Genome-wide association study of advanced age-related macular degeneration identifies a role of the hepatic lipase gene (*LIPC*)

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Advanced age-related macular degeneration (AMD) is the leading cause of late onset blindness. We present results of a genome-wide association study of 979 advanced AMD cases and 1,709 controls using the Affymetrix 6.0 platform with replication in seven additional cohorts (totaling 5,789 unrelated cases and 4,234 unrelated controls). We also present a comprehensive analysis of copy-number variations and polymorphisms for AMD. Our discovery data implicated the association between AMD and a variant in the hepatic lipase gene (*LIPC*) in the high-density lipoprotein cholesterol (HDL) pathway (discovery $P = 4.53 \times 10^{-5}$ for rs493258). Our *LIPC* association was strongest for a functional promoter variant, rs10468017, ($P = 1.34 \times 10^{-8}$), that influences *LIPC* expression and serum HDL levels with a protective effect of the minor T allele (HDL increasing) for advanced wet and dry AMD. The association we found with *LIPC* was corroborated by the Michigan/Penn/Mayo genome-wide association study; the locus near the tissue inhibitor of metalloproteinase 3 was corroborated by our replication cohort for rs9621532 with $P = 3.71 \times 10^{-9}$. We observed weaker associations with other HDL loci (*ABCA1*, $P = 9.73 \times 10^{-4}$; cholesteryl ester transfer protein, $P = 1.41 \times 10^{-3}$; *FADS1-3*, $P = 2.69 \times 10^{-2}$). Based on a lack of consistent association between HDL increasing alleles and AMD risk, the *LIPC* association may not be the result of an effect on HDL levels, but it could represent a pleiotropic effect of the same functional component. Results implicate different biologic pathways than previously reported and provide new avenues for prevention and treatment of AMD.

complex disease | HDL | lipid pathway | retinal degeneration

Age-related macular degeneration (AMD) is a common, late-onset neurodegenerative disorder that is modified by covariates including smoking and body mass index, and it has a 3- to 6-fold higher recurrence ratio in siblings than in the general population (1). The burden of AMD is increasing among the growing elderly population. The advanced form of AMD is clinically significant, and it causes visual loss and reduces quality of life.

The role of the alternative complement pathway in disease pathogenesis was documented by the discovery (2–5) and replication (6, 7) of the *CFH* association as well as reports of three additional risk loci in this pathway: *CFB/C2* on chromosome 6 (6, 8), *C3* on chromosome 19 (9, 10), and *CFI* on chromosome 4 (11). Together with strongly associated variants in the *ARMS2/HTRA1* region on chromosome 10 (12, 13), these multiple loci have been

estimated to explain approximately one-half of the heritability of AMD (6), and combined with demographic, ocular, and environmental factors, they have potential predictive power (14). To date, however, no large sample genome-wide association study (GWAS) has been undertaken to attempt to explain the remaining heritability of AMD and to identify other susceptibility loci. We present here such a GWAS involving 979 cases of advanced AMD in the discovery phase with multiple stages of replication.

Samples were genotyped on the Affymetrix 6.0 platform, which contains probes for 906,000 SNPs and an additional 946,000 SNP-invariant probes to enhance copy-number variation (CNV) analysis; it captures 82% of the variation at an $r^2 \geq 0.8$ for Europeans in the 3.1 million SNPs of HapMap phase 2 (15). Our study has uncovered several new AMD susceptibility loci. Intriguingly, our most significant, replicated association is a functional variation in the hepatic lipase gene (*LIPC*), a gene involved in triglyceride hydrolysis and high-density lipoprotein cholesterol (HDL) function (16), thus revealing another candidate pathway for AMD pathogenesis.

