Relationship between cholesteryl ester transfer protein and LDL heterogeneity in familial hypercholesterolemia

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Abstract Small, dense LDL particles have been associated with an increased risk of coronary artery disease, and cholesteryl ester transfer protein (CETP) has been suggested to play a role in LDL particle remodeling. We examined the relationship between LDL heterogeneity and plasma CETP mass in familial hypercholesterolemia (FH). LDL particles were characterized by polyacrylamide gradient gel electrophoresis in a total of 259 FH heterozygotes and 208 nonFH controls. CETP mass was measured by enzyme-linked immunosorbent assay in a subgroup of 240 participants, which included 120 FH patients matched with 120 controls. As compared with controls, FH subjects had an 11% higher CETP mass. Moreover, LDL-peak particle diameter (LDL-PPD) was significantly smaller in FH heterozygotes than in controls (258.1 ± 4.8 vs. 259.2 ± 4.1 Å; $P = 0.01$) after adjustment for covariates. There was also an inverse relationship between LDL-PPD and CETP mass ($R = -0.15$; $P = 0.02$), and this relationship was abolished by adjustment for the FH/control status, indicating that LDL-PPD changes in FH are mediated, at least in part, by an increase in plasma CETP mass concentrations. These results suggest that increased plasma CETP mass concentrations could lead to significant LDL particle remodeling in FH heterozygotes and could contribute to the pathogenesis of atherosclerosis.—Hogue, J-C., B. Lamarche, D. Gaudet, M. Larivière, A. J. Tremblay, J. Bergeron, I. Lemieux, J-P. Després, C. Gagné, and P. Couture. **Relationship between cholesteryl ester transfer protein and LDL heterogeneity in familial hypercholesterolemia.** *J. Lipid Res.* 2004. 45: 1077–1083.

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Familial hypercholesterolemia (FH) is an autosomal codominant single-gene disorder caused by mutations in the LDL receptor (LDLR) gene that disrupt the normal clearance of LDL (1). Phenotypic features characteristic of the disease's heterozygous form are a 2- to 3-fold rise in plasma LDL-cholesterol (LDL-C) concentrations, tendinous xanthomatosis, and premature atherosclerotic coronary artery disease (CAD), usually occurring between the ages of 35 years and 55 years. Homozygous or compound heterozygous patients have plasma LDL concentrations 6- to 8-fold higher than normal and usually manifest a CAD event before the age of 20 years. FH is also one of the most common inherited diseases in the world, with a frequency of 1 in 500 for heterozygotes and 1 per million for homozygotes (1). In the Province of Québec, the homozygote prevalence is 6-fold higher, and the minimal heterozygote frequency ranges from 1:81 to 1:154 (2). Nine mutations are responsible for 90% of the heterozygous FH cases in the French-Canadian population, defined on the basis of clinical and biochemical criteria (3).

Cholesteryl ester transfer protein (CETP) plays a major role in the remodeling of lipoprotein particles by mediating the transfer of cholesteryl ester from HDL to apolipoprotein B (apoB)-containing lipoproteins in exchange for triglycerides, and several lines of evidence support the notion that CETP is linked to LDL size heterogeneity (4). Small, dense LDL particles have been associated with CAD in a number of studies (5–7). These previous results, however, were obtained in nonFH subjects exhibiting lipoprotein profiles very different from the extremely elevated LDL-C seen in FH patients. To date, only a few studies have examined the heterogeneity of LDL particles in FH patients (8–11), but their limited small sample size precluded any definitive conclusions. As characterization of

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Abbreviations: apoE3, apolipoprotein E3; BMI, body mass index; CAD, coronary artery disease; CETP, cholesteryl ester transfer protein; FH, familial hypercholesterolemia; LDL-C, LDL cholesterol; LDL-PPD, LDL-peak particle diameter; LDLR, LDL receptor.

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LDL size could be relevant for the understanding of the variability in CAD risk among FH patients, the objective of the present study was to examine LDL size heterogeneity and its relationship to CETP in a large cohort of genetically-defined FH heterozygotes and controls.

METHODS

FH subjects

A total of 259 FH heterozygotes (122 men and 137 women) from Québec City and Saguenay (Canada) were enrolled. All participants were at least 18 years-of-age. Subjects were excluded if they: had a history of cardiovascular disease; were pregnant or nursing; had acute liver disease, hepatic dysfunction, or persistent elevations of serum transaminases; had plasma triglyceride levels >4.5 mmol/l or homozygous FH; had a secondary hyperlipidemia due to any cause; had a recent history of alcohol or drug abuse; had diabetes mellitus; had a history of cancer; or had hormonal treatment.

