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Decreased High-Density Lipoprotein (HDL) Particle Size, Pre β -, and Large HDL Subspecies Concentration in Finnish Low-HDL Families

Relationship With Intima-Media Thickness

Hiroshi Watanabe, Sanni Söderlund, Aino Soro-Paavonen, Anne Hiukka, Eeva Leinonen, Corradina Alagona, Riitta Salonen, Tomi-Pekka Tuomainen, Christian Ehnholm, Matti Jauhiainen, Marja-Riitta Taskinen

Objective—High-density lipoprotein (HDL) cholesterol correlates inversely with the risk of coronary heart disease (CHD). The precise antiatherogenic mechanisms of HDL subspecies are not thoroughly elucidated. We studied the relationship between carotid intima-media thickness (IMT) and HDL subspecies distribution in Finnish families with low HDL cholesterol and premature CHD.

Methods and Results—Altogether, 148 members of Finnish low-HDL families and 133 healthy control subjects participated in our study. HDL particle size was significantly smaller in affected family members (HDL \leq 10th Finnish age-sex specific percentile) compared with unaffected family members and control subjects (9.1 ± 0.04 nm versus 9.5 ± 0.05 nm, $P < 0.0001$, versus 9.8 ± 0.03 nm, $P < 0.0001$ [mean \pm SE]). Large HDL_{2b} particles as well as pre β -HDL concentration were significantly decreased among the affected family members. Mean IMT was significantly higher in the affected family members than in the control subjects (0.85 ± 0.01 mm versus 0.79 ± 0.01 mm; $P < 0.0001$). Age, HDL_{2b}, systolic blood pressure, and pre β -HDL were significant independent determinants of mean IMT.

Conclusions—The decreased levels of HDL_{2b} and pre β -HDL reflect the potentially efflux-deficient HDL subspecies profile in the affected low-HDL family members. Decreased HDL particle size caused by the decrease of plasma concentration of HDL_{2b} and decreased pre β -HDL levels correlate with increased IMT. (*Arterioscler Thromb Vasc Biol.* 2006;26:897-902.)

Key Words: HDL particle size ■ HDL subspecies ■ pre β -HDL ■ intima-media thickness ■ atherosclerosis

Epidemiological studies have shown that low level of high-density lipoprotein (HDL) cholesterol is an independent predictor of coronary heart disease (CHD).^{1,2} Already, a small 0.03-mmol/L (1 mg/dL) increase in total HDL cholesterol (HDL-C) level is associated with a 2% to 3% decline in cardiovascular risk.^{3,4} HDL particles are classified into several subspecies differing in their composition, size, charge and function. Based on the electric charge, HDL can be separated into pre β -HDL, α -HDL, and pre α -HDL. Pre β -HDL can be separated into 3 major subspecies, pre β ₁, pre β ₂, and pre β ₃-HDL, whereas α -HDL can be separated into HDL_{2b}, _{2a}, _{3a}, _{3b}, and _{3c} according to particle size.^{5,6} At present, the reverse cholesterol transport (RCT) process, which removes excess cholesterol from peripheral cells to the liver for

excretion, is considered one of the most important antiatherogenic functions of HDL.^{7,8} The initial step of RCT is cholesterol efflux, in which the ATP-binding cassette transporter A1 (ABCA1) mediates efflux of cellular cholesterol to pre β -HDL,⁹ and ATP-binding cassette transporter G1 (ABCG1) stimulates efflux to larger HDL particles, especially to HDL₂.¹⁰ The discovery of ABCG1 directing cellular cholesterol to large HDL particles¹⁰ has focused current attention on the role of HDL subspecies in the prevention of atherosclerosis.

Evaluation of carotid intima-media thickness (IMT) using ultrasonography is a validated quantitative method for assessing the presence and severity of atherosclerosis,¹¹ and a close relationship has been observed between IMT and the inci-

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dence of CHD.^{12–14} To investigate the association of different HDL subspecies with early atherosclerosis in Finnish low-HDL family members, we measured the distribution of HDL subspecies HDL_{2b}, 2a, 3a, 3b, and 3c using polyacrylamide native gradient gel electrophoresis, quantitated proportion of pre β -HDL particles by crossed immunoelectrophoresis, and measured carotid IMT by ultrasonography. Altogether, 148 low-HDL family members and 133 normolipidemic control subjects participated in the study.

Materials and Methods

Study Subjects

The study subjects were recruited from multigenerational, carefully characterized pedigrees with low HDL-C and premature CHD. The pedigrees were recruited in Helsinki and Turku University Central Hospitals in Finland as described previously.^{15,16} For the inclusion criteria of the probands, please see online data supplement I, available at <http://atvb.ahajournals.org>.

