

LDL particle size in familial combined hyperlipidemia: effects of serum lipids, lipoprotein-modifying enzymes, and lipid transfer proteins

Juha Vakkilainen,* Matti Jauhiainen,[†] Kati Ylitalo,* Ilpo O. Nuotio,[§] Jorma S. A. Viikari,[§] Christian Ehnholm,[†] and Marja-Riitta Taskinen^{1,*}

Department of Medicine,* Helsinki University Central Hospital, Haartmaninkatu 4, P.O. Box 340, 00029 HUCH, Helsinki, Finland; Department of Molecular Medicine,[†] Biomedicum, National Public Health Institute, Helsinki, Finland; and Department of Medicine,[§] Turku University Central Hospital, Turku, Finland

Abstract Small, dense LDL particles are typical for FCHL. Intravascular lipid exchange and net transfer among HDL, LDL, and triglyceride-rich lipoproteins as well as lipolysis in the VLDL-IDL-LDL cascade regulate properties of LDL. We investigated postheparin plasma activities of hepatic lipase (HL) and LPL, and plasma activities of CETP and phospholipid transfer protein (PLTP) in 191 individuals from 37 Finnish FCHL families. LDL peak particle diameter (LDL size) was measured with 2–10% gradient polyacrylamide gel electrophoresis. LDL size was significantly smaller in affected FCHL family members ($n = 68$) as compared with nonaffected FCHL family members ($n = 78$) or spouses ($n = 45$) (25.3 ± 1.5 nm, 26.8 ± 1.2 nm, and 26.6 ± 1.2 nm, respectively, $P < 0.001$ for both). In affected FCHL family members, serum triglycerides were the strongest correlate for LDL size ($r = -0.71$, $P < 0.001$). In univariate correlation analysis LDL size was not associated with HL, LPL, CETP, and PLTP activities. In multivariate stepwise regression analysis, however, serum triglycerides, CETP activity, HL activity, and HDL cholesterol were significant predictors of LDL size in affected FCHL subjects (adjusted $r^2 = 0.642$). We conclude that serum triglyceride concentration is strongly correlated with LDL size in affected FCHL subjects. After adjustment for serum triglycerides, HL and CETP activities are associated with LDL size in FCHL.—Vakkilainen, J., M. Jauhiainen, K. Ylitalo, I. O. Nuotio, J. S. A. Viikari, C. Ehnholm, and M-R. Taskinen. **LDL particle size in familial combined hyperlipidemia: effects of serum lipids, lipoprotein-modifying enzymes, and lipid transfer proteins.** *J. Lipid Res.* 2002. 43: 598–603.

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FCHL is a common hereditary dyslipidemia with a prevalence of 1–2% in general population (1) and 14% among subjects with premature coronary artery disease (2). The diagnosis of FCHL is based on the occurrence of different types of dyslipidemia within the family: high cho-

lesterol (type IIA), high triglycerides (type IV), or both high cholesterol and high triglycerides (type IIB) (3, 4). Preponderance of small, dense LDL particles (5) and insulin resistance (6) are also characteristic for FCHL patients with hypertriglyceridemia. The pathophysiologic abnormalities behind FCHL are currently unknown. Suggested disturbances in lipoprotein metabolism are hepatic overproduction and impaired peripheral clearance of apolipoprotein B (apoB) containing lipoprotein particles (7) that tie FCHL to elevation of serum apoB. A genetic linkage of FCHL (combined hyperlipidemia trait) to locus Iq21-q23 has been demonstrated and confirmed, but the precise gene defect is currently unknown (8, 9). In addition, a genomewide screen has suggested that several additional loci influence triglyceride, cholesterol, and apoB levels in FCHL families (10).

LDL particles are heterogeneous and vary according to size, density, and lipid composition (11, 12). Differences in physicochemical properties of LDL particles seem to affect their ability to induce or progress human atherosclerosis. Small, dense rather than large, buoyant LDL particles are more common among subjects with coronary artery disease (13–15). Some publications also suggest that the risk associated with small LDL is higher in young than old subjects (16–18). The increased atherogenic potential of small LDL particles may result from their decreased binding to LDL receptors and increased binding to arterial wall proteoglycans (19–21) or their increased susceptibility to oxidative modification (22).

