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A Cluster of Proteins Implicated in Kidney Disease Is Increased in High-Density Lipoprotein Isolated from Hemodialysis Subjects

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Abstract

Cardiovascular disease is the leading cause of death in end-stage renal disease (ESRD) patients treated with hemodialysis. An important contributor might be a decline in the cardioprotective effects of high-density lipoprotein (HDL). One important factor affecting HDL's cardioprotective properties may involve the alterations of protein composition in HDL. In the current study, we used complementary proteomics approaches to detect and quantify relative levels of proteins in HDL isolated from control and ESRD subjects. Shotgun proteomics analysis of HDL isolated from 20 control and 40 ESRD subjects identified 63 proteins in HDL. Targeted quantitative proteomics by isotope-dilution selective reaction monitoring revealed that 22 proteins were significantly enriched and 6 proteins were significantly decreased in ESRD patients. Strikingly, six proteins implicated in renal disease, including B2M, CST3, and PTGDS, were markedly increased in HDL of uremic subjects. Moreover, several of these proteins (SAA1, apoC-III, PON1, etc.) have been associated with atherosclerosis. Our observations indicate that the HDL proteome is extensively remodeled in uremic subjects. Alterations of the protein cargo of HDL might impact HDL's proposed cardioprotective properties. Quantifying proteins in HDL may be useful in the assessment of cardiovascular risk in patients with ESRD and in assessing response to therapeutic interventions.

Graphical Abstract

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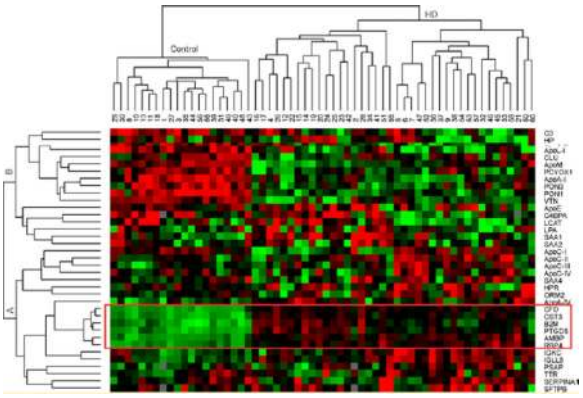
ASSOCIATED CONTENT

Supporting Information

Transition list of targeted SRM proteomics analysis, the relative intensity of 37 HDL proteins quantified by SRM analysis, the correlations between two peptides in a protein by SRM analysis, the correlations between shotgun proteomics analysis and targeted SRM analysis, the relative levels of regular HDL proteins that are increased or decreased in HDL isolated from ESRD subjects, the odds ratios for ESRD status for selected HDL proteins, and the correlations between the six proteins that were dramatically enriched in HDL isolated from ESRD subjects. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/ acs.jproteome.5b00060.

The authors declare the following competing financial interest(s): Dr. Heinecke is named as a coinventor on patents from the U.S. Patent Office on the use of oxidation and protein markers to predict the risk of cardiovascular disease. Dr. Heinecke has served as a consultant for Merck, Bristol Meyer Squibb, Amgen, KOWA, and Insilicos and is the recipient of research support from GSK and Bristol Meyer Squibb. Dr. Himmelfarb has served as a consultant to Thrasos, Inc. and Biogen Idec and has received research support from Blood Purification Technologies Incorporated. All other authors declare no competing financial interest.



Keywords

cardiovascular diseases; end-stage renal disease; high-density lipoprotein; hemodialysis; mass spectrometry; proteomics; selected reaction monitoring; shotgun proteomics

INTRODUCTION

Cardiovascular disease (CVD) is the most common cause of death in uremic subjects on dialysis,[1–4] a group of patients with substantially higher mortality rates than the general population. However, this large increase cannot be completely explained by the conventional risk factors frequently seen in this population. Also, statin use appears to have limited utility for reducing CVD events in uremia,[5–7] suggesting that factors other than LDL are relevant to atherogenesis in this disease. One important factor could be high-density lipoprotein (HDL).

