Consequently, levels of HDL-C are factored into many cardiovascular risk assessments, and HDL has been intensively pursued as a secondary goal for risk reduction after low-density lipoprotein cholesterol (LDL-C) lowering. The belief that levels of HDL-C have a causal relationship to the prevention of CAD has been referred to as the HDL cholesterol hypothesis.3

Approach and Results—Fifty-five subjects with very high HDL-C (mean, 86 mg/dL) and onset of CAD at the age of ≈60 years with no known risk factors for CAD (cases) were identified through systematic recruitment. A total of 120 control subjects without CAD, matched for race, sex, and HDL-C level (controls), were identified. In all subjects, HDL composition was analyzed and HDL cholesterol efflux capacity was assessed. HDL phospholipid composition was significantly lower in cases (92±37 mg/dL) than in controls (109±43 mg/dL; P=0.0095). HDL cholesterol efflux capacity was significantly lower in cases (1.96±0.39) than in controls (2.11±0.43; P=0.04).

Conclusions—In people with very high HDL-C, reduced HDL phospholipid content and cholesterol efflux capacity are associated with the paradoxical development of CAD. (Arterioscler Thromb Vasc Biol. 2015;35:1515-1519. DOI: 10.1161/ATVBAHA.115.305504.)

Key Words: ABC transporters • coronary artery disease • high-density lipoprotein cholesterol

Plasma high-density lipoprotein cholesterol (HDL-C) levels are strongly inversely correlated with the incidence of coronary artery disease (CAD).1 It has been estimated that for each milligrams per deciliter increase in HDL-C, the risk of cardiovascular events is decreased by 2% to 3%.2 Consequently, levels of HDL-C are factored into many cardiovascular risk assessments, and HDL has been intensively pursued as a secondary goal for risk reduction after low-density lipoprotein cholesterol (LDL-C) lowering. The belief that levels of HDL-C have a causal relationship to the prevention of CAD has been referred to as the HDL cholesterol hypothesis.3

There have been recent challenges to the HDL-C hypothesis. Common variations associated with small changes in HDL levels are not associated with protection from coronary disease, in contrast to variants that affect LDL-C and triglycerides.4,5 Recently, several clinical trials using agents that raise HDL-C have failed to show any clinical benefit. In the dal-OUTCOMES trial of the cholesteryl ester transfer protein inhibitor dalcetrapib, patients received dalcetrapib in addition to other agents that lower LDL-C. Although a significant elevation in HDL-C levels was noted in patients treated with dalcetrapib, the trial was terminated because of futility of the study.6 The HPS2-THRIVE trial was designed to assess cardiovascular outcomes in patients treated with extended release niacin and laropiprant, an antiflushing agent, in addition to a statin. However, HPS2-THRIVE missed its primary end point of reducing the risk of myocardial infarction, stroke, or coronary revascularizations compared with statin therapy alone.7 These studies have fueled the debate about a causal role of HDL-C in heart disease, and whether raising HDL-C levels is a viable therapeutic strategy.

HDL has several properties that may offer protection against CAD, including its role in promoting cholesterol efflux and reverse cholesterol transport.8 Genetic and pharmacological manipulations of HDL that increase reverse cholesterol transport in animal models are generally protective against atherosclerosis.9 However, HDL-C concentration does not always reflect its functionality. For example, even after controlling for HDL-C the cholesterol efflux capacity of HDL was inversely
associated with prevalent carotid and coronary atherosclerosis\textsuperscript{10} and with incident cardiovascular events.\textsuperscript{11}

Extremely high HDL-C levels are generally associated with reduced risk of CAD. However, an unusual phenotype is that of very high HDL-C with development of CAD in the absence of traditional risk factors. We hypothesized that these individuals have altered composition and reduced function of their HDL that may predispose them to increased risk of CAD. We systematically recruited individuals with very high HDL both with and without CAD and compared the composition and function of HDL. We found that the HDL from high HDL-C subjects with CAD had reduced phospholipid content and reduced cholesterol efflux capacity when compared with the HDL from high HDL-C subjects without CAD.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Clinical Characteristics and Plasma Lipids and Apolipoproteins

The clinical characteristics of the 55 cases with high HDL-C and CAD and the 120 matched controls with high HDL-C and no CAD are shown in Table 1. Mean age was 64±11 years for the cases and 69±12 years for the controls with ±40% of the subjects being women. The mean age of onset of CAD was ≈60 years in the cases for both men and women, although this was not reliably ascertained in all subjects.