Altogether, 281 subjects participated in the study, including 148 family members from 50 low-HDL families and 133 normolipidemic healthy subjects. The mean number of family members from each participating family was 3 (range 1 to 10); 41 families contributed ≤ 4 members to this study. Based on their HDL-C level, family members were categorized as affected (HDL-C ≤ 10 th Finnish age-gender specific percentile;^{17,18} $n=83$) or unaffected ($n=65$). In 5 families, we allowed the proband to be hypertriglyceridemic. These families contributed 12 subjects altogether to this study. The control group contained 10 healthy spouses from low-HDL families and 123 healthy volunteers. At the time of the sample collection, 34 affected family members, 4 unaffected family members, and 2 control subjects were using β -blockers; 19 affected family members and 2 unaffected family members were using statins. Subjects using other medications potentially affecting HDL metabolism were excluded. Each study subject gave written informed consent before participating in the study. All samples were collected in accordance with the Helsinki declaration and the ethics committees of the participating centers approved the study design.

Measurements

Venous blood samples were obtained after an overnight fast. Serum and EDTA plasma were separated by centrifugation and stored at -80°C until analysis. For the measurements of lipids, apolipoproteins and glucose, please see online data supplement II.

We measured the relative distribution of HDL subspecies 2b, 2a, 3a, 3b, and 3c by gradient gel electrophoresis in 78 affected and 59 unaffected low-HDL family members and in 127 control subjects. We used a lipoprotein fraction separated from plasma by ultracentrifugation in density of 1.210 g/mL. Samples of 5 μL were loaded into a native 4% to 22% polyacrylamide gradient gel (10×10.5 cm). All gels were prepared in the laboratory. We used high molecular weight (HMW) calibration kit for native electrophoresis from Pharmacia for standardization. Gels were stained 1 hour with Coomassie blue G-250 and destained overnight with acetic acid. Stained gels were analyzed with ImageMaster 1D software (version 4.00; Amersham Pharmacia Biotech). The diameter of each lipoprotein band was calibrated by computing a log-linear standard curve of the protein-stainable HMW standards as a function of their relative migration distance.^{5,6}

The quantification of pre β -HDL was performed by crossed immunoelectrophoresis¹⁹ in 63 affected and 46 unaffected family members and in 89 control subjects. Areas under the immunoprecipitate curves were measured after staining. The pre β -HDL area is expressed as a percentage of the sum of α -HDL and pre β -HDL areas. Pre β -HDL concentration is given in absolute amount (milligrams of apolipoprotein A-I [apoA-I] present in pre β -HDL particles per liter serum).

B-mode ultrasound imaging was used to measure the intima-media complex of carotid artery wall with a Hewlett Packard Image Point

M2410A ultrasound system (Hewlett Packard) as previously described.^{16,20} IMT was measured in 65 affected and 47 unaffected family members and in 105 control subjects. The Prosound software (Caltech) was used to measure IMTs from ultrasound images.²¹ Intrareader variability was assessed by reading the scanings of 10 study subjects twice. The coefficient of variation between the mean IMT of the paired scans was 1.0%, and the absolute difference 0.014 ± 0.018 mm, respectively. The readers were blinded for the identities of the study subjects.

Statistical Methods

The statistical comparisons of clinical and biochemical parameters were performed with JMP 5.01 for Windows (SAS Institute Inc.). Results are expressed as mean \pm SE for continuous variables and as frequencies or percentages for categorical variables. The normality of each variable distribution was tested by Kolmogorov–Smirnov test and variables with skewed distribution were \log_{10} transformed before the analyses, but the values in text, tables, and figure are presented as nontransformed. Continuous variables were compared between control subjects and family members by general linear model, univariate ANCOVA, and between unaffected and affected family members by random effects model to adjust for dependence among the family members. $P < 0.05$ was considered significant (2-tailed). The frequency distribution of the categorical variables was compared between the groups with the χ^2 test. The relationships of the characteristics were examined by Pearson correlation and Spearman correlation analysis as appropriate. Multivariate stepwise linear regression analysis was performed to determine the relative contribution of different parameters to the mean IMT. Independent variables were removed from the model until the best-fitting model with the maximum adjusted multiple R^2 was achieved. All values used in the comparisons with control subjects and in correlation analyses were adjusted for age and gender because of the differences between the study groups.

Results

Table 1 shows the biochemical and clinical characteristics of the study subjects. Altogether, 35 family members had CHD. Among these, 33 were affected (31 probands) and 2 were unaffected. The affected family members had significantly higher body mass index (BMI), waist circumference, triglycerides (TGs), and apoB and significantly lower HDL-C, apoA-I, apoA-II, and total cholesterol (TC) than the control subjects. Compared with the unaffected family members, the affected family members were older and had significantly higher BMI, waist circumference, TGs, apoB, and fasting glucose and significantly lower TC, HDL-C, apoA-I, and apoA-II.