Results

Case and Control Sample Development. Our initial study consisted of 1,057 unrelated cases with geographic atrophy or neovascular AMD and 558 unrelated controls without AMD who were phenotyped based on clinical examination and ocular photography (6, 9, 11). The AMD grade in the worse eye was used in the analyses (17). All individuals were of European ancestry (*Methods* and *Table S1*). To enhance the

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Conflict of interest statement: Tufts Medical Center (J.M.S.) and Massachusetts General Hospital (M.J.D.) have filed a joint patent application for materials related to this manuscript.

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power of our study (18), we included additional unrelated control samples that were genotyped on the same platform in the same laboratory, and we conducted stringent quality control to ensure the technical and population compatibility of these datasets (*Methods* and [Table S2](#)). The final discovery sample consisted of 979 unrelated cases and 1,709 controls. Because the additional controls were unscreened for AMD status, we evaluated their impact on established associations with AMD. We examined 159 SNPs that were in linkage disequilibrium (LD) with the most positively associated variants, and of these SNPs, 137 showed an improvement in the χ^2 with the addition of these controls. After quality control, the average ratio of the final study χ^2 to the initial study χ^2 was 1.82—nearly identical to the expected improvement in χ^2 based on theoretic power calculations of 1.84.

Genome-Wide Association Discovery Phase. Association was assessed using logistic regression as implemented in PLINK (19). We plotted our genome-wide association findings from the final dataset in quantile–quantile (QQ) plots. The top end of the QQ plot (Fig. S14) is dominated by the strong associations of previously reported SNPs, which were excluded to more fully examine the remaining results (Fig. S1B). The regions of previously reported association are near *CFH* (rs572515 proxy for rs1061170 with $r^2 = 0.654$ and $P < 10^{-55}$; rs10737680 proxy for *CFH* rs1410996 with $r^2 = 1$ and $P < 10^{-47}$), *CFI* (rs7690921 proxy for rs10033900 with $r^2 = 0.403$ and $P < 10^{-4}$), *CFB*/*C3* (rs222162 proxy for rs641153 with $r^2 = 1$ and $P < 10^{-6}$), and the *ARMS2/HTRA1* locus (rs10490924 with $P < 10^{-59}$) (Table S3). There were no adequate proxies for the C3 locus. Although no other SNPs reached genome-wide significance of $P < 5 \times 10^{-8}$ (20), we did identify several SNPs of interest in new regions with P values between 10^{-4} and 10^{-6} in our discovery scan (Table 1), including rs493258 (*LIPC*) with $P = 4.5 \times 10^{-5}$ as discussed in more detail below.

Replication Phases. For all SNPs with $P < 10^{-3}$ in our GWAS, we received results for those SNPs from the Michigan/Penn/Mayo (MPM) scan (21). For all SNPs with $P < 10^{-3}$ in the MPM scan, we sent the results for those SNPs from our GWAS to the MPM group. We excluded overlapping samples and we combined the GWAS results for advanced cases as equally weighted Z scores. We then genotyped SNPs with $P < 10^{-4}$ or better in our independent local replication sample of advanced cases and controls from Tufts Medical Center, Tufts University School of Medicine, and Massachusetts General Hospital (Tufts/MGH). These samples were obtained from individuals unrelated to those in our GWAS. We also provided replication data for the MPM study using this cohort, in addition to sharing data from our discovery GWAS sample. For replication in independent samples, subsets of promising SNPs were tested in samples from Johns Hopkins University (JHU), Columbia University (COL), University of Utah (UT), Hôpital Intercommunal de Creteil (FR-CRET), and Washington University (WASH-U). In total, there were 5,789 cases and 4,234 controls in the replication cohorts (Table S1). The most significantly associated SNPs from our overall analyses are presented in Tables 1 and 2. We did not find evidence for other reported candidate gene associations with AMD (22), including *TLR3*, *SERPING1*, *LRP6*, *PEDF*, *VEGF*, *TLR4*, *CX3CR1*, *ELOVL4*, *PON1*, and *SOD2* (Table S4).

