Fractional esterification rate of cholesterol in high density lipoprotein is correlated with low density lipoprotein particle size in children

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Abstract Small low density lipoprotein (LDL) particles are thought to be more atherogenic than larger LDL particles, although this association may depend on plasma triglyceride (TG) and high density lipoprotein (HDL) levels. To help prevent coronary artery disease (CAD), it may be useful to understand risk factors during childhood and adolescence. In the present study, we evaluated low density lipoprotein particle size (LDL-size) by 2–16% gradient gel electrophoresis in 70 healthy children (30 boys and 40 girls) along with conventional lipid and lipoprotein parameters which are thought to affect LDL-size. The fractional and molar esterification rates (FER and MER) of cholesterol in plasma and HDL were also determined. As expected, plasma levels of TG, HDL-cholesterol (HDL-C) and apoA-I were closely associated with LDL-sizes in both sexes (boys: $r = -0.694$, 0.708 and 0.701, girls: $r = -0.579$, 0.551 and 0.539, $P < 0.001$). However, a closer association was found between FER in HDL (FER_{HDL}) and LDL-size (boys: $r = -0.874$, girls: $r = -0.642$, $P < 0.001$). In a stepwise multiple regression analysis, FER_{HDL} alone accounted for 76% and 41% of the variability in LDL-size in boys and girls, respectively. MER in HDL accounted for additional 4% and 19% in boys and girls, respectively. Other parameters, including plasma TG, HDL-C and apoA-I had no significant additional effects. Thus, the determination of FER_{HDL} is useful to predict the particle size of LDL in children.—**Ohta, T., Y. Kakiuti, K. Kurahara, K. Saku, N. Nagata, and I. Matsuda.** Fractional esterification rate of cholesterol in high density lipoprotein is correlated with low density lipoprotein particle size in children. *J. Lipid Res.* 1997. **38:** 139–146.

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Low density lipoprotein (LDL) particles vary in size and hydrated density (1–3). Several LDL subfractions can be identified on gradient gel electrophoresis. Among these LDL subfractions, small LDL particles possess a lower binding affinity for cellular LDL receptor and are more easily oxidized in vitro (3–6). These data suggest that small LDL particles are atherogenic because their lower binding affinity for LDL-receptor reflects a longer plasma residence time for them to be oxidized and taken up by macrophages in extravascular spaces. In accordance with these in vitro studies, epidemiological studies have shown that plasma concentrations of small dense LDL particles are associated with coronary artery disease (CAD) (7–10). This association is independent of age, sex, beta-blocker use, insulin resistance, and environmental background, which have been reported to affect LDL-size (10–12). However, this association is not independent of plasma triglyceride (TG), high density lipoprotein (HDL) cholesterol (HDL-C) or apoA-I concentrations. Plasma LDL-size and plasma TG and HDL-C levels are closely interrelated and 50–57% of the variance in plasma LDL-size can be explained by plasma TG and HDL-C levels (8). These data suggest that the presence of small LDL could reflect metabolic changes in triglyceride-rich lipoproteins or HDL which predispose to CAD.

Recent studies in human apoA-I transgenic mice and double-transgenic mice expressing human apoA-I and apoA-II clearly indicate that LpA-I (HDL containing apoA-I but not apoA-II) is the anti-atherogenic fraction of HDL (13, 14). With regard to the function of LpA-

Abbreviations: FER_{HDL}, fractional esterification rate of cholesterol in HDL; MER_{HDL}, molar esterification rate of cholesterol in HDL; FER_{plasma}, fractional esterification rate of cholesterol in plasma; MER_{plasma}, molar esterification rate of cholesterol in plasma; LCAT, lecithin:cholesterol acyltransferase; HDL-C, high density lipoprotein cholesterol; LpA-I, lipoprotein containing apoA-I but not apoA-II; CAD, coronary artery disease; LDL, low density lipoprotein.