All FH subjects were carriers of one of the nine previously known French-Canadian mutations in the LDLR gene (3) and were apoE3 homozygotes. Of those 259 heterozygous subjects selected, 123 had the deletion >15 kb at the 5′ end of the gene (12), 112 had the W66G mutation in exon 3 (13), 13 had the Y468× mutation in exon 10 (14), six had the C646Y mutation in exon 14 (15), one had the C347R mutation in exon 8 (3), one had the E207K mutation in exon 4 (15), one had the C152W mutation in exon 4 (3), one had the R329× mutation in exon 7 (3), and one had the 5 kb deletion in exons 2 and 3 (15). All eligible FH participants had to withdraw lipid-lowering medications for at least 6 weeks before a blood sample was taken. The study was approved by the Laval University Medical Center ethical review committee and informed consent was obtained from each patient.

Controls

A total of 208 controls (115 men and 93 women) were selected among the 2,056 participants of the Québec Health Survey, which was comprised of noninstitutionalized men and women, excluding aboriginal populations, selected from health insurance files (16). As previously described (16), the Québec Health Survey was designed to obtain relevant information on the prevalence and distribution of cardiovascular disease risk factors in the Québec population. All controls selected for the purpose of this study were apoE3 homozygotes.

Plasma lipids and lipoproteins

Blood samples were collected after a 12 h fasting period in tubes containing disodium EDTA and benzamidine (0.03%) (17). Samples were then immediately centrifuged at 4°C for 10 min at 3,000 rpm to obtain plasma and were stored at 4°C until processed. Cholesterol and triglyceride levels were determined in plasma and in lipoprotein fractions by enzymatic methods (Randox Co., Crumlin, UK) using an RA-500 analyzer (Bayer Corporation, Inc., Tarrytown, NY), as previously described (18). Plasma VLDLs (d < 1.006 g/ml) were isolated by preparative ultracentrifugation and the HDL fraction obtained after precipitation of LDL in the infranatant (d > 1.006 g/ml) with heparin and MnCl₂. The cholesterol and triglyceride contents of the infranatant fraction were measured before and after the precipitation step.

LDL particle size characterization

Nondenaturing 2% to 16% polyacrylamide gradient gel electrophoresis was performed as described previously (19). Briefly,

LDL particle size was determined on 8 cm × 8 cm polyacrylamide gradient gels prepared in batches in our laboratory. Aliquots of 3.5 μl of whole plasma samples were mixed in a 1:1 vol ratio, with a sampling buffer containing 20% sucrose and 0.25% bromophenol blue, and loaded onto the gels. A 15 min prerun at 75 V preceded electrophoresis of the plasma samples at 150 V for 3 h. Gels were stained for 1 h with Sudan black (0.07%) and stored in a 0.81% acetic acid/4% methanol solution until analysis by the Imagemaster 1-D Prime computer software (Amersham Pharmacia Biotech). LDL size was extrapolated from the relative migration of four plasma standards of known diameter. The estimated diameter for the major peak in each scan was identified as the LDL-peak particle diameter (LDL-PPD). An integrated (or mean) LDL diameter was also computed by using a modification of the approach described by Tchernof et al. (20). This integrated LDL particle size corresponds to the weighed mean size of all LDL subclasses in one individual. It was calculated as a continuous variable and was computed as the sum of the diameter of each LDL subclass multiplied by its relative area. Analysis of pooled plasma standards revealed that measurement of LDL-PPD was highly reproducible, with an interassay coefficient of variation of <2%. The relative proportion of LDL having a diameter <255 Å (LDL%$_{<255 Å}$) was ascertained by computing the relative area of the densitometric scan <255 Å (21). The absolute concentration of cholesterol among particles <255 Å (LDL-C$_{<255 Å}$) was calculated by multiplying the plasma LDL-C levels by the relative proportion of LDL with a diameter <255 Å (21). A similar approach was used to assess the relative and absolute concentrations of cholesterol in particles with a diameter between 255 Å and 260 Å, or >260 Å (LDL%$_{255-260 Å}$, LDL-C$_{255-260 Å}$ and LDL%$_{>260 Å}$, LDL-C$_{>260 Å}$, respectively).

