Associations of *LPL* and *APOC3* gene polymorphisms on plasma lipids in a Mediterranean population: interaction with tobacco smoking and the *APOE* locus

Dolores Corella,* Marisa Guillén,* Carmen Sáiz,* Olga Portolés,* Antonio Sabater,* José Folch,* and Jose M. Ordovas[1,*,†]

Genetic and Molecular Epidemiology Unit,* Department of Preventive Medicine and Public Health, University of Valencia, Valencia, Spain; and JM-USDA Human Nutrition Research Center on Aging at Tufts University,† Boston, MA

Abstract We conducted a cross-sectional study in a Spanish population (n = 1,029) to investigate associations between the *LPL* and *APOC3* gene loci (*LPL-Hind*III, *LPL*-S447X, and *APOC3-Sst*I) and plasma lipid levels and their interaction with *APOE* polymorphisms and smoking. Carriers of the H^- or the X447 allele had higher levels of HDL cholesterol (HDL-C), and lower levels of TG, after adjustment for age, body mass index, alcohol, smoking, exercise, and education ($P < 0.01$). The *APOC3* polymorphism presented additive effects to the *LPL* variants on TG and HDL-C levels in men, and on TG in women. The most and the least favorable haplotype combinations were H^-/X447/S1 and H^+/S447/S2, respectively. These combinations accounted for 7% and 5% of the variation in HDL-C and TG in men, and 3% and 4% in women. There was a significant interaction between *APOE* and *LPL* variants and HDL-C levels in both genders ($P < 0.05$). The increases in HDL-C observed for the rare alleles were higher in ε4 than in ε3 subjects, and absent in ε2 individuals. This effect was modulated by smoking (interaction *Hind*III-*APOE*-smoking, $P = 0.019$), indicating that smoking abolished the increase in HDL-C levels observed in ε4/H^- subjects. Understanding this gene-gene-environmental interaction may facilitate preventive interventions to reduce coronary artery disease risk.—Corella, D., M. Guillén, C. Sáiz, O. Portolés, A. Sabater, J. Folch, and J. M. Ordovas. **Associations of *LPL* and *APOC3* gene polymorphisms on plasma lipids in a Mediterranean population: interaction with tobacco smoking and the *APOE* locus. *J. Lipid Res.* 2002.** 43: **416–427.**

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The etiology of coronary artery disease (CAD) involves complex interactions between genetic and environmental factors (1, 2). Therefore, an approach to dissecting individual CAD risk is to examine specific intermediate phenotypes to identify the underlying factors. Plasma TG and HDL cholesterol (HDL-C) levels are now considered established risk factors for CAD (3). Therefore, association of common gene variants at candidate genes with changes in TG and HDL-C levels might be important determinants of CAD risk. The *LPL* gene represents one such gene (4, 5). LPL is a multifunctional protein that hydrolyses core TG from circulating chylomicrons and VLDL, which are then either degraded by the liver or converted to LDL particles by hepatic lipase (6, 7). During this process, surface free cholesterol and phospholipids are transferred to HDL particles, increasing the concentration of HDL-C (8). Numerous sequence variants within the *LPL* gene have been identified (9–13). Two of these variants are the *Hind*III polymorphism in intron 8 (12) and the Serine-447-Stop (S447X) mutation in exon 9, which truncates the LPL protein by two amino acids (Ser-Gly) (13). Both of them are common in the general population and have been associated with TG and HDL-C concentrations in several studies (11, 14–17). However, other investigations did not replicate such associations (18, 19). Another candidate gene that plays an important role in the metabolism of TG is the *APOC3*, which codes for apolipoprotein C-III (apoC-III). ApoC-III is a major component of TG-rich lipoproteins (chylomicrons and VLDL), and a minor component of HDL (20); and it has been shown in vitro that apoC-III is an inhibitor of LPL (21, 22). A *Sst*I polymorphism in the 3′ untranslated region of the *APOC3* gene has been reported to be associated with TG levels in a number of studies (23–25), but not in others (26), as has been the case for *LPL* gene variations. Therefore, it appears that additional genetic and/or environmental factors have an impact on the potential associations of these polymorphisms

Abbreviations: CAD, coronary artery disease; CI, confidence interval; TRL, triglyceride-rich lipoproteins.

[1] To whom correspondence should be addressed at the Lipid Metabolism Laboratory, JM-USDA Human Nutrition Research Center on Aging at Tufts University, 711 Washington St., Boston, MA 02111.
e-mail: ordovas@hnrc.tufts.edu

depending on the ethnic-geographical origin of the studied population.

Among those additional genetic factors, the apolipoprotein E (*APOE*) gene plays a significant role (27, 28). The *APOE* gene has three common alleles: ε2, ε3, and ε4. These alleles have been consistently related with LDL-C levels; however, the association between these alleles and HDL-C or TG concentrations is less clear (27, 29, 30). ApoE is the major ligand involved in the binding and degradation of VLDL and remnant particles. In this regard, a number of in vitro studies have reported that apoC-III interacts with apoE, resulting in a displacement of apoE from VLDL (31, 32). Moreover, apoE interacts with LPL in the binding and lipolysis of the VLDL (33, 34). Therefore, the *LPL*, *APOC3*, and *APOE* genes codify for three highly interrelated proteins that have a key role in the metabolism of TG rich lipoproteins (TRL). The combined study of their naturally and highly prevalent occurring variations, as well as the possible interactions with other environmental factors, are essential to understand the impact of their genetic variation on lipid traits at the population level.

Among the environmental factors, tobacco smoking has been largely related to lipid metabolism. There is a large body of literature reporting that smokers have lower HDL-C and higher TG concentrations than non-smokers (35, 36). In addition, a reduced LPL activity has been demonstrated in smokers (36). Therefore, the aims of our study were: *1*) to investigate the combined effect of the *LPL-Hind*III, *LPL*-S447X, and the *APOC3-Sst*I gene variants on plasma lipids in a large and well-characterized Mediterranean population, *2*) to examine the interaction between *LPL* and *APOE* polymorphisms as modulators of TG and HDL-C concentrations, and *3*) to assess the influence of a common behavioral factor in this population (tobacco smoking) as the effect modifier of these potential gene-gene interactions.

MATERIALS AND METHODS

Subjects and study design

This work is part of a broader population survey on cardiovascular risk factors in the Valencia Region, on the East Mediterranean coast of Spain, aimed to ascertain the prevalence of both genetic and environmental CAD risk factors in this population. The Ethics Committee on Human Research of the Valencia University approved the study protocol and all subjects provided informed consent for participation. In this article, we present data obtained from 1,029 individuals attending the first cross-sectional examination in 1998–2000. Participants were healthy, unrelated subjects residing and working in the region. These subjects were randomly selected from more than 5,000 employees examined in a medical center. Using a continuously updated computerized population register, a sex-stratified random sample was drawn in two groups (50% male and 50% female), with 850 subjects selected from each stratum. Of 1,700 eligible subjects asked to participate, 467 men (55%) and 595 women (70%) agreed. Previously validated questionnaires were distributed at the time of the medical examination, and participants were invited to fill them. Non-Caucasian individuals (three men and one woman) were excluded from the study at this stage. Of the 1,058 individuals who completed the questionnaire, 1,029 (449 men and 580 women aged from 18 to 66 years) had a DNA sample isolated from blood; the remaining 29 were excluded because they did not report for blood samples.