Plasma lipid and apolipoprotein values for the cases and controls are depicted in Table 2. There was no difference in the mean HDL-C between the cases and controls as they were matched for HDL-C level by study design. Triglyceride levels were also not different between the 2 groups. LDL-C was matched for HDL-C level by study design. Triglyceride levels between the cases and controls as they were matched for HDL-C level by study design. Triglyceride levels were also not different between the 2 groups. LDL-C was not reliably ascertained in all subjects.

Table 1. Basic Demographics

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>55</td>
<td>120</td>
<td>...</td>
</tr>
<tr>
<td>Age (mean±SD), y</td>
<td>64±11</td>
<td>69±12</td>
<td>0.07</td>
</tr>
<tr>
<td>Female, %</td>
<td>36.4</td>
<td>40</td>
<td>0.65</td>
</tr>
<tr>
<td>Black, %</td>
<td>3.6</td>
<td>6.1</td>
<td>0.95</td>
</tr>
<tr>
<td>BMI</td>
<td>24.6±3.5</td>
<td>24.4±3.3</td>
<td>0.81</td>
</tr>
<tr>
<td>Mean age of onset of CAD (% reported)</td>
<td>60 (44)</td>
<td>NA</td>
<td>...</td>
</tr>
<tr>
<td>Statin users,* %</td>
<td>80</td>
<td>32</td>
<td>...</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; CAD, coronary artery disease; and NA, not applicable.

*A total of 64% of cases and 79% of controls provided information on statin usage.

Table 2. Plasma Lipids and Apolipoproteins

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
<th>PVal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides</td>
<td>80±34</td>
<td>85±38</td>
<td>0.34</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>201±47</td>
<td>228±37</td>
<td>0.0003</td>
</tr>
<tr>
<td>Total phospholipid</td>
<td>253±55</td>
<td>274±52</td>
<td>0.017</td>
</tr>
<tr>
<td>HDL-C</td>
<td>86±21</td>
<td>86±20</td>
<td>0.97</td>
</tr>
<tr>
<td>LDL-C</td>
<td>97±38</td>
<td>125±33</td>
<td>0.00016</td>
</tr>
<tr>
<td>ApoAI</td>
<td>195±42</td>
<td>194±40</td>
<td>0.91</td>
</tr>
<tr>
<td>ApoAII</td>
<td>43±11</td>
<td>40±15</td>
<td>0.40</td>
</tr>
<tr>
<td>ApoCIII</td>
<td>15±5</td>
<td>13±5</td>
<td>0.89</td>
</tr>
<tr>
<td>ApoE</td>
<td>5±2</td>
<td>6±2</td>
<td>0.046</td>
</tr>
<tr>
<td>GlycA</td>
<td>332±65</td>
<td>319±61</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Lipid and apolipoprotein parameters are mg/dL. Apo indicates apolipoprotein; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; and Lp(a), lipoprotein.

*Data reported as median.

HDL Cholesterol Efflux Capacity, Cholesterol Esterification Rate, and Phospholipid Transfer Protein Activity

The capacity of HDL to promote cholesterol efflux from J774 macrophages in the presence and absence of cAMP is shown in Table 4. After adjusting for age, sex, and BMI, total HDL cholesterol efflux capacity was significantly lower in cases than in controls (P=0.03). The ratio of cholesterol efflux/HDL-C was also significantly lower in cases (P=0.006). Furthermore, cAMP-inducible cholesterol efflux capacity was significantly lower in cases (P=0.025). HDL-PL was a significant predictor of total cholesterol efflux capacity (P=0.009; slope 0.0025; R$^2$ 0.06). No differences were observed between
Recently, Rohatgi et al\textsuperscript{11} showed that HDL efflux capacity was associated with prevalent carotid and coronary atherosclerosis even after adjusting for HDL-C, a finding confirmed by Li et al.\textsuperscript{13} These findings add to the growing body of data linking HDL composition and function to clinical cardiovascular disease as distinct from HDL-C concentrations.

