Complement Component 3 (*C3*) Haplotypes and Risk of Advanced Age-Related Macular Degeneration

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Purpose. Nonsynonymous coding single nucleotide polymorphisms (nsSNPs) in complement component 3 (*C3*) alter the risk of age-related macular degeneration (AMD). This was a study of the effect of haplotypes across *C3* on AMD risk.

Methods. Nine SNPs tagging haplotypes across *C3* were genotyped on 738 subjects at the Mayo Clinic. Haplotype analyses were performed with and without conditioning on individual SNPs. Replication studies were performed using 1541 subjects from the age-related eye disease study (AREDS).

Results. Two nsSNPs located 5125 bp apart in the 5′ end of *C3* showed the highest association (rs1047286 or P314L, P = 9.2E-05; rs2230199 or R102G, P = 4.1E-05) with AMD. The minor alleles of both SNPs tagged a single risk haplotype. The effects of the two nsSNPs could not be distinguished due to high linkage disequilibrium. The risk SNPs preferentially promoted the development of advanced AMD relative to early AMD in both the Mayo and AREDS subjects. Haplotypes in the 3′ end of the *C3* locus were associated with AMD in both the Mayo and AREDS subjects. The effect persisted after conditioning on the nsSNPs only in the Mayo subjects. No interaction was found between rs2230199 and smoking or other AMD loci.

Conclusions. nsSNPs in *C3* increased the risk of developing AMD 1.8-fold for 1 risk allele or 2.4-fold for two risk alleles and were preferentially associated with advanced AMD. Further study is needed to determine whether haplotypes in the 3′ end of C3 have an independent association with AMD. (*Invest Ophthalmol Vis Sci.* 2009;50:3386–3393) DOI:10.1167/iovs.08-3231

Age-related macular degeneration (AMD) is a complex trait influenced by aging, genetic risks, and environmental factors such as smoking and diet.[1] By 2020, three million people in the United States are expected to have advanced AMD, which often leads to severe vision loss.[2] Genetic variation altering the risk of AMD is established for the regulation of complement activation (RCA) locus, including the Y402H variation in complement factor H (*CFH*),[3–6] the complement component 2/complement factor B (*C2/CFB*) locus,[7] and a segment of DNA called the age-related maculopathy susceptibility 2 (*ARMS2*) locus that contains a hypothetical gene known as *LOC387715* and the promoter and beginning of the high temperature requirement A1 (*HTRA1*) gene (hereafter referred to as *LOC387715/HTRA1*).[8–11] Complement factor H (CFH) and factor B (FB) regulate the alternative pathway of complement activation, whereas C2 is involved in the classic pathway. The biological role of *LOC387715* and *HTRA1* in the retina are unknown. In combination with the presence of innate immunity proteins in drusen and other subretinal deposits of AMD,[12,13] the genetic variants in the alternative complement pathway provide strong support for abnormal regulation of the innate immune system in AMD.

Recently, two research groups reported variation in the complement component 3 (*C3*) locus that increased the risk of AMD.[14,15] This observation has been replicated by others.[16,17] Two nonsynonymous coding (ns)SNPs near each other were highly associated with AMD in the original studies.[14,15] Conditional analyses using two of the datasets suggested that rs2230199 (R102G) better explained the risk for AMD than did rs1047286 (P314L).[15,16] No additional effect of haplotypes in the region defined by these two SNPs near the 5′ end of *C3* was observed, suggesting that the nsSNPs explained the risk. In the present study, we performed a detailed study of common haplotypes across the *C3* locus to determine whether other variations could alter the risk of AMD. We also re-evaluated the relative contribution of R102G and P314L on AMD risk in an attempt to confirm previous observations. We observed that haplotypes in the 3′ region of *C3* are associated with AMD and that the observation cannot be completely explained by the two nsSNPs (P314L or R102G). Further, we observed that conditional analyses could not separate the effects of R102G and P314L in our subjects, suggesting that functional studies are needed to separate the effect of these two nsSNPs. We also report the relative effect of the nsSNPs on subtypes of AMD.

Methods

Subjects

The study adhered to the tenets of the Declaration of Helsinki and was approved by the institutional review board of the Mayo Clinic. Written informed consent was obtained from all subjects. The Mayo subjects were composed of 738 Caucasian individuals (439 AMD cases and 299 controls without AMD). Diagnosis was determined by review of fundus photographs, as described previously.[3,18,19] Briefly, all subjects diagnosed with AMD had large drusen with sufficient drusen area to fill a 700-μm circle or more advanced findings. Controls had five or fewer hard drusen without pigment changes or more advanced findings. Geographic atrophy and exudation were defined with the Wisconsin age-related maculopathy grading system.[20]

Replication studies were performed on 1541 Caucasian subjects (1241 with AMD and 300 controls without AMD) from the AREDS study genetic repository that were graded as previously reported.[21,22] The age, sex, and AMD subtype distributions for both subject groups are provided in Table 1.