Sample and data collection

Participants were instructed to fast for at least 12 h before the morning examination. Venous blood was collected during the medical check-up into EDTA-containing glass tubes. Plasma total cholesterol and TG were determined by a Technicon Chem 1 assay (Technicon Instruments, Tarrytown, NY), and HDL-C was measured in the supernatant after precipitation of apoB-containing lipoproteins with heparin-manganese chloride. Coefficients of variation for total cholesterol, HDL-C, and TG measurements were each less than 5%. LDL-C was calculated according to the equation of Friedewald et al. (37) for samples with serum TG concentrations below 400 mg per deciliter. Anthropometric measurements were taken using standard techniques: weight with light clothing by digital scales and height without shoes by fixed stadiometer. Body mass index (BMI) was calculated as weight (kg)/height (m^2).

DNA extraction and genotyping

Genomic DNA was isolated from white blood cells by phenol-chloroform extraction. DNA samples were subjected to amplification by the PCR in an Eppendorf DNA thermal cycler. Amplification of the region flanking the *Hind*III site was carried out as previously described (16). Amplified products were digested with *Hind*III at 37°C, and the resulting fragments were separated on 2% agarose gels. The S447X polymorphism was identified by the introduction of a forced *Hinf*I restriction enzyme site into the PCR product (17). The restriction site was present in the X447 allele. The fragments were separated by electrophoresis on a 4% agarose Metaphor® gel. The 3′-untranslated region of the *APOC3* gene, which contained the polymorphic *Sst*I site, was amplified by PCR as previously reported (38). PCR products were digested with *Sac*I at 37°C, and the resulting fragments were resolved by electrophoresis on 2% agarose gel. The presence of the *Sst*I restriction site constitutes the S2 allele. For the common *ApoE* polymorphism, a 244 bp of the *APOE* gene including the two polymorphic sites was amplified by PCR, and genotyping was carried out as previously described (39).

Questionnaire

Data on gender, date of birth, nationality, place of residence, marital status, type of education, profession, medication, possible pregnancies or hysterectomies, health problems, family history, tobacco use, alcohol consumption, and physical activity, were assessed by a self-administered questionnaire as previously indicated (40). Current smokers were defined as those smoking at least one cigarette a day. Former smokers were defined as those who smoked regularly at least one cigarette per day but had not smoked for over 1 month before the examination. Alcohol intake (in g/day) was carefully evaluated by a set of 22 questions about the use of alcoholic beverages during workdays and weekends. Alcohol consumption was further categorized as a drinker variable: non-drinkers (alcohol consumption = 0) and drinkers (subjects with any amount of alcohol consumed). Physical activity was estimated from questions about regularly leisure-time physical sports, as well as the average number of hours per week spent in each activity as previously reported (40). For regression analyses, physical exercise was also dichotomized as sedentary (no physical exercise) versus active (moderate plus high). Education was classified into four categories: primary, secondary, university I (3 years), university II (5 years or more); and after recoding into two: university and non-university.

Statistical analysis

Allele frequencies were estimated by gene counting, and 95% confidence intervals (CIs) were calculated. χ^2 tests (Pearson, Fisher exact test, or the Monte Carlo approach) were used to test differences between observed and expected frequencies, assuming Hardy-Weinberg equilibrium, and to test differences in percentages between men and women. Haplotype frequencies were computed by direct counting. Linkage disequilibrium parameters D and D′ (D/D_{max} if $D > 0$) were estimated. Normal distribution for all continuous variables was checked. TG and alcohol intake were markedly skewed, and these variables were logarithmically and square-root-transformed, respectively, to improve normality. Statistical analyses with these variables were performed on transformed data. To assess mean differences between genders and genotypes, Student's *t*-test was used. For multiple comparisons of means between genotypes, one-way ANOVA was performed. When the number of cases in each subgroup was small, nonparametric tests (Mann-Whitney or Kruskal-Wallis) were applied to compare means. Multivariate linear regression analysis with dummy variables for categorical terms was used to test the null hypotheses of no association between genetic variants, and lipid and lipoprotein levels. Regression coefficients and the proportion of variance attributable to each predictor were estimated from the models. Finally, homogeneity of allelic effects according to environmental (smoking) or genetic factors was tested by introducing the corresponding terms of interaction (in a hierarchical way) in the more parsimonious linear regression model. Standard regression diagnostic procedures were used to ensure the appropriateness of these models. Analyses were done using the SPSS and the LINKAGE programs.

RESULTS

Characteristics of the 1,029 study subjects (449 men and 580 women) are presented in **Table 1**. All of them were genotyped for the two *LPL* gene polymorphisms: *Hind*III (alleles designated H^+ and H^-) and S447X (alleles designated S447 and X447), and for the *Sst*I polymorphism in the *APOC3* gene (alleles S1 and S2). **Table 2** shows genotypes and allele frequencies for the observed alleles. For all polymorphisms in both men and women, the distribution of genotypes was as expected from the Hardy-Weinberg equilibrium ($\chi^2 = 0.045$, 1 df, $P = 0.832$ for *LPL-Hind*III; $\chi^2 = 0.005$, 1 df, $P = 0.943$ for *LPL*-S447X; and $\chi^2 = 0.007$, 1 df, $P = 0.933$ for *APOC3-Sst*I). Combined association analysis of genotype distribution indicated that *LPL-Hind*III and *LPL*-S447X were strongly associated ($P < 0.001$). **Table 3** shows prevalence of the specific combination of the *LPL* polymorphisms in this population. From these data, it can be derived that only three haplotypes existed: H^+S447, H^-S447, and H^-X447. Therefore, haplotypes could be assigned unambiguously, even for those individuals' heterozygotes for both polymorphisms. From the 1,005 subjects in whom the two variants were successfully determined, the estimated haplotype frequencies were: 0.686 (1379/2010) for H^+S447, 0.174 (350/2010) for H^-S447, and 0.140 (281/2010) for H^-X447. The linkage disequilibrium parameter (D) was +0.096, and $D/D_{max} > 0.999$; $P < 0.001$, indicating a very strong linkage disequilibrium between the two sites in this Mediterranean population. However, no statistically significant associations between *LPL-Hind*III and *APOC3-Sst*I ($P = 0.869$) or between *LPL*-S447X and *APOC3* genotypes ($P = 0.760$) were found.