TABLE 1. Demographic, genotypic, and biochemical characteristics of participants according to FH/control status

Variable	Controls	FH	P
	N = 208	N = 259	—
Age (y)	35.6 ± 16.2	37.0 ± 12.5	0.32
Gender			0.08
Men N, (%)	115 (55.3)	122 (47.1)	
Women N, (%)	93 (44.7)	137 (52.9)	
BMI (kg/m²)	24.7 ± 4.2	25.2 ± 4.3	0.25
Waist circumference (cm)	82.3 ± 12.2	81.9 ± 12.8	0.74
LDLR mutations			
Deletion >15kb N, (%)	—	123 (47.5)	—
W66G N, (%)	—	112 (43.2)	—
Y468X N, (%)	—	13 (5.0)	—
C646Y N, (%)	—	6 (2.3)	—
C347R N, (%)	—	1 (0.4)	—
E207K N, (%)	—	1 (0.4)	—
C152W N, (%)	—	1 (0.4)	—
R329X N, (%)	—	1 (0.4)	—
Deletion 5kb N, (%)	—	1 (0.4)	—
Smoking			
Ever N, (%)	151 (72.6)	125 (48.3)	<0.0001
Never N, (%)	57 (27.4)	134 (51.7)	
Total Plasma Cholesterol (mmol/l)	5.07 ± 0.89	8.48 ± 1.71	<0.0001*
LDL-C (mmol/l)	3.13 ± 0.80	6.75 ± 1.62	<0.0001*
HDL-C (mmol/l)	1.31 ± 0.30	1.09 ± 0.28	<0.0001*
Triglycerides (mmol/l)	1.39 ± 0.67	1.48 ± 0.74	0.21*

apoE3, apolipoprotein E3; BMI, body mass index; FH, familial hypercholesterolemia; LDL-C, LDL cholesterol; LDLR, LDL receptor. Results are listed as mean ± SD. All participants included were apoE3 homozygotes.
* Probability levels were adjusted for age, gender, BMI, and smoking.

TABLE 2. Plasma CETP mass concentration and electrophoretic characteristics of LDL according to FH/control status

Variable	Controls	FH	P
	N = 120	N = 120	—
CETP mass (μg/ml)	1.52 ± 0.45	1.68 ± 0.45	0.009^a
	N = 208	N = 259	—
LDL-PPD (Å)	259.2 ± 4.1	258.1 ± 4.8	0.01^b
Integrated LDL size (Å)	258.9 ± 4.3	259.2 ± 4.2	0.09^b
LDL%$_{<255Å}$	31.2 ± 13.7	27.5 ± 14.9	0.01^b
LDL%$_{255-260Å}$	20.2 ± 4.8	23.2 ± 7.3	$<0.0001^b$
LDL%$_{>260Å}$	48.6 ± 14.7	49.3 ± 15.3	0.98^b
LDL-C$_{<255Å}$ (mmol/l)	0.98 ± 0.53	1.86 ± 1.07	$<0.0001^b$
LDL-C$_{255-260Å}$ (mmol/l)	0.64 ± 0.04	1.35 ± 0.04	$<0.0001^b$
LDL-C$_{>260Å}$ (mmol/l)	1.51 ± 0.60	3.39 ± 1.37	$<0.0001^b$

CETP, cholesteryl ester transfer protein; LDL-PPD, LDL-peak particle diameter. Results are listed as mean ± SD. All participants included were apoE3 homozygotes.

^{a} Probability levels were adjusted for plasma triglyceride levels.

^{b} Probability levels were adjusted for age, gender, BMI, smoking, and plasma triglyceride levels.

Measurement of CETP mass

Plasma CETP mass concentration was used to assess plasma CETP activity because they are strongly correlated (22). However, sample handling and storage conditions may influence the stability of lipid transfer activity and, consequently, could have a significant impact on the relationship between CETP mass and activity. In the present study, CETP mass determination was used to minimize the potential effect of storage and sample handling on the stability of the lipid transfer activity. Plasma CETP mass concentration was determined by a commercial sandwich enzyme-linked immunosorbent assay kit (Wako Chemicals, Inc., Richmond, VA) in a subgroup of 240 participants, including 120 FH subjects matched for age, gender, body mass index (BMI), and smoking habits, with 120 controls.