Clinical and epidemiological studies show a robust, inverse association of HDL cholesterol (HDL-C) levels with cardiovascular disease risk in the general population.[8] One key cardioprotective function of HDL is to accept cholesterol from macrophages in the artery wall.[9–12] HDL's anti-inflammatory and antioxidant properties may also make significant contributions to cardioprotection.[13–15] An elevated triglyceride level and a decreased level of HDL cholesterol are characteristic of subjects with uremia and end-stage renal disease.[16–20] Low levels of HDL-C, altered HDL composition, and changes in HDL function may contribute to the increased risk of atherosclerosis in these subjects.[15,19,21–23] Indeed, recent studies demonstrated that the cholesterol efflux capacity of HDL isolated from dialysis patients is impaired due to the altered HDL composition, especially elevated levels of SAA1 and other inflammatory proteins.[24,25] Thus, alterations of HDL protein composition may make important contributions to the increased risk of CVD in uremic subjects.

HDL is a circulating, noncovalent assembly of amphipathic proteins and lipids composed of a monolayer of phospholipid (PL) and free cholesterol and a core of hydrophobic triglycerides (TG) and cholesteryl esters (CE). The major HDL protein is apolipoprotein A-I (apoA-I), which accounts for ~70% of protein mass in HDL. The second most abundant HDL protein is apoA-II (~15% of HDL protein mass). Mass-spectrometry-based analysis of

HDL has demonstrated a wide array of low abundance proteins that are associated with HDL, including proteins involved in lipid metabolism, complement regulation, inhibition of proteolysis, and acute inflammation.[26] Subsequent studies have demonstrated over 80 proteins that are reliably detected in HDL.[27] Importantly, HDL isolated from CVD patients exhibits altered protein composition,[26] which is normalized by aggressive lipid lowering therapy.[28] These observations suggest that HDL contains proteins that may serve as markers, and perhaps mediators, of CVD risk.[29]

Shotgun proteomics is a powerful mass spectrometric approach that can detect hundreds or even thousands of proteins in one sample.[30] It uses liquid chromatography in concert with electrospray ionization and tandem mass spectrometric analysis (LC–ESI–MS/MS) to identify peptides in proteolytic (usually tryptic) digests; however, this approach is semiquantitative. In contrast, targeted proteomics analysis based on selective reaction monitoring (SRM) with isotope dilution provides precise relative quantification of proteins in complex mixtures. Indeed, using HDL as a model system, Hoofnagle et al.[31] demonstrated that LC–SRM–MS/MS with either isotope-labeled internal standard peptides or a single internal standard protein (^{15}N-apoA-I) provides accurate, linear, and reproducible quantification of HDL proteins. Moreover, a recent study has demonstrated good reproducibility of SRM-based protein quantification by different laboratories.[32] A major advantage of LC–SRM–MS/MS analysis is that multiple proteins can be readily quantified in a single analysis over a wide range of relative concentrations without cross-reacting interferences often seen in multiplexed immunoassays.[31,33–37]

Three groups have investigated the HDL proteome in ESRD patients,[24,25,38] but none of them applied a targeted, quantitative SRM approach. Using shotgun proteomics, Holzer et al.[24] and Weichhart et al.[25] identified 35 and 49 proteins, respectively, in HDL isolated from ESRD patients. Applying iTRAQ labeling and LC–MS analysis, Mangé et al.[38] identified a total of 122 proteins in HDL isolated from ESRD subjects; however, this study quantified proteins in only seven ESRD subjects against one pooled control sample.

In the current studies, we initially used shotgun proteomics in an unbiased manner to identify candidate proteins that might be enriched or depleted in HDL of uremic subjects. We then used targeted isotope dilution MS/MS with ^{15}N-labeled apoA-I to quantify relative levels of 37 proteins in HDL of 20 control and 40 uremic subjects on hemodialysis. Our observations confirm previous studies demonstrating that the protein cargo of HDL is markedly altered in uremic subjects. Importantly, they also demonstrate that multiple proteins linked to end stage renal disease are enriched in the HDL of uremic subjects.

EXPERIMENTAL SECTION

Subjects

All studies were approved by the Human Studies Committee at the University of Washington. Hemodialysis subjects (n = 40) were from the Provision of Antioxidant Therapy in Hemodialysis Study (PATH Study, NCT 00237718).[39] Plasma samples were collected at the baseline of the study. Control subjects (n = 20) were healthy volunteers recruited at the Kidney Research Institute (UW Harborview Medical Center). Control

subjects had no history of kidney disease, no family history of premature CAD, no diabetes, and were not receiving lipid-lowering therapy.

