Composition-function analysis of HDL subpopulations: influence of lipid composition on particle functionality

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Abstract The composition-function relationship of HDL particles and its effects on the mechanisms driving coronary heart disease (CHD) is poorly understood. We tested the hypothesis that the functionality of HDL particles is significantly influenced by their lipid composition. Using a novel 3D-separation method, we isolated five different-sized HDL subpopulations from CHD patients who had low preβ-1 functionality (low-F) (ABCA1-dependent cholesterol-efflux normalized for preβ-1 concentration) and controls who had either low-F or high preβ-1 functionality (high-F). Molecular numbers of apoA-I, apoA-II, and eight major lipid classes were determined in each subpopulation by LC-MS. The average number of lipid molecules decreased from 422 in the large spherical α-1 particles to 57 in the small discoid preβ-1 particles. With decreasing particle size, the relative concentration of free cholesterol (FC) decreased in α-mobility but not in preβ-1 particles. Preβ-1 particles contained more lipids than predicted; 30% of which were neutral lipids (cholesterol ester and triglyceride), indicating that these particles were mainly remodeled from larger particles not newly synthesized. There were significant correlations between HDL-particle functionality and the concentrations of several lipids. Unexpectedly, the phospholipid:FC ratio was significantly correlated with large-HDL-particle functionality but not with preβ-1 functionality. There was significant positive correlation between particle functionality and total lipids in high-F controls, indicating that the lipid-binding capacity of apoA-I plays a major role in the cholesterol efflux capacity of HDL particles. Functionality and lipid composition of HDL particles are significantly correlated and probably both are influenced by the lipid-binding capacity of apoA-I. — Niisuke, K., Z. Kuklenyik, K. V. Horvath, M. S. Gardner, C. A. Toth, and B. F. Asztalos. Composition-function analysis of HDL subpopulations: influence of lipid composition on particle functionality. J. Lipid Res. 2020. 61: 306–315.

Supplementary key words high density lipoprotein • pre-beta • phospholipids • lipid ratios

Although epidemiological studies indicate that HDL plays a significant role in coronary heart disease (CHD) (1–3), clinical trials of HDL-targeted therapies have recently failed (4–6). Therefore, there is a need to better understand the mechanisms by which HDL influences CHD risk and how the anti-atherogenic properties of HDL can be manipulated for preventive and therapeutic purposes. HDL is the most complex lipoprotein class comprised of various particles that differ in size, composition, and function, including cholesterol efflux and anti-oxidative and anti-inflammatory activities. HDL’s functions are determined, or at least significantly influenced, by the size and chemical composition of its particles. We have shown that the concentrations of apoA-I-containing HDL particles (e.g., large α-1 and small preβ-1) are significantly better predictors of CHD risk than HDL-C levels in both primary and secondary prevention and that an increase in α-1 level is significantly associated with decreased progression of coronary artery stenosis as assessed by angiography (7–9). Recently, it has been documented that the cell-cholesterol efflux capacity (CEC) of HDL predicts incident and prevalent CVD risk more accurately than HDL-C (10–12). CEC, via ABCA1 and scavenger receptor BI (SR-BI), is HDL-particle specific; preβ-1 particles are responsible for about 60% of cell-cholesterol efflux via the ABCA1 pathway, and large-HDL-particles (α-1 and α-2) are responsible for about

Abbreviations: CE, cholesteryl ester; CEC, cholesterol efflux capacity; CHD, coronary heart disease; FC, free cholesterol; high-F, high preβ-1 functionality; low-F, low preβ-1 functionality; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; SR-BI, scavenger receptor BI; TG, triglyceride.

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80% of cell-cholesterol efflux via the SR-BI pathway (13, 14). CEC depends not only on the concentration but also on the functionality of specific HDL particles. For example, CHD patients have higher than normal preβ-1 concentrations but lower than normal preβ-1 functionality (ABCA1-dependent CEC normalized for preβ-1 concentration) (13).

The relationship between HDL composition and function is not fully understood. We tested the hypothesis that the functionality of HDL particles is significantly influenced by their lipid composition. We isolated and purified five different-sized apoA-I-containing HDL subpopulations and determined the molecular numbers of apoA-I, apoA-II, and eight major lipid classes per particle in each subpopulation. Then we analyzed how the lipid composition correlated with the functionality of the small (preβ-1) and the large (α1 + α2) HDL particles, the mediators of cell-cholesterol efflux via the ABCA1 and SR-BI pathways, respectively.