Results of Combined Scan and Replication Analysis. With additional replication, the SNP that we identified on chromosome 15, rs493258, showed significant association with $P = 1.61 \times 10^{-8}$ (independent of the MPM GWAS data $P = 4.92 \times 10^{-7}$). This SNP is located 35 kb upstream of the *LIPC* gene on chromosome 15q22 and is in LD ($r^2 = 0.33$; $D' = 0.847$) with the previously described functional variant (rs10468017) reported to influence LIPC expression and serum HDL levels (23, 24).

To evaluate whether or not this association represented an effect of that same functional variant, we genotyped rs10468017 in our

Table 1. Meta-analysis association results from GWAS discovery sample and replication genotyping

[illegible]

SNP, single nucleotide polymorphism; Chr, chromosome; position/base-pair position in National Center for Biotechnology Information 36.1; A1, minor allele; A2, major allele; MAF, minor-allele frequency; number of associated SNPs, number of other SNPs in the region showing association in our discovery scan; Tufts/MGH *P*, *P* value of our original GWAS scan; Tufts/MGH replication *P*, *P* value of Tufts/MGH replication cohort; MPM *P*, *P* value of Michigan/Penn/Mayo GWAS; replication cohort *P*, *P* value of total combined weighted average of the meta-analysis *Z* score across the individual replication cohort sites; Meta *Z* score, weighted *Z* score used to combine the association from all cohorts calibrated on the theoretic power for a balanced case-control study design; meta OR, odds ratio for AMD combining all cohorts - A1 compared with A2; 95% CI, confidence interval for meta OR; final *P*, *P* value of all cohorts listed in the table plus the replication cohorts based upon the meta *Z* score.

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Table 3. Association between AMD and previously reported HDL genetic loci

Gene(s) of interest within or near associated interval	Chr	Position	SNP	HDL-raising allele	Minor allele	MAF	Z score	Meta <i>P</i>	OR (minor allele)	95% CI
<i>ABCA1</i>	9	106704122	rs1883025	C	T	21.4%	3.298	9.73E-04	0.77	0.66–0.90 ^{*†‡§¶**}
<i>CETP</i>	16	55545545	rs3764261	A	A	35.1%	3.193	1.41E-03	1.12	1.04–1.20 ^{*†‡§¶**}
<i>FADS1-3</i>	11	61327359	rs174547	T	C	32.5%	2.213	2.69E-02	0.93	0.88–0.99 ^{*†‡§¶**}
<i>LPL</i>	8	19888502	rs12678919	G	G	10.9%	1.794	0.073	0.85	0.713–1.02 ^{†‡§}
<i>TTC39B</i>	9	15279578	rs471364	T	C	10.5%	1.405	0.16	1.16	0.94–1.42 ^{*†‡§¶}
<i>ANGPTL4</i>	19	8375738	rs2967605	C	T	20.5%	1.104	0.27	1.11	0.92–1.33 ^{*†‡§¶}
<i>LIPG</i>	18	45421212	rs4939883	C	T	16.2%	1.052	0.29	0.96	0.88–1.04 ^{*†‡§¶}
<i>MMAB, MVK</i>	12	108379551	rs2338104	G	C	47.0%	0.962	0.34	0.94	0.84–1.06 ^{*†‡§¶}
<i>LCAT</i>	16	66459571	rs2271293	A	A	13.1%	0.724	0.47	1.04	0.93–1.16 ^{*†‡§¶**}
<i>APOA1-APOC3-APOA4-APOA5</i>	11	116154127	rs964184	C	G	13.5%	0.503	0.62	0.97	0.88–1.08 ^{*†‡§¶}
<i>PLTP</i>	20	44009909	rs7679	T	C	18.1%	0.395	0.69	1.02	0.94–1.10 ^{*†‡§¶**}
<i>HNF4A</i>	20	42475778	rs1800961	C	T	3.1%	0.078	0.94	0.99	0.74–1.31 ^{*†‡§¶}
<i>GALNT2</i>	1	228362314	rs4846914	A	G	39.5%	0.059	0.95	1.01	0.81–1.24 ^{*†‡§¶}

No consistent trend for HDL-raising or -lowering alleles conferring risk of AMD was seen, suggesting that the *LIPC* association may be a consequence of pleiotropy. Chr, chromosome; position, base-pair position in NCBI 36.1; HDL-raising allele, allele reported in Kathiresan et al. (25) as the allele responsible for raising HDL; MAF, minor-allele frequency; Z score, weighted average and direction of minor allele signal; meta *P*, *P* value for the association between the minor allele and AMD; OR, odds ratio for the minor allele; 95% CI, confidence interval for the OR.

