Component Analysis of HPLC Profiles of Unique Lipoprotein Subclass Cholesterols for Detection of Coronary Artery Disease

Mitsuyo Okazaki,1* Shinichi Usui,2 Akio Fukui,3 Isao Kubota,3 and Hitonobu Tomoike4

Background: Patients with coronary artery disease (CAD) are known to have several lipoprotein abnormalities. We examined plasma cholesterol concentrations of major lipoproteins and their subclasses, using a gel permeation HPLC, to establish an association between a lipoprotein subclass pattern and the presence of CAD.

Methods: We performed a simple and fully automated HPLC, followed by mathematical treatment on chromatograms, for measuring cholesterol concentrations of major lipoproteins and their subclasses in 62 male patients (45 with CAD and 17 controls without CAD) who underwent cardiac catheterization.

Results: For major lipoprotein classes, the patient group had a significantly (P < 0.05) higher LDL-cholesterol (LDL-C) and lower HDL-cholesterol (HDL-C), but no difference in VLDL-cholesterol (VLDL-C) concentrations. For lipoprotein subclasses, the patient group had a significantly higher small VLDL-C (mean particle diameter of 31.3 nm, P < 0.001), small LDL-C (23.0 nm, P < 0.05), and very small LDL-C (16.7–20.7 nm, P < 0.001), but a significantly lower large HDL-C (12.1 nm, P < 0.001) concentrations. Combined variables of “small VLDL-C / small LDL-C / very small LDL-C – large HDL-C” differentiated the patient from the control group more clearly than single-subclass measurements or calculated traditional lipid markers.

Conclusions: These results suggest the usefulness of multiple and simultaneous subclass analysis of proatherogenic and antiatherogenic lipoproteins and indicate that HPLC and its component analysis can be used for easy detection and evaluation of abnormal distribution of lipoprotein subclasses associated with CAD.

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Patients with coronary artery disease (CAD)5 have several lipoprotein abnormalities, such as increased triglyceride (TG) and LDL-cholesterol (LDL-C), and decreased HDL-cholesterol (HDL-C) (1, 2). In epidemiological studies, calculated non–HDL-C concentration [the difference of total cholesterol (TC) and HDL-C] was demonstrated to be a stronger predictor of cardiovascular events than plasma TC alone (3, 4). Partially catabolized TG-rich lipoproteins such as intermediate-density lipoproteins and remnant-like particles (RLP) are considered to be highly atherogenic (5). The atherogenic lipoprotein phenotype has been defined as the presence of a predominance of small, dense LDL particles and high TG and low HDL-C concentrations (6, 7). Prospective studies have reported the small LDL phenotype to be an important predictor of subsequent CAD (8, 9). Therefore, detailed analysis of both major lipoproteins and their subclasses, including remnant lipoproteins, might be required for more effective assessment of CAD risk status. Many techniques have been used for lipoprotein subclass analysis; analytical ultracentrifugation (10), sequential separation at various densities (11), rate zonal ultracentrifugation (12), and density-gradient ultracentrifugation (13). All these techniques are laborious and not available in routine clinical laboratories.

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6 Received March 12, 2006; accepted August 24, 2006.
7 Previously published online at DOI: 10.1373/clinchem.2006.070094

† Nonstandard abbreviations: CAD, coronary artery disease; TG, triglyceride; LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; TC, total cholesterol; RLP, remnant-like particle; MI, myocardial infarction; AUC, area under the curve.
Lipoprotein particle size analysis is usually performed with nondenaturing gradient gel electrophoresis (8, 9). Alternative techniques include a Lipoprint LDL system with a nongradient (3%) polyacrylamide gel electrophoretic method (14) and nuclear magnetic resonance (15–17). In addition, Hirano et al. (18) reported a new technique for small LDL-C measurement that combines selective precipitation and a homogeneous LDL-C assay. Techniques for remnant lipoprotein measurement include a homogeneous assay developed by Miyauchi et al. (19) and an immunoseparation method by Nakajima et al. (20). Detailed HDL subclass analysis has been performed with 2-dimensional gel electrophoresis (21, 22).

HPLC with gel permeation columns is an alternative method for classifying and quantifying lipoproteins on the basis of differences of particle size (23–25). Component analysis of cholesterol profiles after HPLC can provide useful information about almost all of the above-mentioned atherogenic lipoprotein subclasses (26, 27).

