The negative correlation between plasma high density lipoprotein (HDL) and the risk of development of coronary heart disease (CHD) was observed as early as 1951 by Russ et al. They noted that women during their reproductive years, when their susceptibility to CHD is lower than that of men, have higher levels of alpha lipoprotein which is equivalent to HDL. Many epidemiological studies have since established the negative association between plasma HDL levels and CHD risk, so that it is not uncommon to include a HDL estimate in an individual’s lipid profile. Furthermore, the severity of CHD defined by coronary angiography was found to have a strong relation to the level of an HDL subtraction, HDL₂. Therefore, as well as being able to indicate the presence and severity of existing CHD, measurement of plasma HDL or its subfractions has been demonstrated to be a powerful predictor of CHD risk.

The power of the HDL level as a negative predictor is even greater than the plasma low density lipoprotein (LDL) level as a positive predictor. In men and women, the negative correlation between HDL levels and CHD holds at any given level of LDL and is therefore an independent predictor. In the Framingham Study it was found that, for men and woman ages 49 to 82 years, the major lipid risk factor for CHD was a low HDL level. At these ages a weaker association with the incidence of CHD was observed for LDL cholesterol, and total plasma cholesterol was not associated with CHD. In the Tromso Heart Study it was found that in men ages 20 to 49 years, the dependence of coronary risk on HDL cholesterol was threefold greater than that on the density <1.063 cholesterol concentration.

Recent advances in apolipoprotein (apo) quantification have led to numerous case-controlled studies providing consistent evidence that plasma apo A-I and apo B levels are useful in discriminating between healthy subjects and CHD patients and could therefore be predictors of CHD risk. As with any new potential risk factors for CHD, these apoproteins require long-term, population-based, epidemiological studies to evaluate their predictive capacity.

This work is a study of HDL cholesterol (HDL-c) and the seldom assayed HDL free cholesterol (HDL-fc) and HDL choline-containing phospholipids (HDL-pc) in healthy controls and in patients with CHD.

Methods

Subjects

Blood specimens were obtained from 50 consecutive male hospital inpatients scheduled for coronary artery bypass surgery for CHD. Their mean age was 59 years (range, 35 to 74; SD = 9.4). Control blood specimens were also obtained from 50 consecutive male blood transfusion donors who had no history of CHD. Their mean age was 58 years (range 36 to 84, SD = 10.9). Blood for the determination of the effect of plasma storage at 4°C on HDL-fc was obtained from healthy male and female hospital staff volunteers.

Informed consent was given by all subjects in accordance with institutional guidelines and, in the majority of cases, only surplus plasma from routine procedures was used.

Analytical Techniques

Plasma or serum was treated with equal volumes of 20% (wt/vol) buffered polyethylene glycol (PEG) to precipitate LDL and VLDL. This method of producing HDL plasma supernatant correlates well with ultracentrifugation and other precipitation techniques such as heparin/manganes. HDL-c, HDL-fc, and HDL-pc were quantitated...
enzymically by modifications of published methods. All analyses were performed on a Roche Cobas Bio centrifugal analyzer with good precision and all analytical methods satisfied the criterion that the analytical variance should be less than a quarter of the population variance. The plasma HDL esterified cholesterol level (HDL-ec) was calculated by subtracting HDL-fc from the HDL-c level. Commercial quality control sera were used to assess the interassay precision for each analytical method. Coefficients of variation (CV) ranged from 2.42% to 8.74%.

All plasma specimens obtained from subjects were prepared with lithium heparin anticoagulant and were stored at 4°C. Analysis of all specimens was completed within 10 days after venepuncture on stored refrigerated plasma. The control bloods were analyzed on Day 4 after venepuncture. The majority of the CHD plasma specimens (35) were analyzed on Day 4, as were the control group; the rest were analyzed within 10 days of venepuncture.

The effect of storage on plasma HDL-fc levels at 4°C was determined by analyzing the blood of male and female controls and male hospital inpatients scheduled for coronary artery bypass surgery over a period of 10 days. Blood from five males, females, and CHD patients of similar age to the CHD and control study groups was chilled in ice-water promptly after venepuncture and centrifuged at 4°C to obtain plasma which was then separated and immediately analyzed for HDL-fc. The remaining plasma of each individual subject was stored at 4°C and re-analyzed daily for 10 days. The within-group plasma HDL-fc mean for each daily analysis was used to construct Figure 1.

