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Genetic Variants and Systemic Complement Activation Levels Are Associated With Serum Lipoprotein Levels in Age-Related Macular Degeneration

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ger-related macular degeneration (AMD) is a multifactorial, progressive disease and a leading cause of blindness in the elderly population.1,2 The strong genetic underpinnings of AMD based on genome-wide association studies (GWAS) broadly point toward the involvement of three systems in the pathogenesis of AMD: the complement system, lipid metabolism, and the extracellular matrix.3 Investigating the pathways identified by genetic associations has proven to be a fruitful research strategy in the past. A higher rate of systemic complement activation levels was demonstrated in patients compared with controls.4,5 bringing systemic physiological consequences in line with the genetic associations. For the second major system involved in AMD, the lipid metabolism, such congruency is not immediately apparent.

Lipids, due to their insoluble nature, are transported through the circulation by lipoproteins.6 Two major lipoproteins of this process are low-density lipoprotein (LDL) and high-density lipoprotein (HDL).7 Low-density lipoprotein is responsible for transporting cholesterol from the liver to the periphery, while HDL transports peripheral cholesterol back to the liver in a process called reverse cholesterol transport (RCT).8

In a recent GWAS on AMD, three genes involved in the lipoprotein transport system (CETP, APOE, and LIPC) reached genome-wide significance.3 In addition, two earlier association studies also reported associations for LPL, FADS1, and ABCA1.9,10 All of the proteins encoded by these genes, are either enzymes, coenzymes, or transporters within the lipid metabolism. Thus, ample genetic evidence exists for the involvement of lipid metabolism in the etiology of AMD.

In the pathology of AMD, aberrant lipid homeostasis has also been observed. Particularly, approximately 40% of drusen composition (one of the major hallmarks of AMD) is made up of esterified cholesterol, unesterified cholesterol, and phosphola-
Genetic Variants Associated With AMD

The secondary aim was to determine if there is a correlation between the previously described complement measurements. Serum was obtained by a standard protocol of the Cologne Image Reading Center (CIRCL) by certified graders (TR, LE). The classification of AMD was performed as described previously.27

Demographic data and nongenetic parameters including smoking status (current/past/never), regular alcohol intake (current/past/never), body mass index (BMI), exercise/physical activity (never, almost never, 1–2 times a week, 3 or more times a week), and daily fat consumption (more than 35 g oil per day: Yes/No) were obtained by standardized interviewer-assisted questionnaires.

Serum Measurements and Genetic Analysis

Serum samples were used for the various lipid and systemic complement activation. Serum was obtained by a standard coagulation/centrifugation protocol, after which the samples were stored at −80°C within 1 hour after collection. Serum levels of apolipoprotein B (Apo-B), apolipoprotein AI (Apo-AI), total cholesterol, triglycerides (TG), and HDL-cholesterol (HDLC) were measured in all patients and controls using standard procedures by a clinical chemistry laboratory (Architect Analyzer; Abbott Diagnostics Hoofddorp, The Netherlands). Non-HDL cholesterol (NHDL) was calculated by subtracting HDLC from total cholesterol; and low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula.28

Complement component C3 and the activation fragment C3d were measured in serum samples as previously described29 and C3d/C3 was calculated as a measure of complement activation.

Genomic DNA was extracted from peripheral blood samples using standard procedures. Eight SNPs in the LIPC, CETP, APOE, FADS1, LPL, and ABCA1 genes (see Supplementary Table S1) were genotyped using the KAsPar SNP Genotyping System by LGC Genomics.

Statistical Analysis

All calculations were performed using SPSS software version 20.0 (IBM Software and Systems, Armonk, NY, USA). Associations between lipid levels, SNP genotypes and disease status were analyzed using general linear models with each lipid, in turn, set as the dependent variable. The first model was built to find the association between lipids and disease status, the model was corrected for all possible confounders (see Table 3). The second model was built to find the association between lipids and SNPs, again the model corrected for all possible confounders (see Table 4). In literature smoking status, alcohol intake, BMI, and dietary fat intake are reported to significantly influence lipid and lipoprotein levels,50–54 for this reason they were selected as correction factors for the models to eliminate any possible confounding. Additionally, age, sex, and exercise/physical activity were significantly different between patients and controls, thus they were also added as correction factors.