*Tufts/MGH GWAS; †Tufts/MGH replication; ‡JHU; §COL; ¶UT; †FR-CRET; **WASH-U.

retinal pigment epithelium and plays a central role in the pathogenesis of AMD (31).

CNVs. We used CANARY (32) to evaluate 554 common segregating copy-number polymorphisms (CNPs) in advanced cases and controls from the phenotyped sample, and observed no CNPs with $P < 0.001$ with the exception of the previously reported deletion variant at *CFHR1* that showed strong association ($P = 1 \times 10^{-19}$) (33). This observation is explained by other previously reported *CFH* variants associated with AMD, particularly the intronic SNP rs1410996 (6), and does not constitute an independent association. We also used Birdseye to examine the potential role of rare CNVs (32). After stringent QC, we observed a set of 150 rare deletions and 278 rare duplications greater than 100 kb. However, these events were not enriched across the genome among cases compared with controls nor were there specific regions showing association of a rare CNV (Fisher's exact test $P < 0.01$). We also did not find evidence for any rare CNVs disrupting genes at the six confirmed associated loci.

Immunoprecipitation Real-Time PCR and Immunohistochemistry of LIPC. We examined sequence conservation for *LIPC* in evolution and determined approximately 75% amino acid identity across mammals (Fig. S2A). To investigate whether the LIPC protein was expressed in the eye and if expression levels change over time, we performed immunoprecipitation using a monoclonal LIPC antibody and quantitative RT-PCR. Despite LIPC being considered a hepatic enzyme, we were able to detect low levels of LIPC protein in bovine retinas (Fig. S2B) and *LIPC* RNA in the retinas of C57BL/6 mice across a broad range of ages (Fig. S2C–D). Consistent with our expression data from mouse retina, we were also able to amplify *LIPC* message from total RNA isolated both from human retinal epithelial transformed cells (RPE19) and human eyes. Next, using a rabbit polyclonal antibody raised against amino acids 91–160 of human LIPC, we were able to show strong labeling of human macula and RPE/choroid and weaker labeling of peripheral retina at the appropriate molecular weight of ~55 kDa observed in human liver (Fig. 1). Bands of mobility ~80 kDa likely represented glycosylated hepatic lipase. This same antibody was then used to immunolocalize LIPC in sections of perfusion-fixed monkey retina (Fig. 2). Prominent labeling of all retinal neurons was observed, especially in retinal ganglion cells of the central retina. However, Mueller glial cells were unlabeled, and RPE cells

appeared to exhibit significant fluorescence signal beyond intrinsic autofluorescence. Thus, our immunoblot and immunohistochemical data corroborate the findings of Tserentsoodol et al., (34) regarding the existence of intraretinal proteins (e.g., ABCA1 transporter, apoA1, SR-BI, and SR-BII) that mediate lipid transport and processing.

Discussion

We found a significant association between advanced AMD and the *LIPC* locus in our GWAS. *LIPC*, which encodes hepatic triglyceride lipase, has been shown to catalyze hydrolysis of phospholipids, monoglycerides, diglycerides, triglycerides, and acyl-CoA

Fig. 1. Immunoblot of hepatic lipase antibody (H-70, SC-21007) against human whole-protein extracts. Lane 1, macular retina; lane 2, peripheral retina; lane 3, RPE-choroid; lane 4, liver. Lanes 1–3 were normalized against actin.