LPL polymorphisms and lipids

To remove the influence of some relevant confounding factors in the association study between these genetic variants and lipid traits, subjects taking any lipid-lowering drug (24 men and 12 women), pregnant women (n = 3),

TABLE 1. Demographic, biochemical, and life-style characteristics of the study subjects in the Mediterranean Spanish population, 1998–2000

	Men (n = 449) Mean (SD)	Women (n = 580) Mean (SD)	P^a
Age (years)	37.6 (10.3)	37.0 (10.4)	0.371
BMI (kg/m^2)	26.2 (3.7)	23.6 (4.2)	<0.001
Total-C (mmol/l)	5.3 (1.1)	5.0 (0.9)	<0.001
LDL-C (mmol/l)	3.5 (0.9)	3.2 (0.8)	<0.001
HDL-C (mmol/l)	1.1 (0.3)	1.4 (0.3)	<0.001
TG (mmol/l)	1.5 (1.2)	0.9 (0.5)	<0.001
Current smokers (%)	41.9	41.7	0.949
Past smokers (%)	21.5	16.9	0.124
Alcohol users (%)	90.3	57.0	<0.001
Physical exercise (%)			<0.001
Sedentary	49.5	60.6	
Moderate	28.6	28.3	
High	21.9	11.1	
Education (%)			<0.001
Primary	32.4	28.9	
Secondary	27.4	26.6	
University I	17.5	33.2	
University II	22.7	11.3	
Premenopausal (%)	—	81.6	
Oral contraceptive users (%)	—	15.8	
Taking lipid lowering drugs (%)	5.2	2.2	0.018
Individuals with any missing data (%)	11.0	3.2	0.011

Students *t*-test for comparison of means, and Chi square tests for percentages. BMI, body mass index; University I, 3 years; University II, 5 years or more.

[a] *P* value in the comparison between men and women.

TABLE 2. Genotype distribution and allele frequencies of the *LPL-Hind*III and S447X, and *APOC3-Sst*I, polymorphisms by gender in the Mediterranean Spanish population

	Total (n = 1,029) n (%)	Men (n = 449) n (%)	Women (n = 580) n (%)
*LPL-Hind*III			
H+H+	474 (46.8)	216 (48.8)	258 (45.3)
H+H−	440 (43.3)	188 (42.4)	252 (44.2)
H−H−	99 (9.8)	39 (8.8)	60 (10.5)
LPL-S447X			
S447/S447	752 (74.1)	341 (76.8)	411 (72.0)
S447/X447	243 (23.9)	96 (21.6)	147 (25.7)
X447/X447	20 (2.0)	7 (1.6)	13 (2.3)
*APOC3-Sst*I			
S1S1	875 (86.2)	379 (86.1)	496 (86.3)
S1S2	135 (13.3)	58 (13.2)	77 (13.4)
S2S2	5 (0.5)	3 (0.7)	2 (0.3)
Allele frequency and 95% CI			
H+	0.68 (0.66–0.71)	0.70 (0.67–0.73)	0.67 (0.65–0.70)
H−	0.32 (0.30–0.34)	0.30 (0.27–0.33)	0.33 (0.30–0.35)
S447	0.86 (0.84–0.87)	0.88 (0.85–0.90)	0.85 (0.83–0.87)
X447	0.14 (0.12–0.15)	0.12 (0.10–0.15)	0.15 (0.13–0.17)
S1	0.93 (0.92–0.94)	0.93 (0.91–0.94)	0.93 (0.91–0.94)
S2	0.07 (0.06–0.08	0.07 (0.06–0.09)	0.07 (0.06–0.08)

The genotyping of *LPL-Hind*III, *LPL*-S447X, and *APOC-Sst*I polymorphisms could not be determined in 16, 14, and 14 cases, respectively. Differences by gender across *LPL-Hind*III, *LPL*-S447X and *APOC3-Sst*I genotypes were non significant. Chi square *P* values = 0.452, 0.204, and 0.751, respectively.

and individuals with TG levels higher than 400 mg/dl (15 men) were excluded in the subsequent analyses. There were also 69 exclusions due to partial missing data on genetic, biochemical, or lifestyle variables. The final group size in the association analyses was 906 subjects (360 men and 546 women). There were no differences in demographic, biochemical, life-style, or genetic variables between this final group and those reported in Tables 1 and 2 for the whole sample. **Table 4** shows age, BMI, plasma lipid, and lipoprotein levels by *LPL-Hind*III and *LPL*-S447X polymorphisms in men and women. Because of the small number of subjects' homozygotes for the rare alleles, heterozygotes and homozygotes were grouped as H^- and X447 carriers. No significant difference in age or BMI was found by *LPL* variants in any gender. Likewise, there were no significant differences for total cholesterol and LDL-C concentrations. In men, the H^- allele was associated with an overall lowering effect on mean TG levels of 13.6% ($P = 0.011$), and with an increasing effect on mean HDL-C of 8.3% ($P = 0.001$). In women, although there was not heterogeneity of genotype effects, the differences were smaller, and only the increasing effect on HDL-C levels reached statistical significance. In both men and women, the *LPL*-S447X polymorphism showed a higher association with HDL-C and TG levels than the *LPL-Hind*III polymorphism. X447 carriers clearly exhibited both the lowest TG and the highest mean HDL-C concentrations.

TABLE 3. Distribution of subjects according to *LPL-Hind*III and *LPL*-S447X combined genotypes in the Mediterranean Spanish population

	LPL-S447X		
*LPL-Hind*III	S447S447 n (%)	S447X447 n (%)	X447X447 n (%)
H^+/H^+	472 (47)	0 (0)	0 (0)
H^+/H^-	242 (24)	192 (19)	0 (0)
H^-/H^-	29 (3)	49 (5)	20 (2)

The distribution was not significantly different between men and women ($P = 0.897$). $P < 0.001$ in the Monte Carlo test for the association between the two sites.

To learn the proportion of variance of HDL-C and TG levels in the population accounted for each polymorphism, and to estimate the quantitative effect of the genetic variants after adjustment by sex, age, BMI, tobacco smoking, alcohol consumption, physical activity, education, menopausal status, and oral contraceptive use, several linear regression models were fitted. **Table 5** shows adjusted regression coefficients for each variable by combining men and women. After the adjustment for covariates, the association of the *LPL* variants with HDL-C or TG levels remained statistically significant in all cases. No statistically significant gene-gender interactions were obtained for the *LPL* variants. In this population, *LPL-Hind*III and *LPL*-S447X polymorphisms accounted for 2.5% and 3.7%, respectively, of the variance in HDL-C concentrations ($P < 0.05$), and for 1.4% and 2.1%, respectively, of the variation in TG concentrations ($P < 0.05$). Interestingly, the adjusted regression coefficients (B) for the *LPL* gene variants showed in Table 5, were similar in magnitude to those obtained for smoking or drinking, indicating a comparable effect of these *LPL* genetic variants on TG and HDL-C concentrations.

