Distinct Proteomic Signatures in 16 HDL (High-Density Lipoprotein) Subspecies

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Objective—HDL (high-density lipoprotein) in plasma is a heterogeneous group of lipoproteins typically containing apo AI as the principal protein. Most HDLs contain additional proteins from a palate of nearly 100 HDL-associated polypeptides. We hypothesized that some of these proteins define distinct and stable apo AI HDL subspecies with unique proteomes that drive function and associations with disease.

Approach and Results—We produced 17 plasma pools from 80 normolipidemic human participants (32 men, 48 women; aged 21–66 years). Using immunoaffinity isolation techniques, we isolated apo AI containing species from plasma and then used antibodies to 16 additional HDL protein components to isolate compositional subspecies. We characterized previously described HDL subspecies containing apo AII, apo CIII, and apo E; and 13 novel HDL subspecies defined by presence of apo AIV, apo CI, apo CII, apo J, α-1-antitrypsin, α-2-macroglobulin, plasminogen, fibrinogen, ceruloplasmin, haptoglobin, paraoxonase-1, apo LI, or complement C3. The novel species ranged in abundance from 1% to 18% of total plasma apo AI. Their concentrations were stable over time as demonstrated by intraclass correlations in repeated sampling from the same participants over 3 to 24 months (0.33–0.86; mean 0.62). Some proteomes of the subspecies relative to total HDL were strongly correlated, often among subspecies defined by similar functions: lipid metabolism, hemostasis, antioxidant, or anti-inflammatory. Permutation analysis showed that the proteomes of 12 of the 16 subspecies differed significantly from that of total HDL.

Conclusions—Taken together, correlation and permutation analyses support speciation of HDL. Functional studies of these novel subspecies and determination of their relation to diseases may provide new avenues to understand the HDL system of lipoproteins.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2018;38:2827-2842. DOI: 10.1161/ATVBAHA.118.311607.)**

Key Words: apolipoproteins ■ cardiovascular diseases ■ enzyme-linked immunosorbent assay ■ humans ■ lipoproteins, HDL ■ peptides ■ proteomics

Although the plasma concentrations of HDL (high-density lipoprotein)-cholesterol (HDL-C) and apo AI are established inverse risk factors for cardiovascular disease (CVD), raising HDL-C or apo AI by pharmaceutical intervention has not so far lowered CVD.[1–5] Similarly, genetic variation associated with altered HDL-C levels did not associate with CVD.[6] It is possible that the treatments or genes associated with higher HDL-C or apo AI levels did not augment protective HDL functions, such as reverse cholesterol transport from peripheral tissues to the liver.[7] This may be explained by compositional and functional diversity among types of HDL. HDL-C (which only accounts for about 20% of HDL mass) reflects mainly the concentration of the large HDL subspecies and does not capture the protein and lipid species profile that likely drive HDL function.[8] HDL is composed of many types, which may have different functions related to CVD and other diseases.

Alaupovic proposed in 1964 that proteins on lipoproteins, including HDL, determined their diverse functions, and that lipoproteins may be advantageously classified from their protein components,[9] thus originating the subspeciation concept. In 1971, 2 groups independently demonstrated the concept of protein-based HDL subspecies.[10,11] About two-thirds of plasma apo AI in normolipidemic people is on HDL that also has apo AII, whereas the rest of apo AI is on HDL that is free of apo AII[12]. Apo AI HDL particles with apo AII differ metabolically from those without[13,14] in part because of their differential interaction with scavenger receptor B1[15] and it has been shown that they have distinct proteomes.[16]

HDL speciation has been explored according to content of apo CIII and apo E. About 5% to 10% of the total plasma apo AI in HDL is associated with apo CIII or apo E in people with normal body weight, and more in obesity.[17] With a plasma

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Nonstandard Abbreviations and Acronyms

A1AT	α-1-antitrypsin
A2M	α-2-macroglobulin
CoC3	complement C3
CP	ceruloplasmin
CVD	cardiovascular disease
FBG	fibrinogen
HDL-C	high-density lipoprotein-cholesterol
HP	haptoglobin
IAC	immunoaffinity column chromatography
ICC	intraclass correlation coefficient
KNG1	kininogen
PAI-1	plasminogen activator inhibitor 1
PLMG	plasminogen
PON1	paraoxonase 1
VTNC	vitronectin

HDL concentration of apo CIII of about 5 mg/dL, there are enough molecules of apo CIII to be present on most HDL particles rather than just 5% to 10% of them, providing additional support for the speciation concept. A similar argument can be made for HDL that contains apo E.[17] Several studies show that certain HDL proteins occur on particles in specific size ranges, such as larger sizes for apo E and smaller for apo AIV.[18–21] Enrichment of HDL with the acute-phase protein serum amyloid A may impair HDL function.[22–25]

Thus, proteins cluster into certain HDL particles and form subspecies rather than being distributed randomly throughout all HDL particles in plasma. The functional significance of subspecies is exemplified by HDL containing apo E and apo CIII. HDL containing apo E is active in size expansion and contraction, and clearance from the circulation, pathways of reverse cholesterol transport; and the simultaneous presence of apo CIII obliterates these metabolic effects.[26] HDL containing apo CIII is associated with higher risk of CVD and type 2 diabetes mellitus compared with HDL lacking apo CIII.[27,28] Finally, the apo E content of HDL lacking apo CIII is associated with lower risk of CVD.[26] These results show that proteins interact on HDL affecting metabolism and risk of disease.

Over the past decade, proteomics studies have identified many proteins associated with HDL.[20,25,29–42] The HDL Proteome Watch has compiled a list of these HDL proteins (http://homepages.uc.edu/~davidswm/HDLproteome.html), designating 95 as likely based on the criteria of appearing in reports from 3 independent laboratories. While it has been shown by immunoaffinity chromatography that apo AII, apo CIII, and apo E define apo AI containing HDL subspecies,[12,17] it seems likely that other proteins which are located in the HDL size or density range of plasma form unique subspecies of apo AI containing HDL. The properties of HDL-associated proteins include not only lipid metabolism and transport but also complement regulation, inflammation or acute-phase response, antioxidation, antithrombosis, hemostasis, and protease inhibition.[43] HDL-associated proteins overlap among HDL protein-defined subspecies. For example, apo CIII and apo E define individual HDL subspecies but also may coexist on HDL. HDL lacking apo AII (LpA1) has a much more diverse protein content than HDL containing apo AII (LpA1A2).[16,20,34] The overall proteomic profile of each HDL subspecies may direct its function.

We hypothesized that particular proteins define stable apo AI HDL subspecies that differ from each other and from HDL particles that lack the protein. We also posited that HDL subspecies grouped by the dominant function of their defining protein will exhibit more highly correlated proteomes reflecting similar functionality. From 95 HDL-associated proteins, we selected 16 proteins (Table I in the online-only Data Supplement) that are associated in the literature with one or more of the major HDL functions and, therefore, are likely to define metabolically important subspecies. We chose proteins to cover a range of HDL functions, including lipid metabolism, immunity, hemostasis, antioxidation, and anti-inflammation. The proteins included had to comprise at least 1% of the total HDL and could be reliably measured. We developed immunoassays to measure the subspecies and found remarkable similarities in proteomes between those with hypothesized overlap in function, as well as stability for up to 2 years within individuals.

Materials and Methods

The data that support the findings of this study are available from the corresponding author upon request.

This study was conducted in 3 phases to address the following aims: identification of HDL subspecies suitable for study, characterization of the concentrations of these HDL subspecies, and analysis of their proteomic profiles. First, large volume plasma pools were created to provide samples for the identification of HDL subspecies and the development of the measurement protocols. Pools that combined plasma from several individuals were chosen over individual samples to maximize volume of homogenous samples and to reduce the risk of including individuals with atypical subspecies profiles rather than representing typical values in the general population. The pools were designed to include men and women; and white, Hispanic, and black individuals. Next, we measured the apo AI concentrations of these subspecies. Finally, 3 of the pools were used for intensive study of the proteomic profiles of the HDL subspecies.

Creation of Plasma Pools

Seventeen plasma pools of ≈1 L each were created from plasma donated by 80 healthy men and women volunteers aged 21 to 66 years who were recruited at the Harvard T. H. Chan School of Public Health. Individuals fasted overnight and blood samples were collected in EDTA-treated tubes. Samples were immediately centrifuged to isolate plasma and all plasma from a single volunteer was combined and stored at −80°C. The plasma samples were immediately anonymized. All volunteers gave informed consent and samples were collected in compliance with a blood collection protocol reviewed and approved by the Harvard School of Public Health Institutional Review Board. A small aliquot of plasma from each of the 80 individuals was removed from cryogenic storage and thawed at room temperature. Total cholesterol, HDL cholesterol (after precipitation of apo B–containing lipoproteins with dextran sulfate and magnesium chloride), and triglycerides were measured by enzymatic methods (Infinity Kit, Thermo Fisher Scientific, Waltham, MA). Plasma of the individuals was assigned to pools based on the content of these lipids, self-reported race, or ethnicity of the volunteer (black, white, Hispanic), and date of sample collection. Characteristics of these 17 pools are described in Table II in the online-only Data Supplement.

Identification of HDL Subspecies

Immunoaffinity column chromatography (IAC) followed by ELISA (IAC/ELISA) is the laboratory standard for quantification of lipoprotein subspecies. This set of techniques uses antibodies to a particular protein of interest coupled to resin beads to bind intact lipoprotein complexes that contain that protein, separating them from plasma. We conducted an extensive screening of over 150 antibodies to establish successful capture-detection antibody pairs that would bind the proteins of interest for fractionation by IAC, as well as measurement by ELISA in both intact, HDL-associated and dissociated forms. Identification of an HDL subspecies was considered successful when the concentration of apo AI in an HDL subspecies was >1% of total HDL. HDL subspecies were isolated and measured as follows.