In this study, we investigated a paradoxical phenotype, extremely high HDL-C associated with CAD. We hypothesized that individuals with this phenotype have altered HDL composition and function that put them at greater risk for CAD in the setting of high HDL-C. We found that individuals with very high HDL-C and CAD have reduced levels of HDL phospholipids and reduced HDL cholesterol efflux capacity. These findings add to the growing body of data linking HDL composition and function to clinical cardiovascular disease as distinct from HDL-C concentrations.

Cholesterol efflux is the first step of the reverse cholesterol pathway that can be assessed ex vivo by a method first developed by Rothblat and colleagues.\textsuperscript{12} Khera et al\textsuperscript{10} demonstrated that HDL cholesterol efflux capacity was inversely associated with prevalent carotid and coronary atherosclerosis even after adjusting for HDL-C, a finding confirmed by Li et al.\textsuperscript{13} Recently, Rohatgi et al\textsuperscript{14} showed that HDL efflux capacity was inversely associated with incident cardiovascular events after adjusting for HDL-C. Similarly, Hafiane et al\textsuperscript{14} and Shao et al\textsuperscript{15} have demonstrated impaired cholesterol efflux capacity in acute coronary syndrome. In contrast, Li et al\textsuperscript{13} reported a positive association of cholesterol efflux capacity with incident cardiovascular events in an angiographic cohort. Thus, there remain questions about the relationship of efflux capacity to cardiovascular disease.

The HDL from subjects with high HDL-C and CAD was reduced in phospholipid content. An inverse relationship has been noted between HDL-PL and the CAD.\textsuperscript{16} Furthermore, HDL-PL composition has been positively associated with cholesterol efflux capacity.\textsuperscript{17–21} In our study, HDL-PL was a significant predictor of total cholesterol efflux capacity. Taken together, the reduction in HDL-PL levels may play a causative role in the reduced cholesterol efflux capacity of HDL. The reduced HDL-PL was not associated with a difference in HDL subfraction particle numbers or size. A more thorough lipidomic analysis of the HDL particles is of interest but outside the scope of this study.

Lecithin-cholesterol acyltransferase hydrolyzes HDL phospholipids and could influence HDL-PL content as well as potentially cholesterol efflux capacity. We measured the cholesterol esterification rate as an assay of endogenous lecithin-cholesterol acyltransferase that is also influenced by the endogenous lipoproteins, but found no evidence of a difference between cases and controls. Prior studies have demonstrated an inverse association between phospholipid transfer protein activity and cholesterol efflux capacity.\textsuperscript{22} We measured phospholipid transfer protein activity and found no significant differences between the 2 groups. One limitation inherent in the phospholipid transfer protein activity assay is that it measures transfer between synthetic donor and acceptor particles, rather than between native lipoproteins where lipid and protein composition may influence activity. It is possible that increased phospholipase activity (ie, hepatic and endothelial lipase) may underlie the lower phospholipid content in the HDL of these patients, but measurement of these lipases requires postheparin plasma, which was not available.

A limitation of this study is incomplete data on medical history (such as date of onset of CAD) and lack of prospective data. To minimize this problem, we selected controls that were either the same age or older than the cases. Furthermore, prior studies have demonstrated that CAD can reduce cholesterol efflux capacity.\textsuperscript{14,15} Another limitation in our study is the assessment of only one of the major functions of HDL. HDL is a heterogeneous particle that has additional antioxidant and anti-inflammatory functions, as well as potentially cholesterol efflux capacity. We measured the cholesterol esterification rate as an assay of endogenous lecithin-cholesterol acyltransferase that is also influenced by the endogenous lipoproteins, but found no evidence of a difference between cases and controls. Prior studies have demonstrated an inverse association between phospholipid transfer protein activity and cholesterol efflux capacity.\textsuperscript{22} We measured phospholipid transfer protein activity and found no significant differences between the 2 groups. One limitation inherent in the phospholipid transfer protein activity assay is that it measures transfer between synthetic donor and acceptor particles, rather than between native lipoproteins where lipid and protein composition may influence activity. It is possible that increased phospholipase activity (ie, hepatic and endothelial lipase) may underlie the lower phospholipid content in the HDL of these patients, but measurement of these lipases requires postheparin plasma, which was not available.