DNA analysis

Genotyping of apoE was done by PCR-amplification of a 244 bp fragment of the exon 4 of the apoE gene with oligonucleotides F4 and F6 and digestion of PCR fragments with the restriction enzyme *Hha*I (23).

Statistical analyses

Data from FH patients were compared with data from control patients using Chi-square tests for categorical measures and ANOVA tests for continuous measures. Plasma triglycerides were log-transformed to normalize their distribution. Pearson correlation coefficients were determined to assess the significance of associations of LDL-PPD and other parameters. Stepwise multiple linear regression analysis was used to interpret the relationship of these associations. Nominal logistic regression was used to assess the relative risk of having LDL-PPD <255 Å based on plasma triglyceride levels and CETP mass. All analyses were performed using JMP Statistical Software (version 5.01a, SAS Institute, Cary, NC).

RESULTS

Demographic, genotypic, and biochemical characteristics

Results were analyzed from a total of 467 subjects (259 FH heterozygotes and 208 control subjects) who participated in the study and who had lipid values available off lipid-altering medication. The demographic, genotypic, and biochemical characteristics of the two groups are presented in **Table 1**. There was no significant difference between the control group and FH group for age, gender, BMI, and waist circumference. The deletion >15 kb and the W66G missense mutation were largely predominant over the other seven French-Canadian mutations because they were present in more than 90% of the cases. The percentage of smokers was significantly lower among FH heterozygotes ($P < 0.0001$). FH heterozygotes had significantly greater plasma concentrations of total cholesterol (67%; $P < 0.0001$) and LDL-C (116%; $P < 0.0001$) and lower HDL-C levels (17%; $P < 0.0001$) compared with controls. There was no significant difference in plasma triglyceride levels between the two groups ($P = 0.21$).

CETP mass concentration and electrophoretic characteristics of LDL particles

Plasma CETP mass concentration and electrophoretic characteristics of LDL according to FH/control status are
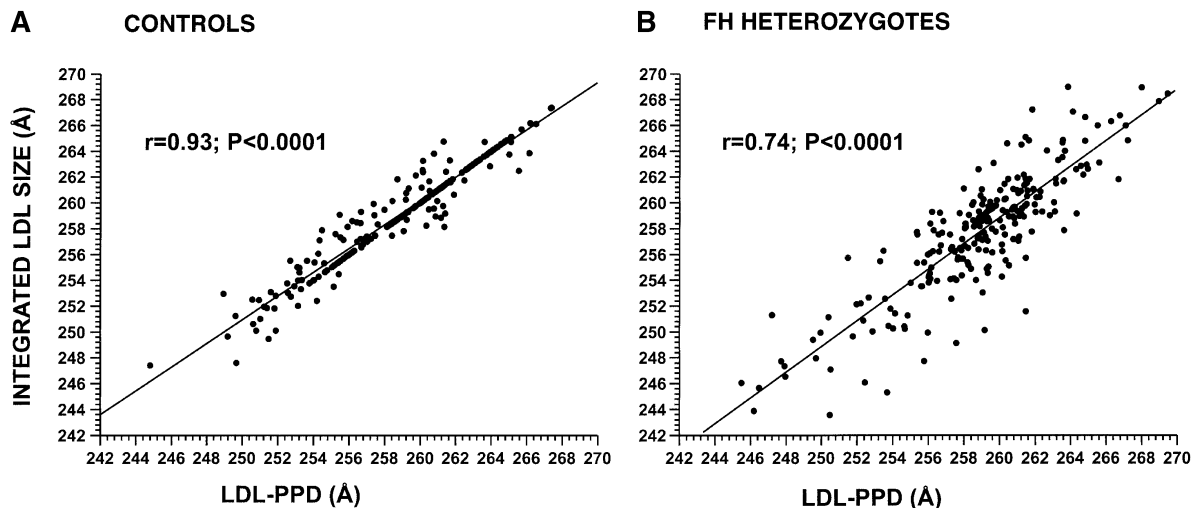

Fig. 1. Correlation between LDL-peak particle diameter (LDL-PPD) and the integrated LDL size among (A) controls and (B) familial hypercholesterolemic (FH) heterozygotes.

presented in **Table 2**. Plasma CETP mass concentration was measured in a subgroup of 240 subjects, including 120 FH subjects matched for age, gender, BMI, and smoking with 120 controls, and was 11% higher in FH patients than in controls. This difference remained highly significant after adjustment for plasma triglyceride levels ($P = 0.009$).