Plasma Levels of Total Cholesterol, HDL Cholesterol, and Triglycerides

The plasma values for total cholesterol, HDL cholesterol, and triglycerides were analyzed by a DXC 600 chemistry analyzer (Beckman Coulter). A timed end-point method was used to determine the concentration for each of the respective chemistries.

HDL Isolation

Blood was collected from overnight fasted subjects into ice-cold tubes containing EDTA (6 mM final concentration). Plasma was prepared immediately by centrifugation (2500*g* for 15 min) and frozen at −80 °C until analysis. HDL (density 1.063 to 1.210 g/mL) was isolated by sequential ultracentrifugation from freshly thawed plasma[40] using buffers supplemented with 100 μM diethylenetriaminepentaacetic acid (DTPA), 100 μM butylated hydroxytoluene (BHT), and a protease inhibitor cocktail (Sigma, St. Louis, MO).

Isotope-Labeled ApoA-I

[^{15}N]ApoA-I was prepared by growing bacteria expressing human apoA-I in minimal medium supplemented with [^{15}N]ammonium chloride.[41]

Shotgun Proteomic Analyses

The protein concentration of HDL was determined using the Lowry assay (BioRad), with albumin as the standard. Following the addition of freshly prepared Met (10 mM final concentration), proteins were reduced with dithiothreitol and alkylated with iodoacetamide. Then, HDL was incubated overnight at 37 °C with 20:1 (w/w) of sequencing grade modified trypsin (Promega) in 50 mM NH_4HCO_3, pH 7.8. Digestion was halted by acidifying the reaction mixture (pH 2 to 3) with trifluoroacetic acid. Proteolytic digests were desalted with solid-phase extraction using an Oasis HLB Cartridge (1 mL, 30 μm; Waters) prior to MS analysis. Following the desalting, HDL tryptic digests (2 μg protein) were directly injected onto an analytical reverse-phase column (0.15 × 150 mm, 5 μm beads; Magic C18AQ, Michrom Bioresources) and separated at a flow rate of 1 μL/min over 175 min, using a linear gradient of 0% to 35% buffer B (90% acetonitrile, 0.1% formic acid) in buffer A (0.1% formic acid in water). Mass spectra were acquired in the positive-ion mode, using an LC–ESI–MS/MS system (a Michrom Bioresources MS4B Paradigm Capillary LC coupled to a Thermo LTQ linear ion trap instrument). The spray voltage was 1.8 kV, and the temperature of the heated capillary was 250 °C. A survey scan from *m/z* 200 to *m/z* 2000 was followed by data-dependent MS/MS of the nine most abundant ions at 35% collision energy. Dynamic exclusion was set to repeat the same precursor ion twice within a 30 s window and followed by excluding it for 45 s. This approach facilitated extensive data-dependent MS/MS sampling and the generation of an adequate number of peptide counts to reproducibly reflect relative peptide abundance. MS/MS spectra were searched against the UniProtKB human database (uniprot.sptr.human.20130503, which contains a total of 134 137 proteins/sequences) using the SEQUEST search engine (Thermo) with fixed Cys alkylation and variable Met oxidation modifications.[26] One incomplete cleavage site was

allowed in peptides for trypsin-restricted searches. The SEQUEST results were further validated using PeptideProphet and ProteinProphet,[42] using an adjusted probability of >0.90 for peptides and >0.95 for proteins.

At least two peptides unique to the protein of interest had to be detected in at least five subjects in any group. Requiring at least two unique peptides with a high confidence score markedly decreases the false-positive rate of protein identification.[43] Each charge state of a peptide was considered a unique identification. Proteins identified by ≤3 unique peptides were inspected manually to verify the results. We used spectral counting and the peptide index[26,44] to initially quantify the proteins in the HDL samples and to identify candidate proteins for targeted proteomic analysis. The peptide index (PI)[26,44] was calculated as

$$\mathrm{PI}=\frac{\overline{P_{\mathrm{ESRD}}}\times\frac{N^{\mathrm{D}}_{\mathrm{ESRD}}}{N^{\mathrm{T}}_{\mathrm{ESRD}}}-\overline{P_{\mathrm{Ctrl}}}\times\frac{N^{\mathrm{D}}_{\mathrm{Ctrl}}}{N^{\mathrm{T}}_{\mathrm{Ctrl}}}}{\overline{P_{\mathrm{ESRD}}}+\overline{P_{\mathrm{Ctrl}}}}$$