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TABLE 1. Demographic Features of the Mayo and AREDS Subjects

	Mayo Subjects			AREDS Subjects		
	n	Age (Mean ± SD)	Male/Female Ratio	*n*	Age (Mean ± SD)	Male/Female Ratio
Control subjects	299	69.5 ± 8.3	0.87	300	77.6 ± 4.3	0.79
AMD subjects*	439	77.2 ± 9.2	0.56	1241	79.9 ± 5.1	0.68
Early AMD	199	74.5 ± 9.8	0.47	583	79.0 ± 4.9	0.65
Exudation	153	79.2 ± 8.8	0.59	324	80.6 ± 5.0	0.76
Geographic atrophy†	87	79.9 ± 6.6	0.71	247	80.8 ± 5.3	0.65

* An additional 87 AREDS subjects with the "questionable advanced AMD" grade were utilized in studies including all types of advanced AMD, but are not included in this table.

† 25 Mayo subjects and 85 AREDS subjects who had both geographic atrophy and exudative AMD (category "Both") were included in the geographic atrophy group of this table.

Selection of Tag and Functional SNPs

Genotyping scores (Illumina, San Diego, CA) were obtained for SNPs in *C3* and those with a score greater than 0.6 were included for further analyses. After merging the files containing the scores with a list of candidate tag-SNPs generated by using ldSelect,[23] an algorithm for tag-SNP selection was developed so that a single-tag SNP would be selected for each linkage disequilibrium (LD) bin. Only those SNPs deemed candidate tag-SNPs by ldSelect with a score greater than or equal to 0.60 and an MAF of greater than or equal to 0.05 (to provide adequate power for statistical analysis) were considered for further selection. Tag-SNPs were ultimately selected based on a functional ranking system wherein nonsynonymous coding SNPs were preferentially selected among the tag-SNP candidates in each LD bin, followed by synonymous coding SNPs, SNPs from 5′ untranslated regions (UTRs), SNPs from 3′ UTRs, SNPs from 5′ flanking UTRs, SNPs from 3′ flanking UTRs, and finally SNPs from intronic regions. If an LD bin contained more than one candidate tag SNP with the same highest functional ranking, the SNP with the highest MAF was selected as the tag for that bin. Seven SNPs were needed to tag the common *C3* haplotypes and were selected for genotyping. Two additional nonsynonymous SNPs (rs1047286; P314L and rs2230199; R102G) in *C3* with scores greater than or equal to 0.60 and MAFs greater than or equal to 0.05 were added to the list of tag-SNPs, because they might alter protein function and have been studied previously.[14–17]

Genotyping

The nine SNPs (seven tag SNPs and two nsSNPs) were genotyped by using genomic DNA extracted from peripheral blood leukocytes. A commercial genotype assay was used at Mayo Clinic according to the manufacturer's protocol (GoldenGate; Illumina). The 384 SNP option was used to achieve sample conservation and cost reduction across several projects. Genotype calls were made using the genotyping module of the software (BeadStudio 3; Illumina). Genotype clusters were reviewed by using the replicate and heritability information of 16 control CEPH trios to refine clustering. Initial laboratory quality assurance relied on the genotype (GenCall; Illumina) score, a quality metric indicating the reliability of called genotypes that is generated by the software. The 10th percentile genotype score in a particular distribution of scores is referred to as GenCall_10. For loci, it represents the 10th percentile rank for all scores for that locus. Samples with GenCall_10 scores below 0.4 and/or call rates below 95% and SNPs with call rates below 95% were failed. Quality control for genotype call was assessed by concordance for the control CEPH trio DNA replicates and the sample replicates within each plate (two per 96-well plate).

Replication Studies

For the replication of the AMD subtype and haplotype effects, we genotyped the 3′ haplotype SNPs (rs11569536, rs344542, and rs3745565) and the nsSNPs (rs1047286 and rs2230199) on the AREDS subjects (Taqman assays; Applied Biosystems, Foster City, CA).

Statistics

On receipt of genotype intensities and processing, the SNPs were evaluated for Hardy-Weinberg equilibrium and all were in equilibrium ($P \geq 0.05$). Single SNP analyses on genotype distributions, were performed in R statistical software (www.r-project.org) by using logistic regression assuming a log-additive genetic model where the SNPs were coded as 0, 1, or 2 for the number of minor alleles and the corresponding *P*-values were calculated based on the score test statistics. Fisher's exact tests were also performed on genotype distributions. Haplotype analyses were completed using the score test with a three-SNP-sliding-window approach, as implemented in haplo.stats.[24] Additional haplotype analyses were performed with the two nsSNPs (rs1047286 and rs2230199). The interaction effect between C3 SNPs and smoking status on AMD status was evaluated in a log-additive model with logistic regression, using two main-effect terms (genotype for each SNP and smoking status) and their interaction term, and was assessed with a likelihood ratio test. To investigate the effect of the 5′ nsSNP (rs2230199) on AMD subtypes, a baseline-category logit model was used, considering early AMD as the baseline. Odds ratios (ORs) are reported with 95% confidence intervals (CIs). Haplotype analyses for AMD subtypes were also performed using a likelihood ratio test to explore for an effect of 3′ haplotypes (rs11569536-rs344542-rs3745565) on the three subtypes. Both age and sex were different between all AMD cases and controls ($P < 2E\text{-}16$ for age and $P = 0.004$ for sex). However, neither of them had any significant effect on single SNP analyses, and each SNP effect on disease status was not changed in either the direction of association or statistical significance after adjustment for age and/or sex. Thus, analyses were not corrected for age or sex. Nominal *P*-values are reported.