Isolation of HDL

HDL was isolated from plasma based on apo AI content by IAC. Two milliliters of plasma was incubated 16 hours overnight with gentle swirling at 4°C on 4 mL of Sepharose 4B resin (GE Healthcare, Marlborough MA, Catalog no. 17012001) coupled to a polyclonal anti–apo AI antibody (Academy Biomedical, Houston, TX, Catalog no. 11A-G2b, 10 mg antibody per 1 mL of resin) in 20 mL Econo-Pac chromatography columns (Bio-Rad Laboratories, Hercules, CA). HDLs were bound to the column by their content of apo AI. The unbound fraction not containing apo AI was eluted by gravity flow by washing 3× with 4 mL PBS. The bound HDL fraction was eluted by washing 3× with 4 mL of 3 M sodium thiocyanate followed by a single wash with 4 mL of PBS. Sodium thiocyanate was immediately removed by buffer exchange to PBS and restored to the initial load volume of 2 mL.

Subspeciation of HDL

0.1 mL of the HDL isolate was loaded into each of 16 different 5 mL Econo-Pac chromatography columns (Bio-Rad Laboratories, Hercules, CA), 1 per HDL subspecies to be isolated. These columns contained 0.5 mL of resin bound to antibody to 1 of the 16 proteins defining the HDL subspecies of interest (0.1 mg of antibody per 1 mL of resin). The details of these antibodies are described in Major Resources Table III in the online-only Data Supplement. HDL was incubated for 16 hours at 4°C with gentle swirling. The unbound HDL that lacked the subspecies-defining protein of interest was eluted by gravity flow washing 3× with 0.2 mL PBS. Bound fractions were eluted by washing 3× with 0.2 mL of 3 M NaSCN followed by a single wash with 0.2 mL of PBS. Sodium thiocyanate was immediately removed by buffer exchange to PBS and both the bound and unbound fractions were restored to the initial load volume of 0.1mL.

Measurement of Apo AI and the Subspecies-Defining Protein

Apo AI and the subspecies-defining protein of interest were measured in the bound and unbound fractions, as well as in the unfractionated plasma by sandwich ELISA. Absence of the subspecies-defining protein in the unbound fraction confirmed that the columns were functioning properly and not overloaded. The sources of coating antibodies and detection antibodies, as well as the ELISA antibody concentrations used for each assay, are described in Major Resources Table III in the online-only Data Supplement).

Concentrations of HDL Subspecies

The set of procedures that comprise IAC/ELISA is time consuming and complex, making it unsuitable for high-throughput analysis. We developed a series of 16 assays based on a novel ELISA protocol to allow measurement of the concentrations of the HDL subspecies without the need for IAC. These protocols are more efficient than IAC/ELISA in that they are faster and less costly, yet exhibit increased reliability.

Reference Concentrations

We first established reference concentrations of apo AI in the 16 HDL subspecies using the laboratory standard IAC/ELISA protocol described above in 4 of the 17 plasma pools (C3-7, C3-8, C3-9, C3-10; Table II in the online-only Data Supplement). This protocol was repeated 5× to 10× per HDL subspecies and the mean values were set as the reference values for the 4 plasma pools. One of these pools was used as the calibration standard curve for the ELISAs that measured the 16 HDL subspecies, whereas the other 3 were run in each assay batch as control samples.

Novel ELISA-Based Procotol

The 16 ELISA-based immunoassays we developed to isolate HDL subspecies and measure the concentration of apo AI containing and lacking the protein of interest differ from each other only in the antibodies used and the sample dilutions as described in Major Resources Table III in the online-only Data Supplement. Here we describe in detail the protocol for HDL containing apo CIII as an example. This assay procedure measures the concentration of plasma total apo CIII, apo AI in HDL that lacks apo CIII, and apo AI in HDL that contains apo CIII (Figure 1). In addition, plasma total apo AI was measured. The percentage of apo AI in HDL that contains apo CIII was calculated by dividing the concentration of apo AI in HDL that contains apo CIII by the concentration of total apo AI. We assessed sensitivity, specificity, validity, and reproducibility through a series of experiments with clear a priori criteria for acceptance. The novel ELISA-based protocol met all prespecified criteria for acceptance and exhibited better reproducibility than the laboratory standard IAC/ELISA protocol.

Fractionation by Apo CIII Content and Measurement of Apo CIII

A 96-well microplate (Greiner Bio-One MICROLON 600, VWR Catalog no. 82050–734) was coated by incubation for 1 hour at 37°C with rabbit antihuman apo CIII antibody (Academy Biomedical, Catalog no. 33A-R1b, 10 µg/mL in 1× PBS) and blocked with casein blocking buffer. This apo CIII plate binds lipoproteins that contain apo CIII and then is used to measure plasma total apo CIII. One of the reference plasma pools described above whose concentration of plasma total apo CIII, plasma total apo AI, apo AI containing apo CIII, and apo AI lacking apo CIII were previously established by the standard IAC/ELISA protocol was chosen for the calibration curve. This calibration curve was prepared at a starting dilution of 1:2000 and serially diluted 3× to 1:4.4 million times with PBS containing 0.5% BSA. The test samples consisted of the 17 plasma pools created for this study which were prepared at 1:10 000 dilutions, a dilution that was chosen empirically during methods development to maximize protein concentration yet ensure complete binding of all apo CIII by the plate. Importantly, the diluent did not contain surfactant or detergent to ensure that the lipoprotein particles remained intact. The prepared calibration curve and pooled samples were loaded into the apo CIII plate and incubated overnight at 4°C. On the second day, the liquid in each well comprising the unbound fraction of lipoproteins lacking apo CIII was removed from the apo CIII plate and reserved for subsequent analysis (apo AI lacking apo CIII). The apo CIII plate was washed 3× gently with 1× PBS with no surfactant or detergent to remove all traces of the unbound fraction yet leave bound lipoproteins intact, then incubated for 1 hour at 37°C with Tween-containing ELISA diluent (2% BSA, 0.05% Tween 20 in 1× PBS) to dissociate the lipid and protein components of the lipoproteins from the apo CIII that was bound by antibody to the plate. The dissociated fraction was removed from the apo CIII plate and reserved for subsequent analysis (apo AI containing apo CIII). The plate was washed 3× with ELISA wash buffer (0.1% Tween 20 in 1× PBS) to remove all traces of the dissociated fraction and then incubated 1 hour at 37°C with anti–apo CIII antibody conjugated to horseradish peroxidase (Academy Biomedical, Catalog no. 33H-G2b, 1 µg/ml in 1× PBS). After 3 more washes, o-phenylenediamine (Sigma Aldrich, Catalog no. P9187) was added to each well of the apo CIII plate, color was allowed to develop for 1 hour and 20 minutes at room temperature, and the absorbance was read at 450 nm.

Measurement of HDL Lacking Apo CIII

HDL lacking apoCIII was quantified by the concentration of apo AI in lipoproteins lacking apo CIII as fractionated by the apo CIII plate.

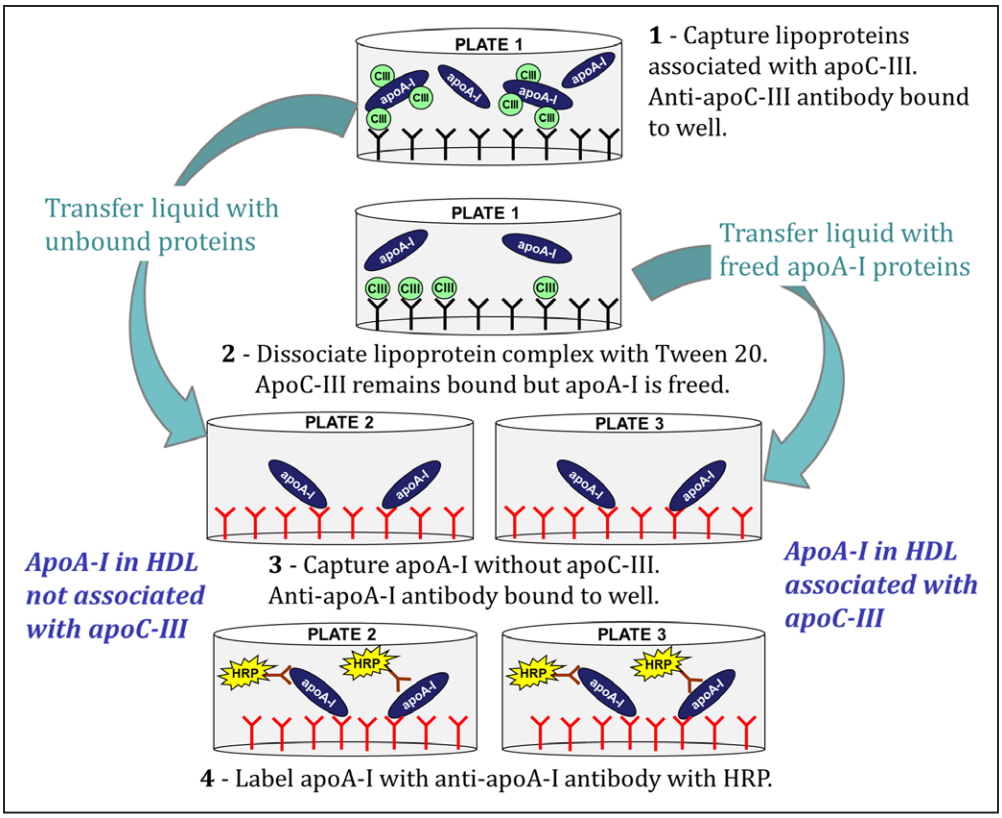

Figure 1. The novel sandwich ELISA procedure, using apo AI of HDL (high-density lipoprotein) associated and not associated with apo CIII as an example.

A 96-well microplate was coated by incubation for 1 hour at 37°C with goat antihuman apo AI antibody (Academy Biomedical, Catalog no. 11A-G2b, 5 µg/ml in 1× PBS) and blocked with casein blocking buffer. This apo AI lacking apo CIII plate was loaded with the unbound fraction of lipoproteins lacking apo CIII from the apo CIII plate diluted 10× with Tween-containing ELISA diluent (2% BSA, 0.05% Tween 20 in 1× PBS). Tween is used in this diluent to dissociate the lipoprotein complexes, which renders the proteins more amenable to binding to antibodies. The apo AI lacking apo CIII plate was then incubated for 1 hour at 37°C and washed 3× with ELISA wash buffer (0.1% Tween 20 in 1× PBS). Anti–apo AI antibody conjugated to biotin (Academy Biomedical, Catalog no. 11B-G2b, 1 µg/mL with 1× PBS) was added and the plate was incubated for 1 hour at 37°C and washed 3× with ELISA wash buffer. Avidin-peroxidase (Sigma Aldrich, Catalog no. A7419-2ML, 0.01 µg/mL in 1× PBS) was added and the plate was incubated for 1 hour at 37°C. After 3 more washes, o-phenylenediamine was added, color was allowed to develop for 1 hour and 20 minutes at room temperature, and the absorbance was read at 450 nm.

Measurement of HDL Containing Apo CIII

HDL containing apo CIII was quantified by the concentration of apo AI in lipoproteins containing apo CIII as fractionated by the apo CIII plate. A 96-well microplate was coated identically to the apo AI lacking apo CIII plate. This apo AI containing apo CIII plate was loaded with the fraction of lipids and proteins dissociated from the apo CIII plate with Tween-containing diluent. The plate was then processed identically to the apo AI lacking apo CIII plate, with a 1-hour incubation at 37°C followed by consecutive incubations with biotinylated anti–apo AI, avidin-peroxidase, and o-phenylenediamine separated by washing steps.

Measurement of Plasma Total Apo AI

A 96-well microplate was coated identically to the apo AI lacking apo CIII plate. The plasma pool used as a calibration curve was diluted starting at 1:20 000 and then serially 2× through 1:1.28 million with Tween-containing ELISA diluent. Samples and controls were diluted 1:140 000 with the same diluent. The plate was incubated 1 hour at 37°C, washed 3× with ELISA wash buffer, and incubated 1 hour at 37°C with anti–apo AI antibody conjugated to horseradish peroxidase (Academy Biomedical, Catalog no. 11H-G1b, 1 µg/mL in 1× PBS). After 3 more washes, o-phenylenediamine was added, color was allowed to develop for 1 hour and 20 minutes at room temperature, and the absorbance was read at 450 nm.

In all cases, ELISAs were judged on the quality of the calibration curve, the correlation of obtained and expected values of the control samples (required $r>0.7$), and the coefficients of variation for the unknown samples. Extreme outliers could be removed from calibration curves, but curves had to be produced from at least 4 of the 7 calibration curve points and show a fit of $r^2>0.95$. The average coefficient of variation (%CV) for all replicate samples could not exceed 15% for a plate to be accepted. Individual replicates whose %CV exceeded 20% were repeated.

Proteome Analysis of HDL Subspecies

HDL Subspecies Isolation

Three of the control pools (C3-8, C3-9, and C3-10; Table II in the online-only Data Supplement) were fractionated into the 16 HDL subspecies by IAC using the procedure previously described in the Isolation of HDL and Subspeciation of HDL sections above. Samples were frozen at −80°C and shipped overnight on dry ice to the University of Cincinnati for analysis.

Preparation of HDL Subspecies for Analysis

HDL subspecies were delipidated by organic solvent as previously described[16] with minor modifications. Briefly, samples were dialyzed into 50 mmol/L ammonium bicarbonate (NH_4HCO_3) at pH 8.1 and lyophilized to dryness. Lipids were extracted by addition of 0.75 mL of ice-cold chloroform:methanol (2:1 v/v) and incubation on ice for 30 minutes. Ice-cold methanol was added to a final chloroform:methanol ratio of 1:1 (v/v) and protein was pelleted by centrifugation at 8000*g* for 30 minutes. Solvent was decanted, and

the protein was resuspended in 1.5 mL of ice-cold methanol and mixed by vortexing and sonication. Protein was pelleted by centrifugation and resuspended in 90 μL of 20% methanol/80% 50 mmol/L NH_4HCO_3 at pH 8.1. Samples were reduced with dithiothreitol at 10 mmol/L for 30 min at 42°C. Reduced protein was carbamidomethylated for 30 minutes at room temperature in the dark. Sequencing grade trypsin (Promega) was added to a final protease:protein ratio of 1:20 (w/w) and sample was digested for 16 hours at 37°C. Additional trypsin was added to bring the final protease:protein ratio to 1:10 (w/w) and samples were digested for an additional 2 hours at 37°C. Samples were lyophilized to dryness and stored at −20°C until MS analysis.

Mass Spectrometry Analysis of Peptides

Methods are given in the online-only Data Supplement.

Data Reduction

Raw spectral counts were obtained for 209 proteins in total HDL and each of the 16 HDL subspecies across the 3 pools analyzed. We reduced this list to 150 proteins by eliminating keratin proteins (generally considered sample preparation contaminants) and IgG proteins. We further reduced the protein list to 42 by eliminating proteins whose inclusion was based on identification in a single sample or whose mean spectral count in the 3 total HDL samples was <4. The average spectral count for the 42 proteins was 47 with a range of 4 to 150 and an SD of 70.

Computation of Relative Proteomes of the 16 HDL Subspecies

The raw spectral counts within each HDL subspecies were normalized to the apo AI spectral counts in that subspecies to equalize HDL abundance among the 16 HDL subspecies and total HDL, given that apo AI was the defining protein for HDL in this study. We then calculated the ratio of the apo AI normalized values in each HDL subspecies to the apo AI normalized values in the total HDL sample for each of the 3 pools. This data set is referred to as the relative proteome (relative to total HDL). We calculated the mean and SE across the 3 pools for each of the 16 HDL subspecies (mean relative proteome). For each of the 42 proteins in each HDL subspecies proteome, enrichment relative to total HDL is defined as a mean value >1 whose 2(SE) range does not include 1, while depletion is defined as mean value <1 whose 2(SE) range does not include 1.

Statistics

We examined stability of HDL subspecies using intraclass correlation coefficients (ICC) in 2 different settings. First, for ICC1 (Table 1) we measured 8 of the 16 HDL subspecies in 2 samples collected from the same individual at dates 1 to 2 years apart. At the time this was performed, assays for 8 of the 16 HDL subspecies had not yet been developed. We then measured all 16 HDL subspecies in 2 samples collected from the same individual at dates 3 months apart (ICC2; Table 1). In both cases, the ICC were calculated by dividing the between-person variance by the sum of the between- and within-person variances obtained using SAS PROC MIXED models as described by Hankinson et al[44] and Pai et al.[45]

The proteomes of each subspecies were compared by 2 complementary methods. First, similarities between proteomes were assessed by Pearson correlation coefficients calculated among all HDL subspecies' relative proteomes. Significant correlations were defined as $P<0.0004$ ($\alpha=0.05$ with a Bonferroni correction for 120 comparisons). Second, the uniqueness of each HDL subspecies in relation to the others was evaluated using a permutations analysis in which we test the null hypothesis that there is no difference between pairwise comparisons of proteomes among the HDL subspecies. A significant P value rejects the null hypothesis. The statistic we used to compare the different HDL subtype proteomes is the maximum scaled mean differences between any 2 subtypes. For 2 HDL subspecies i and j, the mean difference of the kth measurement is defined as $\Delta_k ij$ and the estimated standard variance is $\sigma_k ij$. The test statistic is max $|(\Delta_k ij)/(\sigma_k ij)|$. To find the distribution of our test statistics, we assume there is no difference between HDL subspecies i and HDL subspecies j. Under this assumption, random shuffle between categories can create the new mean difference $\Delta^*_k ij$ and would not change the distribution of our test statistics and we can simulate this distribution by repeatedly shuffling 10 000×. Then, to illustrate functional groupings suggested by proteomic similarities, we prepared a network analysis linking HDL subspecies with statistically significant correlations that were in concordance with the permutations analysis results. All statistical analyses were performed using the SAS software package, version 9.4 (SAS Institute Inc, Cary, NC) or R (R Core Team [2013]).

Table 1. Intraclass Correlation Coefficients Show Within-Subject Stability of HDL Subspecies Over Time

	ICC1	ICC2
Apo AI	0.75	0.77
HDL cholesterol	0.71	
HDL that contains		
α-1-antitrypsin	…	0.37*
Apo AII	0.42	0.75
Apo AIV	…	0.62
α-2-macroglobulin	…	0.53
Apo CI	0.67	0.73
Apo CII	0.69	0.59
Apo CIII	0.75	0.58
complement C3	0.60	0.51
ceruloplasmin	…	0.33
Apo E	0.69	0.86
fibrinogen	…	0.60
haptoglobin	0.55	0.76
Apo J	…	0.37
Apo LI	…	0.74
plasminogen	…	0.37
paraoxonase-1	0.52	0.75

ICC1, intraclass correlation coefficients calculated from repeated samples from 60 individuals from the Nurses' Health Study collected 1–2 y apart. Eight of the 16 HDL subspecies were included in this evaluation.

ICC2, intraclass correlation coefficients calculated from repeated samples from 76 individuals collected 3 mo apart.

HDL indicates high-density lipoprotein.

*n=36 individuals.

Results

HDL Subspecies Concentrations

HDL subspecies were determined by the novel ELISA-based protocols described in the methods section and Figure 1. Briefly, HDL containing the subspecies-defining protein was bound to a 96-well plate coated with antibody to that protein (plate 1). The unbound fraction containing HDL without the protein was removed and its apo AI concentration was measured by ELISA (plate 2). The HDL bound to the first plate was dissociated by surfactant and components of HDL other than the antibody-bound protein were released into solution, which was removed and its apo AI concentration was measured by ELISA (plate 3).

HDL Subspecies Stability

The stability of each of the 16 HDL protein-based subspecies was established by assessment of reproducibility of the concentration of HDL containing the protein of interest within independent aliquots made from a single blood draw, as well as reproducibility within an individual across independent blood draws over time. In an externally administered blinded validation study, the coefficient of variation for the 16 HDL subspecies in blinded replicate aliquots averaged 8% indicating low variability from 1 aliquot to another of the same sample. Biological stability over time was established by comparing subspecies concentrations in 2 samples from the same individuals sampled 3 months apart (all 16 subspecies) and 1 to 2 years apart (8 subspecies). The ICC shown in Table 1 (0.33–0.86; mean 0.62) indicate good stability of these subspecies over time. In studies of biomarkers, an ICC<0.4 indicates poor reproducibility, 0.4<ICC<0.75 indicates fair to good reproducibility, and ICC>0.75 indicates excellent reproducibility.[46]

HDL Subspecies Concentrations

The concentrations of blood lipids and apo AI for the 17 plasma pools are shown in Table II in the online-only Data Supplement and Figure 2. Apo AII was associated with 60% of the total apo AI (range 48%–71%), apo E with 9% (range 4%–18%), and apo CIII with 6% (range 5%–8%), consistent with previous reports.[12,17] Among the novel HDL subspecies, the most abundant was apo CI HDL (HDL containing apo CI),

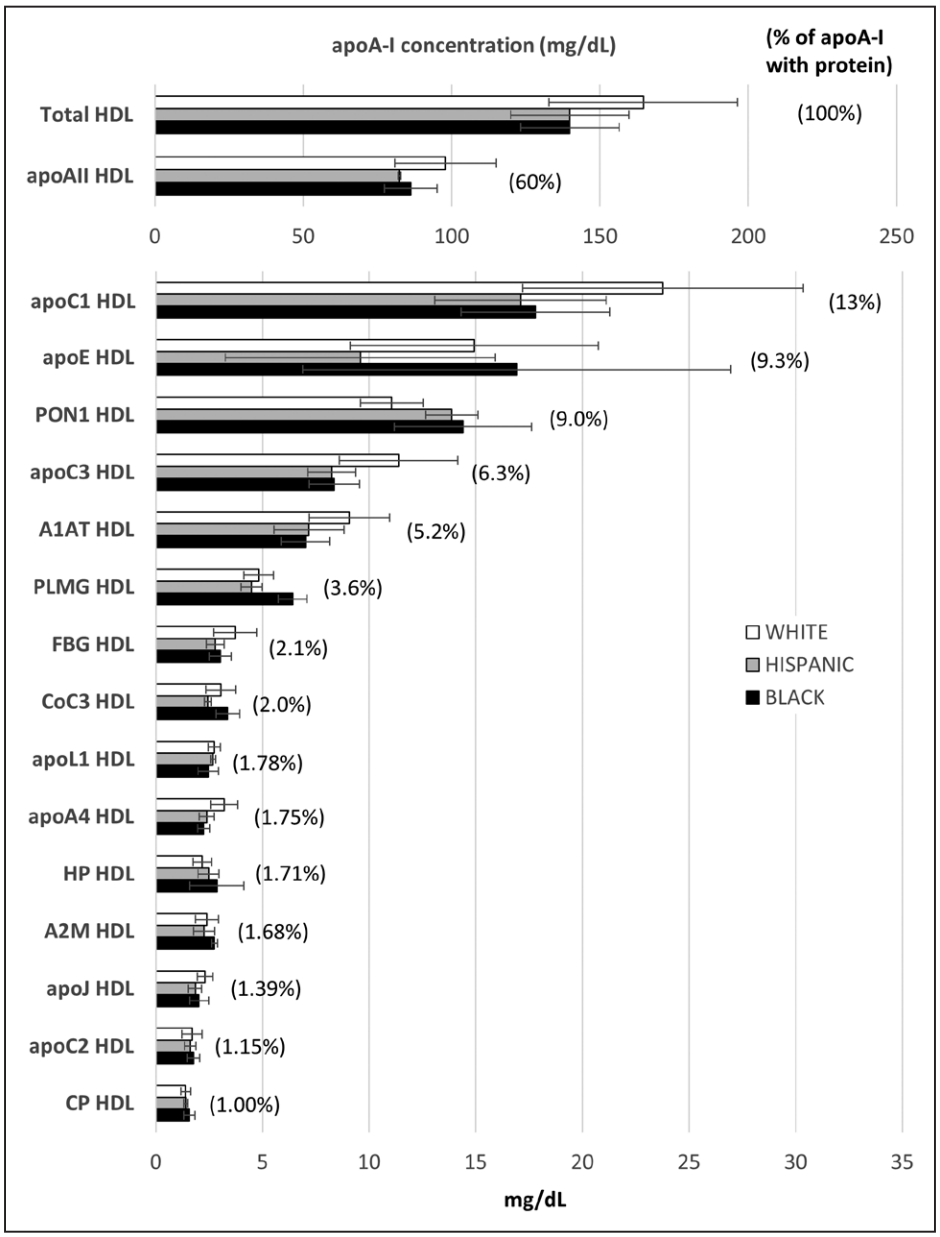

Figure 2. The apo AI concentration in plasma and in each HDL (high-density lipoprotein) subspecies by race. White (n=10 pools; n=59 individuals), Black (n=4 pools; n=13 individuals), Hispanic (n=3 pools; 8 individuals). Each data point is the average of the pools (mean±SD). To the right of each is the overall mean percentage of apo AI associated with each HDL protein. A1AT indicates α-1-antitrypsin; Apo AII HDL, HDL containing apo AII; apo CI HDL, HDL containing apo CI; CoC3, complement C3; CP, ceruloplasmin; FBG, fibrinogen; HP, haptoglobin; PLMG, plasminogen; and PON1, paraoxonase-1.

with 11% to 18% of apo AI HDL in association with apo CI (21±6 mg/dL). HDL subspecies containing PON1 (paraoxonase 1; PON1 HDL) or A1AT (α-1-antitrypsin; A1AT HDL) were relatively prevalent subspecies with 9% and 5% of total apo AI associated with them, respectively. The other 9 subspecies were associated with 1% to 5% of total apo AI.

HDL Subspecies Relative Proteomes

Proteomes of the 16 HDL subspecies were analyzed in 3 of the control pools containing a total of 18 individuals (C3-8, n=4; C3-9, n=5; and C3-10, n=9) (Table II in the online-only Data Supplement). The 42 selected proteins of HDL are described in Table IV in the online-only Data Supplement. They have been sorted into 7 groups by their purported functions: protease inhibition, hemostasis, lipid metabolism and transport, immune system, complement system, antioxidant, and acute-phase response/inflammation. Proteomes of each HDL subspecies relative to that of total HDL are shown in Figures 3 through 7. The 7 groups by purported function are indicated to the left of the graph. For each of the 42 proteins

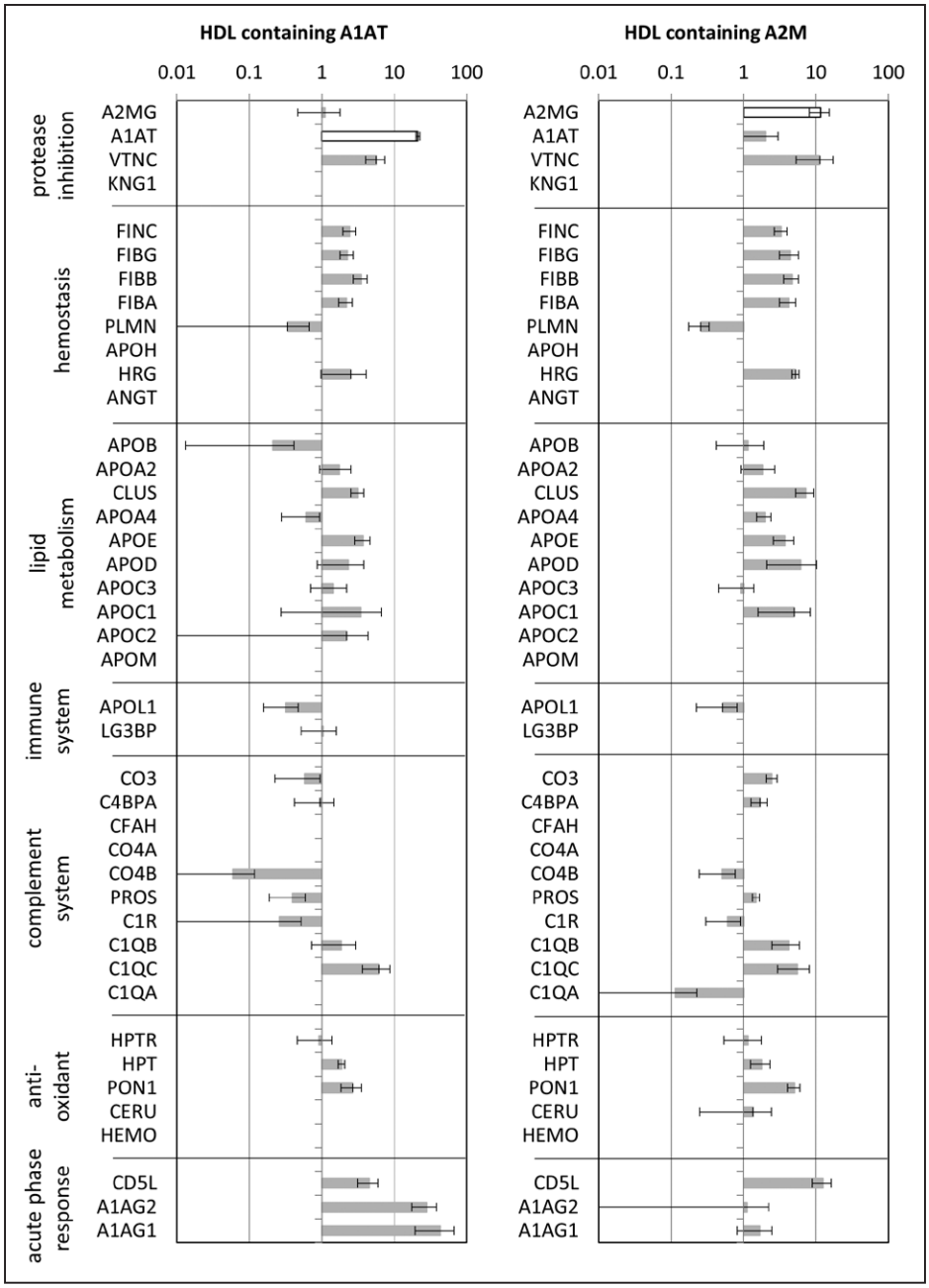

Figure 3. Protease inhibition. Proteomics of apo AI HDL (high-density lipoprotein; HDL containing apo AI) subspecies that are defined by proteins involved in protease inhibition. Data are mean of 3 pools representing 18 individuals with standard error (SE) bars. Missing bars indicate that protein was not detected in any of 3 replicates of that HDL subspecies. Graphs are in logarithmic scale. Proteins are expressed relative to the total HDL proteome with each protein's spectral counts normalized by apo AI spectral count within each pool. Enrichment relative to total HDL is defined as a mean value greater than 1 whose 2(SE) range does not include 1, while depletion is defined as mean value <1 whose 2(SE) range does not include 1. A1AT indicates α-1-antitrypsin; and A2M, α-2-macroglobulin. Proteins are listed using standard abbreviations. Full names can be found in Table IV of the online-only Data Supplement and https://www.uniprot.org.

in each HDL subspecies proteome, enrichment relative to total HDL is defined as a mean value >1 whose 2(SE) range does not include 1, while depletion is defined as mean value <1 whose 2(SE) range does not include 1. For example, Figure 3 presents the relative proteome of A1AT HDL (HDL containing A1AT). Looking at the Protease inhibition group, the A1AT HDL subspecies does not differ from total HDL in A2MG (α-2-macroglobulin), while it is enriched in A1AT and VTNC (vitronectin) by 21- and 6-fold, respectively. KNG1 (kininogen) was not detected.

Protease Inhibitors and Hemostasis (FBG HDL, PLMG HDL, A1AT HDL, A2M HDL)

These HDL subspecies (Figures 3 and 4) are enriched in both protease inhibitor and hemostasis panel proteins; most of the antioxidant and acute-phase response proteins; and apo E and apo J. A1AT HDL, A2M HDL, and FBG (fibrinogen) HDL are depleted in plasminogen, and all are depleted in apo LI.

Lipid Metabolism (Apo A2 HDL, Apo A4 HDL, Apo C1 HDL, Apo C2 HDL, Apo C3 HDL, Apo E HDL, Apo J HDL)

Apo A2 HDL is depleted in nearly all of its proteins, except for enrichment in apo CI, apo CII, and apo M (Figure 5). The relative proteomes of apo C1 HDL, apo C2 HDL, apo C3 HDL, and apo E HDL are similar to each other. They are enriched in lipid metabolism proteins and in vitronectin but depleted in kininogen and the immune system proteins. Apo CII is depleted in apo C3 HDL. Apo J HDL shows marked enrichment in the protease inhibition, immune system, and hemostasis proteins.

Antioxidation (CP HDL, PON1 HDL, HP HDL)

These subspecies have similar relative proteomic profiles (Figure 6). They are enriched in the protease inhibition and hemostasis proteins. HP HDL and PON1 HDL are enriched in the acute-phase response proteins, HP (haptoglobin), and PON1, but depleted in CP (ceruloplasmin).

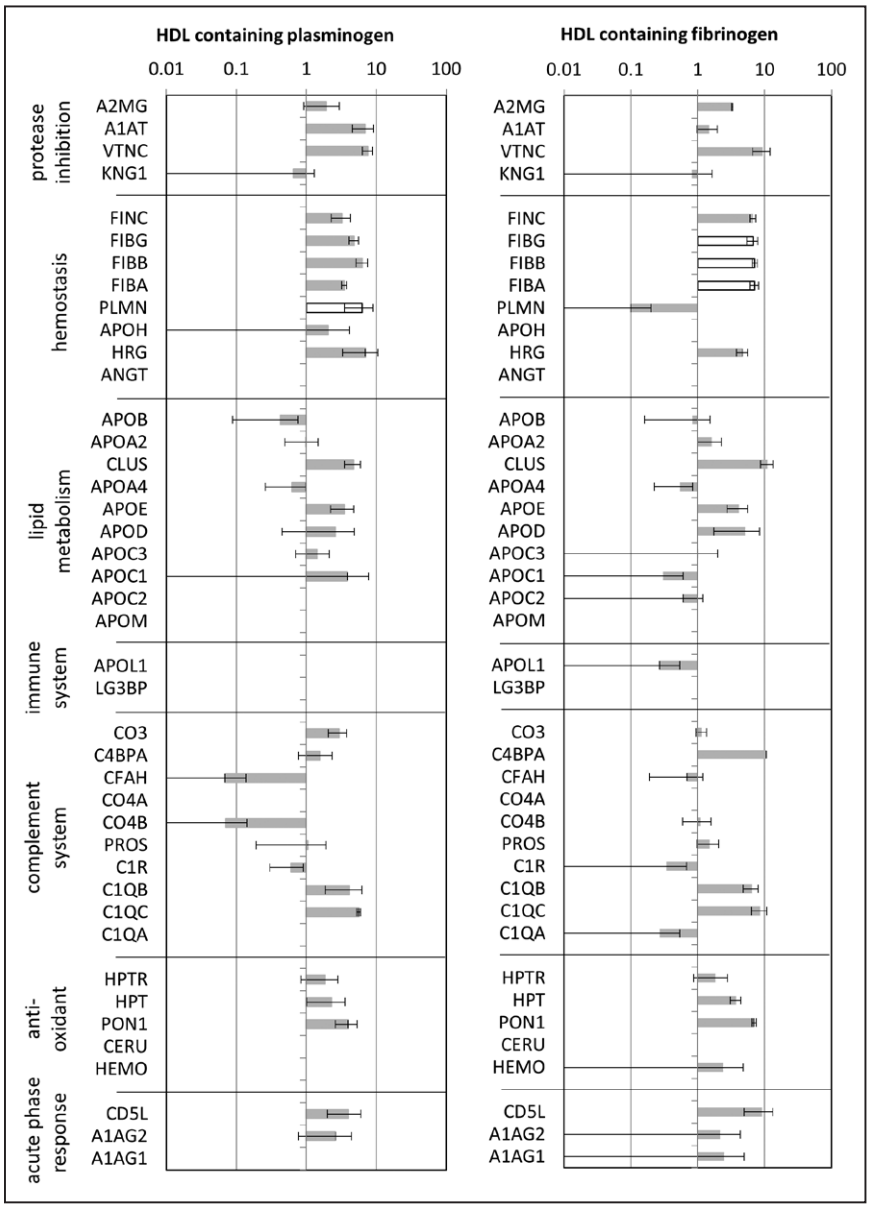

Figure 4. Hemostasis. Proteomics of apo AI HDL (high-density lipoprotein; HDL containing apo AI) subspecies that are defined by proteins involved in hemostasis. See Figure 3 for experimental details and explanation.

Immunologic Functions (CoC3 HDL, Apo L1 HDL)

CoC3 (complement C3) HDL and apo L1 HDL show an indistinct pattern across all panels with few proteins differing in enrichment from total HDL (Figure 7).

Comparing HDL Subspecies' Relative Proteomes.

The relative proteomes of the 16 HDL subspecies were compared by correlation analysis adjusted for multiple comparisons (Table 2). When comparing the relative enrichment values of the 42 proteins we studied between A1AT HDL and A2M HDL, the correlation was poor at 0.046, whereas that between apo E HDL and apo C1 HDL was high at 0.895. Most of the HDL subspecies defined by lipid metabolism proteins had relative proteomes that were highly correlated with each other. However, the proteomes of apo J HDL and apo A4 HDL did not correlate with proteomes of other lipid metabolism subspecies. The relative proteomes of the HDL subspecies defined by protease inhibition (A1AT HDL and A2M HDL) were not significantly correlated with each other. The subspecies related to hemostasis (PLMG [plasminogen] HDL and FBG HDL) were correlated with each other. Of the antioxidant HDL subspecies, CP HDL more closely resembled the proteomes of PLMG HDL, CoC3 HDL, and apo J HDL than those of the other antioxidant subspecies (PON1 HDL and HP HDL).

Differences between the proteomes of the HDL subspecies were assessed by permutations analysis (Table V in the online-only Data Supplement). One hundred thirty-six pairwise comparisons were made and 58 statistically significant differences were found. Of the 58 differences, 48 were concordant with the correlation data (Table 2) in that the correlation coefficients were not statistically significant ($P>0.0004$), whereas 10 were discordant with the correlation data. The proteomes of 12 of the 16 subspecies differed significantly from that of total HDL.

In Figure 8, we present a network analysis of the 16 HDL subspecies. We used the connections indicated by correlation and permutation analysis as described in the Methods to revise the initial hypothetical groupings (indicated by similar color spheres in the figure) that had been based on purported function of the defining protein. In this figure, blue lines indicate significant correlations between subspecies proteomes within hypothesized groups (thus expected) and red lines indicate unexpected significant correlations. The weight of the line is proportional to the degree of correlation. Only connections supported by both the correlation and permutation analyses were used. The revised groupings are indicated by gray boxes: antioxidant/anti-inflammatory group (PON1 HDL, HP HDL, A1AT HDL), hemostasis group (CP HDL, CoC3 HDL, PLMG HDL, A2M HDL, FBG HDL, apo J HDL, apo C2),

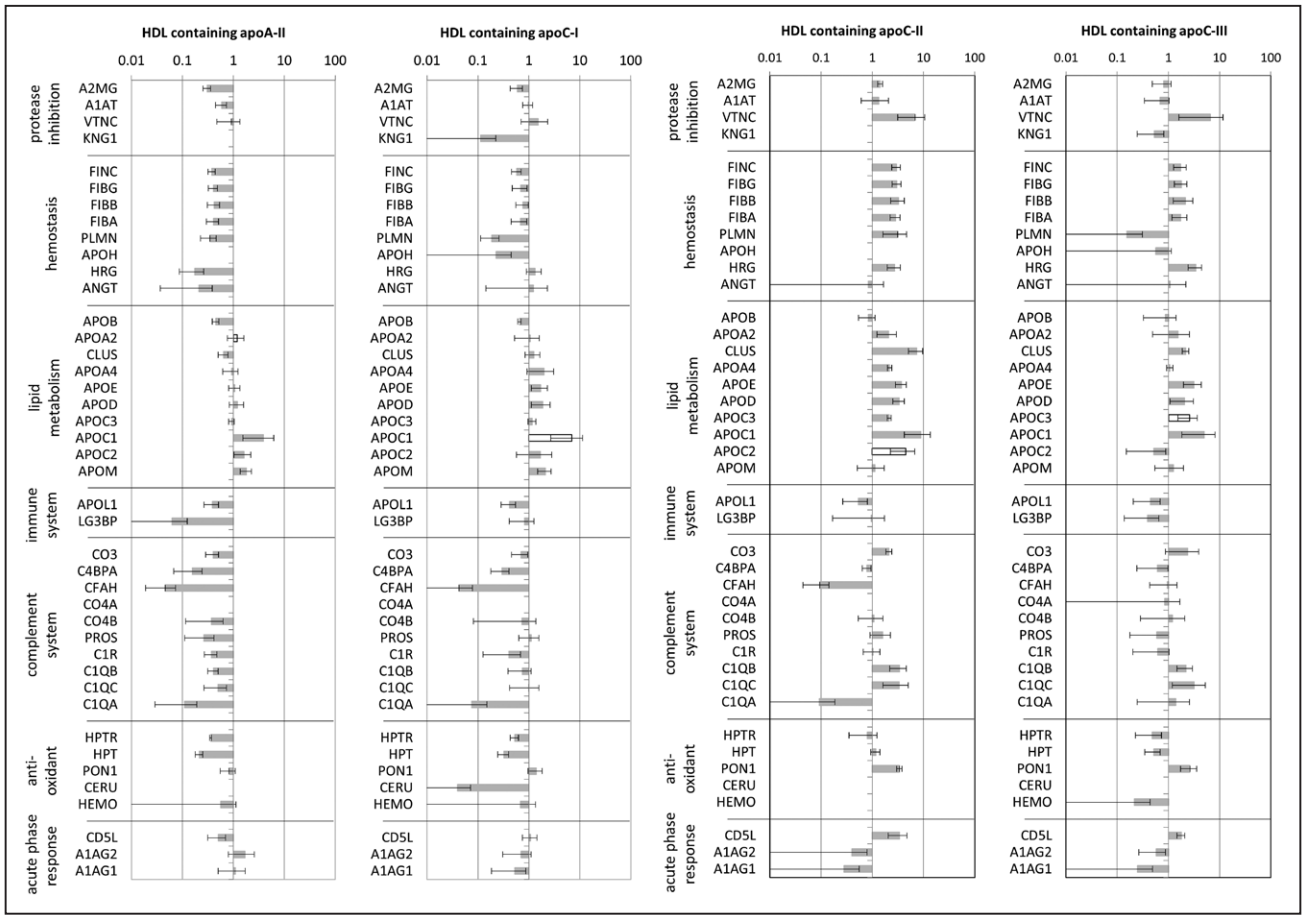

Figure 5. Lipid Metabolism. Proteomics of apo AI HDL (high-density lipoprotein; HDL containing apo AI) subspecies that are defined by proteins involved in lipid metabolism. (*Continued*)

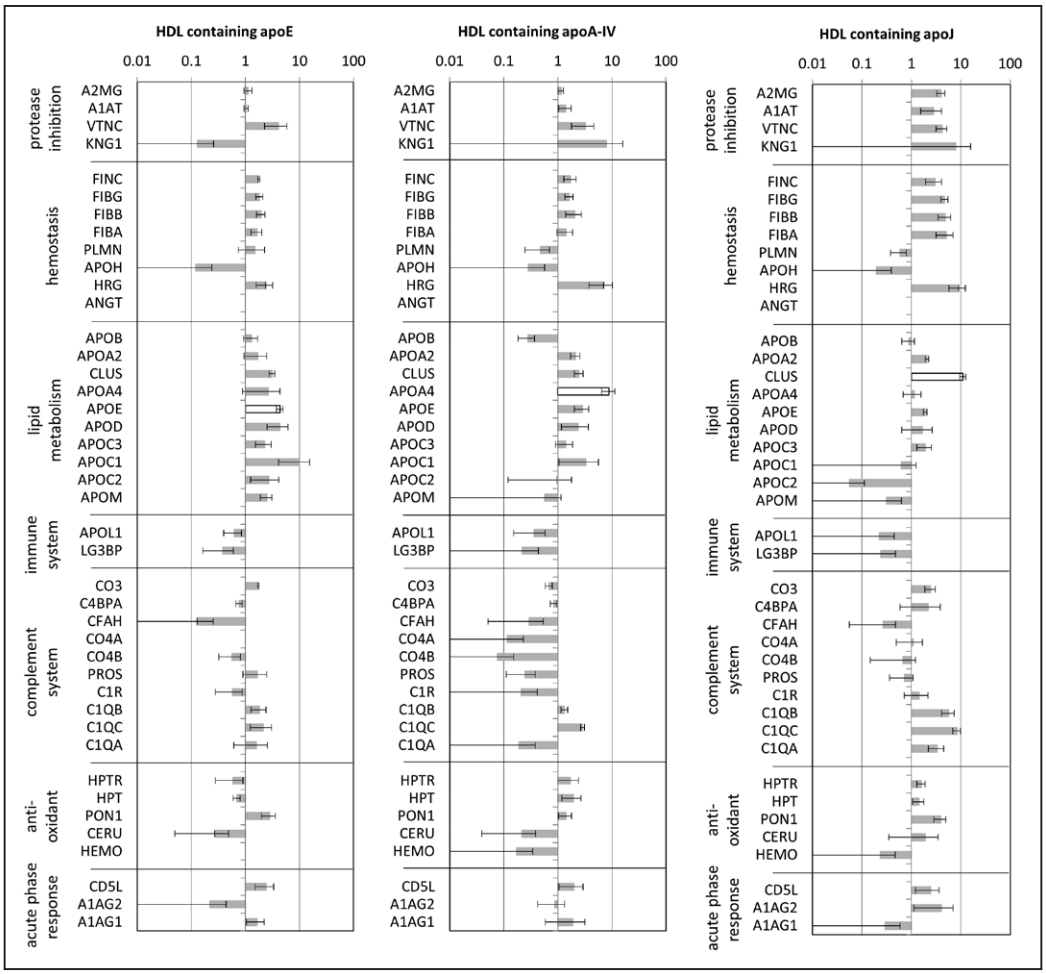

Figure 5 Continued. See Figure 3 for experimental details and explanation.

and lipid metabolism group (Apo C2 HDL, apo E HDL, apo A2 HDL, apo C3 HDL, apo C1 HDL). Apo L1 HDL and apo A4 HDL do not seem to connect with the other subspecies. Apo C2 HDL seems to overlap in the hemostasis and lipid metabolism groups.

Discussion

We have shown that HDL particles can be speciated by their protein content and that these subspecies are stable within individuals. Additionally, certain proteins cosegregate during the immunoaffinity isolation, suggesting that a disequilibrium exists that targets certain proteins to certain particles. Furthermore, proteins with similar known functions tend to segregate together. Taken together, these results support the hypothesis that different classes of HDL exist which are likely tasked to certain biological activities in metabolism and disease. Given the small size of HDL and large variety of peptides identified in each subspecies, it is not physiologically plausible that the HDL subspecies are composed of a set of identical HDL particles containing exact amounts of each of the proteins in our panel. Rather, the HDL subspecies are likely to be groups of HDLs all containing the defining protein plus varying arrays of other HDL proteins that emphasize a subset of these proteins (a proteomic profile) that is characteristic to that particular subspecies, together driving that subspecies' function.

We developed an ELISA-based method to measure the concentrations of 16 HDL subspecies that is faster and more precise than standard immunoaffinity chromatography followed by ELISA. This is imperative for the measurement of HDL subspecies in larger scale research studies, such as prediction of disease and effects of treatments. These subspecies differ from each other in their proportions of total HDL, ranging from about 1% to 20% (except 60% for apo A2 HDL). The subspecies existed in relatively narrow ranges of concentration preserved across the various plasma pools. The apo AI concentrations of the subspecies are stable within individuals, as shown by good ICC, supporting the use of the subspecies as potential biomarkers for studies of disease prediction. The modest sample size limits the power to detect small to moderate differences between sexes and among race and ethnic groups.

For many subspecies, there is enough of the defining protein in plasma to be present on all or nearly all HDL particles, yet accumulate on only a fraction of the total HDL (Figure 2; Table I in the online-only Data Supplement). Thus, the proteins that define subspecies are clearly not randomly distributed across the entire range of plasma HDL. It is likely that

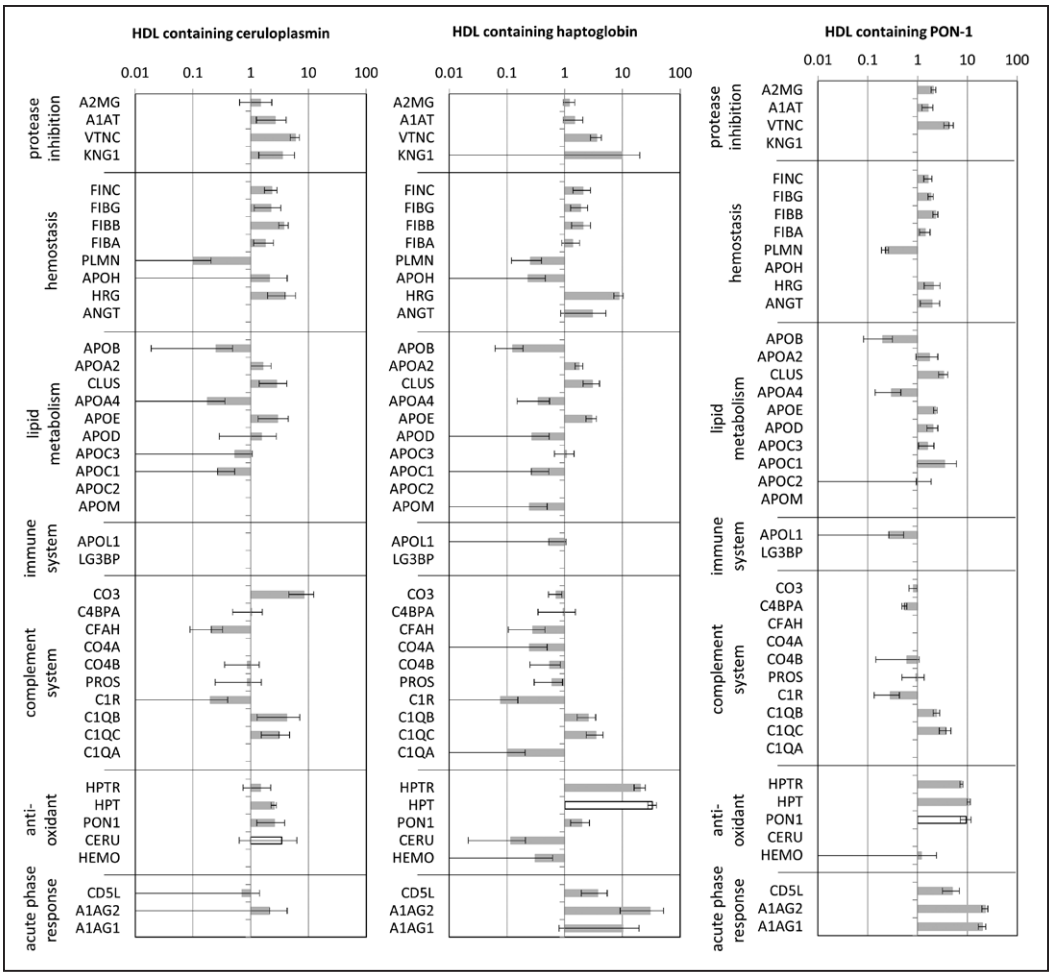

Figure 6. Antioxidant. Proteomics of apo AI HDL (high-density lipoprotein; HDL containing apo AI) subspecies that are defined by proteins involved in antioxidant functions. See Figure 3 for experimental details and explanation.

there is something unique about the portion of HDL which attracts a particular protein and keeps it there (size, lipidome, protein conformation, presence of certain other proteins, etc).[34] In turn, this clustering of certain proteins may elicit metabolic and functional uniqueness. These properties may impart differential associations with disease that render some HDL subspecies protective against disease and others risk factors for the disease.

The functional properties of the 16 HDL subspecies that we studied could be driven solely by the protein defining that subspecies, but also modified by other proteins that coexist on the same HDL. Thus, knowledge of the proteome of each subspecies facilitates the ability to derive testable hypotheses of their function. Indeed, it has already been shown that apo C3 HDL is dysfunctional compared with HDL lacking apo CIII in that the former is associated with increased rather than decreased risk of CVD; and apo CIII in HDL nullifies the beneficial effect of apo E on HDL metabolism and relation to CVD.[26]

The relative proteomes of the subspecies defined by protease inhibitor proteins (A1AT, A2M) and hemostasis (FBG, PLMG) proteins are similar. The activities of the protease inhibitors are involved in hemostatic mechanisms,[47,48] supporting overlap of function of these HDL subspecies. These HDL subspecies are mutually enriched in the defining proteins except for plasminogen protein. The depletion of plasminogen protein in A1AT HDL, A2M HDL, and FBG HDL could stem from their enrichment with vitronectin, another protease inhibitor panel protein. Vitronectin interacts with PAI-1 (plasminogen activator inhibitor 1) to enhance the conversion of plasminogen to plasmin, which could account for the depletion.[49] A1AT HDL is more strongly correlated with the subspecies defined by proteins involved in protection against oxidation and inflammation (PON1 HDL and HP HDL), which supports previous reports of the anti-inflammatory actions of HDL containing A1AT.[50]

Apo A2 HDL is unique among the HDL subspecies in that it comprises the majority of total HDL. There is nearly as much apo AII per apo AI in total HDL as there is in apo A2 HDL because only a small portion of total HDL does not have apo AII, thus the relative proteome of apo A2 HDL as compared with total HDL is only marginally enriched in apo AII. This is the only subspecies whose relative proteome is not strongly enriched in the defining protein. Interestingly, apo A2 HDL is actually depleted in all but the lipid metabolism panel proteins. This result implies that most of the 42 proteins that lie outside the lipid metabolism panel, including 10 of the HDL subspecies we have characterized, are predominantly

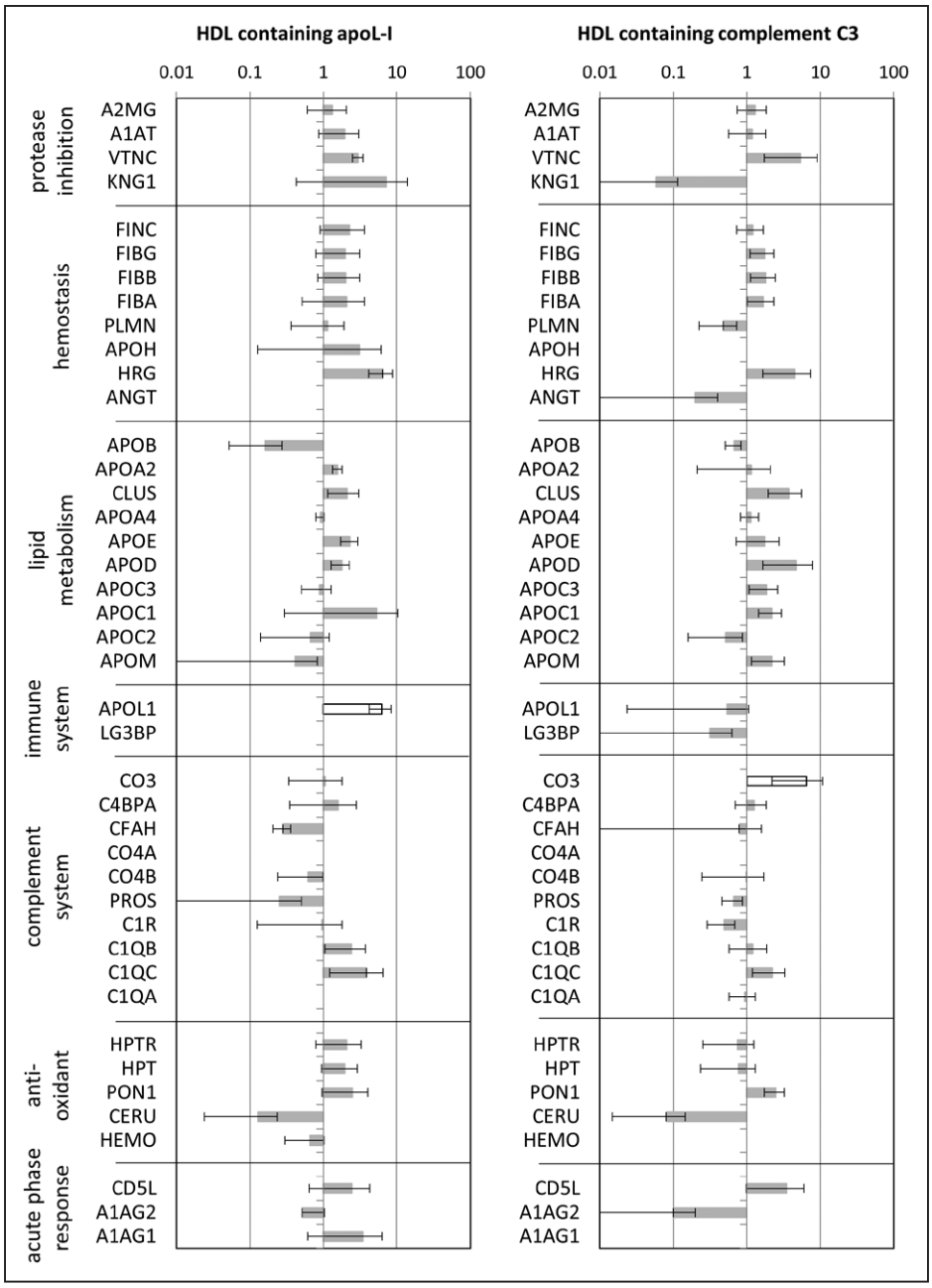

Figure 7. Immune Function. Proteomics of apo AI HDL (high-density lipoprotein; HDL containing apo AI) subspecies that are defined by proteins involved in immune functions (apo LI) or the complement system (complement C3). See Figure 3 for experimental details and explanation.

associated with HDL that lacks apo AII, the minor fraction of HDL. This data are concordant with results recently published by Melchior et al,[16] which shows that of 64 proteins identified in that study, 48 (75%) were depleted in the apo A2 HDL compared with the apo AI HDL that lacks apo AII. Apo CI, apo CII, and apo M are enriched in our apo A2 HDL as they were in Melchior et al.[16] The depletion of all but the lipid metabolism panel proteins coupled with data from Melchior et al[16] showing enrichment of LCAT (lecithin-cholesterol acyltransferase) and CETP (cholesteryl ester transfer protein) suggests that apo AII could delineate between HDL that facilitate lipid metabolism and transport from those involved in other functionalities.

Several members of the lipid metabolism grouping had similar proteomic profiles: apo CI HDL, apo C2 HDL, apo C3 HDL, and apo E HDL. The apo CI HDL proteome is enriched in lipid metabolism panel proteins. Apo C2 HDL and apo C3 HDL exhibit relative proteomic profiles nearly identical to apo E HDL. Similarities between apo C3 HDL and apo E HDL are not surprising given the large overlap between these 2 HDL subspecies described by Talayero et al.[17] These patterns coupled with strong correlations among these proteins and with other lipid metabolism panel proteins (and, in most cases, only lipid metabolism panel proteins) along with colocalization in the α-2 and α-3 size fractions in the study by Singh et al[21] suggests an overlapping family of HDL

Table 2. Correlation Coefficients Between the Relative Proteomes of the 16 HDL Subspecies

Protease inhibition		Lipid metabolism and transport							Immunologic	Hemostatic		Complement	Antioxidant			
A1AT	A2M	apoA2	apoA4	apoC1	apoC3	apoC3	ApoE	ApoJ	apoL1	PLMG	FBG	CoC3	CP	HP	PON1	
															Protease inhibition	
1	0.05	0.27	0.03	0.00	-0.06	-0.06	0.01	0.03	0.12	0.13	0.06	-0.10	0.01	0.39	0.80	A1AT
	1	0.13	0.26	0.25	0.61	0.60	0.46	0.48	0.27	0.61	0.70	0.64	0.38	-0.04	0.12	A2M
															Lipid metabolism and transport	
		1	0.15	0.88	0.58	0.43	0.80	-0.15	0.16	0.09	-0.07	0.18	-0.14	0.03	0.29	apoA2
			1	0.28	0.28	0.34	0.33	0.48	0.59	0.29	0.18	0.27	0.26	0.16	0.03	apoA4
				1	0.70	0.57	0.90	-0.07	0.26	0.19	-0.01	0.30	-0.08	-0.14	0.02	apoC1
					1	0.77	0.85	0.36	0.29	0.62	0.50	0.58	0.28	-0.16	-0.01	apoC2
						1	0.74	0.39	0.36	0.61	0.47	0.72	0.48	-0.13	-0.01	apoC3
							1	0.10	0.32	0.40	0.23	0.51	0.10	-0.18	0.02	ApoE
								1	0.45	0.58	0.63	0.41	0.55	0.15	0.08	ApoJ
															Immunologic	
									1	0.38	0.24	0.22	0.29	0.15	0.10	apoL1
															Hemostatic	
										1	0.61	0.56	0.57	0.08	0.10	PLMG
											1	0.50	0.40	0.06	0.00	FBG
															Complement	
												1	0.63	-0.12	-0.06	CoC3
															Antioxidant	
													1	0.16	0.06	CP
														1	0.74	HP
															1	PON1

For all $r>0.305$, P value <0.05; for all $r>0.6$, P value <0.0001. ssssssSignificant correlations are colored red which darkens as correlations approach 1.