In the current study, we applied simple and fully automated HPLC, followed by mathematical treatment analysis of chromatograms, to identify lipoprotein subclass patterns associated with the presence of CAD in men who underwent cardiac catheterization.

Materials and Methods

STUDY PARTICIPANTS

The 62 study patients, [mean (SD) age 62 (9) years, range 41–76 years], were selected from 609 consecutive male patients who underwent cardiac catheterization in Yamagata University Hospital. We excluded patients who had diabetes mellitus, renal or liver disease, or were receiving any lipid-lowering medication. All participants gave their informed consent before entering the study. The study was carried out according to guidelines of the institutional review board.

The presence of CAD was assessed by coronary angiography. Narrowing of the luminal diameter of the coronary artery by ≥75% was considered significant. Coronary vasospasm was induced by a provocation test with ergonovine (2–20 μg), and only total or subtotal occlusion was considered positive. According to these assessments, the CAD patient group (n = 45) comprised 21 patients who were myocardial infarction (MI) survivors (acute MI, 3; MI within 1 month, 11; previous MI, 7), 22 patients with angina (effort angina, 7; unstable angina, 4; vasospastic angina, 11), and 2 patients with silent myocardial ischemia with considerable coronary stenosis. The non-CAD group (n = 17) comprised patients without substantial coronary stenosis (atypical chest pain, 4; cardiomyopathy, 6; aortic valve disease, 4; electrocardiogram abnormality, 3).

After patients had fasted overnight, venous blood was drawn and placed in tubes containing disodium EDTA (1 g/L). Plasma samples were kept in a refrigerator and analyzed within 7 days after blood collection.

HPLC METHOD

Plasma lipoproteins were analyzed by HPLC, as previously described (24, 27, 28). We defined 3 VLDL subclasses, 4 LDL subclasses, and 5 HDL subclasses according to lipoprotein particle size (diameter) from 20 component peaks [see Table 1. in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue11] determined on the basis of mean profiles of healthy and hyperlipidemic persons (27).

OTHER CLINICAL AND LIPID PARAMETER ANALYSIS

We used commercial enzymatic reagent sets (Kyowa Medex) to measure plasma TG. Fasting blood sugar was measured by the enzymatic method (Arkray Inc.). We used medical records and questionnaires to obtain data on age, weight, height, smoking history, family history, hypertension, previous MI, angina pectoris, diabetes mellitus, and medication use. Body mass index was calculated from weight and height as weight (kg) per [height (m)]².

STATISTICAL ANALYSIS

Data were analyzed with SPSS (Version 10.0, SPSS Inc.). Continuous measures are expressed as mean (SD), and judged by Student t-test. Dichotomous variables were tested by means of χ² analysis. Area under the curve (AUC) of ROC curve was calculated to describe the power of variables to distinguish the CAD patients from the control individuals. A value of P <0.05 was considered to be statistically different.

Results

Clinical characteristics, lipid, and major lipoprotein concentrations in 62 men in this study are shown in Table 1. The patient group had significantly (P <0.05) higher LDL-C and lower HDL-C, but there was no difference in VLDL-C concentrations.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients with CAD, n = 45</th>
<th>Control participants without CAD, n = 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>63.7 (8.7)</td>
<td>58.8 (9.1)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.6 (3.1)</td>
<td>22.4 (2.9)</td>
</tr>
<tr>
<td>FBS, mg/dL</td>
<td>98.6 (16.2)</td>
<td>93.8 (13.1)</td>
</tr>
<tr>
<td>Current smoker, %</td>
<td>23 (51.1%)</td>
<td>5 (29.4%)</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>21 (46.7%)</td>
<td>10 (58.8%)</td>
</tr>
<tr>
<td>Family history, %</td>
<td>14 (31.1%)</td>
<td>7 (41.1%)</td>
</tr>
<tr>
<td>Plasma TG, mg/dL</td>
<td>103.8 (51.4)</td>
<td>84.9 (48.9)</td>
</tr>
<tr>
<td>Plasma TC, mg/dL</td>
<td>193.1 (36.6)</td>
<td>175.8 (33.1)</td>
</tr>
<tr>
<td>VLDL-C, mg/dL</td>
<td>27.9 (11.2)</td>
<td>22.4 (12.7)</td>
</tr>
<tr>
<td>LDL-C, mg/dL</td>
<td>121.7 (29.6)</td>
<td>103.2 (24.9)</td>
</tr>
<tr>
<td>HDL-C, mg/dL</td>
<td>43.4 (10.0)</td>
<td>50.1 (10.0)</td>
</tr>
</tbody>
</table>

* Data represent mean (SD) or the number (%) of participants.