where $N^{\mathrm{D}}_{\mathrm{ESRD}}$ is the number of ESRD subjects with peptides ≥2, $N^{\mathrm{T}}_{\mathrm{ESRD}}$ is the total number of ESRD subjects, $N^{\mathrm{D}}_{\mathrm{Ctrl}}$ is the number of control subjects with peptides ≥2, and $N^{\mathrm{T}}_{\mathrm{Ctrl}}$ is the total number of control subjects. $\overline{P_{\mathrm{ESRD}}}$ is the average peptides in ESRD subjects, $\overline{P_{\mathrm{ESRD}}}=P^{\mathrm{T}}_{\mathrm{ESRD}}/N^{\mathrm{T}}_{\mathrm{ESRD}}$, where $P^{\mathrm{T}}_{\mathrm{ESRD}}$ is the total peptides in ESRD subjects and $\overline{P_{\mathrm{Ctrl}}}$ is the average peptides in control subjects, $\overline{P_{\mathrm{Ctrl}}}=P^{\mathrm{T}}_{\mathrm{Ctrl}}/N^{\mathrm{T}}_{\mathrm{Ctrl}}$, where $P^{\mathrm{T}}_{\mathrm{Ctrl}}$ is the total peptides in control subjects.

Quantification of HDL Proteins by Isotope-Dilution and Selected Reaction Monitoring (SRM) with ^{15}N-Labeled ApoA-I

To quantitatively measure the relative levels of candidate proteins in HDL, we used targeted proteomics with isotope-dilution selective reaction monitoring (SRM) approach, which was recently shown to measure multiple HDL proteins as accurately as biochemical approaches.[31] Peptide digests of HDL were analyzed with a nano-LC–MS/MS Thermo TSQVantage coupled to a Waters nanoACQUITY UltraPerformance liquid chromatography system as previously reported. [40,45] Isotope-labeled [^{15}N]apoA-I (0.1 μg per 10 μg HDL) was added into each HDL sample prior to digestion as the internal standard.[40]

At least two peptides per protein were selected and three or more SRM transitions of each peptide were chosen for quantitative analysis.[40,45] A list of all the transitions in the targeted analysis together with the protein names, the peptide sequences, precursor *m/z*, charge states, and relative collision energies are provided in Supplemental Table 1 in the Supporting Information (SI). SRM data was analyzed with Skyline, an open source program,[46] to obtain the peak area of each transition. To obtain the relative levels of a peptide, we calculated the ratio of the total peak area of the transitions from the peptide to the total peak area of the transitions from [^{15}N]THLAPYSDELR peptide (the internal standard peptide derived from [^{15}N]apoA-I). To calculate the relative levels of the peptide between control and ESRD groups, we set the average ratio of the peptide in control subjects as an arbitrary unit of one. To obtain the relative levels of a protein, the relative levels of peptides from the protein were

averaged. We used the "abundance index" (the counterpart of "peptide index" in shotgun proteomics analysis) to assess the enrichment or depletion of a protein in ESRD group compared with control group in SRM analysis. The abundance index (AI) was calculated as

$$\mathrm{AI}=\frac{\overline{A_{\mathrm{ESRD}}}\times\frac{N^{\mathrm{D}}_{\mathrm{ESRD}}}{N^{\mathrm{T}}_{\mathrm{ESRD}}}-\overline{A_{\mathrm{Ctrl}}}\times\frac{N^{\mathrm{D}}_{\mathrm{Ctrl}}}{N^{\mathrm{T}}_{\mathrm{Ctrl}}}}{\overline{A_{\mathrm{ESRD}}}+\overline{A_{\mathrm{Ctrl}}}}$$

where $\overline{A_{\mathrm{ESRD}}}$ is the average protein abundance in ESRD subjects, $\overline{A_{\mathrm{Ctrl}}}$ is the average protein abundance in control subjects, $N^{\mathrm{D}}_{\mathrm{ESRD}}$ is the number of ESRD subjects the protein was detected, $N^{\mathrm{T}}_{\mathrm{ESRD}}$ is the total number of ESRD subjects, $N^{\mathrm{D}}_{\mathrm{Ctrl}}$ is the number of control subjects the protein was detected, and $N^{\mathrm{T}}_{\mathrm{Ctrl}}$ is the total number of control subjects.