A1AT indicates α-1-antitrypsin; A2M, α-2-macroglobulin; CoC3, complement C3;CP, ceruloplasmin; FBG, fibrinogen; HDL, high-density lipoprotein; HP, haptoglobin; PLMG, plasminogen; and PON1, paraoxonase-1.

subspecies participating predominantly in lipid metabolism and transport.

Apo J HDL and apo A4 HDL differed markedly from other lipid metabolism subspecies. The relative proteomic profile of apo J HDL is strikingly similar to those of the hemostasis panel subspecies. The proteome suggests functionality in hemostasis, antioxidant activity, and the acute-phase response rather than lipoprotein metabolism and transport. Despite a great deal of focus in the literature on apo J as a transporter of lipids in CVD and Alzheimer disease, it has also been implicated in these other functions.[51–54] Apo AIV is one of the only HDL proteins that resides primarily on pre-β HDL, a small discoidal particle.[21] Therefore, the unique proteome of apo A4 HDL may reflect its unique particle size. Apo A4 HDL was also mildly enriched with other lipid metabolism panel proteins such as apo AII and apo E. The presence of these proteins on apo A4 HDL supports its role as a lipid metabolism species, as apo AIV, apo AI, apo AII, and apo E have all be shown to activate cholesterol efflux from cholesterol-loaded cells.[16,55]

The uniqueness of the apo LI–containing HDL proteome is not surprising given its well-established and highly specific function as the trypanosome lytic factor.[56] Indeed, the emergence of this HDL subspecies as different and unique from the other subspecies and its enrichment in haptoglobin-related protein have been shown previously.[57] Complementary to this, apo LI protein is depleted in all other HDL subspecies, implying that apo LI protein tends to isolate on to its own HDL subspecies with minimal overlap of proteins with other subspecies.

We assessed proteomic similarities by correlation and proteomic differences by permutations analysis as described in the Methods and combined these results to produce a network analysis (Figure 8). The result is organization into 3 functional clusters: hemostasis, antioxidant/anti-inflammatory, and lipid metabolism; and 2 independent subspecies. The hemostasis group is composed of PLMG HDL, CoC3 HDL, A2M HDL, FBG HDL, CP HDL, apo J HDL, and apo C2 HDL. CoC3 is indeed involved in hemostasis.[58,59] CP improves aggregation of human platelets[60] and prevents hemostatic disorders in rats.[61] The antioxidant/anti-inflammatory group is formed by A1AT HDL, PON1 HDL, and HP HDL.[50] The lipid metabolism group is composed of apo A2 HDL, apo E HDL, apo C1 HDL, apo C2 HDL, and apo C3 HDL. This grouping agrees with those suggested by Davidson et al,[20] which showed strong correlations between apo AII, apo CI, apo CII, apo CIII, and apo E in HDL.

This study has several limitations. Despite the plasma pools analyzed for proteomics being composed of 18 individuals, combining them into 3 pools restricts the variation.

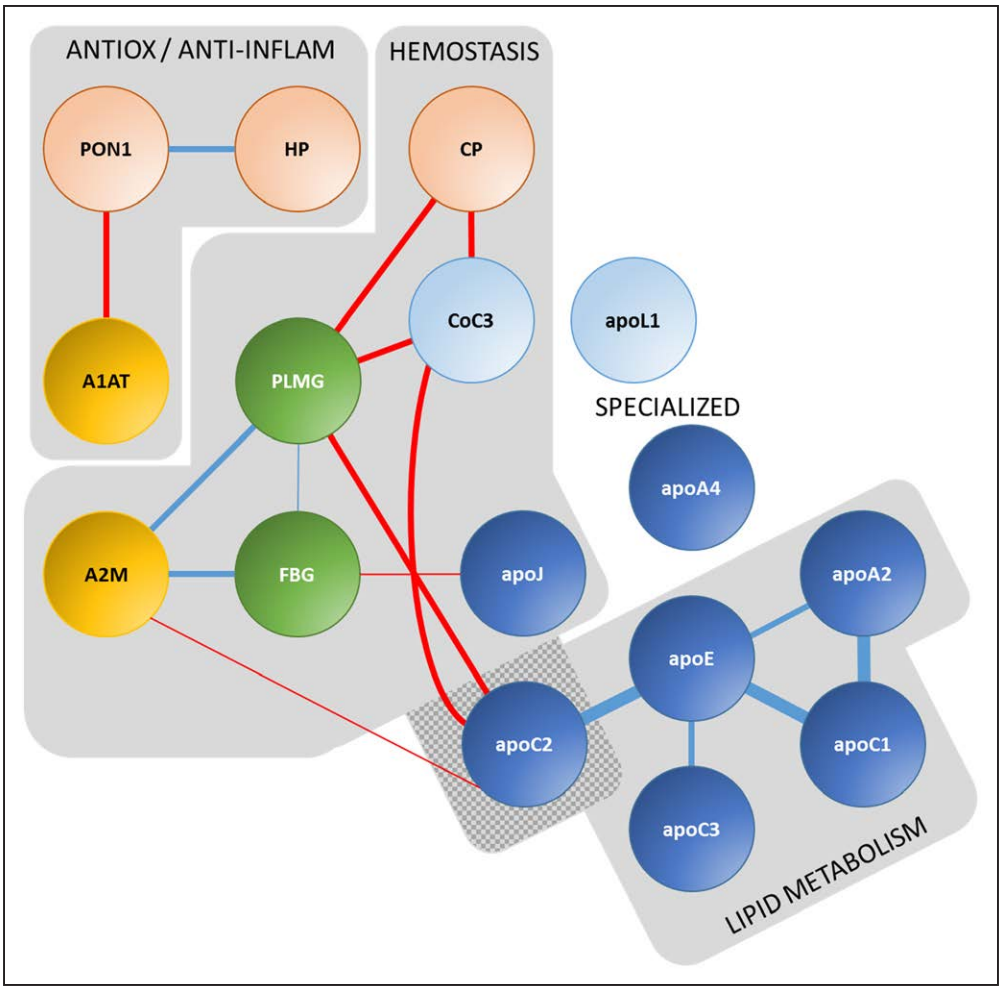

Figure 8. Network plot linking HDL (high-density lipoprotein) subspecies. Like-colored HDL subspecies were hypothesized to have similar functionality. Lines link subspecies with strong correlation (r>0.55; P<0.0004) with weight of line proportional to strength of correlation. Blue lines link subspecies that were hypothesized to have similar functionality. Red lines link subspecies that were not hypothesized to have similar functionality. Gray patches represent revised groupings of subspecies with similar purported functionality based on proteomic profile results. A1AT indicates α-1-antitrypsin; A2M, α-2-macroglobulin; CoC3, complement C3;CP, ceruloplasmin; FBG, fibrinogen; HDL, high-density lipoprotein; HP, haptoglobin; PLMG, plasminogen; and PON1, paraoxonase-1.

The pooled samples do not allow for assessment of true variability across individuals, which is likely greater than what we see across the 3 pools. The pools that were used in proteomic analysis only included white participants and so we cannot assess racial or ethnic variation in the HDL proteome of the subspecies. The proteome assay uses spectral counting of peptides unique to the protein and, therefore, is not a quantitative assay of mass. Spectral counts measure peptide fragments rather than the intact protein,[31] though these may exert the functions of the intact protein. We have done direct comparisons between peptide counting profiles and more quantitative techniques like full scan filtering and showed that both methods were similar. Thus, while not fully quantitative, the method is good for comparing relative differences between samples as we have done here. While we hypothesize that proteome drives function, ultimately this study is on the structure of the HDL system.

In conclusion, we have identified 16 stable HDL subspecies that exist in abundance with potential biological importance whose proteomic profiles exhibit cohesive patterns that may direct their specific functionality. An important next step in this research would be the investigation of associations between plasma levels of these subspecies and CVD outcomes. More study is needed to understand the composition and stoichiometry of the protein components and how they interact to form the various HDL subspecies. Further, assessment of the subspecies for differential capacity for the hallmark HDL functions such as reverse cholesterol transport, prevention of oxidation, reduction of inflammation, and hemostasis is needed to provide mechanistic support for functional speciation.

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Disclosures

F. Sacks was a consultant to Pfizer on drug development and was an expert witness on cases involving Aegerion and Pfizer (significant). F. Sacks and J. Furtado are inventors on patents awarded to Harvard University pertaining to HDL: US 8,846,321 B2 and US 9,494,606 B2 (modest).

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Highlights

- We identified and characterized 16 stable HDL (high-density lipoprotein) subspecies defined by proteins with functions that may drive the known actions of HDL: lipid metabolism, hemostasis, immunity, antioxidation, or anti-inflammation.
- Here we publish for the first time the plasma apo AI concentration and proteomic profile for these 16 HDL subspecies.
- We have compared the proteomes of these 16 HDL subspecies to total HDL and to each other using correlation and permutation analyses and used these results to suggest functional groupings of the HDL subspecies.