In order to assess the cumulative effect of CETP and APOE SNPs on lipid/lipoprotein levels, a new variable was created that had all nine possible genotype combinations (see Table 4). The association with the lipid/lipoprotein levels was tested in a general linear model also corrected for age, sex, BMI, smoking status, alcohol intake, exercise/physical activity, daily fat consumption, and disease status. The significance threshold was corrected for multiple testing, P values less than or equal to 0.006 (0.05/8 associations per experiment) were considered statistically significant for the associations to AMD of both SNP genotypes and serum lipid levels. For the associations of the SNPs and serum lipid levels we have corrected for 12 associations (4 genetic variables against 3 lipid/lipoprotein levels). The P values less than or equal to 0.004 (0.05/12 studied associations) were considered significant.

Associations between AMD phenotype and genotypes were evaluated using cross tabulation, P values were calculated with Pearson χ² and odds ratios were generated using logistic regression. Pearson correlations were used to investigate the relationship between lipids and complement activation levels.

All power calculations were performed using CaTS - Power Calculator v0.0.2 (Center for Statistical Genomics, University of Michigan, Ann Arbor, MI, USA) as previously described.35 For the calculation we assumed a multiplicative model, a disease prevalence of 10% and a significance level of 0.006 (0.05/8 SNPs). The disease allele frequency and genotype relative risk were extracted from the papers that first described the associations (Supplementary Table S1).

Results

Summary of the demographics for the subjects included in the present study are shown in Table 1.

Eight SNPs in genes of the lipid metabolism, previously shown to be associated with AMD, were selected from literature (see Supplementary Table S1). Out of the eight SNPs that were analyzed, genotypes in CETP (rs5764261; P = 0.002), APOE (rs4420638; P = 0.005), and FADS1 (rs174547; P = 0.005), were significantly associated with AMD after correcting for multiple testing (P < 0.006). A summary of all associations and genotype frequencies are presented in Table 2.
Mean serum levels of Apo-B, Apo-AI, total cholesterol, HDLC, LDL, and triglycerides of AMD patients compared to controls are presented in Table 3. After adjusting for age, sex, BMI, smoking status, alcohol intake, exercise/physical activity, and daily fat consumption, AMD patients had significantly higher Apo-AI \((P = 0.002)\) and HDLC levels \((P = 4.4 \times 10^{-5})\) compared with controls. In contrast, patients had significantly lower serum levels of TG \((P = 1.9 \times 10^{-4})\) compared with controls. Significant positive correlations were observed between Apo-AI and HDLC, and negative correlations between HDLC and TG (Supplementary Fig. S1). The association between Apo-AI and AMD was independent of HDLC and TG. Similarly, the association of TG with AMD was independent of HDLC and Apo-AI. When correcting for Apo-AI and TG, the association of HDLC with AMD was negated, which is in line with the correlation of HDLC with Apo-AI and TG levels. No significant associations were found for any of the other measurements.

Stratifying for the different AMD stages revealed significant associations only with the intermediate AMD stage. The observed effect directions were similar to the comparison of all AMD stages versus controls, with only Apo-AI, HDLC, and TG being significantly associated with intermediate AMD after correction for multiple testing (Supplementary Table S2).

The SNPs that were significantly associated with AMD in the EUGENDA cohort, were analyzed for association with the lipid/lipoprotein levels that significantly differed between patients and controls. Only APOE (rs4420638) and CETP (rs3764261) genotypes displayed significant associations with Apo-AI and HDLC serum levels. APOE (rs4420638) genotypes were moderately associated with TG levels \((P = 0.026)\), however the association did not remain significant after correcting for multiple testing. A summary of the results is presented in Table 4.

Because both CETP and APOE SNP genotypes were significantly associated with Apo-AI and HDLC serum levels,
The genetic analyses from the present case-control study confirm previously described associations for CETP (rs3764261), APOE (rs4420638), and FADS1 (rs174547) with AMD. However, no associations were observed for APOE (rs2075650), LIPC (rs493258 and rs10468017), LPL (rs12678919), and ABCA1 (rs3758294). The SNPs were selected from recent large GWAS (see Supplementary Table S1). For the SNPs in ABCA1 (rs1883025) and LIPC (rs493258 and rs10468017) our study was underpowered with 52%, 59%, and 53% chance of detection, respectively. Therefore, we cannot exclude the possibility that these SNPs may be associated to AMD in a larger cohort. On the other hand, for LPL (rs12678919) this study had the cumulative effect of carrying multiple risk genotypes on the lipid levels was investigated. Mean levels for each genotype combination of CETP and APOE are displayed in Table 4 and visualized in Figure 1. In both cases, carriers of double high-risk genotypes for CETP and APOE showed significantly elevated levels of Apo-AI and HDL-C compared with low-risk genotype carriers.

