Higher order lipase gene association with plasma triglycerides

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Abstract **Lipoprotein lipase, HL, and endothelial lipase (EL) are proteoglycan-bound enzymes that regulate plasma lipoprotein levels through coordinated triglyceride (TG) lipase and phospholipase activity. We hypothesized that single nucleotide polymorphisms (SNPs) in lipase genes would have higher order impact on plasma lipoproteins beyond the influence of individual SNPs. In a sample of asymptomatic Caucasian subjects (n = 738), we used a two-stage approach, first identifying groups of subjects with similar multilocus lipase genotypes and then characterizing the relationships between genotype groups and plasma lipids. Using complementary methods, including a permutation test procedure and a mixed-effects modeling approach, we found a higher order interaction between four SNPs in three lipase genes (EL 2,237 3′ untranslated region, EL Thr111Ile, HL −514C/T, and LPL *Hind*III) and plasma TG levels. Subjects who were heterozygous for all four lipase SNPs had significantly higher plasma TG levels beyond the effect of individual lipase SNPs and environmental factors, even after correcting for multiple comparisons. In conclusion, lipase genes had synergistic association with plasma TG beyond individual gene effects. Higher order multilocus genotype contributions to dyslipidemia and atherosclerotic cardiovascular disease need to be considered a priori because they may have an important effect even in the absence of significant main effects of the individual genes.**—Reilly, M. P., A. S. Foulkes, M. L. Wolfe, and D. J. Rader. **Higher order lipase gene association with plasma triglycerides. *J. Lipid Res.* 2005.** 46: **1914–1922.**

Supplementary key words lipoproteins • lipases • higher order gene interactions

Plasma lipoproteins are major determinants of the risk for atherosclerotic cardiovascular disease (CVD) (1). Although a wealth of biochemical and genetic evidence implicates multiple individual genes in the regulation of plasma lipids (2), defining the genetic basis of these complex traits across community-based populations has proven difficult. This reflects, in part, the modest main effects of individual gene variations and complex higher order interactions (3). Identifying gene-gene and gene-environment interactions may be of particular importance because the molecular mechanisms that control lipid levels may provide greater insight into the risk of developing CVD than the absolute lipid values.

Lipoprotein lipase (LPL by HUGO nomenclature; also called LPD), HL (LIPC by HUGO nomenclature), and endothelial lipase (EL; LIPG by HUGO nomenclature), members of the same lipase gene family, are extracellular proteoglycan-bound enzymes that influence plasma lipoprotein levels through coordinated hydrolysis of lipoprotein triglyceride (TG) and phospholipid (**Fig. 1**). LPL preferentially hydrolyzes TGs in chylomicrons and VLDLs; HL hydrolyzes lipoprotein TG and phospholipids, particularly in HDLs and LDLs; and EL preferentially hydrolyzes HDL phospholipids (4). Gene deletion and overexpression studies in animal models have confirmed the lipoprotein-specific effects of these lipases in vivo (4).

In humans, lipase gene variants are associated with changes in plasma lipoproteins, particularly TG and HDL-cholesterol (HDL-C). Deficiency of LPL in humans, even heterozygous forms, results in increased TGs and low HDL-C (5). The relatively common LPL variant Ser447Stop and variants that are in linkage disequilibrium (e.g., *Hind*III) (6) are associated with increased LPL levels, decreased TG, and increased HDL-C (7, 8). HL deficiency is associated with increased HDL levels and particle size (9). A common variant in the HL promoter (−514), which is in linkage disequilibrium with three other common HL polymorphisms (10), is associated with reduced HL activity, increased HDL-C levels, and, in some studies, higher TGs (10–12). Early data suggest an association between common EL variants [T111I and 2,237 3′ untranslated region (UTR)] and HDL-C (13–16).

Given distinct, but complementary, tissue distribution and lipoprotein substrates, we hypothesized that common

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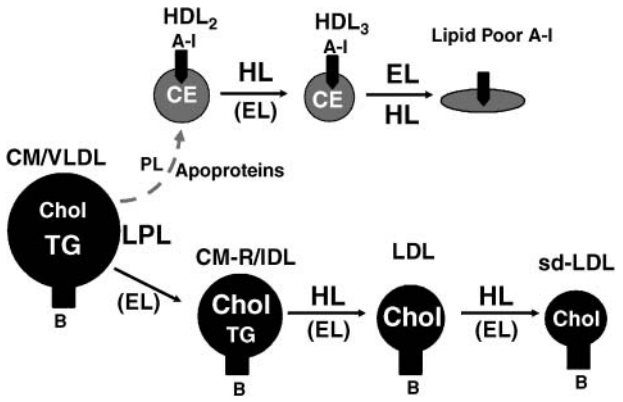

Fig. 1. LPL hydrolyzes triglycerides (TGs) in chylomicrons (CMs) and VLDL, decreasing plasma TG levels and transferring phospholipids and apolipoproteins to HDL. The LPL *Hind*III variant is associated with increased plasma LPL levels, lower TG levels, and higher HDL levels. HL, predominantly localized to hepatic sinusoids, hydrolyzes TGs and phospholipids in CM remnant particles (CM-R), intermediate density lipoproteins (IDL), LDLs, and HDLs, generating smaller lipid-depleted LDL and HDL particles. The HL −514 promoter variant is associated with lower HL activity and increased plasma levels of remnant particles, LDLs, and HDLs. Endothelial lipase (EL), expressed throughout the endothelium, preferentially hydrolyzes HDL phospholipids, promoting HDL turnover to lipid-poor apolipoprotein A-I (apoA-I) particles but also has lipolytic activity for apoB-containing lipoproteins. Early data suggest an association between the EL 2,237 3′ untranslated region and Thr111Ile EL variants and HDL-cholesterol levels. A-I, apolipoprotein A-I; B, apolipoprotein B; Chol, cholesterol; CE, cholesteryl ester; PL, phospholipids; sd-LDL, small dense LDL. The broken arrow depicts the transfer of apolipoproteins and phospholipids from VLDL/CM particles to HDL particles. Nonlipase pathways, including cholesteryl ester transfer protein and phospholipid transfer protein, also contribute to lipoprotein remodeling through the transfer of cholesteryl ester, TGs, and phospholipids between lipoproteins.