MATERIALS AND METHODS

Study design and study population

To test the hypothesis that HDL-particle functionality is significantly influenced by the lipid composition of the particle, we studied 66 subjects in three groups: 33 subjects had CHD and low preβ-1 functionality (low-F) (CHD group); 16 subjects had no CHD and low-F (low-F control group); and 17 subjects had no CHD and high preβ-1 functionality (high-F) (high-F control group). Preβ-1 functionality was calculated as ABCA1-mediated CEC divided by apoA-I concentration in preβ-1 particles. Low-F controls were selected with preβ-1 functionality not higher than 2 SD of the mean preβ-1 functionality of the CHD group and high-F controls were selected with preβ-1 functionality at least 2 SD higher than the mean preβ-1 functionality of the CHD group.

CHD patients (30 males and 3 females) were randomly selected from the baseline samples of a randomized clinical trial (15). Participants in this trial had established stable coronary artery disease (including previous myocardial infarction (>6 months prior), coronary artery bypass surgery (>12 months prior), or angioplasty, stable angina, or evidence of CHD on prior imaging), an abnormal exercise-tolerance test, or ischemia by nuclear imaging deemed by the patients’ clinical cardiologist not to require current intervention, and at least one evaluable segment with plaque by immunochemical staining. Additional inclusion criteria were age 21–75 years, BMI >25 and <35 kg/m² in women and <40 kg/m² in men, a stable regimen of statin dosing, and an estimated creatinine clearance of at least 60 ml/min per 1.73 m².

Age-matched controls (27 males and 6 females) were selected from a large outpatient sample pool having no history of cardiovascular, kidney, thyroid, or liver disease, or diabetes mellitus, BMI >25 and <35 kg/m² in women and <40 kg/m² in men, no use of lipid-lowering medication, an LDL-C level <200 mg/dl, and a triglyceride (TG) level <300 mg/dl.

Laboratory measurements

Blood samples were collected after an overnight fast. Total cholesterol, TG, LDL-C, and HDL-C concentrations were measured using enzymatic colorimetric methods, and apoA-I concentration was measured using an immunoturbidimetric method. apoA-I concentrations in five different-sized HDL subpopulations [α-1 (11.0 nm), α-2 (9.4 nm), α-3 (8.4 nm), α-4 (7.7 nm), and preβ-1 (5.6 nm)] were determined by 2D native agarose-PAGE, followed by immunoblot for apoA-I and image analysis as described previously (16).

HDL CEC and anti-oxidative capacity were measured at Vascular Strategies (Plymouth Meeting, PA) using methods previously described (14, 17). Prior to the analyses, plasma was converted to serum by adding 25 mM CaCl₂ to plasma for clotting, and then the clot was removed by low-speed centrifugation. ABCA1-dependent CEC was measured in [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 and unstimulated cells. Liquid scintillation counting was used to quantitate the effluxed radialized cholesterol in the medium. Total (global) efflux was defined as the efflux measured from cAMP-stimulated [³H]-cellular cholesterol in the medium. Non-ABCA1-specific (basal) efflux was measured from unstimulated [³H]-cells. ABCA1-specific efflux was calculated as the difference between cAMP-stimulated (total) and unstimulated (non-ABCA1) cells. SR-BI-mediated CEC was measured as the fraction of radialized cholesterol released from radialized Fu5AH cells to the medium containing 2.8% apoB-free serum. Efflux data were expressed as percent cholesterol efflux per 4 h. HDL anti-oxidative capacity was measured by assessing the ability of apoB-depleted serum to inhibit or enhance the oxidation of LDL in the presence of a fluorescent organic substrate dichlorofluorescin (2’,7’-dichlorohydrofluorescin diacetate) (17). Dichlorofluorescin was dissolved in methanol at 2.0 mg/ml and incubated at room temperature in the dark for 1 h with isolated LDL (5 μg/ml as final concentration) in the presence or absence of apoB-depleted serum as surrogate for HDL (20 μl). Fluorescence intensity was determined with a spectrofluorometer set at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The anti-oxidative capacity of apoB-depleted serum was expressed in arbitrary units: values >1.0 indicated anti-oxidative capacity while values <1.0 indicated that the serum was pro-oxidative. To correct for inter-assay variability, a pool of human serum was tested in parallel with the test samples in every efflux and anti-oxidative capacity assay. For inter-day comparability, measurements were normalized to a standard pool.