Table 2 displays the HDL subspecies distribution. HDL particle size was markedly smaller in the affected family members than in the unaffected family members and control subjects. (For a figure, please see online data supplement III.) The amount of protein (as percentage) of integration area of HDL_{2b} was significantly lower, whereas that of HDL_{3a}, HDL_{3b}, and HDL_{3c} was significantly higher in the affected family members than in the unaffected family members and in the control group. The greatest difference was demonstrated in HDL_{2b}, with a reduction of $\approx 50\%$ in the affected family members compared with the control group. Interestingly, when the unaffected family members were compared with the control subjects, they had significantly smaller HDL particle size, and their relative amount of HDL_{2b} was significantly reduced. Moreover, the relative amounts of HDL_{3a}, HDL_{3b}, and HDL_{3c} subspecies were significantly increased in

TABLE 1. Characteristics of the Study Subjects

	Control Subjects n=133 (91 men/42 women)	Unaffected Family Members n=65 (32 men/33 women)	Affected Family Members n=83 (60 men/23 women)	P Value 1	P Value 2	P Value 3
CHD, n (%)	0 (0)	2 (3.1)	33 (39.8)			
Age, years	50.5±1.1	40.6±1.6	47.8±1.5	<0.0001	NS	<0.0005
BMI, kg/m ²	24.7±0.3	24.7±0.4	27.2±0.4	NS	<0.0001	<0.001
SBP, mm Hg	125±1	131±2	127±2	<0.005	NS	<0.05
DBP, mm Hg	78±1	82±1	80±1	<0.005	<0.05	NS
Waist, cm	85.3±0.8	87.6±1.2	93.9±1.0	<0.05	<0.0001	<0.001
TC, mmol/L	5.03±0.08	5.24±0.12	4.76±0.10	NS	<0.05	<0.01
TG, mmol/L	0.93±0.06	1.17±0.08	1.68±0.07	<0.005	<0.0001	<0.01
HDL-C, mmol/L	1.55±0.03	1.43±0.04	0.94±0.04	<0.05	<0.0001	<0.0001
LDL-C, mmol/L	3.05±0.07	3.29±0.10	3.06±0.09	<0.05	NS	NS
ApoA-I, mg/dL	143±2	139±2	115±2	NS	<0.0001	<0.0001
ApoA-II, mg/dL	36±1	36±1	32±1	NS	<0.0001	<0.0001
ApoB, mg/dL	89±2	96±3	106±3	<0.05	<0.0001	<0.05
Glucose, mmol/L	4.94±0.06	4.70±0.09	5.05±0.08	<0.01	NS	<0.05

DBP indicates diastolic blood pressure.

P value 1: Between control subjects and unaffected; P value 2: between control subjects and affected; P value 3: between unaffected and affected.

Values are mean±SE. All values used in the comparisons with control subjects were adjusted for age and gender. Means, SEs, and P-values 1 and 2 are from ANCOVA; P value 3 from random effects model.

the unaffected family members compared with the control subjects.

Pre β -HDL concentration was significantly lower in the affected family members compared with the levels detected in the unaffected family members and in the control group (Table 2; for a figure, please see online supplement III).

Mean IMT was significantly higher in the affected family members than in the control subjects (0.85±0.01 mm versus 0.79±0.01 mm; $P<0.0001$). However, no significant difference was observed in mean IMT between the affected and unaffected family members (0.85±0.01 mm versus 0.82±0.02 mm; see the figure in online data supplement III).

Table 3 shows the results of the correlation analyses between mean IMT and clinical parameters in all subjects. Mean IMT showed a significant positive correlation with age, systolic blood pressure (SBP), HDL_{3a}, and HDL_{3b} (Table 3; Figure, D). It correlated inversely with HDL-C, HDL particle size, HDL_{2b}, and pre β -HDL level (Table 3; Figure, A through C).

Stepwise regression analysis was performed in the pooled study sample to test the relative contributions of different variables to IMT. Age, gender, blood pressure, BMI, TC, TGs, HDL-C, pre β -HDL, HDL particle size, the amount of protein (as percentage) of integration area of HDL subspecies, low-density lipoprotein (LDL) cholesterol, apoA-I, apoA-II, apoB, and fasting glucose were used for independent variables. The remaining variables in the final model included age, HDL_{2b}, SBP, and pre β -HDL concentration, with the adjusted multiple R^2 of 0.62 (Table 4).

Discussion

We measured HDL particle size and the proportions of different HDL subspecies in low-HDL family members and control subjects to clarify the influence of HDL subspecies distribution on the degree of atherosclerosis as measured by IMT. To the best of our knowledge, this is the first study to quantitate specific HDL subspecies, HDL particle size, and pre β -HDL concentrations simultaneously in the same cohort.

TABLE 2. HDL Species of the Study Subjects

	Control Subjects	Unaffected Family Members	Affected Family Members	P Value 1	P Value 2	P Value 3
Percentage of pre β -HDL, %	15.3±0.5	15.0±0.6	13.2±0.5	NS	<0.005	NS
Pre β -HDL, mg/L	221.8±8.3	216.7±10.7	153.9±8.5	NS	<0.0001	<0.0001
HDL particle size, nm	9.8±0.03	9.5±0.05	9.1±0.04	<0.0001	<0.0001	<0.0001
HDL _{2b} , %	37.9±1.0	29.1±1.5	18.2±1.3	<0.0001	<0.0001	<0.0001
HDL _{2a} , %	28.1±0.4	28.7±0.6	26.8±0.6	NS	NS	NS
HDL _{3a} , %	23.7±0.6	26.9±0.8	31.6±0.7	<0.005	<0.0001	<0.0001
HDL _{3b} , %	7.9±0.4	11.7±0.6	17.9±0.6	<0.0001	<0.0001	<0.0001
HDL _{3c} , %	2.3±0.2	3.5±0.3	5.5±0.3	<0.0001	<0.0001	<0.001

P value 1: Between control subjects and unaffected; P value 2: between control subjects and affected; P value 3: between unaffected and affected.