APOC3 polymorphism and lipids

Regarding the *APOC3-Sst*I polymorphism, it shows a strong association with TG levels in both genders (Table

TABLE 4. Plasma lipid and lipoprotein levels by LPL-HindIII, and S447X, and APOC3-SstI polymorphisms and gender in the Mediterranean Spanish population

	*LPL-Hind*III			*LPL*-S447X			*APOC3-Sst*I		
	H^+/H^+[a]	H^- carriers[b]	*P*[g]	S447/S447[c]	X447 carriers[d]	*P*[g]	S1S1[e]	S2 carriers[f]	*P*[g]
Age (years)									
Men	38.53 (10.56)	37.37 (10.21)	0.290	38.15 (10.34)	37.47 (10.42)	0.593	38.23 (10.43)	37.37 (10.38)	0.585
Women	36.99 (10.41)	36.86 (10.33)	0.884	36.98 (10.38)	37.43 (10.24)	0.648	37.35 (10.38)	35.27 (10.11)	0.106
BMI (Kg/m^2)									
Men	26.29 (3.54)	25.92 (3.69)	0.319	26.19 (3.43)	25.87 (4.21)	0.482	26.07 (3.43)	26.41 (4.75)	0.547
Women	23.77 (3.89)	23.38 (4.30)	0.274	23.73 (4.01)	23.08 (4.24)	0.100	23.52 (3.97)	23.65 (4.89)	0.806
Total-C (mmol/l)									
Men	5.22 (0.89)	5.22 (1.04)	0.919	5.23 (0.94)	5.22 (1.05)	0.961	5.22 (0.99)	5.28 (0.86)	0.718
Women	5.03 (0.85)	5.00 (0.93)	0.629	5.01 (0.89)	5.03 (0.91)	0.805	5.00 (0.89)	5.08 (0.90)	0.471
LDL-C (mmol/l)									
Men	3.46 (0.80)	3.48 (0.93)	0.784	3.48 (0.84)	3.49 (0.93)	0.932	3.48 (0.88)	3.53 (0.75)	0.736
Women	3.19 (0.75)	3.14 (0.82)	0.403	3.16 (0.79)	3.18 (0.79)	0.739	3.17 (0.79)	3.13 (0.77)	0.696
HDL-C (mmol/l)									
Men	1.08 (0.22)	1.17 (0.26)	0.001	1.09 (0.23)	1.21 (0.27)	<0.001	1.13 (0.24)	1.04 (0.26)	0.013
Women	1.39 (0.26	1.45 (0.28)	0.025	1.41 (0.27)	1.47 (0.27)	0.046	1.43 (0.26)	1.43 (0.32)	0.929
TG (mmol/l)									
Men	1.47 (0.81)	1.27 (0.69)	0.011	1.43 (0.77)	1.17 (0.65)	0.002	1.32 (0.71)	1.62 (0.85)	0.009
Women	0.92 (0.47)	0.88 (0.48)	0.160	0.93 (0.48)	0.81 (0.46)	0.003	0.87 (0.45)	1.03 (0.62)	0.019
Log (TG/HDL-C)									
Men	1.02 (0.62)	0.81 (0.63)	0.002	0.99 (0.62)	0.69 (0.63)	<0.001	0.87 (0.61)	1.15 (0.68)	0.004
Women	0.32 (0.53)	0.23 (0.53)	0.048	0.31 (0.54)	0.15 (0.51)	0.001	0.24 (0.52)	0.37 (0.62)	0.058

[a] n for men are 175, n for women are 249, and numbers are mean (SD).
[b] n for men are 185, n for women are 297, and numbers are mean (SD).
[c] n for men are 275, n for women are 395, and numbers are mean (SD).
[d] n for men are 85, n for women are 151, and numbers are mean (SD).
[e] n for men are 309, n for women are 471, and numbers are mean (SD).
[f] n for men are 51, n for women are 75, and numbers are mean (SD).
[g] *P* value obtained in the Student's *t*-test for the comparison between genotypes for each polymorphism.

4) with those bearing the S2 allele having the highest mean concentrations (23% increase; $P = 0.009$, in men, and 18% increase; $P = 0.019$, in women). By contrast, the *APOC3-Sst*I polymorphism was only related to mean HDL-C levels in men (8% decrease; $P = 0.013$, in S2 carriers), but there was no evidence of association in women. Table 5, shows regression coefficients after adjustment for covariates as described above. The association of *APOC3-Sst*I polymorphism with TG levels remained highly independent of the adjustment for covariates (Table 5), and no statistically significant gene-gender interaction was obtained for this association. However, when the homogeneity of the allelic effect by sex was assessed in the case of HDL-C levels, a statistically significant interaction term between the *APOC3-Sst*I polymorphism and gender was obtained ($P = 0.047$). In men, but not in women, the S2 variant remained statistically related to lower HDL-C levels even after multivariate adjustment. In men, the *APOC3-Sst*I polymorphism accounted for 1.8% and 1.9% of variance in HDL-C and TG levels, respectively. In women, these polymorphisms explained 0% and 1% of variation in HDL-C and TG levels.

Interaction between the *LPL* and *APOC3* polymorphisms

Next, we examined the combined effects of the *LPL* and *APOC3* genetic variants on HDL-C and TG concentrations. All possible combinations of these three genotypes were considered, and mean lipid levels were compared. Taking into account the small size of some groups, subjects were further grouped as: H^- carriers/SS/S1S1, H^- carriers/X carriers/S1S1, H^+H^+/SS/S2 carriers, H^- carriers/SS/S2 carriers, and H^- carriers/X carriers/S2 carriers. Each group was compared with the reference category (H^+H^+/SS/S1S1). **Figure 1** shows the effects associated with each genotype combination (expressed as a percentage difference in TG and HDL-C levels) relative to the reference category in men (Fig. 1A) and women (Fig. 1B). The most favorable genotype combination in both genders was H^-/X/S1S1, and the least was H^+H^+/SS/S2. Compared with the H^-/X/S1S1 genotype, the H^+H^+/SS/S2 group was associated with a significant mean increase in TG levels (61%; $P < 0.001$ in men, and 36%; $P = 0.003$ in women), and with a significant decrease in HDL-C levels (−21%; $P < 0.001$ in men, and −10.7%; $P = 0.01$ in women). There were no differences by age, BMI, or environmental variables across combinations of the three genotypes. These combined genotypes accounted for 7% and 5% of variance of HDL-C and TG levels, respectively, in men. In women, they accounted for 3% and 4% of variance of HDL-C and TG levels.