Fig. 2. (A) Distribution of hepatic lipase C (red) in central monkey retina using a rabbit polyclonal antibody (Cat. #SC-21007) raised against amino acids 91–160 of human origin. This antibody labels all retina neurons, especially ganglion cells of central monkey retina, but does not label Mueller cells (identified by glutamine synthetase; green). (Scale bar, 20 μ m.) (B) Distribution of hepatic lipase C (red) in peripheral monkey retina. (Scale bar, 20 μ m.) (C) High-magnification images of photoreceptors show immunoreactivity for antihepatic lipase C (red). (Scale bar, 10 μ m.) (D) High-magnification images of ganglion cells show immunoreactivity for antihepatic lipase C (red). (Scale bar, 10 μ m.) Cones, defined by primate cone arrestin labeling (mAb 7G6; green in C), are positive for hepatic lipase C. However, rods reveal a strong punctate label (red) in the outer nuclear layer as well as over inner and outer segments. In D, strong immunoreactivity for hepatic lipase C (red) observed in ganglion-cell somata and axons of the nerve fiber layer (NFL) contrasts with that of a Mueller cell marker (green; glutamine synthetase; BD Transduction).

thioesters, and is a critical enzyme in HDL metabolism (16). Hepatic lipase also binds heparin and has the dual functions of triglyceride hydrolase and ligand/bridging factor for receptor-mediated lipoprotein uptake (16). LIPC has been shown through our experiments to be expressed in the retina, and CETP and ABCA1 have previously been shown to be expressed in the retina (34).

The pattern of results from the previously reported HDL loci was inconsistent with a straightforward correlation between HDL levels and AMD. If the association between *LIPC* and AMD was driven by this phenotypic correlation, then all SNPs associated with HDL would likely show some level of association with AMD. Additionally, the direction of the effects would be consistent. The T allele of the *LIPC* SNP has been shown to increase HDL levels, and our data suggest that it decreases risk for AMD. In contrast, data from our scan suggest that the HDL-raising alleles of *ABCA1* and *CETP* may increase the risk of AMD, although these results are not currently genome-wide significant. Thus, the association between advanced AMD and *LIPC* may not represent phenotypic correlation to or a causal effect of serum HDL but could indicate a shared underlying biologic mechanism involving the cholesterol pathway.

If the relationship of the *LIPC* polymorphism to AMD is not mediated by variation in HDL levels, alternative mechanisms may play a role. Hepatic lipase has been shown to have an impact on atherogenesis (35), and cardiovascular risk factors are associated with AMD (36, 37). The vascular intima in atherosclerosis and Bruch's membrane in macular degeneration may undergo similar age-related changes (38). These diseases may represent parallel responses to tissue injury induced by multiple factors, including genetic variation, impaired immune responses, and oxidative stress. Whether *LIPC* genetic variation has parallel or distinct roles in AMD and atherogenesis remains to be determined.

Data regarding the association between serum HDL levels and AMD are conflicting (37, 39–44). Some studies found no relationship (37, 43), whereas others found that increased risk of AMD or a subphenotype was associated with increased HDL levels (39, 41). Three studies have shown an inverse relationship with either decreased HDL levels in AMD cases or decreased incidence of advanced AMD with higher HDL (40, 42, 44).

Another possibility regarding the role of the *LIPC* polymorphism in AMD involves the role of HDL as the major lipoprotein transporter of lutein and zeaxanthin. Reduced dietary intake of these two carotenoids has been associated with an increased risk of AMD (45). Variation in the uptake and transport into the retina of carotenoids by HDL has been implicated in AMD pathogenesis (46). Changes in HDL-related efficiency of carotenoid delivery are other possible mechanisms by which *LIPC* variation could impact the risk of AMD. It is also interesting to note that drusen, deposits in the macula that are the hallmark of AMD, also contain cholesterol deposits (47).