To exclude the possibility that the associations of HDL-C and Apo-AI with AMD were mainly a consequence of the underlying genetic associations of CETP and APOE that drive HDL-C and Apo-AI levels, all lipid analyses were corrected for CETP and APOE genotypes and all the other genotyped SNPs. After doing so, HDL-C and Apo-AI remained significantly associated with AMD, independent of the genotypes ($P = 1.4 \times 10^{-4}$ and 0.003, respectively).

Finally, because serum complement activation levels were previously shown to be associated to AMD, we tested whether a relation exists between lipid levels and complement activation levels (C3d/C3 ratio). This analysis revealed significant positive correlations between Apo-AI, HDL, and complement activation and a significant negative correlation for TG (Fig. 2). All $P$ values were less than $1.9 \times 10^{-9}$. A general linear model corrected for disease status and other variables confirmed the association of C3d/C3 to lipids/lipoproteins ($P = < 1.9 \times 10^{-9}$) and revealed that the association to disease status is independent of lipid levels ($P = 9 \times 10^{-6}$).

### DISCUSSION

The genetic analyses from the present case-control study confirm previously described associations for CETP (rs3764261), APOE (rs4420638), and FADS1 (rs174547) with AMD. However, no associations were observed for APOE (rs2075650), LIPC (rs493258 and rs10468017), LPL (rs12678919), and ABCA1 (rs3758294). The SNPs were selected from recent large GWAS (see Supplementary Table S1). For the SNPs in ABCA1 (rs1883025) and LIPC (rs493258 and rs10468017) our study was underpowered with 52%, 59%, and 53% chance of detection, respectively. Therefore, we cannot exclude the possibility that these SNPs may be associated to AMD in a larger cohort. On the other hand, for LPL (rs12678919) this study had the cumulative effect of carrying multiple risk genotypes on the lipid levels was investigated. Mean levels for each genotype combination of CETP and APOE are displayed in Table 4 and visualized in Figure 1. In both cases, carriers of double high-risk genotypes for CETP and APOE showed significantly elevated levels of Apo-AI and HDL-C compared with low-risk genotype carriers.

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77% detection power, suggesting that not all genetic associations may be reliably replicated between different populations.

In this study, we observed significant differences in the serum levels of Apo-AI, HDLC, and TG of AMD patients compared with controls. Triglycerides were significantly lower, while Apo-AI and HDLC were significantly higher in patients compared with controls. No statistically significant associations with AMD were detected for any of the other measured lipids/lipoproteins.

In the literature, there are inconsistent associations of AMD with serum lipid levels. Comparing the mean lipid/lipoprotein levels observed in the present study with values previously reported (Supplementary Table S3), is challenging since the measurements were performed differently across the various reports, different correction factors were applied, and different populations were studied. All of these factors can influence the mean levels, making it difficult to pinpoint the cause of the different study outcomes. However, if we compare the main effects, our findings of high HDLC levels in patients compared with controls are consistent with several previous studies.12–18 The positive association of HDLC with only the intermediate AMD stage confirms the finding reported by Cougnard-Grégoire et al.12 On the other hand, other publications have reported inverse or no association between HDLC and AMD.19–25,26 When results were pooled in a meta-analysis, no associations have been detected.1,24,38 To our knowledge, for Apo-AI this is the first large study to report a positive association with AMD, and for TG other studies reported opposite or no associations with AMD.12,20,26 The reasons for these inconsistencies are not fully understood, however in a recent publication high levels of HDLC were associated with risk for AMD only after a stringent multivariate correction.12 Because our study, and others,39–41 show a clear effect of genotype on lipid levels, correcting for these genotypes may improve the insight into the associations of lipid levels with AMD and the direction of their effect. This is especially important because our study, although appropriately powered, had failed to detect associations with \(LPL\) (rs12678919), suggesting that population- or cohort-specific genetic substructures may account partly for the observed inconsistencies. Another reason could be related to sample size, which in some studies might not be large enough to allow for the necessary adjustments and still have sufficient power to detect significant associations. In our cohort, higher levels of Apo-AI and HDLC were associated with risk genotypes in \(CETP\) (rs5764261; TT) and \(APOE\) (rs4420658; AA). A cumulative effect was observed for these two SNPs, with a risk-allele dose dependent increase in both HDLC and Apo-AI serum levels (Fig. 1). The \(CETP\) and \(APOE\) loci have previously been linked to lipid metabolism in cardiovascular studies.40 In the context of AMD, few studies have looked into the relation of AMD lipid SNPs and serum lipid levels. Our results for \(CETP\) were consistent with a recent report from the Alienor study.12 Another study observed that individuals carrying the \(LPL\) (rs12678919) GG genotype, TG levels were significantly lower and HDLC levels were significantly higher.42 Moreover, one study reported that the \(LIPC\) (rs10468017) T allele was associated with higher levels of HDLC.43 Our study does not describe an association of \(LPL\) and \(LIPC\) genotypes with lipid levels, because no significant difference between patients and controls was observed.