It has been suggested that generation of small, dense LDL particles requires that serum triglyceride concentration is increased above the threshold level of approxi-

Abbreviations: HDL-C, HDL-cholesterol; HL, hepatic lipase; IDL-C, IDL-cholesterol; LDL-C, LDL-cholesterol; PLTP, phospholipid transfer protein; TC, total cholesterol; VLDL-C, VLDL-cholesterol.

¹ To whom correspondence should be addressed.

e-mail: mataskin@helsinki.fi

mately 1.5 mmol/l concomitantly with a high hepatic lipase (HL) activity (23). Other regulatory proteins such as CETP also participate in the modelling of LDL particles in the circulation (23). Genetic linkage between HL and CETP and small, dense LDL has been reported in several (24–27) but not in all (28) studies. Phospholipid transfer protein (PLTP) modifies HDL particles, but it also influences the CETP-mediated cholesteryl ester transfer (29). Thus, PLTP could potentially have an effect on serum LDL in addition to HDL. LPL is one of the key enzymes that hydrolyse triglyceride-rich particles and regulate serum triglyceride concentration. A genetic linkage of LDL size to LPL gene has been reported in subjects with heterozygous LPL gene mutation (30). So far there are little data on the associations of serum HL, LPL, CETP, and PLTP activities and LDL particle size in subjects with FCHL. The aim of the present study was to evaluate these associations in Finnish FCHL patients.

MATERIALS AND METHODS

Subjects

The study subjects comprise a subgroup of the European Multi-center Study on Familial Dyslipidemias (EUFAM) study (10). Study subjects ($n = 191$) were recruited from 37 Finnish FCHL families. The study group consisted of 68 affected and 78 nonaffected FCHL family members and 45 spouses. The inclusion criteria for the probands and affection status criteria for relatives were as previously described (10). Briefly, 90th percentiles age-sex specific serum total cholesterol (TC) and total triglyceride (TG) concentrations derived from large Finnish population studies (31, 32) were used as cut points of the affection status determination for both probands and family members. Probands were also required to have a verified coronary artery disease before the age of 55 years (men) or 65 years (women). Subjects using lipid-lowering medication were asked to discontinue the medication for 4 weeks before collecting the blood samples. We excluded subjects with diabetes, and hepatic or renal disease, and those using oral contraceptives or hormone replacement therapy. Blood samples were collected after a 10–12 h overnight fast. The study was approved by the ethics committees of the participating centers and all participants gave their informed consent.

LDL peak particle size determination

LDL peak particle diameters were determined using 1 mm thick 2% to 10% nondenaturing polyacrylamide linear gradient gels. The vertical slab gels were run in the Bio-Rad Mini-Protean II Electrophoresis Cell. Pre-electrophoresis (20 min, 30 V) and electrophoresis (18 h, 125 V) were performed at 4°C by using Tris-Glycine buffer, pH 8.3 (24 mM Tris and 192 mM Glycine). Serum samples stored at –80°C were thawed, diluted with sample buffer (0.6 M Tris pH 8.3, 8% sucrose, 0.035% bromphenol blue), and applied (10 μ l) to wells. The edge wells were not used to avoid possible aberrant migration. After electrophoresis gels were stained with newly prepared Sudan Black B lipid stain (0.3% Sudan Black B and 1% Zn-acetate in 30% methanol, 30% 2-propanol) for 1 h and destained with 30% 2-propanol for 24 h, gels were kept in 5% acetic acid for 4–6 h and dried with Bio-Rad's GelAir Drying System for 4 h. Dried gels were photographed with a Kodak Digital Science DC120 camera and analyzed with a Kodak Digital Science Electrophoresis Documentation and

Analysis System 120. Two isolated LDL samples were used as size standard on each gel. Control LDLs were isolated by ultracentrifugation and dialysed against 0.9% NaCl containing 0.01% EDTA. After dialysis, sucrose was added to a final concentration of 8%. Particle diameters of the two standard LDL preparations were measured by electron microscopy (EM) with negative staining. The diameter of each standard was calculated by measuring the diameter of at least 100 LDL particles from the EM photograph and calculating their median value. The calculated median diameters of standard LDL particles were 27.9 nm and 23.9 nm. The sample LDLs' major peak diameter (LDL size) was determined by comparing the mobility of the sample to the mobility of the two standard LDL preparations run on each gel. In addition, a control LDL sample was run on each gel in order to measure the coefficient of variation (CV) between different gels. The CV was 1.4%.

Lipolytic enzyme and lipid transfer protein activities

Hepatic lipase and lipoprotein lipase activities were measured as previously described (33). CETP and PLTP activities were determined by using radiometric assays (34, 35). PLTP mass was analyzed with the ELISA method (36).

