Common genetic variation in multiple metabolic pathways influences susceptibility to low HDL-cholesterol and coronary heart disease[S]

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Abstract **A low level of HDL-C is the most common plasma lipid abnormality observed in men with established coronary heart disease (CHD). To identify allelic variants associated with susceptibility to low HDL-C and CHD, we examined 60 candidate genes with key roles in HDL metabolism, insulin resistance, and inflammation using samples from the Veterans Affairs HDL Intervention Trial (VA-HIT; cases, n = 699) and the Framingham Offspring Study (FOS; controls, n = 705). VA-HIT was designed to examine the benefits of HDL-raising with gemfibrozil in men with low HDL-C (≤40 mg/dl) and established CHD. After adjustment for multiple testing within each gene, single-nucleotide polymorphisms (SNP) significantly associated with case status were identified in the genes encoding *LIPC* (rs4775065, $P < 0.0001$); *CETP* (rs5882, $P = 0.0002$); *RXRA* (rs11185660, $P = 0.0021$); *ABCA1* (rs2249891, $P = 0.0126$); *ABCC6* (rs150468, $P = 0.0206$; rs212077, $P = 0.0443$); *CUBN* (rs7893395, $P = 0.0246$); *APOA2* (rs3813627, $P = 0.0324$); *SELP* (rs732314, $P = 0.0376$); and *APOC4* (rs10413089, $P = 0.0425$). Included among the novel findings of this study are the identification of susceptibility alleles for low HDL-C/CHD risk in the genes encoding *CUBN* and *RXRA*, and the observation that genetic variation in *SELP* may influence CHD risk through its effects on HDL.**—Peloso, G. M., S. Demissie, D. Collins, D. B. Mirel, S. B. Gabriel, L. A. Cupples, S. J. Robins, E. J. Schaefer, and M. E. Brousseau. **Common genetic variation in multiple metabolic pathways influences susceptibility to low HDL-cholesterol and coronary heart disease. *J. Lipid Res.* 2010.** 51: **3524–3532.**

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Coronary heart disease (CHD) is a major cause of death and disability in our society, resulting in approximately 450,000 deaths per year in the United States (2009 Heart and Stroke Statistical Update). Numerous population studies have shown that a strong inverse relationship exists between plasma high density lipoprotein cholesterol (HDL-C) concentrations and CHD risk (1–4). Approximately 50% of men with CHD have a low level of HDL-C, and not elevated low density lipoprotein cholesterol (LDL-C), as their primary lipid abnormality (5). In fact, a study in men has shown that a low level of HDL-C better distinguishes CHD status than a high level of LDL-C (6).

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Abbreviations: ABC, ATP-binding cassette transporter; AIM, ancestry informative marker; Apo, apolipoprotein; AUC, area under the curve; BMI, body mass index; CARDIA, Coronary Artery Risk Development in Young Adults (study); CETP, cholesteryl ester transfer protein; CHD, coronary heart disease; CUBN, cubilin; FDR, false discovery rate; FOS, Framingham Offspring Study; HWE, Hardy Weinberg Equilibrium; LIPC, hepatic lipase; MAF, minor allele frequency; OR, odds ratio; PC, principal component; PCA, principal components analysis; RXRA, retinoid X receptor alpha; SBP, systolic blood pressure; SELP, P-selectin; SNP, single-nucleotide polymorphism; VA-HIT, Veterans Affairs HDL Intervention Trial.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of two tables.

Environmental and metabolic factors that are commonly associated with low HDL-C concentrations include alcohol consumption, dietary saturated fat, decreased exercise, cigarette smoking, obesity, and diabetes (7–9). Low HDL-C is commonly seen in association with the metabolic syndrome, which is characterized by visceral obesity, elevated glucose and blood pressure, dyslipidemia, and frequently, insulin resistance and a pro-inflammatory state (10). In addition to environmental factors, strong evidence also exists for the role of genetics in the determination of HDL-C levels. HDL-C level is a heritable characteristic, with heritability estimates in the range of 40-60% (11). Most of the variation in HDL-C observed at the population level is of multifactorial origin and is the result of the complex interaction between genetic and environmental factors.

In the present study, we utilized a candidate gene approach to extend our earlier work (12–14) with cases recruited through the Veterans Affairs HDL Intervention Trial (VA-HIT) and controls selected from the Framingham Offspring Study (FOS). VA-HIT was designed to examine the benefits of HDL-raising with gemfibrozil in men with low HDL-C ($\leq$40 mg/dl) and prevalent CHD (15). To identify susceptibility loci for the low HDL-C/CHD trait, we examined the allelic variation of 1,114 single-nucleotide polymorphisms (SNP) in 60 candidate genes using cases from VA-HIT before treatment and controls selected from the FOS. We hypothesized that susceptibility to the low HDL-C/CHD trait would be associated with common variants derived from genes involved in HDL metabolism, insulin resistance, and inflammation. We further compared the cases to controls having an HDL-C of $<$ 40 mg/dl, as well as to controls having an HDL-C of $\geq$ 40 mg/dl. These comparisons allowed us to evaluate whether the genetic variants were influencing CHD through HDL-C levels or via alternative mechanisms.