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1, we previously reported two new findings (15); first, the plasma concentration of LpA-I is inversely correlated with the cellular cholesterol-reducing capacity of LpA-I (which is the first step in reverse cholesterol transport mediated by HDL), and second, plasma concentrations of LpA-I are inversely correlated with the rate of cholesterol esterification in HDL. As most cholesterol esterification takes place in the HDL fraction, these data suggest that LpA-I regulates the reactivity of HDL to lecithin:cholesterol acyltransferase (LCAT) and that the anti-atherogenic nature of LpA-I may be linked to this regulation of plasma cholesteryl ester production. Increased reactivity of HDL to LCAT has been reported in patients with CAD (16).

Fractional esterification rate of cholesterol in HDL (FER_{HDL}), which reflects the reactivity of HDL to LCAT, is closely correlated with plasma TG and HDL-C levels (15, 16). Furthermore, FER_{HDL} shows a strong positive correlation with plasma levels of $HDL_{3b,c}$ particles and a strong negative correlation with the concentration of HDL_{2b} and LpA-I (15, 17). Therefore, as a first step in clarifying the underlying mechanism which could explain the link between increased reactivity of HDL to LCAT and CAD, we investigated the relation between variations in FER_{HDL} and LDL particle size (LDL-size) in children. Children were used as study subjects because, to help prevent CAD, it may be useful to understand risk factors during childhood and adolescence. In addition, the effects of medication, smoking, alcohol, and chronic disease on lipid metabolism and LDL-size (10–12) need not to be considered in most children.

SUBJECTS AND METHODS

Subjects

The present study has been approved by the Review Board of Kumamoto University School of Medicine. The subjects consisted of outpatients (30 boys: aged between 9 and 18 years and 40 girls: aged between 9 and 18 years) of the Department of Pediatrics, Kumamoto University School of Medicine, who had visited the hospital for a routine check-up. We obtained informed consent from the study subjects and their parents. None of the subjects had clinical signs or symptoms of atherosclerotic coronary heart disease or were receiving medication. None of the subjects were obese (body weight > 120% of ideal body weight) or smoked.

Preparation methods

Blood samples. Freshly drawn venous blood (10 ml) from subjects who had fasted overnight was collected into sterile tubes containing EDTA (final concentration, 1 mg/mL), and plasma was isolated from this blood by low-speed centrifugation (1000 *g*, 20 min at 4°C). Plasma samples were used in the following experiments immediately after isolation.

Isolation of LDL. LDL was isolated by single vertical-spin density-gradient ultracentrifugation (18). Briefly, the density of 1 mL of the plasma was adjusted to 1.21 g/mL by the addition of KBr. This plasma was then pipetted into an ultracentrifuge tube and overlaid with a 1.006 g/mL solution. The tubes were sealed and placed in a RP 80 AT-313 rotor in a HITACHI himac CS 120 and spun at 80,000 rpm for 60 min at 4°C. After ultracentrifugation, LDL formed a visible band in the middle of the centrifuge tube. The ultracentrifuge tube was punctured and LDL was aspirated.

Determination of LDL-size

LDL-size was evaluated by electrophoresis in nondenaturing polyacrylamide gradient gels on Pharmacia precast PAA 2/16 gels according to the procedure specified by the manufacturer. Standards used for size calibration purposes included latex beads (37 nm) (Dow Chemical Company) and Pharmacia high-molecular-weight standards (Pharmacia). The stained gels were scanned with a laser scanning densitometer (model CS-9000, Shimadzu) to provide a quantitative measurement of the size of the peak and its distance from the origin. Particle diameter was calculated from a plot of the log of the known diameters of the standards (latex beads 37 nm, thyroglobulin 17 nm, apoferritin 12.2 nm) on the y-axis against their positions from the origin of the gel (R_f) on the x-axis.

