)	50 (96)	1.0		47 (92)	1.0		97 (94)	1.0	
CT/TT	11 (19)	2 (4)	0.2 (0.03-0.6)	0.03	4 (8)	0.28 (0.08-1.0)	0.05	6 (6)	0.23 (0.08-0.70)	0.01
CFH:rs1061170 (Y402H)										
TT	28 (50)	12 (23)	1.0		8 (16)	1.0		20 (19)	1.0	
CT	18 (32)	21 (40)	2.5 (1.0-6.3)		22 (43)	5.4 (1.8-16.0)		43 (42)	3.4 (1.5-7.8)	
CC	10 (18)	19 (37)	4.4 (1.6-12.6)		21 (41)	9.2 (2.8-30.0)		40 (39)	5.7 (2.3-14.4)	

	Control (n = 60)	GA (n = 58)			NV (n = 62)			Total Advanced AMD (n = 120)		
	n (%)	n (%)	OR (95% CI)*	P	n (%)	OR (95% CI)*	P	n (%)	OR (95% CI)*	P
CFH:rs1410996				0.004†			0.0002†			0.0002†
TT	6 (11)	3 (6)	1.0		2 (4)	1.0		5 (5)	1.0	
CT	32 (58)	12 (23)	0.9 (0.2-42)		11 (23)	1.1 (0.2-7.3)		23 (23)	0.8 (0.2-3.1)	
CC	17 (31)	37 (71)	4.5 (1.0-21)	0.001†	34 (72)	8.0 (1.3-50)	0.0002†	71 (72)	4.9 (1.3-18.1)	0.0001†
C2:rs9332739 (E318D)										
GG	48 (84)	52 (100)	1.0		44 (88)	1.0		96 (94)	1.0	
CG/CC	9 (16)	0 (0)	0‡	0.048§	6 (12)	0.6 (0.2-1.8)	0.32	8 (6)	0.3 (0.1-0.80)	0.02
LOC387715: rs10490924 (A69S)										
GG	33 (58)	20 (38)	1.0		15 (31)	1.0		35 (35)	1.0	
GT/TT	24 (42)	53 (62)	2.2 (1.0-4.7)	0.05	33 (69)	3.7 (1.6-8.9)	0.003	66 (65)	2.8 (1.4-5.8)	0.004
C3:rs2230199 (R102H)				0.02†			0.13†			0.02†
CC	36 (62)	21 (42)	1.0		23 (49)	1.0		44 (45)	1.0	
CG	19 (33)	22 (44)	2.4 (1.0-5.6)		19 (40)	1.7 (0.7-4.1)		41 (42)	2.1 (1.0-4.3)	
GG	3 (5)	7 (14)	4.2 (0.9-19.0)		5 (11)	2.6 (0.5-12.9)		12 (12)	3.4 (0.8-13.4)	
CFH:rs10033900				0.23†			0.09†			0.08†
CC	20 (36)	19 (36)	1.0		10 (20)	1.0		29 (28)	1.0	
CT	28 (51)	20 (38)	0.8 (0.3-2.0)		30 (61)	2.5 (0.9-6.6)		50 (49)	1.4 (0.6-3.0)	
TT	7 (13)	14 (26)	2.3 (0.7-7.3)		9 (18)	2.7 (0.7-9.9)		23 (23)	2.6 (0.9-7.3)	

Frequency counts for some genotypes and pack years may not add up to the total sample size due to missing information for some subjects.

*
ORs reflect comparisons between GA vs. controls, NV vs. controls, and total AMD (GA and NV vs. controls). ORs are adjusted for age (60-79, 80+) and sex.

† P (trend).

‡ORs not calculated due to 0 events in CG/CC group.

§P calculated with Fisher's exact test.