RESULTS

Genotyping of SNPs across *C3*

Among the nine SNPs studied across the *C3* locus (Table 2), the highest association with AMD was with the two nsSNPs rs1047286 ($P = 9.2E\text{-}05$) and rs2230199 ($P = 4.1E\text{-}05$). Two other SNPs including rs11569536 ($P = 0.009$) and rs3745565 ($P = 0.014$) were nominally associated with AMD. The genotype distributions of these four SNPs (Table 3) were consistent with an additive effect of each allele and the minor allele conferring AMD risk except for rs3745565 where the minor allele was protective. After conditioning on rs2230199 or rs1047286, no SNP showed significant association with AMD, suggesting that either one of these SNPs explained the association with AMD (Table 2). The effect of the two nsSNPs could

TABLE 2. Association between AMD and Nine SNPs across the *C3* Locus in the Mayo Subjects before and after Conditioning on rs1047286 or rs2230199

SNP	Function	Location	Major/Minor Allele	Minor Allele Frequency	Hardy-Weinberg Equilibrium *P* in controls	Fisher *P*	Log-Additive *P* Conditioned on rs1047286	Log-Additive *P* Conditioned on rs2230199
rs11569536	Intron 29	6637089	G/A	0.07	0.36	0.02	0.102	0.144
rs344542	Intron 27	6638517	A/G	0.38	0.79	0.99	0.763	0.663
rs3745565	Intron 26	6641982	C/G	0.11	0.10	0.04	0.070	0.085
rs423490	Exon 21 (A915A)	6648406	G/A	0.23	0.38	0.29	0.200	0.231
rs428453	Exon 19 (V807V)	6653157	C/G	0.35	0.42	0.72	0.609	0.632
rs2230205	Exon 14 (T612T)	6660704	G/A	0.12	0.70	0.48	0.789	0.842
rs1047286	Exon 9 (P314L)	6664262	G/A	0.23	0.05	2.8E-04	—	0.889
rs2230199	Exon 3 (R102G)	6669387	C/G	0.24	0.10	1.3E-04	0.163	—
rs339392	5′ UTR	6673022	A/C	0.20	0.22	0.14	0.801	0.863

Log-additive *P*-values for each SNP are shown in Table 3.

not be distinguished with conditional analysis because of high LD as discussed in the next section.

Linkage Disequilibrium

To define the genomic regions of association, we investigated the LD among the nine SNPs with Haploview.[25] As would be expected based on our selection of tag-SNPs, moderate to minimal LD was observed across most of *C3*. Strong LD was found between rs1047286 and rs2230199 (r^2 = 0.85, Fig. 1), consistent with their similar association with AMD (Table 2). All SNPs in Table 2 except rs1047286 were available in HapMap.[26] The LD across *C3* in HapMap subjects was similar to the pattern observed in the Mayo subjects for the tag-SNPs (Fig. 1). Examination of the 102 SNPs across the *C3* locus genotyped in the HapMap project identified only one SNP in high LD with rs2230199, a synonymous coding variation at amino acid 518 (rs2230203, r^2 = 0.7). Thus, the LD pattern across *C3* suggests that the genetic variation explaining the association with AMD arose primarily in the vicinity of rs1047286 and rs2230199,

TABLE 3. Genotype Distributions of the Four SNPs in the *C3* Gene Significantly Associated with AMD in the Mayo Subjects and SNPs Studied in Both the Mayo and AREDS Subjects