variants in lipase genes may have higher order effects on plasma lipoproteins, that is, that multilocus combinations of lipase single nucleotide polymorphisms (SNPs) would have effects above and beyond the influence of the individual SNPs. In a sample of asymptomatic Caucasian subjects (n = 738), we used a two-stage approach, first identifying groups of subjects with similar multilocus genotypes and then characterizing the relationships between these genotype groups and plasma lipids. Using complementary methods, including a permutation test procedure and mixed-effects modeling, we characterized higher order relationships, controlling for demographic and environmental factors, and tested genotype group-sex interactions that were motivated by the reported influence of sex (6, 10, 17) on lipase gene-lipoprotein associations.

MATERIALS AND METHODS

Study subjects

The Study of Inherited Risk of Coronary Atherosclerosis is a cross-sectional study of risk factors for coronary atherosclerosis in healthy volunteer subjects and families enriched for family history of premature coronary artery disease (CAD). The study design and preliminary findings have been published (18–21). Inclusion criteria included *1*) men aged 30–65 years and women aged 35–70 years and *2*) at least one first-degree relative with premature CAD (males younger than 60 years and females younger than 70 years). Exclusion criteria included established clinical CAD and extremes of major established CAD risk factors (known diabetes, total cholesterol > 300 mg/dl, cigarette smoking of more than one pack per day, and serum creatinine > 3.0) to facilitate the discovery of novel genetic influences on CVD. This report focuses on 738 unrelated Caucasian subjects with complete genotype data for lipase SNPs. The University of Pennsylvania Institutional Review Board approved the study protocol. Written informed consent was given by all subjects.

Evaluated parameters

Study subjects were evaluated at the General Clinical Research Center of the University of Pennsylvania Medical Center after a 12 h overnight fast. A questionnaire regarding medical, family, and social history and medication use was collected. Height, weight, waist circumference, resting systolic and diastolic blood pressures, and electrocardiogram data were recorded. Blood was drawn and plasma, serum, and DNA were isolated. Plasma total cholesterol, HDL-C, TG, and apolipoprotein A-I (apoA-I) and apoB levels were measured enzymatically on a Cobas Fara II (Roche Diagnostic Systems, Inc.) using Sigma reagents (Sigma Chemical Co., St. Louis, MO) in a Centers for Disease Control and Prevention-certified lipid laboratory. LDL-C was calculated using the Friedewald formula.

For this study, we chose SNPs in lipase genes that have been associated previously with plasma lipids. These included EL 2,237 3′UTR, EL Thr111Ile, HL −514, and LPL *Hind*III. Genotyping of lipase SNPs was performed on an Orchid 25K SNPstream™ platform (Orchid Bioscience, Princeton, NJ) using a single-base primer extension method. A set of three primers was chosen for each SNP: two PCR primers selected to amplify a 100–200 bp product, and a 25 bp single-base primer extension primer. For EL 2,237 3′UTR, AGTGCAACCCAWGAGAWCCCCAACAGC, GTGTTCAATAGACATTTGCTCAATTAA, and GTACTCTGCCTGACGAGGAAC; for EL Thr111Ile, GGGGAGCCAGTCAACCAC, AACTACATTGGCGTCTTTCTCTCTT, and TGCAGATGAGCGGTATCTTTG; for HL −514C/T, GTGTGZTGCAGAAAACCCTTZACCCCC, CAAATTTCTGTTGGGTTCAGTGA, and GTCACTTGGCAAGGGCATC; for LPL *Hind*III, AACATTACCCAGZTTGATCATGTA, AAAATGGATGTGAATATGCCATG, and ATTCTGATGTGGCCTGAGTGT. SNP-IT primers were extended by one base at the polymorphic site of interest using DNA polymerase I. The extension mixes contained two labeled terminating nucleotides (one fluorescein, one biotin). An ELISA technique was used for detection of the extension product: anti-fluorescein-alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) for fluoresceinated nucleotides (allele 1) and anti-biotin-horseradish peroxidase conjugate (Zymed, San Francisco, CA) for biotinylated nucleotides (allele 2). Raw optical density data were analyzed by GetGenos software, and genotypes were corroborated by visual inspection.

Statistical analyses

Our goal was to examine higher order, multilocus lipase genotype effects on plasma lipids. TG and HDL-C were the primary lipid end points, but total cholesterol, LDL-C, apoA-I, and apoB data are presented also. Medians, ranges, and proportions (categorical variables) are reported. Plasma TG values were natural log transformed (ln-TG) to normalize the data. LPL, HL, and EL SNP alleles were considered to be in Hardy-Weinberg proportions if the observed homozygote frequencies did not differ sig-

nificantly ($P > 0.05$) from those expected based on a Chi-square test. The D′ estimate of linkage disequilibrium between the EL 2,237 3′UTR and EL Thr111Ile SNPs and the association of EL haplotypes with lipid outcomes were determined using Qhapipf (Mander et al., MRC Biostatistics Unit, Cambridge, UK; adrian.mander@mrc-bsu.cam.ac.uk). This method uses an expectation maximization algorithm to resolve phase in determining haplotypes and in turn assesses the association between haplotype and a quantitative trait.

