An inverse relationship between plasma levels of high density lipoprotein cholesterol (HDL-C) and the risk for coronary artery disease (CAD) has been well established (1,2). Direct evidence for the antiatherogenic effects of HDL has recently been obtained in studies of the over- or under-expression of apo A-I using genetic animal models of reverse cholesterol transport (3–7). HDL particles differ in size, structure and function (8). Both HDL₂ and HDL₃ levels are reduced in patients with CAD (9,10). However, although some studies have reported altered relative proportions of the HDL₂ and HDL₃ subclasses, the functional aspects of HDL did not attract much attention until Dobiasova and Frohlich (8) established a functional assessment of HDL: the fractional esterification rate in low density lipoprotein (LDL)- and very low density (VLDL)-depleted plasma (FER₇₅). They indicated that FER₇₅ is a functional test of HDL particle interaction (8), and suggested that angiographically proven CAD subjects had higher FER₇₅ values than controls (11).

A major hypothesis for explaining the antiatherogenic properties of HDL involves the role of HDL in reverse cholesterol transport (RCT) (12). Reverse cholesterol transport is a multistep process that results in the net movement of cholesterol from peripheral tissues back to the liver via the plasma compartment (3). The efflux of cholesterol from the plasma membrane of peripheral cells to HDL is the first step in the RCT pathway (13). Promotion of this step may be antiatherogenic because it reduces the possibility of the overaccumulation of cellular cholesterol, and this hypothesis has been supported by studies using genetic animal models of RCT (4–6). Two kinds of nascent HDL particles are believed to be secreted...
the HDL 3 also be released from HDL 2 to produce pre-
other lipid parameters.
controlling for age, gender, conventional risk factors and
from 1994 to 1996. This study was approved by the ethics
known coronary atherosclerosis or for other reasons (mostly
diagnostic coronary angiography (CAG) for suspected or
Patients.
This study included 259 patients who underwent
METHODS
3
HDL cycle and pre-
1 HDL cycle are
b
1 HDL (22).
(14) that migrate with pre-β mobility on agarose gel electrophoresis
(16). These two subfractions of HDL remove cellular free
cholesterol by distinct mechanisms (17–19): diffusion-based or
receptor-dependent (15).

Cholesterol ester that has accumulated in HDL may then transfer from HDL to apolipoprotein (apo)-B containing lipoproteins (LDL and VLDL) in exchange for triglycerides (TG) as a result of the activity of cholesterol ester transfer protein (CETP) with subsequent uptake of TG-rich lipoprotein remnants by the liver. In humans, this second step of RCT is illustrated by the dramatic accumulation of HDL in subjects with CETP deficiency (20,21). TG-rich HDL2 particles are subjected to hydrolysis by hepatic lipase and perhaps lipoprotein lipase (14) and are converted back to a small HDL3-like particle. Apolipoprotein (Apo) A-I can also be released from HDL2 to produce pre-β1 HDL (22). Thus, the HDL3 → HDL2 → HDL2 cycle and pre-β1 HDL → HDL3 → HDL2 → pre-β1 HDL cycle are completed. Promotion of this second step of RCT is probably proatherogenic because CETP transfers cholesterol ester from “good” or “safe” lipoprotein HDL to atherogenic apo-B containing LDL and VLDL, thereby promoting cholesterol ester deposition (22). Therefore, we propose that both the quantity of HDL, as measured by HDL-C and the function of HDL in RCT, as quantitatively measured by FERHDL, play important roles in proatherogenic properties of HDL.

In this case-control study, we tested the association of FERHDL with CAD and its interaction with HDL-C, after controlling for age, gender, conventional risk factors and other lipid parameters.

