Influence of HDL particles on cell-cholesterol efflux under various pathological conditions

Bela F. Asztalos, Katalin V. Horvath, Michael Mehan, Yuya Yokota, and Ernst J. Schaefer

Cardiovascular Nutrition Laboratory, Human Nutrition Research Center on Aging at Tufts University; Boston Heart Diagnostics

Abstract It has been reported that low cell-cholesterol efflux capacity (CEC) of HDL is an independent risk factor for CVD. To better understand CEC regulation, we measured ABCA1- and scavenger receptor class B type I (SR-BI)-dependent cell-cholesterol efflux, HDL anti-oxidative capacity, HDL particles, lipids, and inflammatory- and oxidative-stress markers in 122 subjects with elevated plasma levels of triglyceride (TG), serum amyloid A (SAA), fibrinogen, myeloperoxidase (MPO), or \( \beta \)-sitosterol and in 146 controls. In controls, there were strong positive correlations between ABCA1-dependent cholesterol efflux and small pre\( \beta \)-1 concentrations (\( R^2 = 0.317 \)) and SR-BI-dependent cholesterol efflux and large (\( \alpha \)-1 + \( \alpha \)-2) HDL particle concentrations (\( R^2 = 0.774 \)). In high-TG patients, both the concentration and the functionality (pre\( \beta \)-1 concentration-normalized ABCA1 efflux) of pre\( \beta \)-1 particles were significantly elevated compared with controls; however, though the concentration of large particles was significantly decreased, their functionality (large HDL concentration-normalized SR-BI efflux) was significantly elevated. High levels of SAA or MPO were not associated with decreased functionality of either the small (pre\( \beta \)-1) or the large (\( \alpha \)-1 + \( \alpha \)-2) HDL particles. HDL anti-oxidative capacity was negatively influenced by high plasma \( \beta \)-sitosterol levels, but not by the concentrations of HDL particles, TG, SAA, fibrinogen, or MPO. Our data demonstrate that under certain conditions CEC is influenced not only by quantitative (concentration), but also by qualitative (functional) properties of HDL particles.—Asztalos, B. F., K. V. Horvath, M. Mehan, Y. Yokota, and E. J. Schaefer. Influence of HDL particles on cell-cholesterol efflux under various pathological conditions. J. Lipid Res. 2017. 58: 1238–1246.

Supplementary key words pre\( \beta \)-1 • cardiovascular disease risk • \( \beta \)-sitosterol • adenosine triphosphate binding cassette transporter A1 • scavenger receptor class B type I • apolipoprotein A1

In the last several decades, cross-sectional and intervention trials have indicated that HDL cholesterol (HDL-C) concentration is inversely associated with CVD risk (1–3). Although a large spectrum of HDL functions has been described, the underlying mechanisms of how HDL protects against CVD are not fully understood. Moreover, randomized drug-intervention trials have failed to prove that increasing HDL-C level decreases CVD risk (4–6). It has been shown that apoA-I concentration in individual HDL subpopulations is superior to HDL-C concentration in predicting CVD risk (7–10). Recently, several HDL functionality markers, most importantly the cell-cholesterol efflux capacity (CEC) of HDL, have emerged as better CVD-risk markers than HDL-C and/or apoA-I concentration. Several studies reported that, independent of HDL-C level, HDL CEC had a strong inverse association with both carotid intima-media thickness and the likelihood of angiographic coronary artery disease (11–13). Unfortunately, CEC studies are hard to compare due to methodological differences. In addition, although HDL removes cholesterol from cells by multiple mechanisms [the two major pathways are the ABCA1- and the scavenger receptor class B type I (SR-BI)-mediated pathways], in most studies, only ABCA1-dependent cholesterol efflux was measured.

We have previously documented that cell-cholesterol efflux via the ABCA1 pathway has a strong positive association with the small pre\( \beta \)-1 HDL particles, while cell-cholesterol efflux via the SR-BI pathway has strong positive association with the large \( \alpha \)-1 and \( \alpha \)-2 HDL particles (14). It is worth noting that, in vivo, net cholesterol flux depends not only on acceptors (e.g., HDL particles), but also on...
the expression of cell-surface proteins, including ABCA1 and SR-BI. ABCA1 mediates cholesterol efflux from cells to acceptors, while SR-BI mediates bi-directional lipid flux. apoA-I, one of the building blocks of HDL, is produced in the liver and intestine. Liver cells highly express both ABCA1 and SR-BI surface proteins. Macrophages, the precursors of foam cells and fatty streaks in the vessel wall, also express both ABCA1 and SR-BI. It has been documented that coronary heart disease (CHD) patients have significantly higher concentrations of preβ-1 particles than controls and that these particles have a strong positive correlation with ABCA1-dependent efflux (15–18). Considering these results, one would expect that CHD patients would have higher CEC via ABCA1 than healthy subjects.

In this study, we tested the hypothesis that HDL CEC depends not only on quantitative properties (i.e., the concentration), but also on qualitative properties (i.e., the functionality) of HDL particles. We measured cell-cholesterol efflux via the ABCA1 and SR-BI pathways and calculated HDL particle concentration-normalized efflux capacities in control subjects and in subjects with a wide range of pathological conditions [high plasma levels of triglyceride (TG), serum amyloid A (SAA), fibrinogen, myeloperoxidase (MPO), or β-sitosterol] in order to assess the influence of these conditions on HDL subpopulation profile and HDL functionality.