This *LIPC* locus may greatly enhance our biologic understanding by opening up another pathway for consideration in the pathogenesis of AMD. This finding could lead to insights regarding disease progression, ways to modify risk of AMD, and new treatments.

Methods

Tufts/MGH Study-Sample Descriptions. This study conformed to the tenets of the Declaration of Helsinki and received approval from Institutional Review Boards; informed consent was signed by all participants. Tufts participants were unrelated and had geographic atrophy, neovascular disease, or no sign of AMD, based on fundus photography and ocular examination (17). Subjects were derived from ongoing AMD study protocols as described previously (6, 9, 11, 48–51). The Tufts/MGH replication dataset included DNA samples from unrelated Caucasian individuals not included in the GWAS, including 868 advanced AMD cases and 410 examined controls identified from the same Tufts cohorts as well as 379 unexamined controls (52).

Genotyping, Analysis, and Replication. Genotyping was performed at the Broad Institute using the Affymetrix SNP 6.0 GeneChip and the Sequenom MassARRAY system for iPLEX assays (32). We started with 1,057 cases and 558 examined controls and studied 906,000 genotyped SNPs and 946,000 CNVs using the Affymetrix 6.0 GeneChip, which passed QC filters. Then, 43,562 SNPs were removed for low call rate, 4,708 were removed for failing the Hardy–Weinberg test at 10^{-3} , 8,332 SNPs were removed because of failing a differential missing test between cases and controls at 10^{-3} , and 126,050 SNPs were removed for having allele frequency less than 1%. We removed 73 individuals for lower than expected call rate, resulting in 1,006 cases and 536 controls. All QC steps were performed using PLINK (19). We conducted a preliminary χ^2 association analysis to determine the extent to which population stratification and other biases were affecting the samples and observed $\lambda \sim 1.05$, indicating that the samples were generally matched well for population ancestry but with some minor inflation remaining (explanation and visual representation in Fig. S3). We added Myocardial Infarction Genetics Consortium (MIGen) shared controls (53), which were genotyped on the same Affymetrix 6.0 GeneChip product, and conducted population stratification analyses using multidimensional scaling in PLINK (19). These analyses identified 27 cases, 12 AMD controls, and 223 MIGen controls for a total of 262 individuals who were outliers in the principal component analysis. The final genomic control λ for the logistic regression included seven significant (for prediction of phenotype status) principal components as covariates, and was 1.036 for 632,932 SNPs. This dataset was used for our official GWAS analysis.

SNPs with $P < 10^{-3}$ from our GWAS discovery sample ($n = 720$ SNPs excluding previously associated regions) were exchanged with the MPM study group, which enabled us to use the two scans as primary replication efforts, and enhanced the power of each study. We performed genotyping of all SNPs with combined $P < 10^{-4}$ using Sequenom iPLEX with our Tufts/MGH replication sample. Focusing on sites that continued to show association with $P < 10^{-4}$ after this local replication, we performed a third stage of replication in which our collaborators at JHU and COL genotyped SNPs with ABI 7900 Taqman genotyping, samples from WASH-U were genotyped using the Sequenom platform, and samples from UT and FR-CRET were sent for genotyping to the Broad Institute (Table S1).

CNVs. Using the Birdsuite (32) family of algorithms, we called rare CNV and common CNV in 459 controls and 838 cases after removing those individuals

failing SNP clustering that had an excessive number of singleton CNVs or an excessive length of total length of singleton CNVs. The study was well-powered to detect CNV events (Fig. S4). For rare variants, we identified all singleton CNVs that were larger than 100 kb, had logarithm of the odds scores greater than 10, and relied on at least 10 probes. We also looked at a smaller set of rare variants (>20 kb) appeared in <1% of individuals, and we used PLINK's permutation function to assess if there were any regions that had significant differences between cases versus controls. For common variants, we examined 554 variants that had at least one allele <99%. We used logistic regression in PLINK to test if copy number significantly predicted case-control status.

See *SI Methods* for methods related to PCR and immunohistochemistry.

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