METHODS

Study design

The rationale, design, and methods for VA-HIT have been described elsewhere in detail (15). Briefly, men were recruited at 20 Veterans Affairs (VA) medical centers throughout the United States. Eligibility for the trial required a documented history of CHD (defined as a history of myocardial infarction, angina corroborated by objective evidence of ischemia, coronary revascularization, or angiographic evidence of stenosis greater than 50% of the luminal diameter in one or more major epicardial coronary arteries); age $<$ 74 years; HDL-C level $\leq$ 40 mg/dl (1.0 mmol/l); LDL-C level $\leq$ 140 mg/dl (3.6 mmol/l); plasma triglyceride concentration $\leq$ 300 mg/dl (3.4 mmol/l); and absence of coexisting conditions (cancer, excluding skin; other life-threatening diseases, such as chronic pulmonary or kidney disease; alcohol or substance abuse; cholelithiasis; or marked left ventricular dysfunction). Information on age, alcohol consumption, smoking status, blood pressure, body mass index (BMI), and diabetes was available for all subjects enrolled in VA-HIT. Based on the existence of informed consent for genotyping analysis, 870 subjects from the VA-HIT study were available for the present study as cases. Subjects were self-identified as Caucasian (n = 808) or non-Caucasian race (n = 58) (4 missing information). Data used in our statistical analyses were obtained at baseline, before treatment with gemfibrozil. The characteristics of the cases at baseline, as well as basic demographic information, are provided in **Table 1**.

The Framingham Offspring Study is a longitudinal study designed to identify risk factors for cardiovascular disease in subjects from Framingham, MA (16). A total of 866 males from FOS were selected for use as controls in our case-control analysis. Controls were free of CHD, at least 39 years old at exam six (data collected between 1995 and 1998), and self-reported Caucasian. Plasma lipid and demographic information for the controls at exam six are provided in Table 1.

All participants in both cohorts provided written informed consent. The VA-HIT study was approved by the Human Rights Committee of the Cooperative Studies Program Coordinating Center and by the Institutional Review Board (IRB) of each VA medical center. Study protocols were approved by the IRB for human research at Tufts University, Boston University, and the Broad Institute.

Biochemical analyses

Plasma lipid concentrations were determined from blood samples collected from subjects, after a 12-14 h fast, into tubes containing 0.1% EDTA. Plasma was isolated and frozen for subsequent analysis of plasma lipid, lipoprotein, and apolipoprotein (apo) concentrations. Plasma total cholesterol and triglyceride concentrations were determined using enzymatic assays (17). Plasma HDL-C concentrations were measured after dextran sulfate-magnesium precipitation of apoB-containing lipoproteins (18). LDL-C levels were calculated with the equation of Friedewald et al. (19).

SNP selection and genotyping

Because HDL-C is a complex trait, we considered it important to explore common variation not only in genes whose products directly influence HDL metabolism but also in genes whose products may indirectly influence HDL concentrations. Thus, candidate genes included those associated with diabetes and/or insulin resistance, as well as those involved in inflammatory pathways. As illustrated in **Fig. 1**, many of the candidate genes have been associated with more than one phenotype (i.e., *LIPG* with lipoprotein metabolism and inflammation). For each candidate gene, the entire coding length of the gene (from the first 5′ and 3′ UTR) plus 10 kb upstream and downstream of the gene were downloaded from HapMap for the CEU sample (Genome Build 36, and Version 126 of dbSNP), allowing us to search for variation in the promoter and other regulatory regions. SNPs were selected using the Tagger algorithm (www.broad.mit.edu/mpg/tagger) to identify SNPs (tagSNP) within each gene having a pair-wise $r^2 > 0.9$ (20). Priority was given to SNPs that had higher design scores for Illumina genotyping. A list of all the candidate genes and number of tagSNPs selected in each gene is presented in supplementary Table I. To address potential population structure, 274 ancestry informative markers (AIM) were selected for genotyping (21–23). Inclusion criteria for AIMs included 1) not within 60 base pairs (bp) of another AIM SNP; 2) not in a candidate gene; 3) a minor allele frequency $>$ 0.05; 4) an Illumina design score $>$ 0.6; and 5) an appropriate Illumina validation class.

Genomic DNA was extracted from whole blood samples using either QIAamp mini kits (Qiagen) or Generation Capture Column kits (Gentra Systems). DNA isolated from the case samples was of low concentration and was whole-genome-amplified (WGA) to provide sufficient DNA for genotyping. For consistency,

TABLE 1. Baseline characteristics of subjects

Characteristic	Cases (n = 699)	Controls (n = 705)	Controls HDL < 40 (n = 270)	Controls HDL ⩾ 40 (n = 435)	P^a
Age (years)	64.29 ± 7.2	58.67 ± 8.82	58.8 ± 8.7	58.59 ± 8.9	<0.0001
Age category (years)					<0.0001
⩽50	41 (5.87)	132 (18.72)	45 (16.67)	87 (20.00)	
51–60	133 (19.03)	294 (41.7)	121 (44.81)	173 (39.77)	
61–70	375 (53.65)	201 (28.51)	73 (27.04)	128 (29.43)	
Over 70	150 (21.46)	78 (11.06)	31 (11.48)	47 (10.80)	
Body mass index (kg/m^2)	29.28 ± 4.56	28.47 ± 4.3	29.6 ± 4.4	27.77 ± 4.09	0.0006
Alcohol (>1 drink/day)	44 (6.29)	49 (7.05)	4 (1.5)	45 (10.51)	0.5718
Current smoker	123 (17.6)	99 (14.06)	42 (15.61)	57 (13.1)	0.0697
Diabetic	190 (27.18)	54 (7.66)	29 (10.74)	25 (5.75)	<0.0001
Hypertensive	636 (90.99)	290 (41.19)	125 (46.47)	165 (37.93)	<0.0001
Cholesterol					
Total (mg/dl)	178.75 ± 24.37	201.56 ± 34.2	196.4 ± 34.87	204.77 ± 33.42	<0.0001
LDL (mg/dl)	114.45 ± 22.36	130.11 ± 30.7	128.29 ± 30.62	131.21 ± 30.73	<0.0001
HDL (mg/dl)	31.64 ± 4.99	44.29 ± 11.96	33.03 ± 4.63	51.27 ± 9.54	<0.0001
HDL < 40		270 (38.3)			–[b]
HDL ⩾ 40		435 (61.7)			–[b]
Triglycerides (mg/dl)	163.94 ± 63.86	140.97 ± 101.46	186.06 ± 130.52	112.99 ± 63.95	<0.0001
Blood pressure					
Systolic (mmHg)	131.95 ± 18.48	130.28 ± 16.88	130.78 ± 16.61	129.97 ± 17.06	0.0782
Diastolic (mmHg)	76.64 ± 9.82	77.97 ± 8.96	78.69 ± 8.82	77.52 ± 9.03	0.008
Drug therapy					
Diabetes medication	114 (16.31)	28 (3.97)	18 (6.67)	10 (2.3)	<0.0001
Antihypertensive	570 (81.55)	180 (25.53)	81 (30)	99 (22.76)	<0.0001