MATERIALS AND METHODS

Study population

The study protocol was approved by the Institutional Review Board of Tufts University. We studied 269 subjects: 91 male and 55 female control subjects selected with no history of CVD, LDL cholesterol (LDL-C) <200 mg/dl, and TG <200 mg/dl; 30 subjects selected with elevated plasma TG level (>300 mg/dl); 30 subjects with elevated MPO level (>500 pmol/l); 28 subjects with increased SAA level; 22 subjects with increased β-sitosterol level (>3.5 μg/ml); and 13 subjects with increased fibrinogen (>300 mg/dl), but not SAA, level. It is worth noting that significantly elevated levels of these parameters were associated with unique/abnormal HDL subpopulation profiles (described in Fig. 1), which were also accounted for in the selection of patients.

Laboratory measurements

Plasma and serum samples were collected after an overnight fast. Laboratory measurements were conducted either in fresh samples (within 48 h of blood draw) or in samples stored at −80°C until analysis. Blood chemistries were determined by standard methods at Boston Heart Diagnostics (Framingham, MA) in plasma or serum samples, as required by the specific protocols. Total cholesterol (TC), TG, LDL-C, HDL-C, apoA-I, apoB, and high-sensitive C-reactive protein (hsCRP) were measured on a COBAS 8000 analyzer using kits from Roche (Indianapolis, IN). Small-dense LDL-C (sdLDL-C) was measured using kits from Denka-Seiken (Tokyo, Japan). Fibrinogen was measured using immunoturbidimetric kits from Medtest (Cortland Manor, NY). MPO was measured on a Dimension clinical chemistry system using kits from Siemens (Newark, DE). SAA was measured by ELISA (Invitrogen, Thermo Fisher) at Tufts University. HDL subpopulation profile was measured in plasma by 2D native gel electrophoresis, immunoblot, and image analysis, as described (16, 19) and shown in supplemental Fig. S1. Noncholesterol sterols in plasma were measured by GC/MS as described (20). In samples with high β-sitosterol, apoB-containing lipoproteins (d ≤ 1.063 g/ml), HDL2 (d = 1.063–1.125 g/ml), and HDL4 (d = 1.125–1.21 g/ml) were separated by ultracentrifugation (UC) and β-sitosterol was measured in each fraction.

HDL cell-cholesterol efflux and anti-oxidative (anti-ox) capacities were measured at Vascular Strategies (Plymouth Meeting, PA) using methods previously described (11, 14, 21). In short, ABCA1-dependent cell cholesterol efflux was measured in J774 cells radiolabeled with 2 μCi of 3H-cholesterol per milliliter. ABCA1 was upregulated by 6 h incubation with 0.3 mM of 8-(4-chlorophenylthio)-cyclic-AMP. Medium containing 2.8% apoB-depleted serum was then added for 4 h to cAMP-stimulated

Fig. 1. apoA-I-containing HDL subpopulation profile of a representative subject from each control and affected group. Compared with males, females have higher concentrations of large particles (α-1 and α-2) and lower concentration of smaller particles (α-3 and α-4). High TG level is associated with reduced levels of large particles (α-1 and α-2), increased levels of small particles (α-3 and preβ-1), and the appearance of larger preβ particles (prefix, marked by *). High SAA level is associated with an HDL particle profile dominated by α-2 particles. Subjects with high fibrinogen level have apoA-I primarily in a smaller than normal size α-2 subpopulation and have multiple-size preβ-1 particles. Subjects with a high MPO level do not have a signature HDL subpopulation profile. High β-sitosterol level is associated with disproportionally increased apoA-I concentration in the large α-1 particles compared with the rest of HDL particles.
and unstimulated cells. Liquid scintillation counting was used to quantify the effluxed radio-labeled cholesterol to the medium. Total (global) efflux was defined as the efflux measured from cAMP-stimulated J774 cells. Non-ABCA1-specific (basal) efflux was measured from unstimulated J774 cells. ABCA1-specific efflux was calculated as the difference between cAMP-stimulated (total) and unstimulated (non-ABCA1) cells. SR-BI-mediated cell-cholesterol efflux was measured as the fraction of radio-labeled cholesterol released from radio-labeled Fu5AH cells to the medium containing 2.8% apoB-free serum. Efflux data are expressed as percent cholesterol efflux per 4 h.

In the efflux assays, cells had to be incubated with serum. For the high-TG, high-SAA, high-fibrinogen, and high-β-sitosterol groups and their controls, we had only plasma samples available for the efflux assays. In these cases, plasma was converted to serum by adding 25 mM CaCl₂ to plasma for clotting, and then the clot was removed by low-speed centrifugation.

HDL inflammatory index (HII) was measured to assess the ability of apoB-depleted plasma or serum to inhibit or enhance the oxidation of LDL in the presence of a fluorescent organic substrate (21). HII measurements were expressed in arbitrary units. HDL anti-ox capacity is the inverse of HII; values >1.0 indicate that HDL has anti-oxidative. Vascular Strategies indicated that there are significant differences between measurements of HDL anti-ox capacity in plasma and serum. In the case of the high MPO group, we only had access to serum samples; therefore, we provided serum samples for the efflux and oxidation assays for the controls for this group as well.

To correct for inter-assay variability, a pool of human serum was tested in parallel with the test samples in every assay. For inter-day comparability, measurements were normalized to a standard pool.

