High Density- and Beta-Lipoprotein Screening for Risk of Coronary Artery Disease in the Context of New Findings on Reverse Cholesterol Transport

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Abstract. This article considers how high density lipoproteins (HDL) act as anti-arteriosclerotic agents, examines the usefulness of HDL indexes alone and in conjunction with other markers of coronary artery disease (CAD), and discusses how HDL markers compare with what one might expect from known metabolic mechanisms. This is accomplished by: (i) an overview of mechanisms associated with CAD, especially new findings regarding reverse cholesterol transport; (ii) a brief review of the clinical literature on biochemical markers for automated use; and (iii) analysis of data for persons with or without angiographically documented CAD. Based on these considerations, the ratio of optimized apo A-I/apo B appears superior to lipoprotein lipid markers for predicting the risk of CAD. Yet the ratio shows poor diagnostic accuracy by itself; it yields poorer diagnostic accuracy than would be expected from assessing the metabolic pathways. Discrimination is improved by using the ratio in conjunction with risk factors defined by the National Cholesterol Education Program (NCEP). Based on receiver-operator characteristic (ROC) curve data, this approach increases the accuracy by 13-14% above that obtained with current lipid markers; it improves discrimination more than the use of inflammatory markers. Apolipoprotein testing is better related to the mechanisms of cholesterol transport, is widely available, and requires only two tests, compared to three, to improve discrimination. However, inclusion of inflammatory markers may need to be considered in the future, when more information is available about their functions and clinical value. (received 3 December 2001; accepted 22 December 2001)

Keywords: apolipoproteins, arteriosclerosis, C-reactive protein, reverse cholesterol transport

Introduction

High density lipoproteins (HDL) are operationally defined as those within the density range of 1.063 - 1.21 g/ml. It has long been known that elevation of high density lipoprotein cholesterol (HDLC) is inversely associated with coronary artery disease (CAD), and a more powerful statistical predictor of CAD than low density lipoprotein cholesterol (LDL) [1]. This inverse association has led to the concept of reverse cholesterol transport (RCT). RCT is a process whereby cholesterol is removed from peripheral tissues directly by HDL and in conjunction with triglyceride-rich lipoproteins deposited in the liver for disposal in the bile. Accumulation of lipids in macrophages is considered an early event in arteriosclerosis, and RCT removes cholesterol from these cells. Although HDL metabolism is not as clearly understood as LDL metabolism, during the past decade considerable information has been acquired regarding HDL species, receptors, and transporters. This has resulted in better knowledge of HDL metabolism and RCT. Several assays for HDL markers are now available for the clinical laboratory, either as kits or in convenient procedures for automated or semi-automated testing. These markers include total HDLC, HDL₂C, HDL₃C, apo A-I, apo A-II, and apo E.
The purposes of this article are: (i) to determine which HDL marker(s) best predict the risk of CAD; (ii) to consider whether clinical studies support this premise; (iii) to identify which marker of beta-lipoproteins, used in conjunction with the selected HDL marker, best predicts the risk of CAD; (iv) to decide whether or not these markers meet theoretical expectations; and (v) to examine how the markers compare with the proposed inflammatory markers.

To achieve these goals, my initial step will be to present new information in the form of receiver-operator characteristic (ROC) curves and multivariate analyses from our studies of HDLC, subspecies of HDLC, apo A-I, and beta-lipoprotein markers. Second, I shall review current knowledge about the basic process of arteriosclerosis, about HDL metabolism and RCT, about the clinical usefulness of HDLC, the subspecies of HDLC, and the apolipoproteins associated with HDL as markers for CAD, and about the proposed anti-atherogenic attributes of HDL. Finally, I shall consider the meaning of the theoretical and actual relationships.

Methods

Subjects, blood collection, and angiography. Parameters regarding the patients, blood samples, and angiography are similar to those previously described [2]. Briefly, the patients were males, 40- to 70-yr old, who entered the hospital for clinically indicated angiographic studies. Blood samples were drawn from consecutively examined patients, excepting those with the following exclusion criteria: persons who received known lipid-altering medications (gemfibrozil, HMG CoA reductase inhibitors, bile acid resins, niacin, and heparin), diabetics, persons with chronic kidney disease, and persons who had myocardial infarction within 3 mo. The study was approved by the Hospital and University Committees on Protection of Human Rights.

