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Identification of Unique Lipoprotein Subclasses for Visceral Obesity by Component Analysis of Cholesterol Profile in High-Performance Liquid Chromatography

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Objective—The contribution of visceral fat accumulation to the development of coronary heart disease was previously reported, but the relation between visceral fat accumulation and serum lipoprotein subclasses was unknown.

Methods and Results—We examined the relation of lipoprotein subclasses with visceral fat accumulation in 62 male subjects (aged 22 to 67 years) with visceral fat syndrome or obesity. Cholesterol levels in very low–density, low-density, and high-density lipoprotein subclasses (VLDL, LDL, and HDL) were determined by computer-assisted high-performance liquid chromatography. Subcutaneous fat area and visceral fat area were measured by computed tomographic scanning. There was no significant correlation between the subcutaneous fat area and the cholesterol levels in all lipoprotein subclasses. In contrast, the visceral fat area was correlated positively ($P<0.002$) with VLDL and LDL subclasses, except for large LDL, but negatively ($P<0.001$) with those in large and medium HDL subclasses. The observed positive correlations of small and very small LDL subclasses remained significant ($P<0.005$) after adjustment for serum cholesterol, triglycerides, HDL cholesterol, and LDL cholesterol, respectively, but a significant negative correlation ($P<0.005$) of large LDL was obtained after adjustment for LDL cholesterol.

Conclusion—These findings indicate that this simple high-performance liquid chromatography method may be applied for easy detection and evaluation of abnormal distribution of lipoprotein subclasses. (***Arterioscler Thromb Vasc Biol.* 2005; 25:578-584.**)

Key Words: obesity ■ risk factors ■ particle size ■ triglyceride

Lipoprotein profiles are well accepted as predictors of risk for coronary heart disease (CHD). The important relationship between elevated low-density lipoprotein cholesterol (LDL-C) or decreased high-density lipoprotein cholesterol (HDL-C) and the increased risk of CHD is definitely established in many epidemiological studies.[1,2] Another established risk factor for cardiovascular disease is obesity, which was confirmed to be a strong positive predicting factor of CHD in the Framingham Heart Study, and the risk for CHD is particularly increased when abdominal obesity is present.[3] Although measurement of LDL-C and HDL-C has been recommended by the U.S. National Cholesterol Education Program for initial classification of CHD risk status,[4] obesity is not considered to be a major risk factor, because the incremental risk imparted by obesity independently of accompanying risk factors is uncertain. Atherogenic dyslipidemia frequently found in abdominal obesity is a combination of high serum triglycerides (TG) and low HDL-C, which is a strong correlate of the small dense LDL phenotype.[5] Cholesterol synthesis is increased in men with visceral obesity, and this may be partly related to insulin resistance, and the reduction in visceral fat is associated with a decrease in the hepatic secretion of VLDL apolipoprotein B (apoB).[6] We also previously reported that the contribution of visceral fat accumulation to the development of CHD is partially through progression of insulin resistance in nonobese men.[7] The visceral fat area (VFA) had significant positive correlations with serum total cholesterol (TC), serum TG, apoB, and apoE levels and the concentrations of VLDL-C, intermediate density lipoprotein cholesterol (IDL-C), and LDL-C.

Many studies showed that LDL subclasses, characterized by variations in their density, size, and chemical composition, might be clinically significant.[8,9] Although earlier studies for lipoprotein subclass analysis have been done by analytical ultracentrifugation,[10] density analysis by various preparative ultracentrifugation methods have also been used: sequential separation at various density,[11] rate zonal ultracentrifugation,[12] and density gradient ultracentrifugation.[13] The particle

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size analysis by nondenaturing gradient gel electrophoresis has been used,[14,15] and recently the Lipoprint LDL system (Quantimetrix) using nongradient (3%) polyacrylamide gel electrophoretic method has been developed.[16] More recently, a rapid and convenient method using nuclear magnetic resonance (NMR) has been developed[17,18] and used widely in clinical subjects.[19,20] Many methods for detection and quantification of LDL subclasses based on their particle size, density, shape, and charge were reported, but it is unclear which method is clinically useful.[21]

High-performance liquid chromatography (HPLC) with gel permeation columns is an alternative method for classifying and quantifying lipoproteins on the basis of differences of particle size.[22,23] We successfully applied this technique to compare the effects of bezafibrate and pravastatin on lipoprotein subclasses in type 2 diabetes.[24] The HPLC method, similar to the NMR, measures all lipoprotein subclasses at a single analysis from very small amount of whole serum or plasma in a very short time. The HPLC, however, may be superior because of direct cholesterol determination available in major lipoproteins and their subclasses.

In this study, a new simple and fully automated method for analyzing lipoprotein subclasses by HPLC with gel permeation columns followed by mathematical treatment on chromatograms was applied to examine the relationship of cholesterol levels in lipoprotein subclasses with visceral fat accumulation in men with obesity or nonobese subjects with accumulation of visceral fat. The clinical significance for measurements of the cholesterol levels in all lipoprotein subclasses by HPLC method will be evaluated on the basis of visceral fat accumulation, which plays an important role in the occurrence of CHD associated with a cluster of multiple risk factors.

Methods

Subjects

Sixty-two men (aged 22 to 67 years) were enrolled in this study, which included 15 healthy volunteers and 47 hospitalized patients in Osaka University Hospital. All of the subjects gave their informed consent before entering the study according to the Osaka University Hospital ethics committees. All patients had no severe hepatic or renal diseases, and none of them had any medication known to affect insulin action or serum lipoprotein levels. Venous blood was drawn after an overnight fasting. Serum samples were kept in a refrigerator and analyzed within 7 days after blood collection.

HPLC Method

Serum lipoproteins were analyzed by HPLC, as previously described.[23,25] In brief, 5 μL whole serum sample was injected into 2 connected columns (300×7.8 mm) of TSKgel LipopropakXL (Tosoh) and eluted by TSKeluent Lp-1 (Tosoh). The effluent from the columns was continuously monitored at 550 nm after an online enzymatic reaction with a commercial kit, Determiner L TC (Kyowa Medex). The cholesterol concentration in major lipoproteins and their subclasses was calculated by our own computer program, which was designed to process the complex chromatograms with the modified Gaussian curve fitting for resolving the overlapping peaks by mathematical treatment.

We determined the number, position, and width of each Gaussian component peak for subclass analysis to carry out a sufficient curve fitting analysis of various samples from animals and humans under the constant condition in which the peak width and position of each Gaussian curve were not changed. For this purpose, we first took priority to refer the mean particle size of VLDL and LDL of healthy normolipidemic men (n=28). Therefore, the positions of component peaks 6 and 9 corresponded to those of VLDL (36.8±2.5 nm) and LDL (25.5±0.4 nm) of healthy subjects, respectively. Similarly, the positions of peaks 5 and 10 were those of VLDL (44.5±2.1 nm) and LDL (23.0±0.5 nm) of extremely hypertriglyceridemic subjects >1000 mg/dL (n=7) with or without lipoprotein lipase (LPL), respectively. Peak 7 corresponded to LDL (or VLDL; 31.3±1.0 nm) of type III hyperlipidemia with apoE2/2 (n=5). Peak 15 was HDL (13.5±0.4 nm) of cholesterol ester transfer protein deficiency (n=6). Other component peaks (peaks 16 to 20) of HDL subclasses were based on the 5 subclasses determined by HPLC using a gel permeation column (G3000SW) with a separation range for only HDL.[23,26] In addition to the 11 component peaks determined by some experimental background as described above, 9 additional peaks (peaks 1 to 4, 8, and 11 to 14) were introduced to obtain the best curve fitting analysis by changing only peak height of each Gaussian curve. The position of peak 8 (28.6 nm) was determined as the middle point between peak 7 and peak 9, representing a transition component from TG-rich remnant lipoproteins to LDL. Four peaks (peaks 11 to 14) were regularly inserted between peak 10 and peak 15 to make similar intervals from peak 8 to peak 20. Moreover, 3 peaks (peaks 2 to 4) at least needed to be introduced between a void volume (peak 1) and peak 5 to perform the best curve fitting. Alternative setting of additional peaks resulted in the decrease of the degree of curve fitting analysis on the original chromatogram. The conversion of elution time to particle diameter was performed using a column calibration curve, a plot of logarithm of the particle diameter of standard samples, latex beads (Magsphere Inc) 25 and 37 nm in diameter, and a high molecular weight calibrator (Pharmacia Biotech) containing thyroglobulin (17 nm), ferritin (12.2 nm), catalase (9.2 nm), albumin (7.1 nm), and ovalbumin (6.1 nm) against their elution times.

Other Clinical and Lipid Parameter Analysis

Serum TC and TG were determined enzymatically using commercial kits (Kyowa Medex). HDL-C was quantified by the heparin-Ca^{2+} precipitation method.[27] LDL-C was calculated from the formula of Friedewald et al.[28] Uric acid (UA), fasting immunoreactive insulin (IRI), and plasminogen activator inhibitor (PAI)-1 were measured by enzymatic methods and by a double antibody radioimmunoassay, respectively.

Body fat distribution was determined by computed tomographic (CT) scanning (General Electric CT/T scanner, General Electric Co) in the supine position as described previously.[29] The fat layer to the

TABLE 1. Clinical Characteristics, Lipid, and Lipoprotein Profiles of 62 Men

	Mean±SD	Min/Max
Age, years	43.8±11.3	22/67
Height, cm	169.8±6.3	152/181
Weight, kg	77.5±15.1	56/135
BMI, kg/m^2	26.8±4.6	21.0/43.1
VFA, cm^2	129.2±50.4	24.0/255.0
SFA, cm^2	195.5±97.9	55.0/512.0
UA, mg/dL	6.4±1.7	3.8/12.5
IRI, μU/mL	9.3±7.7	2.0/42.8
PAI-1, ng/mL	25.4±18.6	5.0/75.7
TC, mg/dL	212.6±35.2	135.0/308.7
TG, mg/dL	146.3±88.3	41.5/416.0
HDL-C, mg/dL*	45.0±12.4	22.6/70.7
LDL-C, mg/dL†	138.3±34.5	61.1/211.5

*Determined by the precipitation method.
†Calculated by the Friedwald equation.

TABLE 2. Definition for Major and Subclasses of Serum Lipoproteins and Within-Day Precision (n=5) for Measurement of Their Cholesterol Levels

Component Peak No.	Particle Diameter (nm)	Major and Subclass Name	Pool 1 (mg/dL)			Pool 2 (mg/dL)		
			Mean	SD	CV%	Mean	SD	CV%
1	>90		NA	NA	NA	3.69	0.04	1.03
2	75		NA	NA	NA	2.06	0.06	3.13
3	64	large VLDL	0.06	0.01	20.69	1.95	0.04	2.30
4	53.6	large VLDL	1.16	0.04	3.07	1.65	0.06	3.86
5	44.5	large VLDL	2.37	0.09	3.76	7.58	0.24	3.14
6	36.8	medium VLDL	9.78	0.12	1.27	16.12	0.29	1.83
7	31.3	small VLDL	7.45	0.31	4.21	12.71	0.23	1.78
8	28.6	large LDL	27.92	0.98	3.51	18.56	0.46	2.46
9	25.5	medium LDL	38.95	0.14	0.37	23.28	0.23	0.97
10	23.0	small LDL	19.22	0.30	1.54	18.60	0.58	3.09
11	20.7	very small LDL	4.90	0.05	1.06	9.76	0.28	2.91
12	18.6	very small LDL	1.39	0.02	1.69	3.52	0.16	4.41
13	16.7	very small LDL	0.27	0.01	4.22	1.29	0.05	3.91
14	15.0	very large HDL	0.95	0.03	2.64	1.20	0.05	4.25
15	13.5	very large HDL	1.49	0.06	4.02	2.07	0.08	3.98
16	12.1	large HDL	20.27	0.68	3.35	11.51	0.27	2.37
17	10.9	medium HDL	24.64	0.27	1.09	14.30	0.20	1.38
18	9.8	small HDL	11.92	0.36	3.05	8.86	0.15	1.73
19	8.8	very small HDL	3.03	0.09	3.02	2.59	0.09	3.47
20	7.6	very small HDL	1.25	0.03	2.03	1.68	0.07	3.94
1–20		total	177.0	1.13	0.64	163.0	0.66	0.41
1–2	>80	CM	NA	NA	NA	5.75	0.10	1.75
3–7	30–80	VLDL	20.81	0.52	2.51	40.01	0.52	1.29
8–13	16–30	LDL	92.64	0.56	0.60	75.02	0.61	0.81
14–20	8–16	HDL	63.55	0.13	0.20	42.22	0.33	0.78

NA indicates not available; CV, coefficient of variation; CM, chylomicrons.
Pool 1, normolipidemic pooled serum (TG=56 mg/dL); Pool 2, hyperlipidemic pooled serum (TG=428mg/dL).

extraperitoneal region between skin and muscle was defined as subcutaneous fat area (SFA), with an attention range from −40 to −140 Hounsfield units. The intraperitoneal region, with the same density as SFA, was defined as the VFA. The SFA and VFA were measured at the level of the umbilicus.

Statistical Analysis

Data are expressed as mean±SD, unless stated otherwise. Correlations between various variables were presented as the Pearson correlation coefficient (r-value) with a P-value <0.05 considered to be statistically different.

Results

Clinical Characteristics and Lipid Levels of Studied Subjects

Clinical characteristics and lipid levels in 62 men in this study are shown in Table 1. A considerably wide range of anthropometric values was obtained, because they were recruited to cover a large spectrum of body fat values: body mass index (BMI) from 21 to 43 kg/cm^2, VFA from 24 to 255 cm^2, and SFA from 55 to 512 cm^2. Metabolic parameters showed a variation as compared reference values: UA from 3.8 to 12.5 mg/dL, IRI from 2 to 43 μU/mL, and PAI-1 from 5.0 to 75.7 ng/mL.

Analytical Performance of HPLC for Determination of Serum Cholesterol Levels in Major Lipoproteins and Their Subclasses

We defined 3 VLDL subclasses (large, medium, and small), 4 LDL subclasses (large, medium, small, and very small), and 5 HDL subclasses (very large, large, medium, small, and very small) on the basis of lipoprotein particle size (diameter), as shown in Table 2: chylomicrons (>80 nm, peaks 1 to 2), VLDL (30 to 80 nm, peaks 3 to 7), LDL (16 to 30 nm, peaks 8 to 13), and HDL (8 to 16 nm, peaks 14 to 20). The representative chromatograms for curve fitting analysis of normolipidemic (TC=131 mg/dL, TG=39 mg/dL) and hyperlipidemic subjects (LPL deficiency, TC=219 mg/dL, TG=1420 mg/dL) are presented in Figure 1.

Within-run reproducibility (n=5) for the cholesterol determination of 20 subclasses and major classes was determined on 2 different pooled samples (pool 1: TC=177 mg/dL, TG=56 mg/dL; pool 2: TC=163 mg/dL, TG=428 mg/dL) as shown in Table 2.

Within-run reproducibility (n=5) of LDL and HDL particle sizes was 25.20±0.07 nm (coefficient of variation [CV], 0.27%) and 11.25±0.04 nm (CV, 0.36%) for pool 1 and 25.63±0.14 nm (CV, 0.56%) and 11.03±0.05 nm (CV, 0.45%) for pool 2, respectively.

Sum area of the 20 Gaussian curves was 100.2±0.4% (99.1 to 101.7%, n=62) of the area under the original chromatogram. Sum of the peak area corresponding to HDL (peaks 14 to 20) was 99.7±1.1% (98.3 to 103.9%, n=62) of the HDL peak area under the original chromatogram.

A good correlation between HDL-C determined by the precipitation method (x) and total HDL (all HDL subclasses) by HPLC (y) was obtained: $y=0.975x+5.29$ ($r=0.973$, n=62, $P<0.0001$). Moreover, a good correlation between

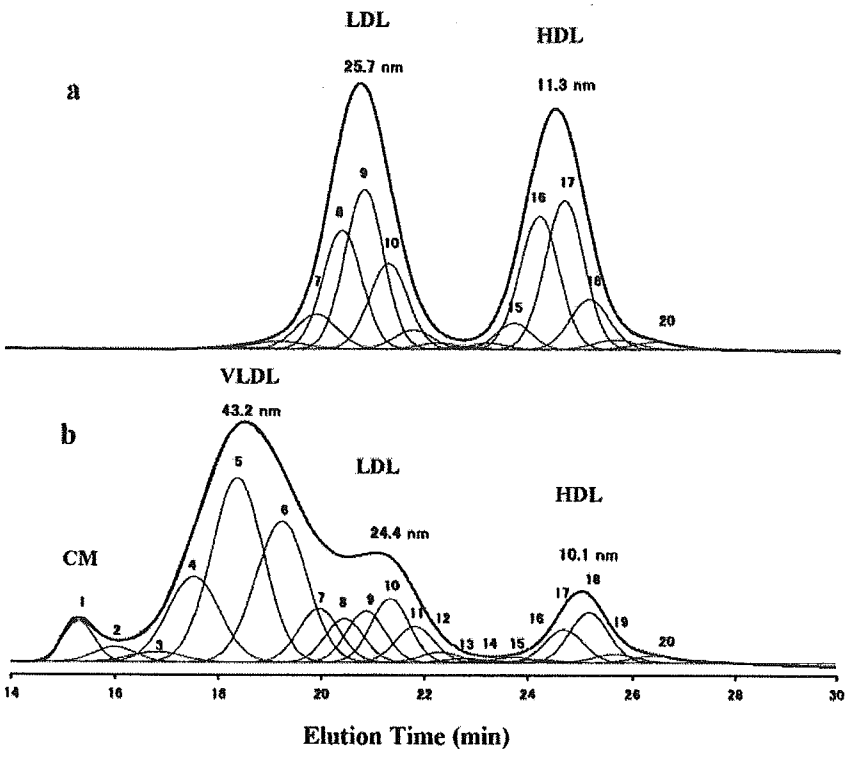

Figure 1. Representative HPLC patterns of (a) a healthy woman and (b) a patient with LPL deficiency. A 5-μL serum sample was injected onto 2 tandem gel permeation columns (TSKgel LipopropakXL) and eluted with TSKeluent LP-1 at a flow rate of 0.7 mL per min. Solid line is real HPLC pattern detected by online enzymatic reaction for TC reagent. Dashed lines are individual subclasses and their sum of Gaussian curves, which are determined curve fitting using Gaussian summation method. Serum TC and serum TG levels are 131 mg/dL and 39 mg/dL (a) and 219 mg/dL and 1420 mg/dL (b), respectively. Particle sizes (nm) determined from observed peak times are also presented.

LDL-C calculated by Friedewald equation (x) and total LDL (all LDL subclasses) by HPLC (y) was also obtained: $y=0.903x+6.28$ ($r=0.977$, n=62, $P<0.0001$).

Correlation of Cholesterol Profiles by HPLC With Clinical Parameters

Simple correlations of cholesterol levels in major lipoproteins and their subclasses with various clinical parameters (age, BMI, VFA, SFA, UA, IRI, and PAI-1) and serum TG levels are summarized in Table 3. Moreover, the correlations of LDL and HDL particle sizes are also presented in Table 3.

As for age, significant negative correlations ($P<0.01$) for medium and small HDL-C were obtained. As for BMI, significant negative correlations were observed only for HDL parameters: large HDL-C ($P<0.01$) and HDL particle size ($P<0.01$). Although no correlations were observed between SFA and all of the lipoprotein subclasses, there were significant positive correlations ($P<0.01$) of VFA with VLDL-C subclasses (large, medium, and small) and LDL-C subclasses (medium, small, and very small) and negative correlations ($P<0.01$) with large and medium HDL-C, LDL, and HDL particle sizes.

As for UA, positive correlations ($P<0.01$) for VLDL-C subclasses (medium and small) and negative correlations ($P<0.01$) for large HDL-C and HDL particle size were obtained. In the case of IRI, positive correlations ($P<0.01$) for small and very small LDL-C and negative correlations ($P<0.01$) for large HDL-C and HDL particle size were obtained. As for PAI-1, positive correlations ($P<0.01$) were observed for small HDL-C and very small HDL-C.

As for serum TG levels, there were significant positive correlations ($P<0.01$) of VFA with VLDL-C subclasses (large, medium, and small) and LDL-C subclasses (small and very small) and negative correlations ($P<0.01$) with large LDL-C, HDL-C subclasses (large and medium), LDL, and HDL particle sizes.

Influences of Traditional Lipid Parameters on the Correlation Between VFA and Lipoprotein Subclasses

Among the anthropometric values in Table 3, VFA levels showed most strong correlations with lipoprotein subclasses. Therefore, influences of traditional lipid parameters on the correlation between VFA and lipoprotein subclasses were examined by adjustment for serum TG, serum TC, HDL-C, and LDL-C levels, respectively (Table 4). Positive correlations of VFA with small LDL-C and very small LDL-C remained significant ($P<0.01$) after adjustment for TG, TC, HDL-C, and LDL-C, respectively.

As for VLDL subclasses, simple correlation analysis showed all VLDL subclasses were positively correlated with VFA, but large VLDL and small VLDL were correlated negatively and positively with VFA, respectively, after adjustment for serum TG level. In the case of LDL subclasses, adjustment for LDL-C gave a significant negative correlation ($P<0.01$) between large LDL and VFA but removed a significant positive correlation between medium LDL and VFA.

Effects of LDL-C on the Correlations Between VFA and LDL Subclasses

The studied subjects were divided into subgroups by the median value of total LDL-C levels (sum of all LDL subclasses) into the low LDL-C (n=31, LDL-C<130 mg/dL) and high LDL-C groups (n=31, LDL-C≥130 mg/dL). In the total population (n=62), a significant positive correlation

TABLE 3. Simple Correlations of Cholesterol Profiles by HPLC Method With Clinical Parameters (n=62)

Clinical Parameters	Age	BMI	VFA	SFA	UA	IRI	PAI-1	TG
Total VLDL	0.100	0.208	0.508†	0.055	0.368‡	0.283§	0.171	0.943†
Large VLDL	0.013	0.245	0.405‡	0.120	0.242	0.185	0.224	0.930†
Medium VLDL	0.126	0.210	0.488†	0.066	0.385‡	0.254§	0.192	0.951†
Small VLDL	0.119	0.070	0.434†	−0.063	0.327‡	0.318§	0.002	0.531†
Total LDL	0.229	0.146	0.431‡	0.090	0.220	0.349‡	0.010	0.057
Large LDL	0.079	0.009	0.115	0.016	0.083	0.183	−0.121	−0.329‡
Medium LDL	0.219	0.144	0.386‡	0.098	0.190	0.311§	0.006	−0.014
Small LDL	0.243	0.202	0.571†	0.111	0.268§	0.380‡	0.122	0.406‡
Very small LDL	0.240	0.219	0.556†	0.105	0.292§	0.375‡	0.164	0.577†
Total HDL	−0.230	−0.281§	−0.528†	−0.180	−0.269§	−0.295§	0.091	−0.418†
Very large HDL	0.200	−0.191	−0.095	−0.232	−0.139	−0.149	−0.180	−0.151
Large HDL	0.057	−0.334‡	−0.426†	−0.212	−0.356‡	−0.385‡	−0.277	−0.400‡
Medium HDL	−0.329‡	−0.251§	−0.502†	−0.159	−0.182	−0.220	0.203	−0.378‡
Small HDL	−0.382‡	0.240	−0.022	0.145	0.178	0.241	0.617†	0.126
Very small HDL	−0.303§	0.153	−0.029	0.238	0.031	0.010	0.369‡	0.045
LDL size*	−0.071	−0.246	−0.389‡	−0.188	−0.158	−0.170	−0.325§	−0.577†
HDL size*	0.166	−0.371‡	−0.368‡	−0.236	−0.354‡	−0.393‡	−0.325§	−0.364‡

Values are Pearson correlation coefficients.
*Average particle diameters (nm) obtained from LDL and HDL peak time by HPLC.
†$P<0.001$; ‡$P<0.01$; §$P<0.05$.

($r=0.431$, $P<0.001$) between VFA and total LDL-C was obtained as presented in Table 3. There was no correlation between VFA and total LDL-C in the low LDL-C group but a significant positive correlation ($r=0.546$, $P<0.002$) in the high LDL-C group.

Scattered plots between VFA and LDL subclasses are presented in Figure 2. Large LDL-C showed no significant correlations with VFA in total population and the high LDL-C group but showed a significant negative correlation ($r=-0.446$, $P<0.02$) in the low LDL-C group. Small LDL-C and very small LDL-C showed significant positive correlations with VFA in total population and both subgroups, except for very small LDL-C in high LDL-C group.

TABLE 4. Partial Correlations of Cholesterol Profiles by HPLC Method With VFA (n=62)

Controlling Factor	TG	TC	HDL-C	LDL-C
Total VLDL	0.009	0.362‡	0.363‡	0.495†
Large VLDL	−0.301§	0.278§	0.253§	0.427†
Medium VLDL	−0.084	0.386‡	0.336‡	0.522†
Small VLDL	0.257§	0.227	0.344‡	0.324§
Total LDL	0.476†	0.016	0.317§	0.141
Large LDL	0.366‡	−0.259§	0.155	−0.356‡
Medium LDL	0.466†	−0.026	0.266§	−0.035
Small LDL	0.458†	0.346‡	0.403†	0.437†
Very small LDL	−0.357‡	0.348‡	0.389‡	0.424†
Total HDL	−0.397‡	−0.514	−0.044	−0.460
Very large HDL	−0.016	−0.126	0.074	−0.106
Large HDL	−0.274§	−0.348‡	−0.082	−0.337‡
Medium HDL	−0.383‡	−0.497†	−0.089	−0.445†
Small HDL	−0.107	−0.157	0.134	−0.061
Very small HDL	−0.063	−0.047	0.292§	0.044
LDL size*	−0.116	−0.313§	−0.221	−0.379‡
HDL size*	−0.220	−0.277§	−0.081	−0.278§

Values are Pearson partial correlation coefficients. TC, HDL-C, and LDL-C are the values obtained by enzymatic method, precipitation method, and Friedewald equation.
*Average particle diameters (nm) obtained from LDL and HDL peak time by HPLC.
†$P<0.001$; ‡$P<0.01$; §$P<0.05$.

Discussion

It is well known that measurement of lipoprotein subclasses other than LDL-C and HDL-C is very important for prediction of risk for CHD. Recently, the Adult Treatment Panel (ATP)-III claimed that one component of atherogenic dyslipidemia is small LDL particles[4] but did not recommend a routine clinical measurement of small LDL particles because of lack of standard and inexpensive methodologies. We offer in this study another alternative method superior to NMR and other methods, giving cholesterol levels for all lipoprotein subclasses simultaneously. Size exclusion HPLC has been used for decades in lipoprotein research applications[23,30] but only recently has become sufficiently robust for consideration as a routine method.

Analytical precision of HPLC was demonstrated for the first time to be acceptable in the determination of 20 component peaks for 3 VLDL, 4 LDL, and 5 HDL subclasses as shown in Table 2, which is well comparable to major lipoprotein quantification reported previously.[25,30,31]

Our HPLC and the traditional methods gave a good agreement in LDL-C and HDL-C values ($r>0.97$) for 62 men in this study. Because Friedewald equation and precipitation methods were used for determination of LDL-C and HDL-C in large-scale epidemiological studies, our HPLC method could be used as an alternative technique in clinical studies. We already compared this method with ultracentrifugation, and very high correlations were obtained.[23] Each of the ultracentrifugally isolated fractions consisted of several sub-

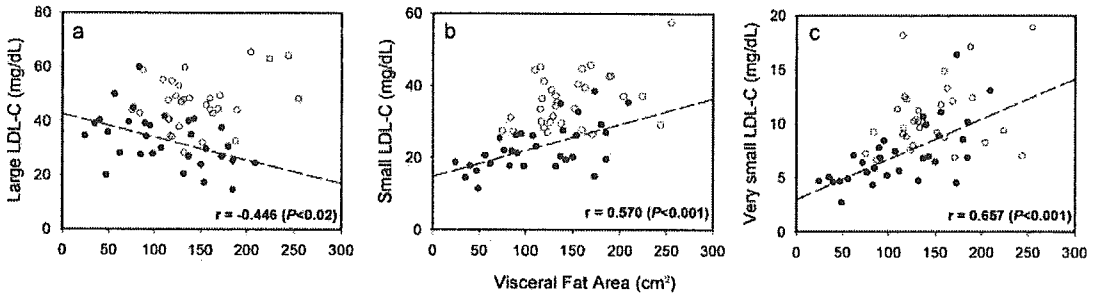

Figure 2. Scatter plots of VFA against (a) large LDL-C, (b) small LDL-C, and (c) very small LDL-C for high LDL-C group (○) and low LDL-C group (●). Dashed lines represent a linear regression for low LDL-C group. Correlation coefficients and *P*-values are also presented for low LDL-C group (n=31).

classes as follows: VLDL fraction, large VLDL (10% to 30%)+medium VLDL (45%)+small VLDL (10% to 25%); IDL fraction, small VLDL (40%)+large LDL (35%)+medium LDL (25%); LDL fraction, large LDL (10% to 30%)+medium LDL (35% to 40%)+small LDL (20% to 35%)+very small LDL (5% to 15%); HDL_2 fraction, very large HDL (5% to 10%)+large HDL (50%)+medium HDL (30% to 40%); and HDL_3 fraction, medium HDL (55%)+small HDL (35% to 40%).

LDL subclass profiles were compared between HPLC and an electrophoretic method using 3% polyacrylamide gels (Lipoprint LDL system)[16] on non–insulin-dependent diabetes mellitus patients (n=87), and LDL-score values determined by the Lipoprint system were positively correlated with small LDL-C (r=0.356, P<0.001) and very small LDL-C (r=0.604, P<0.0001), respectively (M. Okazaki et al, unpublished data, 2004).

Obesity is a major cause of atherosclerotic vascular disease in industrial countries. Obesity is a heterogeneous phenotype, and there is some confusion in the fat distribution literature regarding measurements and indices used to assess regional fat distribution. Subcutaneous skinfolds, skinfold ratio, circumferences, or circumference ratios have been used, and more recently CT has been used to distinguish between measurements of subcutaneous and visceral fat accumulation at any site of the body.[29] We examined the relationship of cholesterol profiles in major lipoproteins and their subclasses by HPLC to various clinical parameter in 62 men with a wide range of anthropometric values to cover a large spectrum of body fat variation (Table 1).

Recent advances in the biology of adipose tissue have revealed that adipose tissue is not simply an energy storage organ, but it also secretes a variety of molecules which affect the metabolism of the whole body.[32] It has been clarified that adipose tissue development and the extent of subsequent fat accumulation are closely associated with the occurrence of advancement of the metabolic syndrome. The presence of obesity increases the risk of thrombotic vascular diseases. Plasma PAI-1 levels were closely correlated with VFA but not with SFA in human subjects.[33] Moreover, visceral fat accumulation is well known to be associated with insulin resistance through the increase of serum free fatty acid followed by the increase of VLDL production by liver.[34,35] In the subjects of this study, there was a weak correlation between VFA and PAI-1 (r=0.261, P<0.05; data not shown) but a strong positive correlation between VFA and IRI (r=0.443, P<0.001; data not shown). As presented in Table 3, the degree of correlations of major lipoproteins and their subclasses with IRI and PAI-1 were different. The strong positive correlations of small and very small HDL with PAI-1 were observed, although positive correlations of small LDL and very small LDL and negative correlation of large HDL with IRI were obtained. Similar correlations were obtained between subclasses and UA, which is increased under overnutrition state in obesity.

Visceral obesity causes various metabolic abnormalities including the increase of serum TG, and there was a strong positive correlation between VFA and serum TG levels in this study (r=0.536, n=62, P<0.0001; data not shown). The increase of serum TG was the result of the increased VLDL as shown by very strong positive correlations of TG with total VLDL-C and all VLDL-C subclasses (Table 3). The increased TG in VLDL results in its flow into LDL and HDL by cholesterol ester transfer protein (in exchange with cholesterol ester), and TG is kept hydrolyzed in LDL and HDL,[36,37] which clearly were demonstrated in our data by the results of negative correlations of serum TG levels with LDL and HDL particle size and by the positive correlation with small and very small LDL-C and negative correlations with large and medium HDL-C. Among anthropometric values, VFA showed a strong positive correlation with total VLDL, all VLDL subclasses, total LDL, and LDL subclasses, except for large LDL, and a negative correlation with total HDL and large and medium HDL as presented in Table 3. These statistical correlations between lipoprotein subclasses and VFA may be the consequence of the increase of TG. But in this study, these significant correlations remained after adjustment for serum TG level (Table 4). Therefore, we did not think the increase of small LDL or decrease of HDL might simply be the consequence of high serum TG in this study. Moreover, the large VLDL and small VLDL were correlated positively and negatively with VFA, respectively, after adjustment for serum TG levels. In our study, the influence of TG increase in fasting state could be evaluated, but the contributions of TG increase in postprandial state to lipoprotein subclass distributions need to be examined in future studies.

As shown in Table 4, partial correlation analysis by controlling LDL-C showed a different contribution of visceral fat accumulation to the cholesterol levels in LDL subclasses. In this study, large LDL-C was negatively associated with visceral fat accumulation in low LDL-C groups as presented in Figure 2. Increased CHD risk associated with

reductions of HDL could be related to reciprocal increases of IDL or smaller, denser LDL, or related considerably to parallel reduction of a component within the larger LDL subclasses. Different contribution of visceral fat accumulation to large LDL-C level demonstrated only by a new subclass analysis with HPLC might reflect the difference of atherogenicity of LDL subclasses. Our new approach demonstrating the presence of unique LDL particles by component analysis will help to discover new lipoprotein particles contributing to atherosclerotic diseases.

Our HPLC method can provide the cholesterol levels of major lipoproteins and their subclasses in a small amount of whole serum or plasma (<10 μL) within 16 minutes; this technique might be very useful in a large-scale clinical study.

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