METHODS

Patients. This study included 259 patients who underwent diagnostic coronary angiography (CAG) for suspected or known coronary atherosclerosis or for other reasons (mostly atypical chest pain) at the Fukuoka University Hospital from 1994 to 1996. This study was approved by the ethics committee of Fukuoka University Hospital, and informed consent was obtained from each patient. Controls (CAD− patients) were defined as those with less than 25% luminal narrowing, and cases (CAD+ patients) were those who had one, two or three stenosed (>50% luminal narrowing) epicardial coronary arteries. Patients with luminal narrowing of between 25% and 50% were excluded. Patients with spastic angina pectoris, i.e., acetylcholine-positive, were excluded from the controls and none of the controls had myocardial infarction (MI). Patients with acute MI (within three weeks after onset), heart failure (Killip Class ≥2 after myocardial infarction), vascular disease (aortitis treated by prednisolone), hepatic dysfunction (virus and nonvirus, transaminases more than three times the normal value) or uncontrollable diabetes mellitus were excluded from the study. Patients with systolic or diastolic blood pressure >160 mm Hg or 95 mm Hg or who were under anti-hypertensive treatment were considered to have hypertension (HT). Patients under treatment for diabetes mellitus (DM) and/or with symptoms of DM and a fasting glucose concentration ≥126 mg/dL were considered to have DM. Otherwise, the results of a 75 gm glucose tolerance test were used to give a diagnosis of DM. About 98% of the women were in menopause but none were receiving hormone replacement therapy. None of the patients were being treated with lipid-lowering agents at the time of sampling.

Coronary angiography. Coronary arteries were cannulated by the Judkins technique (23) with 5F catheters, and recorded on Kodak 35 mm cinefilm at a rate of 25 frames/s. Coronary arteries were divided into 15 segments, according to the classification of the American Heart Association Grading Committee. In this study, reliable and reproducible measurements were obtained. Coronary artery segments were carefully selected by two expert cardiologists on the basis of smooth luminal borders and the absence of stenotic changes. The presence of stenosis was determined using a computer-assisted coronary angiography analysis system (Mipron 1; Kontron Co., Tokyo, Japan) after the direct intracoronary injection of isosorbide dinitrate (ISDN) (2.5 mg/5 mL solution), as described previously (24). Arterial stenosis, that produced more than 50% luminal narrowing, was considered significant.

Determination of serum lipids, lipoproteins, and apolipoproteins. Blood was drawn in the morning after an overnight fast. Serum total cholesterol (TC) and triglyceride (TG) concentrations were determined enzymatically. HDL-C was determined by the heparin Ca2+ precipitation method (25). HDL fraction (HDL2 and HDL3) were separated by standard sequential preparative ultracentrifugation techniques (26). Apo A-I, apo A-II, apo B, apo C-II, apo C-III and apo E were determined by the turbidity immunoassay method (27). Serum lipoprotein (a) (Lp[a]) levels were measured by an enzyme-linked immunosorbent assay using Tint Eliza Lp(a) (Biopool Co., Stockholm, Sweden) (28). For all measurements in our laboratory, the
coefficients of interassay and intraassay variation were less than 5.0%, and blinded quality-control specimens were included in each assay.

**Assay for FER<sub>HDL</sub> in plasma.** VLDL-LDL-depleted plasma was prepared by precipitating apolipoprotein B-containing lipoproteins with phosphotungstate-MgCl<sub>2</sub> (11). FER<sub>HDL</sub> was determined according to the method of Ohta et al. (29) with minor modifications. [3H] Free cholesterol (FC) was incorporated onto polystyrene tissue-culture wells (Corning, New York, New York) as follows: absolute ethanol (100 μL) containing 1 μCi of [3H] FC was placed in wells and dried off by flushing with nitrogen. Next, 100 μL of the VLDL- and LDL-depleted plasma samples, in 400 μL of PBS was added to each well and [3H] FC was equilibrated with the FC in each sample by incubation at 4° C for 16 h. [3H] FC-labeled VLDL- and LDL-depleted plasma samples were incubated in a shaking water bath for 3 h at 37° C. 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 was then dissolved in 60 μL of isopropanol. Aliquots (20 μL) of lipid extracts were spotted in duplicate on a thin-layer chromatography plate (Merck, West Point, Pennsylvania) and developed in n-hexane/diethyl ether/acetic acid/methanol (85:20:1:1, v/v). Spots corresponding to FC and cholesteryl ester (CE) were cut from the plate and the radioactivities were determined. The increase in [3H] CE was expressed as the difference between the percentage of radioactive cholesterol esterified before and after incubation at 37° C. The samples were measured in triplicate and the coefficient of variation of the assay was 0.75%. The coefficient of variation for the interassay variability was 6.2%.

**Statistical analysis.** Statistical analysis was performed using the SAS Software Package (Version 6, Statistical Analysis System, SAS Institute Inc., Cary, North Carolina). Categorical variables (such as gender) were compared between cases and controls by a chi-square analysis. Differences between cases and controls or among patients with 1-, 2-, and 3-vessel diseases were examined by an analysis of variance (ANOVA). Comparisons of 1-vessel and 2- or 3-vessel disease patients were performed with the multiple comparison test of Dunnett (30). Age and gender were adjusted for by an analysis of covariance (ANCOVA) (30). The logistic model was used to evaluate linear associations between CAD and lipid variables (continuous). In addition, odds ratios were simultaneously adjusted for age, gender and potentially confounding variables by a multiple logistic regression analysis (30). For all of the odds ratios, we calculated 95% confidence intervals (CI). For logistic regression coefficients, we show either 95% CI or the standard error. A multiple regression analysis was used to test the correlation between HDL-C or FER<sub>HDL</sub> and lipid variables while controlling for age, gender and other lipid parameters (30). Because some variables were not normally distributed, we used rank scores in the regression analysis to simplify the calculation (31). All p values are two-tailed. The significance level was considered to be 5% unless otherwise indicated.

**RESULTS**

Table 1 shows the patient characteristics. There were significantly more men (especially in patients younger than 63 years) and smokers among CAD+ patients than among CAD− patients. On the other hand, the prevalence of diabetes mellitus and hypertension was not significantly different between the two groups. The prevalences of smokers and diabetes in both CAD+ and CAD− patients were both higher than those seen in the United States (32).

In Table 2 serum lipids, lipoproteins, apolipoproteins, FER<sub>HDL</sub> and HDL-FC were compared between CAD+ and CAD− patients after controlling for age and gender. Serum levels of HDL-C, HDL<sub>2</sub>-C, HDL<sub>3</sub>-C, apo A-I, apo A-II, apo E and HDL-FC were significantly lower, and serum LP(a) levels and FER<sub>HDL</sub> values were significantly higher in CAD+ patients than in CAD− patients. However, these variables (except for HDL<sub>2</sub>-C levels) were not associated with the extent of stenosis as judged by the number of vessels affected (data not tabulated). As shown in Table 2, serum levels of total cholesterol in CAD+ patients were much lower than those in equivalent American patients with proven atherosclerotic disease but similar to those reported by another Japanese study group (33). The levels of LDL and HDL-C in CAD+ patients were almost the same as those in the Cholesterol and Recurrent Events (CARE) Study (34).

Table 3 shows the results of the test for the linearity of the associations of CAD−related variables (continuous) after controlling for age and gender, and with (indicated by †)
and without also adjusting for smoking, hypertension, diabetes and body mass index. For each variable shown in Table 3, the association with CAD was significantly linear: The relative risk of CAD (odds ratio) decreased with increasing serum levels of HDL-C, HDL3-C, apo A-I, apo A-II, apo E, and HDL-FC and with decreasing serum Lp(a) levels and FERHDL values. After adjusting for conventional risk factors (Table 3, right columns), the association of HDL-C, apo A-I, apo E, Lp(a), and FERHDL with CAD remained significant, suggesting that these variables are independently associated with CAD.

Table 3. Age- and Gender-Adjusted Linear Trends Across Fractional Ranks of Coronary Atherosclerosis-related Variables, as Tested by a Multiple Logistic Regression Analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression Coefficient (Standard Error)</th>
<th>Wald chi-square</th>
<th>(p value)</th>
<th>Regression Coefficient (Standard Error)</th>
<th>Wald chi-square</th>
<th>(p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjusted for Age and Gender</td>
<td></td>
<td></td>
<td></td>
<td>Adjusted for All†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL-C</td>
<td>−2.5 (0.57)</td>
<td>19.3</td>
<td>(&lt; 0.001)</td>
<td>−0.45 (0.12)</td>
<td>13.7</td>
<td>(&lt; 0.001)</td>
</tr>
<tr>
<td>HDL2-C</td>
<td>−2.19 (0.63)</td>
<td>12.1</td>
<td>(&lt; 0.001)</td>
<td>−0.11 (0.09)</td>
<td>1.18</td>
<td>(0.28)</td>
</tr>
<tr>
<td>HDL3-C</td>
<td>−2.15 (0.62)</td>
<td>12.2</td>
<td>(&lt; 0.001)</td>
<td>−0.14 (0.09)</td>
<td>2.03</td>
<td>(0.15)</td>
</tr>
<tr>
<td>Apo A-I</td>
<td>−2.52 (0.57)</td>
<td>19.4</td>
<td>(&lt; 0.001)</td>
<td>−0.44 (0.11)</td>
<td>14.4</td>
<td>(&lt; 0.001)</td>
</tr>
<tr>
<td>Apo A-II</td>
<td>−1.24 (0.52)</td>
<td>5.66</td>
<td>(0.017)</td>
<td>−0.2 (0.10)</td>
<td>3.45</td>
<td>(0.06)</td>
</tr>
<tr>
<td>Apo E</td>
<td>−1.3 (0.51)</td>
<td>5.78</td>
<td>(0.008)</td>
<td>−0.31 (0.11)</td>
<td>7.23</td>
<td>(0.007)</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>1.3 (0.51)</td>
<td>6.44</td>
<td>(0.011)</td>
<td>0.28 (0.10)</td>
<td>7.06</td>
<td>(0.008)</td>
</tr>
<tr>
<td>FERHDL</td>
<td>1.38 (0.64)</td>
<td>4.5</td>
<td>(0.033)</td>
<td>0.3 (0.11)</td>
<td>7.14</td>
<td>(0.008)</td>
</tr>
<tr>
<td>HDL-FC</td>
<td>−2.99 (0.74)</td>
<td>16.2</td>
<td>(&lt; 0.001)</td>
<td>−0.13 (0.09)</td>
<td>1.77</td>
<td>(0.18)</td>
</tr>
</tbody>
</table>

Fractional ranks rather than original values are used for the regression analysis and were calculated by dividing the rank by the number of observations (31). †Adjusted for age, gender, smoking, hypertension, diabetes and body mass index.

HDL-C = high density lipoprotein cholesterol; Apo = apolipoprotein; Lp(a) = lipoprotein (a); FERHDL = fractional esterification rate in the HDL fraction of plasma; HDL-FC = free cholesterol content in HDL.

Table 4 shows the influence of HDL-C on the association between FERHDL and CAD and the effect of interaction between FERHDL and HDL-C. The association of FERHDL with CAD varied with the serum level of HDL-C: FERHDL was significantly associated with CAD in the low tertile of HDL-C (Wald χ² = 6.20, p = 0.01) but not in the middle and high tertiles (Wald χ² = 0.08 and 0.03, n.s.). Figure 1 shows a plot of the prevalence of CAD, FERHDL (in tertiles), and HDL-C (in tertiles). These results show that the association of FERHDL with CAD was modified by HDL levels. It is
also possible that some other factor(s) mediate the relationship of both HDL-C and FER HDL with coronary atherosclerosis.

We tested the relationships between FER HDL and lipid parameters in patients with and without CAD by a multiple regression analysis after controlling for age and gender. As shown in Table 5, the age- and gender-adjusted parameter estimates were significant between FERHDL and HDL-C, HDL2-C, apo A-II, apo B, apo C-III, TG, and HDL-FC in both groups, and between FERHDL and apo A-I, apo C-II, apo E, TC, and LDL-C in the CAD+ patients.

The dependence of the association of FER HDL with CAD on HDL-C was tested by a multiple logistic regression analysis (Table 6, upper panel). As shown in Models 1 and 2, HDL-C (in tertiles) and FERHDL (in tertiles) were significantly and linearly associated with CAD after controlling for age and gender. When FERHDL was added to Model 1 (Model 3), the fit was improved, as judged by the model fitting criterion, $-2 \log \text{Likelihood}$ (Table 6). Addition of the HDL-C-by-FERHDL interaction term to Model 3 (Model 4) further improved the fit, with the association of FERHDL. These results suggest that the association of FER HDL with CAD is independent of HDL-C levels. Adding smoking, HT and DM to Model 4 (Model 5) slightly improved the model fit, and adding Lp(a), TG, apo E and apo C-II to Model 5 (Model 6) further improved the fit.

Table 6 (lower panel) also shows the odds ratio for each combination of FERHDL (two levels) and HDL-C (two levels) after controlling for age and gender. As shown, patients with low FERHDL-low HDL-C had a significantly higher relative risk than patients with low FERHDL-high HDL-C, and patients with high FERHDL-low HDL-C had the highest relative risk. Patients with high-FERHDL-low HDL-C also had the highest prevalence of CAD (Fig. 1). These results suggest that FERHDL modified the risk associated with HDL levels, and when HDL-C was low, high FERHDL values further increased the risk of CAD.

**DISCUSSION**

In the present study, we investigated the relationship between the function of HDL as quantitatively measured by FER HDL and coronary atherosclerosis.
FERHDL and CAD and its interaction with the quantity of HDL as measured by serum levels of HDL-C. Our findings seem to support the most recent hypothesis of reverse cholesterol transport and the atherogenic remnant hypothesis which are beginning to be seen as a single concept (12). Figure 2 shows a schematic view of this model for HDL metabolism. We propose that the process by which cholesterol moves from the periphery to apo B-containing particles consists of two steps: the first step is antiatherogenic, as measured by HDL-C levels, and the second is proatherogenic, as measured by FERHDL. The antiatherogenic role that HDL plays in the first step of RCT has been well-established, and direct evidence has been obtained in studies of the over- or under-expression of apo A-I using genetic animal models of reverse cholesterol transport (3–7). Our finding that HDL-C levels are linearly and inversely related to HDL-C and that the association of FERHDL with CAD is modified by HDL levels. The finding is supported by several facts that suggest that increased CETP activity may be atherogenic and reduced activity may protect against the development of atherosclerosis. Mice that lack CETP are resistant to the deleterious effects of high-cholesterol diets, and humans and rabbits with CETP are susceptible to hypercholesterolemia and atherosclerosis when fed diets high in fat and cholesterol. Elevated levels of CETP in transgenic mice containing simian CETP result in the formation of atherosclerotic lesions (36). Increased CETP activity is associated with acceleration of the rate of atherosclerotic plaque formation in human dyslipidemias such as dysbetalipoproteinemia (37), familial hypercholesterolemia (38) and hypercholesterolemia (39).

HDL is remodeled during the process of RCT, with changes in structure and size (40), which are the most important factors in determining the rate of LCAT-catalyzed cholesterol esterification (8) and CETP-mediated transport of cholesterol ester (40). This is reflected in our findings that FERHDL is inversely related to HDL-C and that the association of FERHDL with CAD is modified by HDL levels. The net flux of cellular cholesterol to HDL particles is generated by a gradient of the cholesterol content between cells and the lipoprotein surface and provided by an LCAT reaction (41). Because the size of circulating HDL particles as reflected by FERHDL (8) depends on the balance between the opposing processes of cholesterol acceptance (which increases particle size) and lipolytic digestion (which reduces it), we can speculate.

![Figure 2](image-url)

**Figure 2.** Model for HDL metabolism in the two-steps reverse cholesterol transport process. The first step is the efflux of cellular free cholesterol to HDL, which is an antiatherogenic step. The second step is the transfer of cholesterol ester from HDL to apo B-containing lipoproteins (LDL and VLDL) via CETP, which may be proatherogenic and is what FERHDL measures.
that an increased FER\textsubscript{HDL} value reflects a detrimental condition that facilitates accumulation of intracellular cholesterol via LDL. This is confirmed by our finding that increased FER\textsubscript{HDL} is associated with an increased prevalence of CAD. However, a sufficient number of HDL particles may overcome this condition by retaining cholesterol esters in the HDL fraction. This is reflected in our finding that when HDL-C levels were high, increased FER\textsubscript{HDL} did not significantly increase the risk of CAD (Table 4, Fig. 1). Because low HDL-C levels also tended to be linked to a reduction in the expression of lipoprotein lipase and a rise in hepatic lipase, both of which are enzymatic changes that would lead to a decrease in triglyceride-rich particle lipolysis (12), increased FER\textsubscript{HDL} may cause an increased accumulation of cholesteryl ester-rich remnants of VLDL and chylomicra under conditions of low HDL-C and then confer an increased risk of CAD. This is reflected in our findings that patients with both low HDL-C and high FER\textsubscript{HDL} had the highest risk of CAD (Table 6, Fig. 1) and that the combination of both FER\textsubscript{HDL} and HDL is more strongly related to CAD than is either measure alone (Table 6).