SNP	Subject Group	Genotype	AMD Cases *n* (%)	Controls *n* (%)	Log-Additive *P*
rs11569536	Mayo	GG	365 (84)	268 (90)	0.009
		GA	69 (16)	30 (10)	
		AA	3 (1)	0 (0)	
	AREDS	GG	1030 (85)	264 (90)	0.023
		GA	174 (14)	26 (9)	
		AA	9 (1)	2 (1)	
rs344542	Mayo	AA	172 (40)	115 (39)	0.891
		AG	198 (46)	136 (46)	
		GG	63 (15)	43 (15)	
	AREDS	AA	489 (40)	120 (41)	0.614
		AG	568 (46)	124 (42)	
		GG	170 (14)	50 (17)	
rs3745565	Mayo	CC	361 (82)	226 (76)	0.014
		CG	73 (17)	64 (21)	
		GG	5 (1)	9 (3)	
	AREDS	CC	956 (78)	221 (75)	0.220
		CG	256 (21)	66 (23)	
		GG	13 (1)	6 (2)	
rs1047286	Mayo	GG	240 (55)	208 (70)	9.2E-05
		GA	159 (37)	76 (26)	
		AA	36 (8)	14 (5)	
	AREDS	GG	682 (56)	199 (68)	1.54E-04
		GA	471 (39)	84 (29)	
		AA	60 (5)	8 (3)	
rs2230199	Mayo	CC	233 (53)	204 (68)	4.1E-05
		CG	167 (38)	80 (27)	
		GG	38 (9)	14 (5)	
	AREDS	CC	668 (55)	196 (66)	1.7E-04
		CG	477 (39)	92 (31)	
		GG	71 (6)	8 (3)	

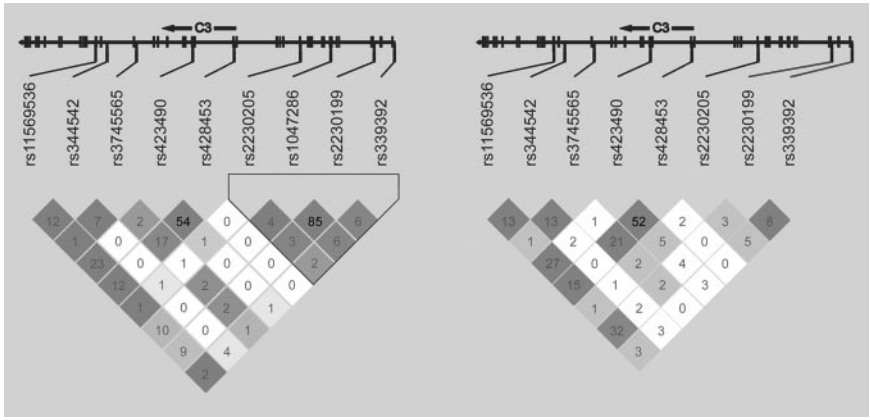

FIGURE 1. LD plots of nine tag SNPs and two nsSNP in the Mayo (*left*) and HapMap subjects (*right*). Strong LD was observed only between rs1047286 and rs2230199 in the Mayo subjects (r^2 = 0.85). Other SNPs showed moderate to minimal association in both the Mayo and HapMap subjects as expected from the study design. Numbers in the squares represent r^2 estimates, whereas the gray scale represents D′ estimates from low (*white*) to high (*dark*).

between rs2230205 and rs339392, a maximum distance of 12 kb (Table 2; Fig. 1, triangle).

Haplotype Analysis

Because rs1047286 and rs2230199 were strongly associated with AMD, haplotype analysis of these two SNPs was performed. Haplotypes of the two SNPs showed significant association with AMD (global simulation P = 7.0E-04; Table 4). However, this global haplotype effect was abolished after conditioning on rs1047286 or rs2230199 (global simulation P = 0.569 and P = 1.0, respectively). Thus, the association with AMD in the 5′ region of *C3* appears to be driven primarily by risk haplotypes containing the minor alleles for both rs2230199 and rs1047286 (Table 4).

Haplotypes containing only one common allele of either SNP were rare, and there was no difference in the major/minor allele haplotypes (GG and AC haplotypes, Table 4). Thus, two-SNP haplotypes could not separate the effects of the two nsSNPs on AMD risk.

Because of the limited LD between the tag-SNPs studied across *C3* and the association observed in the 3′ region of *C3*, we sought to determine whether other haplotypes in *C3* were associated with AMD. We used a three-SNP sliding window haplotype analysis with the nine SNPs across *C3* locus. In addition to haplotypes containing the two nsSNPs, haplotypes from the three SNPs (rs11569536, rs344542, and rs3745565) in the 3′ end of *C3* showed significant association with AMD (global simulation P = 0.001, Table 5). The global haplotype effect was maintained after conditioning on rs2230199 (global simulation P = 0.05) and on rs1047286 (global simulation P = 0.05). An individual haplotype of the combination (A-G-C) increased the risk of AMD (P = 0.007). Because the effect of the 3′ haplotypes persisted after conditioning on either rs2230199 or rs1047286, a contribution from variants in the 3′ region of *C3* gene on the risk of AMD cannot be excluded in the Mayo subjects.

Replication Study

To replicate the association between these 3′ haplotypes and AMD, we genotyped the first three SNPs of the 3′ end of *C3* and the two nsSNPs on 1541 Caucasian AREDS samples and performed genotype and haplotype analysis (Tables 3, 5). The replication study showed a trend for a haplotype effect of the first three SNPs on AMD risk (global simulation P = 0.06; Table 5). After conditioning on rs2230199 or rs1047286, the haplotype effect was not statistically significant (global simulation P = 0.39 and P = 0.43, respectively). Although the direction and magnitude of the effect was similar between the Mayo and AREDS subjects (Table 5), the haplotype effects did not reach statistical significance in the AREDS cohort and thus were not replicated.