To further evaluate the independent effect of *LPL-Hind*III, *LPL*-S447X and *APOC3-Sst*I variants on HDL-C or TG concentrations, they were simultaneously added to the multivariate regression models, and adjusted for the effect of covariates (age, BMI, tobacco smoking, alcohol consumption, physical activity, education, menopausal status, and

TABLE 5. Association between *LPL-Hind*III, *LPL*-S447X, and *APOC3-Sst*I polymorphisms and HDL-C or TG levels after adjustment for covariates in the Mediterranean Spanish population. Multiple linear regression analysis

	*LPL-Hind*III				*LPL*-S447X				*APOC3-Sst*I			
	HDL-C		TG		HDL-C		TG		HDL-C[a]		TG	
	B (SE)	*P*	B (SE)	*P*	B (SE)	*P*	B (SE)	*P*	B (SE)	*P*	B(SE)	*P*
Polymorphism												
Carriers of the rare allele	0.05 (0.02)	0.003	−0.06 (0.03)	0.078	0.06 (0.02)	0.005	−0.14 (0.04)	<0.001	0.05 (0.03)	0.079	−0.16 (0.05)	0.001
Reference	H+/H+		H+/H+		S/S		S/S		S1S1		S1S1	
BMI (kg/m^2)	−0.02 (0.00)	<0.001	0.03 (0.00)	<0.001	−0.02 (0.00)	<0.001	0.03 (0.00)	<0.001	−0.02 (0.00)	<0.001	0.03 (0.00)	<0.001
Nonsmoking versus smoking	0.08 (0.02)	<0.001	−0.09 (0.03)	0.006	0.08 (0.02)	<0.001	−0.09 (0.03)	0.006	0.08 (0.02)	<0.001	−0.09 (0.03)	0.006
Nondrinker versus drinker	−0.04 (0.02)	0.037	0.03 (0.04)	0.401	−0.04 (0.02)	0.033	0.03 (0.04)	0.386	−0.04 (0.02)	0.033	0.03 (0.04)	0.366
Men versus women	−0.27 (0.02)	<0.001	0.36 (0.04)	<0.001	−0.27 (0.02)	<0.001	0.36 (0.04)	<0.001	−0.27 (0.02)	<0.001	0.36 (0.04)	<0.001
R^2 of the model[b]	0.34	<0.001	0.30	<0.001	0.35	<0.001	0.32	<0.001	0.35	<0.001	0.31	<0.001

Statistical analysis was performed on log-transformed values for TG. B, Regression coefficient (in mmol/l); SE, Standard error.

[a] The interaction term between sex and the *APOC3* polymorphism was statistically significant, indicating a heterogeneity of the genotype effects between men and women as pointed out in the text.

[b] Regression models were additionally adjusted by age, education, physical activity, menopausal status, and oral contraceptive use.

oral contraceptives). Adjusted regression coefficients for each variant are shown in **Table 6**. The statistical significance of the interaction terms between *LPL* and *APOC3-SstI* variants was also assessed (Table 6). The results revealed that there were independent and additive effects of the *LPL* and the *APOC3* polymorphisms on HDL-C and TG levels in men, and on TG in women. Analysis of the possible additive effects of the specific *LPL* gene variants is complicated by the high degree of linkage disequilibrium that exists between these two sites. When both dummy variables were analyzed in the multiple linear regression models, only the *LPL*-S447X polymorphism remained statistically significant, implying a higher effect for this variant. However, this result may be interpreted with caution because of the high colinearity between the two *LPL*

Fig. 1. Percentages of differences from the mean of HDL-C and TG levels according to combined *LPL-Hind*III, *LPL*-S447X , and *APOC3-Sst*I genotypes in men (A) and in women (B). The H+H+/SS/S1S1 genotype was considered as reference. * $P < 0.05$ in the comparison of the effect of the corresponding genotype with the reference group.

TABLE 6. Combined association between *LPL-Hind*III, *LPL*-S447X, and *APOC3-Sst*I polymorphisms and HDL-C or TG levels in the Mediterranean Spanish population. Multiple linear regression analysis with interaction terms by gender

	Men				Women			
	HDL-C		TG		HDL-C		TG	
	B	*P*	B	*P*	B	*P*	B	*P*
Polymorphisms[a]								
H^- carriers versus H+H+	0.04	0.206	−0.10	0.437	0.04	0.189	0.04	0.746
X447 carriers versus S447S447	0.09	0.010	−0.17	0.058	0.02	0.498	−0.16	0.006
S2 carriers versus S1S1	−0.08	0.033	0.32	0.006	0.02	0.651	0.16	<0.001
Interaction terms[b]								
*LPL-Hind*III**APOC3-Sst*I		0.888		0.407		0.024		0.567
LPL-S447X**APOC3-Sst*I		0.837		0.797		0.699		0.057
Interaction terms[c]								
*LPL-Hind*III**APOC3-Sst*I*smoking		0.215		0.613		0.815		0.636
LPL-S447X**APOC3-Sst*I*smoking		0.728		0.831		0.979		0.761

Statistical analysis was performed on log-transformed values for TG. B, Regression coefficients (in mmol/l).

[a] Regression coefficients were additionally adjusted by age, BMI, tobacco smoking, alcohol consumption, physical activity, education, menopausal status, and oral contraceptives use (in women).

[b] Statistical significance of the first order interaction terms was assessed in separate models after estimation of the previously indicated regression coefficients.

[c] Statistical significance of the second order interaction terms were estimated in separate hierarchical models, including the corresponding first order interactions.

polymorphisms. For the same reason, the interaction term between *LPL-Hind*III and *LPL*-S447X was not obtained.

In women, the interaction between the *APOC3-Sst*I and the *LPL-Hind*III polymorphisms was statistically significant in determining HDL-C concentrations ($P = 0.025$). These results were in agreement with the stratified analysis previously presented in Fig. 1. When high order interaction terms with tobacco smoking were considered (Table 6), there was no evidence of interaction between either genotype combination and smoking status or genders in modulating HDL-C or TG levels.

Interactions with the *APOE* polymorphism and tobacco smoking

Finally, to examine the hypothesis that the *APOE* polymorphism can interact with the *LPL* variants in determining HDL-C or TG concentrations, multiple linear regression models with interaction terms were fitted. *APOE* genotype was successfully determined in a random sample of 801 individuals (329 men and 472 women). Subjects were grouped as ε2 carriers (ε2/2 + ε2/3), ε3 homozygotes (ε3/3), and ε4 carriers (ε3/4 + ε4/4). ε2/4 subjects (n = 4 men and n = 2 women) and one E2 Christchurch man were excluded from the subsequent analyses. Prevalence was 10.5%, 76.5%, and 13.2% for the ε2, ε3, and ε4 groups, respectively. Taking into account that the results for men and women concerning the *LPL* variation were in the same direction, both genders were analyzed together. First, the interaction term between the *LPL-Hind*III and the *APOE* polymorphisms was tested in a regression model including the main effects, the *APOC3* polymorphism, and all the control variables (age, sex, tobacco smoking, drinking, physical activity, BMI, education, menopausal status, and oral contraceptives). We obtained a statistically significant interaction term between the *LPL-Hind*III and the *APOE* polymorphisms in determining HDL-C concentrations ($P = 0.006$), and near the statistical significance in predicting TG levels ($P = 0.099$).

