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The effects of *ABCG5/G8* polymorphisms on plasma HDL cholesterol concentrations depend on smoking habit in the Boston Puerto Rican Health Study

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Abstract Low HDL-cholesterol (HDL-C) is associated with an increased risk for atherosclerosis, and concentrations are modulated by genetic factors and environmental factors such as smoking. Our objective was to assess whether the association of common single-nucleotide polymorphisms (SNPs) at *ABCG5*/G8 (i18429G>A, i7892T>C, Gln604GluC>G, 5U145A>C, Tyr54CysA>G, Asp19HisG>C, i14222A>G, and Thr400LysC>A) genes with HDL-C differs according to smoking habit. *ABCG5/G8* SNPs were genotyped in 845 participants (243 men and 602 women). *ABCG5/G8* (i7892T>C, 5U145A>C, Tyr54CysA>G, Thr400LysC>A) SNPs were significantly associated with HDL-C concentrations ($P < 0.001$–0.013) by which carriers of the minor alleles at the aforementioned polymorphisms and homozygotes for the Thr400 allele displayed lower HDL-C. A significant gene-smoking interaction was found, in which carriers of the minor alleles at *ABCG5/G8* (Gln604GluC>G, Asp19HisG>C, i14222A>G) SNPs displayed lower concentrations of HDL-C only if they were smokers ($P = 0.001$–0.025). Also, for *ABCG8*_Thr400LysC>A SNP, smokers, but not nonsmokers, homozygous for the Thr400 allele displayed lower HDL-C ($P = 0.004$). Further analyses supported a significant haplotype global effect on lowering HDL-C ($P = 0.002$) among smokers. In conclusion, *ABCG5/G8* genetic variants modulate HDL-C concentrations, leading to an HDL-C-lowering effect and thereby a potential increased risk for atherosclerosis only in smokers.—Junyent, M., K. L. Tucker, C. E. Smith, A. Garcia-Rios, J. Mattei, C-Q. Lai, L. D. Parnell, and J. M. Ordovas. **The effects of *ABCG5/G8* polymorphisms on plasma HDL cholesterol concentrations depend on smoking habit in the Boston Puerto Rican Health Study. *J. Lipid Res.* 2009.** 50: **565–573.**

Supplementary key words interaction • atherosclerosis • reverse cholesterol transport

Low concentrations of HDL-cholesterol (HDL-C) have been associated with an increased risk for coronary heart disease (CHD) (1). Smoking, another major risk factor for CHD, exerts negative effects on plasma lipids, particularly a decrease of HDL-C (2, 3). One of the most likely mechanisms by which low HDL-C promotes atherosclerosis is through the impairment of cholesterol clearance via the reverse cholesterol transport (RCT) pathway (4).

ABCG5 and *ABCG8* are cholesterol half-transporters that function together as a heterodimer (5). Expression of these transporters mediates the efflux of cholesterol and plant sterols from enterocytes back into the intestinal lumen and their excretion into the bile, thus limiting their accumulation in the body and promoting RCT (5, 6). In humans, deleterious mutations in either of these genes cause the genetic disease sitosterolemia (7), characterized by highly elevated plasma plant sterols in blood and tissues, with an increased risk for atherosclerosis and CHD that is independent of plasma cholesterol concentrations (8).

Most of our mechanistic knowledge concerning the role of *ABCG5/G8* genes in lipid metabolism comes from animal models. In mice, *ABCG5/G8* deficiency has been associated with reduced biliary cholesterol secretion and enhanced sterol absorption (9), whereas overexpression of those genes promotes biliary cholesterol secretion, reduces dietary cholesterol absorption, and increases fecal neutral sterol excretion (10). Moreover, it has recently been

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Abbreviations: AIM, ancestral informative marker; BMI, body mass index; BP, blood pressure; CHD, coronary heart disease; FXR, farnesoid X receptor; HDL-C, HDL-cholesterol; LD, linkage disequilibrium; LXR, liver X receptor; RCT, reverse cholesterol transport; SNP, single-nucleotide polymorphism; TG, triglyceride.

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shown that these genes play a key role in the RCT pathway and the prevention of atherosclerosis through their up-regulation by liver X receptor (LXR) agonists (11, 12).

In humans, previous small studies have investigated the effect of several *ABCG5/G8* single-nucleotide polymorphisms (SNPs) on lipids with controversial results (13–18). Some of these studies reported significant associations between *ABCG5/G8* SNPs (Gln604Glu, Thr400Lys, and Tyr54Cys) and total cholesterol and LDL-C, including 154 females undergoing weight loss (13), 112 subjects after consumption of plant stanol esters (14), and 263 mildly hypercholesterolemic patients (17). However, Miwa et al. (15) reported no significant associations with lipids in 100 Japanese patients with hypercholesterolemia. Only two studies in patients with gallstone disease reported significant associations with HDL-C (16) and triglyceride (TG) concentrations (16, 18), but not with total cholesterol and LDL-C, for *ABCG5* Gln604Glu and *ABCG8* Thr400Lys SNPs, respectively. Previously, our group investigated the aforementioned polymorphisms in different populations in relation to LDL-C concentrations, without assessment of potential associations with HDL-C concentrations (19, 20). Therefore, the effect of ABCG5/G8 SNPs on lipids remains to be elucidated.