Subtype Analysis

Subjects with AMD were stratified by subtype (early, geographic atrophy, exudative) and the analyses with the 5′ nsSNP (rs2230199) and the 3′ haplotype (rs11569536, rs344542, and rs3745565) was repeated (Table 6). Using early AMD as the reference, likelihood ratio testing of the 3 × 3 table (three subtypes × the three genotypes) showed a trend for a difference between the three AMD types (P = 0.12), with the trend driven by a nominally significant difference between early AMD and geographic atrophy (OR = 1.49, 95% CI = 1.02–2.18). Analysis of the AREDS subjects (Table 6) showed a significant difference between the three AMD subtypes (P = 0.009) and increased risk for both geographic atrophy (OR = 1.30, 95% CI = 1.01–1.66) and exudative AMD (OR = 1.39, 95% CI = 1.11–1.75) compared with early AMD. The data of the Mayo and AREDS subjects were pooled and the increased risk of the nsSNPs for advanced AMD became more apparent with the larger sample size (geographic atrophy: OR = 1.35, 95% CI = 1.10–1.67; and exudative AMD: OR = 1.29, 95% CI = 1.12–1.55). These results are illustrated in Fig. 2 and

TABLE 4. Haplotype Analysis of the Two nsSNPs at the 5′ End of the *C3* Locus for AMD

Haplotype		Haplotype Frequency			
rs1047286	**rs2230199**	**AMD case**	**Control**	**OR (95% CI)**	***P***
G	C	0.714	0.809	Reference	<1.0E-5
A	G	0.258	0.167	1.69 (1.31–2.19)	5.0E-5
G	G	0.019	0.014	1.55 (0.65–3.70)	0.443
A	C	0.009	0.010	1.00 (0.34–2.93)	0.999

Global haplotype *P*-values were 7.0E-4 before, 1.0 after adjusting for rs2230199 and 0.569 after adjusting for rs1047286.

TABLE 5. Three SNP Haplotypes in the 3′ End of *C3* before and after Conditioning on rs2230199 in the Mayo and AREDS Subjects

Subjects	Observed Haplotypes	rs11569536	rs344542	rs3745565	Haplotype Frequency		OR (95% CI)	Simulation *P*	Simulation *P* Conditioned on rs2230199
					AMD Case	Control			
Mayo*	1	G	A	G	0.095	0.137	0.65 (0.47–0.91)	0.012	0.061
	2	G	G	C	0.287	0.330	0.81 (0.64–1.02)	0.108	0.210
	3	G	A	C	0.532	0.482	Reference	0.073	0.118
	4	A	G	C	0.086	0.048	1.61 (1.01–2.57)	0.007	0.093
AREDS†	1	G	A	G	0.115	0.133	0.81 (0.61–1.07)	0.214	0.493
	2	G	G	C	0.292	0.330	0.83 (0.68–1.02)	0.073	0.180
	3	G	A	C	0.513	0.485	Reference	0.222	0.233
	4	A	G	C	0.078	0.051	1.44 (0.96–2.16)	0.025	0.334

* Global haplotype *P*-values were 0.001 before, 0.05 after adjusting for rs2230199 and 0.05 after adjusting for rs1047286.
† Global haplotype *P*-values were 0.06 before, 0.391 after adjusting for rs2230199 and 0.427 after adjusting for rs1047286.

demonstrate that the nsSNPs preferentially increased the risk of advanced AMD compared with early AMD at baseline.

Given the increased effect on advanced AMD subtypes compared with early AMD, we compared each AMD subtype to the control. The minor allele of rs2230199 increased the risk of early (OR = 1.5, 95% CI = 1.11–2.02), geographic atrophy (OR = 2.2, 95% CI = 1.52–3.19), exudative (OR = 1.7, 95% CI = 1.21–2.29), and advanced (both geographic atrophy or exudative; OR = 1.8, 95% CI = 1.40–2.45) disease in the Mayo subjects. The minor allele of rs2230199 increased the risk of early (OR = 1.3, 95% CI = 1.04–1.73), geographic atrophy (OR = 1.7, 95% CI = 1.29–2.33), exudative (OR = 1.9, 95% CI = 1.41–2.46), and advanced (OR = 1.8, 95% CI = 1.41–2.33) disease in the AREDS subjects. The minor allele of rs2230199 increased the risk of early disease (OR = 1.4, 95% CI = 1.15–1.69), geographic atrophy (OR = 1.9, 95% CI = 1.50–2.39), and exudative (OR = 1.8, 95% CI = 1.47–2.22) and advanced disease (OR = 1.8, 95% CI = 1.52–2.21) in the pooled Mayo/AREDS subjects. Plots illustrating the comparison of each AMD subtype to controls are also shown in Figure 2.