The direction of the effect of this interaction was the same in both genders. Subjects carrying the ε4 allele had lower HDL-C levels than ε2 or ε3 individuals did, only if they were H^+/H^+ for the *LPL* polymorphism. However, ε4 carriers bearing the H^- allele had higher HDL-C levels than the corresponding ε2 or ε3. Moreover, when HDL-C mean levels were compared by the *LPL-Hind*III polymorphism considering the *APOE* genotype, the raising effect of the H^- allele on HDL-C levels was larger in ε4 individuals than in the ε3 group, and it was not observed for ε2 individuals. **Figure 2A** shows the interaction effect between the *LPL-Hind*III and the *APOE* polymorphisms on HDL-C levels. Similar results were obtained when the interaction term between the *LPL*-S447X and the *APOE* polymorphism was tested in the multivariate model (*P* for the interaction = 0.014). Figure 2B shows the interaction effect between the *LPL*-S447X and the *APOE* polymorphism on HDL-C levels. The interaction term between the *LPL*-S447X and the *APOE* polymorphisms in determining TG levels was not statistically significant ($P = 0.101$). However, the lack of statistical significance was mainly related to the higher variance of TG, because when the specific effects were examined (results not shown), they were opposite, reflecting the known inverse relation between fasting concentrations of TG and HDL-C. Thus, the decreasing effect of the H^- allele on TG levels was higher in the ε4 individuals than in the other groups, reaching statistical significance ($P = 0.003$).

When the effect of tobacco smoking on the interaction between the *LPL* and the *APOE* polymorphisms in predicting HDL-C levels was tested in the multivariate regression models in men and women, we obtained a statistically significant interaction effect ($P = 0.019$ for the term *LPL-HindIII*APOE**smoking: $P = 0.019$). Thus, in non-smokers

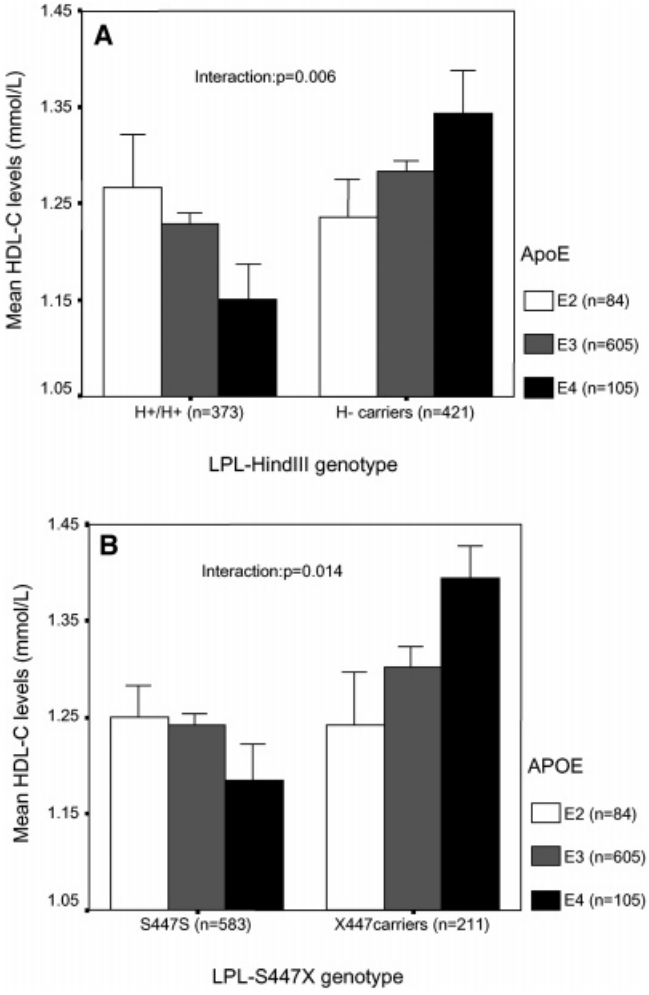

Fig. 2. Interaction between *APOE* polymorphism (ε2, ε3, and ε4) and LPL gene variants (A: *LPL-Hind*III and B: *LPL*-S447X) in predicting HDL-C levels in men and women. Estimated means for HDL-C were adjusted for age, body mass index, tobacco smoking, alcohol consumption, physical activity, education, menopausal status, and oral contraceptives as indicated in Materials and Methods. The reported *P* values ($P = 0.006$ in A and $P = 0.014$ in B) correspond to the statistical significance for the interaction terms between *APOE* and *LPL* polymorphisms in the multivariate regression models. A: Mean HDL-C levels differed statistically by the *Hind*III polymorphism in carriers of the ε4 allele ($P = 0.006$), and in the ε3 group ($P = 0.025$), but not in the ε2 carriers ($P = 0.925$). Likewise, for the LPL-S447X polymorphism (B), mean HDL-C levels were statistically different by the S447X gene variants in the ε4 carriers ($P = 0.003$) and in the ε3 group ($P = 0.001$), but not in the ε2 carriers ($P = 0.828$). Error bars: standard errors of means.