Among the behavioral factors affecting lipoprotein concentrations, smoking has been consistently reported to decrease HDL-C concentrations. To date, no large population studies have reported interactions between common polymorphisms in *ABCG5/G8* genes, lipid concentrations, and smoking. Given the impact of cigarette smoking on HDL-C concentrations and the relevant role of *ABCG5*/G8 genes in the RCT pathway, the aim of the present study was to assess whether the association between *ABCG5/ G8* polymorphisms and lipids, particularly with HDL-C concentrations, differs depending on smoking habit.

PARTICIPANTS AND METHODS

Participants

The initially estimated sample size for the Boston Puerto Rican Health Study was ∼1,000 participants who were self-identified as Puerto Ricans living in the greater Boston metropolitan area. Adult Puerto Ricans who live on the US mainland have been identified as a vulnerable group at increased risk for age-related chronic diseases. Health disparities affecting a high percentage of this population include diabetes, hypertension, and prior CHD as main risk factors for the development of atherosclerosis. Participants were recruited from the Greater Boston area and surrounding areas, primarily using year 2000 census data to identify high-density blocks containing Hispanics from the target age range. Randomly selected census blocks with 10 or more Hispanics aged 45 years and older were enumerated door to door. Blocks were visited at least three times and up to six times, on different days of the week, weekends, and at varying times of day in an attempt to reach those who were not at home during initial enumeration. Households with at least one eligible adult were identified, and one participant per qualified household was invited to participate.

Complete demographic, biochemical, and genotype data were available in 845 participants (243 men and 602 women, age 58 ± 7 years). Participants aged 45–75 years were recruited from the Boston Center for Population Health and Health Disparities to participate in the Boston Puerto Rican Health Study, a longitudinal cohort study on stress, nutrition, health, and aging (http://hnrcwww.hnrc.tufts.edu/departnebts/labs/prchd/). The design of the study was approved by the Institutional Review Board, and all participants provided informed consent. The detailed design and methodology of the study have been described previously (21).

Data collection

Information on sociodemographics, health status and history, and behavior was collected by home interview administered by bilingual interviewers. CHD was defined as a positive response to the question "Have you ever been told by a physician that you had a heart attack or angina." Anthropometric and blood pressure (BP) measurements were collected using standard methods. Weight was measured with a beam balance and height with a fixed stadiometer. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. BP was measured in duplicate at three points during the interview with an oscillometric device (Dinamap Pro Series 100, GE Medical Systems) while participants were seated and had rested for at least 5 min. Reported systolic and diastolic BP values were the mean of the last two measurement points. Smoking and alcohol intake were determined by questionnaire and defined for this analysis as current versus never or past smoking and alcohol use. Physical activity was estimated as a score based on the Paffenbarger questionnaire of the Harvard Alumni Activity Survey (22). The physical activity score was constructed by weighting time spent in various activities by their respective energy costs. We used a weighted 24 h score of typical daily activity, based on hours spent doing heavy, moderate, light, or sedentary activity as well as sleeping, that was categorized as follows: 0–29, sedentary; 30–39, light activity; 40–49, moderate activity; and >50, heavy activity. Using American Diabetes Association criteria, participants were classified as having diabetes if fasting plasma glucose concentration was ≥126 mg/dl or if use of insulin or diabetes medication was reported.

Laboratory methods

Blood samples were drawn after an overnight fast. Plasma samples were stored and analyzed together. Total cholesterol was measured using a cholesterol esterase-cholesterol oxidase reaction on an Olympus AU400e autoanalyzer (Olympus America, Inc., Melville, NY). HDL-C was measured with the same reaction after precipitation of non-HDL cholesterol with magnesium-dextran and before plasma samples were frozen. LDL-C was measured by use of a homogeneous direct method (LDL Direct Liquid Select Cholesterol Reagent; Equal Diagnostics). TGs were measured by a glycerol-blanked enzymatic method on the Olympus AU400e centrifugal analyzer (Olympus America, Inc.).

Genetic analyses

DNA was extracted from blood samples and purified using commercial Puregene reagents (Gentra Systems) following the manufacturer's instructions. Three *ABCG5* SNPs (i18429G>A, rs4148189; i7892T>C, rs4131229; and Gln604GluC>G, rs6720173) and five *ABCG8* SNPs (5U145A>C, rs3806471; Tyr54CysA>G, rs4148211; Asp19HisG>C, rs11887534; i14222A>G, rs6709904; and Thr400LysC>A, rs4148217) were genotyped. SNPs were selected using two criteria: bioinformatics functional assessment and linkage disequilibrium (LD) structure. Computational analysis of *ABCG5/G8* SNPs (http://www.ncbi.nlm.nih.gov/SNP/buildhistory.cgi) ascribed potential functional characteristics to each variant allele. Given that SNP rs3806471 maps to the 5′

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untranslated region (5′-UTR) of *ABCG8* but also lies approximately 216 bp upstream of the *ABCG5* mRNA start, this SNP sequence was analyzed by MAPPER (23), which identified an allele-specific farnesoid X receptor (FXR) (NR1H4) transcription factor binding site. Intronic SNPs were also analyzed with MAPPER and manually checked for altered mRNA splice donor and acceptor sites and transversions affecting the poly-pyrimidine tract near splice acceptors. Assessing LD structure at the *ABCG5/G8* loci facilitated the selection of tag SNPs representing different LD blocks. In our experience, genotyping more SNPs across such a relatively small genetic region (~60 kbp) is not likely to add value to the phenotype-genotype association analysis. Genotyping was performed using a TaqMan® assay with allele-specific probes on the ABIPrism 7900 HT Sequence Detection System (Applied Biosystems) according to routine laboratory protocols (24). The description of *ABCG5/G8* SNPs, probes, and sequences, as well as ABI assay-on-demand ID, is presented in supplementary Table I.