Other biochemical analyses

Serum lipoproteins were isolated by sequential ultracentrifugation (37) using the following cut-off densities: VLDL $d < 1.006$ g/ml, IDL $d = 1.006$ – 1.019 g/ml, LDL $d = 1.019$ – 1.063 g/ml, HDL $d = 1.063$ – 1.210 g/ml. Triglyceride and cholesterol concentrations in serum and lipoprotein fractions were determined with enzymatic spectrophotometric methods (Hoffman-La Roche, Basel, Switzerland) using a Cobas Mira autoanalyser. Serum apoB was measured by immunoturbidimetric methods (Orion Diagnostica).

Statistical analyses

The statistical tests were performed with SPSS 10.0 statistical software (SPSS Inc., Chicago, Illinois). Logarithmic transformations were applied before analyses, when appropriate, to approach Gaussian distribution, but the values given in text and tables are untransformed. Subject characteristics, lipolytic enzyme, and lipid transfer protein activities between affected and nonaffected FCHL family members and spouses were compared by analysis of covariance (ANCOVA) followed by Bonferroni (equal variances) or Tamhane (nonequal variances) post hoc tests when appropriate. Age was used as a covariate because the three groups had significantly different mean age. Associations between LDL size and other variables were determined using partial correlation procedure controlling for age. Multivariate stepwise linear regression analysis was performed to identify the independent associations of LDL size. Variables showing significant correlation with LDL size in univariate analysis or considered a priori having possible metabolic significance on LDL size distribution were used as independent variables. VLDL cholesterol (VLDL-C) and IDL cholesterol (IDL-C) were excluded because they were closely correlated with triglycerides, and LDL cholesterol (LDL-C) because it was closely correlated with TC. Of the three closely correlated PLTP variables (i.e., activity, mass, and specific activity) PLTP activity was used because it was considered to be biologically the most relevant measure. There were one to six (two on average) affected subjects from each pedigree in the study. Because they were related, the data are not entirely independent. However, a recent study suggests that this relatedness does not cause major problems in association analyses (38), and therefore we have not used mathematical models to treat the nonindependence. To confirm the results obtained from family data with nonindependent subjects, we recalculated the correla-

tion and regression analyses using only one affected subject from each family. These results were very similar to the original ones. $P < 0.05$ was considered significant (two-tailed). The data are shown as mean \pm SD.

RESULTS

Subject characteristics are shown in **Table 1**. There were no significant differences in gender distribution among the three groups (affected FCHL family members, nonaffected FCHL family members, and spouses). Affected FCHL family members were on average 10 years older than nonaffected family members, and 8 years younger than spouses ($P < 0.001$ for both). Body mass index (BMI) was higher in affected FCHL family members. LDL size was significantly smaller in affected FCHL family members than in nonaffected FCHL family members or spouses (25.3 ± 1.5 nm, 26.8 ± 1.2 nm, and 26.6 ± 1.2 nm, respectively, $P < 0.001$). Serum TC and triglyceride concentrations were by definition higher among the affected family members in comparison to nonaffected individuals. Affected subjects also had higher VLDL-, IDL-, and LDL-C as well as apoB concentrations. HL, LPL, CETP, and PLTP activities were similar among affected FCHL family members, nonaffected FCHL family members, and spouses (**Table 2**).

Univariate correlations between LDL size and other variables in affected FCHL subjects are shown in **Table 3**. LDL size was strongly associated with triglycerides, VLDL-C, IDL-C, HDL-C, and apoB. HL, LPL, CETP, or PLTP activities did not have significant univariate associations with LDL size, but PLTP specific activity tended to have an association with LDL peak particle size. Serum triglycerides were significantly associated with HL activity ($r = 0.35$, $P = 0.004$), PLTP mass ($r = -0.26$, $P = 0.04$), and PLTP specific activity ($r = 0.39$, $P = 0.001$), but not with LPL activity, CETP activity, or PLTP activity. These results were similar when men and women were analyzed separately (data not shown).

To identify independent associations between LDL size and other variables (age, gender, BMI, TG, TC, HDL-C, and apoB concentrations, and HL, LPL, CETP, and PLTP

activities), linear regression analyses were performed in FCHL patients (**Table 4**). In the first model, age was used as the only independent variable (adjusted $r^2 = 0.064$). Other variables were added in the model using the stepwise method. Serum triglyceride concentration was the first variable to enter the model (r^2 change = 0.469), followed by CETP activity (r^2 change = 0.061), HL activity (r^2 change = 0.022), and HDL-C (r^2 change = 0.026). The adjusted r^2 of the final model was 0.642.