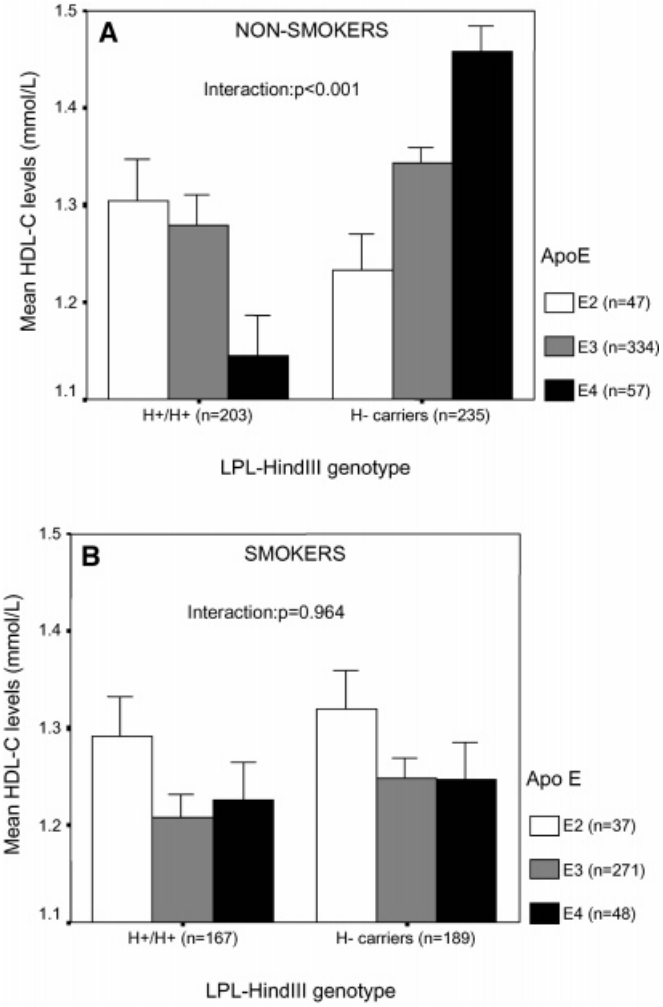

Fig. 3. Interaction of tobacco smoking on the effects of the interaction between *LPL-Hind*III and *APOE* polymorphisms in determining mean HDL-C levels. Stratified analysis by smoking status. A: Non-smokers. B: Smokers. The reported p values ($P < 0.001$ in A and $P = 0.964$ in B) correspond to the statistical significance for the interaction terms between *APOE* and *LPL* polymorphisms in non-smokers and smokers, respectively. Error bars: standard errors of means.

(**Fig. 3A**), the effect of the interaction between the *LPL* and *APOE* polymorphisms, was increased (P for interaction = 0.0001). In contrast, in smokers (Fig. 3B) the raising effect in HDL-C levels observed for the ε4 subjects carrying the H^- allele was abolished, and the interaction between *LPL-Hind*III and *APOE* polymorphisms was not statistically significant (*P* for the interaction = 0.964). Moreover, in non-smokers we also found a statistically significant interaction effect ($P = 0.031$) between *LPL-Hind*III and *APOE* polymorphisms in determining TG concentrations. In this group, the adjusted means of TG levels in ε2 individuals were: 1.08 ± 0.13 and 1.10 ± 0.10 mmol/l if they were H^+/H^+ or H^- carriers, respectively. By contrast, a statistically significant mean decrease (−23%) in TG levels was observed in ε4 individuals depending on their *LPL* genotype (1.52 ± 0.09 and 1.17 ± 0.10 mmol/l, in H^+/H^+ or in H^- carriers, respectively). However, the important lowering effect in TG levels of the H^- allele in ε4 individuals disappears if they smoke.

When these analyses were carried out by sex, the interaction effect between tobacco smoking, *LPL-Hind*III, and *APOE* polymorphisms was observed in both men and women, adding evidence to support this association.

DISCUSSION

The results of this observational study, conducted in a large sample of young and healthy individuals to minimize the effects of aging and medication on plasma lipid

profiles, have shown significant associations of *LPL* and *APOC3* gene variants with TG and HDL-C levels in both genders. However, the magnitude of this association appears to be modulated by the *APOE* locus and its interaction with tobacco smoking, demonstrating the complexity of multiple genetic and environmental effects on lipid traits. Consistent with this complexity, conflicting results have been reported regarding the associations of these gene variants with lipid, lipoprotein levels, and clinical phenotypes in a number of studies (11, 26, 41, 42). Differences in sample size, health status, gender, ethnic-geographical origin, age of subjects, and criteria used to select healthy individuals are also possible causes of the heterogeneity.

We have studied a well-characterized and randomly selected sample from the eastern Mediterranean coast in Spain. This population has been influenced by many immigrations starting with the Paleolithic Iberian population. North Africans entered this Mediterranean region between 20,000 and 8,000 B.C. They were followed by the Saharans, the Greeks, the Phoenicians, the Central Europeans, the Romans, and the Arabs at the beginning of the 8th century (43). This genetic background explains the actual differences with other northern and central European populations. The European Atherosclerosis Research Study (EARS) (17) has demonstrated a statistically significant gradient in frequency of the most favorable H^-X447 haplotype across Europe, from Finland (0.119 ± 0.024) to the south (0.143 ± 0.024). The frequency that we have found in the Mediterranean Spanish population for the H^-X447 haplotype (0.140 ± 0.008) confirms its higher prevalence in this southern European region. In addition to this *LPL* haplotype, a lower frequency of the S2 allele in the *APOC3* gene has been found in this Mediterranean population (24–26, 38). Indeed, the high frequency of the most protective haplotype combination ($H^-/X447/S1$) observed in this study is compatible with the low mortality rate for CAD registered in Spain in comparison with northern European countries (44).

APOC3 polymorphisms and plasma lipid concentrations

Although the S2 allele has been related to hipertriglyceridemia and CAD in several studies (23, 24, 45, 46), the molecular bases of this association are still unknown because the *Sst*I polymorphism is located in the *APOC3* 3′ untranslated region. Several mechanisms have been proposed (25, 38, 45, 47, 48). One of them takes in consideration that the S2 allele is in disequilibrium with the −625 and the −482 polymorphisms located in the promoter region of the *APOC3* gene (45). The presence of these mutations has been related to an impaired regulation of the decrease in apoC-III by insulin, resulting in an increase in apoC-III concentrations (49). It is known that overexpression of human *APOC3* gene in transgenic mice results in hypertriglyceridemia with a positive association between apoC-III levels and TG concentrations (50). In humans, several epidemiological studies have shown that the S2 allele is associated with elevated plasma levels of apoC-III (25, 51, 52) or with TG concentrations (25, 53). However, Kee et al. (26) failed to find such associations in men participating in the Etude Cas-Temoins de l'Infarctus du Myocarde study. Similarly, Dallongeville et al. (41), in a recent study carried out in 590 men and 579 women randomly selected from the population of northern France, showed a lack of association between the *Sst*I polymorphism and TG or HDL-C levels in men. On the other hand, they found an association of the S2 allele with higher TG concentrations in women suggesting a gene-gender interaction for this polymorphism. Our own data from the Framingham Offspring Study showed that male subjects carrying the S2 allele had increased TG concentrations; however, the magnitude of this effect was about half of that observed in the current study, and it did not reach statistical significance. Likewise, the S2 allele was associated in men with lower HDL-C levels in the Framingham Offspring Study, but the magnitude of this effect was less than in the Spanish population (54).

