Associations of the HDL\textsubscript{2} and HDL\textsubscript{3} Cholesterol Subfractions With the Development of Ischemic Heart Disease in British Men

The Caerphilly and Speedwell Collaborative Heart Disease Studies

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**Background** The relative importance of HDL\textsubscript{2} and HDL\textsubscript{3} cholesterol as risk factors for ischemic heart disease (IHD) is still uncertain. Their associations with the incidence of IHD in the Caerphilly and Speedwell prospective studies are described.

**Methods and Results** The two studies have a common core protocol and are based on a total of 4860 middle-aged men from the general population. The first follow-up was at a nearly constant interval of 5.1 years in Caerphilly and 3.2 years in Speedwell; 251 major IHD events had occurred. Lipid levels were measured on fasting samples. Different laboratories were used by the two studies. Each laboratory used ultracentrifugation to separate HDL\textsubscript{2} and HDL\textsubscript{3}. Both subfractions were inversely associated with risk of IHD. Standardized relative odds of developing major IHD were 0.95 (95% confidence interval [CI], 0.80 to 1.14) for HDL\textsubscript{2} cholesterol and 0.83 (95% CI, 0.68 to 1.00) for HDL\textsubscript{3} cholesterol in Caerphilly and 0.76 (95% CI, 0.57 to 1.01) for HDL\textsubscript{2} and 0.64 (95% CI, 0.49 to 0.83) for HDL\textsubscript{3} in Speedwell. The association with incident IHD appeared to be stronger for HDL\textsubscript{3} in both areas. No linear combination of the two subfractions was a better predictor of IHD than total HDL cholesterol alone.

**Conclusions** In British men, both HDL\textsubscript{2} and HDL\textsubscript{3} cholesterol are inversely associated with the incidence of IHD. However, the prediction of the risk of IHD from total HDL cholesterol alone could not be improved upon by measurement of the two HDL subfractions. The relative value of the two HDL subfractions as predictors of risk is still unresolved. The uncertainty may be due, at least in part, to problems associated with their measurement. (Circulation. 1994;90:769-774.)

**Key Words** • coronary disease • lipoproteins
ultracentrifugation. Total HDL and HDL₃ cholesterol were assayed directly, and HDL₂ cholesterol was calculated by subtraction. Both total HDL and HDL₂ cholesterol had inverse associations with risk of acute MI. HDL₃ cholesterol also showed an inverse association with IHD, but statistical significance was lost after adjustment for HDL₂ cholesterol.

Thus, the evidence on the relative importance of HDL₂ and HDL₁ cholesterol is conflicting. Furthermore, no study has yet been able to show that prediction of IHD is improved by replacing total HDL cholesterol with some combination of its subfractions of differing densities or flotation rates.

HDL cholesterol has been shown to be an independent predictor of IHD in the Caerphilly and Speedwell studies. In both cohorts, HDL₂ and HDL₃ cholesterol were also measured. In this report, we consider whether the HDL cholesterol effect is mediated preferentially through HDL₂ or HDL₃ and whether our ability to predict IHD is improved by replacing total HDL cholesterol (the sum of HDL₂ and HDL₃) with some other combination of the two subfractions.

**Methods**

**Study Populations**

In Caerphilly, the study population was all men between 45 and 59 years old who were resident within a given area. A total of 2512 men were seen, 89% of the 2818 found to be eligible. In Speedwell, the population was selected from the age/sex registers of 16 general practitioners working in two neighboring health centers. All men 45 to 59 years old were chosen. A total of 2346 men were seen, 92% of those eligible.

**Survey Methods and Follow-Up Procedures**

The two studies had a common core protocol and procedures. These have been described in detail elsewhere. Briefly, at recruitment, the men attended a clinic at which a standard medical and smoking history was obtained; the London School of Hygiene and Tropical Medicine Chest Pain Questionnaire was administered; height, weight, and blood pressure were measured; and a 12-lead ECG was recorded. The men returned, after an overnight fast, to an early morning clinic, where a blood sample was taken with minimal venous stasis and anticoagulated with disodium EDTA (1 mg/mL). Fasting samples were obtained from 4641 men.