Statistical analyses

SPSS software (version 15.0) was used for statistical analyses. A logarithmic transformation was applied to measures of plasma TG to normalize the distribution of the data. Data were presented as means ± SD for continuous variables and as frequencies or percentages for categorical variables. Differences in mean values were assessed by ANOVA and unpaired *t*-tests. Categorical variables were compared by using the Pearson χ^2 test or Fisher's exact test. Potential confounding factors were age, sex, BMI, physical activity, smoking habit (current vs. never and past smokers), alcohol consumption (current vs. never and past drinkers), medications (treatment for hypertension, diabetes, hyperlipidemia, and use of hormone therapy by women), and prior CHD. All analyses were further adjusted by population admixture estimated using the program STRUCTURE 2.2 (see below). Potential interactions between *ABCG5/G8* polymorphisms and smoking in determining lipid values (as continuous variables) were tested using the ANOVA test. Two-sided P values <0.05 were considered statistically significant.

LD and haplotype analysis

The pairwise LD between SNPs was estimated as correlation coefficient (R) using the HelixTree software package (Golden Helix). For haplotype analysis, we estimated haplotype frequencies using the expectation-maximization algorithm (25). The major goals of haplotype analysis were to explore the interaction among variants and to increase the power to detect associations between genotypes and phenotypes. In this regard, we selected SNPs on the basis of significant individual associations with the phenotypes to ensure reasonable statistical power. To determine the association between haplotypes and phenotypes, we used haplotype trend regression analysis implemented in HelixTree (26). The regression coefficient (β) determines the effect of the haplotype on the phenotype in which inferred haplotypes are considered as predictors, and the aforementioned confounding factors as covariates. Analyses were adjusted for potential confounders and population admixture (see below). P values were further adjusted for multiple tests by a permutation test involving all possible shufflings of all estimated haplotypes versus all phenotypes under a null hypothesis. The permutation P value gave the probability that the significant P value was not observed simply by chance in this study.

Population admixture

Population admixture was estimated based on the genotypes of 100 ancestral informative markers (AIMs) using two programs: STRUCTURE 2.2 (27) and IAE3CI (28), with reference to three ancestral populations: European settlers, native Taíno Indians, and West Africans (29). The existence of genetic subgroups or substructure in a population may lead to spurious associations. To estimate individual ancestry, several panels of AIMs have been developed for Hispanic populations. For the Puerto Rican population, a panel of 100 AIMs was found to be necessary to properly estimate ancestral proportions by using a combination of simulated and applied data (28). Using the estimated admixture of each subject as a covariate, we adjusted for population admixture in all statistical analyses.

RESULTS

Characteristics of the participants and genotype frequencies by smoking status are shown in **Table 1**. Smokers were younger and had lower BMI than nonsmokers. As expected, smokers displayed lower HDL-C and higher TG concentrations than nonsmokers. Smokers were more likely to drink alcohol and less likely to receive treatment

TABLE 1. Demographic, biochemical, and genotypic characteristics of participants by smoking status

	Nonsmokers (n = 640)	Smokers (n = 205)	*P*
Age, years	58 ± 7.3	56 ± 6.9	<0.001
Body mass index, kg/m^2	32.6 ± 6.5	29.6 ± 6.3	<0.001
Systolic blood pressure, mmHg	136 ± 18.0	135 ± 20.8	0.723
Diastolic blood pressure, mmHg	81 ± 10.6	82 ± 11.3	0.100
Total cholesterol, mg/dl	184 ± 42.2	183 ± 42.5	0.452
LDL cholesterol, mg/dl	108 ± 34.9	105 ± 34.7	0.219
HDL cholesterol, mg/dl	46 ± 12.4	43 ± 13.7	0.026
Log (triglyceride, mg/dl)	2.14 ± 0.22	2.19 ± 0.25	0.009
Cigarettes/day	0	11 ± 9	<0.001
Current drinkers, n (%)	208 (33)	110 (54)	<0.001
On diabetes treatment, n (%)	248 (39)	61 (30)	0.020
On hypertension treatment, n (%)	349 (55)	89 (43)	0.006
On lipid-lowering treatment, n (%)	147 (23)	30 (15)	0.010
On hormone treatment, n (%)	292 (46)	65 (32)	<0.001
Prior coronary heart disease, n (%)	136 (21)	32 (16)	0.087
ABCG5/G8 polymorphisms, n (%)			
*ABCG5*_i18429G>A			
GG	353 (55)	106 (52)	0.421
GA+AA	287 (45)	99 (48)	
*ABCG5*_i7892T>C			
TT	302 (47)	94 (46)	0.748
TC+CC	338 (53)	111 (54)	
*ABCG5*_Gln604GluC>G			
CC	374 (58)	108 (53)	0.168
CG+GG	266 (42)	97 (47)	
*ABCG8*_5U145A>C			
AA	300 (47)	99 (48)	0.748
AC+CC	340 (53)	106 (52)	
*ABCG8*_Tyr54CysA>G			
AA	322 (50)	100 (49)	0.748
AG+GG	318 (50)	105 (51)	
*ABCG8*_Asp19HisG>C			
GG	559 (87)	178 (87)	0.904
CG+CC	81 (13)	27 (13)	
*ABCG8*_i14222A>G			
AA	445 (70)	134 (65)	0.263
AG+GG	195 (31)	71 (35)	
*ABCG8*_Thr400LysC>A			
CC	388 (61)	126 (62)	0.870
CA+AA	252 (39)	79 (39)	

All values are mean ± SD.