Because the SRM analysis is more sensitive than shotgun proteomics, all candidate HDL proteins were detected in all HDL samples in both control and ESRD groups, that is, $N^{\mathrm{D}}_{\mathrm{ESRD}}=N^{\mathrm{T}}_{\mathrm{ESRD}}$ and $N^{\mathrm{D}}_{\mathrm{Ctrl}}=N^{\mathrm{T}}_{\mathrm{Ctrl}}$. Therefore, abundance index can be simplified as

$$\mathrm{AI}=\frac{\overline{A_{\mathrm{ESRD}}}-\overline{A_{\mathrm{Ctrl}}}}{\overline{A_{\mathrm{ESRD}}}+\overline{A_{\mathrm{Ctrl}}}}$$

Using this approach we reliably quantified 37 HDL proteins in a single analysis.

ABCA1 Cholesterol Efflux Capacity of Serum HDL

Serum was derived from plasma by adding calcium.[47] Polyethylene glycol (PEG) was then used to precipitate lipoproteins containing apolipoprotein B, and the supernatant was centrifuged to generate serum HDL.[47,48] ABCA1-specific sterol efflux to serum HDL was quantified using baby hamster kidney (BHK) cells expressing mifepristone-inducible human ABCA1, as previously reported.[45,49]

Cluster Analysis

Hierarchical clustering of proteins quantified by SRM analysis was performed using the average linkage clustering method and the centered correlation metric by the open access programs Clustering and TreeView.[50]

Statistical Analysis

Continuous variables are presented as means and standard deviations (SDs) and categorical variables as frequencies and percentages. If the levels of proteins exhibited a non-normal distribution in our study population, we used logarithmic transformation for the analyses. Correlation analysis with continuous variables used Pearson's coefficient. Logistic regression was used to estimate the association between the levels of HDL proteins, and ESRD (on hemodialysis) status. Odds ratios are reported for one SD change for continuous variables and were calculated with SPSS (Windows version 19, Chicago, IL) or OriginPro (version 8.6, Origin Lab, Northampton, MA).

RESULTS

Table 1 shows the baseline demographic data for the 40 ESRD subjects and 20 healthy control subjects we studied. The average age was 58 ± 14 years in ESRD group and 40 ± 11 in control group ($P < 0.0001$); 50% of subjects were female in both groups. None of the control subjects in control group was hypertensive, diabetic, smoked, or had known CVD. In contrast, 75% of subjects in ESRD group were hypertensive, 55% were diabetic, 55% were smokers, and 37.5% had established CVD. For ESRD subjects, the average time on hemodialysis (vintage) was 2.2 years. The ESRD group had significantly lower HDL-C levels (41 ± 13 mg/dL) than the control group (57 ± 15 mg/dL; $P = 0.0003$). Low-density lipoprotein cholesterol (LDL-C) were significantly lower in the ESRD group ($P < 0.0001$). The triglyceride levels of ESRD subjects were also elevated compared with control subjects, although this was of borderline statistical significance (175 ± 96 mg/dL vs126 ± 81 mg/dL, $P = 0.049$).

Identification of Candidate HDL Proteins by Shotgun Proteomics analysis

We used shotgun proteomics and spectral counting (the number of peptides unique to a protein) to investigate the protein composition of HDL isolated by ultracentrifugation in control and ESRD subjects. An example of this analysis is shown in Figure 1, which shows the identification of 4 peptides derived from CST3 (Figure 1A). Manual inspection of the MS/MS spectra confirmed the sequence of the peptides, as shown for the anticipated series of b and y ions for ALDFAVGEYNK (Figure 1B) and LVGGPMDASVEEEGVR (Figure 1C). This approach identified 63 proteins in HDL (Table 2).

Among the 63 proteins we identified, 35 proteins were observed in at least half of the control subjects and 43 proteins were present in at least half of the ESRD subjects (Table 2). This is in contrast with other studies where peptides were only detected in a few samples.[24,25] Nine HDL proteins have not been previously reported: cathelicidin antimicrobial peptide (CAMP), Ig α-2 chain C region (IGHA2), Ig κ-chain C region (IGKC), Ig κ-2 chain C regions (IGLC2), immunoglobulin λ-like polypeptide 5 (IGLL5), MENT, Profilin-1 (PFN1), pro-activator polypeptide (PSAP), and prostaglandin-H2 D-isomerase (PTGDS) (Table 2).

Gene ontology (GO) analysis of the HDL proteins is shown in Figure 2. Similar to previous reports,[26,27] 28 of the 63 HDL proteins were linked to lipid and lipoprotein metabolism: 14 were apolipoproteins (such as apoA-I, apoA-II, apoCs). Nine proteins are involved in regulation of complement system, such as complement component C3 (C3) and complement factor D (CFD). Seven of the nine complement proteins are also involved in immune response. We identified 20 proteins implicated in immune regulation, including α-1-microglobulin/ bikunin precursor (AMBP) and β-2-microglobulin (B2M). Another major category (18 proteins) centered on proteins involved in the acute phase response and inflammation, including serum amyloid A (SAA1, SAA2, and SAA4). Fourteen proteins were linked to proteolysis inhibition, such as cystatin C (CST3), AMBP, CDF, and alpha-1-antitrypsin inhibitor. Eleven proteins regulate platelet activation (e.g., fibrinogens FGA and FGB, CFD) and six proteins regulate hemostasis (FGA, FGB, A1AT, etc.). We also found four proteins involved in vitamin binding and transport, including group-specific component

(GC, also known as vitamin D binding protein) and retinol binding protein 4 (RBP4). Consistent with previous studies,[25–27] our observations implicate HDL in the acute-phase response, immunity, regulation of complement activation, proteolysis inhibition, platelet activation, and vitamin binding and transport.

HDL Isolated from Healthy Subjects and ESRD Patients Carry Distinct Protein Cargoes

Spectral counting or summing all of the peptides derived from a single protein in an LC–ESI–MS/MS analysis can assess relative protein abundance.[26,44,51] We used the peptide index,[26,44] an empiric test based on the number of unique peptides detected by MS/MS, to provide a semiquantitative measure of relative protein abundance in different groups of subjects (Figure 3). The average number of peptides detected for each protein, the number of subjects in each group with at least two detectable peptides, and the peptide index for each protein are shown in Table 2. Five proteins were exclusively detected in HDL isolated from hemodialysis subjects (defined as detection of at least two peptides [our criteria for protein identification] in >50% of the subjects in the group). These proteins were B2M, CFD, CST3, IGLL5, and PTGDS (Table 2). Moreover, three proteins were markedly enriched in ESRD subjects: AMBP, α-1-acid glycoprotein 2 (ORM2), and RBP4. Statistical analysis of peptide abundance indicated that eight additional proteins were enriched significantly in the ESRD patients (Table 2 and Figure 3; $P < 0.01$). The eight proteins were: alpha-1-antitrypsin (A1AT), apoA-IV, apoC-II, apoC-III, haptoglobin-related protein (HPR), IGKC, SAA1, and transthyretin. In contrast, six proteins were enriched in control group ($P < 0.01$, Table 2 and Figure 3). The six proteins were: apoA-I, apoA-II, apoL-I, apoM, PCYOX1, and PON1. Collectively, these observations suggest that HDL of ESRD subjects is relatively enriched or depleted in specific proteins.

Quantitative Analysis of HDL Proteins by Selected Reaction Monitoring (SRM)

To confirm our shotgun proteomics results, we used MS/MS analysis with SRM, an approach that provides precise and accurate relative quantification of HDL proteins.[31] Three or four of the most abundant SRM transitions were chosen empirically for quantitative analysis. We used [^{15}N]THLAPYSDELR peptide from [^{15}N]apoA-I as the internal standard because it is an abundant ion and the peptide exhibited minimal variations between different samples and in different analyses (data not shown). To further increase confidence in the quantification of the isotope-labeled peptide, we monitored six SRM transitions. Figure 4 shows quantification of peptide ALDFAVGEYNK derived from CST3 protein in HDL isolated from a control and an ESRD patient. The peak areas of peptide [^{15}N]THLAPY-SDELR of [^{15}N]apoA-I were similar in HDL from the control subject (Figure 4A) or from the ESRD patient (Figure 4B) because the same amount of [^{15}N]apoA-I was added to each HDL sample. Slightly reduced peak areas were observed for peptide THLAPYSDELR derived from endogenous apoA-I in HDL isolated from ESRD patient (Figure 4D) than in HDL from control subject (Figure 4C). In contrast, there was a marked increase in the peak area for peptide ALDFAVGEYNK derived from CST3 in HDL isolated from ESRD patient (Figure 4F) relative to the control subject (Figure 4E).