There were on average two affected FCHL subjects from each pedigree; thus the data are not independent. In order to avoid possible influence of this nonindependence on the results, we recalculated the linear regression analysis using only one affected subject from each pedigree ($n = 34$). The results remained similar. Serum triglycerides and HL activity entered the regression model (adjusted $r^2 = 0.653$), but CETP activity ($P = 0.051$) and HDL-C ($P = 0.26$) remained outside the model.

DISCUSSION

In the present study we provide novel data on the roles of HL and CETP as modifiers of LDL size in FCHL and confirm the importance of hypertriglyceridemia as a predictor of LDL size. In previous genetic studies, HL and CETP have been linked with small, dense LDL trait (24, 25). In this study, their activities were measured and found to be associated with LDL size in subjects with FCHL.

Hypertriglyceridemia, preponderance of small, dense LDL particles, and low HDL-C concentration are often present together (39, 40). There is also a strong inverse correlation between the change in serum triglyceride concentration and the change in LDL size (41). CETP mediates lipid exchange between triglyceride-rich lipoproteins (e.g., VLDL), LDL, and HDL. Specifically, it transports cholesterol esters from, and triglycerides to, LDL and HDL particles making them enriched with triglycerides but decreasing their cholesterol content. It has been proposed that serum triglyceride concentration over 1.3–1.5

TABLE 1. Subject characteristics

	FCHL Affected n = 68 (35m/33f)	FCHL Nonaffected n = 78 (46m/32f)	Spouse n = 45 (26m/19f)	P^a
Age, years	42.3 \pm 12.4 ^{b,c}	32.4 \pm 12.3	50.2 \pm 7.6	<0.001
BMI, kg/m ²	27.0 \pm 3.9	24.7 \pm 4.0	25.9 \pm 4.4	0.03
TC, mmol/l	6.47 \pm 0.98	4.92 \pm 0.92	5.54 \pm 1.02	–
VLDL-C, mmol/l	0.89 \pm 0.71 ^{b,c}	0.30 \pm 0.19	0.34 \pm 0.22	<0.001
IDL-C, mmol/l	0.32 \pm 0.18 ^{b,c}	0.14 \pm 0.08	0.20 \pm 0.09	<0.001
LDL-C, mmol/l	3.98 \pm 0.90 ^{b,c}	3.12 \pm 0.74	3.61 \pm 0.87	<0.001
HDL-C, mmol/l	1.26 \pm 0.30 ^b	1.35 \pm 0.29	1.38 \pm 0.29	0.03
TG, mmol/l	2.50 \pm 1.50	1.10 \pm 0.47	1.25 \pm 0.47	–
apoB, g/l	12.7 \pm 2.7 ^{b,c}	8.5 \pm 2.3	9.7 \pm 2.4	<0.001
LDL size, nm	25.3 \pm 1.5 ^{b,c}	26.8 \pm 1.2	26.6 \pm 1.2	<0.001

^a ANCOVA with age as covariate.

^b $P < 0.05$ FCHL affected versus FCHL nonaffected.

^c $P < 0.05$ FCHL affected versus spouse, post hoc test with Bonferroni adjustment for multiple comparisons.

BMI, Body mass index.

TABLE 2. Results of lipolytic enzyme and lipid transfer protein analyses

	FCHL Affected n = 68 (35m/33f)	FCHL Nonaffected n = 78 (46m/32f)	Spouse n = 45 (26m/19f)	<i>P</i> ^a
HL activity	340 ± 164	330 ± 140	295 ± 127	0.38
LPL activity	219 ± 65	207 ± 67	235 ± 67	0.72
CETP activity	20.5 ± 3.8	21.6 ± 6.9	22.3 ± 5.9	0.22
PLTP activity	4598 ± 1437	4208 ± 1345	4455 ± 1040	0.09
PLTP mass	12.0 ± 3.8 ^b	13.7 ± 4.3	13.7 ± 3.9	0.012
PLTP specific activity	424 ± 195	346 ± 191	366 ± 188	0.04

^a ANCOVA with age as covariate.