After adjustment for age, gender, BMI, smoking, and plasma triglyceride levels, LDL-PPD, which represents the diameter of the most abundant subclass of LDL particles, was found to be significantly smaller in FH heterozygotes than in control subjects (258.1 ± 4.8 vs. 259.2 ± 4.1; $P = 0.01$). **Figure 1** shows that the correlation between LDL-PPD and the integrated LDL size, which corresponds to the weighed mean size of all LDL subclasses in each individual, was stronger among controls ($r = 0.93$; $P < 0.0001$) than in FH subjects ($r = 0.74$; $P < 0.0001$), suggesting that the distribution of LDL particle size differs between the two groups. Despite the presence of a significantly smaller proportion of LDL with a diameter <255 Å in FH subjects, the integrated LDL size of FH subjects did not differ significantly from that of controls (259.2 ± 4.2 vs. 258.9 ± 4.3 Å; $P = 0.09$). It is important to note, however, that the smaller proportion of LDL <255 Å found in FH was associated with a reciprocal increase in the relative proportion of LDL with a diameter between 255 Å and 260 Å. No significant difference in the relative proportion of large LDL (>260 Å) was observed between FH heterozygotes and controls. The distribution of integrated LDL size among FH subjects and controls is depicted in **Fig. 2**. As expected, the LDL-C$_{<255\,Å}$, LDL-C$_{255-260\,Å}$, and LDL-C$_{>260\,Å}$ were significantly higher in FH heterozygotes than in controls.

Factors associated with LDL-PPD variability

Univariate analyses revealed that LDL-PPD was inversely correlated with plasma triglyceride levels ($r = -0.45$; $P = < 0.0001$) and plasma CETP mass concentrations ($r = -0.15$; $P = 0.02$). Furthermore, LDL-PPD was found to be significantly smaller in males than in females (257.4 ± 4.7 vs. 259.8 ± 4.0; $P < 0.0001$). The significance of the association between LDL-PPD and plasma CETP mass concentrations was abolished after adjustment for the FH/control status, indicating that the LDL-PPD changes in FH were mediated, at least in part, by CETP.

Stepwise multiple linear regression analyses were performed to identify independent contributors to the LDL-PPD variability. We found that 26.7% of the variability in LDL-PPD was attributable to plasma triglyceride levels (23.3%, $P < 0.0001$), plasma CETP mass concentrations (1.9%, $P = 0.02$), and gender (1.5%, $P = 0.03$). Age, BMI, and plasma LDL-C did not contribute significantly to LDL-PPD variance after adjustment for covariates.

The combined impact of concomitant variations in plasma CETP mass concentrations and plasma triglyceride levels on the risk of having LDL-PPD <255 Å is shown in **Fig. 3**. Triglyceride levels ≥ 1.20 mmol/l (median of the cohort) were associated with a significant increase in the risk of having LDL-PPD <255 Å, and this risk was further increased in subjects with CETP mass concentration above

median. The presence of plasma CETP concentrations above median was not associated with a higher risk of having small LDL in subjects with low triglyceride levels.

DISCUSSION

To the best of our knowledge, this was the first study to examine the role of CETP as the determinant of LDL size heterogeneity in a large cohort of FH heterozygotes and controls. Our results suggested that LDLR gene mutations leading to FH are associated with significant variations in electrophoretic characteristics of LDL particle size; FH heterozygotes having smaller LDL-PPD associated with an accumulation of mid-size LDL particles (255–260 Å). Our results also showed that plasma triglyceride levels and CETP mass concentrations, as well as gender, are independent predictors of LDL-PPD in this cohort of FH and control subjects.