There was no haplotype effect of the three 3′ SNPs on subtypes of AMD ($P = 0.998$). We also examined haplotype effects of all nine SNPs with three SNP sliding-window approach on AMD subtypes and no significant effects were noted ($P = 0.55$–0.99).

Interaction Analysis and Comparison to Other Genetic Risks for AMD

We observed no interaction between smoking (ever, never) and AMD for the *C3* SNPs using logistic regression and a log-additive model ($P = 0.40$–0.78). We also found no interaction between the two nsSNPs in *C3* ($P = 0.452$–0.843 for rs1047286; $P = 0.542$–0.784 for rs2230199) and other major genetics risks for AMD including *CFH* (Y402H), *LOC387715/HTRA1* (tagging SNP A69S), and *C2/CFB* (*CFB*, L9H; *C2*, rs547154). The impact of *C3* R102G and P314L on AMD risk is compared with these other genetic risks for AMD in the Mayo subjects in Table 7.

DISCUSSION

Association between nsSNPs in *C3* (rs1047286 and rs2230199) and AMD has been established in multiple studies.[14–17] Most of the effect on AMD risk appears to localize to a maximum 12 kb region spanning these two nsSNPs. However, because of high LD and the low frequency of recombinant haplotypes, conditional SNP and haplotype analyses with rs1047286 and rs2230199 could not determine which nsSNP explains the effect on AMD risk (Tables 2, 4). Thus, our observations in the Mayo and AREDS subjects differed from studies of other subject groups that concluded that rs2230199 (R102G) explained the association with AMD.[14–16] Yates et al.[15] performed stepwise logistic regression analysis and showed significant association with AMD adding R102G in the P314L model ($P = 0.02$). However, this association was not observed adding P314L in the R102G model ($P = 0.90$). Spencer et al.[16] evaluated the effect of C3 R102G in C3 P314L carriers and vice versa. They also proposed that P314L ($P = 0.2$) was not, whereas R102G ($P = 0.01$) was significantly associated with AMD. Maller et al.[14] did not genotype rs1047286, but examined publically available resequencing data from (http://pga.gs.washington.edu) across this region of *C3* in 23 CEPH samples, and reported no other nsSNPs in high LD. Thus, although genetic studies do not allow for clear separation between the effects of these two nsSNPs on AMD risk, no other nearby variation is known that could explain the effect on AMD.

Unlike previous studies, we observed that haplotypes from the three SNPs (rs11569536, rs344542, and rs3745565) in the

TABLE 6. Differential Effect of nsSNP rs2230199 (R102G) on a Baseline–Category Logit Model of AMD Containing All Disease Subtypes

Subject Group	Genotype	Early AMD (%)	Geographic Atrophy (%)	Exudative AMD (%)	Geographic Atrophy vs. Early		Exudative AMD vs. Early	
					OR (95% CI)	*P*	OR (95% CI)	*P*
Mayo	CC	113 (57.1)	39 (44.8)	81 (52.9)	1.49 (1.02–2.18)	0.039	1.12 (0.80–1.55)	0.520
	CG	70 (35.4)	37 (42.5)	60 (39.2)				
	GG	15 (7.6)	11 (12.6)	12 (7.8)				
AREDS	CC	331 (58.2)	128 (52.9)	161 (50.5)	1.30 (1.01–1.66)	0.039	1.39 (1.11–1.75)	0.004
	CG	217 (38.1)	95 (39.3)	132 (41.4)				
	GG	21 (3.7)	19 (7.9)	26 (8.2)				

Overall *P* of rs2230199 with AMD subtype is 0.118 from Mayo subjects and 0.009 from AREDS subjects.