The following demographic variables were also assessed: smoking, alcohol intake, family history of myocardial infarction, age, medications (ie, beta-adrenergic antagonists, calcium channel blockers, ACE inhibitors, diuretics), and body mass index (BMI = body weight (kg)/[height (m)]²). Nominal variables were classified as yes or no, except alcohol intake, which was classified as “none,” “light-moderate,” and “heavy,” based on 0, 1 to 6, or >6 alcoholic drinks per day.

Patients were fasted over night. Blood for lipoprotein studies was drawn, without preservatives, from the femoral artery prior to infusion of angiographic contrast medium. Serum was obtained by centrifugation at room temperature. Cholesterol and triglyceride assays were performed within 48 hr on serum stored at 4°C. Aliquots were frozen at -70 °C for apo A-I and apo B measurements.

Angiography was performed by standard methods. Patients were categorized into two groups for ROC curve analysis and three groups for discriminant analysis [3,4]. Group NCA comprised patients with normal coronary arteries (<20% stenosis). In the great majority of cases, these patients showed no detectable stenosis. Group CAD comprised patients with severe CAD (≥70% stenosis in at least one major vessel). For discriminant analysis, a third group (Group ICS) comprised the subjects with intermediate degrees of coronary artery stenosis (ie, >20% and <70% in any vessel).

Assays. Serum cholesterol, HDLC, and triglycerides were assayed by routine methods with a discrete sample analyzer (Eastman Kodak, Rochester, NY). HDLC was separated by precipitation with dextran sulfate-magnesium ion, with magnetic removal of LDL and VLDL (Polymedco, Courtland, NY) [5]. HDL₃₃ was precipitated using dextran sulfate 50,000 (Genzyme, Boston MA), and HDL₂₃ was calculated (HDL₂₃ = total HDLC - HDL₃₃) [6]. LDLC was calculated by the Friedewald equation on samples with triglycerides ≤ 4 g/L.

Serum apo A-I and apo B levels were measured by automated rate immunonephelometry using kits with the Array analyzer (Beckman Instruments, Inc., Brea, CA) [7]. The apo A-I kit was modified by addition of 0.5% Tween 20 [8]. Although results obtained with this kit did not show a statistically significant difference with or without addition of Tween 20, the assay was modified to ensure comparability with our prior study [2].

Statistics. The Kruskal-Wallis test and contingency tables (likelihood ratio) were used for determining
possible significant confounders (JMP, SAS Institute, Cary, NC). Based on the contingency tables and Kruskal-Wallis test, the only variables that showed significant relationships with CAD were age and beta-adrenergic antagonists. Age showed a strong relationship (p <0.0001); beta-adrenergic antagonists showed a weak relationship (p 0.05). These variables were considered as possible confounders in stepwise discriminant analysis.

The Kolmogorov-Smirnov test was used for goodness-of-fit testing (Statview 4, SAS Institute). Results of goodness-of-fit testing indicated that HDL cholesterol assays and apo A-I results were significantly different from a Gaussian distribution. When these tests were transformed logarithmically, they fit well. Consequently, for discriminant analysis, log-converted values were used with HDL results.

Stepwise discriminant analysis (SPSS 6.1, SPSS, Inc., Chicago, IL) used Wilks' lambda with a p for entry of 0.05 and a p for removal of 0.1. Receiver operating characteristic (ROC) curves were calculated by the Rockit program (kindly provided by Dr. Charles E. Metz, Department of Radiology, University of Chicago Medical Center, Chicago, IL; http://www-radiology.uchicago.edu/sections/roc). This program uses the maximum-likelihood-estimation technique for estimating the ROC curve. When logistic regression was combined with ROC analysis, coefficients for logistic regression were obtained from JMP (SAS Institute).

The ninety-fifth percentile significance levels (p ≤ 0.05) were based on two-tail cutoffs (JMP), except for the ROC areas, where one-tail 95% confidence intervals (CI) were used.

Results

**ROC analysis.** Comparisons of the areas and confidence intervals (CI) for the CAD and NCA groups and plots derived from ROC curve analysis are shown in Table 1 and Fig. 1, respectively. Based on the areas and CI values in Table 1, HDL\(_2\)C shows

Table 1. Areas under the receiver-operator characteristic (ROC) curves for the various lipoprotein tests and selected test ratios, based on data from patients in groups NCA (normal coronary arteries) and CAD (severe coronary disease).