Values are mean±SE. All values used in the comparisons with control subjects were adjusted for age and gender. Means, SEs, and P-values 1 and 2 are from ANCOVA; P value 3 from random effects model.

TABLE 3. Relationship of IMT With Clinical Parameters by Univariate Regression Analysis

	R	P Value
Age	0.76	<0.0001
HDL-C	-0.23	<0.05
SBP	0.32	<0.005
Pre β -HDL	-0.29	<0.01
HDL particle size	-0.26	<0.05
HDL _{2b}	-0.31	<0.01
HDL _{3a}	0.25	<0.05
HDL _{3b}	0.28	<0.05

All values used in the analyses were adjusted for age and gender.

HDL particle size was markedly smaller in the affected family members than in the unaffected family members or control subjects. Pre β -HDL concentration and percentage of HDL_{2b} were significantly lower, whereas percentages of HDL_{3a}, HDL_{3b}, and HDL_{3c} were significantly higher in the affected family members compared with the 2 other groups. This is in accordance with previous studies, which have shown that large HDL particles are decreased in subjects with low HDL.²²⁻²⁴ Large HDL particles were also decreased in hypertriglyceridemic subjects.^{23,25} However, the present affected low-HDL family members showed only a moderate elevation of TGs, suggesting that the changes in HDL subspecies may not be explained by elevation of serum TGs only. This is further supported by the fact that the unaffected family members showed qualitatively similar changes in HDL profile despite a comparable TG level with control subjects. We propose that the observed changes of HDL profile may reflect also an exposure to genetic components regulating the distribution of HDL subspecies. Interestingly, recent data suggest that changes of HDL subspecies may provide better phenotypes than only HDL-C levels.^{26,27}

HDL particle size was markedly smaller in the affected low-HDL family members than in the control subjects. Recently, larger HDL particle size has been shown to associate with exceptional longevity.²⁸ In contrast, reduced HDL particle size is a feature of the high-TG/low-HDL profile in subjects with abdominal obesity.²⁹ Thus, HDL particle size may be a relevant marker of the atherogenic lipid profile.

The most important antiatherogenic function of HDL is considered to be its role in RCT,^{7,8} in which ABCA1 mediates efflux of cellular cholesterol to pre β -HDL⁹ and ABCG1

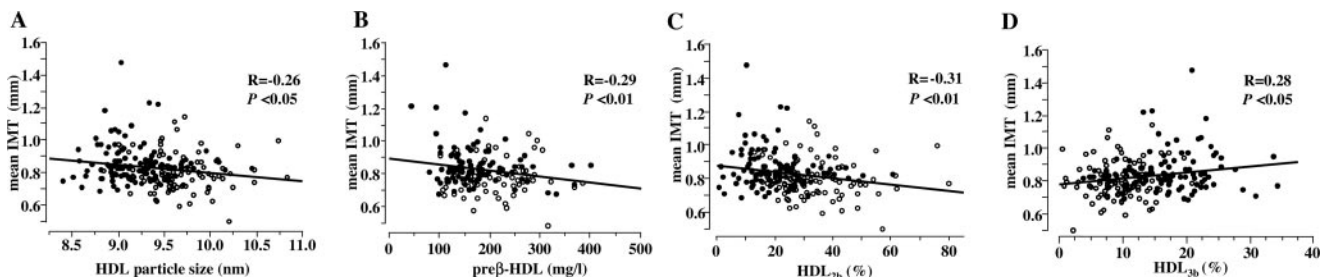
TABLE 4. Relationship of Mean IMT With Clinical Parameters by Multivariate Regression Analysis

	β	SE	P Value
Age	0.0096	0.0008	0.0001
HDL _{2b}	-0.0071	0.0021	0.0002
SBP	0.0024	0.0006	0.0046
Pre β -HDL	-0.0003	0.0001	0.0359
HDL particle size	-0.0042	0.0066	0.0759

Age, gender, blood pressure, BMI, TC, TGs, pre β -HDL, HDL-C, HDL particle size, the protein percentage of integration areas of HDL subclasses, LDL cholesterol, apoA-I, apoA-II, apoB, and fasting glucose were used as predictor variables.

stimulates efflux to larger HDL particles, especially to HDL₂.¹⁰ Wang et al hypothesize that ABCG1-mediated efflux could be more important in preventing CHD because most of the plasma HDL consists of such mature HDL particles.¹⁰ Our affected low-HDL family members had decreased proportions of large HDL particles and pre β -HDL concentration, supporting the theory of compromised efflux explaining the elevated risk of CHD in subjects with low HDL. Furthermore, the greatest reduction was seen in HDL_{2b} (\approx 50%). This finding further supports the hypothesis of ABCG1-mediated efflux to large HDL species being an important mechanism for the protective action of HDL against atherosclerosis.¹⁰ In addition, ABCG1 prevents cellular lipid accumulation in hepatocytes and macrophages in several tissues.³⁰ Thus, RCT-defective HDL subspecies distribution might be associated with increased risk of atherosclerosis in Finnish low-HDL family members.