Heterogeneity of LDL particles was reported before in FH patients, albeit in very small cohorts. Slack and Mills
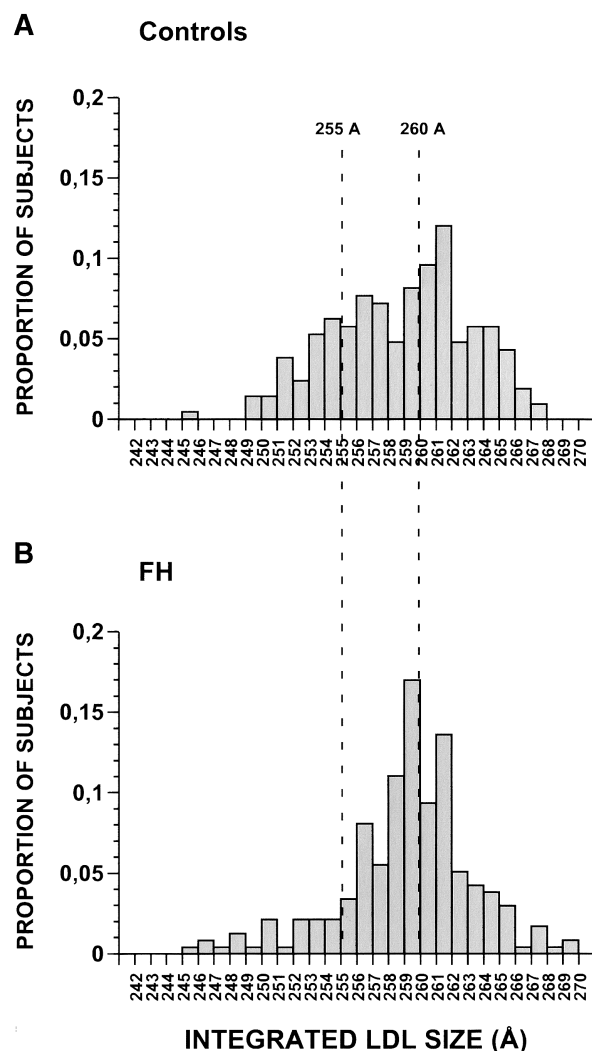
Fig. 2. Distribution of integrated (mean) LDL size among (A) controls and (B) FH heterozygotes.
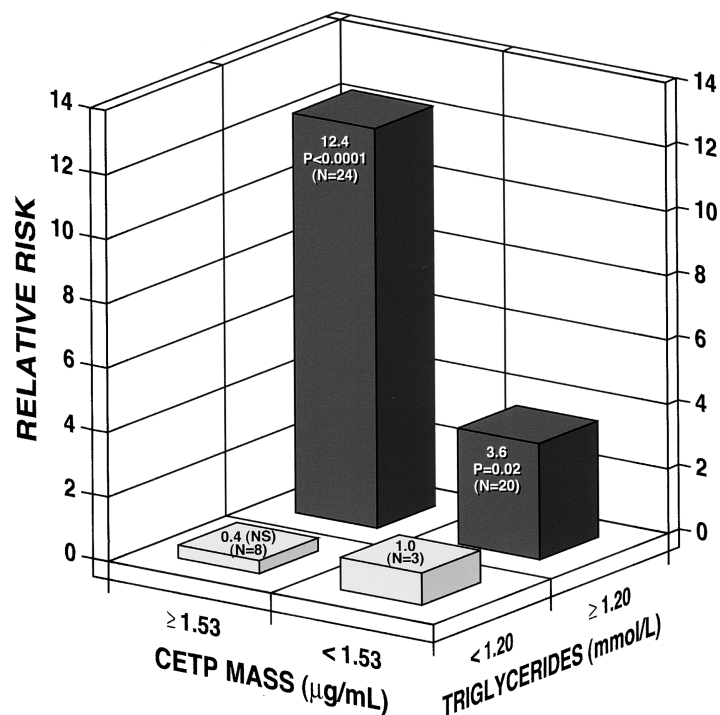
Fig. 3. Relative risks of LDL-PPD < 255 Å according to baseline plasma triglyceride levels (above or below median of 1.20 mmol/l) and cholesteryl ester transfer protein (CETP) mass (above or below median of 1.53 μg/ml) in a subgroup of 120 FH heterozygotes matched for age, gender, body mass index, and smoking with 120 controls. Number of subjects with LDL-PPD < 255 Å in each group is shown in parentheses.

(8) examined LDL particle density in 18 FH heterozygotes compared with 20 controls and found higher LDL peak flotation rate in FH patients (8.2 S_f vs. 7.1 S_f), thus indicating less dense LDL particles. Patsch et al. (9) also found that, as compared with LDL particles of control subjects, the LDL of seven FH heterozygotes were cholesterol-enriched and triglyceride-poor, suggesting decreased density, increased size, or both. Similarly, Bagnall and Lloyrd (10) and Teng et al. (11) observed that the LDL particles of FH heterozygotes had an increased cholesterol content and were depleted in triglycerides. The present study expanded the latter observations by showing that the distribution of LDL particle size in FH is characterized by a decreased proportion of small LDL (<255 Å) associated with a reciprocal increase in the proportion of LDL particles with a diameter between 255 Å and 260 Å. Furthermore, this was the first study to demonstrate that LDL-PPD, representing the most abundant subclass of LDL, is smaller in FH heterozygotes than in controls.