Table 2

Levels of Plasma Complement Components/Fragments in the Control and Maculopathy Groups

Complement Components/ Fragments	Control (*n* = 60) Median (10th, 90th Percentile)	GA (*n* = 58) Median (10th, 90th Percentile)	*P*[†]	NV (*n* = 62) Median (10th, 90th Percentile)	*P*[†]	Total Advanced AMD* (*n* = 120) Median (10th, 90th Percentile)	*P*[†]
Bb, μg/mL	0.8 (0.5–1.4)	1.0 (0.6–2.0)	0.03	0.8 (0.5–1.4)	0.30	0.9 (0.6–1.4)	0.06
C3, mg/dL	110 (86–155)	113 (82–154)	0.89	115 (86–155)	0.85	114 (83–155)	0.97
C3a, ng/mL	1498 (768–2154)	1567 (959–2899)	0.03	1647 (704–2613)	0.22	1594 (758–2739)	0.05
C5a, ng/mL	14 (8–20)	17 (9–21)	0.02	16 (9–24)	0.09	16 (9–23)	0.02
CFB, μg/mL	228 (183–311)	249 (182–352)	0.21	251 (189–341)	0.19	251 (185–352)	0.14
iC3b, ng/mL	10 (5–23)	11 (5–29)	0.42	10 (6–25)	0.97	10 (5–26)	0.63
SC5b-9, ng/mL	403 (176–624)	332 (164–716)	0.50	335 (188–615)	0.26	333 (184–652)	0.29
CFI, μg/mL	40 (27–84)	40 (28–56)	0.66	43 (22–57)	0.97	42 (23–56)	0.82
CFH, μg/mL	312 (253–420)	289 (231–417)	0.008	295 (246–389)	0.06	294 (238–392)	0.009
Factor D, μg/mL	3.2 (2.3–4.6)	3.4 (2.6–4.7)	0.42	3.5 (2.6–4.6)	0.25	3.4 (2.6–4.6)	0.25

*
Patients with AMD who had GA or NV disease in at least one eye.

[†]
P by Wilcoxon rank sum test. All reflect comparison between the maculopathy and the control groups.

Table 3

Association between Plasma Complement Components/Fragments and Advanced AMD, with and without Adjustment for Genotypes

Plasma Complement Component/Fragment	Model A without Genotype[*]		Model B with Genotype[†]	
	OR (95% CI)[‡]	P (trend)	OR (95% CI)[‡]	P (trend)
Bb	3.3 (1.3-8.6)	0.01	5.4 (1.3-22.9)	0.02
C3	0.8 (0.3-2.1)	0.65	1.5 (0.4-6.2)	0.70
C3a	2.1 (0.8-5.6)	0.28	2.2 (0.5-9.4)	0.75
C5a	3.6 (1.2-10.3)	0.01	25.2 (3.7-171.7)	0.0003
CFB	1.6 (0.6-4.2)	0.18	3.3 (0.8-12.7)	0.08
iC3b	0.8 (0.3-2.1)	0.79	0.7 (0.2-2.7)	0.81
SC5b-9	0.7 (0.3-1.8)	0.12	0.8 (0.2-2.6)	0.29
CFI	0.9 (0.3-2.3)	0.90	0.9 (0.2-3.4)	0.92
CFH	0.3 (0.10-0.75)	0.01	0.6 (0.1-2.6)	0.50
Factor D	1.4 (0.5-3.7)	0.63	3.1 (0.7-12.6)	0.21

[*] Adjusted for age (60-79, 80+), sex, smoking (ever smoked vs. never smoked), body mass index (<25, 25-29.9, >29.9).

[†] Adjusted for all variables in A plus CFB (CC, CT/TT), CFH Y402H (TT, CT, CC), CFHrs1410966 (TT, CT, CC), C2 (GG, CG/CC), LOC387715 (GG, GT, TT), C3 (CC, CG, GG), CFI (CC, CT, TT).

[‡] Comparison between cases and controls in the 4th quartile vs. 1st quartile of plasma complement factor.