Values are either mean ± SD or n (%).
[a]A two sample *t*-test was used to obtain the *P* value for continuous variables, and a chi-square test was used to obtain significance for categorical variables to compare cases (n = 699) to all controls (n = 705).
[b]Cannot perform test due to missing information.

control DNA samples underwent WGA as well. Genotyping was performed using Illumina GoldenGate Bead Array platform with a 1536-SNP OPA (GS0010268-OPA). Sentrix Array Matrix (SAM) fiber-optic bundles manufactured at Illumina using two lots of oligo-beads (beadtype v4 or v5) were used to image the genotypes. The use of different beadtypes is not expected to affect the performance of SNP assays. No duplicated samples were processed on both beadtypes, but allele frequencies were similar between beadtypes. Individuals with a call rate of < 90%, missing phenotype information, or ⩾ 50% genotype data inconsistent with previous genotyping were omitted from analysis (supplementary Table II). A total of 1,404 individuals (cases, n = 699; controls, n = 705) met quality control criteria and were included in the final analysis. SNPs were excluded based on 1) call rate of < 0.90; 2) minor allele frequency (MAF) < 0.05; or 3) Hardy Weinberg Equilibrium (HWE) *P* value in the control sample < 0.001. A total of 1,355 SNPs (1,114 candidate gene SNPs and 241 AIMs) met these quality control criteria and were used in our analysis.

Assessment of population stratification

Population structure, or stratification, is the presence of a systematic difference in allele frequencies between subpopulations in a sample, possibly due to differences in ancestry. Population structure can cause bias in the results of an association study and can lead to false positive findings. The smartpca package in the software EIGENSTRAT (24, 25) was used on the AIMs to determine population structure within the sample. Smartpca performs efficient principal components analysis (PCA) on the genotype matrix and provides principal components (PC) that describe the genetic variability in the sample. A modified PCA (26), described below, was conducted on the study sample plus the four HapMap samples [Utah residents with Northern and Western European ancestry from the CEPH collection (CEU); Yoruba in

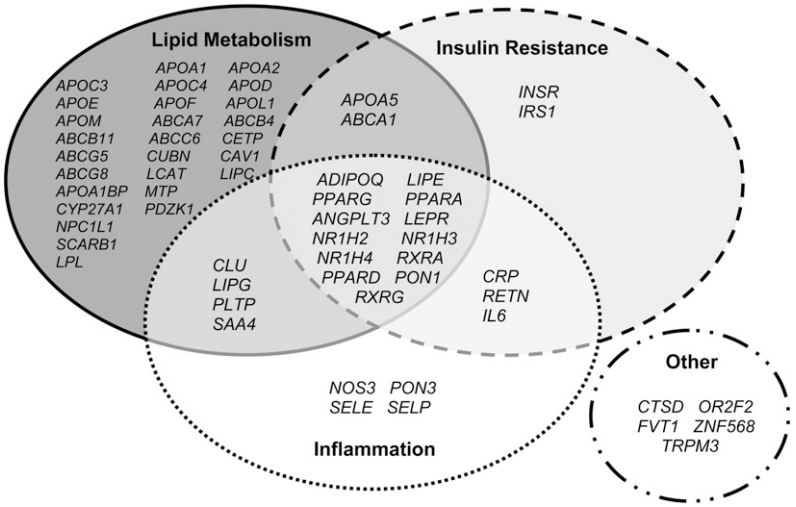

Fig. 1. Candidate genes selected for analysis. Because HDL-C is a complex trait, we explored common variations in genes whose products directly influence HDL metabolism, as well as in genes whose products may indirectly influence HDL concentrations (diabetes, insulin resistance, and inflammation).

Ibadan, Nigeria (West Africa) (YRI); Japanese in Tokyo, Japan (JPT); and Han Chinese in Beijing, China (CHB)] using the selected AIMs. Plotting PC1 versus PC2 shows that some subjects in our sample are near the YRI individuals (**Fig. 2**). Subjects clustering near the YRI sample (PC1 > 0.03), denoted with a nonCaucasian race, or removed based on outlier detection (see below), were excluded from analysis to obtain a homogeneous sample of Caucasian individuals for analysis (supplementary Table II). The rationale for excluding these subjects is based on the fact that, due to a lack of control subjects having a PC1 of > 0.03, we cannot adequately adjust for the nonCaucasian subjects in the cases. Including multiple population groups in a sample without appropriate adjustment leads to an increase in Type I error (27); therefore, only Caucasian individuals were included in the analyses.

A modified PCA on the study subjects was performed (26). A few family members in a sample are known to bias a PCA, and the entire control sample may have extended family members. To take this into account, we performed PCA on the case sample and a subset of unrelated controls based on the full Framingham pedigree file, and then inferred the PCs for the remaining individuals based on the SNP weights. Thirty-three subjects were removed during EIGENSTRAT's default outlier removal process, which iteratively performs PCA, removing subjects that are more than six standard deviations from the mean for the top ten PCs over five iterations.