The results reported in this article refer to the first follow-up. This was at nearly constant intervals that averaged 61 months in Caerphilly and 38 months in Speedwell. At follow-up, a second ECG was recorded, and each man was asked whether he had been in hospital with severe chest pain. This latter, together with Hospital Activity Analysis notifications of admissions with a diagnosis of IHD, was used as the basis for a search of hospital notes. For men who died before the follow-up, a copy of the death certificate was automatically received. From this information, three categories of incident IHD events were defined: IHD death (cause of death coded as 410-414 on the International Classification of Diseases), clinical nonfatal MI (an event satisfying WHO criteria for definite acute myocardial infarction), and electrocardiographic MI (the appearance of major or moderate Q/QS waves on the follow-up ECG) when there were no Q/QS waves of any severity on the recruitment ECG). The development, or incidence, of major IHD is defined as the occurrence of any one of these three types of events. No separate assessment has been made of the relation between the HDL subfractions and the three individual categories of incident IHD. Such an assessment would require a larger number of events than had occurred by the time of the first follow-up.

**Laboratory Methods and Reproducibility**

Because of the heavy workload, two laboratories (one for each area) were used for the lipid analyses. Plasma samples were transported at 4°C by rail to the two laboratories on the day of venipuncture. Cholesterol and triglyceride concentrations were measured by enzymatic procedures. The HDL fraction was isolated by precipitation of the other lipoproteins with sodium phosphotungstate and magnesium chloride (Caerphilly) or with heparin and manganese chloride (Speedwell). The HDL subclasses were isolated by ultracentrifugation of the supernatant in a Beckman Airfuge, after the background density had been adjusted to 1.125 g/mL. In the Caerphilly samples, the HDL₃ class was isolated, total HDL and HDL₂ cholesterol were measured directly, and HDL₂ cholesterol was calculated by subtraction. In the Speedwell samples, the HDL₁ subclass was isolated, total HDL and HDL₂ cholesterol were measured directly, and HDL₃ cholesterol was calculated by subtraction.

From both field centers, at least 1 in every 20 blood samples were sent to the laboratories as split sample duplicates to assess reproducibility. For the major lipids, reproducibility in the two laboratories was very similar. Coefficients of variation were 7% (Caerphilly) and 10% (Speedwell) for total cholesterol, 14% and 15%, respectively, for total triglyceride, and 20% in both laboratories for total HDL cholesterol. As would be expected, the reproducibility of the directly measured HDL subclass cholesterols was worse than that of total HDL cholesterol, the coefficients of variation being 36% for HDL₁ cholesterol in Caerphilly and 43% for HDL₁ cholesterol in Speedwell. The coefficient of repeatability of Bland and Altman was 0.75 mmol/L for HDL₃ cholesterol in Caerphilly and 0.25 mmol/L for HDL₂ cholesterol in Speedwell.

Although the mean concentrations of total HDL cholesterol were almost identical in the two areas, the concentrations of the two subfractions differed. The directly measured HDL₁ in Speedwell was substantially lower than the HDL₁ in Caerphilly, which was calculated by difference. The converse was true for HDL₃. Because of these differences in mean concentrations and because of the difference in which subfraction was directly measured and which was calculated by subtraction, the results from the two cohorts are presented separately.

**Statistical Methods**

The pooled, age-adjusted mean difference in HDL cholesterol between men who developed IHD and those who did not was 0.095 mmol/L. On further adjustment for diastolic blood pressure, body mass index, smoking habits, evidence of IHD at baseline, and total cholesterol, this mean difference decreased only slightly, to 0.074 mmol/L. Thus, the independent contribution of HDL cholesterol to IHD is closely approximated by the simple age-adjusted estimate. For this reason, in this article we ignore all IHD risk factors other than age and concentrate only on trying to assess whether the HDL effect is mediated principally through one or another of the subfractions and on whether we can improve the prediction of IHD by replacing total HDL cholesterol concentration by some linear combination of the two subfractions other than the straightforward sum.