**Statistical analysis**

The statistical significance of group differences was assessed using a linear model with gender as a covariate. All correlations reported are Pearson correlation coefficients. Regression analysis was performed using Deming regression to avoid biasing the fit toward the errors of one of the test results. All analyses were performed using the R Language for Statistical Computing version 3.2.2.

## RESULTS

### Cell-cholesterol efflux and HDL particles in control subjects

We summarized data on global, basal, ABCA1-dependent, and SR-BI-dependent cell-cholesterol efflux in control subjects (n = 146) in supplemental Fig. S2. ABCA1-dependent efflux, calculated as the difference between global and basal efflux from J774 cells, was very strongly correlated with global efflux (r² = 0.879), but not with basal efflux (r² = 0.140). There was a very strong correlation between basal and SR-BI-dependent efflux (r² = 0.808), although they were measured in two different cell lines (J774 and Fu5AH, respectively). Global efflux was modestly associated with SR-BI-dependent efflux (r² = 0.243).

The characteristics of the control group are shown in Table 1. In supplemental Table S1, we compared the controls by gender. There were no significant differences between males and females in the concentration of preβ-1 HDL particles, but there were significant differences in the concentrations of HDL-C, apoA-I, α-1, α-2, and α-4 HDL particles. Mean total and preβ-1-normalized efflux rates via the ABCA1 pathway were similar between the two gender groups. In females, in line with the increased levels of large-HDL particles (α-1 + α-2) (115.8 vs. 91.4 mg/dl),

| Control | TG | SAA | Fibrinogen | β-Sitosterol |
|---------|----|-----|------------|-------------|
| Malefemale (n) | 56/30 | 27.7 | 19.9 | 8.5 | 12.10 |
| Age (years) | 56 ± 16 | 55 ± 12 | 62 ± 14 | 60 ± 9 | 60 ± 13 |
| TC (mg/dl) | 168 ± 50 | 281 ± 191* | 163 ± 44 | 182 ± 55 | 181 ± 60 |
| LDL-C (mg/dl) | 98 ± 25 | 114 ± 69 | 89 ± 36 | 104 ± 44 | 98 ± 53 |
| sLDL-C (mg/dl) | 19 ± 10 | 59 ± 35* | 20 ± 10 | 33 ± 20* | 16 ± 15 |
| TG (mg/dl) | 80 ± 36 | 951 ± 1088* | 127 ± 59* | 247 ± 264* | 127 ± 99* |
| apoB (mg/dl) | 81 ± 17 | 111 ± 34* | 80 ± 24 | 100 ± 34* | 79 ± 36 |
| HDL-C (mg/dl) | 58 ± 13 | 14 ± 14* | 10 ± 17 | 10 ± 13* | 60 ± 28 |
| apoA-I (mg/dl) | 157 ± 23 | 125 ± 34* | 108 ± 48 | 71 ± 29* | 152 ± 42 |
| SAA (mg/l) | 25 ± 16 | 32 ± 21* | 63 ± 3* | 25 ± 21 | 32 ± 24 |
| Fibrinogen (mg/dl) | 310 ± 63 | 604 ± 151* | 575 ± 188* | 478 ± 108* | 253 ± 83* |
| MPO (pmol/l) | 282 ± 16 | 692 ± 489* | NA | 326 ± 95 | 883 ± 433* |
| CRP (mg/l) | 1.9 ± 2.5 | 7.1 ± 14.6* | 55.3 ± 42.7* | 4.2 ± 2.9 | 5.9 ± 6.9* |
| Preβ-1 (mg/dl) | 8.0 ± 5.2 | 15.3 ± 15.2* | 19.5 ± 8.2 | 11.6 ± 5.7* | 10.1 ± 6.8 |
| α-1 (mg/dl) | 34.0 ± 11.9 | 12.0 ± 7.8* | 23.5 ± 12.8* | 22.3 ± 11.0* | 51.7 ± 19.8* |
| α-1 + α-2 (mg/dl) | 63.7 ± 16.2 | 44.9 ± 17.8* | 76 ± 24.0* | 61.5 ± 15.9 | 42.7 ± 14.5* |
| α-3 (mg/dl) | 97.6 ± 20.4 | 56.9 ± 24.4* | 99.7 ± 31.9 | 83.9 ± 21.4* | 94.5 ± 30.6 |
| α-4 (mg/dl) | 21.5 ± 5.9 | 25.2 ± 8.1* | 18.6 ± 11.0* | 19.4 ± 5.9 | 16.7 ± 5.4* |
| ABCA1-dependent efflux (%/4 h) | 6.0 ± 2.2 | 17.7 ± 7.0 | 9.6 ± 6.1* | 14.1 ± 4.9 | 20.6 ± 5.4* |
| Preβ-1-normalized ABCA1 efflux | 0.61 ± 0.29 | 1.03 ± 0.52* | 0.70 ± 0.88 | 0.98 ± 0.50* | 0.89 ± 0.51* |
| SR-BI-dependent efflux (%/4 h) | 3.4 ± 0.7 | 2.8 ± 0.8* | 3.3 ± 0.9 | 2.8 ± 0.6* | 4.2 ± 1.4* |
| Large-HDL-normalized SR-BI efflux | 0.035 ± 0.004 | 0.056 ± 0.026* | 0.034 ± 0.006 | 0.035 ± 0.009 | 0.045 ± 0.008* |
| HDL anti-ox capacity | 3.5 ± 0.5 | 3.1 ± 0.8* | 3.8 ± 0.5 | 3.1 ± 0.3 | 2.1 ± 0.6* |

*Significant difference (P < 0.05) between patients and controls after controlling for gender.