Most of the reported cardioprotective properties of HDL have been associated with HDL₂ rather than HDL₃ fraction.^{31,32} Importantly, low levels of HDL_{2b} together with high levels of HDL_{3b} have been reported to associate with high CHD risk.^{32,33} Thus, changes of specific HDL subspecies may have more significant roles in CHD risk prediction than generally recognized. To investigate the influence of HDL subspecies distribution on the degree of atherosclerosis in Finnish low-HDL families, we measured IMT by ultrasonography. The affected low-HDL family members had significantly higher mean IMT than the control subjects. Mean IMT tended to be higher in the affected family members than in the unaffected family members, but the difference was not significant. Of the traditional risk factors of CHD, age, SBP, and HDL-C appeared to be the most important determinants



Relationship between mean IMT and HDL particle size, pre β -HDL concentration, HDL_{2b}, and HDL_{3b} in low-HDL family members (●) and control group (○). A, Correlation between mean IMT and HDL particle size. B, Correlation between mean IMT and pre β -HDL concentration. C, Correlation between mean IMT and HDL_{2b}. D, Correlation between mean IMT and HDL_{3b}.

of IMT. Most important, adding HDL subspecies as independent variables in the stepwise regression analysis, the data demonstrated that among HDL subspecies, HDL_{2b} was the most important determinant of carotid atherosclerosis.

Moreover, stepwise regression analysis showed that pre β -HDL is also an important determinant of IMT. As discussed, pre β -HDL acts as the primary acceptor of cellular cholesterol in RCT. Decreased pre β -HDL concentration is one of the major reasons to diminished cholesterol efflux capacity and thus would promote progression of atherosclerosis.⁷ Due to the important role of RCT in maintaining the cholesterol homeostasis and lowering the risk of atherosclerosis, the regulation of HDL and HDL subspecies distribution may directly influence the atherogenic process.

One interesting finding was the difference in HDL subspecies between the unaffected low-HDL family members and the control subjects. Although having similar HDL-C levels, the unaffected family members had significantly smaller HDL particle size and less HDL_{2b}. This may reflect their risk of developing the characteristic low-HDL profile later in life. The unaffected family members were younger than the affected members, and thus at least some subjects may have been exposed to the same genotype as the affected members. We speculate that the observed changes in HDL subspecies distribution may be the first changes in the lipoprotein profile leading to the lowering of HDL-C under long-term exposure. In the context of RCT process and HDL subspecies distribution, the next important step will be to measure cholesterol efflux from macrophages to serum withdrawn from low-HDL family members. It would be also interesting to compare serum cholesterol removal capacity between unaffected family members and control subjects having similar HDL-C levels but different HDL subspecies distribution.

One potential limitation of our study is that the correlation analyses as well as the stepwise regression analyses were performed in a pooled study sample. Therefore, these results do not necessarily show the actual causality. On the other hand, the pooling of the different groups together gives us a wider range of these biological parameters, which, in turn, facilitates the interpretation of the outcome in the present study. Another drawback is the relatively small sample size. This is partly because of the exclusion of a large number of women using estrogen. However, the family criteria were strict and the subjects were well characterized and therefore "analysis-wise" behaved similarly.

The effect of distribution of HDL subspecies on the development of atherosclerosis has not been elucidated thoroughly. However, HDL subspecies may become a target in the context of drug development designed to increase the antiatherogenic species of circulating HDL levels. In this context, the discovery of the human genetic cholesterol ester transfer protein (CETP) deficiency typically characterized by markedly increased HDL levels and moderately reduced LDL launched the design of drugs that will inhibit CETP.^{34–37} A mechanistically interesting result was the observed significant increase in HDL particle size and a decrease in small dense LDL particles in subjects treated with the CETP inhibitor torcetrapib.³⁵ One relevant mechanism to explain the protective effect of large-size HDL particles might be that

they act as ideal free cholesterol acceptors mediated by ABCG1.¹⁰ This suggests that rising the concentration of large HDL particles might reflect mechanisms that may enhance RCT and consequently reduce the risk of CHD.

In summary, our results suggest that the risk for the development of atherosclerosis in low-HDL family members is increased because of decrease of HDL particle size and a general shift in HDL subspecies distribution toward small-size HDL. The decreased levels of HDL_{2b} and pre β -HDL reflect the potentially efflux-deficient HDL subspecies distribution in the affected low-HDL family members. These novel findings support the hypothesis of impaired cholesterol efflux being the mechanism underlying the importance of HDL subspecies in preventing atherosclerosis.

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References

- Genest J, Jr, Marcil M, Denis M, Yu L. High density lipoproteins in health and in disease. *J Invest Med*. 1999;47:31–42.
- Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. *Am J Med*. 1977;62:707–714.
- Gordon T, Kannel WB, Castelli WP, Dawber TR. Lipoproteins, cardiovascular disease, and death. The Framingham Study. *Arch Intern Med*. 1981;141:1128–1131.
- Assmann G, Schulte H. Relation of high-density lipoprotein cholesterol and triglycerides to incidence of atherosclerotic coronary artery disease (the PROCAM experience). Prospective Cardiovascular Munster study. *Am J Cardiol*. 1992;70:733–737.
- Perusse M, Pascot A, Despres JP, Couillard C, Lamarche B. A new method for HDL particle sizing by polyacrylamide gradient gel electrophoresis using whole plasma. *J Lipid Res*. 2001;42:1331–1334.
- Blanche PJ, Gong EL, Forte TM, Nichols AV. Characterization of human high-density lipoproteins by gradient gel electrophoresis. *Biochim Biophys Acta*. 1981;665:408–419.
- Stein O, Stein Y. Atheroprotective mechanisms of HDL. *Atherosclerosis*. 1999;144:285–301.
- Assmann G, Nofer JR. Atheroprotective effects of high-density lipoproteins. *Annu Rev Med*. 2003;54:321–341.
- Wang N, Silver DL, Costet P, Tall AR. Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. *J Biol Chem*. 2000;275:33053–33058.
- Wang N, Lan D, Chen W, Matsuura F, Tall AR. ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proc Natl Acad Sci U S A*. 2004;101:9774–9779.
- Poli A, Tremoli E, Colombo A, Sirtori M, Pignoli P, Paoletti R. Ultrasonographic measurement of the common carotid artery wall thickness in hypercholesterolemic patients. A new model for the quantitation and follow-up of preclinical atherosclerosis in living human subjects. *Atherosclerosis*. 1988;70:253–261.
- Davis PH, Dawson JD, Mahoney LT, Lauer RM. Increased carotid intimal-medial thickness and coronary calcification are related in young and middle-aged adults. The Muscatine Study. *Circulation*. 1999;100:838–842.
- O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK Jr. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. *N Engl J Med*. 1999;340:14–22.

14. Hodis HN, Mack WJ, LaBree L, Selzer RH, Liu CR, Liu CH, Azen SP. The role of carotid arterial intima-media thickness in predicting clinical coronary events. *Ann Intern Med.* 1998;128:262–269.
15. Soro A, Pajukanta P, Lilja HE, Ylitalo K, Hiekkalinna T, Perola M, Cantor RM, Viikari JS, Taskinen MR, Peltonen L. Genome scans provide evidence for low-HDL-C loci on chromosomes 8q23, 16q24.1–24.2, and 20q13.11 in Finnish families. *Am J Hum Genet.* 2002;70:1333–1340.
16. Alagona C, Soro A, Ylitalo K, Salonen R, Salonen JT, Taskinen MR. A low high density lipoprotein (HDL) level is associated with carotid artery intima-media thickness in asymptomatic members of low HDL families. *Atherosclerosis.* 2002;165:309–316.
17. Vartiainen E, Puska P, Jousilahti P, Korhonen HJ, Tuomilehto J, Nissinen A. Twenty-year trends in coronary risk factors in north Karelia and in other areas of Finland. *Int J Epidemiol.* 1994;23:495–504.
18. Vartiainen E, Jousilahti P, Alfthan G, Sundvall J, Pietinen P, Puska P. Cardiovascular risk factor changes in Finland, 1972–1997. *Int J Epidemiol.* 2000;29:49–56.
19. van Haperen R, van Tol A, Vermeulen P, Jauhainen M, van Gent T, van den Berg P, Ehnholm S, Grosveld F, van der Kamp A, de Crom R. Human plasma phospholipid transfer protein increases the antiatherogenic potential of high density lipoproteins in transgenic mice. *Arterioscler Thromb Vasc Biol.* 2000;20:1082–1088.
20. Ylitalo K, Syvanne M, Salonen R, Nuotio I, Taskinen MR, Salonen JT. Carotid artery intima-media thickness in Finnish families with familial combined hyperlipidemia. *Atherosclerosis.* 2002;162:171–178.
21. Selzer RH, Hodis HN, Kwong-Fu H, Mack WJ, Lee PL, Liu CR, Liu CH. Evaluation of computerized edge tracking for quantifying intima-media thickness of the common carotid artery from B-mode ultrasound images. *Atherosclerosis.* 1994;111:1–11.
22. Yang Y, Yan B, Fu M, Xu Y, Tian Y. Relationship between plasma lipid concentrations and HDL subclasses. *Clin Chim Acta.* 2005;354:49–58.
23. Xu Y, Fu M. Alterations of HDL subclasses in hyperlipidemia. *Clin Chim Acta.* 2003;332:95–102.
24. Asztalos BF, Lefevre M, Foster TA, Tulley R, Windhauser M, Wong L, Roheim PS. Normolipidemic subjects with low HDL cholesterol levels have altered HDL subpopulations. *Arterioscler Thromb Vasc Biol.* 1997;17:1885–1893.
25. Syvanne M, Ahola M, Lahdenpera S, Kahri J, Kuusi T, Virtanen KS, Taskinen MR. High density lipoprotein subfractions in non-insulin-dependent diabetes mellitus and coronary artery disease. *J Lipid Res.* 1995;36:573–582.
26. Yang Q, Lai CQ, Parnell L, Cupples LA, Adiconis X, Zhu Y, Wilson PW, Housman DE, Shearman AM, D'Agostino RB, Ordovas JM. Genome-wide linkage analyses and candidate gene fine mapping for HDL3 cholesterol: the Framingham Study. *J Lipid Res.* 2005;46:1416–1425.
27. Almsay L, Hixson JE, Rainwater DL, Cole S, Williams JT, Mahaney MC, VandeBerg JL, Stern MP, MacCluer JW, Blangero J. Human pedigree-based quantitative-trait-locus mapping: localization of two genes influencing HDL-cholesterol metabolism. *Am J Hum Genet.* 1999;64:1686–1693.
28. Barzilai N, Atzmon G, Schechter C, Schaefer EJ, Cupples AL, Lipton R, Cheng S, Shuldiner AR. Unique lipoprotein phenotype and genotype associated with exceptional longevity. *J Am Med Assoc.* 2003;290:2030–2040.
29. Pascot A, Lemieux I, Prud'homme D, Tremblay A, Nadeau A, Couillard C, Bergeron J, Lamarche B, Despres JP. Reduced HDL particle size as an additional feature of the atherogenic dyslipidemia of abdominal obesity. *J Lipid Res.* 2001;42:2007–2014.
30. Kennedy MA, Barrera GC, Nakamura K, Baldan A, Tarr P, Fishbein MC, Frank J, Francone OL, Edwards PA. ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. *Cell Metab.* 2005;1:121–131.
31. Lamarche B, Moorjani S, Cantin B, Dagenais GR, Lupien PJ, Despres JP. Associations of HDL2 and HDL3 subfractions with ischemic heart disease in men. Prospective results from the Quebec Cardiovascular Study. *Arterioscler Thromb Vasc Biol.* 1997;17:1098–1105.
32. Johansson J, Carlson LA, Landou C, Hamsten A. High density lipoproteins and coronary atherosclerosis. A strong inverse relation with the largest particles is confined to normotriglyceridemic patients. *Arterioscler Thromb.* 1991;11:174–182.
33. Williams PT, Krauss RM, Vranizan KM, Stefanick ML, Wood PD, Lindgren FT. Associations of lipoproteins and apolipoproteins with gradient gel electrophoresis estimates of high density lipoprotein subfractions in men and women. *Arterioscler Thromb.* 1992;12:332–340.
34. Brousseau ME, Diffenderfer MR, Millar JS, Nartsupha C, Asztalos BF, Welty FK, Wolfe ML, Rudling M, Bjorkhem I, Angelin B, Mancuso JP, Digenio AG, Rader DJ, Schaefer EJ. Effects of cholesteryl ester transfer protein inhibition on high-density lipoprotein subspecies, apolipoprotein A-I metabolism, and fecal sterol excretion. *Arterioscler Thromb Vasc Biol.* 2005;25:1057–1064.
35. Brousseau ME, Schaefer EJ, Wolfe ML, Bloedon LT, Digenio AG, Clark RW, Mancuso JP, Rader DJ. Effects of an inhibitor of cholesteryl ester transfer protein on HDL cholesterol. *N Engl J Med.* 2004;350:1505–1515.
36. Clark RW, Sutfin TA, Ruggeri RB, Willauer AT, Sugarman ED, Magnus-Aryitey G, Cosgrove PG, Sand TM, Wester RT, Williams JA, Perlman ME, Bamberger MJ. Raising high-density lipoprotein in humans through inhibition of cholesteryl ester transfer protein: an initial multidose study of torcetrapib. *Arterioscler Thromb Vasc Biol.* 2004;24:490–497.
37. de Grooth GJ, Kuivenhoven JA, Stalenhoef AF, de Graaf J, Zwinderman AH, Pasma JL, van Tol A, Kastelein JJ. Efficacy and safety of a novel cholesteryl ester transfer protein inhibitor, JTT-705, in humans: a randomized phase II dose-response study. *Circulation.* 2002;105:2159–2165.

Supplementary data I

The inclusion criteria for the low HDL probands were as follows: age 30-60 years, verified coronary heart disease, HDL-C concentration below the 10th age-gender specific Finnish population percentile (<0.9 mmol/l in men and <1.1 mmol/l in women), total cholesterol (TC) <6.3 mmol/l in men and <6.0 mmol/l in women, and triglycerides (TG) <2.3 mmol/l in both men and women. The age-gender specific percentile criteria used for the classification of the subjects were derived from the population-based survey FINRISK ^{1,2}. Exclusion criteria for the probands were diabetes, significant hepatic or renal disease, untreated hypothyroidism, or body mass index (BMI) >30 Kg/m² ^{3,4}. The proband was required to have at least 3 accessible first-degree relatives and at least 2 affected members were required to be in each family.