Statistical analyses

The primary outcome was the combined trait of low HDL-C/CHD. Low HDL-C was defined as HDL-C < 40 mg/dl. Subgroup analysis included comparing 1) low HDL-C/CHD and control subjects with HDL-C < 40 mg/dl; 2) low HDL-C/CHD and control subjects with HDL-C ≥ 40 mg/dl; and 3) control subjects with HDL-C < 40 mg/dl and control subjects with HDL-C ≥ 40 mg/dl. These comparisons allowed us to evaluate whether the genetic variants were influencing CHD through HDL-C levels or via alternative mechanisms. The first subgroup analysis provided a comparison of those with low HDL-C and CHD to those who have neither, whereas the second analysis provided a comparison of those who have CHD versus those without CHD among men with low HDL-C. The intra-cohort comparison within FOS allowed us to identify SNPs associated with HDL-C levels. Three covariate adjustments were made: 1) age and PC1-4 (labeled "MinAdj"); 2) age, PC1-4, body mass index (BMI), systolic blood pressure (SBP), diabetes status, smoking status, and alcohol use ("MultiAdj"); and 3) age, PC1-4, BMI, SBP, diabetes status, smoking status, alcohol use, HDL-C, triglycerides, and total cholesterol ("LipidAdj"). Adjusting for lipid levels allowed us to compare CHD cases to controls, after controlling for the differences in lipid levels. We note that differences in unmeasured exposures are always a concern and recognize this as a limitation of our work. All analyses used logistic regression in PLINK (28) with a general genetic model. A general model was used to avoid power loss due to mis-specifying the genetic model (29). We did not account for family structure in the association analyses, because only extended familial relationships (i.e., cousins) existed among some of the control subjects.

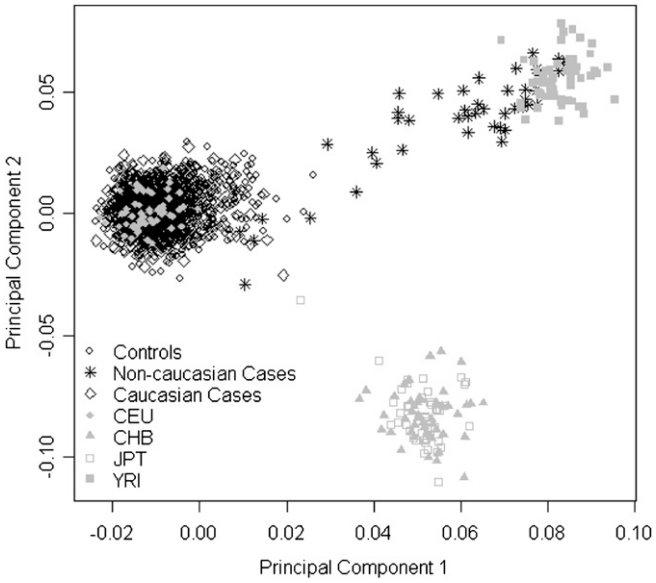

Fig. 2. Plot of PC1 and PC2 from PCA of study subjects and HapMap samples using ancestry informative markers to detect population structure. CEU, Utah residents with Northern and Western European ancestry from the CEPH collection; CHB, Han Chinese in Beijing, China; JPT, Japanese in Tokyo, Japan; YRI, PC, principal component; PCA, principal component analysis; Yoruba in Ibadan, Nigeria.

Statistical significance was determined using an empirical *P* value (AdjP) obtained by a permutation strategy (30) to adjust for testing multiple SNPs within each gene. A null distribution of minimum *P* values was generated based on permuting the outcome phenotype (keeping the corresponding covariate values) and performing the analysis as described above. We did not permute the genotypes to maintain the linkage disequilibrium within the genes intact. The minimum *P* value across all the SNPs in the same gene was documented for 10,000 permutations of the phenotype and compared with the observed *P* value. This approach provides correction for testing multiple SNPs within each gene. To take into account multiple outcomes, we only conducted subgroup analysis for SNPs that were significant in the primary analysis, as an approach to refine the association. False discovery rate (FDR) (31) was calculated in SAS based on the 60 genes (1,114 candidate gene SNPs) analyzed using the observed *P* values.

SNP interaction with age analyses were conducted with SNPs that were significantly different between cases and controls, after adjustment for multiple testing within each gene ($P_{Adj} < 0.05$), and in silico function predictions were obtained (32).

RESULTS

Baseline characteristics of the cases (VA-HIT) and controls (FOS) are presented in Table 1. Significant differences in baseline characteristics were observed between the cases (n = 699) and controls (n = 705). Cases tended to be older and have higher BMI; there are a greater percentage of diabetic and hypertensive subjects relative to controls. Consistent with the selection criteria of VA-HIT, cases also had lower plasma concentrations of total, LDL-, and HDL-cholesterol and higher triglyceride levels compared with controls. Cases were also more likely to be on drug therapy (diabetes medication or antihypertensive medication) than their control counterparts. Note that statistical adjustments were made to account for the differences in these measures when testing for associations between each candidate gene and case status.