<table>
<thead>
<tr>
<th>Test</th>
<th>Area under ROC curve</th>
<th>Confidence Interval</th>
<th>Number of NCA patients</th>
<th>Number of CAD patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>High density lipoproteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL(_2) cholesterol</td>
<td>0.56</td>
<td>0.50-0.62</td>
<td>131</td>
<td>228</td>
</tr>
<tr>
<td>HDL(_3) cholesterol</td>
<td>0.62</td>
<td>0.55-0.67</td>
<td>131</td>
<td>228</td>
</tr>
<tr>
<td>Total HDL cholesterol</td>
<td>0.60</td>
<td>0.54-0.65</td>
<td>136</td>
<td>246</td>
</tr>
<tr>
<td>Apo A-I</td>
<td>0.63</td>
<td>0.57-0.68</td>
<td>136</td>
<td>246</td>
</tr>
<tr>
<td>HDL(_2)C/HDL(_3)C</td>
<td>0.51</td>
<td>0.45-0.57</td>
<td>131</td>
<td>228</td>
</tr>
<tr>
<td>Low and very low density lipoproteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.57</td>
<td>0.51-0.62</td>
<td>136</td>
<td>240</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.57</td>
<td>0.51-0.62</td>
<td>136</td>
<td>246</td>
</tr>
<tr>
<td>Apo B</td>
<td>0.63</td>
<td>0.52-0.64</td>
<td>136</td>
<td>246</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.58</td>
<td>0.52-0.64</td>
<td>136</td>
<td>246</td>
</tr>
<tr>
<td>Ratio of high density to low density lipoproteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDLC/total cholesterol</td>
<td>0.62</td>
<td>0.57-0.68</td>
<td>136</td>
<td>246</td>
</tr>
<tr>
<td>HDLC/LDLC</td>
<td>0.62</td>
<td>0.57-0.68</td>
<td>136</td>
<td>240</td>
</tr>
<tr>
<td>Apo A-I/apo B</td>
<td>0.70</td>
<td>0.64-0.75</td>
<td>136</td>
<td>246</td>
</tr>
<tr>
<td>HDLC/total cholesterol plus triglycerides</td>
<td>0.63</td>
<td>0.57-0.68</td>
<td>136</td>
<td>246</td>
</tr>
<tr>
<td>HDLC/LDLC plus triglycerides</td>
<td>0.62</td>
<td>0.56-0.67</td>
<td>136</td>
<td>240</td>
</tr>
<tr>
<td>Apo A-I/apo B plus triglycerides</td>
<td>0.68</td>
<td>0.62-0.73</td>
<td>136</td>
<td>246</td>
</tr>
</tbody>
</table>
the poorest discrimination among the individual HDL markers. The ratio of HDL_2C/HDL_3C is the poorest marker, overall, and its CI substantially overlaps 0.5 (area of no discrimination), indicating that this ratio is nugatory. On the basis of the ROC areas, although apo A-I appears to be the best predictor, its area is similar to HDL_3C; total HDLC is third. Based on the overlap of the respective CI values, these differences are not statistically significant.

Table 1 indicates that apo B is a better discriminator than LDLC or total cholesterol. Although there is slight overlap between the upper CI of triglycerides and apo B, serum triglycerides have usually been shown by multivariate analysis to provide no additional predictive value when HDL and LDL markers are combined [9]. This feature of triglycerides can be seen from the entries near the bottom of Table 1, where ROC analysis from ratios of HDL and beta-lipoprotein markers were combined with triglycerides from the output of logistic regression analysis [10]. The combined area is no greater than the areas for HDL and beta-lipoprotein markers without triglycerides.

As seen in Table 1, ROC analysis indicates the ratio of apo A-I/apo B is a superior marker, compared to the ratios of HDLC/total cholesterol or HDLC/LDLC. Examination of plots in Fig. 1 shows that the curve for the ratio of HDLC/LDLC crosses the curve for apo A-I, so that apo A-I by
Table 2. Discriminant analysis of HDL markers, based on data from 130 patients in group NCA (normal coronary arteries), 225 patients in group CAD (severe coronary disease), and 55 patients in group ICS (intermediate coronary stenosis). The discriminate analysis was performed after logarithmic transformation of the data.

<table>
<thead>
<tr>
<th>Variables in analysis after first step*</th>
<th>F to enter</th>
<th>Entered</th>
<th>Not entered at any step</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comparison #1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lnapo A-I</td>
<td>9.4</td>
<td>Lnapo A-I</td>
<td>LnHDL₃ cholesterol</td>
</tr>
<tr>
<td>LnHDL₃ cholesterol</td>
<td>7.6</td>
<td></td>
<td>LnHDL cholesterol</td>
</tr>
<tr>
<td>Ln HDL cholesterol</td>
<td>7.5</td>
<td></td>
<td>LnHDL₂ cholesterol</td>
</tr>
<tr>
<td>LnHDL₂ cholesterol</td>
<td>4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comparison #2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LnHDL₃ cholesterol</td>
<td>6.7</td>
<td>LnHDL₃ cholesterol</td>
<td>LnHDL cholesterol</td>
</tr>
<tr>
<td>Ln HDL cholesterol</td>
<td>6.7</td>
<td></td>
<td>LnHDL₂ cholesterol</td>
</tr>
<tr>
<td>LnHDL₂ cholesterol</td>
<td>3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comparison #3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ln HDL cholesterol</td>
<td>6.7</td>
<td>LnHDL cholesterol</td>
<td>LnHDL₂ cholesterol</td>
</tr>
<tr>
<td>LnHDL₂ cholesterol</td>
<td>3.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Age was the most powerful variable and entered alone in the first step; it was included in all comparisons, although not shown in the table. Use of beta-blockers did not enter any equation and was not included in any comparison.

itself shows better discrimination in the useful range (ie, below a false positive frequency of about 20%).

**Discriminant analysis.** The data were also analyzed by multivariate discriminant analysis. Logistic regression and ROC analysis permit comparisons of only two groups. On the other hand, discriminant analysis allows simultaneous comparison of the three groups (ie, NCA, ICS, and CAD), while also correcting for possible confounders. The results of discriminant analysis are listed in Table 2. The conclusions are similar to those obtained by ROC analysis. In comparison #1, apo A-I is the best marker. In comparison #2, the other markers were tested in the absence of apo A-I. Here, HDL₃C was the better marker, but total cholesterol showed a similar “F to enter” value, indicating that the two tests give similar information. In comparison #3, total HDLC proved to be a better marker than HDL₂C. These results show that apo A-I is the best discriminator. This confirms the conclusions from ROC analysis that apo A-I is the superior marker and indicates that the ROC analysis was not overly affected by confounding variables or by the absence of the intermediate (ICS) group.

**Literature Review**

**Current view of the process of arteriosclerosis.** The process of atherosclerosis that leads to myocardial infarction involves endothelial cell injury and dysfunction, lipid deposition, abnormal cell migration, chronic inflammation, and thrombosis [11,12]. This process, outlined in Fig. 2, occurs largely in the subendothelial space, with rupture into the blood stream. Injury causes trapping of lipoproteins within the vessel wall. Among other causes, injury to the vascular wall may be due to blood pressure fluctuations, toxic products from smoking, diabetic complications, bacterial or viral insults, and elevated lipoproteins. It appears that trapped LDL and other apo B containing lipoproteins are oxidized due to interactions with monocytes, other white blood cells, and oxidizing agents. Macrophages incorporate oxidized LDL in preference to normal LDL. Unlike normal LDL, which is taken up by the well-regulated LDL receptor, oxidized LDL is taken up by scavenger receptors (SR) that appear not to be subject to feedback inhibition. This group of multifunctional receptors, which are capable of taking up a variety of lipoprotein subfractions, is known to be upregulated in atherosclerosis.
of ligands, are homologs of CD36. They include CD36, SR-A, the LDL receptor-related protein (LRP), and SR-BI, of which, as will be discussed, the latter two appear most important in HDL metabolism [13-15]. As a result of this unregulated uptake, macrophages form lipid-laden foam cells; this early event in atherosclerosis initiates the fatty streak [16,17].

Endothelial cells and white blood cells release diverse cytokines, growth factors, and other pro-inflammatory mediators that stimulate cell migration and inflammation. This gives rise to a fibrous cap with cholesterol crystals and debris within the deep necrotic layer, while inflammatory cells form a dynamic outer edge. Oxidized lipoproteins may facilitate many of these processes. Consequently, a cycle of oxidation, inflammation, thrombosis, and further oxidation is perpetuated [11,12,16]. Mechanical forces predispose the soft outer layer of the atheromatous plaque to rupture at sites of structural weakness. Rupture of plaques causes thrombosis and incorporation of thrombi into the plaque. Ultimately, the appearance of a large thrombus in an obstructed vessel can lead to sudden ischemia and unstable coronary syndromes.

**HDL species and subclasses.** Table 3 lists the major and minor apolipoproteins that comprise serum HDL, along with their sites of synthesis, and their known functions. Apo A-I is the major protein in HDL, consisting of ~70% of the total protein. Apo A-II comprises approximately 20–25% of the protein, and several minor proteins, of which apo E is the largest, comprise the rest [18]. Several species of HDL have been identified. These include lipid-free nascent apo A-I chains, lipid-poor apo A-I chains...
containing cholesterol and phospholipid, lipid-rich discoid particles containing apo A-I, spherical particles containing only apo A-I, and spherical particles containing apo A-I and apo A-II [19]. Some spherical particles also contain apo E. Lipid-poor species that contain apo E and apo A-IV have also been described [19]. Most HDL migrates in the alpha region of the gel upon agarose electrophoresis, but lipid-free and lipid-containing apo A-I chains, and discoid particles migrate in the pre-beta region and are referred to as “pre-beta HDL” [18-20].

Table 3. Apolipoproteins that comprise HDL.

<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>Lipoprotein</th>
<th>Synthesis site</th>
<th>Major functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo A-I</td>
<td>HDL</td>
<td>Liver</td>
<td>Reverse cholesterol transport, LCAT activation, facilitates cholesterol efflux</td>
</tr>
<tr>
<td>Apo A-II</td>
<td>HDL, VLDL, chylomicrons</td>
<td>Liver</td>
<td>Inhibits LCAT, binds phospholipids, inhibits cholesterol efflux</td>
</tr>
<tr>
<td>Apo A-IV</td>
<td>HDL, chylomicrons</td>
<td>Liver, intestine</td>
<td>Poorly defined</td>
</tr>
<tr>
<td>Apo C-I</td>
<td>HDL, VLDL, chylomicrons</td>
<td>Liver, intestine</td>
<td>Inhibits hepatic uptake of lipoproteins</td>
</tr>
<tr>
<td>Apo C-II</td>
<td>HDL, VLDL, chylomicrons</td>
<td>Liver, intestine</td>
<td>LPL activation</td>
</tr>
<tr>
<td>Apo C-III</td>
<td>HDL, VLDL, chylomicrons</td>
<td>Liver, intestine</td>
<td>LPL inhibition</td>
</tr>
<tr>
<td>Apo E (exists in E2, E3, &amp; E4 isoforms)</td>
<td>chylomicrons, remnants</td>
<td>Intestine, liver, brain, macrophages, other peripheral cells</td>
<td>LDL, VLDL, and remnant receptor recognition</td>
</tr>
</tbody>
</table>

Particles that contain apo A-I alone are called “LpA-I”; those that contain apo A-I and apo A-II are called “LpA-I:A-II.” The LpA-I:A-II particles contain apo A-I (two to four copies per particle) and apo A-II in a ratio of 2:1 [21]. LpA-I particles appear to promote cholesterol efflux from cells, while LpA-I:A-II particles do not [22,23]. HDL can be assigned to subclasses based on particles of various density [24], of which the main two are the less dense, larger HDL₂ (d. 1.063-1.125 g/mL) and the more dense, smaller HDL₃ (d. 1.125-1.21 g/mL). HDL₂ is richer in particles containing apo A-I without apo A-II, whereas HDL₃ is richer in particles containing both apo A-I and apo A-II [25].

**Mechanisms of HDL metabolism and reverse cholesterol transport.** Tangier disease is an autosomal dominant disorder in which homozygotes typically show HDLC and apo A-I levels of ~5% and 1% of normal and heterozygotes show levels of ~50% of normal. Homozygotes show 4- to 6-fold increase in the incidence of CAD [26,27]. These patients show
increased accumulation of cholesterol esters in macrophages of the reticuloendothelial system [26,27]. It appears that a defect in the ATP-binding cassette transporter (ABC A1) is responsible for this disease [27]. Evidence indicates that ABCA1 transports cholesterol and phospholipid to nascent apo A-I chains from many types of cells and tissues, including liver, lung, adrenal glands, and macrophages [19,27]. This evidence, along with new findings on the roles of phospholipid transport protein (PLTP) and SR-BI in HDL metabolism, has enhanced our understanding of reverse cholesterol transport (RCT).

Fig. 3 outlines current concepts regarding HDL metabolism. ABCA1 transports cholesterol and phospholipid from tissues to nascent apo A-I. This lipid-free (or lipid-poor) apo A-I may come from the liver or may dissociate from mature particles [28]. Especially interesting is an in vitro finding that ABC A1 from macrophage lines, but not from...
other tissues, can be up-regulated [29]. Lipid-poor HDL takes up additional cholesterol from tissues, probably by diffusion, facilitated by the phospholipid-rich surface of HDL [21,28]. Lipid-rich apo A-I forms discoid particles that are associated with lecithin:cholesterol acyltransferase (LCAT) which catalyses esterification of free cholesterol for deposit in the particle core [21,30]. Since apo A-I is a powerful activator of LCAT, discoid and apo A-I-rich spherical particles probably accumulate cholesterol ester by diffusion of free cholesterol from tissue to the outer layer with esterification into the core [21,23,28].

LCAT is not associated with larger spherical particles, but these particles can exchange cholesterol with phospholipid from triglyceride-rich lipoproteins, such as very low density lipoprotein (VLDL), and various remnants [21,28]. The lipid exchange is mediated by cholesterol ester transfer protein (CETP) and phospholipid transfer protein (PLTP) [31]. These triglyceride-rich particles containing apo E may be converted to intermediate density lipoprotein (IDL), which is rapidly taken up by the LDL receptor, and apo E-containing remnants that can be taken up by the LRP [32]. In either case, cholesterol can be removed from HDL by exchange with triglyceride-rich lipoproteins and disposed of through the liver [23,31]. PLTP also mediates the splitting of lipid-poor apo A-I from smaller HDL3-like spherical particles and the subsequent fusion of the unstable residues into a larger HDL2-like particle [19,33]. Hepatic lipase can also split apo A-I from larger HDL particles, but this remodeling does not give rise to larger HDL3-like particles; rather, in conjunction with CETP larger particles can be converted to smaller ones [19]. Thus, as illustrated by the solid arrows in Fig. 3, a cycle is perpetuated whereby cholesterol can be transferred from tissue to lipid-poor apo A-I and then to discoid and spherical particles, and finally back to lipid-poor apo A-I, while cholesterol is removed for elimination through triglyceride-rich lipoproteins. A final event in this process seems to be the selective uptake of cholesterol ester from spherical HDL2-like particles by SR-B1, which is highly expressed in liver and steroidogenic tissues [19,27]. In this two-step process, high affinity lipoprotein binding is followed by a transfer of lipid from HDL through the receptor to the cell membrane, after which the depleted particle is released [14]. The evacuated cholesterol may be eliminated in the bile [34].

Although the foregoing information advances our understanding of how RCT proceeds, it is less than complete. The dashed arrows in Fig. 3 illustrate some of many points that remain unclear. What is the role of apo E in RCT? Apo E in HDL has been hypothesized to be transferred from macrophages, providing a mechanism by which HDL can bind to an apo E receptor. It has been suggested that in this way whole HDL particles can be removed from the circulation. This proposed holo-HDL mechanism may proceed through an apo E receptor or binding to proteoglycans, or by another mechanism [28,31]. In any case, the role of apo E in HDL remains unclear. In fact, there is evidence that apo E-containing particles play little or no role in normal RCT [21]. How does apo A-II enter spherical particles and what is its role in RCT? Lipid-poor apo A-I is reclaimed by the kidney through cubilin, a protein that also acts to reclaim vitamin B12 [19]. What role does this play?

Clinical findings associated with RCT. HDL subtypes. In the 1980’s, it was suggested that the more dense HDL2 accepts cholesterol esters and is converted to the less dense HDL3 because the HDL3 subtype accepts cholesterol more readily than HDL2 [35,36]. It was suggested that apo E from macrophages might facilitate cholesterol transfer to the less dense HDL subtypes [36]. Studies suggested that HDL2C was a more powerful predictor of CAD than total HDLC or HDL3C [37-41], and that the ratio of HDL2C/HDL3C might be an even more powerful marker for risk of CAD. These findings seemed to support the notion that heavier HDL3 takes up apo E and cholesterol from peripheral tissue and macrophages to form HDL2. Consequently, a high ratio of HDL2C/HDL3C would suggest more active RCT [38,39]. As found here (Table 1, Fig. 1), subsequent studies did not confirm these findings, but showed that total HDLC and HDL3C are equal or more powerful markers for CAD than HDL2C [42-45]. These reports included one study
by investigators who had previously concluded that HDL₂C was better, but altered their conclusions based on additional data [44].

**Apolipoprotein A-I.** Automated assay kits for apo A-I are available with standardized calibrators [46], but it is unclear that results obtained by all methods give comparable results. While some studies indicated that apo A-I is a better marker for CAD than HDL₃C [3,4,47], other studies showed no difference between HDL₃C and apo A-I [43,48,49]. An important reason for this disagreement may be that the antigenic sites of apo A-I are difficult to access by immunological methods, because as much as 75% is hidden by lipids [50]. The problems of immunological non-reactivity and diagnostic inaccuracy seem related. Studies indicated that a detergent, Tween 20, was effective for exposing the antigenic groups of apo A-I [4,51,52]. We showed that treatment with Tween 20 substantially and significantly improved the discriminating power with one assay kit, but caused only small improvement with another kit [8]. After treatment with Tween 20, the apo A-I results obtained by both kits were better than HDL₃C as a marker for CAD. We concluded that the nature of the detergents used is important in ensuring optimal diagnostic performance with apo A-I testing. Recent studies suggest that optimized apo A-I may be better than lipoprotein lipids for predicting recurrent CAD events in patients treated with statin therapy [53] and may predict CAD in persons without other major risk factors [54].

**Apo A-II and apo E.** Based on current thinking regarding RCT, neither apo A-II nor apo E would be expected to be good markers for CAD. Apo A-II inhibits cholesterol uptake from tissue, and LpA-I generally appears to be a better marker for CAD [55]. Although the role of apo E in RCT is unclear, apo E may possibly facilitate cholesterol uptake by the liver. Only a small fraction of the total apo E is found in HDL, so even if HDL-apo E proved to be a useful marker, it would be necessary to develop new tests to measure HDL-apo E. Both of these apolipoproteins appear inferior to apo A-I or HDL₃C as a risk marker [42,43,56].

**Table 4.** Anti-arteriosclerotic activities other than reverse cholesterol transport (RCT) that are attributed to HDL.

- Inhibits LDL oxidation (paraoxidase)
- Anticoagulant activity
- Inhibits platelet thrombosis
- Antiviral activity
- Inhibits membrane lysis
- Inhibits expression of adhesion molecules
- Stabilizes endothelial cell integrity

**Other mechanisms by which HDL may act.** Besides RCT, other potential anti-atherogenic mechanisms have been attributed to HDL [18,57]. Some of these are outlined in Table 4. The antioxidant activity of HDL has been well studied. Lipid peroxide accumulation due to copper-mediated oxidation of LDL was shown to be reduced in the presence of HDL [58]. Apo A-I and LCAT were also found to have some antioxidant potential, and paraoxynase-1 (PON1), an aryldialkylphosphatase, had substantially more.

PON1 is a enzyme (43 kDa, 354 amino acids) that splits organophosphate compounds [59]. It is synthesized in the liver and is largely associated with HDL. The ability of PON1 to protect LDL from oxidation involves a cysteine residue at position 283 and is separate from its organophosphatase activity. PON1 has two amino acid polymorphisms—one at position 55 (L→M) and the other at 192 (Q→R). Although it was thought that these polymorphisms are associated with CAD, recent evidence indicates this may not be so [60]. A mechanism was suggested whereby HDL diminishes the accumulation of lipid peroxides by enzymatic hydrolysis of phospholipid hydroperoxides from unsaturated fatty acids of LDL. This produces fragments that may be bound to HDL; such a mechanism would remove toxic products from LDL and inhibit autooxidation [59]. It is uncertain whether non-RCT mechanisms are clinically important. For example, paraoxonase is decreased in persons with Tangier disease, yet these persons have a lower frequency of CAD than would be expected [26].
Discussion

In the present study, ROC analysis indicated that HDL₃C is nearly equal to apo A-I in predicting the risk of CAD, whereas discriminant analysis, which examined a more diverse group of patients, indicated that apo A-I is a more powerful marker than HDL-C or its subfractions. Consequently, the data presented here point to apo A-I as the superior marker for assessing CAD risk. This conclusion is supported by other clinical studies using optimized apo A-I assays [3,4,47,53,54].

This conclusion is also supported by our current understanding of RCT mechanisms (Fig. 3). Apo A-I appears largely responsible for cholesterol uptake from the ABCA1 transporter. Apo A-I activates LCAT, recycling of spherical particles involves the splitting off of apo A-I, and LpAI particles promote cellular cholesterol efflux, while LpAI:LpAII do not. Yet apo A-I gave an ROC curve area of 0.63, which provides poor diagnostic accuracy [10]. This result is surprising, in view of the widespread participation of apo A-I in RCT. Consequently, despite the recent advances in understanding HDL metabolism, some important mechanisms still need to be elucidated.

Among these, the role of LpAI:LpAII particles remains unclear. It has been suggested that measuring apo A-I in LpAI particles, but not LpAI:LpAII, may enhance diagnostic discrimination [55]. Tall et al [61] suggested that the flux of cholesterol from macrophage foam cells is the critical index in RCT. An increase of apo A-I, HDL-C, or ABCA1 that leads to decrease in foam cell cholesterol would be only a small change in the overall transport of cholesterol between HDL particles and peripheral tissues. This “macrophage cholesterol efflux” would be too slight to be detected by ordinary methods [61]. The flux of cholesterol through the CETP pathway and its relationship to HDL metabolism is unclear. Men with null mutations in the CETP gene have massively elevated HDL levels and heterozygotes have moderately elevated levels. Men with very high HDL levels showed reduced risk of heart disease irrespective of the mutation, but those with moderately elevated levels showed increased risk [27]. Consequently, it appears that understanding the exact interactions between the pathways may be important, and it may be necessary to measure exact fluxes of different components.

In practice, the problem of poor discrimination of markers for CAD can be moderated by assessing several risk factors at once. The additional inclusion of the Framingham risk factors (age, sex, blood pressure, glucose intolerance, and smoking) significantly improved the area under the ROC curve, compared to that obtained using only HDL-C and LDL-C [62]. With a computer-assisted model, the area under the ROC curve was increased above 0.8. The recent report of the Adult Treatment Panel-III (ATP-III) of the National Cholesterol Education Program estimates the 10-yr risk of CAD on the basis of age, smoking status, and blood pressure, along with HDL-C and total cholesterol [63].

Apo B is a better predictor of coronary risk than total cholesterol or LDL-C [64-66]. Theoretically, this would be expected because apo B is found in all fractions of atherogenic lipoproteins; apo B is better than LDL-C as a measure of the number of particles of potentially atherogenic small, dense LDL [63,65,66]. Despite the less than desirable area under the ROC curve for apo A-I/Apo B (Fig. 1), shifting from the use of HDL-C and total cholesterol or LDL-C to the use of apo A-I and apo B would improve diagnostic sensitivity by 13-14% at false positive frequencies of 15-20%. This would identify thousands of additional persons at risk.

There has recently been a search for markers to identify more persons at high risk for CAD [67]. Because of evidence that links inflammation to arteriosclerosis, inflammatory markers have been examined [67-69]. Acute phase proteins, especially C-reactive protein (CRP), have been suggested as a marker [68-70], although their linkage to arteriosclerosis is far less clear than apo A-I and apo B. In one study, the addition of CRP to the ratio of total cholesterol/HDL-C increased the ROC curve area from 0.64 to 0.68 [71]. In the present study, substitution of apo A-I/apo B for HDL-C/total cholesterol (Table 1) increases the ROC area from 0.62 to 0.70. This is a comparable or greater increase than that reported by addition of CRP.

The present author suggests that a collaborative study should be initiated to compare apo A-I and apo B kits from different manufacturers, to define...
the conditions for optimal antigen exposure and performance, and to normalize methods if necessary. This would be a simple way to improve discrimination since the procedures are widely available and only two tests are required. In the future, when more information is known about the value of inflammatory markers, a further evaluation could be performed to see if the addition of a third test (e.g., CRP) enhances the discrimination of CAD risk.

Acknowledgments

This work was supported by the Department of Veterans Affairs and by a generous gift of reagents from Beckman Instruments, Inc.

References