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for diabetes, hypertension, hyperlipidemia or, for women, hormone replacement therapy, than were nonsmokers. No significant differences in other variables examined were observed. Analysis of these characteristics did not differ significantly by sex (see supplementary Tables II and III). Given the higher prevalence of men who reported smoking compared with women (33% vs. 21%, $P < 0.001$), all performed statistical analyses were adjusted by sex.

For all *ABCG5/G8* polymorphisms, there was no departure from Hardy-Weinberg equilibrium ($P > 0.05$). The pairwise LD in correlation coefficients of all eight SNPs is presented in supplementary Table IV. Given that all pairwise LDs were <0.80, all eight SNPs were retained for further analysis. Because of low genotype frequencies of individuals homozygous for the minor alleles, and because the analysis did not suggest a recessive mode of action, we analyzed all SNPs using two genotype categories. Considering the homogeneity of the effect observed by sex for all variables examined, men and women were pooled together for subsequent analyses.

We examined associations between *ABCG5/G8* SNPs and lipids (**Table 2**). For the *ABCG5*_i7892T>C SNP, C allele carriers had lower HDL-C than TT participants ($P = 0.013$). Lower HDL-C concentrations were also observed in carriers of the minor alleles at *ABCG8* (5U145A>C and Tyr54CysA>G) SNPs ($P < 0.001$ for both) and homozygotes for the major allele at *ABCG8*_Thr400LysC>A SNP ($P = 0.012$). For *ABCG8* (Asp19HisG>C and 14222A>G) SNPs, carriers of the minor alleles displayed lower LDL-C concentrations than did those homozygous for the major alleles ($P = 0.016$ and $P = 0.046$, respectively). No other significant associations were found between these SNPs and other lipid variables.

To understand the combined effects of genetic variants at *ABCG5/G8*, we conducted haplotype analysis using a subset of *ABCG5/G8* SNPs according to their association with the phenotypes as individual variants. We selected four ABCG5/G8 SNPs (i7892T>C, 5U145A>C, Tyr54CysA>G, and Thr400LysC>A) significantly associated with HDL-C concentrations as individual variants. There were seven haplotypes with frequencies ranging from 2% to 36% accounting for 99% of all haplotypes in this population (**Table 3**). After adjustment for covariates, *ABCG5/G8* haplotypes were significantly associated with HDL-C concentrations (global significance, $P = 0.005$). Based on a permutation test, the probability for observing such association was $P = 0.007$. For individual haplotypes, carriers of the haplotype C-C-G-C showed significantly lower HDL-C concentrations ($\beta = -3.43$, $P = 0.002$), whereas carriers of T-A-A-A and C-A-A-C exhibited significantly higher HDL-C concentrations ($\beta = 1.94, 5.25$; $P = 0.014, 0.012$, respectively). Haplotype T-C-A-C was associated with lower HDL-C concentrations when compared with all other haplotypes combined; however, the association did not reach statistical significance ($P = 0.073$).

We next examined plasma lipid concentrations by genetic variation at *ABCG5/G8* in a stratified analysis by smoking status (see supplementary Table V). A significant interaction between *ABCG5*_Gln604GluC>G SNP and smoking was found for HDL-C ($P = 0.009$), in which smokers with G alleles displayed lower values than CC participants (40.4 ± 11.5 mg/dl vs. 44.1 ± 14.1 mg/dl; $P = 0.024$), whereas no significant differences were seen in nonsmokers (46.6 ± 12.9 mg/dl for G allele carriers vs. 45.4 ± 12.2 mg/dl for CC; $P > 0.2$) (**Fig. 1**). A significant interaction between *ABCG8*_Asp19HisG>C SNP and smoking was found for HDL-C ($P = 0.025$), in which C allele carriers showed a trend toward lower values in smokers (38.4 ± 16.6 mg/dl vs. 43.0 ± 12.3 mg/dl; $P = 0.113$) and higher concentrations in nonsmokers (47.4 ± 14.1 mg/dl vs. 45.6 ± 12.3 mg/dl; $P = 0.189$) compared with GG participants (Fig. 1). A significant interaction between *ABCG8*_i14222A>G SNP and smoking was found for total cholesterol and HDL-C concentrations ($P = 0.028$ and $P = 0.001$, respectively), in which smoking G allele carriers displayed lower values than AA participants for total cholesterol (169.9 ± 36.5 mg/dl vs. 186.4 ± 44.2 mg/dl; $P = 0.005$) and HDL-C (39.5 ± 11.0 mg/dl vs. 43.9 ± 13.8 mg/dl; $P = 0.028$), whereas nonsmoking G allele carriers displayed similar