Molar and fractional esterification rates in plasma and very low density lipoprotein (VLDL)- and LDL-depleted plasma

The molar and fractional esterification rates of cholesterol in plasma (MER_{plasma} and FER_{plasma}) and in VLDL- and LDL-depleted plasma (MER_{HDL} and FER_{HDL}) were determined as described previously (15,16). VLDL- and LDL-depleted plasma was prepared by precipitating VLDL and LDL with phosphotungstate–$MgCl_2$ (19, 20). Free [^{3}H]cholesterol (FC) was incorporated onto polystyrene tissue culture wells (Corning) as follows: absolute ethanol (100 μL) containing 0.2 μCi of [^{3}H]FC was placed in wells and then dried off by flushing with nitrogen. One hundred μL of either plasma samples, for determining MER_{plasma} and FER_{plasma}, or VLDL- and LDL-depleted plasma samples, for determining MER_{HDL} and FER_{HDL} in 400 μL of PBS was added to each well and [^{3}H]FC was equilibrated with the FC in each sample by incubation at 4°C for 16 h. [^{3}H]FC-labeled plasma or VLDL- and LDL-depleted

plasma samples were then incubated at 37°C for 30 min. The enzyme reaction was stopped by immersing the sample tubes in an ice bath. The lipids in incubation samples were extracted with methanol–chloroform 2:1 (v/v). The extract was dried by flushing it with nitrogen and then dissolved in 60 μL of isopropanol. Aliquots (20 μL) of lipid extracts were spotted in duplicate on a thin-layer chromatography (TLC) plate (Merck) and developed in n-hexane–diethyl ether–acetic acid–methanol 85:20:1:1 (v/v). Spots corresponding to FC and CE were cut out from the plate and their radioactivities were determined. The increase in [^{3}H]CE was linear over 30 min of incubation. The fractional esterification rate was expressed as the difference between the percentage of radioactive cholesterol esterified before and after incubation at 37°C and the molar esterification rate of cholesterol (μmol/h/L) was calculated based on the specific activity (dpm/nmol FC) of each sample.

Apolipoproteins, lipoproteins and lipids analysis

ApoA-I, apoA-II, and apoB concentrations in plasma were measured by radial immunodiffusion assay. ApoA-II level is a major factor regulating the distribution of apoA-I among HDL subclasses LpA-I and LpA-I/A-II (lipoprotein containing both apoA-I and apoA-II) in normolipidemic humans (21). LpA-I/A-II appears to have a fixed molar ratio of 3:2 for these apolipoproteins (22, 23). In our preliminary experiments, plasma concentrations of LpA-I isolated by immunoaffinity chromatography (15) were correlated well with those of LpA-I calculated based on a fixed molar ratio of apoA-I to apoA-II (n = 30, $r = 0.91$, $P < 0.001$). Thus, we calculated the plasma concentrations of apoA-I in LpA-I and LpA-I/A-II in the present study. Concentrations of total cholesterol (TC), FC, and TG in these samples were determined by enzymatic methods using commercial kits. HDL-C was measured by selective precipitation of LDL using phosphotungstate–$MgCl_2$ (19, 20). The protein content of LDL was determined by the method of Lowry et al. (24).

Statistical evaluation

Group differences were determined by a two-tailed Student's *t*-test. The effects of parameters on LDL-size or FER_{HDL} as adjusted for other parameters were determined by multiple regression analysis. Pearson correlation coefficients were computed to assess the associations between parameters. A stepwise multiple regression analysis was performed by entering the independent variable with the highest partial correlation coefficient at each step, until no variable remained with an F value of ≥ 4. Group differences or correlations with $P < 0.05$ were considered to be statistically significant.

TABLE 1. Age, LDL particle size, plasma lipid and apolipoprotein characteristics in 30 male and 40 female subjects

	Males	Females
Age (years)	15 ± 1 (5)	16 ± 1 (6)
LDL particle size (nm)	26.6 ± 0.1 (0.6)	27.0 ± 0.1[b] (0.6)
Total cholesterol (mg/dL)	189 ± 7 (38)	202 ± 5 (31)
Triglyceride (mg/dL)	115 ± 5 (27)	87 ± 5[a] (31)
HDL-cholesterol (mg/dL)	57 ± 4 (21)	65 ± 2 (12)
ApoA-I (mg/dL)	129 ± 5 (27)	138 ± 5 (31)
ApoA-II (mg/dL)	33 ± 1 (5)	31 ± 1[a] (6)
ApoB (mg/dL)	89 ± 4 (21)	89 ± 3 (19)

Data are presented as mean ± SEM (SD).
[a] $P < 0.05$.
[b] $P < 0.01$.

RESULTS

As shown in **Table 1,** LDL-size was significantly smaller in boys than that in girls ($P < 0.01$). In contrast, plasma TG and apoA-II levels were significantly higher in boys than in girls ($P < 0.05$). The HDL-C level was slightly lower in boys than in girls, but this difference was not significant. When we accounted for plasma TG and HDL-C levels, the gender-related difference in LDL-size was not significant. Gender-related differences were not found in FER_{plasma}, MER_{plasma}, FER_{HDL}, or MER_{HDL} (**Table 2**).

Age- and gender-related differences in lipid and apolipoprotein levels have been reported in children, especially in parameters related to HDL (22). In the present study, we found gender-related differences in association of LDL-size or FER_{HDL} with parameters studied, but age-related difference was not found. Thus, we did not consider the age in the following studies.

Correlations of LDL-size with cholesterol esterification, lipids and apolipoproteins

Figures 1A and 1B show the relationship between LDL-size and FER_{HDL} and plasma TG and HDL-C levels in boys and girls, respectively. Plasma TG and HDL-C levels correlate well with LDL-size in both boys and girls. However, FER_{HDL} is more closely associated with LDL-size in both cases.

Table 3 shows Pearson correlation coefficients between LDL-size and selected variables overall, and separately for boys and girls. Overall, LDL-size was inversely correlated with plasma levels of TG and apoB, FER_{plasma}, MER_{plasma}, and FER_{HDL} and positively correlated with plasma HDL-C and apoA-I levels. In boys, the correlations between LDL-size and plasma apoB levels or

TABLE 2. Fractional esterification rates in plasma and low density lipoprotein/very low density lipoprotein-depleted plasma in 30 male and 40 female subjects

	FER_{plasma}	MER_{plasma}	FER_{HDL}	MER_{HDL}
	%/h	*μmol/h/L*	*%/h*	*μmol/h/L*
Males	2.30 ± 0.08 (0.44)	23.63 ± 1.89 (10.33)	7.55 ± 0.56 (3.06)	17.21 ± 0.81 (4.43)
Females	2.13 ± 0.08 (0.51)	25.25 ± 1.24 (7.84)	6.34 ± 0.37 (2.36)	17.20 ± 0.68 (4.30)

Data are presented as mean ± SEM (SD). FER, fractional esterification rate; MER, molar esterification rate.

MER_{plasma} were not significant. In girls, the correlations between LDL-size and plasma apoB or FER_{plasma} were not significant. In all cases, FER_{HDL} was most strongly correlated with LDL-size. As each of these parameters can potentially contribute directly to the regulation of LDL-sizes, we performed a stepwise multiple regression analysis with LDL-size as the dependent variable and the other parameters as independent variables (Model I: the independent variables included all of the variables listed in Table 1; Model II: the independent variables excluded parameters regarding cholesterol esterification). In the Model I analysis (Step 1), FER_{HDL} was most significantly associated with LDL-size, and accounted for 76%, 41%, and 57% of the variability in this parameter in boys, in girls and overall, respectively (**Table 4**). MER_{HDL} had a small additional effect. FER_{HDL} and MER_{HDL} could explain 60–81% of the variability in LDL-size (Step 2). Plasma TG, HDL-C, and apoA-I had no significant additional effects. To compare our data with those previously reported (7–10), parameters for cholesterol esterification were excluded from the independent variables (Model II). As shown in Table 4, similar to previously reported data, TG, HDL-C, and apoA-I were significant predictors of LDL-size. All of these parameters, taken together, accounted for 42–65% of the variability in LDL-size.