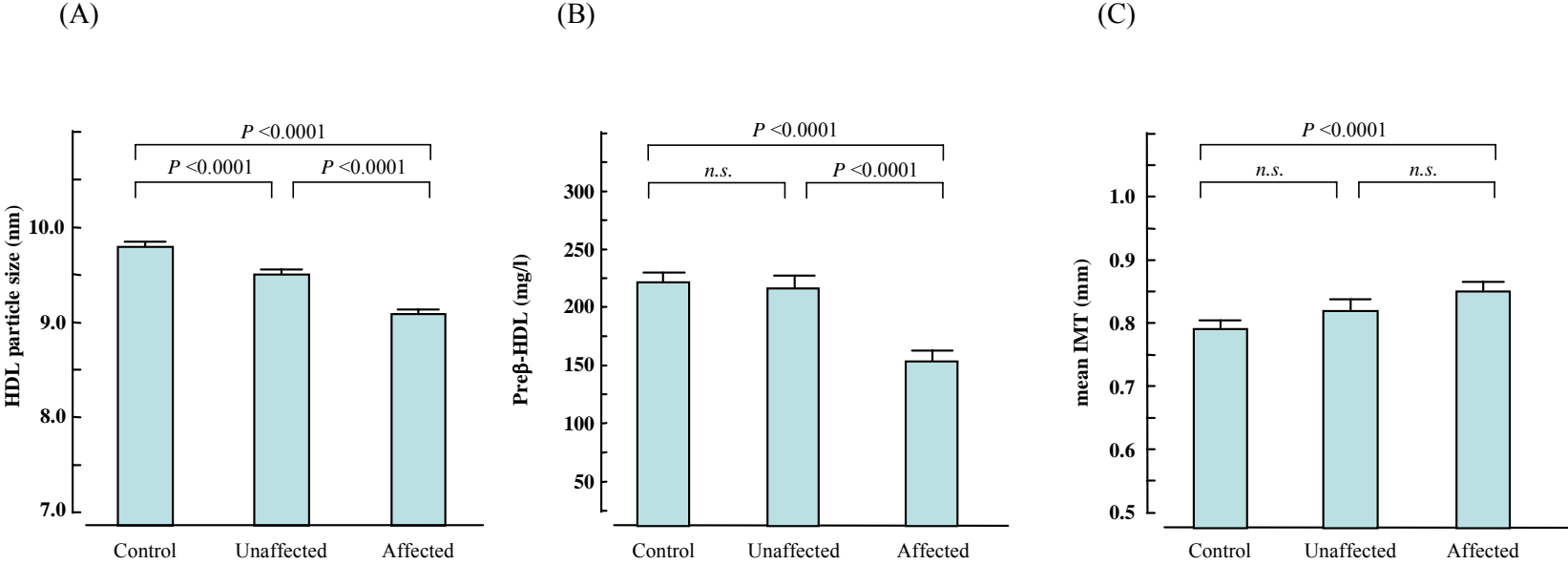
1. Vartiainen E, Puska P, Jousilahti P, Korhonen HJ, Tuomilehto J, Nissinen A. Twenty-year trends in coronary risk factors in north Karelia and in other areas of Finland. *International Journal of Epidemiology*. 1994;23:495-504.
2. Vartiainen E, Jousilahti P, Alfthan G, Sundvall J, Pietinen P, Puska P. Cardiovascular risk factor changes in Finland, 1972-1997. *Int J Epidemiol*. 2000;29:49-56.
3. Soro A, Pajukanta P, Lilja HE, Ylitalo K, Hiekkalinna T, Perola M, Cantor RM, Viikari JS, Taskinen MR, Peltonen L. Genome scans provide evidence for low-HDL-C loci on chromosomes 8q23, 16q24.1-24.2, and 20q13.11 in Finnish families. *Am J Hum Genet*. 2002;70:1333-1340.
4. Alagona C, Soro A, Ylitalo K, Salonen R, Salonen JT, Taskinen MR. A low high density lipoprotein (HDL) level is associated with carotid artery intima-media thickness in asymptomatic members of low HDL families. *Atherosclerosis*. 2002;165:309-316.

Supplementary data II

Serum total cholesterol (TC) and triglycerides (TG) were determined with an automated Cobas Mira analyser (Hoffman-La Roche, Basel, Switzerland) by fully enzymatic methods (Hoffman-La Roche kits #0722138 and #0715166, respectively). Serum high-density lipoprotein cholesterol (HDL-C) was quantified by phosphotungstic acid/magnesium chloride precipitation procedures (Hoffman-La Roche kit #0720674). Serum low-density lipoprotein (LDL) was calculated from the Friedewald formula $[\text{LDL}=\text{TC}-(\text{HDL-C})-\text{TG}/2.2]^1$. Concentrations of apolipoprotein A-I (apoA-I), apolipoprotein A-II (apoA-II) and apolipoprotein B (apoB) were measured by immunoturbidometric methods (for apoA-I; Wako Chemicals GmbH, Neuss, Germany, for apoA-II; Wako Chemicals GmbH and own polyclonal antibody produced in rabbits against human apoA-II, and for apoB; Orion Diagnostica, Espoo, Finland). Plasma glucose concentration was analysed by glucose dehydrogenase method (Precision-G Blood Glucose Testing System, Medisense, Abbott, Illinois, USA).

1. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem.* 1972;18:499-502.

Supplementary figure (supplementary data III).



Supplementary figure legend.

Comparison of HDL particle size, pre β -HDL concentration and mean IMT in affected family members, unaffected family members and control group.

(A) HDL particle size in affected family members (n = 78), unaffected family members (n = 59) and control group (n = 127). (B) Pre β -HDL concentration in affected family members (n = 63), unaffected family members (n = 46) and control group (n = 89). (C) Mean IMT in affected family members (n = 65), unaffected family members (n = 47) and control group (n = 105)