Table 2 shows the SNPs that were significantly different between cases and controls, after adjustment for multiple testing within each gene ($P_{Adj} < 0.05$). SNPs significantly associated with case status were located in the

TABLE 2. SNPs significantly associated with low HDL-C/CHD

Chr	Location	Gene	SNP	Major/ Minor Allele	Minor Allele Frequency in VA-HIT	Minor Allele Frequency in FOS	OR_{het} [a]	OR_{hom} [b]	P_{2df} [c]	P_{Adj} [d]	P_{FDR} [e]	$P_{lipidAdj}$ [f]	P_{HWE} [g]
15	56589235	*LIPC*	rs4775065	G/A	0.33	0.23	2.36 (1.83, 3.05)	1.54 (0.82, 2.89)	3.82E-10	< 0.0001	4.26E-07	1.15E-06	0.075
16	55573593	*CETP*	rs5882	A/G	0.32	0.40	0.63 (0.48, 0.82)	0.39 (0.26, 0.60)	1.06E-05	0.0002	5.90E-03	0.0101	0.673
9	136399813	*RXRA*	rs11185660	T/C	0.16	0.24	0.72 (0.55, 0.94)	0.22 (0.10, 0.50)	0.0002	0.0021	0.0623	2.42E-04	0.835
9	106664063	*ABCA1*	rs2249891	A/G	0.14	0.13	0.61 (0.45, 0.85)	2.7 (1.26, 5.94)	0.0003	0.0126	0.0656	3.40E-04	0.235
16	16159501	*ABCC6*	rs150468	A/C	0.13	0.19	0.70 (0.53, 0.92)	0.34 (0.15, 0.73)	0.0017	0.0206	0.1840	0.0500	0.469
10	16945506	*CUBN*	rs7893395	C/T	0.17	0.23	0.58 (0.45, 0.76)	0.77 (0.39, 1.55)	0.0003	0.0246	0.0658	0.0010	0.029
1	159461772	*APOA2*	rs3813627	G/T	0.33	0.34	1.52 (1.18, 1.97)	1.08 (0.73, 1.60)	0.0045	0.0324	0.3119	0.1051	0.065
1	167865878	*SELP*	rs732314	G/A	0.49	0.47	1.71 (1.28, 2.30)	1.43 (1.01, 2.01)	0.0017	0.0376	0.1840	0.1695	0.082
19	50147428	*APOC4*	rs10413089	T/C	0.19	0.19	0.68 (0.52, 0.88)	1.15 (0.59, 2.23)	0.0109	0.0425	0.3900	0.0792	0.282
16	16188277	*ABCC6*	rs212077	C/G	0.14	0.20	0.72 (0.55, 0.93)	0.38 (0.18, 0.81)	0.0036	0.0443	0.2899	0.1555	0.818

Statistical significance defined as a multiple testing-adjusted *P* value (P_{Adj}) < 0.05 (adjusted for testing multiple SNPs in a gene). 2df, two degrees of freedom; ABC, ATP-binding cassette transporter; Apo, apolipoprotein; BMI, body mass index; CETP, cholesteryl ester transfer protein; CUBN, cubilin; FOS, Framingham Offspring Study; FDR, false discovery rate; HWE, Hardy Weinberg Equilibrium; LIPC, hepatic lipase; OR, odds ratio; SBP, systolic blood pressure; RXRA, retinoid X receptor alpha; SELP, P-selectin; SNP, single-nucleotide polymorphism; VA-HIT, Veterans Affairs HDL Intervention Trial.

[a] OR comparing heterozygote genotype to the major allele homozygote genotype (95% CI).

[b] OR comparing the minor allele homozygote genotype to the major allele homozygote genotype (95% CI).

[c] *P* value for a 2df genotypic test of association between the SNP and case status, adjusting for age, PC1-PC4, BMI, SBP, diabetes status, smoking status, and alcohol use.

[d] *P* value adjusted for multiple testing within the gene for a 2df genotypic test of association between the SNP and case status, adjusting for age, PC1-4, BMI, SBP, diabetes status, smoking status, and alcohol use.

[e] FDR *P* value for the observed 2df genotypic test of association between the SNP and case status, adjusting for age, PC1-4, BMI, SBP, diabetes status, smoking status, and alcohol use based on the 60 candidate genes.

[f] *P* value for a 2df genotypic test of association between the SNP and case status, adjusting for age, PC1-4, BMI, SBP, diabetes status, smoking status, alcohol use, HDL-C, triglycerides, and total cholesterol.

[g] *P* value for test of HWE in control subjects.

genes encoding hepatic lipase (*LIPC*; rs4775065; $P < 0.0001$); cholesteryl ester transfer protein (*CETP*; rs5882; $P = 0.0002$); retinoid X receptor α (*RXRA*; rs11185660; $P = 0.0021$); ATP-binding cassette transporter A1 (*ABCA1*; rs2249891; $P = 0.0126$); ATP-binding cassette transporter C6 (*ABCC6*; rs150468 and rs212077; $P = 0.0206$ and $P = 0.0443$); cubilin (*CUBN*; rs7893395; $P = 0.0246$); apolipoprotein A-II (*APOA2*; rs3813627; $P = 0.0324$); P-selectin (*SELP*; rs732314; $P = 0.0376$); and apoC-IV (*APOC4*; rs10413089; $P = 0.0425$). All SNPs in Table 2 were in HWE, and similar results were seen with the minimal adjustment model (results not shown). None of the reported SNPs had a significant interaction with age (all interaction *P* values > 0.37). A model fit with the ten reported variants for the primary outcome of low HDL-C/CHD indicated a generalized-R^2 of 0.14, indicating a 14% improvement over a null model for low HDL-C/CHD, with an area under the curve (AUC) of 0.687. Addition of risk factors (age, BMI, SBP, diabetic status, smoking status, alcohol consumption) to the ten reported variants provides a 37% improvement over the null model with an AUC of 0.810, while a model with just risk factors has a 24% improvement over the null model and an AUC of 0.751. Note, in this context a generalized-R^2 should not be confused with the percent of variability explained in ordinary least square regression.

In addition to comparing CHD cases to all control subjects, we conducted three subgroup analyses, in attempt to determine if the SNP associations with low HDL-C/CHD were acting through the HDL pathway or via other mechanisms. We compared 1) cases (n = 699) to controls with a HDL-C ⩾ 40 mg/dl (n = 435); 2) cases to controls with a HDL-C < 40 mg/dl (n = 270); and 3) controls with a HDL-C < 40 mg/dl to controls with a HDL-C ⩾ 40 mg/dl. Subgroup analysis was only conducted for SNPs that reached the significance threshold in the primary analysis. The results of the subgroup analyses are provided in **Tables 3–5**. All SNP associations identified in the primary analysis remained significant when comparing cases to controls having a HDL-C ⩾ 40 mg/dl.