To verify our SRM method, we tested the assumption that the relative amount of two peptides derived from each protein was correlated. Supplemental Figure I in the SI shows

the data for four proteins: apoA-I, PON1, AMBP, and CST3. These data demonstrated that the two peptides from each individual protein correlated strongly ($r > 0.9$ for all four proteins; $P < 0.0001$). For most proteins, relative protein quantification by shotgun proteomics correlated very well with the amount quantified by SRM (Supplemental Figure II in the SI). Moreover, the peptide indexes of those 37 proteins by shotgun and the abundance indexes (see Experimental Section) by SRM analyses were highly correlated ($r = 0.92$, Supplemental Figure III in the SI). These observations strongly suggest that our quantitative approach was giving a reliable estimate of the relative amounts of proteins in HDL of control and ESRD subjects by SRM analysis.

Targeted, quantitative SRM analysis confirmed that the relative abundance of 18 proteins increased significantly in HDL isolated from ESRD subjects compared with control subjects (all $P < 0.001$, Supplemental Table 2 and Supplemental Figure IV in the SI). In contrast, six proteins had increased abundance in HDL-isolated control subjects ($P < 0.001$, Supplemental Table 2 and Supplemental Figure V in the SI) Taken together, our shotgun proteomics and targeted SRM analyses provide strong evidence that there are marked differences in the HDL proteome of control and ESRD subjects.

Compared with control subjects, ESRD subjects had markedly higher levels of AMBP, B2M, CFD, CST3, PTGDS, and RBP4 in their HDL (Supplemental Table 2 in the SI). For those six proteins, the lowest levels in ESRD subjects were higher than the highest levels in the control group (Figure 5). The levels of many other proteins in HDL were also altered in ESRD subjects (Supplemental Figure VI in the SI shows 12 of those proteins). For example, the levels of PON1 protein in HDL were significantly lower in ESRD subjects compared with the control group (mean value, 56% of control group; $P < 0.0001$; Supplemental Table 2 in the SI). Increased levels of PON1 in HDL associated with a decreased likelihood of being on ESRD (odds ratio [OR] per 1-SD change, 0.07; 95% confidence interval, 0.02–0.27; $P = 0.0001$; Supplemental Figure VI in the SI). In contrast, as previously shown[24,25] the levels of SAA1 in HDL were significantly higher in ESRD subjects compared with the control group (mean values, 6.4-fold higher; $P = 0.001$). Increased levels of SAA1 in HDL associated with an increased likelihood of being an ESRD subject (OR 13.7; 95% CI, 3.36–55.6; $P = 0.0003$; Supplemental Figure VI in the SI).

Recent studies suggest that the cholesterol efflux capacity of HDL is impaired in patients with ESRD.[52] One potential cause might be alterations of HDL's protein cargo by SAA1;[24] however, we found no significant differences between ESRD and control subjects in efflux capacity of serum HDL in our study (data not shown). This likely reflects the relatively low number of subjects in our study. On the basis of the intra-assay variability for efflux capacity of HDL in our studies and the 15% difference in efflux capacity between control and CVD subjects,[45,48] power calculations suggested that we would need 100 subjects per group to find a 15% difference in efflux capacity with 80% sensitivity.

A Cluster of Proteins Linked to Renal Disease Is Markedly Increased in HDL Isolated from ESRD Subjects

The relative levels of eight proteins were elevated more than 4-fold in HDL isolated from ESRD subjects compared with the control subjects (Supplemental Table 2 in the SI; AMBP,

B2M, CFD, CST3, PTGDS, RBP4, SAA1, and SAA2). The levels of CST3, AMBP, B2M, and CFD in HDL were 9- to 14-fold higher in ESRD patients than in control subjects. Remarkably, the levels of PTGDS in HDL were 29 times higher in ESRD patients than in control subjects. Importantly, for six proteins (AMBP, B2M, CFD, CST3, PTGDS, and RBP4), there was no overlap between the relative abundance of the proteins in the two groups of subjects (Figure 5). The P values for all six proteins were $<1 \times 10^{-10}$ (two-tailed Student's *t* test).