* BMI, body mass index; FBS, fasting blood sugar.

* P <0.05 (vs control participants).
The difference or the ratio of increased subclasses (Vs/Ls represented the sum of small LDL and very small LDL, as Vs/Ls) and a decreased subclass (large HDL) was expressed as a significant decrease in large HDL-C ($P < 0.001$). The patient group, were combined and expressed as Vs/Ls, which were significantly increased in the patient group, and Vs + Ls − Hs showed the highest significant differences ($P < 0.001$) in the 2 groups. All the traditional risk markers, non-HDL-C, TC/HDL-C, and LDL-C/HDL-C ratios, were significantly higher in the patient group.

On ROC analysis, AUCs for the derived HPLC variables and the calculated traditional risk markers were estimated to describe the power of the variables to distinguish between the 2 groups. AUCs and the range of 95% confidence intervals are presented in Table 2. From ROC analysis, the derived HPLC variable Vs + Ls − Hs produced the greatest AUC, indicating that this variable was a more powerful discriminator than any other derived variable, including traditional risk markers.

### Discussion

In this study, we compared for the first time lipoprotein cholesterol profiles between CAD patients and controls and propose a unique lipoprotein subclass pattern for identifying the patients at increased risk for CAD. HPLC has been used for decades in lipoprotein research applications and as a routine method (24, 25, 27), but the present study is a first report for clinical and diagnostic testing of lipoprotein subclass analysis by HPLC in CAD patients. Analytical precision of HPLC was previously demonstrated to be acceptable in the determination of 3 VLDL, 4 LDL, and 5 HDL subclasses, with CV values of 1%–4% (n = 5), results that were comparable to those obtained with major lipoprotein quantification (27, 28). Our HPLC and the traditional methods [Friedewald equation for LDL-C (29) and the precipitation method for calculated traditional risk markers, non-HDL-C, TC/HDL-C, and LDL-C/HDL-C ratios, were compared between the patient and the control groups. All derived HPLC variables were significantly higher in the patient group, and Vs + Ls − Hs showed the highest significant differences ($P < 0.001$) in the 2 groups. All the traditional risk markers, non-HDL-C, TC/HDL-C, and LDL-C/HDL-C ratios, were significantly higher in the patient group.

#### Table 2. Comparison of derived HPLC and traditional variables between the patients with CAD and the control participants without CAD.

<table>
<thead>
<tr>
<th>Derived variables$^a$</th>
<th>Patients with CAD, n = 45</th>
<th>Control participants without CAD, n = 17</th>
<th>AUC$^b$ n = 62</th>
<th>95% CI$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vs + Ls − Hs (mg/dL)</td>
<td>42.9 (16.7)$^d$</td>
<td>25.1 (16.8)</td>
<td>0.773$^d$</td>
<td>0.645−0.900</td>
</tr>
<tr>
<td>Vs + Ls (mg/dL)</td>
<td>51.3 (14.5)$^a$</td>
<td>39.6 (10.2)</td>
<td>0.749$^a$</td>
<td>0.620−0.878</td>
</tr>
<tr>
<td>Ls (mg/dL)</td>
<td>38.8 (13.4)$^a$</td>
<td>30.9 (7.9)</td>
<td>0.677$^f$</td>
<td>0.543−0.811</td>
</tr>
<tr>
<td>(Vs + Ls)/Hs</td>
<td>9.5 (6.9)$^a$</td>
<td>4.2 (3.4)</td>
<td>0.759$^b$</td>
<td>0.631−0.888</td>
</tr>
<tr>
<td>Ls/Hs</td>
<td>7.3 (5.7)$^a$</td>
<td>3.2 (2.6)</td>
<td>0.749$^f$</td>
<td>0.618−0.880</td>
</tr>
<tr>
<td>Traditional</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-HDL-C (mg/dL)</td>
<td>149.6 (34.1)$^f$</td>
<td>125.7 (34.7)</td>
<td>0.710$^f$</td>
<td>0.555−0.865</td>
</tr>
<tr>
<td>TC/HDL-C</td>
<td>4.6 (1.1)$^a$</td>
<td>3.7 (1.1)</td>
<td>0.745$^e$</td>
<td>0.596−0.894</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>2.9 (0.8)$^a$</td>
<td>2.2 (0.8)</td>
<td>0.748$^b$</td>
<td>0.592−0.903</td>
</tr>
</tbody>
</table>

$^a$ Vs + Ls − Hs, small VLDL-C + small LDL-C + very small LDL-C − large HDL-C; Vs + Ls, small VLDL-C + small LDL-C + very small LDL-C; Ls, small LDL-C + very small LDL-C; (Vs + Ls)/Hs, (small VLDL-C + small LDL-C + very small LDL-C)/large HDL-C ratio; Ls/Hs, (small LDL-C + very small LDL-C)/large HDL-C ratio; non-HDL-C, the difference of TC and HDL-C.

$^b$ The area under the ROC curve (AUC) discriminating the patients with CAD from the control participants without CAD.

$^c$ CI, confidence interval.

$^d$ $P < 0.001$, $e$ $P < 0.01$, $f$ $P < 0.05$. 

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Fig. 1. Comparison of cholesterol concentrations in lipoprotein subclasses between the CAD patients (n = 45) and the control participants (n = 17).

The values are shown as mean (SD). Closed and open columns represent the CAD patient and the control groups, respectively.

In Table 2, these derived variables by HPLC and the analytically precision of HPLC was previously demonstrated to be acceptable in the determination of 3 VLDL, 4 LDL, and 5 HDL subclasses, with CV values of 1%–4% (n = 5), results that were comparable to those obtained with major lipoprotein quantification (27, 28).
HDL-C (30) were in agreement for LDL-C and HDL-C values (r > 0.97), as described previously (27).

As presented in Tables 1 and 2, significantly higher LDL-C and lower HDL-C concentrations (P < 0.05) were observed in the patient group, and these significant differences were more clearly differentiated by computed values of TC/HDL-C or LDL-C/HDL-C (P < 0.01). Non-HDL-C has been recently recognized as one of the calculated risk markers for CAD (3, 4) but was less significant (P < 0.05) in this study (Table 2).

In the subclass analysis by HPLC (Fig. 1), small VLDL-C, small LDL-C, very small LDL-C, and large HDL-C were found to be significantly different between the 2 groups. Increased small VLDL-C in the CAD group might represent an increase in remnant lipoproteins, although neither intermediate-density lipoprotein-C nor RLP-C was measured in this study. Our previous study showed that RLP fractions isolated by an immunoaffinity separation were very heterogeneous (31), but the particle size of RLP from Type III hyperlipidemia corresponded mainly to small VLDL (peak 7). In another previous study, all VLDL subclasses were positively correlated with visceral fat area, and small VLDL remained considerable after adjustment for serum TG concentration (27).

In conjunction with these previous studies, the increase of small VLDL-C in CAD patients also supports the concept that smaller, partially catabolized triglyceride-rich lipoprotein (VLDL remnants) and/or a part of intermediate-density lipoprotein are atherogenic.

Although HDL subclasses are also heterogeneous and their atherogenic properties differ between subclasses (32), many investigators suggest that measuring HDL subclasses may provide additional information about risk for the development of CAD. In this study, AUC for large HDL was larger than total HDL-C (results not shown), indicating the potential usefulness of HDL subclass analysis.

Increased small LDL-C and very small LDL-C in the CAD patients were consistent with atherogenic profiles of increasing remnant lipoproteins as well as small, dense LDL reported by Cohn et al. (33, 34), but their ability to differentiate between the CAD patients and the controls was not as strong when compared with small VLDL-C, judged by AUC (results not shown).

To more clearly differentiate between the 2 groups, several derived variables were calculated from each subclass, as shown in Table 2. AUC for all derived HPLC variables except for Ls (small LDL + very small LDL) was larger than that for traditional risk marker, and Vs + Ls − Hs produced the largest AUC. These observations indicate that the new parameter, Vs + Ls − Hs, might be useful for total interpretation of both proatherogenic and antiatherogenic lipoproteins and provide additional clinical information to evaluate the risk status for CAD.

In conclusion, component analysis after HPLC provided the cholesterol concentrations of major lipoproteins and their subclasses within 16 min with a small volume of plasma or serum (<10 μL). Our results support the general concept of the usefulness of lipoprotein subclass analysis for diagnostic testing. Larger clinical trials are needed to establish the diagnostic significance of our proposed parameter, Vs + Ls − Hs, for identifying the patients at increased risk for CAD.

We gratefully acknowledge Kyowa Medex, Japan for providing enzyme reagents for the cholesterol measurement by HPLC. We greatly thank Skylight Biotech Inc. (Akita, Japan) for technical assistance in this study.

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