^b *P* < 0.05 FCHL affected versus spouse, post hoc test with Bonferroni adjustment for multiple comparisons.

mmol/l is required for significant CETP mediated lipid exchange between LDL and VLDL (23, 42). These LDL particles, which are depleted in cholesteryl esters but enriched with triglycerides, can be further modified by HL, which hydrolyses both triglycerides and phospholipids in LDL particles. This pathway is a reasonable explanation for the associations between triglycerides, HL activity, CETP activity, and small, dense LDL trait reported in the present and previous studies.

We found a strong inverse correlation between serum triglycerides and LDL size. Although CETP and HL activities did not correlate with LDL size in univariate analyses, they were significantly associated with LDL size in the multivariate regression analysis, i.e., when the influence of triglycerides was taken into account. This may be explained by results of Mann et al., who discovered that CETP mass and activity are the rate limiting factor in net cholesteryl ester transfer from LDL to VLDL only in hypertriglyceridemic subjects (43). Based on these and our own data, we propose that hypertriglyceridemia is the obligatory element that allows LDL to be modified by other factors, such as CETP and HL.

LPL activity was not associated with LDL size or serum triglyceride concentration in univariate or multivariate analyses in affected FCHL subjects, but in nonaffected family members and spouses there was a significant negative correlation between LPL activity and serum triglycerides. On the other hand, the mean LPL activities were

similar in affected and nonaffected family members and spouses. These results suggest that 1) whereas in the general population LPL is an important modifier of serum triglyceride concentration, in FCHL the gene(s) causing FCHL are more important than LPL in determining serum triglyceride concentration, and 2) genetic polymorphisms affecting LPL activity are as frequent in Finnish FCHL subjects as in the general population. These data are in agreement with the study by Pajukanta et al., who reported that the LPL gene is not linked to FCHL in Finnish subjects (44). In familial LPL deficiency, the LPL gene is linked to hypertriglyceridemia and small LDL particles (30), which emphasizes the importance of hypertriglyceridemia in formation of small, dense LDL particles.

Plasma PLTP activity was similar in affected and nonaffected FCHL family members and spouses. However, affected family members had lower PLTP mass and, as a consequence, higher PLTP specific activity than nonaffected subjects. These data and the correlations between triglyceride concentration and PLTP mass and specific activity are in agreement with previous results (36). PLTP activity was not associated with LDL size in either dyslipidemic or normolipidemic subjects. Taken together, these data suggest that the univariate associations between PLTP mass and specific activity and LDL size in affected FCHL subjects are likely to result from their common associations with serum triglycerides.

Based on segregation analysis, it has been suggested that the small, dense LDL trait in FCHL is determined by a major gene and an additional polygenic component (5, 45). In both of these studies hypertriglyceridemia and small, dense LDL trait were associated, and Bredie et al. estimated that serum lipid and lipoprotein concentrations would explain approximately 60% of the variation of the LDL density profile (5). The genetic complexity of FCHL is also shown in studies that indicate more than one locus influences small LDL trait, apoB concentration, and insulin resistance (46, 47).

In conclusion, serum triglyceride concentration is the most important predictor of LDL size in Finnish FCHL patients. HL and CETP activities are associated with LDL size in subjects with FCHL after the effect of serum triglycerides has been taken into account. These data support the idea that LDL particle size in FCHL is regulated by several factors. ■

TABLE 3. Univariate correlations between LDL size and other variables

	FCHL Affected (n = 68)	
	<i>r</i>	<i>P</i>
TG	-0.71	<0.001
TC	-0.12	0.33
VLDL-C	-0.68	<0.001
IDL-C	-0.53	<0.001
LDL-C	0.27	0.03
HDL-C	0.52	<0.001
apoB	-0.49	<0.001
HL activity	-0.05	0.67
LPL activity	-0.00	0.97
CETP activity	-0.14	0.28
PLTP activity	-0.06	0.63
PLTP mass	0.21	0.10
PLTP specific activity	-0.26	0.04

r = partial correlation controlling for age.

TABLE 4. Multivariate stepwise linear regression analysis

Dependent variable: LDL size			
All FCHL patients, n = 68		One FCHL patient from each pedigree, n = 34	
Independent variable(s)	Adjusted R ²	Independent variable(s)	Adjusted R ²
Age	0.064	Age	0.114
Age, TG	0.533	Age, TG	0.501
Age, TG, CETP	0.594	Age, TG, HL	0.653
Age, TG, CETP, HL	0.616		
Age, TG, CETP, HL, HDL-C	0.642		

HL, postheparin HL activity.

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