**Statistics**

The Mann-Whitney U test (M-W) was carried out on HDL-c, HDL-ec, HDL-pc, and HDL-fc data to determine the significance between the two groups. Significance between the study groups was tested for each HDL parameter using a z value derived from the U score, and the standard deviation of its sampling distribution was corrected for tied values.

Stepwise discriminant function analysis (DFA) was performed using the logarithms of the individual plasma lipid variables of the control and CHD males. Wilk's lambda was used to test the degree of separation produced by the discriminant function. SPSS, a computerized statistical software package, was used to process the data.

**Results**

**HDL Total Cholesterol**

The mean (± SD) plasma HDL-c level (Table 1) in healthy men was calculated to be 41.2 (± 13.9) and in

<table>
<thead>
<tr>
<th>Group</th>
<th>HDL-c</th>
<th>HDL-ec</th>
<th>HDL-fc</th>
<th>HDL-pc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control males (n = 50)</td>
<td>41.2 ± 13.9</td>
<td>34.6 ± 11.6</td>
<td>6.6 ± 2.4</td>
<td>84.0 ± 21.8</td>
</tr>
<tr>
<td>CHD males (n = 50)</td>
<td>39.1 ± 9.8</td>
<td>34.7 ± 8.6</td>
<td>4.4 ± 1.5</td>
<td>78.7 ± 15.4</td>
</tr>
</tbody>
</table>

| Significance | p = 0.730 | p = 0.497 | p < 0.001 | p = 0.216 |

HDL-c, HDL cholesterol; HDL-ec, HDL esterified cholesterol; HDL-fc, HDL free cholesterol; HDL-pc, HDL choline-containing phospholipids. Values are ± SD, mg/dl.

Figure 1. The effect of plasma storage at 4°C on mean HDL free cholesterol values in five CHD males, control males, and females analyzed over a period of 10 days. The error bars denote the standard error of the daily mean values of each group.

CHD men this value was 39.1 (± 9.8) mg/dl. This difference was not statistically significant (M-W, p > 0.05).

**HDL Phospholipids**

The mean (± SD) plasma HDL-pc level (Table 1) in healthy males was found to be 84.0 (± 21.8) and in CHD males, 78.7 (± 15.4) mg/dl. The difference was not significant (M-W, p > 0.05).

**HDL Free Cholesterol**

HDL-fc levels decrease soon after venepuncture in healthy males and females and in males with CHD (Figure 1). The difference of the plasma HDL-fc time paired means between the CHD and control plasmas stored at 4°C remained approximately 2.0 mg/dl after venepuncture on each respective day for at least 10 days (Figure 1). Therefore, these results indicate that the in vivo differences between groups would be represented by the differences found between their group means determined from plasma stored at 4°C.

The mean (± SD) plasma HDL-fc levels (Table 1) in male controls and CHD males were 6.6 (± 2.4) mg/dl and 4.4 (± 1.5) mg/dl, respectively, a difference of 33% (M-W, z = 5.0, p < 0.001). Although some of the plasmas of the CHD group used in the comparison were not analyzed on Day 4 after venepuncture as were all the control males, the Mann-Whitney U test on the 50 controls and on 35 CHD HDL-fc results obtained on Day 4 also resulted in a highly
significant difference (M-W, z = 4.5, p < 0.001). The mean (± SD) level for the 35 patients analyzed on Day 4 was 4.3 (± 1.4) mg/dl. These results indicate that there was little difference between those CHD specimens analyzed on Day 4 and those analyzed on Day 10.

Figure 2 shows a combined frequency distribution plot of plasma HDL-fc values for all CHD and control males. Both distributions are skewed.

**HDL Esterified Cholesterol**

The mean (± SD) plasma HDL-ec level in control males was 34.6 (±11.6) mg/dl which is almost the same value found for the CHD group (Table 1). Thus, the HDL-ec means between control males and CHD males were practically the same, whereas their HDL-fc means were different. Figure 3 shows a combined frequency distribution plot of plasma HDL-ec values for control and CHD males. There is considerable overlap between the two distributions.

**Correlations between Analytes**

Correlation coefficients were determined for each study group between different combinations of HDL-c, HDL-ec, HDL-fc, and HDL-pc. All HDL variables correlated positively and significantly with each other (p < 0.001). When data from both CHD and healthy subjects were combined, the combined HDL-c versus HDL-fc correlation coefficient for both groups was 0.95.