The main statistical method used was multiple logistic regression analysis, with the occurrence or nonoccurrence of a major IHD event as the dependent variable. Logistic regression takes no account of the length of follow-up. However, this will be immaterial, since the length of follow-up was virtually constant within each area. Any model involving time would also face the problem that no “time to event” is available for ECG-defined MI. In the logistic regression analysis, the various HDL cholesterol concentrations were treated in two ways. First, they were entered into the model as continuous variables, and these results are presented as standardized relative odds, defined as the proportionate change in the odds of developing major IHD associated with an increase of 1 SD in the cholesterol concentration. Second, the cholesterol distri-
TABLE 1. Mean Levels of HDL Cholesterol and Its Subfraction Concentrations and Incident IHD

<table>
<thead>
<tr>
<th></th>
<th>No IHD</th>
<th>IHD</th>
<th>Age-Standardized Mean Difference (95% confidence interval)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean (SD)</td>
<td>N</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caerphilly</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>2180</td>
<td>1.12 (0.33)</td>
<td>136</td>
<td>1.06 (0.33)</td>
</tr>
<tr>
<td>HDL2 cholesterol</td>
<td>2093</td>
<td>0.39 (0.30)</td>
<td>132</td>
<td>0.39 (0.30)</td>
</tr>
<tr>
<td>HDL3 cholesterol</td>
<td>2096</td>
<td>0.73 (0.28)</td>
<td>132</td>
<td>0.68 (0.26)</td>
</tr>
<tr>
<td>Speedwell</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>2119</td>
<td>1.11 (0.38)</td>
<td>87</td>
<td>0.99 (0.33)</td>
</tr>
<tr>
<td>HDL2 cholesterol</td>
<td>1969</td>
<td>0.20 (0.14)</td>
<td>82</td>
<td>0.17 (0.10)</td>
</tr>
<tr>
<td>HDL3 cholesterol</td>
<td>1959</td>
<td>0.92 (0.34)</td>
<td>80</td>
<td>0.82 (0.34)</td>
</tr>
</tbody>
</table>

HDL indicates high-density lipoprotein; IHD, ischemic heart disease. Units are mmol/L. To convert to mg/dL, multiply by 38.7. In Caerphilly, HDL3 was measured directly, and HDL2 was calculated by subtraction. In Speedwell, HDL2 was measured directly, and HDL3 was calculated.

Results

A total of 251 major IHD events occurred during the period of follow-up: 153 in Caerphilly and 98 in Speedwell. The average annual incidence was 1.2% in Caerphilly and 1.3% in Speedwell. The distribution of the three types of IHD events was similar in the two areas: 50% were fatal, 39% were clinical nonfatal MI, and 11% were ECG-defined MI.

Fasting blood samples were available from 2368 (94%) of the men in Caerphilly and from 2273 (97%) of the men in Speedwell. HDL cholesterol was successfully measured on 2316 men in Caerphilly and on 2206 men in Speedwell. The technology for the HDL subclass separation was not available for the first few weeks of the recruitment phase. As a result, HDL subfraction concentrations are available for about 90 fewer men in Caerphilly and for 160 fewer men in Speedwell.

Mean concentrations of total HDL cholesterol and of HDL2 and HDL4 cholesterol are shown in Table 1 for the men who developed major IHD and for those who did not. These results and all others are given separately for the two areas. In every case, the men who developed major IHD had lower concentrations. Also given in Table 1 are the age-standardized mean differences in concentration between the men who developed IHD and those who did not. For total HDL, HDL2, and HDL3 cholesterol, these mean differences are larger in Speedwell than in Caerphilly. However, there is no evidence of heterogeneity between the two areas, since the differences between areas do not approach the conventional level of statistical significance.