TABLE 2. Associations between *ABCG5/G8* SNPs and fasting lipid profiles

*ABCG5*_i18429G>A	GG (n = 459)	GA+AA (n = 386)	*P*
Total cholesterol	183.1 ± 43.6	184.2 ± 40.8	0.971
LDL cholesterol	106.6 ± 35.7	107.2 ± 33.9	0.807
HDL cholesterol	45.0 ± 12.8	45.0 ± 12.5	0.936
Log triglycerides	2.15 ± 0.23	2.14 ± 0.23	0.605
*ABCG5*_i7892T>C	TT (n = 396)	TC+CC (n = 449)	*P*
Total cholesterol	183.1 ± 43.4	184.1 ± 41.3	0.734
LDL cholesterol	105.8 ± 35.9	107.8 ± 34.0	0.391
HDL cholesterol	46.1 ± 13.7	44.0 ± 11.7	**0.013**
Log triglycerides	2.14 ± 0.24	2.15 ± 0.22	0.541
*ABCG5*_Gln604GluC>G	CC (n = 482)	CG+GG (n = 363)	*P*
Total cholesterol	183.1 ± 43.7	184.2 ± 40.5	0.698
LDL cholesterol	105.8 ± 34.6	108.3 ± 35.2	0.304
HDL cholesterol	45.0 ± 12.6	45.0 ± 12.8	0.966
Log triglycerides	2.15 ± 0.24	2.15 ± 0.22	0.970
*ABCG8*_5U145A>C	AA (n = 399)	AC+CC (n = 446)	*P*
Total cholesterol	185.2 ± 43.0	182.2 ± 41.7	0.299
LDL cholesterol	107.5 ± 35.9	106.4 ± 34.0	0.631
HDL cholesterol	46.6 ± 13.8	43.6 ± 11.4	**<0.001**
Log triglycerides	2.14 ± 0.24	2.15 ± 0.23	0.392
*ABCG8*_Tyr54CysA>G	AA (n = 422)	AG+GG (n = 423)	*P*
Total cholesterol	184.0 ± 41.8	183.2 ± 42.8	0.773
LDL cholesterol	106.4 ± 35.3	107.4 ± 34.5	0.661
HDL cholesterol	46.6 ± 13.6	43.4 ± 11.4	**<0.001**
Log triglycerides	2.14 ± 0.24	2.16 ± 0.22	0.248
*ABCG8*_Asp19HisG>C	GG (n = 737)	CG+CC (n = 108)	*P*
Total cholesterol	184.8 ± 42.2	175.6 ± 42.1	**0.029**
LDL cholesterol	108.0 ± 35.3	99.5 ± 30.8	**0.016**
HDL cholesterol	45.0 ± 12.3	45.2 ± 15.0	0.857
Log triglycerides	2.15 ± 0.23	2.14 ± 0.24	0.691
*ABCG8*_i14222A>G	AA (n = 579)	AG+GG (n = 266)	*P*
Total cholesterol	185.2 ± 42.6	180.2 ± 41.5	0.097
LDL cholesterol	108.5 ± 35.8	103.5 ± 32.7	**0.046**
HDL cholesterol	44.8 ± 12.7	45.4 ± 12.5	0.463
Log triglycerides	2.15 ± 0.23	2.14 ± 0.23	0.436
*ABCG8*_Thr400LysC>A	CC (n = 514)	CA+AA (n = 331)	*P*
Total cholesterol	184.1 ± 42.1	182.8 ± 42.7	0.645
LDL cholesterol	107.0 ± 35.0	106.7 ± 34.7	0.916
HDL cholesterol	44.2 ± 12.4	46.3 ± 13.0	**0.012**
Log triglycerides	2.16 ± 0.22	2.12 ± 0.24	**0.018**

Values are mean ± SD. *P* values were adjusted for age, sex, body mass index (BMI), physical activity, smoking habit, alcohol consumption, medications, prior coronary heart disease, and population admixture. Boldface type indicates statistically significant ($P < 0.05$).

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TABLE 3. Associations between *ABCG5/G8* haplotypes and plasma HDL-C concentrations

Haplotype	Polymorphism				Frequency[a]	Coefficient β^b	*P*
	*G5*_i7892T>C	*G8*_5U145A>C	*G8*_Tyr54CysA>G	*G8*_Thr400LysC>A			
H1	T	A	A	C	0.36	−0.53	0.766
H2	C	C	G	C	0.24	−3.43	**0.002**
H3	T	A	A	A	0.22	1.94	**0.014**
H4	C	A	A	C	0.06	5.25	**0.012**
H5	T	C	A	C	0.06	−3.47	0.073
H6	T	A	G	C	0.03	1.18	0.805
H7	T	C	G	C	0.02	−1.89	0.510

HDL-C, HDL-cholesterol; *G5, ABCG5; G8, ABCG8. P* values were adjusted for age, sex, BMI, physical activity, smoking habit, alcohol consumption, medications, prior coronary heart disease, and population admixture. These haplotypes showed global association with HDL-C levels at $P = 0.007$ after permutation correction for multiple tests. Boldface type indicates statistically significant ($P < 0.05$).

[a] Haplotype frequencies were estimated using the expectation-maximization algorithm (25).