**Discriminant Function Analysis**

Discriminant function analysis reduced the overlap between the CHD group and controls. Of the four group variables, HDL-c, HDL-ec, HDL-pc, and HDL-fc, only HDL-fc and HDL-ec achieved a significantly high F value (> 1.0) for further inclusion by the stepwise DFA method. A discriminant function was obtained using these two variables which produced a high degree of separation (p < 0.001). The standardized discriminant function coefficients, which represent the relative contributions of the associated variables to the discriminant function, were 1.799 (p < 0.001) for HDL-fc and 1.531 (p < 0.001) for HDL-ec. Classification of CHD patients and controls using this function yielded an error rate of 11%. Classification using the discriminant function obtained for HDL-fc alone by direct DFA yielded an error rate of 27% (p < 0.001).

**Discussion**

Lower means were obtained for all the HDL analytes in the CHD group. The difference between the plasma HDL-pc means and their control group of 5.3 mg/dl was not statistically significant. By comparison, Fruchart et al. determined the plasma means (± SD) of a healthy group and a CHD group and found them to be 98 (± 20) and 70 (± 17) mg/dl, respectively. The differences between the results in their study and this present one is probably due to the possible inclusion of men and women of various ages within their control and diseased groups. While the healthy subjects had significantly higher levels of HDL-pc, there was a 23% overlap between the two groups. They suggested it was preferable to measure HDL-pc rather than HDL-c because HDL phospholipids are basically structural lipids and their concentration in HDL is relatively constant.

It was expected that significantly lower HDL-c would be found in the CHD patients. This did not occur. The difference between the CHD and control group means of 2.1 mg/dl was not statistically significant despite an analytical error much lower than this difference. However, the sample size involved in the comparison was small and this may explain the result.

Of all HDL comparisons between the CHD group and control group, only the HDL-fc value demonstrated a large and statistically significant difference. This difference between the control and CHD males was seven times the interassay low level control serum analytical standard deviation of 0.3 mg/dl. Thus, despite the good correlation found between HDL-c and HDL-fc, it is evident that there is enough individual variation to account for the reason only...
one of the analyte comparisons produced a statistically significant difference.

Optimal discrimination between the CHD and control group was obtained, with the discriminant function derived from stepwise DFA employing HDL-fc and HDL-ec. This equation yielded a misclassification rate of only 11%. Direct DFA of HDL-fc alone produced a discriminant function which yielded a classification error rate of 27%. HDL-c and HDL-pc were eliminated in the stepwise method as they contributed relatively little to group discrimination. Thus, it appears that it is necessary to perform only two assays, HDL-c (to derive HDL-ec) and HDL-fc, to obtain good discrimination for CHD. Larger control and CHD subject numbers would be required to produce a reliable function, and its classification ability to predict CHD in healthy people should be evaluated over many years.

Since the free cholesterol content of HDL₂ is about four to five times the concentration found in HDL₃, a high HDL-fc level should therefore reflect the antiatherogenic HDL₂ level. The results of this study are consistent with this assertion. Shifts in the amounts of the HDL subfractions (and therefore, CHD risk) would account for differences found for the CHD and control group.

According to one hypothesis, HDL exerts its antiatherogenic effect by transporting cholesterol away from tissues to the liver. If this is correct, then it would appear that HDL-fc would play a central role since there is evidence that over other lipids, it is preferentially taken up by the liver for bile synthesis.

If HDL-fc is only serving as a marker of HDL₂ levels, its estimation is technically easier than separation of HDL₂ from HDL₃. The assay for HDL-fc on the centrifugal analyzer was simple, accurate, rapid, and relatively cheap. The direct reagent costs per specimen (allowing for a proportion of standards and controls) currently amounts to only $0.13 (U.S. $0.08). Apolipoprotein assays, on the other hand, are expensive, the methodology is complex, and the assays are not easily standardized. According to Kostner, long-term prospective studies of known apolipoproteins could be spoiled by the detection of new apolipoproteins. In view of the heterogeneity of the lipoprotein classes, this is a possibility.

It should be noted that the lower HDL-fc values in CHD may be a result of the disease rather than an independent risk factor. Long-term prospective epidemiological studies are required to determine whether it is a superior predictor of CHD than estimations of HDL₂, LDL, apo A-I or apo B. Studies are planned that will compare HDL-fc with apo A-I and apo B, but the results obtained in the present study provide indications for further evaluation of HDL-fc as a candidate for inclusion in long-term population based epidemiological studies.

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References


Index Terms: HDL total, free, and esterified cholesterol • HDL phospholipids • coronary heart disease • discriminant function analysis • apolipoprotein • apo A-I • apo B
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