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as unknown differences in lipidomic and proteomic compositions between the cases and controls, which when all considered together may give a more comprehensive picture of HDL functionality.

The significant differences in LDL-C and apoB levels between the cases and controls are attributed to higher use of statins in the cases than in the controls. Of the subjects who provided information on statin usage, 80% of the cases and 32% of controls reported being on statins. Recent studies have shown conflicting data on the effect of statins on cholesterol efflux capacity making it difficult to assess the nature of their effect on our study population.10–23 However, it is unlikely that statins from the polyethylene glycol precipitated supernatants would have a substantial effect on the cells during a 2-hour efflux assay. Recently, Miyamoto-Sasaki et al27 determined cholesterol efflux capacity in dyslipidemic patients before and after treatment with pitavastatin. The statin increased serum HDL-C levels, HDL-PL levels, and enhanced cholesterol efflux capacity, suggesting it is unlikely that statins decreased the HDL-PL content and efflux capacity of the cases. In fact, it is possible that the observed differences may actually have been greater if measurements were taken before statin therapy.

In conclusion, individuals with the paradoxical phenotype of very high HDL-C and CAD were found to have taken before statin therapy. The enhanced cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. N Engl J Med. 2011;364:127–135. doi: 10.1056/NEJMo1001669.


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The present study describes characterization of the high-density lipoprotein (HDL) in individuals with a paradoxical phenotype of very high HDL cholesterol and coronary artery disease. We demonstrate that the HDL from these individuals has reduced HDL phospholipid content and reduced cholesterol efflux capacity compared with individuals with comparably high HDL cholesterol but no coronary artery disease. These data add to the growing body of data suggesting that HDL quality, not quantity, is important in influencing risk of coronary artery disease.

Significance
High-Density Lipoprotein (HDL) Phospholipid Content and Cholesterol Efflux Capacity Are Reduced in Patients With Very High HDL Cholesterol and Coronary Disease
Anandita P. Agarwala, Amrith Rodrigues, Marjorie Risman, Mary McCoy, Kevin Trindade, Liming Qu, Marina Cuchel, Jeffrey Billheimer and Daniel J. Rader

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**Material and Methods:**

Materials and Methods are available in the online-only Data Supplement

**Study design:** Patients included in this study were selected from the following studies at the University of Pennsylvania: University of Pennsylvania (UPenn) High HDL Cholesterol Study (HHDL), UPenn Catheterization cohort (PennCATH), High HDL and CHD study (HCAD), and Philadelphia Area Metabolic Syndrome Network (PAMSyN). HHDL is a cross-sectional study of genetic factors contributing to elevated HDL-C levels. Individuals with HDL-C levels ≥ 90th percentile for age, race, and gender were identified by physician referrals or through the Hospital of the UPenn clinical laboratory. PennCATH is a study of subjects undergoing coronary angiography at UPenn Health System hospitals and has been previously described. HCAD is a cross-sectional, observational study of subjects with HDL-C > 90th percentile and coronary heart disease. PAMSyN is a cross-sectional study of individuals with varying numbers of metabolic syndrome criteria, from none to all 5.

We identified patients that previously participated in clinical studies at the University of Pennsylvania and selected individuals with HDL-C levels above the 90th percentile with documented cardiovascular disease defined as either a history of heart attack, angioplasty, coronary artery bypass surgery, coronary calcium score above the 90th percentile, or greater than 50% stenosis on CT angiogram. We excluded subjects with plasma LDL-C level greater than 190 mg/dL, triglycerides greater than 400 mg/dL, diabetes (type I and type II), history of liver disease with LFTs greater than twice the upper limit of normal, history of kidney disease or chronic renal insufficiency, and use of medications known to significantly affect HDL-C levels, specifically including niacin doses greater than 1500 mg daily. Controls were selected based on the absence of CAD and matched to cases for race, gender, and HDL-C level within 10 mg/dL. Controls were selected to be the same age or up to 10 years older than the cases. Additional exclusion criteria for controls included history of stroke, transient ischemic attack, and history of abdominal aortic aneurysm. Cases were included retrospectively and data (i.e. medications, age of onset of coronary disease) was self-reported via a survey.