The results of our study do not support a gene-gender interaction for the *APOC3-Sst*I polymorphism in determining TG levels, because the association of the S2 allele with TG concentrations was present in both genders, even after the control for genetic and environmental factors. However, we found a significant gene-gender interaction for the *APOC3-Sst*I polymorphism in determining HDL-C concentrations. Thus, the S2 allele was associated with lower levels of HDL-C in men. By contrast, in women, in agreement with Dallongeville et al. (41), plasma levels of HDL-C did not significantly differ between S2 carriers and S1S1 homozygotes. These results may indicate that the effect of the S2 allele on plasma TG levels in women is not secondary to variation in plasma HDL-C. Furthermore, we found a statistically significant gene-gene interaction between the *LPL-Hind*III and the *APOC3-Sst*I polymorphisms in determining HDL-C in women. Women bearing the S2 allele had lower HDL-C levels than those S1S1, only if they were H^+/H^+ for the *LPL-Hind*III polymorphism. H^- carriers bearing the S2 allele had higher HDL-C levels than the corresponding S1S1 women did. This is the first time that this gene-gene interaction has been demonstrated in an epidemiological study, and the mechanisms are unknown. However, from the in vitro results concerning the binding and inhibition of *LPL* by apoC-III (25, 55), a possible mechanism could be hypothesized. If the S2 allele is associated with increased plasma apoC-III levels, then this may result in an inhibition of the *LPL* activity with the subsequent decrease in HDL-C levels in women with the wild-type genotype (H^+/H^+). In women with the H^- allele, the inhibition of *LPL* by apoC-III could be defective, resulting in a lack of the decreasing effect in HDL-C levels. However, this mechanism is not compatible with the effects observed on TG concentrations, for which we have shown that the *APOC3* and the *LPL* gene variants acted in an additive fashion, as previously suggested by Peacock et al. (56).

LPL polymorphisms and plasma lipid concentrations

Concerning the effects of *LPL* (*Hind*III and S447X) gene variants on lipid levels, previous studies have reported a remarkable heterogeneity, which is explained by methodological bias or by environmental and genetic in-

teractions. Although there are studies showing effects on total cholesterol, LDL-C, and apoB (18, 42, 57), associations with TG or with HDL-C levels have been reported by the most part of investigations (14, 15, 56, 58–60). Interestingly, in several of these studies, the effects of the *LPL* variants on TG and HDL-C were only seen in men, suggesting a gene-gender interaction (59, 60). In the present report, although the association of the *LPL* gene variants with HDL-C or TG levels was higher in men than in women, there was no evidence for heterogeneity of the effect by gender. In both men and women, the rare alleles for the *LPL* variants were associated with higher HDL-C and lower TG concentrations.

To date, the mechanisms of these genotype effects on lipid traits are still unclear. In addition, alleles H^- and X447 were strongly associated, making it very difficult to examine their independent effect. The *Hind*III polymorphism is located in an intron and it is unlikely to be associated with changes in LPL protein activity or conformation. It may be a marker for a functional mutation elsewhere in the gene or in close proximity to the *LPL* gene. One of these functional variants is the *LPL*-S447X, but other hypotheses have been suggested (61–63). The X447 variant results in a truncation of the C-terminal end of the LPL protein by two amino acids, and it has been associated with increased LPL activity (13, 64, 65). Thus, the mechanism by which these variants exert their effects on lipid traits could be related to the enhanced LPL activity. A higher lipolytic activity results in a decrease of plasma TG levels and an increase in the transfer of protein, phospholipid, and cholesterol from VLDL particles to HDL3 particles, resulting in an increase of HDL2 particles, and a subsequent increase in HDL-C (66). In addition to this functional role of the S447X variant, other possibilities, including modification of endothelial binding, specific interaction with lipoproteins, and modification of binding to TRL have been proposed (7).

Interaction with the *APOE* polymorphism and smoking

Recently, several studies have focused on the interaction between apoE and LPL in lipid metabolism (7, 67), showing that apoE and LPL coordinately enhance binding and uptake of lipoproteins by cells (68), and that *APOE* polymorphisms appears to modulate LPL activity (33). In the present study, we have reported a statistically significant interaction between *LPL* and *APOE* polymorphisms on lipid traits. In this regard, we found that the effect of the rare alleles in the *LPL* polymorphism was different depending on the *APOE* gene variant, with the raising effect on HDL-C levels associated with the H^- allele being seen most strongly in ε4 subjects, and being absent in the ε2 individuals. The interaction effects of these polymorphisms on TG levels were in opposite directions and near statistical significance. One possible reason of this lack of significance may be the higher variance associated with TG concentrations, requiring a greater sample size to detect an effect comparable to that observed on HDL-C levels. Our results are in agreement with those of Salah et al. (69) in the STANISLAS cohort of healthy men and women from France. These authors reported an interaction between *LPL*-S447X and *APOE* polymorphisms determining the TG variation. They found that ε4 individuals with the S447S447 genotype presented the greatest TG levels. However, these authors did not find statistically significant interactions for HDL-C levels concentrations.

The possible mechanism to explain the interaction between the *LPL* and the *APOE* polymorphism in HDL-C concentrations is thought to be complex and involves several biochemical pathways, including different modulation of LPL activity by the apoE isoforms (33) and the different affinity of the apoE isoforms for the receptor that governs the uptake of lipoprotein remnants (27). Thus, the significant decrease of TG and the increase of HDL-C levels in ε4 individuals carrying the H^- allele could be the consequence of an increased rate of clearance of TRL by receptor-mediated pathways. Jong et al. (70) suggested that apoE might inhibit the lypolytic activity of LPL. They demonstrated that hydrolysis of TG from VLDL by LPL is inversely related to the apoE content. ApoE-poor VLDL particles are better substrates of LPL. We have shown that apoE concentrations in *APOE4* carriers are significantly lower than those observed for *APOE2* carriers and *APOE3/3* subjects (71). Therefore, despite the higher affinity of the *APOE4* for TRL (72), the balance of these opposite effects may yield apoE-poor VLDL particles associated with increased lipolytic activity, lower TG concentrations, and conversely higher HDL-C levels.

When the effect of tobacco smoking on the interaction between *LPL* and *APOE* polymorphisms in determining TG and HDL-C levels was assessed, a significant interaction was also found. The raising effect of the ε4 allele on HDL-C levels observed in individuals carrying the H^- allele was abolished in smokers. In smokers, a reduced post-heparin LPL activity has been demonstrated (36). The decreased LPL activity would result in delayed metabolism of TRL, and the less efficient lipolysis of VLDL and chylomicrons will reduce the amount of surface material available for incorporation into nascent HDL particles. Thus, this effect becomes limiting and has a greater effect than those associated with the genetic variability.

In summary, the findings of the present study indicate the presence of a gene-gene-environmental interaction on the effects of the studied gene variants on lipid traits. These findings apply to this population consisting primarily of young, mostly healthy individuals. It is possible that in older subjects, the age-related increases in plasma TG concentrations and the molecular mechanisms associated with these age-related changes may have a modulating effect over the observed associations and interactions. Nevertheless, our data emphasize the importance of analyzing the effect of candidate gene variants on combined strata of genetic and environmental factors. The identification of such gene-environment interactions could result in preventive interventions that may be of crucial importance in public health (73).

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