In Caerphilly, total HDL cholesterol is lower in men who develop major IHD by 0.064 mmol/L (95% confidence interval [CI], 0.006 to 0.122 mmol/L), and HDL2 cholesterol is lower by 0.053 mmol/L (95% CI, 0.004 to 0.102 mmol/L). In Speedwell, HDL cholesterol is lower by 0.128 mmol/L (95% CI, 0.048 to 0.209 mmol/L), and HDL2 cholesterol is lower by 0.110 mmol/L (95% CI, 0.034 to 0.185 mmol/L). All four of these differences are statistically significant. In neither area is the difference in HDL2 cholesterol statistically significant, although it approaches the 5% level of significance in Speedwell.

In both areas, about 80% of the difference in total HDL cholesterol between men who developed IHD and those who did not is accounted for by the difference in HDL3 cholesterol.

Table 2 shows age-adjusted relative odds of developing major IHD by quintile group for HDL, HDL2, and HDL3 cholesterol for both areas. Also given is a P value for a test for trend of decreasing IHD incidence across the quintile groups. In Caerphilly, the odds of developing major IHD for a man in the highest 20% of the HDL cholesterol distribution are 0.48 compared with a man in the bottom 20%. The corresponding relative odds in Speedwell are 0.28. In both areas, the trend of decreasing incidence of IHD with increasing HDL is statistically significant. There is a similar pattern for HDL2 cholesterol. Relative odds for men in the highest 20% of HDL2 are 0.70 and 0.26 in Caerphilly and Speedwell, respectively, and in both areas the trend is statistically significant.

For HDL2 cholesterol, there is little suggestion of a trend in Caerphilly. In Speedwell, the trend approaches statistical significance, but the relative odds of incident IHD for men in the top 20% of the distribution are 0.64, much higher than those for either total HDL or HDL3.

Standardized relative odds of developing major IHD are shown in Table 3 for various combinations of HDL, HDL2, and HDL3 cholesterol. Also shown is the deviance, which indicates the degree of fit for each model. All relative odds are <1.0, reflecting the inverse nature of the relation between the HDL cholesterol and incident IHD. For all models, the standardized odds are lower in Speedwell than in Caerphilly because, as described earlier, the inverse relation is stronger in...
TABLE 3. Standardized Relative Odds of Developing Major Ischemic Heart Disease and HDL, HDL₂, and HDL₃ Cholesterol

<table>
<thead>
<tr>
<th>Variables Included in the Model</th>
<th>Caerphilly</th>
<th>Speedwell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDL₂-C</td>
<td>HDL₃-C</td>
</tr>
<tr>
<td>Caerphilly</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL₂-C only</td>
<td>0.95 (0.80 to 1.14)</td>
<td></td>
</tr>
<tr>
<td>HDL₃-C only</td>
<td>0.83 (0.68 to 1.00)</td>
<td></td>
</tr>
<tr>
<td>HDL₂-C and HDL₃-C</td>
<td>0.87 (0.71 to 1.08)</td>
<td>0.79 (0.64 to 0.97)</td>
</tr>
<tr>
<td>HDL-C only</td>
<td>0.81 (0.67 to 0.99)</td>
<td></td>
</tr>
<tr>
<td>Speedwell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL₂-C only</td>
<td>0.76 (0.57 to 1.01)</td>
<td></td>
</tr>
<tr>
<td>HDL₃-C only</td>
<td>0.64 (0.49 to 0.83)</td>
<td></td>
</tr>
<tr>
<td>HDL₂-C and HDL₃-C</td>
<td>0.79 (0.58 to 1.06)</td>
<td>0.64 (0.49 to 0.84)</td>
</tr>
<tr>
<td>HDL-C only</td>
<td>0.59 (0.45 to 0.79)</td>
<td></td>
</tr>
</tbody>
</table>

HDL indicates high-density lipoprotein; C, cholesterol. In Caerphilly, HDL₃ was measured directly, and HDL₂ was calculated by subtraction. In Speedwell, HDL₃ was measured directly, and HDL₂ was calculated.