A 0.10 difference in minor allele frequency between cases and controls was observed for SNP rs4775065 in *LIPC*, with the minor allele having a detrimental effect. Relative to the major allele homozygous genotype, an increased odds ratio (OR) for low HDL-C/CHD was seen in heterozygotes (OR = 2.36; 95% CI: 1.83-3.05), while an OR of 1.54 (95% CI: 0.82-2.89) was observed in minor allele homozygotes. The association remained significant after adjustment for plasma lipid levels ($P = 1.15E-06$). When controls with a HDL ⩾ 40 mg/dl were compared with controls with a HDL-C < 40 mg/dl, we did not find rs4775065

TABLE 3. Comparison of cases (n = 699) to controls with HDL-C ⩾ 40 mg/dl (n = 435)

SNP	Gene	OR_{het} [a]	OR_{hom} [b]	P_{2df} [c]
rs4775065	*LIPC*	2.29 (1.70, 3.09)	1.32 (0.64, 2.71)	2.75E-07
rs5882	*CETP*	0.62 (0.46, 0.86)	0.33 (0.20, 0.54)	2.02E-05
rs11185660	*RXRA*	0.86 (0.63, 1.18)	0.27 (0.11, 0.68)	0.0170
rs2249891	*ABCA1*	0.57 (0.39, 0.82)	2.12 (0.92, 4.92)	0.0013
rs150468	*ABCC6*	0.62 (0.45, 0.85)	0.28 (0.12, 0.65)	0.0004
rs7893395	*CUBN*	0.62 (0.45, 0.84)	0.69 (0.31, 1.53)	0.0076
rs3813627	*APOA2*	1.74 (1.29, 2.35)	1.05 (0.67, 1.65)	0.0009
rs732314	*SELP*	1.90 (1.35, 2.67)	1.76 (1.18, 2.65)	0.0007
rs10413089	*APOC4*	0.64 (0.47, 0.88)	1.03 (0.49, 2.17)	0.0175
rs212077	*ABCC6*	0.59 (0.44, 0.80)	0.33 (0.14, 0.77)	0.0003

2df, two degrees of freedom; ABC, ATP-binding cassette transporter; Apo, apolipoprotein; BMI, body mass index; CETP, cholesteryl ester transfer protein; CI, confidence interval; CUBN, cubilin; HET, heterozygote; HOM, homozygote; LIPC, hepatic lipase; OR, odds ratio; PC, principal component; RXRA, retinoid X receptor alpha; SBP, systolic blood pressure; SELP, P-selectin; SNP, single-nucleotide polymorphism.

[a]OR comparing heterozygote genotype to the major allele homozygote genotype (95% CI).

[b]OR comparing the minor allele homozygote genotype to the major allele homozygote genotype (95%CI).

[c]*P* value for a 2df genotypic test of association between the SNP and case status, adjusting for age, PC1–4, BMI, SBP, diabetes status, smoking status, and alcohol use.

to be significant ($P = 0.886$). However, in comparing cases to controls with low HDL-C, we observed an increase in the odds ratio, indicating that our top SNP in *LIPC* may not be acting through the HDL pathway to modify CHD risk. Likewise, the *RXRA* SNP (rs11185660), which is significantly associated with HDL-C level ($P = 0.0079$), does not appear to modify CHD risk through the HDL pathway but influences both CHD and HDL-C levels.

For rs732314 in *SELP*, the minor allele also had a detrimental effect. An OR of 1.71 (95% CI: 1.28-2.30) for low HDL-C/CHD was seen for heterozygous carriers of the minor allele versus major allele homozygotes, while an OR of 1.43 (95% CI: 1.01-2.01) was observed in minor allele homozygotes. The effect was enhanced when cases were compared with controls with high HDL-C ($OR_{het} = 1.9$; $OR_{hom} = 1.76$) and diminished when cases were compared with controls with low HDL-C ($OR_{het} = 1.52$; $OR_{hom} = 0.99$, respectively). These data suggest that rs732314 in *SELP* may be influencing CHD via the HDL pathway. After adjustment for lipid levels, the association between case status and rs732314 was not significant ($P = 0.1695$) nor was the association comparing controls with low HDL-C to controls with high HDL-C ($P = 0.205$). Similarly, patterns in associations for SNPs in *CETP* (rs5882) and *ABCC6* (rs150468 and rs212077) indicate that these SNPs may also influence CHD risk through their effects on HDL.

For the SNPs in *ABCA1* (rs2249891), *CUBN* (rs7893395), *APOA2* (rs3813627), and *APOC4* (rs10413089), the influence of HDL-C on CHD status is less clear. The associations observed between case status and *ABCA1* and *APOC4* indicate a heterozygote genotype advantage, while the association with *APOA2* indicates a heterozygote disadvantage. In silico functional predictions (32) were seen for the SNPs in *APOA2* and *APOC4*, indicating that these SNPs may regulate transcription by affecting transcription factor binding sites. However, as these findings may be due to gene-gene, allelic, and/or gene-environment interactions, further biological support is required to confirm these results.

Two SNPs in *ABCC6*, rs150468 and rs212077, reached our significance threshold. In the controls, the linkage disequilibrium (LD) measure (D′) between the two SNPs is 0.9 and the r^2 is 0.77, indicating that these two SNPs are capturing the same variant associated with case status. The

TABLE 4. Comparison of cases (n = 699) to controls with HDL-C < 40 mg/dl (n = 270)

SNP	Gene	OR_{het} [a]	OR_{hom} [b]	P_{2df} [c]
rs4775065	*LIPC*	2.38 (1.70, 3.34)	1.86 (0.80, 4.35)	2.83E-06
rs5882	*CETP*	0.66 (0.46, 0.95)	0.49 (0.28, 0.86)	0.0147
rs11185660	*RXRA*	0.59 (0.42, 0.83)	0.18 (0.07, 0.49)	0.0001
rs2249891	*ABCA1*	0.71 (0.47, 1.09)	5.88 (1.31, 26.38)	0.0166
rs150468	*ABCC6*	0.80 (0.55, 1.14)	0.38 (0.14, 1.02)	0.0921
rs7893395	*CUBN*	0.52 (0.37, 0.74)	0.93 (0.35, 2.48)	0.0010
rs3813627	*APOA2*	1.38 (0.98, 1.93)	1.20 (0.70, 2.04)	0.1737
rs732314	*SELP*	1.51 (1.02, 2.24)	0.99 (0.64, 1.55)	0.0403
rs10413089	*APOC4*	0.73 (0.52, 1.04)	1.70 (0.63, 4.56)	0.0976
rs212077	*ABCC6*	0.91 (0.63, 1.30)	0.39 (0.16, 0.98)	0.1254

2df, two degrees of freedom; ABC, ATP-binding cassette transporter; Apo, apolipoprotein; BMI, body mass index; CETP, cholesteryl ester transfer protein; CUBN, cubilin; HET, heterozygote; HOM, homozygote; LIPC, hepatic lipase; OR, odds ratio; PC, principal component; RXRA, retinoid X receptor alpha; SBP, systolic blood pressure; SELP, P-selectin; SNP, single-nucleotide polymorphism.

[a]OR comparing heterozygote genotype to the major allele homozygote genotype (95% CI).

[b]OR comparing the minor allele homozygote genotype to the major allele homozygote genotype (95% CI).

[c]*P* value for a 2df genotypic test of association between the SNP and case status, adjusting for age, PC1–4, BMI, SBP, diabetes status, smoking status, and alcohol use.

TABLE 5. Comparison of controls with HDL-C < 40 mg/dl (n = 270) to controls with HDL-C ≥ 40 mg/dl (n = 435)

SNP	Gene	OR_{het} [a]	OR_{hom} [b]	P_{2df} [c]
rs4775065	*LIPC*	0.96 (0.68, 1.35)	0.82 (0.35, 1.89)	0.8860
rs5882	*CETP*	0.86 (0.58, 1.26)	0.75 (0.44, 1.29)	0.5329
rs11185660	*RXRA*	1.71 (1.22, 2.41)	1.07 (0.51, 2.25)	0.0079
rs2249891	*ABCA1*	0.77 (0.51, 1.15)	0.32 (0.07, 1.49)	0.1697
rs150468	*ABCC6*	0.79 (0.55, 1.14)	0.54 (0.23, 1.26)	0.2002
rs7893395	*CUBN*	1.16 (0.83, 1.63)	0.80 (0.31, 2.12)	0.5868
rs3813627	*APOA2*	1.19 (0.84, 1.68)	0.88 (0.53, 1.48)	0.4414
rs732314	*SELP*	1.05 (0.71, 1.54)	1.44 (0.92, 2.25)	0.2050
rs10413089	*APOC4*	1.00 (0.71, 1.40)	0.77 (0.28, 2.13)	0.8768
rs212077	*ABCC6*	0.72 (0.50, 1.02)	0.79 (0.35, 1.77)	0.1718

2df, two degrees of freedom; ABC, ATP-binding cassette transporter; Apo, apolipoprotein; BMI, body mass index; CETP, cholesteryl ester transfer protein; CUBN, cubilin; HET, heterozygote; HOM, homozygote; LIPC, hepatic lipase; OR, odds ratio; PC, principal component; RXRA, retinoid X receptor alpha; SBP, systolic blood pressure; SELP, P-selectin; SNP, single-nucleotide polymorphism.

[a]OR comparing heterozygote genotype to the major allele homozygote genotype (95% CI).

[b]OR comparing the minor allele homozygote genotype to the major allele homozygote genotype (95% CI).

[c]*P* value for a 2df genotypic test of association between the SNP and case status, adjusting for age, PC1–4, BMI, SBP, diabetes status, smoking status, and alcohol use.

LD for these SNPs in cases is similar to that seen in controls. A model was fit jointly with both *ABCC6* SNPs, and it was found that neither SNP was significant. We encountered colinearity issues when both SNPs were fit in the model, with a variance inflation factor (VIF) of 3.5. Logistic regression values with a VIF greater than 2.5 indicate colinearity. Therefore, a haplotype analysis of the two *ABCC6* SNPs (rs150468:rs212077) was performed. We found that the haplotype with alleles C G (frequency of 16%) was significantly different from the haplotype with alleles A C (frequency of 81%) (*P* value = 8.12E-05). The *ABCC6* haplotype A C had a 56% increase risk of CHD/low HDL-C compared with haplotype C G.

DISCUSSION

Nearly half of patients with CHD have low HDL-C as their primary lipid abnormality. Strong evidence exists for the role of genetics in the determination of HDL-C levels, with approximately 50% of the variation in HDL-C levels in humans genetically determined (11). However, HDL-C is a complex genetic trait, which involves the action of multiple genes that interact with each other and with environmental factors. Therefore, we examined 60 candidate genes with roles in HDL metabolism, insulin resistance, and/or inflammation to identify allelic variants associated with susceptibility to low HDL-C and CHD. Candidate gene studies are cost-effective, with little loss of power for detecting common variants (33). Moreover, a candidate gene approach has inherent biological plausibility for the associations uncovered.

The majority of susceptibility alleles for the low HDL-C/CHD trait identified in our study were located in genes with established roles in HDL metabolism. For instance, we identified susceptibility alleles for low HDL-C and/or CHD risk in the genes encoding *LIPC* (rs4775065), *CETP* (rs5882), *ABCA1* (rs2249891), *CUBN* (rs7893395), and *APOA2* (rs38136627). The strongest association with case status involved a variant in the *LIPC* gene (rs4775065), with cases having a minor allele frequency of 0.33 compared with 0.23 for controls. *LIPC* has consistently been associated with HDL-C in genome-wide studies of lipid traits (34–39) and has also been implicated as a susceptibility locus for CHD (40). The lack of a significant association between *LIPC* variants and HDL-C in the present study may be due to the limited sample sizes in our subgroup analyses, the dichotomizing of HDL-C levels, or the fact that *LIPC* may be influencing CHD risk via a nonHDL-related mechanism.

We also observed a significant association between case status and rs5882, a nonsynonymous SNP (I405V) in the *CETP* gene. The minor allele of this variant has been associated with reduced CETP activity, increased lipoprotein particle size, and exceptional longevity in Ashkenazi Jewish probands (41). Consistent with this finding, the minor allele had a protective effect in the present study and appears to be influencing CHD through via its effects on HDL. The effect was enhanced when cases were compared with controls with high HDL-C (OR_{het} = 0.62; OR_{hom} = 0.33) and diminished when cases were compared with controls with low HDL-C (OR_{het} = 0.66; OR_{hom} = 0.49).

Included among the novel findings of this study are the identification of susceptibility alleles for low HDL-C/CHD in the genes encoding *CUBN* (rs7893395) and *RXRA* (rs11185660), with the minor allele having a protective effect in each case. The cubilin gene encodes a high affinity receptor for endocytosis of HDL-C and lipid-poor apoA-I (42), while *RXRA* has been implicated in altering the risk for Alzheimer's disease through its effects on cholesterol metabolism (43). In silico functional predictions (32) were not found with the associated *CUBN* (rs7893395) or *RXRA* (rs11185660) SNPs or with SNPs in linkage disequilibrium (>0.7) with the associated SNPs.

Although genetic variation in P-selectin (*SELP*) has previously been associated with atherosclerotic risk in European-American adults from the Coronary Artery Risk Development in Young Adults (CARDIA) Study (44), we provide new information that *SELP* may influence CHD risk through its effects on HDL-C. When cases with low

HDL-C and CHD were compared with controls with HDL-C < 40 mg/dl, the association between *SELP* and case status diminished ($P = 0.0403$). Specifically, the odds ratio for the comparison of minor allele homozygotes to major allele homozygotes was reduced from 1.43 (Table 2) to 0.99 (Table 4) when cases were compared with all controls versus controls with HDL-C < 40 mg/dl, respectively. Conversely, when we compared cases to controls with HDL-C ⩾ 40 mg/dl, the association was enhanced ($OR_{hom} = 1.9$, $OR_{het} = 1.76$, $P = 0.0007$). Finally, when we compared controls having a HDL-C < 40 mg/dl to controls having a HDL-C ⩾ 40 mg/dl (Table 5), no association between HDL status and the SNP in *SELP* was observed ($P = 0.2050$). Since the effect increased when low HDL-C/CHD cases were compared with healthy controls (HDL ⩾ 40 mg/dl), this suggests that the variant in *SELP* may be associated with CHD via HDL or that having the minor allele for provides a higher risk of having both CHD and low HDL-C. It is also important to note that the *SELP* SNP associated with increased risk for case status in our study (rs732314) is in strong linkage disequilibrium with rs2235302, a SNP shown to be significantly associated with both soluble P-selectin and carotid intima-media thickness among European-Americans in CARDIA (44). Finally, in silico analysis demonstrated a functional prediction (transcription factor binding site) for rs732314 in *SELP*, suggesting that this SNP is functionally relevant.

To assess whether any of the top risk loci identified in the present study may be related to survival, we determined whether the genotype distribution of the reported SNPs varied by decade of age. We found no evidence of a survival bias in our study. Therefore, the SNPs identified in this study do not appear to be related to survival time but, rather, are likely associated with the development of the combined trait of low HDL-C/CHD. Given that the inability to evaluate survival time is a limitation of our study, further research is required to confirm this finding.

There are additional limitations to our study. The reported novel findings are preliminary in nature. To fully confirm our findings, the results need to be replicated in independent samples and validated through experimentation. Although we adjusted for potential confounding variables in our analyses, we cannot account for potential differences in unmeasured exposures between the cohorts. Furthermore, this study is based on the common variant-common disease hypothesis, which does not allow us to evaluate the contribution of rare alleles to low HDL-C/CHD risk (45). Finally, while we have replicated the findings of some earlier studies (*LIPC*, *CETP*, *ABCA1*, *APOA2*, *ABCC6*, and *APOC4*), other findings were not replicated in the current study (i.e., *APOA5*, *LPL*, *LCAT*, *LIPG*, and *PON1*). *APOA5* and *LPL* have been associated with HDL-C in European and multi-ethnic samples using the Illumina cardiovascular disease (CVD) BeadChip (45–47). The lack of replication may be due to the relatively modest sample size of the present study or to the distinctiveness of the cohort studied.

The VA-HIT cohort, composed of men with low HDL-C, normal LDL-C, and established CHD, affords a unique opportunity to identify genetic variants associated with CHD and low HDL-C. With this cohort, we used a candidate gene approach to identify novel allelic variants in the genes encoding *CUBN* and *RXRA* that are associated with susceptibility to the low HDL-C/CHD trait, and we provide new information that genetic variation in *SELP* may influence CHD risk through its effects on HDL-C. We also replicated data from previous studies through the identification of susceptibility alleles for low HDL-C and/or CHD risk in the genes encoding *LIPC*, *CETP*, *ABCA1*, and *ABCC6*. The findings of the present study may help to identify individuals at increased risk for the development of CHD.

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