[b] Coefficients and *P* values were estimated based on haplotype trend regression analysis implemented in the HelixTree program.

values for total cholesterol (183.7 ± 42.9 mg/dl vs. 185.0 ± 42.2 mg/dl; $P > 0.6$) and higher concentrations for HDL-C (47.5 ± 12.5 mg/dl vs. 45.1 ± 12.4 mg/dl; $P = 0.019$) than AA participants (Fig. 1). A significant interaction between *ABCG8*_Thr400LysC>A SNP and smoking was also found for HDL-C concentrations ($P = 0.004$), in which, among smokers, those carrying the CC genotype displayed lower values than A allele carriers (39.9 ± 11.0 mg/dl vs. 46.2 ± 15.0 mg/dl; $P < 0.001$), whereas similar values were found in nonsmokers (45.6 ± 12.6 mg/dl vs. 46.3 ± 12.3 mg/dl; $P > 0.4$) regardless of the genotype (Fig. 1). Importantly, these interactions remained significant after further adjustment for TG (*P* values ranging from <0.001 to 0.034) (data not shown). No significant gene-smoking interactions were found in other examined SNPs (see supplementary Table V).

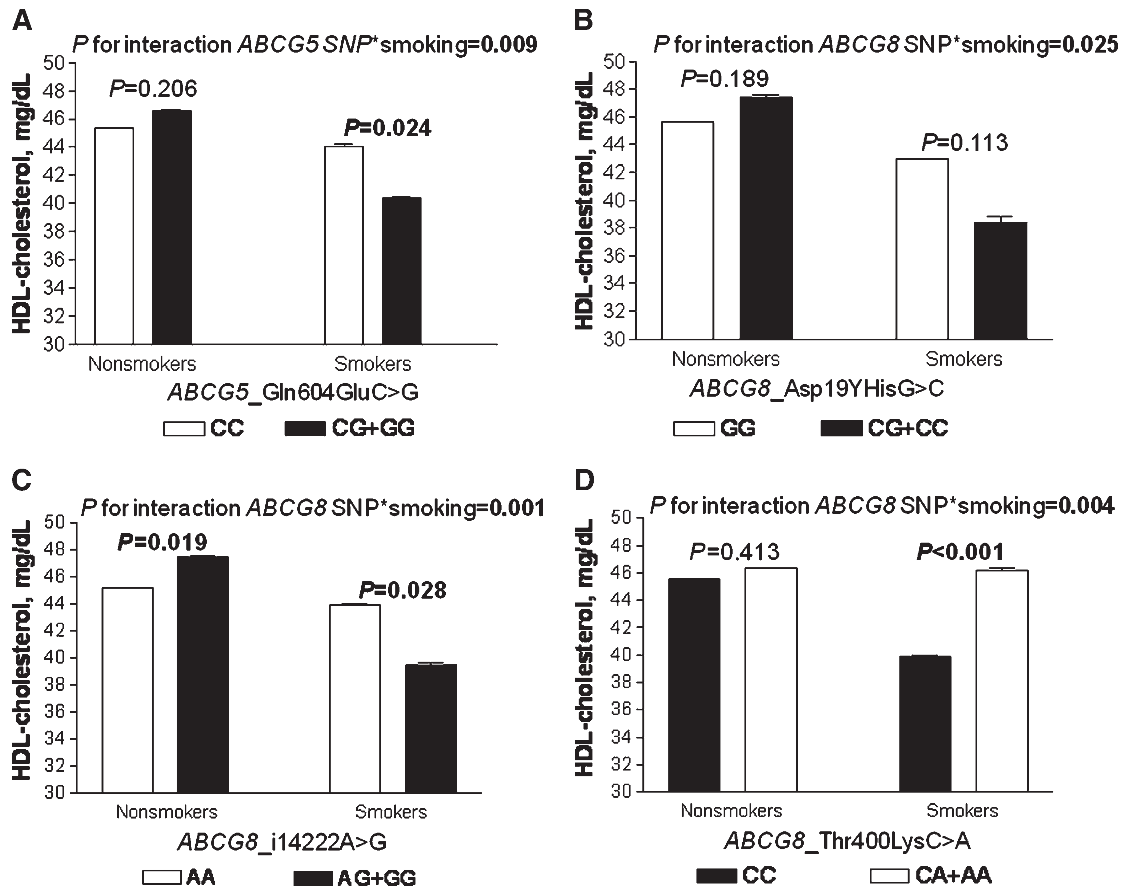

Fig. 1. Age, sex, body mass index, physical activity, alcohol consumption, medications, prior coronary heart disease, and population admixture adjusted HDL-cholesterol (HDL-C) (in mg/dl) concentrations depending on *ABCG5*_Gln604GluC>G (A), *ABCG8*_Asp19HisG>C (B), *ABCG8*_i14222A>G (C), and *ABCG8*_Thr400LysC>A (D) polymorphisms and smoking status. Values are mean ± SD. Probability values were obtained in the multivariate models in predicting differences in HDL-C concentrations for the analyzed polymorphisms.

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We selected the aforementioned *ABCG5/G8* SNPs (Gln604GluC>G, Asp19HisG>C, i14222A>G, and Thr400LysC>A) for haplotype analysis in relation to smoking status (**Table 4**). In smokers, there were eight haplotypes with frequencies ranging from 2% to 47% accounting for 99% of all haplotypes. These ABCG5/G8 haplotypes were significantly associated with HDL-C concentration before and after the permutation test (P = 0.002 for both). For individual haplotypes, carriers of the haplotypes G-C-G-C, C-C-G-C, and C-G-A-A showed significantly lower HDL-C (P = 0.010 for all). Haplotype G-G-A-C was associated with lower HDL-C when compared with noncarriers of such haplotypes; however, the association did not reach statistical significance (P = 0.078). Among nonsmokers, there were seven haplotypes with frequencies ranging from 2% to 45%, which were significantly associated with HDL-C concentrations (P = 0.049). After a permutation test, this probability became marginally significant (P = 0.056). For individual haplotypes, carriers of the haplotype C-G-A-C showed significantly lower HDL-C relative to noncarriers (P = 0.020). Although haplotypes C-G-G-C and G-G-G-C were associated with lower HDL-C, those associations were marginally significant (P = 0.054 and P = 0.088, respectively).