Our results show that low HDL is a better indicator of CAD than high FER\textsubscript{HDL}, as is indicated by a better model fit for HDL than FER\textsubscript{HDL} (−2 log likelihood: 21.31 vs. 16.35, Table 6) and as is apparent in Figure 1. This may be attributed to other functions of HDL particles, e.g., the antioxidant properties of HDL (which inhibits the oxidation of LDL particles) (42) and the ability of HDL particles to inhibit the expression of adhesion molecules on the surface of endothelial cells (43).

Limitations. In this angiographic case-control study, we demonstrated that FER\textsubscript{HDL} is independently associated with CAD and this association was modified by HDL-C levels. However, whether or not FER\textsubscript{HDL} plays a causal role is unclear and cannot be determined from a case-control study. In this case-control study, cases were not matched with controls with regard to the number of patients or gender (Table 1). Although when the cost of sampling for cases and controls is equal and the relation between disease and exposure is not known beforehand, matching 1 case with 1 control can minimize the variance of the estimated odds ratio (44). We did not obtain a suitable number of controls due to limited funds. Differences in the gender ratio between cases and controls may have caused biased estimates of the odds ratio, since HDL-C levels were different (p < 0.05) between males and females among the controls (43.2 ± 2.0 mg/dL vs. 51.1 ± 2.6 mg/dL) and in all of the patients together (38.4 ± 0.90 mg/dL vs. 44.5 ± 1.5 mg/dL) after controlling for age. Although we tried to avoid this possible bias by adjusting for gender in the logistic regression analysis and found no gender interaction (group \* gender: F value = 1.86, p = 0.17; HDL-C \* gender: χ² = 0.01, p = 0.99), the variance of the estimated odds ratio may not be as low as that in a matched study.

We selected angiographically defined normal subjects as controls. However, a selection bias is known to exist: angiographically defined normal subjects generally have more risk factors for coronary disease than patients with clinical symptoms but who have not been selected for angiography, because a person with both a chest pain and a known risk factor, such as smoking, may be more likely to be referred for angiography than a person with just a clinical symptom (45). The lack of significance for the fairly substantial difference in the prevalence of diabetes between cases and controls and the lack of an association between FER\textsubscript{HDL} and the severity of coronary disease as judged by the number of involved vessels (Table 2) in the present study may be due to this bias. Previous angiographic studies such as the Coronary Artery Surgery Study have also failed to show associations between classic lipid risk factors and the severity of disease, presumably due to the biases involved in selecting patients for angiography. The controls were defined as having less than 25% luminal narrowing by conventional coronary angiography. However, since even mild coronary atherosclerotic lesions in the 25% range can result in significant acute events when based on unstable plaque (46), our controls are not absolute controls in the sense of having no significant coronary atherosclerosis or its eventual sequel. Thus, limitations regarding the controls may have limited the power of this study.

Conclusions. FER\textsubscript{HDL}, a quantitative measure of the HDL function, when combined with serum HDL-C levels, is a new epidemiological marker for the risk of CAD that is superior to HDL-C levels alone. Since FER\textsubscript{HDL} values are fairly constant, this test may be of great value in clinical screening. The clinical significance of this finding needs to be demonstrated in a prospective trial.

Reprint requests and correspondence: Keijiro Saku, Department of Internal Medicine, Fukuoka University School of Medicine, 7-45-1 Nanakuma Jonan-ku, Fukuoka 814-80, Japan. E-mail: hls035399@msat.fukuoka-u.ac.jp.

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