For proteomic and lipidomic analysis, HDL particles were isolated from whole plasma utilizing a 3D separation method (supplemental Fig S1). In the first dimension, HDL particles were separated from the majority of apoB-containing lipoproteins by net surface charge into preβ- and α-mobility subfractions. Plasma and 40% sucrose containing a trace amount of bromophenol blue were mixed in a 4:1 ratio. Two hundred microliters of this mixture were applied into a 110 mm wide sample channel on a 3 mm thick 0.7% agarose gel and run at 250 V in 4°C Tris-tetracine buffer (pH 8.6) until the α-front moved 35 mm from the origin. To mark the β- and α-front, BODIPY-labeled plasma was run on each gel (BODIPY FL C5-sphingomyelin; Thermo Fisher) (supplemental Fig S1a). The preβ- and α-mobility bands were cut out on a UV-light table. In the second dimension, HDL particles were further separated by size. The agarose gel pieces containing preβ- and α-HDL were placed separately on the top of two 4-27% concave-gradient native polyacrylamide gels and electrophoresed in 4°C Tris-borate-EDTA buffer (pH 8.3) at 250 V for 17 h. On the first gel, the different size α-mobility HDL subpopulations (α-1, α-2, α-3, and α-4) were localized based on the fluorescently labeled whole plasma marker run in each gel (supplemental Fig S1b). On the second gel, the position of the preβ-1 subpopulation was indicated by a naturally glowing band (not shown in figure). The five localized HDL particle bands were cut out from the gels and the HDL particles were electro-eluted from the gel under none-naturing conditions [25 mM Tris-glycine buffer (pH 8.4), 100 V, 4°C, 24 h] using homemade large-capacity electro-eluters. In the
third dimension, the apoA-I-containing HDL particles in each subfraction were further purified by immunoaffinity chromatography (supplemental Fig. S1c). HDL particles were continually removed from the elution buffer by connecting each elution chamber to an immunoaffinity column (1 ml Sepharose gel containing about 5 mg of immobilized anti-apoA-I polyclonal γ globulin) via a peristaltic pump to prevent the formation of highly concentrated insoluble HDL particles on the surface of the elution membrane. The immobilized HDL particles were eluted from the immunoaffinity columns using pH 2.5 Tris-HCl buffer, which was immediately neutralized with 0.5 M sodium-phosphate buffer (pH 7.4). To test the size and integrity of the isolated HDL particles, each fraction was concentrated to 100 μl (0.5–1.0 μg/ml apoA-I) of which 5 μl were rerun side-by-side with whole plasma on a 4–27% native polyacrylamide gel followed by immunodetection of apoA-I (supplemental Fig. S2).

In each purified subfraction, LC-MS/MS analysis was performed at the CDC (Atlanta, GA). A dilution series of a plasma pool was used for external calibration. For apoA-I and apoA-II, the calibration plasma pool was previously value assigned by standard-addition methodology using purified proteins. For lipids, the calibration plasma pool was value assigned with solvent-based standards prepared from certified pure lipid reference materials. The limits of quantification in the fractions were as follows: apoA-I (20 nmol/ml), apoA-II (20 nmol/ml), cholesteryl ester (CE) (50 nmol/ml), free cholesterol (FC) (50 nmol/ml), TG (50 nmol/ml), phosphatidylecholine (PC) (50 nmol/ml), lysophosphatidylcholine (LPC) (50 nmol/ml), phosphatidylethanolamine (PE) (10 nmol/ml), phosphatidyllysinol (PL) (5 nmol/ml), and SM (50 nmol/ml). The lowest concentrations were measured in the preB-1 and α-4 fractions with <5% of the fraction concentrations under the limit of quantification for some of the analytes. The LC-MS/MS methods were described in previous publications (19–21).

For protein analysis, a 50 μl aliquot of each immunopurified HDL subfraction was diluted 5-fold with buffer containing 10 mM sodium-bicarbonate, 150 mM NaCl (pH 7.4), and 0.15% Zwittergent 3-12 (EMD Millipore) (18). The analysis was performed using an online column-switching system, Perfinity (Shimadzu Scientific Instruments/Perfinity Biosciences Inc.), equipped with a trypsin column (2.1 × 50 mm), a trapping column, and an analytical column (HCLO C18 core shell 5 × 2.1 mm, 2.7 μm particle size and 100 × 2.1 mm, 2.7 μm particle size, respectively). Target peptides for apoA-I (THLAPSYDLER, ATEHLSTLSEK, and AKPALEDLR) and for apoA-II (EQLTPIK) were monitored relative to stable isotope-labeled analogs by multiple reaction monitoring. One class-specific quantitation and one conformation ion transition were monitored for all CE and TG species.

For lipid analysis, the purified fractions were diluted 2-fold with 10 mM sodium bicarbonate and 150 mM NaCl (pH 7.4). For analysis of nonpolar lipids (FC, CE, and TG) (19), a 50 μl aliquot of each diluted fraction was precipitated with 200 μl of ethanol spiked with isotope-labeled internal standards, evaporated to a dry pellet, and then extracted with 50 μl of nonane. From each extract, 6 μl was injected into an Agilent 1290 UHPLC system equipped with a Kinetex HILIC 1.7 μm, 2.1 × 50 mm column (Phenomenex) using hexane/ethanol/isopropanol mobile phase gradient. A 4000 Qtrap mass spectrometer (Sciex, Framingham, MA) was used with atmospheric pressure ionization, in-source collision fragmentation, and multiple reaction monitoring. One class-specific quantitation and one conformation ion transition were monitored for all CE and TG species.

For analysis of phospholipid (PL) classes (PC, SM, LPC, PE, and PI), a 20 μl aliquot of each diluted fraction was precipitated with 200 μl of ethanol/methyl-butyil ether/dichloromethane spiked with one isotope-labeled internal standard per PL class, evaporated to a dry pellet, and then extracted with a 50 μl mix of nonane/isopropanol/water (20). From each sample supernatant, 5 μl were injected into an Acquity UHPLC system (Waters) equipped with a Kinetex HILIC 100 Å pore, 2.1 × 100 mm, 1.7 μm particle column, using an ammonium acetate/water/acetonitrile mobile phase gradient. A 6500 Qtrap (Sciex) was operated in electrospray ionization and multiple reaction monitoring mode. A selected set of PL species were targeted (expected to be most abundant in plasma by carbon chain length and number of double bonds); 19 for PC, 18 for SM, 19 for PE, 8 for LPC, and 15 for PI. Lipid class concentrations were calculated based on the sum of all the peak intensities within PL classes, normalized to the peak of the corresponding internal standard.

The molecular number of apoA-I in the particles of each different-sized subpopulation was estimated by cross-linking experiments using 3,3’-dithiodiisoproponic acid di (Nhydroxysuccinimide ester) (Sigma-Aldrich). The cross-linked proteins were separated on a 4–12% SDS gels (Novus) under nonreduced condition in Quick-Set minigel equipment. Each purified HDL subpopulation was run parallel on two gels for apoA-I and apoA-II determinations. Molecular-weight standard proteins (Amersham Biosciences, UK) and cross-linked immuno-purified and delipidated apoA-I (Molecular Innovations) were run in each gel as size reference. Gels were electro-transferred to nitrocellulose membranes followed by Western blot with polyclonal primary antibodies specific to either human apoA-I or apoA-II and 125I-labled secondary antibody. The 125I-signals were detected in a phosphorimager (Storm 860; Molecular Dynamics) (Fig. 1).

Because we could not use internal standards during the isolation of HDL particles, the apoA-II and lipid concentrations (micromoles per liter) were normalized for apoA-I concentration (micromoles per liter) measured by LC-MS in each isolated HDL fraction. HDL particle number in each of the five HDL subpopulations was calculated by dividing apoA-I concentrations (micromoles per liter) with the number of apoA-I molecules per particle. apoA-II and the various lipids were expressed as molecular number per particle.

**Statistical analyses**

Preβ-1 particle functionality was calculated as ABCA1-mediated cholesterol efflux divided by apoA-I concentration in preβ-1 particles and large-HDL-particle functionality was calculated as SR-BI-mediated cholesterol efflux divided by apoA-I concentration in α-1 + α-2 particles. Data that were not normally distributed were log-transformed for the analyses. Variables were expressed as mean ± SD. Differences in parameters between study groups were compared using two-tailed Student’s t-tests. The correlations between functionality and lipid composition of small (preβ-1) and large (α-1 + α-2) HDL particles were expressed as Pearson correlation coefficients and their differences between study groups were calculated via two-sample Fisher’s z-tests. P values are reported unadjusted, but the false discovery rate method was used to limit the experiment-wide type I error rate for all correlation analyses to 0.05.

### RESULTS

Major characteristics of the three study groups (high-F control, low-F control, and CHD) are presented in Table 1. Compared with control subjects, CHD patients had significantly higher TG (87%) and significantly lower HDL-C (~30%) levels. Although plasma apoA-I concentrations were similar, there were significant differences in the distribution of apoA-I in HDL particles among the groups. Compared with high-F and low-F controls, CHD patients had
significantly higher concentrations of the small preβ-1 particles (168% and 86%, respectively) and significantly lower concentration of the large α-1 particles (−46% and −48%, respectively). ABCA1-dependent CEC was significantly higher (65%) in the CHD group compared with the low-F control group but similar compared with the high-F control group. Preβ-1 functionality was similar in the low-F and CHD groups and significantly lower (−55%) in both compared with the high-F control group. SR-BI-dependent CEC was similar in all three groups. Large-HDL-particle functionality was significantly higher (32%) and the anti-oxidative capacity of apoB-depleted serum was significantly lower (−17%) in the CHD group compared with the control groups.

In order to estimate particle numbers in each of the five different-sized HDL subpopulations, the number of apoA-I molecules per particle was assessed by cross-linking and SDS-PAGE based on the size profile of the purified/cross-linked/delipidated apoA-I and the high-molecular-weight protein standards (Fig. 1). The average number of apoA-II molecules per apoA-I was determined based on the quantitative LC-MS data. We estimated that α-1 particles contained four apoA-I and three apoA-II molecules (apoA-II:apoA-I = 0.7 ± 0.4); α-2 particles contained three apoA-I and four apoA-II molecules (apoA-II:apoA-I = 1.4 ± 0.4); and α-3 particles contained two apoA-I and two apoA-II molecules (apoA-II:apoA-I = 0.9 ± 0.2). The α-4 and preβ-1 particles contained two apoA-I molecules and an insignificant amount of apoA-II detected by LC-MS analysis (apoA-II:apoA-I = 0.1 ± 0.2 and 0.2 ± 0.2, respectively). A small amount of monomeric apoA-I (apoA-I not

![Fig. 1. apoA-I and apoA-II molecules in HDL particles. Proteins were cross-linked within the particles in the isolated and immunopurified HDL subpopulations and the complex proteins were separated on 4–12% SDS-PAGE under nonreduced condition. Proteins were electro-transferred to nitrocellulose membranes and immunoprobed for either apoA-I or apoA-II. High molecular weight protein standards and cross-linked delipidated apoA-I were used as size reference.](image-url)
successfully cross-linked) was present in each isolated HDL subpopulation.

Lipidomic analysis of the five apoA-I-containing HDL subpopulations utilizing data from all subjects (n = 66) are presented in Fig. 2. The average number of total lipid molecules per particle decreased with particle size from 422 in the large α-1 particles to 57 in the small preβ-1 particles. PC and CE were the most abundant lipids in each sub-

| Lipid   | α-1   | α-2   | α-3   | α-4   | preβ-1 |
|---------|-------|-------|-------|-------|--------|
| Total   | 422 (83) | 227 (38) | 112 (22) | 96 (25) | 57 (35) |
| Min     | 239   | 115   | 57    | 63    | 12     |
| Max     | 713   | 359   | 174   | 210   | 179    |
| CE      | 130 (34) | 71 (13) | 38 (8) | 29 (7) | 13 (10) |
| %       | 30.8  | 31.3  | 33.9  | 30.2  | 22.8   |
| Min     | 53    | 41    | 24    | 16    | 2      |
| Max     | 213   | 123   | 61    | 54    | 39     |
| FC      | 35 (12) | 16 (4) | 6 (2) | 3 (3) | 4 (3)  |
| %       | 8.3   | 7.0   | 5.3   | 3.1   | 7.0    |
| Min     | 14    | 6     | 4     | 2     | 1      |
| Max     | 62    | 36    | 16    | 23    | 13     |
| TG      | 32 (22) | 12 (5) | 7 (3) | 8 (5) | 6 (4)  |
| %       | 7.6   | 5.3   | 6.2   | 8.3   | 10.5   |
| Min     | 2     | 1     | 2     | 3     | 0      |
| Max     | 136   | 27    | 18    | 37    | 20     |
| PL      | 156 (37) | 97 (18) | 45 (10) | 36 (11) | 19 (16) |
| %       | 36.9  | 42.7  | 40.2  | 37.5  | 33.3   |
| Min     | 28    | 50    | 17    | 6     | 2      |
| Max     | 273   | 163   | 69    | 63    | 77     |
| LPC     | 23 (14) | 7 (3) | 5 (3) | 8 (5) | 8 (5)  |
| %       | 5.4   | 3.0   | 4.5   | 8.3   | 14.0   |
| Min     | 8     | 2     | 1     | 2     | 2      |
| Max     | 74    | 21    | 16    | 23    | 27     |
| PE      | 10 (4) | 6 (2) | 3 (1) | 2 (1) | 2 (1)  |
| %       | 2.4   | 2.6   | 2.7   | 2.0   | 3.5    |
| Min     | 1     | 2     | 1     | 0     | 0      |
| Max     | 26    | 10    | 5     | 7     | 6      |
| PI      | 3 (2) | 2 (0) | 1 (0) | 1 (1) | 1 (1)  |
| %       | 0.7   | 0.9   | 1.3   | 1.0   | 1.7    |
| Min     | 1     | 1     | 0     | 0     | 0      |
| Max     | 12    | 3     | 2     | 4     | 4      |
| SM      | 37 (10) | 17 (4) | 8 (2) | 8 (3) | 6 (5)  |
| %       | 8.8   | 7.5   | 7.1   | 8.3   | 10.5   |
| Min     | 16    | 7     | 3     | 4     | 0      |
| Max     | 69    | 29    | 15    | 18    | 29     |
| PL/FC   | 7.1 (2.1) | 8.6 (1.5) | 10.1 (2.0) | 11.9 (3.6) | 10.5 (7.9) |
| %       | 4.1   | 5.5   | 5.7   | 4.0   | 3.0    |
| Min     | 4.1   | 12.6  | 12.8  | 13.6  | 18.8   |
| Max     | 12.6  | 12.8  | 13.6  | 18.8  | 50.4   |
| PL/CE   | 1.8 (0.4) | 1.8 (0.3) | 1.7 (0.3) | 2.0 (0.4) | 3.6 (2.3) |
| %       | 1.0   | 1.3   | 1.0   | 0.9   | 1.4    |
| Min     | 1.0   | 3.5   | 2.6   | 2.2   | 3.1    |
| Max     | 3.5   | 3.5   | 2.6   | 2.2   | 3.1    |
| FC/CE   | 0.27 (0.08) | 0.21 (0.03) | 0.17 (0.04) | 0.18 (0.06) | 0.38 (0.11) |
| %       | 0.14  | 0.15  | 0.11  | 0.11  | 0.21   |
| Min     | 0.14  | 0.46  | 0.30  | 0.39  | 0.38   |
| Max     | 0.46  | 0.39  | 0.39  | 0.38  | 0.63   |
| TG/CE   | 0.27 (0.18) | 0.17 (0.06) | 0.17 (0.06) | 0.27 (0.12) | 0.68 (0.75) |
| %       | 0.10  | 0.07  | 0.06  | 0.09  | 0.61   |
| Min     | 0.10  | 1.09  | 0.32  | 0.36  | 0.69   |
| Max     | 1.00  | 4.21  | 0.60  | 0.69  | 4.21   |

Fig. 2. Lipidomic analysis of HDL subpopulations. Values are mean molecular number per particle (SD) and percentile (%) of total lipid in the particle. The figure demonstrates visually and numerically the similarities and differences in the composition of the different-sized HDL subpopulations isolated by a 3D separation method. The lipid composition of the different-sized α particles was very similar. However, the lipid composition of the preβ-1 particles was different: there was less CE and more TG and LPC and substantially higher PL/CE and FC/CE ratios in these particles compared with α particles. There was a large overlap in the number of lipid molecules in the particles (minimum and maximum number of lipids per particle).
with the high-F control group but not when compared with significantly higher in the CHD group when compared to pre-PC (low-F control and CHD groups and significantly lower PL/PC ratio compared with the CE concentration of pre-PC molecules and a higher TG/CE ratio compared with the CHD group. There were several-fold differences in the minimum and maximum numbers of lipid molecules per particle in each HDL subpopulation.

In Table 2, the particle numbers and the lipid compositions of HDL subpopulations are presented in the three subject groups. In α-1 particles, the CHD group had significantly fewer CE and FC molecules and more TG, LPC, and PE molecules and a higher TG/CE ratio compared with both control groups. The PL/CE and PL/FC ratios were significantly higher in the CHD group when compared with the high-F control group but not when compared with the low-F control group. In α-2 particles, the CHD group had significantly higher PL concentration and PL/CE ratio compared with both control groups. In preβ-1 particles, there were no significant differences in the concentrations of lipids among the three groups. The high-F control group had a significantly lower PL/CE ratio compared with the low-F control and CHD groups and significantly lower PL/FC ratio compared with the CHD group.

In Fig. 3, the correlations between the functionality and the lipid content of preβ-1 particles are presented in the three subject groups. There were significant positive correlations between preβ-1 functionality and the amount of SM (r = 0.769), total lipids (r = 0.713), CE (r = 0.704), and PC (r = 0.680) in the high-F control group. In the CHD and low-F control groups, the range of preβ-1 functionality was quite narrow leading to low correlation coefficients. In the CHD group, there were significant positive correlations between the functionality and the TG content (r = 0.458) as well as the TG/CE ratio (r = 0.421) of preβ-1 particles. After adjusting data for multiple comparisons, only the correlation between the functionality and the CE concentration of preβ-1 was significantly different between the high-F control group and the CHD group. There were no significant correlations between preβ-1 functionality and the concentrations of FC, LPC, PE, and PI or the PL/FC, PL/CE, and FC/CE ratios in either group.

The associations between the functionality and the lipid content of large HDL particles (α1 + α2) are shown in Fig. 4. In the high-F control group, there were strong positive correlations between large-HDL-particle functionality and the concentrations of FC (r = 0.580) and total lipids (r = 0.512) as well as the FC/CE ratio (r = 0.525). In the CHD group, large-HDL-particle functionality correlated inversely with the concentrations of FC (r = -0.590) and CE (r = -0.510) and positively with the PL/FC ratio (r = 0.627). After adjustment for multiple comparisons, the correlations between large-HDL-particle functionality and the concentrations of CE and FC as well as the PL/FC ratio were significantly different between the CHD group and the high-F control group. There were no significant correlations between large-HDL-particle functionality and the concentrations of TG, PC, LPC, PE, and PI or the PL/CE and TG/CE ratios in either group.

DISCUSSION

The relationship between HDL composition and function is not fully understood. We tested the hypothesis that the functionality of HDL particles is significantly influenced by their lipid composition. Using a novel 3D-separation method, we isolated and purified five different-sized apoA-I-containing HDL subpopulations and determined the molecular numbers of apoA-I, apoA-II, and eight major lipid classes of the particles in each subpopulation. We associated the lipid content and the functionality of the small (preβ-1) and the large (α1 + α2) HDL particles, the mediators of cell-cholesterol efflux via the ABCA1 and SR-BI pathways, respectively.

HDL particle lipidomics

As expected, the average number of lipid molecules per particle decreased with decreasing particle size (Fig. 2). Unexpectedly, the small discoid-shaped “lipid-poor” preβ-1 particles contained an average of 57 lipid molecules, 19 of which were neutral (core) lipids (CE and TG). It has been disputed whether CE and TG are “true” core lipids, as it has been shown that a substantial proportion (3–27%) of CE and TG is on the surface of HDL particles, and with decreasing particle size, their abundance increases on the surface (21, 22). A high neutral lipid occurrence on the surface makes the particle more hydrophobic and decreases the net surface charge, consistent with slow preβ migration during electrophoresis. The mechanism responsible for the heterogeneity of the lipid composition of the discoid HDL particles is not clear; however, it is speculated that it is regulated by the selective lipid-binding capacity of apoA-I at the disc edge (23). Moreover, we hypothesize that the lipid composition of the different HDL subpopulations is influenced by apoA-I being freely exchangeable among HDL particles and carrying some lipids from the donor to the acceptor particles.

PC and CE were the most abundant lipids in each HDL subpopulation (Fig. 2, supplemental Table S1). In line with previous reports (24), we have found that among PLs, PC was the most abundant followed by SM, LPC, PE, and PI in each particle. However, we could not confirm the findings of Camont et al. (25) who reported a higher percentile of SM in large HDL particles compared with small particles. Preβ-1 particles contained a higher percentage of LPC and TG and a lower percentage of CE compared with α-mobility particles. PC, the major surface lipid, was strongly and positively correlated with both CE (r = 0.719) and TG (r = 0.685) in preβ-1 particles, supporting the concept that the fatty acid chains of PLs are needed for organizing neutral lipids on the surface of lipoprotein particles (23).

Given that the surface area of a particle is a second power of its diameter and core volume a third, the surface area to volume ratio increases linearly with a reduction in particle diameter. Therefore, we assumed that the surface-lipid/core-lipid ratio would continually increase with decreasing particle size. However, the PL/CE ratio was

and sixth in large α-particles (supplemental Table S1). In preβ-1 particles, the PL/CE and FC/CE ratios were significantly higher (100% and 45%, respectively) compared with the average of the α-mobility particles (P < 0.001). There were several-fold differences in the minimum and maximum numbers of lipid molecules per particle in each HDL subpopulation.

There were no significant differences in the concentrations of FC, LPC, PE, and PI or the PL/CE and TG/CE ratios in either group.
The expectation that the PL/CE ratio would inversely correlate with size holds true only if one assumes that the subpopulations are similarly hydrated. ApoA-I on the surface of smaller particles may be less lipidated and more hydrated, while the core size remains proportionate to the hydrated size of the whole particle. Alternatively, some TG that is not accounted for in the surface/core ratio may be among the surface lipids on smaller particles (22). The average PL/CE and FC/CE ratios were the
highest in the small preβ-1 particles (5.6 nm), supporting the findings that these particles are the major acceptors of FC from cells and are not the preferential substrate for LCAT (16, 26). We assume that the CE content of preβ-1 particles originates from the large α-1 particles, as these particles are prone to falling apart due to the concerted activities of CE transfer protein and hepatic lipase, and consequently, apoA-I and its tightly bound lipids are recycled as preβ-1 particles. Alternatively, apoA-I molecules, which are freely exchangeable among HDL particles can also carry lipids from larger size particles to preβ-1 particles.

Fig. 3. Correlations between the lipid content and the functionality of preβ-1 particles in CHD patients and control subjects having high-F or low-F. Preβ-1 functionality was calculated as ABCA1-mediated CEC divided by apoA-I concentration in preβ-1 particles. The r values represent linear correlation coefficients. *Correlation is statistically significant (P < 0.05). †Correlation is significantly different between the CHD group and the High-F control group (P < 0.05).

Fig. 4. Correlations between the lipid content and the functionality of large HDL particles in CHD patients and control subjects having high-F or low-F. Large HDL particle functionality was calculated as SR-BI-mediated CEC divided by apoA-I concentration in α-1 and α-2 particles. The r values represent linear correlation coefficients. *Correlation is statistically significant (P < 0.05). †Correlation is significantly different between the CHD group and the High-F control group (P < 0.05).
The relationship between HDL particle composition and function

Previously, we have shown that preβ-1-particle functionality (ABCA1-dependent CEC normalized for preβ-1-concentration) was significantly lower and less variable in CHD patients as compared with controls, suggesting that specific alteration(s) generated morphologically more similar, although less functional, preβ-1 particles in CHD cases (13). We hypothesized that the lipid composition of preβ-1 particles was altered in CHD patients and this alteration contributed significantly to low-F. However, there was large variability in preβ-1 functionality among controls with a considerable portion of them having as low-F as CHD patients. Therefore, to better understand the relationship between the lipid composition and the functionality of preβ-1 particles, we studied CHD patients who had low-F and compared them to control subjects who had either high-F or low-F (high-F and low-F controls). Contrary to our expectation, there were few significant differences in the lipidome of preβ-1 particles among the three groups (Table 2). When we explored the correlations between the functionality and the lipid content of preβ-1 particles, we found significant positive correlations between preβ-1 functionality and the abundance of total lipids, CE, PC, and SM in the high-F control group; however, only the correlations between preβ-1 functionality and CE concentration were significantly different between the high-F control group and the CHD group after adjustment for multiple comparisons (Fig. 3).

Considering the concept that preβ-1 particles need PL to efflux cell cholesterol efficiently, we hypothesized that the PL-FC ratio in preβ-1 particles would be positively correlated with preβ-1 functionality. However, the findings that the PL-FC ratio was the lowest in the high-F control group (Table 2) and was not significantly correlated with preβ-1 functionality in either group (data not shown) did not support the assumption that a high PL-FC ratio makes preβ-1 particles better acceptors of cell cholesterol. Considering these and recently published data that showed a positive correlation between the lipid-binding capacity and the CEC of apoA1 (27), we assume that both the lipid composition of preβ-1 and the lipid-binding capacity of apoA1 influence the functionality of preβ-1 particles.

We have confirmed that, in contrast to preβ-1, large-HDL-particle functionality (SR-BI-dependent CEC normalized for the concentrations of α-1 and α-2 particles) was significantly higher in CHD patients as compared with controls (13). We found significant inverse correlations between the functionality and the CE and FC concentrations of large HDL particles in the CHD group, which were significantly different from the positive correlations in the high-F control group (Fig. 4). These data support the hypothesis that the lipid binding and the cholesterol efflux capacities of apoA1 were reduced in CHD patients compared with high-F controls. As the FC concentration correlated inversely, the PL:FC ratio correlated positively with the functionality of large HDL particles in CHD patients. Why these apparently functional particles, as assessed by an ex vivo assay, were not effective in effluxing cholesterol in vivo, indicated by the low cholesterol content in the particles, is an unanswered question. It is possible that results from an ex vivo efflux assay might not properly represent how effectively the particles efflux cholesterol in vivo.

The contribution of apoA1 and/or lipids to HDL antioxidative capacity is debated. Previously, we found no associations between the anti-oxidative capacity of apoB-depleted serum (a surrogate of HDL) and the concentrations of either HDL-C or apoA1 (13). In the present study, there was a positive correlation between the anti-oxidative capacity of apoB-depleted serum and the CE content of the large α-1 particles (r = 0.365). It is worth mentioning that besides CE and apoA1, HDLs carry several other lipid and protein molecules with redox capacity and that apoB-depleted serum, a commonly used surrogate for HDL in functional assays, also contains non-HDL-related molecules with potential redox capacity.

The strength of this study is that we have generated novel data on the lipidomic characterization of the major apoA1-containing HDL subpopulations utilizing a high-resolution separation and purification method, which may contribute to the better understanding of the composition-function relationship of HDL. However, this study has several weaknesses. We have studied a relatively small number of subjects and experienced high biological variability that limited the statistical power to show significant associations. Moreover, CHD patients were on stable statin treatment, which may have influenced the lipid composition and/or the efflux capacity of HDL particles compared with control subjects who did not receive statin treatment. Furthermore, several biologically important minor lipids were not measured.

CONCLUSIONS

We have documented a quantitative lipidomic analysis of five different-sized apoA1-containing HDL subpopulations. We have shown that the lipid composition of the different-sized α-mobility particles was very similar and that the small discoid shaped preβ-1 particles were more abundant in LPC and TG and had higher PL-CE and FC-CE ratios compared with α-mobility particles. We have also shown that preβ-1 particles contained more lipids (57 per particle) than predicted, of which 30% were neutral lipids (CE and TG), indicating that these particles were mainly remodeled from larger particles not newly synthesized. When CHD cases were compared with controls, we observed more significant differences in the lipidome of the large HDL particles than that of the small preβ-1 particles. We have found significant correlations between HDL particle functionality and the concentrations of several lipids. There was significant positive correlation between particle functionality and total lipids in high-F controls, indicating that the lipid-binding capacity of apoA1 plays a major role in the CEC of HDL particles. These results indicate a complex interrelationship between the lipid-binding capacity of apoA1 and the lipid composition and the functionality of HDL particles.
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