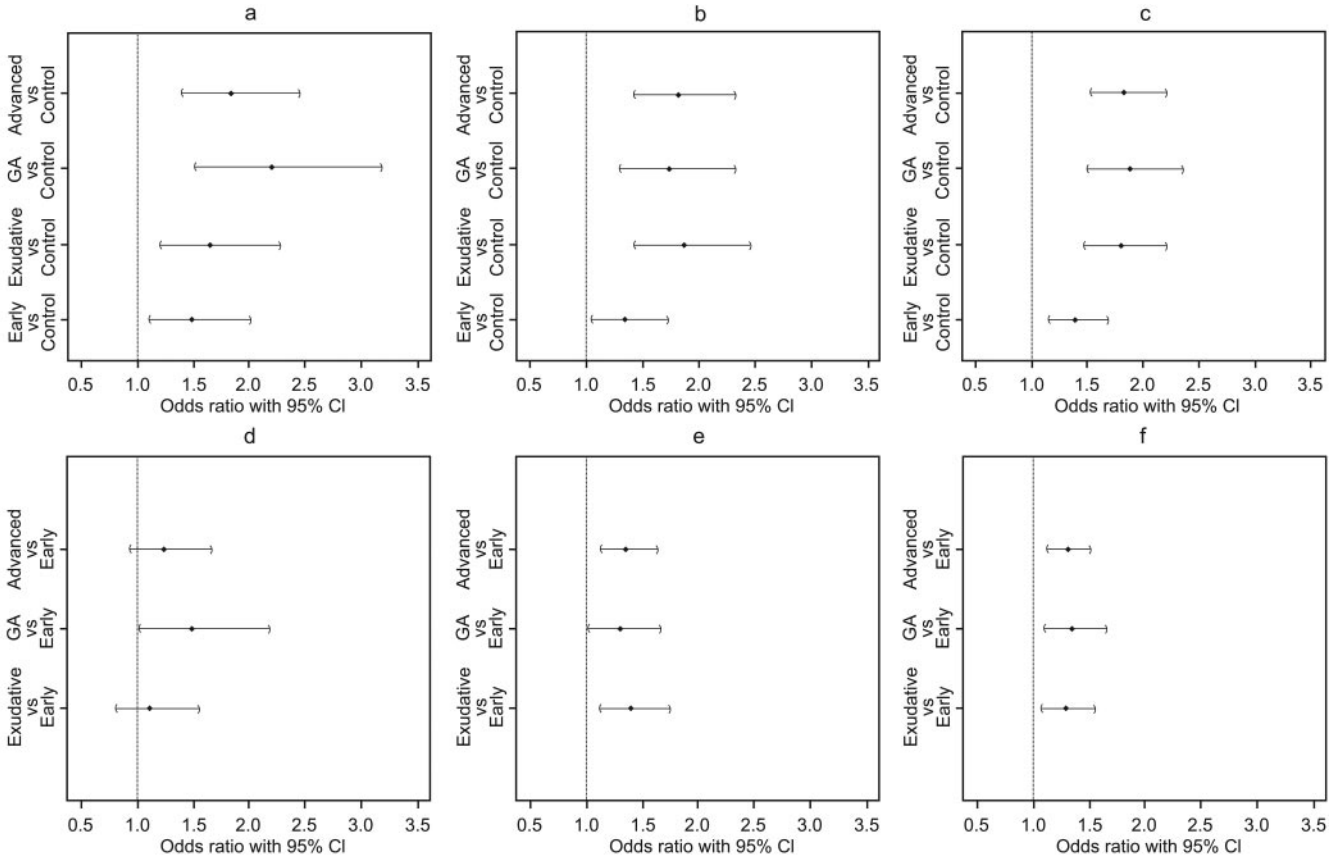

FIGURE 2. Differential effect of nsSNP rs2230199 on AMD subtypes. *Top row*: plots are shown comparing all AMD subtypes to controls for rs2230199. The impact of early AMD, geographic atrophy, and exudative and advanced (combined geographic atrophy and exudative) AMD subtypes compared with the control is shown for the Mayo (**a**), AREDS (**b**), and the pooled Mayo/AREDS (**c**) subjects. *Bottom row*: Plots are shown for the preferential impact on advanced AMD compared with early AMD for rs2230199. A baseline-category logit model was used, considering early AMD as the reference to determine whether the *C3* nsSNPs preferentially predispose to geographic atrophy, exudative, and advanced AMD in the Mayo (**d**), AREDS (**e**), and pooled Mayo/AREDS (**f**) subjects. The analysis demonstrates that the greater association with advanced AMD subtypes in the *top row* is statistically significant.

3′ end of *C3* showed significant association with AMD in the Mayo and AREDS subjects (Table 5). The effect persisted after conditioning on the nsSNPs in the Mayo subjects, but not the AREDS subjects (Table 5). Thus, at this time additional study is needed to determine whether the 3′ end of *C3* has an independent impact on AMD risk. We found no coding DNA sequence variation in publically available SNP databases (dbSNP, HapMap) or 46 Caucasian chromosomes sequenced by Seattle SNPs (http://pga.gs.washington.edu) that would explain the independent AMD risk in the 3′ region of *C3*. There are at least 292 DNA sequence variants across the *C3* locus that could impact splicing or other regulatory functions (http://pga.gs.washington.edu). It is possible that some of these uncommon variants affect *C3* expression, but functional studies would be necessary to evaluate the effects. At this time most, if not all, of the effect of *C3* on AMD can be explained by the two nsSNPs.

Although it has been established that the extent of the maculopathy (drusen, pigment) characterizing AMD is the major predictor of the development of geographic atrophy and exudation, there is great interest in identifying genetic risks

TABLE 7. Comparison of the Impact of the *C3* nsSNP rs2230199 to Other Major Genetic Risks for Any AMD Compared with Controls in the Mayo Subjects

Gene Name	Amino Acid Change	Reference SNP Number	Risk Allele Frequency (Controls)	Genotype OR (95% CI)	
				1 Minor Allele	2 Minor Allele
CFH	Y402H	rs1061170	0.35	2.28 (1.52–3.42)	4.71 (2.82–8.07)
LOC387715/HTRA1	A69S	rs10490924	0.19	2.03 (1.50–2.77)	10.81 (5.19–26.37)
BF/C2	L9H (BF)	rs4151667	0.95	0.54 (0.26–1.12)	Not observed
	Intron (C2)	rs547154	0.91	0.41 (0.23–0.73)	0.37 (0.01–9.43)
C3	R102G	rs2230199	0.18	1.83 (1.32–2.54)	2.38 (1.28–4.66)
	P314L	rs1047286	0.17	1.81 (1.31–2.53)	2.23 (1.19–4.37)

No interaction was observed between loci ($P = 0.54–0.78$), and all loci were consistent with an additive model. Furthermore, additive models showed no interaction between smoking and AMD for the *C3* SNPs ($P = 0.40–0.78$). Smoking did not have an independent effect on any AMD versus controls in the Mayo subjects ($P = 0.17$).

that might promote these two complications of AMD.[27] We used statistical modeling to examine the relative impact of rs2230199 on the common AMD subtypes: early AMD characterized by large drusen, primary geographic atrophy, and exudation. Unlike a previous report,[15] we observed a similar risk for rs2230199 on early and exudative AMD in Mayo subjects. However, we observed an increased risk (1.5-fold) of geographic atrophy compared with early AMD in the Mayo subjects that was not observed in previous studies.[14–16] Others have reported that the *C3* nsSNPs along with *C2/CFB*, *CFH*, and *LOC387715/HTRA1* variants promoted progression to advanced AMD in the AREDS subjects.[28,29]

The replication study with AREDS subjects showed a significantly increased risk of both types of advanced AMD ($P = 0.039$ for geographic atrophy, $P = 0.004$ for exudative AMD) compared with early AMD. We obtained similar results with rs1047286, as would be expected given the tight LD (data not shown). Yates et al.[15] reported that their 189 exudative AMD subjects (but not the 261 early AMD subjects) were associated with this SNP in their Scottish group. Maller et al.[14] reported no difference in association between exudative AMD and GA compared to controls, but did not include subjects with early AMD. Spencer et al.[16] observed no difference between the frequency of the risk allele for rs2230199 (R102G) between all AMD (701 subjects) and neovascular AMD subjects comprising 61% of these subjects. Our analysis of the pooled Mayo and AREDS subjects suggests that *C3* nsSNPs preferentially associate with advanced AMD subtypes (Fig. 2). Thus, variation in *C3* may contribute preferentially to the development of advanced AMD, but additional study in prospective cohorts is warranted.

As noted, genetic studies in the Mayo subjects did not enable us to distinguish the effect of R102G and P314L on AMD risk. POLYPHEN analysis[30] predicted R102G to be a benign alteration, and P314L to be probably damaging. The nsSNP R102G corresponds to the electrophoretically slow (C3S, arginine) and fast (C3F, glycine) forms of *C3*.[31] The other nsSNP P314L has not been reported to affect electrophoretic migration as would be expected based on proline and leucine having the same charge. The P314L substitution is in the third macroglobulin domain and is not near a functional domain in C3 based on the structure reported by Janssen et al.,[32,33] whereas the arginine at amino acid 102 in the first macroglobulin domain is thought to be important in maintaining the structure of the thioester domain.

The C3 allotypes have been associated with other diseases. C3F (102G) was reported to be protective for improved survival of transplanted kidneys, and a risk for Indian childhood cirrhosis, partial lipodystrophy, IgA nephropathy, Chagas disease cardiomyopathy, nephritic factor, type II mesangiocapillary glomerulonephritis, and systemic vasculitis.[34–40] Most of the studies did not look directly at the differences between C3S and C3F and cannot distinguish between the effects of these two nsSNPs on disease risk. Further study to evaluate the functional effects of rs2230199 and rs1047286 variants is needed to understand how the *C3* nsSNPs alter the risk of AMD.

This study has several limitations. Case–control studies are well known to be influenced by non-representative sampling of the underlying population. Although we have not observed such effects in our ongoing work, the possibility cannot be completely excluded. We have performed a large number of exploratory investigations, creating the possibility for random events to reach nominal significance. Nominal *P*-values are reported without correction for multiple testing, but can be readily converted to corrected ones by the reader. Rather than relying on the *P*-values, we have chosen a two-stage analysis method in which findings in one group of subjects were studied in a second independent group of subjects to determine whether the observation could be replicated. This strategy is more rigorous than relying on the magnitude of individual *P*-values. The ultimate value of this study is to generate hypotheses based on the genetic data that can be tested in functional studies.

In summary, we observed that the effect of the nsSNPs rs2230199 and rs1047286 on AMD risk could not be separated. The minor alleles for these two nsSNPs define haplotypes that increase the risk of developing any AMD. The two nsSNPs were significantly associated with advanced AMD compared with early AMD, suggesting that they may promote the development of these complications of early AMD. The possibility that haplotypes in the 3′ end of *C3* locus may be associated with AMD requires further study.

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