Association of individual lipase SNPs with plasma lipids. Multivariable linear regression models were fit to assess the association between plasma lipids and each of the four lipase SNPs and all two-way SNP-SNP and SNP-sex interactions to identify SNPs and two-way interactions for inclusion in subsequent analyses of multilocus genotypes. SNPs were treated as three-level categorical variables (homozygous wild type, heterozygous, homozygous rare). These analyses were adjusted for the effects of age, sex, cigarette smoking (current vs. former and never), exercise (none vs. any), alcohol intake (none vs. any), systolic blood pressure, and use of the following medications (statins, niacin, fibrates, and hormone replacement therapy in women). The likelihood-ratio Chi-square test (LRT) was used to test for sex interactions. We did not correct for multiple testing to be inclusive of all potentially important effects of individual SNPs in our subsequent multilocus modeling.

Multilocus genotype groups. We created genotype groups defined simply as groups of individuals with identical observed multilocus genotypes. In general, this technique is useful for combining human SNP information when the number of SNPs is relatively small, the prevalence of a single SNP is low, or SNPs are highly correlated, as occurs in linkage disequilibrium. In these settings, the number of observed groups may be substantially less than the number of possible multilocus genotypes. In our analysis of four SNPs, there are 81 possible multilocus genotype groups, but only 55 were observed.

Unadjusted analysis of multilocus genotype group effects on lipids. Wilcoxon rank sum statistics for each lipid variable (e.g., ln-TG) were generated for tests comparing each genotype group to the reference group (the most prevalent genotype group). Because of known sex differences in plasma lipids, these analyses were performed separately in men and women. Significance was determined for the association with each lipid outcome controlling for experiment-wide error rate using both Bonferroni adjusted $\alpha = 0.10$ level and a confirmatory permutation-based approach, also $\alpha = 0.10$ level (22). The permutation approach is a straightforward extension of the work of Hoh, Wille, and Ott (23) and proceeds as follows: *1*) the value of each lipid outcome was randomly permuted across individuals; *2*) Wilcoxon rank sum statistics were calculated for each genotype group using the permuted data; *3*) the ordered test statistics were recorded; and *4*) steps 1–3 were repeated 400 times. The ordered observed Wilcoxon test statistics were compared with the distribution of the expected order statistics from these repeated permutations. The corresponding percentile represents the probability that the statistic observed in our data arises from the null distribution.

Multivariable modeling of individual genotype groups. Multivariable linear regression of individual lipid outcomes was performed on genotype groups that were determined to be statistically significant by Bonferroni tests. Unlike the Wilcoxon approach, the goal was to determine whether the association of a specific genotype group, compared with all other groups, with lipid outcome was *1*) independent of environmental influences and individual SNPs, and *2*) modified by sex, as determined by the LRT.

Mixed-effects modeling of all genotype groups simultaneously. We also applied a comprehensive mixed modeling approach, as described by Foulkes et al. (24, 25), that permits the characterization of interactions between demographic factors (e.g., sex) and any multilocus genotypes regardless of whether they have a significant main effect or not. Posterior means of random genotype group-specific effects and corresponding variance estimates were used to assess the significance of multilocus genotypes and genotype-demographic interactions. The observed genotype groups can be considered a random sample from the general population of genotypes and, therefore, treated as random effects in a linear model. The general form of this model has been given (26) and provides a flexible framework for adjusting for potential confounders (through the fixed effects) and allowing genotype effects to vary across values of these variables (through the random effects). The mixed modeling approach allows for two stages of testing: *1*) an omnibus LRT test for overall variability in the multilocus genotype effects on the outcome, and *2*) genotype-specific tests based on conditional means of random effects and corresponding prediction intervals. Note that the significance cutoff for the omnibus test is based on a 50:50 mixture of a Chi-square [1 degree of freedom (d.f.)] and a Chi-square (0 d.f.) distribution, because we are testing the variance parameter at a boundary (27). In the case of a single random effect (e.g., genotype), this equals 1.92. Calculation of empirical Bayes estimates of random effects and corresponding 95% prediction intervals are described in a similar latent variable approach to the analysis of gene expression profiles (28).

RESULTS

Table 1 shows demographic, clinical, and laboratory characteristics of the study sample. Levels of traditional risk factors and plasma lipoproteins were close to the population average in these asymptomatic subjects, reflecting a recruitment strategy that excluded major lipoprotein abnormalities. Lipase SNP alleles were found to be in Hardy-Weinberg proportions in our study sample. There was highly significant linkage disequilibrium (D′ = 0.93, $P <$

TABLE 1. Characteristics of the study sample

Variables	Median (Interquartile Range)	
	Men (n = 425)	Women (n = 313)
Age (years)	47 (41–52)	50 (45–57)
Total cholesterol (mg/dl)	199 (174–223)	211 (183–234)
LDL-C (mg/dl)	126 (102–146)	123 (103–147)
HDL-C (mg/dl)	42 (35–49)	57 (45–68)
TGs (mg/dl)	128 (92–181)	113 (81–152)
ApoB (mg/dl)	99 (86–114)	98 (81–115)
ApoA-I (mg/dl)	115 (102–132)	145 (127–165)
Fasting glucose (mg/dl)	95 (88–104)	92 (85–99)
Body mass index (kg/m^2)	27.6 (25.4–30.6)	26.0 (22.8–30.4)
Waist circumference (cm)	95.9 (88.9–104.1)	82.6 (73.7–92.7)
Blood pressure (mm Hg)		
Systolic	128 (120–138)	126 (115–138)
Diastolic	79 (74–86)	75 (69–82)
Smoking (%)	11.5	11.3
Medications		
Statins (%)	18.1	10.5
Fibrates (%)	1.9	1.0
Niacin (%)	3.3	2.6
Hormone replacement therapy (%)	NA	28.6

ApoB, apolipoprotein B; LDL-C, low density lipoprotein-cholesterol; NA, not applicable; TG, triglyceride.

0.001) between the EL 2,237 3′UTR and EL Thr111Ile SNPs.

Association of individual lipase SNPs with plasma lipoproteins

Unadjusted lipid data by lipase genotypes are shown in **Table 2**. In multivariable linear regression, after controlling for confounders and specifically assessing gene-gender interaction, the strength of lipase genotype associations with plasma lipid levels was modest and often differed for men and women, as reported in previous studies (6, 7, 10, 11, 14, 16). Briefly, *1*) for ln-TGs in stratified analysis, LPL *Hind*III GG was associated with lower TGs in men (P = 0.02) but not in women (P = 0.57) (sex interaction LRT P = 0.05); *2*) for total cholesterol, HL −514 TT was associated with higher total cholesterol in men and women combined (P = 0.03); *3*) for HDL-C, LPL GG was associated with higher HDL in combined analysis (P = 0.006), and EL Thr111Ile CT and TT were associated with lower HDL in women (P = 0.003 and P = 0.05, respectively) but not in men (P = 0.39 and P = 0.48, respectively) (sex interaction LRT P = 0.006). EL haplotypes were not associated with any lipid outcome beyond the effect of the individual EL SNPs (data not shown). Contrary to the report of Xin et al. (8), we did not find an interaction effect of LPL and HL variants on plasma TGs (P = 0.85). These analyses were not the primary focus of our study but a first step to identify individual SNP associations, without penalizing for multiple comparisons, to be included in subsequent modeling of higher order multilocus genotype effects.

Genotype groups

Multilocus genotype groups were defined as groups of individuals with identical genotypes across the four lipase SNPs. To limit the number of tests being performed, our

TABLE 2. Plasma lipoprotein levels by lipase genotypes

	Median (Interquartile Range) for Men					
Genotype	TGs	Total Cholesterol	LDL-C	ApoB	HDL-C	ApoA-I
EL 2,237 3′UTR						
AA (43.4%)	128 (93–173)	196 (173–220)	125 (102–145)	97 (87–111)	42 (35–50)	115 (101–130)
GA (43.7%)	128 (93–193)	203 (176–226)	128 (106–153)	102 (85–116)	41 (34–49)	115 (103–133)
GG (12.9%)	118 (79–176)	199 (174–229)	128 (108–152)	102 (85–115)	44 (37–51)	117 (106–128)
EL Thr111Ile						
CC (52.6%)	128 (91–189)	199 (175–223)	127 (105–147)	100 (86–115)	41 (34–49)	114 (101–131)
CT (39.4%)	128 (93–181)	203 (176–228)	129 (107–152)	101 (86–115)	42 (36–49)	117 (104–132)
TT (8.1%)	113 (78–167)	182 (161–205)	112 (89–135)	92 (85–106)	44 (36–50)	115 (102–124)
HL −514						
CC (63.1%)	126 (91–167)	195 (172–226)	126 (101–146)	98 (85–112)	42 (36–50)	116 (104–133)
CT (32.7%	129 (92–193)	206 (176–226)	129 (110–150)	101 (88–115)	41 (34–48)	114 (100–128)
TT (4.2%)	121 (93–177)	222 (203–234)[a]	142 (124–147)	99 (88–117)	49 (37–58)	126 (112–149)
LPL *Hind*III						
TT (46.4%)	128 (93–186)	199 (173–228)	125 (101–146)	101 (88–109)	41 (34–48)	121 (101–133)
GT (44.1%)	129 (91–189)	198 (176–223)	126 (102–146)	99 (85–113)	42 (37–51)	117 (104–132)
GG (9.5%)	112 (88–138)[a]	201 (185–217)	129 (111–147)	100 (86–117)	43 (36–51)[a]	113 (102–128)
	Median (Interquartile Range) for Women					
Genotype	TGs	Total Cholesterol	LDL-C	ApoB	HDL-C	ApoA-I
EL 2,237 3′UTR						
AA (44.2%)	116 (87–152)	206 (181–237)	120 (102–146)	98 (80–114)	56 (45–67)	145 (126–162)
GA (45.7%)	116 (77–151)	213 (186–242)	128 (102–147)	98 (84–113)	59 (45–68)	147 (126–166)
GG (10.1%)	135 (96–187)	221 (187–234)	128 (108–152)	105 (82–123)	59 (45–73)	150 (129–170)
EL Thr111Ile						
CC (50.8%)	114 (93–149)	211 (185–236)	123 (102–145)	97 (80–115)	60 (48–70)	148 (130–167)
CT (41.0%)	117 (82–169)	213 (184–239)	125 (102–147)	98 (84–114)	56 (44–65)[a]	145 (124–161)
TT (8.2%)	103 (80–143)	204 (175–242)	124 (93–166)	104 (79–121)	58 (42–72)[a]	138 (130–155)
HL −514						
CC (60.1%)	112 (82–165)	209 (184–238)	123 (101–145)	97 (89–113)	59 (47–71)	148 (129–167)
CT (35.8%	120 (82–165)	211 (183–237)	125 (102–147)	99 (81–116)	59 (45–67)	139 (123–159)
TT (4.1%)	127 (93–155)[a]	217 (188–242)[a]	119 (97–146)	105 (85–129)	53 (45–68)	144 (135–173)
LPL *Hind*III						
TT (48.4%)	112 (83–148)	208 (181–240)	124 (99–150)	94 (86–110)	58 (44–67)	154 (124–166)
GT (41.9%)	117 (82–154)	213 (186–237)	127 (105–146)	98 (81–114)	58 (47–67)	144 (128–161)
GG (9.7%)	119 (85–155)	214 (191–227)	118 (107–138)	98 (79–115)	60 (47–74)[a]	147 (127–168)

EL, endothelial lipase; UTR, untranslated region. Plasma levels [median (interquartile range)] of TGs, total cholesterol, LDL-C, HDL-C, apoB, and apoA-I are shown for EL, HL, and LPL genotypes in men and women separately.

[a] In multivariable analysis adjusted for the effects of age, cigarette smoking status (current vs. former and never), exercise (none vs. any), alcohol intake (none vs. any), systolic blood pressure, and use of the following medications (statins, niacin, fibrates, and hormone replacement therapy in women), LPL *Hind*III GG was associated with lower TGs in men (P = 0.02) but not in women (P = 0.57) [sex-genotype interaction likelihood-ratio Chi-square test (LRT) P = 0.05], LPL GG was associated with higher HDL in combined analysis (P = 0.006), EL Thr111Ile CT and TT were associated with lower HDL in women (P = 0.003 and P = 0.05, respectively) but not in men (P = 0.39 and P = 0.48, respectively) (sex-genotype interaction LRT P = 0.006), and HL −514 TT was associated with higher total cholesterol in men and women combined (P = 0.03).

primary analyses included only genotype groups that contained at least 10 individuals, and a confirmatory analysis included genotype groups that contained at least 5 individuals. Of 55 groups observed in our data set, 23 groups contained 10 or more individuals (87% of the sample) (**Table 3**) and 31 groups contained 5 or more subjects (93% of the sample). Genotypes listed in parentheses below refer to genotypes for EL 2,237 3′UTR, EL Thr111Ile, HL −514, and LPL *Hind*III, respectively. The most prevalent group, group 15 (GA, CC, CC, TT), was defined as the referent and contained 57 individuals. Note that an assigned referent group is necessary for the Wilcoxon-based approach but not for the mixed modeling method (see below).

Univariate analysis of genotype groups and plasma lipids

In analysis stratified by sex, we found a significant association (after adjusting for multiple comparisons) between genotype group 13 (GA, CT, CT, GT; representing the heterozygous state for all individual lipase SNPs) and ln-TG in women but not in men (**Fig. 2**, **Table 4**). Interestingly, the Wilcoxon test statistic for the (GA, CT, CT, GT) group compared with the referent group varied markedly between men and women. There was no significant association between genotype groups and any other lipid variable after adjusting for multiple comparisons. Results were similar in sensitivity analyses using genotype groups that contained at least five individuals (data not shown).

TABLE 3. Observed lipase genotype groups with at least 10 observations

	EL 2,237 3′UTR			EL Thr111Ile			HL −514			LPL *Hind*III			
Genotype Group	AA	GA	GG	CC	CT	TT	CC	CT	TT	TT	GT	GG	n
1		X		X			X					X	14
2	X				X		X					X	14
3	X			X			X				X		22
4		X		X			X				X		42
5			X	X			X				X		14
6	X				X		X				X		39
7		X			X		X				X		40
8	X					X	X				X		26
9			X	X				X			X		13
10		X		X				X			X		32
11	X			X				X			X		13
12					X			X			X		23
13					**X**			**X**			**X**		**30**
14	X			X			X			X			30
15		**X**		**X**			**X**			**X**			**57**
16			X	X			X			X			32
17	X				X		X			X			50
18		X			X		X			X			38
19	X					X	X			X			13
20	X			X				X		X			24
21		X		X				X		X			29
22	X				X			X		X			29
23		X			X			X		X			18
Total													642

The most prevalent multilocus genotype group [group 15 (GA, CC, CC, TT); n = 57] and the all-heterozygous group [group 13 (GA, CT, CT, GT); n = 30] are highlighted in boldface.

Multivariable linear regression of the (GA, CT, CT, GT) genotype group and TGs

We used traditional linear regression to test specifically for the potential sex interaction with the (GA, CT, CT, GT) group while controlling for other factors. Notably, in age-adjusted analysis, the (GA, CT, CT, GT) group was associated (fold increase) with ln-TG in women [1.76 (1.33–2.33)] but not in men [1.18 (0.85–1.56)] (interaction LRT $P = 0.05$). In fully adjusted analysis, however, including all individual lipase SNPs, the (GA, CT, CT, GT) group remained significantly associated with ln-TG in women [1.60 (1.22–1.53)] but not in men [1.14 (0.86–1.53)]), but there was no longer a significant interaction with sex (LRT $P = 0.22$). Therefore, results of fully adjusted models are presented for men and women combined (**Table 5**), in which associations with ln-TG were found for the (GA, CT, CT, GT) group [1.34 (1.09–1.64); $P = 0.005$], fasting plasma glucose ($P < 0.001$), waist circumference ($P < 0.001$), statin therapy ($P = 0.01$), systolic blood pressure ($P = 0.02$), and sex ($P = 0.04$), with nonsignificant effects for LPL *Hind*III GG genotype ($P = 0.06$) and cigarette smoking ($P = 0.07$). Adjustment for the LPL-HL SNP interaction, reported to be a significant

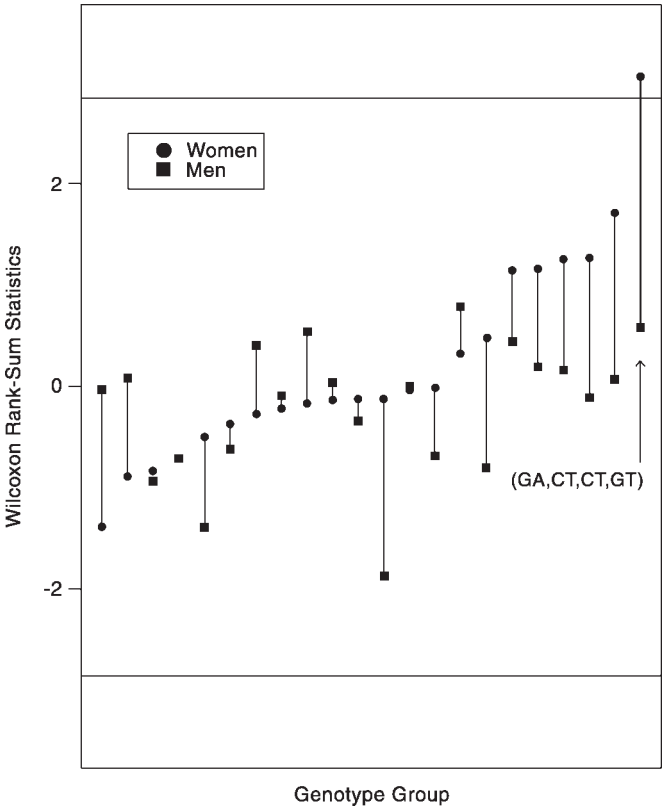

Fig. 2. Wilcoxon rank sum test statistics for log-transformed TG levels in unadjusted analysis for individual multilocus genotype groups with >10 observations for women (circles) and men (squares). Horizontal lines at top and bottom represent the Bonferroni corrected 0.1 boundary and suggest that in women, but not in men, the median TG level in the (GA, CT, CT, GT) group is significantly higher than in the referent group (GA, CC, CC, TT), which is not shown.

TABLE 4. Plasma lipoprotein levels by lipase genotype groups

Genotype Group	Median (Interquartile Range) for Men					
	TGs	Total Cholesterol	LDL-C	ApoB	HDL-C	ApoA-I
All subjects; n = 425	127 (92–181)	198 (174–223)	126 (103–147)	99 (86–114)	42 (35–49)	115 (102–132)
(GA, CC, CC, TT) reference group; n = 35	127 (101–169)	192 (161–210)	125 (99–134)	96 (83–106)	38 (36–44)	112 (102–133)
(GA, CT, CT, GT) group; n = 15	184 (91–290)	210 (184–244)	128 (109–156)	105 (84–119)	40 (33–46)	114 (95–132)
Unadjusted *P*	0.15					
Bonferroni *P*	NA					
Permutation *P*	NS					
	Median (Interquartile Range) for Women					
Genotype Group	TGs[a]	Total Cholesterol	LDL-C	ApoB	HDL-C	ApoA-I
All subjects; n = 313	114 (81–152)	211 (183–239)	124 (102–146)	98 (81–115)	59 (45–68)	145 (127–165)
(GA, CC, CC, TT) reference group; n = 22	110 (73–148)	205 (181–219)	118 (97–145)	97 (77–110)	58 (43–66)	147 (135–167)
(GA, CT, CT, GT) group; n = 15	179 (129–301)	232 (219–263)	131 (116–149)	109 (93–124)	62 (43–68)	152 (120–164)
Unadjusted *P*	0.004					
Bonferroni *P*	0.088					
Permutation *P*	0.01					

NA, not applicable. Plasma levels [median (interquartile range)] of TGs, total cholesterol, LDL-C, HDL-C, apoB, and apoA-I are shown for the (GA, CT, CT, GT) multilocus genotype group compared with the reference genotype group (GA, CC, CC, TT) and the full study sample for men and women separately. Wilcoxon rank sum statistics for lipid variables were compared, in men and women separately, for each genotype group with the reference genotype group, and Bonferroni and permutation-based *P* values were calculated (see Materials and Methods).

[a] In women, a statistically significant difference was observed for TG levels between the (GA, CT, CT, GT) group and the reference group (GA, CC, CC, TT).

predictor of TG levels by Xin et al. (8), did not attenuate the association between the (GA, CT, CT, GT) group and TG ($P = 0.005$).

Mixed-effects modeling of all genotype groups with plasma lipids

As a more comprehensive approach to the association of multilocus genotypes with plasma lipids, we applied linear mixed-effects modeling as described previously (24–26). The unique advantage of the mixed modeling approach is that it allows us to draw from the totality of the genetic data; it differs from the approach described in the previous section because it allows for the identification of an interaction between sex and all multilocus genotype groups, even in the absence of a main effect of individual multilocus genotype groups. Similar to the findings described above, in age-adjusted models, a significant sex-genotype group interaction with ln-TG was observed [global Chi-square (2 d.f.) = 4.86 for test of variance of random genotype effect equal to 0], and again the heterozygote genotype group (GA, CT, CT, GT) was solely identified as having a sex interaction effect that differs from 0 [95% protease inhibitor = (−0.415, −0.103)]. As before, however, in fully adjusted models, there was no longer a significant interaction of genotype groups with sex [global Chi-square (2 d.f.) = 2.45], but the heterozygous genotype was a significant predictor of ln-TG [global Chi-square (1 d.f.) = 2.87; 95% prediction interval = (0.025, 0.27)]. The conditional means and prediction intervals of all random genotype group effects with >10 observations are illustrated in **Fig. 3**. The results were similar after additionally adjusting for individual SNPs as main effects in the model. Neither genotype groups nor their interactions with gender were associated with HDL-C or LDL-C in age-adjusted or fully adjusted mixed-effects models.