Table 4

Evaluation of Change in Genotype-AMD Associations with Addition of Plasma Complement Components/Fragments[*]

| | | | Plasma Complement Components/Fragments[†] | | | | | |
| | No Plasma Complement Factors | | Bb | | C5a | | CFH | |
Genotype	OR (95% CI)	*P* (trend)	OR (95% CI)	*P* (trend)	OR (95% CI)	*P* (trend)	OR (95% CI)	*P* (trend)
CFB:rs641153 (R32Q)								
CC	1.0		1.0		1.0		1.0	
CT/TT	0.26 (0.08-0.80)	0.02	0.28 (0.09-0.91)	0.03	0.24 (0.08-0.74)	0.01	0.25 (0.08-0.81)	0.02
CFH:rs1061170 (Y402H)								
TT	1.0		1.0		1.0		1.0	
CT	3.7 (1.6-8.7)		4.2 (1.8-10.0)		4.3 (1.7-10.6)		3.6 (1.5-8.4)	
CC	6.0 (2.3-15.8)	0.0002	5.9 (2.2-15.7)	0.0002	7.9 (2.8-22.4)	<0.0001	6.6 (2.4-18.0)	0.0002
CFH:rs1410996								
TT	1.0		1.0		1.0		1.0	
CT	0.9 (0.2-3.7)		0.7 (0.2-3.1)		1.0 (0.24-4.6)		1.0 (0.2-3.6)	
CC	5.2 (1.2-21.4)	<0.0001	4.2 (1.0-18.0)	0.0002	7.4 (1.6-33.9)	<0.0001	4.9 (1.2-20.4)	<0.0001
C2:rs9332739 (E318D)								
GG	1.0		1.0		1.0		1.0	
CG/CC	0.20 (0.06-0.67)	0.008	0.23 (0.07-0.78)	0.02	0.19 (0.06-0.63)	0.007	0.22 (0.07-0.74)	0.01
LOC387715:rs10490924(A69S)								
GG	1.0		1.0		1.0		1.0	
GT/TT	2.6 (1.3-5.3)	0.007	2.5 (1.2-5.2)	0.01	3.6 (1.7-7.7)	0.001	2.6 (1.3-5.4)	0.009
C3:rs2230199 (R102H)								
CC	1.0		1.0		1.0		1.0	
CG	2.3 (1.1-4.9)		2.1 (0.96-4.6)		2.0 (0.90-4.4)		2.2 (1.0-4.8)	

	Plasma Complement Components/Fragments[†]							
	No Plasma Complement Factors		**Bb**		**C5a**		**CFH**	
Genotype	**OR (95% CI)**	**P (trend)**	**OR (95% CI)**	**P (trend)**	**OR (95% CI)**	**P (trend)**	**OR (95% CI)**	**P (trend)**
GG	3.8 (0.9-15.8)		3.1 (0.86-14.6)		3.5 (0.83-14.7)		5.2 (1.2-23.2)	
		0.01		0.02		0.03		0.008
CFI:rs10033900								
CC	1.0		1.0		1.0		1.0	
CT	1.6 (0.71-3.4)		1.6 (0.71-3.5)		1.8 (0.78-4.0)		1.5 (0.68-3.4)	
TT	2.6 (0.90-7.7)		2.5 (0.82-7.4)		3.9 (1.2-12.2)		2.1 (0.72-6.4)	
		0.07		0.10		0.02		0.15

[*] Adjusted for age (60-79, 80+), sex, smoking (ever smoked vs. never smoked), body mass index (<25, 25-29.9, >29.9)

[†] Comparison of all cases vs. controls.

[‡] Quartiles.

Table 5

Association between Plasma Complement Components/Fragments, Smoking, and Body Mass Index

Complement Components/ Fragments*	Smoking (Ever vs. Never)† Controls (n = 60)		Smoking (Ever vs. Never)‡ All Subjects (n = 180)			BMI§ Controls (n = 60)		BMI‖ All Subjects (n = 180)		
	Beta ± SE	P¶	Beta ± SE	P¶	P Interaction	Beta ± SE	P¶	Beta ± SE	P¶	P Interaction
Bb	0.05 ± 0.11	(0.65)	0.02 ± 0.10	(0.15)	0.46	−0.02 ± 0.11	(0.83)	−0.11 ± 0.06	(0.08)	0.45
C3	−0.03 ± 0.06	(0.59)	0.05 ± 0.04	(0.18)	0.23	0.09 ± 0.06	(0.17)	0.10 ± 0.03	(0.005)	0.93
C3a	0.001 ± 0.10	(0.99)	0.06 ± 0.07	(0.40)	0.75	0.25 ± 0.09	(0.01)	0.09 ± 0.07	(0.17)	0.07
C5a	−0.03 ± 0.09	(0.76)	0.02 ± 0.06	(0.77)	0.54	0.04 ± 0.09	(0.69)	−0.03 ± 0.06	(0.62)	0.46
CFB	0.05 ± 0.07	(0.51)	−0.006 ± 0.04	(0.88)	0.17	0.13 ± 0.07	(0.06)	0.12 ± 0.04	(0.001)	0.54
iC3b	−0.13 ± 0.16	(0.41)	−0.12 ± 0.09	(0.17)	0.96	0.31 ± 0.16	(0.05)	0.10 ± 0.09	(0.26)	0.11
SC5b-9	−0.15 ± 0.13	(0.24)	−0.12 ± 0.07	(0.17)	0.55	0.11 ± 0.13	(0.39)	−0.05 ± 0.08	(0.56)	0.41
CFI	−0.03 ± 0.09	(0.73)	−0.005 ± 0.05	(0.92)	0.70	0.01 ± 0.09	(0.89)	0.04 ± 0.05	(0.47)	0.82
CFH	−0.07 ± 0.05	(0.18)	−0.02 ± 0.03	(0.60)	0.28	0.12 ± 0.05	(0.03)	0.12 ± 0.03	(0.0006)	0.95
Factor D	0.09 ± 0.08	(0.25)	0.06 ± 0.04	(0.13)	0.50	0.08 ± 0.08	(0.34)	0.03 ± 0.04	(0.44)	0.38

P interaction is the interaction between smoking or BMI and AMD status.

*
Log values.

†
Adjusted for age and sex.

‡
Adjusted for age, sex, and AMD status (total AMD, controls).

§
BMI categories: <25, 25+. Adjusted for age and sex.

‖
BMI categories: <25, 25+. Adjusted for age, sex, and AMD status (total AMD, controls).

¶
P for controls only was calculated by multiple regression of log complement component/fragment on age 80+ vs. <80 and sex. P for all subjects was calculated the same as for the controls, and in addition was adjusted for AMD status.

Table 6

Associations between Complement Components/Fragments[*] and Genotype among All Subjects

Genotype	Bb		C3a		C5a		CFH	
	LS Mean ± SE[†]	P	LS Mean ± SE[†]	P	LS Mean ± SE[†]	P	LS Mean ± SE[†]	P
CFB:rs641153 (R32Q)								
CC	−0.28 ± 0.08		7.41 ± 0.09		2.79 ± 0.08		5.79 ± 0.06	
CT/TT	−0.33 ± 0.12	0.57	7.20 ± 0.14	0.26	2.78 ± 0.12	0.96	5.80 ± 0.07	0.80
CFH:rs1061170 (Y402H)								
TT	−0.23 ± 0.10		7.34 ± 0.12		2.83 ± 0.10		5.78 ± 0.06	
CT	−0.40 ± 0.11		7.32 ± 0.12		2.77 ± 0.10		5.78 ± 0.06	
CC	−0.29 ± 0.11	0.47[†‡]	7.24 ± 0.13	0.43[†‡]	2.75 ± 0.11	0.51[†‡]	5.82 ± 0.06	0.52[†‡]
CFH:rs1410996								
TT	−0.51 ± 10.15		7.40 ± 0.17		2.86 ± 0.14		5.76 ± 0.08	
CT	−0.22 ± 0.10		7.22 ± 0.11		2.76 ± 0.10		5.81 ± 0.06	
CC	−0.19 ± 0.10	0.16[†‡]	7.29 ± 0.11	0.92[†‡]	2.73 ± 0.10	0.45[†‡]	5.81 ± 0.05	0.77[†‡]
C2:rs9332739 (E318D)								
GG	−0.20 ± 0.07		7.22 ± 0.08		2.70 ± 0.07		5.74 ± 0.04	
CG/CC	−0.42 ± 0.14	0.13	7.39 ± 0.16	0.21	2.87 ± 0.14	0.16	5.85 ± 0.08	0.18
LOC387715:rs10490924(A69S)								
GG	−0.35 ± 0.10		7.33 ± 0.11		2.87 ± 0.09		5.77 ± 0.05	
GT/TT	−0.27 ± 0.09	0.40	7.27 ± 0.11	0.49	2.70 ± 0.09	0.02	5.81 ± 0.05	0.30
C3:rs2230199 (R102H)								
CC	−0.33 ± 0.09		7.20 ± 0.10		2.69 ± 0.09		5.75 ± 0.05	
CG	−0.26 ± 0.09		7.47 ± 0.10		2.82 ± 0.09		5.71 ± 0.05	
GG	−0.34 ± 0.14	0.58[†‡]	7.24 ± 0.16	0.07[†‡]	2.84 ± 0.13	0.04[†‡]	5.91 ± 0.08	0.15[†‡]
CFI:rs10033900								
CC	−0.32 ± 0.10		7.39 ± 0.12		2.82 ± 0.10		5.80 ± 0.06	
CT	−0.30 ± 0.09		7.30 ± 0.10		2.82 ± 0.09		5.80 ± 0.05	

Genotype	Bb		C3a		C5a		CFH	
	LS Mean ± SE[†]	P	LS Mean ± SE[†]	P	LS Mean ± SE[†]	P	LS Mean ± SE[†]	P
TT	−0.30 ± 0.11	0.74[‡‡]	7.22 ± 0.13	0.07[‡‡]	2.72 ± 0.11	0.29[‡]	5.77 ± 0.06	0.65[‡]

[*] Log values.

[†] Least squares mean ± SE, adjusted for age, sex, AMD status, CFB (CC, CT, CT/TT), CFH Y402H (TT, CT, CC), CFH:rs1410966 (TT, CT, CC), C2 (GG, CG/CC), LOC387715 (GG, GT, TT), C3 (CC, CG, GG), CFI (CC, CT, TT).

[‡] P for trend.

Table 7

C Statistics for Age-Related Macular Degeneration Based on Models with Demographic, Behavioral, Genetic Factors, and C5a, Bb, and C3a

Model	Variables	C-statistic ±SD
1	Age, sex, smoking, BMI	0.612 ± 0.050
2	Age, sex, smoking, BMI, CFB:rs641153 (R32Q), CFH:rs1061170 (Y402H), CFH:rs1410996, C2:rs9332739 (E318D), LOC387715:rs10490924 (A69S), C3:rs2230199 (R102H), CFI:rs10033900	0.832 ± 0.036
3	Age, sex, smoking, BMI, CFB:rs641153 (R32Q), CFH:rs1061170 (Y402H), CFH:rs1410996, C2:rs9332739 (E318D), LOC387715:rs10490924 (A69S), C3:rs2230199 (R102H), CFI:rs10033900, C3a fragment	0.844 ± 0.035
4	Age, sex, smoking, BMI, CFB:rs641153 (R32Q), CFH:rs1061170 (Y402H), CFH:rs1410996, C2:rs9332739 (E318D), LOC387715:rs10490924 (A69S), C3:rs2230199 (R102H), CFI:rs10033900, Bb fragment	0.870 ± 0.031
5	Age, sex, smoking, BMI, CFB:rs641153 (R32Q), CFH:rs1061170 (Y402H), CFH:rs1410996, C2:rs9332739 (E318D), LOC387715:rs10490924 (A69S), C3:rs2230199 (R102H), CR:rs10033900, C5a fragment	0.895 ± 0.028
6	Age, sex, smoking, BMI, CFB:rs641153 (R32Q), CFH:rs1061170 (Y402H), CFH:rs1410996, C2:rs9332739 (E318D), LOC387715:rs10490924 (A69S), C3:rs2230199 (R102H), CFI:rs10033900, C3a, Bb, C5a fragments	0.944 ± 0.020

*
P (model 1 vs. 2, P = 0.001; 2 vs. 3 = 0.63; 2 vs. 4 = 0.043; 2 vs. 5 = 0.029; 2 vs. 6 = <0.001).