The present study showed that plasma CETP mass concentration was significantly higher in FH heterozygotes than in controls. We also observed that the significance of the inverse correlation between LDL-PPD and plasma CETP mass was abolished by adjustment for the FH/control status, suggesting that LDL-PPD changes in FH are mediated, at least in part, by an increase in plasma CETP mass concentrations. It has been shown that CETP plays a major role in the remodeling of HDL particles. Several groups have also been able to demonstrate that CETP is an important determinant of LDL particle size (4, 24–26), although this is not a unanimous finding (27, 28). Several lines of evidence support the concept that plasma triglyceride levels modulate the role of CETP in lipoprotein metabolism, specifically LDL remodeling (29, 30). In fact,

CETP is thought to facilitate the generation of small dense LDL and, therefore, to decrease LDL-PPD through an indirect mechanism of increased rate of triglyceride transfer from triglyceride-rich lipoproteins in exchange for cholesteryl ester in LDL and HDL (31). Thus, we subsequently examined the extent to which variations in plasma triglyceride levels may be responsible for the differences observed in LDL-PPD between FH subjects and controls. We found that the presence of high plasma CETP concentrations were associated with a higher risk of having small LDL-PPD in subjects with high plasma triglyceride levels only, suggesting that the CETP-induced remodeling of LDL is dependent on the number of plasma triglyceride-rich lipoproteins.

Our results showed that plasma triglyceride levels, plasma CETP mass concentrations, and gender were independent predictors of LDL-PPD and represented nearly 27% of its variance. This finding indicates that a large proportion of the variability in LDL-PPD remained unexplained by our model in this specific cohort of FH and control subjects. In fact, a number of genetic and metabolic determinants have been shown to be associated with LDL heterogeneity and could also contribute to the variability of LDL-PPD in the present study. Heritability studies based on twins indicated that approximately 1/3 to 1/2 of the variation in LDL-PPD can be attributed to genetic influences (32, 33). Recently, a major quantitative trait locus on chromosome 17q21 affecting LDL-PPD has been identified (34). In addition, certain constituents of lipoprotein metabolism, such as lipoprotein lipase activity and hepatic lipase activity, have been shown to contribute to the formation of small, dense LDL particles and could well represent significant determinants of LDL-PPD in FH (22).

Intrinsic properties of small, dense LDL particles have been suggested to be biologically responsible for increas-

ing the risk of developing CAD. In fact, small, dense LDL particles have been shown to be more susceptible to oxidation than large, buoyant LDL (35) and to have a higher capacity to bind to intimal proteoglycans (36), two properties associated with greater atherogenecity. Moreover, small, dense LDL particles have been associated with CAD in a number of studies (5–7). In the present study, the difference in the mean LDL-PPD between FH heterozygotes and controls was 1.1 Å (258.1 ± 4.8 vs. 259.2 ± 4.1 Å). Such a difference in LDL-PPD might play an important role in the acceleration of atherosclerosis in FH. St-Pierre et al. (19) have shown that the difference in the LDL-PPD between the 1,926 CAD-free subjects and the 108 subjects who developed CAD during a 5-year follow-up was only 1.9 Å (257.1 ± 5.8 vs. 255.2 ± 6.4 Å, respectively). Subtle variations in LDL particle composition and diameter have been shown to induce important conformational changes of apoB100, which may alter epitope exposure and cause changes in LDLR binding affinity and susceptibility to oxidation (37, 38). Moreover, in a recent meta-analysis (39), a 10 Å decrease in the LDL-PPD was associated with a 60% increase in CAD risk. Based on these data, the 1.1 Å variation in LDL-PPD between controls and FH subjects found in the present study would be associated with a nonnegligible 6.6% increase in the CAD risk.

In summary, we have shown that heterozygous FH is associated with increased plasma CETP mass concentrations and specific changes in the distribution of LDL particle size, namely a decreased LDL-PPD and an accumulation of midsize LDL particles. It is, therefore, likely that the prolonged residence time of LDL and the increased CETP mass concentrations could lead to significant LDL remodeling in FH and could contribute to the pathogenesis of atherosclerosis in these patients by decreasing LDL-PPD, which represents the diameter of the most abundant subclass of LDL particles.

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