*The standardized relative odds are the proportionate change in odds associated with an increase of 1 SD in the cholesterol level.

A smaller deviance indicates that the model is a better fit (see "Methods").
deviance follow approximately a \( \chi^2 \) distribution with one df. Thus, the addition of HDL\(_2\) significantly improves the fit, as is also shown by the fact that the 95% CI for the standardized relative odds for HDL\(_2\) does not include 1.0. The change in deviance when HDL\(_2\) is added to the model that already includes HDL\(_3\) is only 1.7 in Caerphilly and 2.8 in Speedwell. Thus, in neither area does the addition of HDL\(_2\) significantly improve the fit of the model. Again, this is confirmed by the fact that both 95% CIs for the standardized relative odds for HDL\(_2\) include 1.0.

**Discussion**

We set out to answer two questions. The first is whether we can improve our ability to predict IHD by replacing total HDL cholesterol in any model by some combination of the two subfractions, HDL\(_2\) and HDL\(_3\). The second is whether the association between total HDL cholesterol and IHD is mediated preferentially through HDL\(_2\) or HDL\(_3\). The first question is obviously of practical importance, and the second is relevant to our understanding of the mechanisms of the association between HDL metabolism and IHD.

On the basis of the present data, the answer to the first question is that we cannot materially improve prediction of IHD by replacing total HDL by any other combination of HDL\(_2\) and HDL\(_3\) cholesterol. In Speedwell, there was a trivial difference in the fit of the two models (Table 3). In Caerphilly, the model with the two subfractions provided better prediction, but the improvement was marginal. To illustrate this, in the model including both subfractions, 64% of actual IHD events occurred among men in the top 50% of the distribution of predicted risk compared with 62% of IHD events for the model with total HDL cholesterol alone. This small difference could be due to chance. This finding concurs with that of Stampfer et al., who concluded that HDL subfractions had no predictive value after conventional risk factors and the ratio of total to HDL cholesterol were considered.

This lack of improvement in the predictive power of the model with the two subfractions is, perhaps, surprising. Such a model effectively chooses that linear combination of the two subfractions that maximizes the fit of the model. That such a model is no improvement over one that includes total HDL cholesterol alone appears to suggest that the simple sum of the two subfractions is at least as good as any other linear combination. There are, however, reasons why this conclusion might be incorrect. The main reason concerns errors in the measurements. Over recent years, the effect of measurement imprecision on the estimation and interpretation of relative odds from logistic regression models has received much attention. The emphasis in this work is on the correction of relative odds estimates to allow for the measurement imprecision. Here we are interested in the comparative fit of two models. It is clear that imprecision of measurement will reduce the goodness of fit of both models. It may well be that the proportionately larger errors in the measurement of the individual subfractions, compared with total HDL, will result in a greater decrease in the goodness of fit. However, as yet no methods are available for estimating the effect of imprecision on the overall fit of the models.

The second question that we attempted to answer is whether the association between total HDL and IHD is mediated preferentially through HDL\(_2\) or HDL\(_3\) cholesterol. There is a widely held view\(^5\) that the HDL\(_2\) fraction is the more important in the pathogenesis of IHD. This arose largely as a result of the study by Gofman et al., which for 25 years provided the only prospective data on HDL subclasses, and those of several studies in which HDL subclasses had been measured in IHD patients and control subjects. However, after reviewing the literature in 1987, Miller\(^4\) concluded that, in the absence of a sufficient body of prospective data, the relative importance of HDL\(_2\) and HDL\(_3\) cholesterol was still unanswered. Since then, HDL\(_2\) and HDL\(_3\) cholesterol have been compared prospectively in two studies: the Physicians Health Study\(^6\) and the Kuopio Ischemic Heart Disease Risk Factor Study.\(^7\) In the former, HDL\(_3\) cholesterol had the stronger association with IHD, whereas in the latter, HDL\(_2\) had the stronger association.