TABLE 5. Multivariable analysis of factors associated with plasma TGs

Variable	Fold Increase in ln-TG (CI)	*P*
(GA, CT, CT, GT) genotype group	1.34 (1.09–1.64)	0.005
Glucose (per 10 mg/dl)	1.04 (1.02–1.06)	<0.001
Waist circumference (per 10 cm)	1.14 (1.11–1.18)	<0.001
Statin use	1.15 (1.05–1.27)	0.01
Systolic blood pressure (per 10 mg/dl)	1.03 (1.00–1.05)	0.02
Female sex	1.26 (1.01–1.58)	0.04
LPL *Hind*III GG-male sex interaction	0.82 (0.65–1.01)	0.06
Cigarette smoking	1.11 (0.99–1.24)	0.07

CI, 95% confidence interval. Natural log-transformed TG (ln-TG) was used as the dependent variable in linear regression. The fully adjusted model was forced to include age, sex, smoking, exercise, alcohol intake, medication use (statins, fibrates, niacin), EL 2,237 3′UTR, EL Thr111Ile, HL −514, LPL *Hind*III, LPL *Hind*III-sex interaction, and the (GA, CT, CT, GT) genotype group. Factors associated with ln-TG at $P < 0.1$ are presented. The association of the (GA, CT, CT, GT) genotype group was stronger in women [1.60 (1.22–1.53)] than in men [1.14 (0.86–1.53)] in the fully adjusted model, but there was no significant interaction by sex (interaction $P = 0.22$).

DISCUSSION

We report, for the first time, a higher order interaction between genetic variants in LPL, HL, and EL in the association with plasma TG levels. These lipases, all members of a TG lipase gene family (4), are likely to act synergistically

to regulate plasma levels of lipoproteins and modulate the risk of developing atherosclerosis. Although studies of combined and interaction effects of genetic variants in candidate lipoprotein genes are emerging, these have been mostly restricted to an examination of two-way gene-gene interactions (8, 17, 29) rather than true higher order interaction effects of multiple genes (30). Using a two-stage analysis, with a conservative approach to multiple testing as well as a more flexible mixed-effects modeling approach, we identified a combination of lipase genotypes, the all-heterozygous (GA, CT, CT, GT) genotype group, that was strongly predictive of higher plasma TG levels. This association was independent of all individual lipase SNPs and environmental influences and, notably, was identified in a study sample with average values of plasma lipids. This study provides one of a few examples of a complex genetic effect on CVD risk factor traits that cannot be detected without considering, a priori, the influence of combinations of candidate genes, even when there appears to be weak or no association of individual genes with the traits (30).

It is likely that the lipase gene family (LPL, HL, and EL) has evolved to regulate plasma lipids in a complex yet coordinated and synergistic manner (4) (Fig. 1). LPL, mostly acting in muscle and fat tissue, hydrolyzes TGs in chylomicrons and VLDLs. HL, predominantly localized to hepatic sinusoids, hydrolyzes LDL- and HDL-TGs and phospholipids. EL is expressed throughout the endothelium and hydrolyzes HDL phospholipids and also has modest lipolytic activity toward apoB-containing lipoproteins (31). Importantly, several studies have demonstrated a role for lipase genes in the development of atherosclerosis in mouse models (4, 32) and an association between lipase gene variation and CVD in human samples (11, 33, 34).

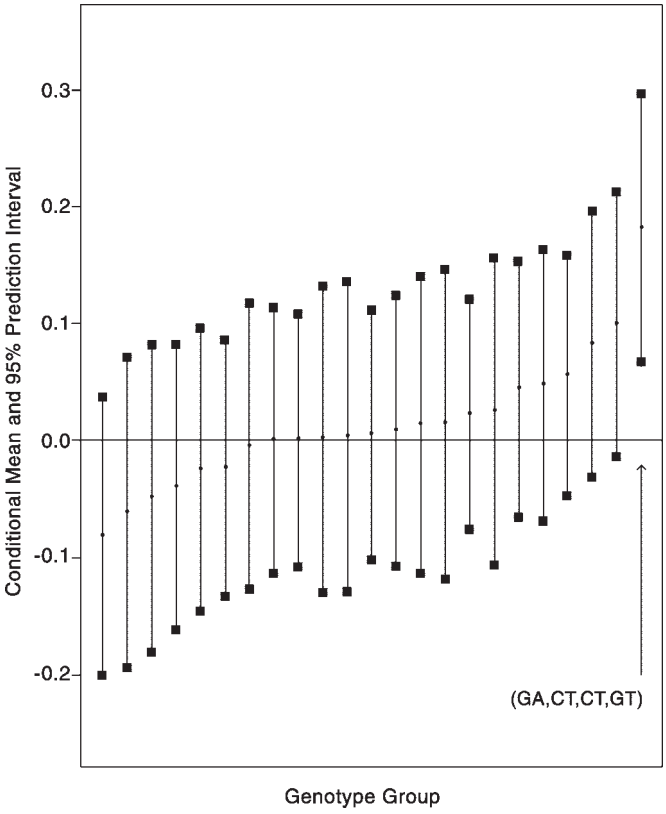

Fig. 3. Empirical Bayes estimates of random genotype group effects and corresponding 95% prediction intervals for log-transformed TG levels in fully adjusted linear mixed-effects models for ordered multilocus genotype groups with >10 observations [including (GA, CC, CC, TT), the most prevalent group]. The 95% interval (0.025, 0.27) for the (GA, CT, CT, GT) genotype group did not cover zero, consistent with significantly higher TG levels in the (GA, CT, CT, GT) group after controlling for demographic and environmental factors.

We selected the specific lipase gene SNPs in this study because they represented strong candidate SNPs based on published literature. Several studies have demonstrated the association of the LPL *Hind*III variant with lower TGs and higher HDL-C (6–8) and of the HL −514 variant with increased HDL-C, reduced small dense LDL-C, and in some reports increased TGs (10–12). Recent papers have reported associations of the EL 2,237 3′UTR and EL Thr111Ile variants with reduced HDL-C levels (14, 16). However, the effects of lipase SNPs have been reported to vary by age (35), sex (6, 10, 17), race (12), body mass (36), and environmental influences (15, 17). Indeed, an interaction of LPL Ser447Stop and HL −514 variants in the association with plasma TG levels has been reported (8). Thus, the modest and inconsistent contribution of candidate genes, including lipases, to plasma lipid levels and CVD events may reflect, in part, the failure to consider higher order gene-gene and gene-environment interactions. Nelson et al. (30) examined the synergistic influence of six distinct loci on plasma TG levels and found that many combinations of loci predicted TG variability in a nonadditive manner, suggesting that traditional methods, which rely on statistically significant single-locus effects, even those considering multiple genes (37), may fail to identify combinations of genes that best predict lipid variability.

In our sample, subjects who were heterozygous for all four lipase SNPs (GA, CT, CT, GT) had significantly higher plasma TG levels than would be expected from the effect of individual lipase SNPs. LPL *Hind*III heterozygosity results in greater chylomicron and VLDL-TG hydrolysis. In this setting, heterozygosity for HL −514 may further promote the accumulation of apoB remnant particles, and EL variants, associated with increased HDL-C levels, also may influence apoB lipoprotein hydrolysis (31). Consistent with these expected individual lipase variant effects, the (GA, CT, CT, GT) group was associated with modest increases in HDL-C and LDL-C as well as TG. However, our cross-sectional study cannot address the mechanistic basis and functional impact of the heterozygous (GA, CT, CT, GT) state on plasma lipids. Metabolic studies in humans and examination of particle size and density will be required to provide additional insights.

Because of reported sex-dependent lipase SNP-lipoprotein associations (6, 10, 17) and the known influence of sex hormones on lipase expression (38), we hypothesized a priori that there would be a sex difference in the mul-

tilocus genotype association with lipids. Although not statistically significant in fully adjusted models (which included factors that may be intermediate in the association), the difference in the strength of association for men and women suggests that the influence of sex on lipase-lipid associations may extend to higher order effects. These analyses also may have limited power with the given sample size (our unpublished results). These apparent sex differences require mechanistic study as well as further assessment in independent large samples with adequate power. Our study is notable for the lack of a multilocus lipase association with HDL-C. This may reflect a lack of synergistic higher order effects, failure to consider other important genetic determinants of HDL (e.g., apoA-I, cholesteryl ester transfer protein), a lack of power in our study, or type II error because of a conservative approach.

Characterizing the association between multiple gene polymorphisms and measures of disease is challenging because of problems associated with multiple testing (inflating type I error) and limitations in current analytic approaches (3). Several papers describe approaches to reduce the number of candidate genes and thus reduce the number of comparisons (23, 37, 39). However, many of these approaches are limited because they fail to consider the interactive effects of SNPs that have modest or no main effects (3). We used a two-stage approach that provides a flexible framework for the analysis of higher order genetic data. Our first stage involved a data dimension reduction step for the generation of genotype groups that does not require the presence of a main effect of individual candidate SNPs. We used a pattern-based approach to define multilocus genotype groups, although this may be useful only in certain settings when using a relatively small number of SNPs. In the second stage of analysis, we initially applied a conservative approach to identify genotype groups with significant association with lipids. Groups with a significant effect in unadjusted analysis, after correction for multiple testing, were selected for multivariable modeling and to test genotype group-sex interaction. Given the limitations of traditional regression approaches, we also applied mixed-effects modeling, a more flexible and robust approach that allows modeling of all multilocus genotypes simultaneously as well as their interaction with demographic factors. Alternative approaches to dimension reduction, including clustering algorithms, and methods for the selection of multilocus genotypes, such as permutation procedures and false discovery rate (40), can be integrated into our framework (24, 25). It is important to note that in analyses requiring multiple tests of association, there is a greater potential for spurious findings resulting from inflation of type I error. To address this concern, we implemented a rigorous, nonparametric permutation-based testing procedure that is particularly useful in the context of correlated tests and notably does not rely on large-sample asymptotic theory. The results of this test imply >90% confidence that our findings are not spurious.

In conclusion, we found a higher order interaction of genetic variants in lipase family members (LPL, HL, and EL) in the association with plasma TG levels. Consistent with a true synergistic multilocus effect, the all-heterozygous lipase group (GA, CT, CT, GT) was predictive of higher TG levels, beyond the influence of individual lipase SNPs. Given the inability of traditional approaches to detect these synergistic, and clinically relevant, genetic effects, our statistical approach and findings have important implications for defining the role of common genetic variations in dyslipidemia and atherosclerotic CVD.

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