DISCUSSION

This study is the first to provide evidence that genetic variation at the *ABCG5/G8* genes modulates plasma HDL-C concentrations depending on smoking habit. The results indicate that carriers of the minor alleles at the *ABCG5/G8* (Gln604GluC>G, Asp19HisG>C, and i14222A>G) SNPs displayed lower HDL-C concentrations than homozygotes for the major alleles only in smokers. A significant gene-smoking interaction was also found for the *ABCG8*_Thr400LysC>A SNP, in which individuals homozygous for the major allele showed lower HDL-C compared with minor allele carriers, if they were smokers. Therefore, carriers of the minor alleles at the aforementioned *ABCG5/G8* SNPs and homozygotes for the Thr400 allele at the *ABCG8* gene exhibit an interaction with smoking that lowers plasma HDL-C concentrations, whereas homozygotes for the major alleles and carriers of the 400Lys allele are resistant to smoking-induced decreases in HDL-C concentrations. Interestingly, the specific *ABCG5/G8* haplotype G-C-G-C was significantly related to the lowest HDL-C concentration in smokers, supporting the reported associations as individual variants.

To date, no large population studies examining the association between genetic polymorphisms at *ABCG5/G8* genes and lipid concentrations have been reported. The present study found a significant association between two common *ABCG8* (Asp19HisG>C and i14222A>G) SNPs and LDL-C concentrations. In contrast to Hubácek et al. (13) and in agreement with Santosa et al. (19) and Gylling et al. (17), we found lower plasma LDL-C concentrations in carriers of the 19His allele compared with homozygotes for the Asp19 allele. Santosa et al. (19) also examined the effect of *ABCG8*_i14222A>G SNP on lipids in 35 young women with mild hypercholesterolemia but found no significant associations with LDL-C concentrations. These discrepancies may be due to small sample size, as well as to differences in age, sex, cholesterol concentrations, and lifestyle across populations.

Despite the lesser studied effect of *ABCG5/G8* genes on HDL-C, the key role of these genes in the last steps of the RCT pathway (11, 12) supports their involvement in HDL metabolism. We found significant associations between

TABLE 4. Associations between *ABCG5/G8* haplotypes and plasma HDL-C levels according to smoking status

	Polymorphism						
	*G5*_Gln604GluC>G	*G8*_Asp19HisG>C	*G8*_i14222A>G	*G8*_Thr400LysC>A	Frequency[a]	Coefficient β[b]	P
Smokers							
H1	C	G	A	C	0.47	−18.37	0.699
H2	G	G	A	C	0.15	−24.63	0.078
H3	C	G	A	A	0.15	−14.84	**0.010**
H4	C	G	G	C	0.08	−20.17	0.894
H5	G	G	A	A	0.05	−12.95	0.176
H6	C	C	G	C	0.04	−34.65	**0.010**
H7	G	G	G	C	0.03	−32.08	0.125
H8	G	C	G	C	0.02	−44.62	**0.010**
Nonsmokers							
H1	C	G	A	C	0.45	−9.16	**0.020**
H2	C	G	A	A	0.16	−7.13	0.825
H3	G	G	A	C	0.15	−8.45	0.763
H4	C	G	G	C	0.09	−3.92	0.054
H5	G	G	A	A	0.06	−6.21	0.434
H6	C	C	G	C	0.05	−12.53	0.405
H7	G	G	G	C	0.02	−1.56	0.088

G5, ABCG5; G8, ABCG8. P values were adjusted for age, sex, BMI, physical activity, alcohol consumption, medications, prior coronary heart disease, and population admixture. These haplotypes showed global association with HDL-C concentrations at P = 0.002 for smokers and P = 0.056 for nonsmokers after permutation correction for multiple tests. Boldface type indicates statistically significant (P < 0.05).

[a] Haplotype frequencies were estimated using the expectation-maximization algorithm (25).

[b] Coefficients and P values were estimated based on haplotype trend regression analysis implemented in the HelixTree program.