In the present study, HDL\(_3\) is the more strongly associated with IHD. Odds of IHD in the top 20% of the HDL\(_3\) distribution relative to the bottom 20% are 0.70 and 0.26 in Caerphilly and Speedwell, respectively. For HDL\(_2\) cholesterol, the corresponding relative odds are 0.87 and 0.64 (Table 2). The standardized relative odds from Table 3 show a similar pattern of stronger associations between HDL\(_2\) and IHD than between HDL\(_2\) and IHD. There is, additionally, the suggestion that the finding of a stronger association between HDL\(_3\) and incident IHD is more clear-cut in Speedwell than in Caerphilly. None of the above relative odds have been corrected for measurement imprecision. Phillips and Davey Smith\(^17,18\) point out that the correction methods are sensitive to the amount of measurement imprecision attributed to each variable. In Caerphilly, only 5% of samples were sent to the laboratory as split samples. In Speedwell, the figure was 10%. The coefficient of variation for split-sample reproducibility changed over the 3 years of the recruitment phase, generally improving with time. In addition, underlying the correction methods is the rare-disease assumption. Rosner et al.\(^19\) suggest that an event probability of \(<.05\) will satisfy that assumption. In Caerphilly, the event probability was .06 (Table 1). Further, the other prospective studies\(^6,7\) with which we wish to compare our results did not correct for measurement imprecision.

Comparison of the prospective studies shows complete disagreement over which is the more important subclass. The Physicians' Health Study\(^5\) concluded that HDL\(_2\) was the more important, a view supported by the current Speedwell study. In the current Caerphilly study, HDL\(_3\) was the more important, but the difference in importance between HDL\(_3\) and HDL\(_2\) was much less marked. The Kuopio study\(^7\) concluded that HDL\(_3\) was the more important. There is, however, a pattern to this apparent disagreement. In the Physicians' Health Study,\(^5\) HDL\(_2\) cholesterol represented only 10% of total HDL cholesterol. In the current Speedwell and Caerphilly studies, HDL\(_2\) represented 18% and 35%, respectively, whereas in the Kuopio study,\(^7\) it represented 66%. Thus, within any study, the greater the proportion of total HDL that is described as HDL\(_2\), the more likely the investigators are to have concluded that HDL\(_2\) is the important subclass. These differences in proportion exist almost entirely in the absolute amount of HDL\(_2\) cholesterol, because
total HDL cholesterol varied only from 1.12 to 1.30 mmol/L across the four studies. Thus, in the Kuopio study, mean HDL<sub>2</sub> cholesterol was 0.85 mmol/L, whereas in the Physicians’ Health Study control group, it was 0.12 mmol/L. It is possible, of course, that these differences are genuine and are due to lifestyle and genetic factors. However, two of the main lifestyle factors associated with HDL subfraction cholesterol concentrations are body mass index and alcohol consumption. Previous work<sup>20</sup> from the current studies has shown that an increase of 200 mL of alcohol per week (equivalent to 20 U) is associated with an increase in HDL<sub>2</sub> cholesterol level of only about 0.03 mmol/L. Similarly, a decrease of 5 in body mass index is associated with an increase of just 0.04 mmol/L in HDL<sub>2</sub> cholesterol. To decrease body mass index by 5, a man 1.8 m tall would have to lose just over 16 kg in weight. Thus, it seems unlikely that lifestyle factors can completely explain the differences. One possibility that must be considered is that the differences are not real but rather reflect differences in measurement methods.

In summary, we have demonstrated that both HDL<sub>2</sub> and HDL<sub>3</sub> cholesterol are inversely associated with the development of IHD in British men, the association with HDL<sub>3</sub> being apparently the stronger. However, as measured in the present studies, no linear combination of the two subfractions is a better predictor of IHD than is total HDL cholesterol alone. Uncertainty over the value of the HDL subfractions as predictors of risk remains and may have arisen because of problems with the measurement of the subfractions.

References
Associations of the HDL2 and HDL3 cholesterol subfractions with the development of ischemic heart disease in British men. The Caerphilly and Speedwell Collaborative Heart Disease Studies.

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