**Data are expressed as average ± SD. Preβ-1-normalized ABCA1-dependent efflux was calculated by dividing efflux values with preβ-1 concentration (mg/dl). Large-HDL-normalized SR-BI efflux was calculated by dividing efflux values with the combined concentration of α-1 + α-2 (mg/dl). NA, not available.**

**TABLE 1. Characteristics of control subjects and patients with increased levels of TG, inflammatory-stress markers, or β-sitosterol**
SR-BI-dependent efflux was significantly higher than in males (4.0% vs. 3.1%); however, the difference disappeared after normalizing the data for large-HDL particle concentrations.

ABCA1-dependent efflux was weakly correlated with HDL-C \( (R^2 = 0.033) \) and apoA1 \( (R^2 = 0.115) \) concentrations (Fig. 2). ABCA1-dependent efflux had a good positive correlation with the concentrations of the small discoidal preβ1-HDL particles \( (R^2 = 0.317) \) and weak correlations with the concentrations of the large spherical \((\alpha-1, R^2 = 0.020 \) and \(\alpha-2, R^2 = 0.084)\) and other small semi-spherical \((\alpha-3, R^2 = 0.070 \) and \(\alpha-4, R^2 = 0.057)\) HDL particles. A scatterplot analysis of ABCA1-dependent efflux demonstrated a 5-fold range of efflux capacity \((1.6–13.5%) \) in controls (supplemental Fig. S3). Subjects in the top 25% of efflux rates mediated on average 3-fold more cholesterol efflux than the bottom 25% \((7.9% \text{ vs. } 2.7\%)\).

Deming regression analysis showed strong positive associations between SR-BI-dependent efflux and the concentrations of HDL-C \( (R^2 = 0.680) \) and apoA1 \( (R^2 = 0.661) \) (Fig. 3). The highest correlation with SR-BI-dependent efflux was observed with the combined concentrations of the two large HDL particles \((\alpha-1 + \alpha-2, R^2 = 0.774)\). In contrast, the associations between small HDL particles and SR-BI-dependent efflux were much weaker \((\text{preβ1-}1, R^2 = 0.099; \alpha-3, R^2 = 0.049; \text{and } \alpha-4, R^2 = -0.004)\). Scatterplot analysis of SR-BI-dependent efflux demonstrated a 3-fold range of efflux capacity \((1.7–5.3\%) \) (supplemental Fig. S4). Subjects in the top 25% of efflux rates mediated on average 75% more cholesterol efflux than the bottom 25% \((4.4\% \text{ vs. } 2.5\%)\). After adjusting data for the combined concentration of the large HDL particles \((\alpha-1 + \alpha-2)\), referred to as large HDL-normalized efflux, the difference between the top and bottom 25% substantially decreased \((0.035\% \text{ vs. } 0.033\%)\), indicating that SR-BI-dependent efflux was primarily influenced by the concentration of large HDL particles.

### Influence of high plasma TG concentration on the subpopulation profile and efflux capacity of HDL

Because plasma TG concentrations play a significant role in HDL metabolism, we tested how plasma TG levels were associated with HDL subpopulation profiles and efflux capacity (total and concentration-normalized efflux rates via the two efflux pathways). In Table 1, we summarize data on 30 high-TG subjects \((\text{TG} >300 \text{ mg/dl})\) compared with controls. The patient group had significantly higher concentrations of TG \((>10\text{-fold})\), TC \((67\%)\), LDL-C \((17\%)\), sLDL-C \((206\%)\), apoB \((37\%)\), fibrinogen \((95\%)\), MPO \((145\%)\), and hsCRP \((275\%)\) than the control group. High-TG patients had significantly lower concentrations of HDL-C \((-41\%)\) and apoA-I \((-20\%)\), and a signature HDL subpopulation profile with 65% lower \(\alpha-1\), 29% lower \(\alpha-2\), and 191% higher preβ1 concentrations compared with controls (Table 1, Fig. 1). In line with the increased preβ1 concentration, ABCA1-dependent efflux was about 3-fold higher in the high-TG group compared with the control group and, unexpectedly, preβ1-normalized ABCA1 efflux was also significantly higher \((69\%)\) (Table 1, supplemental Fig. S3). It is worth noting that the mean value of ABCA1-dependent efflux in patients with the highest TG and preβ1 levels was underestimated, as in four cases, the efflux values exceeded the linear range of the assay \((>20\%)\). High-TG patients had 42% lower mean concentration of large HDL particles \((\alpha-1 + \alpha-2)\), but their large-HDL-normalized efflux was 60% higher than in controls (Table 1, supplemental Fig. S4).

### Influence of high plasma concentrations of inflammatory markers on the subpopulation profile and efflux capacity of HDL

We have also tested how high levels of the inflammatory markers, SAA and fibrinogen, are associated with HDL subpopulation profiles and efflux capacity (total and concentration-normalized efflux rates via the two efflux pathways). We compared 28 subjects with high SAA levels to controls (Table 1). The majority of high-SAA subjects had values at or near the upper limit of the assay \((65 \text{ mg/l})\). Compared with controls, high-SAA subjects had significantly higher concentrations of hsCRP \((29\text{-fold})\), fibrinogen \((86\%)\), and TG \((48\%)\), and a significantly lower concentration of HDL-C \((-13\%)\). As a signature pattern, these patients had significantly increased levels of \(\alpha-2\) HDL particles \((20\%)\) and decreased levels of all other \(\alpha\)-mobility HDL particles \((\text{large } \alpha-1, -31\%; \text{small } \alpha-3, -13\%; \text{and small } \alpha-4, -40\%)\) compared with controls (Table 1, Fig. 1). There were no significant differences in the concentrations of preβ1 and the combined large HDL particles \((\alpha-1 + \alpha-2)\), consequently both ABCA1- and SR-BI-dependent effluxes were similar between the high-SAA and the control groups. In contrast to our expectation, both the preβ1-normalized and the large-HDL particle-normalized effluxes were also normal in these patients (Table 1; supplemental Figs. S3, S4).

Generally, subjects with high fibrinogen levels also have elevated TG, SAA, and MPO levels. In this study, we selected 13 subjects with high fibrinogen level \((\text{fibrinogen} >500 \text{ mg/dl})\), but not significantly elevated SAA and MPO levels. These patients had significantly higher TG \((189\%)\) and significantly lower HDL-C \((-31\%)\) levels than controls (Table 1). Their signature HDL subpopulation profile showed marked concentration of apoA-I in a cluster of particles within the \(\alpha-2\) HDL subpopulation and in multipurpose \(\text{preβ1-1} \) HDL particles (Fig. 1). In line with the elevated TG level, both the concentration and the functionality of preβ1 particles were significantly elevated compared with controls \((45\% \text{ and } 61\%, \text{respectively})\) (Table 1, supplemental Fig. S3). These patients had significantly lower SR-BI-dependent cholesterol efflux capacity \((-18\%)\), due to lower concentration of large HDL particles \((-14\%)\), compared with controls (Table 1, supplemental Fig. S4).

### Influence of high serum MPO concentration on the subpopulation profile and efflux capacity of HDL

MPO is considered to be a major factor in HDL functionality; therefore we compared data on 30 high-MPO subjects \((\text{MPO} >500 \text{ pmol/l})\) to controls (Table 2). High-MPO patients had significantly increased TG \((24\%)\), fibrinogen...
(26%), and hsCRP (500%) levels. They had decreased HDL-C (−17%), apoA-I (−11%), preβ-1 (−17%), and large HDL particle (−14%) levels compared with controls. High MPO concentration was not associated with a specifically altered HDL subpopulation profile (Table 2, Fig. 1). Unexpectedly, high serum MPO level was associated with significant increases in both preβ-1-normalized (60%) and large-HDL-particle-normalized (16%) efflux capacity compared with controls (Table 2; supplemental Figs. S3, S4).

Influence of high plasma β-sitosterol concentration on the subpopulation profile and efflux capacity of HDL

Subjects with an increased level of plasma β-sitosterol had a signature HDL subpopulation profile with significantly
increased concentration of α-1 (LpA-I particle) (52%) and significantly decreased concentration of α-2 (LpA-I:A-II particle) (−33%) (Table 1, Fig. 1).

The distribution of β-sitosterol in various lipoprotein subclasses was measured after separating VLDL, LDL, HDL₂, and HDL₃ by UC. β-Sitosterol was equally split between the LDL and HDL₂ subfractions (data not shown). It is worth noting that after UC separation of plasma, the HDL₂ subfraction runs as α-1 HDL in our 2D native-gel system (16); therefore, α-1 HDL particles in these patients were enriched in β-sitosterol. High-β-sitosterol patients had significantly increased TG (49%), MPO (213%), and hsCRP.
One of the major functions of HDL is preventing LDL oxidation. We summarize the data on HDL anti-ox capacity in Tables 1, 2. HDL anti-ox capacity had very weak or nonexistent associations with HDL-C, apoA-I, HDL particle concentration-normalized (29%) efflux capacities in subjects with a wide range of pathological conditions (high plasma levels of TG, SAA, fibrinogen, MPO, or β-sitosterol) and in control subjects in order to assess the influence of these conditions on HDL subpopulation profile and HDL functionality.

**DISCUSSION**

Over the past 5 years, several studies have documented a significant inverse association between HDL CEC and CVD risk (11–13). However, the underlying mechanisms for this relationship are not completely understood. Cells efflux cholesterol in multiple ways: by nonspecific (e.g., aqueous desorption) and by specific (e.g., ABCA1, ABCG1, and SR-BI) pathways. In this study, we tested the hypothesis that HDL CEC depends not only on quantitative, but also on qualitative, properties of HDL particles. We measured cell-cholesterol efflux via the ABCA1 and SR-BI pathways and calculated HDL particle concentration-normalized efflux capacities in subjects with a wide range of pathological conditions (high plasma levels of TG, SAA, fibrinogen, MPO, or β-sitosterol) and in control subjects in order to assess the influence of these conditions on HDL subpopulation profile and HDL functionality.

**HDL-mediated cell-cholesterol efflux via ABCA1**

We verified that ABCA1-dependent cell cholesterol efflux is the key contributor to global cell-cholesterol efflux \((R^2 = 0.879)\) and that there is a strong positive correlation between ABCA1-dependent efflux and preβ-1 HDL particle concentrations (Fig. 2) (14). Based on the \(R^2\) value of 0.317, we estimate that the concentration of preβ-1 particles explained approximately 32% of the variability in ABCA1-dependent cholesterol efflux. It is well-known that cells can efflux about 30% of cholesterol in nonspecific ways, like aqueous desorption (22). There are other factors, such as the functionality of preβ-1 particles, which can also influence ABCA1-dependent efflux.

Alpha-mobility particles were weakly correlated with ABCA1-dependent efflux, independent of their size, and adding these particles to preβ-1 in the analysis weakened the correlation coefficient. These data support the concept that ABCA1 interacts predominantly with the small lipoprotein preβ-1 HDL particles; therefore, preβ-1 particles are precursors of larger more complex HDL particles, at least via the ABCA1-lipidation pathway.

Preβ-1 HDL particles form in two different ways (23): 1) Newly-synthesized monomer apoA-I molecules spontaneously bind lipids in the circulation. The lipids change the conformation of apoA-I resulting in the formation of dimerized apoA-I molecules (de novo preβ-1 particles). 2) Mature large HDL particles remodel into smaller HDL particles, including preβ-1 (recycled preβ-1), due to the combined action of cholesteryl ester (CE) transfer protein (CETP) and HL. We tested how high plasma TG concentration influenced preβ-1-normalized efflux capacity by comparing high-TG patients (subjects with a higher percentile of recycled preβ-1) to controls (subjects with a higher percentile of de novo preβ-1). Compared with controls, high-TG patients had about a 3-fold increase in ABCA1-dependent efflux, as a result of an almost 2-fold increase in preβ-1 concentration, and a 69% increase in preβ-1 functionality (preβ-1-normalized efflux capacity). These data indicate that ABCA1-dependent cholesterol efflux is influenced not only by quantitative effects (concentration), but also by qualitative properties (functionality) of preβ-1 particles.

Preβ-1 particles consist of two distinct subpopulations (preβ-1a and preβ-1b) (supplemental Fig. S1) (19); therefore, we tested whether preβ-1 was correlated differently for varying preβ-1a versus preβ-1b HDL levels. We noted no significant difference, indicating similar functionality of these two particles (data not shown). In addition,
high-TG patients often have abnormal preβ-1 particles, including preβ-1x particles (Fig. 1); but, again, there was no difference in preβ-1-normalized efflux capacity with or without taking into consideration preβ-1x particles (data not shown).

The concept that inflammation and oxidative stress alter HDL functionality is widely accepted. We tested whether HDL functionality is compromised in subjects with high levels of inflammatory- and oxidative-stress markers (SAA, fibrinogen, and MPO) in blood. The vast majority of SAA in the circulation is carried by HDL. In a concentration-dependent manner, SAA transforms HDL into predominantly SAA-containing α-2-size particles (Fig. 1). The influence of high-SAA-transformed HDL on ABCA1-dependent CEC is not clear (24–26). In this study, a high SAA level was not associated with abnormal preβ-1 concentration. Moreover, the significantly lower level of preβ-1 particles observed in these subjects was proportionally compensated for with higher levels of α-2 HDL particles. High-SAA patients had normal preβ-1 and large-HDL particle-normalized efflux capacities, indicating that high plasma SAA level had no significant influence on HDL functionality. Subjects with high fibrinogen level also had high TG level and their HDL subpopulation profile and ABCA1-dependent efflux capacity were similar to those of high-TG patients, indicating that TG concentration had a dominant effect over fibrinogen on the concentration and functionality of preβ-1 HDL particles.

It is well-documented that MPO oxidizes apoA-I preferentially in HDL (27). Immunohistochemical staining showed that MPO-containing macrophages in human atherosclerotic intimae were colocalized with 3-nitrotyrosine- and HOCl-modified apoA-I (28). It has been reported that the relative proportion of oxidized apoA-I is significantly higher (1:5,000) in plaques than in plasma (28, 29), suggesting that MPO might oxidize apoA-I in the atherosclerotic intimae rather than in the circulation. Despite a significantly increased (6-fold) serum level of MPO, preβ-1-normalized efflux was significantly higher (65%) in high-MPO patients compared with controls, indicating that MPO has no significant detrimental effect on preβ-1 HDL functionality. This result suggests that MPO does not oxidize apoA-I in the circulation.

HDL-mediated cell cholesterol efflux via SR-BI

Similar to our previous findings (14), we observed significant positive correlations between SR-BI-dependent efflux rates and the concentrations of large α-1 and α-2 HDL particles. Based on the $R^2$ value of 0.774, we estimate that the combined level of α-1 and α-2 explained approximately 77% of the variability in cell-cholesterol efflux via the SR-BI pathway (Fig. 3). Moreover, in contrast to published data (30–33), we found no evidence that high plasma levels of SAA or MPO have detrimental effects on the functionality of large HDL particles.

The role of TG in reverse cholesterol transport

The present data support earlier findings that HDL particles are key players in reverse cholesterol transport (RCT) (14). A high TG level increases both the concentration and the functionality of preβ-1 particles and, consequently, enhances the first step of RCT via the ABCA1 pathway. High TG levels are associated with high concentrations of TG-rich lipoproteins (TRLs), which in turn are associated with enhanced LPL activity. Through the action of LPL on TRL, some lipids and apolipoproteins are released from TRLs and are transferred to HDL particles (23). Therefore, a high TG level improves not only the first step of RCT, but also the maturation of small HDL particles into larger more complex particles. However, high TRL concentration is also accompanied by increased CETP activity. CETP exchanges TG for CE between TRL and HDL. The TG-enriched large HDL particles are good targets for HL. The concerted actions of CETP and HL transform large HDL particles into smaller particles (preβ-1, α-3, and α-4) before large particles can transfer CE to the bile via the SR-BI pathway in the liver.

Our data indicate that measuring not only ABCA1-dependent cell cholesterol efflux, but also SR-BI-dependent efflux and concentrations of HDL particles, provides deeper insight into RCT and HDL particle functionality.

HDL anti-ox capacity

Anti-ox capacity is also a major athero-protective function of HDL (33). We did not observe significant associations between HDL anti-ox capacity and cholesterol efflux capacity, either via the ABCA1 or the SR-BI pathway. Moreover, HDL anti-ox capacity was not associated with HDL-C, apoA-I, HDL particles, and inflammatory- and oxidative-stress markers. Therefore, in contrast to previous reports (31–33), we did not see compromised anti-ox capacity in subjects with significantly increased SAA or MPO levels in plasma. However, the anti-ox capacity of HDL in patients with high β-sitosterol level was significantly lower (~39%). This finding may be due to the accumulation of β-sitosterol in the large α-1 HDL particles, although, in very low concentration (about 0.5% of cholesterol in the particles), which in turn influences CETP and/or HL activities on the large α-1 HDL particles. It is worth noting that, in this study, none of the investigated pathological conditions were associated with pro-oxidative HDL (anti-ox capacity <1.0).

The strength of this study is that we correlated HDL CEC and anti-ox function to HDL particles in a diverse population: in normal controls and in subjects with a variety of pathological conditions. The major weakness of this study is that we could not document what type of change(s) alters the functionality of preβ-1 particles. Moreover, further studies are needed to investigate whether apparently healthy subjects with very low preβ-1 functionality have increased risk for CHD.

CONCLUSIONS

RCT is the sum of HDL cell-cholesterol efflux and transport capacities from peripheral cells to the bile. We have generated evidence that RCT is influenced not only by quantitative (concentration), but also by qualitative (functional), properties of HDL particles. We have also documented that the concentration and the functionality of
certain HDL particles are independent of each other and might change independently under different pathological conditions. Our data indicate that high plasma concentrations of TG, or inflammatory- or oxidative-stress markers do not compromise the functionality of either the small preβ-1 or the large (α1- and α2-) HDL particles.27

The authors thank Caitlin Meeks, Mary Ampong, and Carl Klint for their excellent technical support.

REFERENCES

1. Gordon, D. J., J. L. Probstfield, R. J. Garrison, J. D. Neaton, W. P. Castelli, J. D. Kostke, D. R. Jacobs, S. Bangdiwala, and H. A. Tyroler. 1989. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. Circulation. 79: 8–15.

2. Ballantyne, C. M., J. A. Herd, L. L. Fierli, J. K. Dunn, J. A. Farmer, P. H. Jones, J. R. Schein, and A. M. Gotoo. 1999. Influence of low HDL on progression of coronary artery disease and response to flu-

3. Nicholls, S. J., E. M. Tuzcu, I. Sipahi, A. W. Grasso, P. Schoenhagen, T. Hu, K. Wolski, T. Crowe, M. Y. Desai, S. L. Hazen, et al. 2007. Statins, high-density lipoprotein cholesterol, and regression of coro-

4. Kech, A., R. J. Simes, P. Barter, J. Best, R. Scott, M. R. Taskinen, P. Forder, A. Pillai, T. Davis, P. Glazioso, et al; FIELD study investiga-

5. Boden W. E., J. L. Probstfield, T. Anderson, B. R. Chaitman, P. Desvignes-Nickens, K. Koprowicz, R. McBride, K. Teo, and W. Weintaufb. AIM-HIGH Investigators. 2011. Niacin in patients with low HDL cholesterol levels receiving intensive statin therapy. N. Engl. J. Med. 365: 2255–2267. [Erratum. 2012. N. Engl. J. Med. 367: 189.]

6. Schwartz, G. G., A. G. Olsson, M. A., C. M. Ballantyne, P. J. Barter, J. Brumm, B. R. Chaitman, I. M. Holme, D. Kallend, L. A. Leiter, et al; dal-OUTCOMES Investigators. . 2012. Effects of dalteparin in patients with a recent acute coronary syndrome. N. Engl. J. Med. 367: 2089–2099.

7. Ballantyne, F. C., R. S. Clark, H. S. Simpson, and D. Ballantyne. 1982. High density and low density lipoprotein subfractions in survivors of myocardial infarction and in control subjects. Metabolism. 31: 433–437.

8. Asztalos, B. F., I. A. Cupples, S. Demissie, K. V. Horvath, C. E. Cox, M. C. Batista, and J. W. Heinecke. 2004. High-density lipoprotein subpopulation profile and coronary heart disease prevalence in male participants of the Framingham Offspring Study. Arterioscler. Thromb. Vasc. Biol. 24: 2181–2187.

9. Asztalos, B. F., D. Collins, L. A. Cupples, S. Demissie, K. V. Horvath, H. E. Bloomfield, S. J. Robins, and E. J. Schaefer. 2005. Value of high-density lipoprotein (HDL) subpopulations in predicting recurrent cardiovascular events in the Veterans Affairs HDL Intervention Trial. Arterioscler. Thromb. Vasc. Biol. 25: 2185–2191.

10. Williams, P. T. 2012. Fifty-three year follow-up of coronary heart disease and other lipoproteins in Gofman’s Livermore Cohort. J. Lipid Res. 53: 260–272.

11. Khra, A. V., M. Cuchel, M. de la Llera-Moya, A. Rodrigues, M. F. Burke, K. Jafari, B. C. French, J. A. Phillips, M. L. Mucksavage, R. L. Wilensky, et al. 2011. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. N. Engl. J. Med. 364: 127–135.

12. Rohaugi, A. A., Khra, J. D. Berry, E. G. Givens, C. R. Ayers, K. E. Wedin, J. J. Neeland, I. S. Yuhanna, D. R. Rader, J. A. de Lemos, et al. 2014. HDL cholesterol efflux capacity and incident cardiovascular events. N. Engl. J. Med. 371: 2383–2393.

13. Saleheen, D., R. Scott, S. Javad, K. T. Yuan, M. C. Batista, and E. J. Schaefer. 2005. Differential effects of HDL subpopulations on cellular ABCA1- and SR-BI-mediated cholesterol efflux. J. Lipid Res. 46: 2246–2253.

14. Guey, L. T., C. R. Pullinger, B. Y. Ishida, P. M. O’Connor, C. Zellner, O. L. Francone, J. M. Laramie, J. M. Naya-Vigne, K. A. Siradze, P. Deedwania, et al. 2011. Relation of increased preβ-1 high-density lipoprotein levels to risk of coronary heart disease. Am. J. Cardiol. 108: 360–362.

15. Asztalos, B. F., P. S. Roheim, R. L. Milani, M. Lefevre, J. R. McNamara, K. V. Horvath, and E. J. Schaefer. 2000. Distribution of ApoA-I-containing HDL subpopulations in patients with coronary heart disease. Arterioscler. Thromb. Vasc. Biol. 20: 2670–2676.

16. de Vries, R., F. G. Pertson, A. van Tol, and R. P. Dullaart. 2012. Carotid intima media thickness is related positively to plasma pred-

17. Miida, T. Y. Nakamura, K. Inano, T. Matsuto, T. Yamaguchi, T. Tsuda, and M. Okada. 1996. Prebeta 1 high-density lipoprotein increases in coronary artery disease. Clin. Chem. 42: 1992–1995.

18. Asztalos, B. F., C. H. Sloop, L. Wong, and P. S. Roheim. 1993. Two-dimensional electrophoresis of plasma lipoproteins: recognition of new apo A-I-containing subpopulations. Biochim. Biophys. Acta. 1169: 291–300.

19. Matthau, N. R., A. Giovanni, E. J. Schaefer, B. G. Brown, and A. H. Lichtenstein. 2003. Impact of simvastatin, niacin, and/or antioxi-

20. Asztalos, B. F., and J. W. Heinecke. 2013. Translation of high-density lipoprotein function into clinical practice: current prospects and future chal-

21. Banka, C. R., T. Yuan, M. C. de Ber, M. Kindi, L. K. Curtiss, and F. C. de Ber. 2005. Serum amyloid A (SAA): influence on HDL-

22. De DiDonato, J. A., K. Aukal, Y. Huang, M. Wagner, G. Gerstenecker, C. Topolas, V. Gogonea, A. J. De DiDonato, W. H. Tang, R. A. Mehl, et al. 2014. Site-specific nitration of apolipoprotein A1 at tyrosine 166 is both abundant within human atherosclerotic plaque and dys-

23. Van der Westhuysen, D. R., L. Cai, M. C. de Ber, and F. C. de Ber. 2005. Serum amyloid A promotes cellular efflux mediated by scavenger receptor B1. J. Biol. Chem. 280: 35880–35885.

24. Vaisar, T., C. T. Teng, I. Babenko, P. Hutchins, J. W. Heinecke. 2015. Inflammatory remodeling of the HDL proteome impairs cellular efflux capacity. J. Lipid Res. 56: 1519–1530.

25. Shao, B., P. Nemathur, and J. W. Heinecke. 2012. Myeloperoxidase targets apolipoprotein A1, the major high density lipoprotein protein, for site-specific oxidation in human atherosclerotic lesions. J. Biol. Chem. 287: 6375–6386.

26. De DiDonato, J. A., K. Aukal, Y. Huang, M. Wagner, G. Gerstenecker, C. Topolas, V. Gogonea, A. J. De DiDonato, W. H. Tang, R. A. Mehl, et al. 2014. Site-specific nitration of apolipoprotein A1 at tyrosine 166 is both abundant within human atherosclerotic plaque and dys-

27. Li, C. D., M. C. de Ber, F. C. de Ber, and D. R. van der Westhuysen. 2005. Serum amyloid A is a ligand for scavenger receptor class B type I and inhibits high-density lipoprotein binding and selective lipid uptake. J. Biol. Chem. 280: 2954–2961.

28. Annema, W., N. Nijstad, M. Tölle, J. F. de Boer, R. V. Buijs, P. Heeringa, M. van der Giet, and U. J. Tietge. 2010. Myeloperoxidase and serum amyloid A contribute to impaired in vivo reverse cholesterol transport during the acute phase response but not group B1 secretory phospholipase A2. J. Lipid Res. 51: 743–754.

29. Rosenson, R. S., T. H. Brewer, B. Ansell, P. Barter, M. J. Chapman, and J. W. Heinecke. 2013. Translation of high-density lipoprotein function into clinical practice: current prospects and future chal-

30. Shao, B., M. N. Oda, J. F. Oram, and J. W. Heinecke. 2010. Myeloperoxidase: an oxidative pathway for generating dysfunc-

31. Journal of Lipid Research Volume 58, 2017