**Lipid and apolipoprotein measurements:** Plasma concentrations of total cholesterol, HDL-C, triglycerides, and apolipoproteins were measured using blood samples obtained after a minimum of an 8-hour fast using CDC-standardized methods. Measurements were performed on frozen (−80°C) EDTA plasma and serum. The Friedewald equation was used to determine the amount of LDL-C. HDL phospholipid (HDL-PL) was determined by phosphotungstinate precipitation of LDL and VLDL and subsequent measurement of the phospholipid component in the HDL particles in the remaining supernatant. Non HDL-PL was calculated by subtracting the HDL-PL from the total phospholipid content.

**Radiolabeled cholesterol efflux:** Cholesterol efflux capacity was measured in patient samples. J774 mouse macrophage cells were plated and labeled with 2µCi of ³H cholesterol per milliliter overnight. Cells were then incubated for 6 hours in either the
presence or absence of 0.3 mM 8-(4-chlorophenylthio)-cyclic AMP, an upregulator of ATP-binding cassette transporter-1 (ABCA1). ApoB containing proteins were removed from plasma by polyethylene glycol precipitation. Efflux media containing the equivalent of 1% apolipoprotein B–depleted serum or plasma was then incubated for 2 hours at 37°C. Each patient sample was run in duplicate in both the presence and the absence of cyclic AMP (cAMP). Media was collected and radioactivity determined by liquid scintillation counting after passing through a 0.22 µM filter. Efflux to media without serum was used as a baseline control. The quantity of radioactive cholesterol incorporated into cellular lipids was determined after isopropanol extraction. Percent efflux was calculated by the formula: \( \frac{(\text{cpm of } ^3\text{H cholesterol in the media} - \text{cpm of } ^3\text{H cholesterol in serum free media})}{(\text{cpm of } ^3\text{H cholesterol in the cells} + \text{cpm of } ^3\text{H cholesterol in the media})} \times 100 \). A pooled plasma control was included on each plate to which samples from patients were normalized. - mediated cholesterol efflux capacity was determined by subtracting the basal cholesterol efflux capacity (without cAMP) from the total cholesterol efflux capacity (with cAMP).

**Cholesterol esterification rate:** Patient plasma or serum samples were equilibrated overnight at 4°C in the presence of \( ^3\text{H} \) cholesterol in duplicates. Samples were then incubated at 37°C for two hours. Ethanol was added to each sample to terminate the esterification reaction. Free cholesterol and cholesterol ester fractions were subsequently separated by column chromatography. Liquid scintillation counting was used to determine radioactivity in both the free cholesterol and the cholesterol ester fraction of the sample. Cholesterol esterification rate (nmol/hr/ml) = (Free cholesterol concentration) x (% free cholesterol esterified). Each sample was run in duplicates, and a control plasma sample was included in each assay, to which all of the patient samples were normalized to control for inter-assay variation.

**Phospholipid transfer protein (PLTP) activity:** PLTP activity was measured using the Kamiya Biomedical PLTP activity assay (Cat. No. KT-206).

**Nuclear Magnetic Resonance (NMR) spectroscopy:** Particle size and GlycA were measured by NMR spectroscopy using the LipoProfile-3 algorithm at LipoScience, Inc. (Raleigh, NC). VLDL, LDL, IDL, and HDL subclasses of different size were quantified from the amplitudes of their spectroscopically distinct lipid methyl group NMR signals. Large HDL particle subclass diameters range from 9.4 nm to 14 nm, medium HDL particle subclass diameters range from 8.2 nm to 9.4 nm, and small HDL particle subclass diameters range from 7.3 nm to 8.2 nm.

**Statistical Analysis:**

Data was examined using descriptive statistics. Continuous variables were summarized by the min, max, mean, median, and standard error. Categorical variables were summarized by frequency and percentage. Data was modeled using a logistic regression with the case/control as binary response variables. The R software was used for the analysis and the p value was evaluated for each predictor variable. The analysis was performed without any covariate adjustment.
References:


