Genetic variation of PLTP modulates lipoprotein profiles in hypoalphalipoproteinemia

Bradley E. Aouizerat,[1,*,†] Mary B. Engler,[*,†] Yanina Natanzon,[*] Medha Kulkarni,[*,§] James Song,[§] Celeste Eng,[§] Jarkko Huuskonen,[§] Christopher Rivera,[§] Annie Poon,[§] Matt Bensley,[**] Amy Sehnert,[**] Christian Zellner,[§] Mary Malloy,[§] John Kane,[†,§] and Clive R. Pullinger[*,§]

Department of Physiological Nursing, School of Nursing,* Center for Human Genetics,† Cardiovascular Research Institute,§ Department of Pediatrics, School of Medicine,** University of California San Francisco, San Francisco, CA 94143

Abstract Phospholipid transfer protein (PLTP) participates in key processes in lipoprotein metabolism, including interparticle phospholipid transfer, remodeling of HDL, cholesterol and phospholipid efflux from peripheral tissues, and the production of hepatic VLDL. The impact of PLTP on reverse cholesterol transport suggests that the gene may harbor sequence anomalies that contribute to disorders of HDL metabolism. The human PLTP gene was screened for sequence anomalies by DNA melting analysis in 276 subjects with hypoalphalipoproteinemia (HA) and 364 controls. The association with plasma lipid parameters was evaluated. We discovered 18 sequence variations, including four missense mutations and a novel polymorphism (c.-34G>C). In healthy controls, the c.-34G>C minor allele was associated with higher high density lipoprotein-cholesterol (HDL-C) and was depleted in subjects with HA. Linear regression models predict that possession of the rare allele decreases plasma triglyceride (TG) and TG/HDL-C and increases HDL-C independent of TG. Decreased PLTP activity was observed in one (p.R235W) of four (p.E72G, p.S119A, p.S124Y, and p.R235W) mutations in an in vitro activity assay. These findings indicate that PLTP gene variation is an important determinant of plasma lipoproteins and affects disorders of HDL metabolism.—Aouizerat, B. E., M. B. Engler, Y. Natanzon, M. Kulkarni, J. Song, C. Eng, J. Huuskonen, C. Rivera, A. Poon, M. Bensley, A. Sehnert, C. Zellner, M. Malloy, J. Kane, and C. R. Pullinger. **Genetic variation of PLTP modulates lipoprotein profiles in hypoalphalipoproteinemia. *J. Lipid Res.* 2006.** 47: **787–793.**

Supplementary key words dyslipidemia • genetic polymorphism • atherosclerosis • cardiovascular diseases • phospholipid transfer protein

Hypoalphalipoproteinemia [HA (Online Mendelian Inheritance in Man, entry: 604091)] refers to a category of HDL deficiency (less than the population-based 10th percentile) at risk for premature coronary artery disease (1). Whereas ablative mutations in several genes (e.g., ABCA1, APOA1, APOC3, LCAT, and LPL) have been characterized in discrete disorders of HDL metabolism, as a group they explain only a fraction of the cases of primary high density lipoprotein-cholesterol (HDL-C) deficiency (2). Given the estimated heritability of HDL-C levels (~50%) (3), the search for the primary genetic determinants is not only desirable, but tenable.

A combination of in vivo and in vitro studies has demonstrated the participation of phospholipid transfer protein (PLTP) in several key processes in lipoprotein metabolism, including the transfer of phospholipids from triglyceride (TG)-rich lipoproteins to HDL particles (4), the remodeling of HDL (5), the ABCA1-mediated efflux of cholesterol and phospholipids from peripheral tissues to HDL (6), and its participation in hepatic VLDL synthesis (7–9). Thus, the analysis of human genetic variation within the PLTP gene may provide important clues to the importance of PLTP in HDL-associated comorbidities [e.g., dyslipidemia, insulin resistance, and obesity (10)] and outcomes (e.g., type 2 diabetes, metabolic syndrome, and myocardial infarction).

Although the PLTP gene was previously studied in French Canadian (10) and Finnish (11) samples, the inclusion of only 19 and 24 subjects, respectively, for resequencing efforts would be likely to reveal only polymorphisms that are relatively frequent. Although 48 alleles (11) are in theory sufficient to identify variations with 1% frequency (e.g., 1 heterozygote in 24), sampling error can easily mask such discoveries. We chose to conduct a broader mutation detection effort in an attempt to identify both rare and common sequence anomalies within the transcribed region and exon-intron boundaries of PLTP. The aims of this study, therefore, were to screen a case-control cohort for sequence anomalies in the PLTP gene and to test for genetic association with HA and/or biochemical measurements. An additional aim was to test for significant differences in biochemical properties, including lipoprotein compartments, associated with sequence variations. These analyses were conducted in

Manuscript received 31 October 2005 and in revised form 6 December 2005.