\(CETP\) encodes for cholesterol ester transfer protein (CETP), which promotes the transfer of excess cholesterol ester (CE) to the liver through the RCT pathway.44 Several studies have shown that lower CETP activity leads to higher HDLC levels.45–48 \(APOE\) encodes for apolipoprotein E (ApoE), which plays a major role in the metabolism of cholesterol and TG by mediating the clearance of chylomicrons and very low-density-lipoprotein (VLDL) from the bloodstream.47,48 ApoE has been described to have a direct relation with CETP by enhancing the CE and TG transfer between VLDL and HDL in a CETP-dependent manner.49 Despite the direct impact of ApoE and CETP on HDLC metabolism, understanding how the

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/934740/...)

**Figure 1.** Mean lipid levels with standard error bars for CETP/APOE homozygous high-risk genotypes, heterozygous and homozygous low-risk genotypes. All \(P\) values are Bonferroni-corrected. (A) Levels for Apo-AI; (B) levels for HDLC.

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/934740/...)

**Figure 2.** Scatterplots showing the correlations of lipid levels with complement activation levels represented by the log transformed ratio of C3d/C3. Direction of the correlations is indicated by the black regression line. (A) Positive correlation of Apo-AI and logC3d/C3; (B) positive correlation of HDLC with logC3d/C3; (C) negative correlation of TG and logC3d/C3.
risk genotypes of the studied SNPs could have the cumulative HDL-C raising effect is not directly obvious, mainly because both rs3764261 and rs4420638 are located in intergenic regions. One possibility may be a consequence of an effect on CETP expression levels that was reported for rs3764261.50 Traditionally, rs4420638 is reported as an APOE SNP, because it is considered a proxy for rs429358, one of the two coding variants that determine the APOE isoforms (ε2, ε3, and ε4) reported to attenuate binding affinity to the (LDL) receptor.51 and thus affect the entire cholesterol metabolism. However, the $r^2$ value for the linkage disequilibrium of these two SNPs is 0.63,3 indicating that there is not a complete co-inheritance. Also its genomic position is closer to the APOC1 gene, a potent inhibitor of CETP activity,52 thus we cannot exclude the possibility of rs4420638 for being a proxy for a regulatory variant of APOC1 instead.

Understanding the local involvement of lipid and lipoprotein systems at AMD disease sites in the eye is made difficult by the lack of information regarding eye specific function of these molecules. Nevertheless, if we focus on HDLC metabolism, clues can be found. First, key components of the RCT pathway for which the main player is HDLC,53 are expressed in the retina.54–56 In addition, during the normal aging process, an accumulation of Apo-B of unusual composition takes place in the Bruch’s membrane, forming a precursor of basal linear deposit, called the “lipid wall.”57 Moreover, the macromolecular conductivity of the Bruch’s membrane reduces 10-fold between the first and ninth decades of life, which is significant because lipoproteins need to cross the Bruch’s membrane in order to mediate lipid efflux from the RPE.58,59 Furthermore, in vitro, HDL has been observed to mediate efflux of photoreceptor outer segment lipids from the basal surfaces of RPE cells.60 Finally, a retention of cholesterol in drusen, the major lesions of AMD, has been reported.61

Besides the involvement of HDL in lipid and lipoprotein transport, this system has recently been implicated in immune function.62 Recent proteome analyses revealed several types of HDL particles containing complement system components C4a, C4b, C9, and vitronectin53,54,55 in healthy subjects, and C3 in patients with coronary artery disease.63 In our study, we offer support for this emerging concept by demonstrating a significant correlation between HDLC and complement system activation, although it remains to be determined whether the effect is direct or indirect.

One possible limitation of our study may be that the lipid/lipoprotein levels were not overnight fasted blood measurements, which could induce possible artifacts for certain lipids and lipoprotein levels were not overnight fasted blood measurements, which could induce possible artifacts for certain lipids.50 Similarly, the enrichment of drusen in AMD patients might relate to the AMD disease pathogenesis.

In conclusion, the results of our study indicate that patients with high risk CETP/APOE genotypes and high HDLC levels have higher risk of developing AMD, suggesting that they could potentially benefit from HDLC lowering regimens. Further studies are needed to investigate the role of HDLC subfractions and the observed correlation of HDLC with complement activation in the disease pathogenesis of AMD.

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