Correlations of FER_{HDL} with lipids, apolipoproteins and lipoproteins

Table 5 shows Pearson correlation coefficients between FER_{HDL} and selected variables overall, and separately for boys and girls. FER_{HDL} was inversely correlated with plasma levels of HDL-C, apoA-I, and LpA-I and positively correlated with plasma TG and apoB levels in all of the study groups. The relationship between FER_{HDL} and TG or apoB was still significant after adjusting for apoB or TG levels, respectively ($P < 0.01$). Overall, LpA-I was most strongly correlated with FER_{HDL}. As each of these parameters can potentially contribute directly to the regulation of FER_{HDL}, we performed a stepwise multiple regression analysis with FER_{HDL} as the dependent variable and the other parameters as independent variables. In all of the groups, LpA-I had the most significant association with FER_{HDL}, and accounted for 70%, 58%, and 64% of the variability in FER_{HDL} in boys, in girls, and overall, respectively (**Table 6**). TG in girls and TG and apoB overall had additional effects (10% and 8%, respectively).

Correlations of LpA-I and LpA-I/A-II with LDL-size

Plasma concentrations of estimates of LpA-I were significantly correlated with LDL-size (boys: $r = 0.821$, girls: $r = 0.568$, overall: $r = 0.699$, $P < 0.001$), while estimates of LpA-I/A-II were not. However, a stepwise multiple regression analysis with LDL-size as the dependent variable and the other parameters, including LpA-I, as independent variables did not change the result in Table 4 that FER_{HDL} was the strongest predictor of LDL-size.

DISCUSSION

The present study demonstrates that the molar and fractional esterification rates of cholesterol in HDL correlate better with LDL-size than plasma levels of TG and HDL-C. As shown in Model II in Table 4, in which we did not include parameters for cholesterol esterification, plasma TG and HDL-C or apoA-I levels accounted for 40–60% of the variability in LDL-size. These predictive values are equivalent to those in previous studies (7–10). Furthermore, the present results may extend to adult subjects; our preliminary data in adult subjects (n = 50, age: 40–70 years) have also shown that FER_{HDL} is the strongest predictor of LDL-size. Thus, we believe that our present data are not affected by the differences (race, age, and environmental factors) of subjects studied.

The factors that regulate LDL-size are not completely understood. Several processes appear to be involved (25–28); *i*) hepatic production of apoB-containing lipoproteins (25), *ii*) direct hepatic production of small dense LDL (25–27), *iii*) intravascular lipolytic system (26), and *iv*) rates of catabolism of LDL by an LDL receptor-dependent pathway (28). Of the above factors, defective lipolysis resulting in higher triglyceride levels is the most likely explanation for both the change in

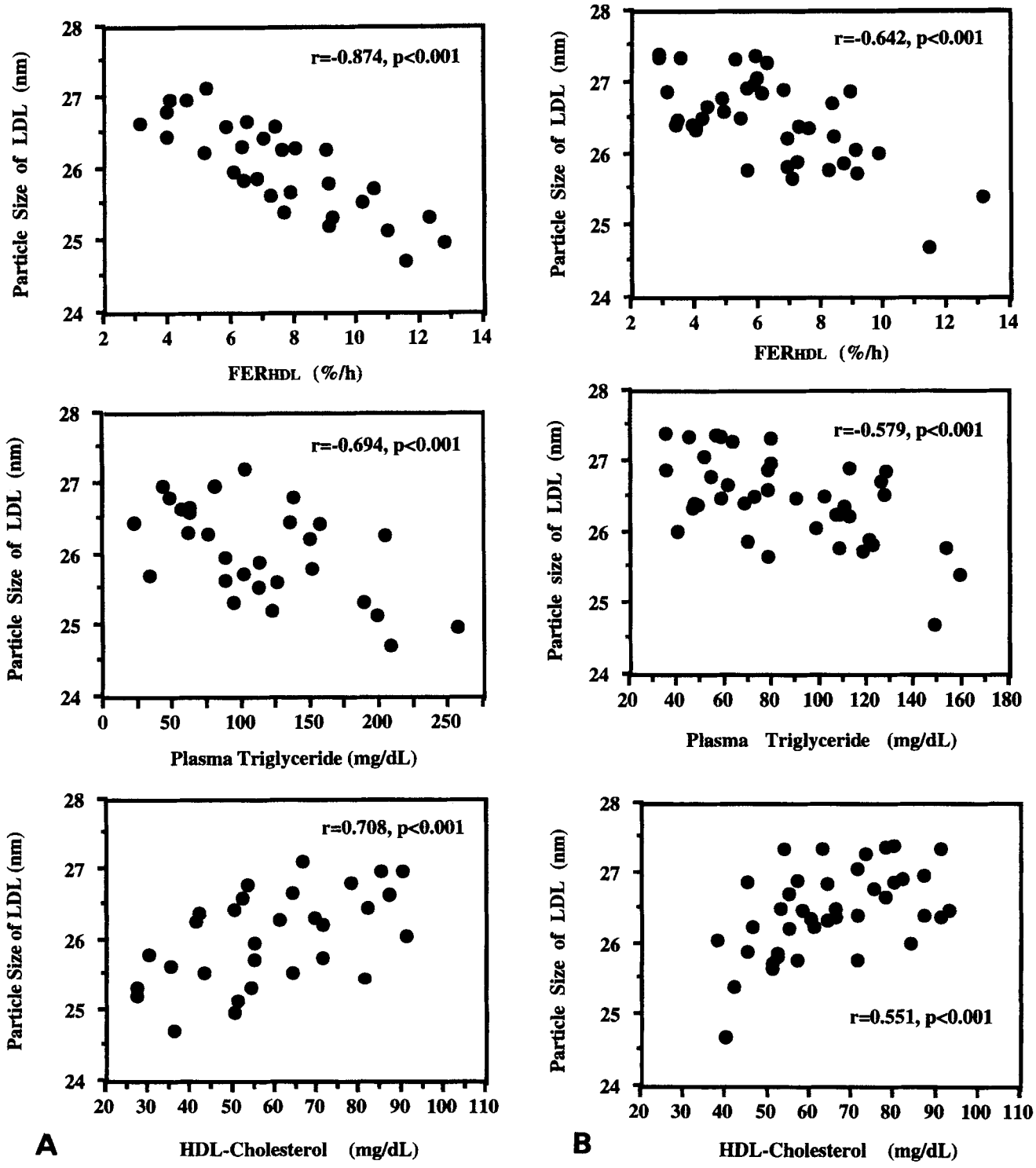

Fig. 1. A: Scatterplots showing relations between plasma LDL-size and FER_{HDL}, plasma TG, or HDL-C in males. B: Scatterplots showing relations between plasma LDL-size and FER_{HDL}, plasma TG, or HDL-C in females.

TABLE 3. Univariate correlation coefficient of LDL particle size with various parameters

	Males	Females	All
Age	0.122	0.094	0.110
Total cholesterol	0.125	−0.007	0.131
Triglyceride	−0.694[c]	−0.579[c]	−0.650[c]
HDL-cholesterol	0.708[c]	0.551[c]	0.629[c]
ApoA-I	0.701[c]	0.539[c]	0.622[c]
ApoA-II	0.044	−0.162	−0.149
ApoB	−0.283	−0.297	−0.248[a]
FER_{plasma}	−0.511[b]	−0.221	−0.350[b]
MER_{plasma}	−0.322	−0.313[a]	−0.276[a]
FER_{HDL}	−0.874[c]	−0.642[c]	−0.753[c]
MER_{HDL}	−0.005	0.130	0.081

All, males and females combined.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

LDL particle size and in FER_{HDL}. The values of FER_{HDL} are strongly related to the particle size distribution in the HDL; it increases with increased relative concentration of the small $HDL_{3b,c}$ particles and decreases in plasma samples containing higher levels of HDL_{2b} and LpA-I (especially large LpA-I particles) (15, 17). Other workers demonstrated the relationship between LDL and HDL particle size and the role of hypertriglyceridemia in lipoprotein remodeling (29, 30). Our results are consistent with these findings and best understood in terms of the increased reactivity of LCAT toward the small HDL particles and the decreased reactivity of LCAT toward HDL_{2b} (large LpA-I particles) (15, 31). Other observations such as the strong positive correlation of FER_{HDL} with plasma triglycerides and the strong negative correlation with LpA-I are in agreement with previous observations (15, 16) and fully consistent with the role of impaired lipolysis in the shift in lipoprotein particle size. Our absolute values for FER_{HDL} as well as the lack of gender effect differ from previous reports (16, 17, 31). The lower values observed in this study may, beside possible subtle differences in methodology, reflect the fact that our subjects were mostly prepubertal and pubertal children; it is likely that the gender effect may become apparent postpubertally.

Is it of practical value to measure FER_{HDL}? It may be argued that assay of plasma triglycerides, HDL-C, or apoA-I (or LpA-I) gives the same information. While this may be true in patients with clearly abnormal lipid values, the majority of patients with coronary artery disease presents with more subtle abnormalities. As shown previously, individuals with similar lipid values may differ markedly in their FER_{HDL} values and/or HDL subspecies distribution (16, 17, 32). Furthermore, FER_{HDL} assay is less complex and more reproducible than the measurement of HDL or LpA-I or LDL subspecies; it also reflects the net cholesterol esterification in apoB-depleted plasma (33).

TABLE 4. Stepwise multiple regression analysis of correlates of LDL particle size

Independent				r	r^2
Model I analysis					
Males					
Step 1	FER_{HDL}			0.874	0.764
Step 2	FER_{HDL}	MER_{HDL}		0.899	0.808
Females					
Step 1	FER_{HDL}			0.642	0.412
Step 2	FER_{HDL}	MER_{HDL}		0.775	0.601
All					
Step 1	FER_{HDL}			0.753	0.568
Step 2	FER_{HDL}	MER_{HDL}		0.827	0.683
Step 3	FER_{HDL}	MER_{HDL}	MER_{plasma}	0.840	0.706
Model II Analysis					
Males					
Step 1	HDL-C			0.731	0.534
Step 2	HDL-C	Triglyceride		0.806	0.650
Females					
Step 1	Triglyceride			0.581	0.338
Step 2	Triglyceride	ApoA-I		0.650	0.420
All					
Step 1	Triglyceride			0.648	0.420
Step 2	Triglyceride	ApoA-I		0.761	0.579

All, males and females combined. For model I, independent variables included all variables listed in Table 3. For model II, independent variables excluded FER_{plasma}, FER_{HDL}, MER_{plasma}, and MER_{HDL}. The r and r^2 values for each stepwise combination of independent variables are given on the right.

TABLE 5. Univariate correlation coefficient of FER_{HDL} with various parameters

	Males	Females	All
Age	0.103	0.097	0.101
Total cholesterol	0.057	0.180	0.052
Triglyceride	0.664[b]	0.676[b]	0.678[b]
HDL-cholesterol	−0.727[b]	−0.673[b]	−0.711[b]
ApoA-I	−0.617[b]	−0.699[b]	−0.674[b]
ApoA-II	0.089	0.281	0.223
ApoB	0.470[a]	0.522[b]	0.470[b]
LpA-I	−0.837[b]	−0.764[b]	−0.803[b]
LpA-I/A-II	0.089	0.281	0.223

All, males and females combined.
[a] $P < 0.05$.
[b] $P < 0.001$.

TABLE 6. Stepwise multiple regression analysis of correlates of FER_{HDL}

Independent Variables				r	r^2
Males					
Step 1	LpA-I			0.837	0.701
Females					
Step 1	LpA-I			0.764	0.584
Step 2	LpA-I	Triglyceride		0.823	0.677
All					
Step 1	LpA-I			0.803	0.644
Step 2	LpA-I	Triglyceride		0.835	0.698
Step 3	LpA-I	Triglyceride	ApoB	0.850	0.723

All, males and females combined. The r and r^2 values for each stepwise combination of independent variables are given on the right.

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