Published, JLR Papers in Press, December 30, 2005.
DOI 10.1194/jlr.M500476-JLR200

* To whom correspondence should be addressed.
e-mail: bradley.aouizerat@nursing.ucsf.edu

two groups: one with HDL-C less than the 10th percentile for gender, and a healthy control group. We report a number of new sequence anomalies within the PLTP transcribed region and provide evidence consistent with a role for variation in the PLTP gene in regulating HDL-C. Importantly, functional studies of four newly discovered missense mutations resulted in the identification of a missense mutant (p.R235W) with altered PLTP activity and support the recent observation that both common polymorphisms (c.-34G>C) and rare functional sequence anomalies are independent sources of HDL deficiency.

METHODS

Study design

This study was a retrospective analysis using a racially and ethnically diverse study population sample. The study population was selected from the University of California San Francisco (UCSF) Genomic Resource in Arteriosclerosis (GRA). Subjects with HA (n = 276) were identified from the GRA as individuals with HDL-C less than the 19th percentile for their age and gender (approximately <35 mg/dl and <45 mg/dl for men and women, respectively). To control for HA secondary to increased transfer of cholesteryl ester to TG-rich lipoproteins in this group, individuals with TG > 3 SD from the mean were excluded. Healthy controls (n = 364) were selected from the GRA as individuals without a primary form of dyslipidemia (e.g., HA). Subjects were of non-Hispanic Caucasian (European) ancestry. All subjects gave informed consent, and the UCSF Committee on Human Research approved the study protocol.

Genotypic and phenotypic studies

Genomic DNA was prepared from whole blood obtained from patients in the GRA population of UCSF (12). Blood was drawn after a 10 h fast, and lipoprotein quantification was carried out using standard protocols (13–16). Standards were provided by the Centers for Disease Control and Prevention (Atlanta, GA). Baseline lipoprotein measurements were obtained when patients had received no lipid-lowering medication for at least 1 month, a standard "wash-out" period for such medications (12).

Molecular gene scan of the PLTP gene

A combination of denaturing high-performance liquid chromatography (dHPLC) (17) and denaturing gradient gel electrophoresis (DGGE) (18) mutation detection was used to scan the 10 amplicons that spanned the 16 exons encoding the full-length PLTP transcript (BC01984) using standard protocols. Exon 5, which is absent in one of the two known PLTP splice variants (19), was included in the analysis. Wave (dHPLC) or electrophoresis (DGGE) profiles consistent with sequence changes were studied further by direct DNA sequencing to identify the underlying DNA variations. Nomenclature guidelines for position and sequence changes described herein are available at http://archive.uwcm.ac.uk/uwcm/mg/docs/mut_nom.html.

Site-directed mutagenesis

A pSVL construct was used for the expression of wild-type and mutant PLTP [wild-type PLTP in pSVL plasmid was a gift from Christian Ehnholm (National Public Health Institute, Helsinki, Finland)]. Site-directed mutagenesis was carried out using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Inc., La Jolla, CA).

Cell culture

COS-7 cells were grown in DMEM supplemented with 10% FBS, L-glutamine, streptomycin, and penicillin. Cells (1.2×10^6) were seeded on 6 cm culture dishes the day before transfection. The cells were then transiently transfected using PolyFect Reagent (Qiagen, Inc., Valenica, CA) according to the manufacturer's protocol and grown in serum-free medium overnight. Supernatants were removed after 72 h, centrifuged to remove detached cells, and used for PLTP transfer activity and mass analysis.

Specific PLTP activity

PLTP activity was measured using a radiometric assay (20, 21). In this assay, the ability of PLTP to transfer phospholipids from [^{14}C]DPPC donor vesicles to ultracentrifugally isolated HDL_3 acceptor particles was measured. PLTP mass was obtained from Western blots. Briefly, culture supernatants were concentrated using acetone precipitation, separated on SDS gels, transferred onto nitrocellulose membranes, and blotted using the monoclonal anti-PLTP antibody JH59 (22) [a gift from Matti Jauhiainen and Christian Ehnholm (National Public Health Institute)]. Relative PLTP mass compared with the wild type was obtained by scanning and quantitation of the PLTP bands. Specific PLTP activity was obtained by dividing the activity by mass and is expressed relative to the wild-type PLTP [wild-type PLTP activity was 727 ± 148 (SD) nmol/ml/h and was set to a value of 1.0]. All mutations were assayed in four replicates and included both positive and negative controls.

Statistical methods

Statistical analyses were conducted using the SPSS for Windows (version 11.0.1) system (23). Allele and genotype frequencies were determined directly by the gene-counting method. Tests for Hardy-Weinberg equilibrium in controls, and allelic or genotypic association in cases versus controls, were evaluated by Chi-square test. Two-group comparisons of means of transformed or normally distributed variables used the independent samples Student's *t*-test (two-tailed). Natural log transformation was found to be appropriate for the normalization of variables. Two-group comparisons of means of untransformed, nonnormally distributed variables used the Wilcoxon two-sample test. The general linear model procedure was used for linear regression models. Power transformations of potential predictor variables were examined to maximize the explanatory power of the overall model (by maximizing the *F* statistic). Selected interaction effects and covariate-adjusted means of the transformed responses for levels of categorical factors were tested using the general linear model procedure. Interaction effects with $P < 0.10$ were retained. Data in the tables are presented as means ± SD.

RESULTS

Baseline characteristics of the clinical populations

The clinical characteristics of subjects with HA and healthy controls are described in **Table 1**. Compared with healthy controls, HA subjects possessed increased VLDL-TG [mean difference, natural log transformed = −0.506 ± 0.6471; 95% confidence interval (CI): −0.6328, −0.3785] and LDL-TG (mean difference, natural log transformed = −0.241 ± 0.0417; 95% CI: −0.3226, −0.1588). In addition, HA subjects possessed increased total cholesterol (mean difference = −17.57 ± 4.806; 95% CI: −27.021, −8.124), VLDL-C (mean difference, natural log transformed =

TABLE 1. Clinical characteristics of the study cohort

Trait	Control (n)	HA (n)	Test Statistic
Age (years)	51.6 ± 20.03 (364)	52.7 ± 20.71 (276)	n.s.
Gender (% female)	0.313 (364)	0.420 (276)	Chi-square = 7.363, 1 df, $P = 0.007$.
TG			
Total	124.7 ± 61.56 (364)	172.4 ± 76.80 (276)	n.a.
VLDL[a]	74.5 ± 57.56 (261)	113.8 ± 72.70 (210)	$t = -7.814$, 467.94 df, $P < 0.001$
LDL[a]	34.8 ± 13.28 (259)	44.3 ± 18.71 (208)	$t = -5.775$, 452.60 df, $P < 0.001$
HDL	16.6 ± 6.35 (364)	16.3 ± 5.80 (276)	n.s.
Cholesterol			
Total	193.3 ± 37.30 (364)	210.9 ± 72.93 (276)	$t = -3.657$, 383.47 df, $P < 0.001$
VLDL[a]	17.9 ± 14.52 (271)	27.0 ± 15.60 (221)	$t = -8.284$, 488.79 df, $P < 0.001$
LDL	128.4 ± 32.90 (271)	152.3 ± 67.64 (221)	$t = -4.824$, 304.60 df, $P < 0.001$
HDL	48.1 ± 13.97 (364)	31.3 ± 6.30 (276)	n.a.
TG/HDL-C	2.89 ± 1.770 (364)	6.14 ± 6.099 (276)	$Z = -14.211$, $P < 0.0001$
LDL-C/HDL-C	2.93 ± 1.125 (271)	5.16 ± 3.212 (221)	$Z = -11.891$, $P < 0.001$
BMI	26.0 ± 4.77 (216)	27.9 ± 3.212 (193)	$Z = -3.351$, $P < 0.001$

BMI, body mass index; df, degrees of freedom; HA, hypoalphalipoproteinemia; HDL-C, HDL-cholesterol; n.a., not applicable; n.s., not significant; TG, triglyceride.
[a] Natural log transformation.

−0.529 ± 0.0639; 95% CI: −0.6544, −0.4035), and LDL-C (mean difference = −23.89 ± 4.951; 95% CI: −33.627, −14.143). The latter findings are explained in part by the preselection bias inherent in the recruitment strategy of subjects into the UCSF GRA (referral to the UCSF Lipid Clinic as a result of increased LDL-C). Given their use in the ascertainment of HA cases, the significance of the differences in TG and HDL-C was not assessed. TG/HDL-C, an emergent marker of insulin sensitivity, and LDL-C/HDL-C, a clinically important cardiovascular risk factor, were found to be significantly increased in HA subjects. Body mass index (BMI) was modestly increased in HA subjects. Subjects in the HA group did not differ significantly with respect to age or HDL-TG concentration.

Results of PLTP molecular gene scan

Mutational analysis of the 16 exons of the PLTP gene in both the HA and healthy control groups resulted in the identification of several sequence anomalies, including a single nucleotide polymorphism in exon 1 (c.-34G>C; **Table 2**). Five missense mutations were identified in four

TABLE 2. PLTP PCR primers and sequence anomalies identified in the PLTP gene

Exon	Primer	Sequence Variants[a]	
		Control Subjects	HA Subjects
1	gagagctgcagagaggagga	c.-34G>C[b]	c.-34G>C[b]
	tagagattctgcctggagcc		AL008726.3.1.86080:g.43974206A>C (at −708 of the ATG)
2–3	tcagataactcctgcgacc	c.200+21T>C	
	ctggtcctgcttccttcttg	c.200+56G>C	
4	gagcagacaaatttcctgtcg	p.E72G, c.215A>G	
	cggaaactcaggaaggctaag	c.329+76C>A p.N94N, p.A95P, c.282T>G, c283G>C	c.329+76C>A
5	gaggtatggtgatgcatggg	p.S119A, c.355T>G	c.485+28C>T
	attgcgctaggcagatgag		p.S124Y, c.371C>A[c]
6–7	ccctagtcactgatttctcc	c.613+118_120delTTG	c.613+118_120delTTG
	caagtgaggaaacaagcac		c.613+84_89delAGTCCT p.Q182Q, c.546G>A
8	caccttatcccacatcacac	p.R235W, c.703C>T	c.614-28C>T
	cttgttaggaagtcagaggtcatac		
9–10	caatgccttttaagagatgtgc		
	cttggtctcactggtgtgc		
11–12	ggcaaaggcatgaaggctag		
	gcaacatccctgtggacag		
13–15	ctgagcctgcaactcagac		c.1218+1G>T
	gtcaggtaatggagggcac		c.1218+79G>C
16	gaatgaataaatgaggctccc		c.1654G>A
	gccctgtacatcccagactg		

PLTP, phospholipid transfer protein. Missense sequence anomalies are represented using standard single letter codes.
[a] Genome build NCBI35:20; sequence variations are denoted according to Human Genome Variation Society conventions (http://www.genomic.unimelb.edu.au/mdi/mutnomen/recs-DNA.html).
[b] Polymorphism displaying >1% allele frequency.
[c] Online Single Nucleotide Polymorphism database (dbSNP) number 11569636.

TABLE 3. Specific PLTP activity in the four PLTP mutants

Mutation	Exon	PLTP Activity
p.E72G (c.215A>G)	4	1.18 ± 0.24
p.S119A (c.355T>G)	5	0.91 ± 0.14
p.S124Y (c.371C>A)	5	0.87 ± 0.26
p.R235W (c.703C>T)	8	0.56 ± 0.09[a]

The results are mean values ± SEM from four independent experiments and are expressed as percentages of the activity of wild-type PLTP.

[a] $P = 0.015$ compared with wild-type PLTP activity (Student's unpaired *t*-test, two-tailed).

subjects, three of which are novel (p.E72G, p.S119Y, and p.R235W; Table 2). One silent mutation and a probable splice site mutation were identified in two subjects with HA (p.Q182Q and c.1218+1G>T; Table 2).

Functional studies of PLTP missense mutations

Four mutations of PLTP were further investigated in vitro. Site-directed mutagenesis was carried out, followed by transient transfection of COS7 cells, subsequent PLTP functional assay (radioisotopic method), and western blotting for protein content to study the defects in lipid transfer activities of mutant proteins.

Three of the mutations showed specific transfer activity comparable to that of wild-type protein (**Table 3**). However, one mutation (p.R235W in exon 8) showed 45% reduced activity in vitro. We were unable to obtain tissue samples for further cholesterol efflux studies from this individual. A sibling of the subject was screened for the mutation by direct sequencing and was found to be normal. All of the mutations were synthesized and secreted from the cells as efficiently as the wild-type PLTP; thus, the reduced specific transfer activity of the R235W mutant is not the result of aberrant folding and secretion but rather displays a true functional defect of the protein.

Genetic association of lipid parameters with PLTP c.-34G>C

On examination of the effects of the c.-34G>C polymorphism on lipoprotein metabolism using static fasting lipoprotein concentrations as a metabolic "snapshot," significant changes in mean measures of plasma lipoprotein compartments were observed. In the control sample, population carriers of the G allele exhibited modest yet significantly increased HDL-C (**Table 4**), whereas these were unchanged in HA subjects (**Table 5**). Suggestive evidence of an association with increased VLDL-TG in carriers of the G allele was observed in healthy controls but not in HA subjects (Tables 4, 5).

Allelic and genotypic frequencies for the c.-34G>C transversion in cases versus controls are listed in **Table 6**. The c.-34G>C genotype distribution did not deviate significantly from Hardy-Weinberg expectations (Chi-square = 0.122, 1 degree of freedom, $P = 0.73$). Compared with healthy controls, the frequency of C allele carriers was modestly decreased in HA subjects (0.0433 vs. 0.0218; Chi-square = 3.429, 1 degree of freedom, $P = 0.064$; odds ratio = 1.904, 95% CI: 0.913, 4.034). No significant gender differences were observed with respect to c.-34G>C allele frequencies. The observed c.-34G>C minor allele frequency was 4.82% and 3.60% in controls and 2.16% and 2.20% in cases for females and males, respectively.

Linear regression analysis of lipoprotein measures in carriers of PLTP c.-34G>C

Adjusting for variables known to be covariates of lipoprotein measures (e.g., gender, age, and BMI) increased the observed associations between c.-34G>C and HDL-C and TG/HDL-C and provided evidence of an association with TG. In contrast, adjusting for these variables weakened the observed association with LDL-C/HDL-C. Regression models for lipid measures included data-driven selection among the polymorphism categories CC and CG+GG (carriers of the minor allele). The effects of the potential predictors included age, gender, BMI, and clinical category (HA or healthy controls). Interaction effects were tested between clinical population and genotype categories. The models always included clinical population. Models in which a polymorphism effect did not achieve $P < 0.10$ are not reported. In the models that follow, the polymorphism effect GC+CC versus GG was evaluated and is presented in **Table 7**.

TABLE 4. Plasma lipid concentrations in the control sample, grouped by genotype

Trait	GG	CG+CC	Statistical Test
Age (years)	51.5 ± 20.19 (334)	51.5 ± 18.32 (29)	n.s.
TG			
Total	126.4 ± 62.88 (335)	104.8 ± 39.09 (29)	n.s.
VLDL	76.6 ± 59.19 (238)	52.9 ± 30.14 (23)	$Z = -1.744$, $P = 0.08$
LDL	34.9 ± 13.40 (236)	33.7 ± 12.22 (23)	n.s.
HDL	16.6 ± 6.44 (335)	16.4 ± 5.41 (29)	n.s.
Cholesterol			
Total	193.0 ± 37.33 (335)	196.6 ± 37.41 (29)	n.s.
VLDL	18.2 ± 14.84 (247)	15.0 ± 10.39 (24)	n.s.
LDL	128.4 ± 33.37 (247)	127.9 ± 28.27 (24)	n.s.
HDL	47.4 ± 13.50 (335)	55.8 ± 16.99 (29)	$Z = -3.026$, $P = 0.002$
TG/HDL-C	2.95 ± 1.757 (335)	2.72 ± 1.835 (29)	$Z = -2.710$, $P = 0.007$
LDL-C/HDL-C	2.95 ± 1.091 (247)	2.66 ± 1.835 (24)	$Z = -2.005$, $P = 0.045$
BMI	26.1 ± 4.84 (198)	24.6 ± 3.72 (18)	n.s.

n.s., not significant. Lipid measurements are expressed in mg/dl.

TABLE 5. Plasma lipid concentrations in the HA sample, grouped by genotype

Trait	GG	CG+CC	Statistical Test
Age (years)	53.1 ± 20.83 (263)	47.9 ± 15.88 (12)	n.s.
TG			
Total[a]	172.9 ± 77.54 (263)	164.3 ± 64.66 (12)	n.s.
VLDL	114.4 ± 73.67 (200)	101.3 ± 56.73 (9)	n.s.
LDL	44.2 ± 18.50 (198)	50.7 ± 21.26 (9)	n.s.
HDL	16.3 ± 5.89 (263)	16.3 ± 3.52 (12)	n.s.
Cholesterol			
Total	210.3 ± 73.39 (263)	226.3 ± 65.63 (12)	n.s.
VLDL	27.0 ± 15.67 (210)	27.6 ± 15.53 (10)	n.s.
LDL	151.5 ± 67.87 (210)	171.1 ± 57.79 (10)	n.s.
HDL[a]	31.2 ± 6.34 (263)	32.6 ± 5.76 (12)	n.s.
TG/HDL-C	6.18 ± 6.224 (263)	5.30 ± 2.512 (12)	n.s.
LDL-C/HDL-C	5.14 ± 3.266 (210)	5.59 ± 2.512 (10)	n.s.
BMI	27.9 ± 5.63 (183)	28.1 ± 5.72 (9)	n.s.

n.s., not significant. Lipid measurements are expressed in mg/dl.
[a] A statistic is not provided for this variable used in the selection of subjects.

For plasma TG (n = 408; $P < 0.001$ for the selected model), selected effects include clinical category ($P < 0.001$), BMI ($P = 0.002$), genotype category ($P = 0.032$), and age ($P = 0.064$); the adjusted R^2 for the model was 0.116. In c.-34G>C minor allele carriers, the difference in TG concentrations was approximately −23.8 mg/dl. For HDL-C (n = 408; $P < 0.001$ for the selected model), selected effects include clinical category ($P < 0.001$), gender ($P < 0.001$), genotype ($P = 0.001$), age ($P = 0.002$), genotype-by-clinical category interaction ($P = 0.021$), BMI ($P = 0.041$), and the natural log of TG ($P = 0.042$); the adjusted R^2 for the model was 0.476. In healthy controls, the difference in HDL-C concentrations between carriers of the C allele (GC+CC) and GG homozygotes was ~12.6 mg/dl. In HA subjects, the difference between the two groups was 2.6 mg/dl. For the ratio TG/HDL-C (n = 408; $P < 0.001$ for the selected model), selected effects include clinical category ($P < 0.001$), gender ($P < 0.001$), genotype ($P = 0.001$), and BMI ($P = 0.003$); the adjusted R^2 for the model was 0.345. In c.-34G>C minor allele carriers, the difference in TG/HDL-C was ~1.121.

DISCUSSION

PLTP's role in phospholipid transfer from TG-rich lipoproteins, phospholipid and cholesterol acquisition from peripheral cells, and participation in hepatic synthesis of VLDL indicate that PLTP is a central effector of HDL metabolism. In this study, we discovered a polymorphism in exon 1 of PLTP that is associated with antiatherogenic changes in lipoprotein profiles in subjects with HA or healthy controls. Individuals in possession of the c.-34G>C minor allele exhibited higher HDL-C. The minor allele was also associated with lower plasma TG and a lower TG/HDL-C ratio. Importantly, decreased PLTP activity was observed in one (p.R235W) of four (p.E72G, p.S119A, p.S124Y, and p.R235W) missense mutations in an in vitro activity assay.

TABLE 6. PLTP c.-34G>C variation allele and genotype frequencies

Genotype	Control	HA
GG	335	264
CG+CC	29	12
Total	365	276
C allele	4.33%	2.18%

Allele and genotype frequencies were determined by gene counting.

TABLE 7. Regression analyses, grouped according to PLTP c.-34G>C genotype

Trait	GG	GC+CC	R^2	P
TG[a]	142.3	118.5	0.116	<0.001
HDL-C[b]				
Cases	32.3	34.9	0.476	<0.001
Controls	48.3	60.9		
TG/HDL-C[c]	3.717	2.596	0.345	<0.001

Lipid measurements are expressed in mg/dl. Covariates appearing in the model were evaluated at the values described in the footnotes.
[a] BMI = 26.92, age = 52.00.
[b] BMI = 26.9, age = 52.00, TG = 139.6.
[c] BMI = 26.92.

Although none of the missense mutations was common in this study population, they have the potential to further our understanding of PLTP structure and function. Predictions regarding the potential impact of these missense mutations on PLTP structure can be made within the context of the structural model proposed by Desrumaux and colleagues (24). The R235W mutation maps to a small stretch of polypeptide between β strands βA3 and βC3. Interestingly, this mutant is predicted to be "probably damaging" when analyzed online by PolyPhen (at http://genetics.bwh.harvard.edu/cgi-bin/pph/polyphen.cgi). This is further supported by structure and function analysis of PLTP using site-directed mutagenesis of the arginine residue at position 235, considered likely to be important in binding to charged lipid surfaces (25). The mutant R235W showed low binding capability for HDL and greatly decreased phospholipid transfer activity (25). In contrast to the ~50% decrease in secretion observed for the PLTP R235E mutant studied by Ponsin and colleagues (25), the PLTP R235W mutant exhibited no such defect. This finding supports the conclusion that there are no gross structural abnormalities affecting the secretion of the mutant protein; rather, the R235W mutation affects the functionality (specific activity) of the protein.

Functional studies are required to formally demonstrate the functional impact of c.-34G>C, although it is likely that it is in linkage disequilibrium with a function variation(s) in the gene's promoter. Although the two promoter polymorphisms reported previously (11) were not captured in the amplicon spanning exon 1 in this study, the relationship between promoter polymorphisms and c.-34G>C should be investigated in subsequent studies. The c.-34G>C minor allele was associated with antiatherogenic changes in HDL-C, TG, and their ratio. It is noteworthy that the occurrence of this polymorphism was appreciably diminished in HA subjects. However, the statistical

significance of this difference can only be considered suggestive in this study. Clearly, validation of this finding in an independent and larger sample is required.

High TG and low HDL-C is a characteristic pattern seen in insulin-resistant individuals (26). Importantly, an increased TG/HDL-C ratio was found to be as least as powerful a predictor of ischemic heart disease as isolated high LDL-C (27). Moreover, similar findings have been reported in other cohort studies (28, 29). Whereas segregation studies (30) and genome-wide linkage scanning (31) have provided evidence for genetic determinants specifically affecting TG/HDL-C, candidate gene studies aimed at identifying the causal variants are few. Given the roles of PLTP in both hepatic VLDL production and HDL metabolism in experimental systems (5–7, 9), the effect of PLTP gene variation on TG, HDL-C, and their ratio demonstrated here represents a critical validation of previous controlled observations and suggests that the role of PLTP in dyslipidemia and cardiovascular risk merits further study.

Potential study limitations include a possibility of preselection bias of primary HA. The UCSF Lipid Clinic is a tertiary clinic to which individuals are most commonly referred for increased LDL-C, which could explain the increased levels observed in this cohort. Although both DGGE and dHPLC have detection sensitivity approaching 100% (32), another potential limitation is the possibility that a small subset of sequence variants were not detected using the described mutation detection methodologies. Although PLTP activity assays have centered on the gene's phospholipid transfer function, an evaluation of the role of PLTP c.-34G>C in this aspect of PLTP function would have been of interest.

The inverse relationship between HDL-C and risk of coronary heart disease is firmly established. The central role of PLTP in HDL metabolism and the fact that increased PLTP activity appears to be atherogenic (33) makes it an attractive target for pharmacological intervention. This report represents the first in-depth sequence analysis of the PLTP gene in humans and identifies a novel polymorphism associated with antiatherogenic alterations in lipoprotein profiles. Analysis of this polymorphism in independent studies and characterization of the functional impact of the missense mutations described here represent the logical next steps in the study of PLTP gene variation in lipid metabolism.

The authors thank the subjects for participating in this study. National Institutes of Health Training Grant in Genetics T32 HL-07731 supported B.E.A. National Institutes of Health Grant K01 NR-07888 supported M.B.E. and Y.N. This work was also supported by grants from Pharmacia Corp. (D91.101) and Hyseq, Inc. (AA11205, HL50779, and HL50782).

REFERENCES

1. Ordovas, J. M., and E. J. Schaefer. 1988. Coronary artery disease, lipid disorders and genetic polymorphisms. *Ann. Biol. Clin. (Paris).* **46:** 24–29.
2. Miller, M., and M. Zhan. 2004. Genetic determinants of low high-density lipoprotein cholesterol. *Curr. Opin. Cardiol.* **19:** 380–384.
3. Austin, M. A., M. C. King, R. D. Bawol, S. B. Hulley, and G. D. Friedman. 1987. Risk factors for coronary heart disease in adult female twins. Genetic heritability and shared environmental influences. *Am. J. Epidemiol.* **125:** 308–318.
4. Jiang, X. C., C. Bruce, J. Mar, M. Lin, Y. Ji, O. L. Francone, and A. R. Tall. 1999. Targeted mutation of plasma phospholipid transfer protein gene markedly reduces high-density lipoprotein levels. *J. Clin. Invest.* **103:** 907–914.
5. Settasatian, N., M. Duong, L. K. Curtiss, C. Ehnholm, M. Jauhiainen, J. Huuskonen, and K. A. Rye. 2001. The mechanism of the remodeling of high density lipoproteins by phospholipid transfer protein. *J. Biol. Chem.* **276:** 26898–26905.
6. Oram, J. F., G. Wolfbauer, A. M. Vaughan, C. Tang, and J. J. Albers. 2003. Phospholipid transfer protein interacts with and stabilizes ATP-binding cassette transporter A1 and enhances cholesterol efflux from cells. *J. Biol. Chem.* **278:** 52379–52385.
7. Lie, J., R. de Crom, T. van Gent, R. van Haperen, L. Scheek, F. Sadeghi-Niaraki, and A. van Tol. 2004. Elevation of plasma phospholipid transfer protein increases the risk of atherosclerosis despite lower apolipoprotein B-containing lipoproteins. *J. Lipid Res.* **45:** 805–811.
8. Jiang, X. C., Z. Li, R. Liu, X. P. Yang, M. Pan, L. Lagrost, E. A. Fisher, and K. J. Williams. 2005. Phospholipid transfer protein deficiency impairs apolipoprotein-B secretion from hepatocytes by stimulating a proteolytic pathway through a relative deficiency of vitamin E and an increase in intracellular oxidants. *J. Biol. Chem.* **280:** 18336–18340.
9. van Haperen, R., A. van Tol, T. van Gent, L. Scheek, P. Visser, A. van der Kamp, F. Grosveld, and R. de Crom. 2002. Increased risk of atherosclerosis by elevated plasma levels of phospholipid transfer protein. *J. Biol. Chem.* **277:** 48938–48943.
10. Bosse, Y., L. Bouchard, J. P. Despres, C. Bouchard, L. Perusse, and M. C. Vohl. 2005. Haplotypes in the phospholipid transfer protein gene are associated with obesity-related phenotypes: the Quebec Family Study. *Int. J. Obes. (Lond.).* **29:** 1338–1345.
11. Tahvanainen, E., M. Jauhiainen, H. Funke, E. Vartiainen, J. Sundvall, and C. Ehnholm. 1999. Serum phospholipid transfer protein activity and genetic variation of the PLTP gene. *Atherosclerosis.* **146:** 107–115.
12. Pullinger, C. R., L. K. Hennessy, J. E. Chatterton, W. Liu, J. A. Love, C. M. Mendel, P. H. Frost, M. J. Malloy, V. N. Schumaker, and J. P. Kane. 1995. Familial ligand-defective apolipoprotein B. Identification of a new mutation that decreases LDL receptor binding affinity. *J. Clin. Invest.* **95:** 1225–1234.
13. Rush, R. L., L. Leon, and J. Turrell. 1971. *In* Advances in Automated Analysis, Technicon International Congress 1970. Vol. 1. Thurman Assoc., Miami, FL, 503.
14. Warnick, G. R., J. Benderson, and J. J. Albers. 1982. Dextran sulfate-Mg^{2+} precipitation procedure for quantitation of high-density-lipoprotein cholesterol. *Clin. Chem.* **28:** 1379–1388.
15. Kane, J. P., M. J. Malloy, T. A. Ports, N. R. Phillips, J. C. Diehl, and R. J. Havel. 1990. Regression of coronary atherosclerosis during treatment of familial hypercholesterolemia with combined drug regimens. *J. Am. Med. Assoc.* **264:** 3007–3012.
16. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
17. O'Donovan, M. C., P. J. Oefner, S. C. Roberts, J. Austin, B. Hoogendoorn, C. Guy, G. Speight, M. Upadhyaya, S. S. Sommer, and P. McGuffin. 1998. Blind analysis of denaturing high-performance liquid chromatography as a tool for mutation detection. *Genomics.* **52:** 44–49.
18. Fischer, S. G., and L. S. Lerman. 1983. DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. *Proc. Natl. Acad. Sci. USA.* **80:** 1579–1583.
19. Schneider, M., B. Verges, A. Klein, E. R. Miller, V. Deckert, C. Desrumaux, D. Masson, P. Gambert, J. M. Brun, J. Fruchart-Najib, et al. 2004. Alterations in plasma vitamin E distribution in type 2 diabetic patients with elevated plasma phospholipid transfer protein activity. *Diabetes.* **53:** 2633–2639.
20. Jauhiainen, M., J. Metso, R. Pahlman, S. Blomqvist, A. van Tol, and C. Ehnholm. 1993. Human plasma phospholipid transfer protein causes high density lipoprotein conversion. *J. Biol. Chem.* **268:** 4032–4036.

21. Damen, J., J. Regts, and G. Scherphof. 1982. Transfer of [^{14}C]phosphatidylcholine between liposomes and human plasma high density lipoprotein. Partial purification of a transfer-stimulating plasma factor using a rapid transfer assay. *Biochim. Biophys. Acta.* **712:** 444–452.
22. Huuskonen, J., M. Jauhiainen, C. Ehnholm, and V. M. Olkkonen. 1998. Biosynthesis and secretion of human plasma phospholipid transfer protein. *J. Lipid Res.* **39:** 2021–2030.
23. SPSS for Windows, release 11.0.1 standard version (November 15, 2001). Chicago, IL, SPSS.
24. Desrumaux, C., C. Labeur, A. Verhee, J. Tavernier, J. Vandekerckhove, M. Rosseneu, and F. Peelman. 2001. A hydrophobic cluster at the surface of the human plasma phospholipid transfer protein is critical for activity on high density lipoproteins. *J. Biol. Chem.* **276:** 5908–5915.
25. Ponsin, G., S. J. Qu, H. Z. Fan, and H. J. Pownall. 2003. Structural and functional determinants of human plasma phospholipid transfer protein activity as revealed by site-directed mutagenesis of charged amino acids. *Biochemistry.* **42:** 4444–4451.
26. Laws, A., and G. M. Reaven. 1992. Evidence for an independent relationship between insulin resistance and fasting plasma HDL-cholesterol, triglyceride and insulin concentrations. *J. Intern. Med.* **231:** 25–30.
27. Jeppesen, J., H. O. Hein, P. Suadicani, and F. Gyntelberg. 1997. Relation of high TG-low HDL cholesterol and LDL cholesterol to the incidence of ischemic heart disease. An 8-year follow-up in the Copenhagen Male Study. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1114–1120.
28. Assmann, G., and H. Schulte. 1992. The importance of triglycerides: results from the Prospective Cardiovascular Munster (PROCAM) Study. *Eur. J. Epidemiol.* **8 (Suppl. 1):** 99–103.
29. Castelli, W. P. 1992. Epidemiology of triglycerides: a view from Framingham. *Am. J. Cardiol.* **70:** 3H–9H.
30. Chien, K. L., H. C. Hsu, T. C. Su, C. Y. Yang, and Y. T. Lee. 2003. Consistency of genetic inheritance mode and heritability patterns of triglyceride vs. high density lipoprotein cholesterol ratio in two Taiwanese family samples. *BMC Genet.* **4:** 7.
31. Shearman, A. M., J. M. Ordovas, L. A. Cupples, E. J. Schaefer, M. D. Harmon, Y. Shao, J. D. Keen, A. L. DeStefano, O. Joost, P. W. Wilson, et al. 2000. Evidence for a gene influencing the TG/HDL-C ratio on chromosome 7q32.3-qter: a genome-wide scan in the Framingham Study. *Hum. Mol. Genet.* **9:** 1315–1320.
32. Cotton, R. 1997. Mutation Detection. Oxford University Press, New York.
33. Albers, J. J., and M. C. Cheung. 2004. Emerging roles for phospholipid transfer protein in lipid and lipoprotein metabolism. *Curr. Opin. Lipidol.* **15:** 255–260.