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ABCG5/G8 (i7892T>C, 5U145A>C, Thr54CysA>G, and Thr400LysC>A) SNPs and HDL-C concentrations, in which carriers of the minor alleles at these polymorphisms and homozygotes for the Thr400 allele displayed lower HDL-C. Consistent with these findings, the C-C-G-C haplotype, representing 24% of observed haplotypes, was significantly associated with an HDL-C-lowering effect (approximately three units), whereas a significant increase in HDL-C (approximately two units) was related to the T-A-A-A haplotype, representing 22% of observed haplotypes. These findings further support the functional importance of those four *ABCG5/G8* SNPs. Several small previous studies have ascertained HDL-C concentrations by genetic variation at *ABCG5/G8* genes (14–18). In contrast to the present study, they did not find significant associations between the Thr400 allele or Thr54 allele and HDL-C. Similarly, Acalovschi et al. (16) reported low HDL-C concentrations in carriers of the Gln604 allele, whereas similar values for common and rare alleles were observed in the present study. The present study is the first to provide consistent and compelling evidence of the involvement of *ABCG5/G8* genes in HDL metabolism in a relatively large population. The mechanism underlying the modulation of genetic variants at *ABCG5/G8* genes on HDL-C concentrations is undefined. One SNP, *ABCG8*_5U145A>C, maps within a transcription factor binding motif for FXR, as reported in Methods, and it is therefore possible that binding of this transcription factor in an allele-specific manner may upregulate *ABCG5/G8* expression and, thus, potentially counteract the accelerated loss of cholesterol from the body with a compensatory increase in HDL-C synthesis. SNP *ABCG8*_5U145A>C, within the 5′-UTR, may exert effects on mRNA folding and interaction with ribosomes. SNP *ABCG5*_i7892T>C, within intron 4, could alter either mRNA splicing or control of gene expression. Finally, SNPs *ABCG8*_Tyr54CysA>G and *ABCG8*_Thr400LysC>A alter the protein sequence, affecting protein structure, and thereby, ABCG8 function. Although these four SNPs have shown the observed associations, it is possible that these are not functional SNPs, but are in strong LD with other variants responsible for the associations observed here.

To our knowledge, this is the first study to demonstrate an interaction between common genetic variants at *ABCG5/G8* genes and smoking habit. Moreover, the haplotype effect appeared to be modulated by smoking habit. Among smokers, there were three haplotypes associated with an HDL-C-lowering effect (C-G-A-A, C-C-G-C, and G-C-G-C). Interestingly, the last haplotype was significantly related to the lowest HDL-C concentration in smokers, supporting the reported associations as individual variants. Among nonsmokers, the haplotype C-G-A-C was associated with lower HDL-C. However, the mechanism underlying the observed interactions is unknown. In mice, *ABCG5/G8* expression is regulated by several transcription factors, such as the LXR and the FXR, as well as their interaction (30–32). The reported presence of the allele-specific FXR (NR1H4) in the *ABCG8*_5U145A>C SNP, as described above, supports that *ABCG5/G8* transcription may be regulated through the LXR pathway. In mice, global LXR activation by synthetic agonists (T0901317 and GW3965) has been shown to increase plasma HDL-C concentrations, mediated by the upregulation of *ABCA1* (31–35), and to inhibit atherosclerosis, through the promotion of direct intestinal transport of HDL into the lumen by *ABCG5/G8* (11, 12, 32, 36, 37). On the basis of these observations, we can hypothesize that the expression of these genes may be downregulated in smokers with low HDL-C, through an inactivation of the LXR pathway.

Interestingly, a compensatory increase in HDL-C synthesis secondary to its accelerated loss from the body has been associated with *ABCG5/G8* overexpression (12). Despite discrepancies among studies, it has also been reported that smoking decreases the activity of other genes related to HDL metabolism, such as LCAT, cholesterol ester transfer protein, and HL (38–42). Taken together, these data support a potential downregulation of *ABCG5/G8* by smoking through an impaired RCT, resulting in low HDL-C concentrations.

As a novelty, the present study suggests that polymorphic variation within the *ABCG5/G8* genes is associated with inter-individual variation in HDL-C, suggesting their potential key role in atherosclerosis risk. Although there are no studies in humans examining the association between *ABCG5/G8* expression and atherosclerosis, several results from animal models are consistent with an anti-atherogenic effect associated with the overexpression of these genes (43, 44). On the basis of these data, we may surmise that smoking, as a major risk factor for atherosclerosis, downregulates their expression.

Although the Paffenbarger questionnaire is a well-validated questionnaire (22) designed to assess leisure physical activity, it has not been validated in our study population. Therefore, a limitation of the current analysis is that the activity score derived from this questionnaire may not accurately reflect its intended measurement. Second, the prevalence of CHD was self-reported, and this may be subject to recall bias. However, several previous large-scale epidemiological studies in the general population have used self-reported CHD to assess its association with low HDL-C and other conditions (21, 45). Another limitation was the lack of measurement of HDL particle size, which may help us to evaluate in more depth the potential mechanisms involved in the reported gene-smoking interactions. Finally, given that *ABCG5/G8* have been related to intestinal sterol absorption (14, 46), measurement of plasma plant sterol concentrations might be useful in understanding the underlying mechanisms responsible for the observed effects. Moreover, replication in other ethnic populations, particularly those with low HDL-C concentrations, is clearly warranted.

In conclusion, the present study demonstrates an interaction between common variants in *ABCG5/G8* genes and smoking on plasma HDL-C concentrations. Understanding the effects of these *ABCG5/G8* SNPs on HDL-C could help to modulate the risk of atherosclerosis in the general population, particularly in smokers. Moreover, recognition of these gene-smoking interactions offers the potential to identify lifestyle changes that, when implemented, may obviate the risk of CHD associated with specific *ABCG5/G8*

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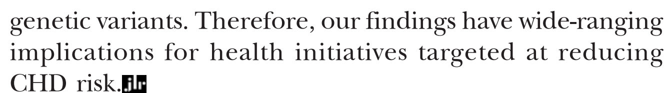

genetic variants. Therefore, our findings have wide-ranging implications for health initiatives targeted at reducing CHD risk.

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