Five of the six proteins markedly enriched in HDL of dialysis patients were also enriched in plasma of ESRD patients,[53–56] raising the possibility that association of these proteins with HDL is nonspecific. To investigate this possibility, we used shotgun proteomics to quantify protein levels in plasma from 20 control subjects and 20 ESRD patients and then compared the relative levels of the proteins in plasma and HDL. Plasma was first fractionated on a MARS-7 column (Agilent) to deplete the seven most abundant plasma proteins (albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, and fibrinogen) to increase the sensitivity of the analysis for minor components of plasma. This analysis detected AMBP and RBP4 in plasma from all 20 control and 20 ESRD subjects. The average spectral counts were 39.5 and 69.1 for AMBP and 56.8 and 93.4 for RBP4 in control and ESRD subjects, indicating about 2-fold increase in ESRD patients. In striking contrast, we observed AMBP in 13 out of 20 control HDL preparations but in all 40 ESRD HDL preparations. The average spectral count was 3.0 in control HDL and 26.9 in ESRD HDL, indicating a 9-fold enrichment in ESRD HDL. Similarly, RBP4 was detected in only 9 of 20 control HDL preparations but in 37 out of 40 ESRD HDL preparations. The average spectral count was 1.8 in control HDL and 14.5 in ESRD HDL, indicating an 8-fold enrichment in ESRD HDL. Our observations strongly suggest that AMBP and RBP4 are present at low levels in HDL of control subjects and that these two proteins are markedly enriched in the HDL of ESRD patients. We observed similar results for B2M, CFD, CST3, and PTGDS. Taken together, these observations support the proposal that the proteins we find elevated in HDL of ESRD subjects are specifically associated with the lipoprotein.

We next created a volcano plot for all 37 proteins quantified by SRM analysis (Figure 6). In this analysis, we plotted the negative log of the *P* value (base 10) for the statistical significance (Student's two-tailed *t* test) of the difference in relative protein abundance for each protein versus the log (base 2) of the fold change in relative protein abundance between the two groups of subjects. This analysis revealed that there were six proteins with both large magnitude fold increases in HDL of ESRD subjects as well as with a high level of statistical significance. To determine whether those six proteins are correlated with each other, we performed correlation analysis between levels of those six proteins in all 60 subjects. These analyses demonstrated that the levels of CST3 protein and other five proteins are significantly correlated with each other (all $r > 0.94$; $P < 10^{-6}$; Supplemental Figure VII and VIII in the SI). These observations suggest that six proteins are greatly enriched in the HDL of ESRD subjects and they form a cluster of proteins in HDL.

Cluster Analysis of HDL Proteins Discriminates between ESRD and Control Subjects

Figure 7 shows the heat map of relative protein abundance in all 60 subjects. The green color and red colors represent low and high relative abundance, respectively. Inspection of the data strongly suggests that certain clusters of proteins are expressed at markedly different levels in the two groups of subjects. To confirm this suggestion, we used hierarchical cluster analysis (using Pearson's coefficient between every possible pair of the proteins quantified by SRM). In this analysis, when expression of two proteins in a sample is correlated highly, they reside in nearby branches of the tree. If protein expression is not correlated, they are located far away from each other.

This approach indicated that the 37 HDL proteins quantified by SRM segregated into two major groups (labeled A and B in Figure 7). CDF, CST3, B2M, PTGDS, AMBP, and RBP4 were markedly enriched in the ESRD subjects and they were clustered together. In contrast, apoM, PCYOX1, apoA-I, PON3, PON1, and VTN were enriched in control subjects and they were clustered together (Figure 7). SAA1 and SAA2 clustered together closely, consistent with the well-established coexpression of these protein isoforms in plasma. This analysis demonstrated a clear separation of the HDL proteome of the 20 control subjects and the 40 ESRD patients (labeled "control" and "HD" in Figure 7). These observations suggest that differences in protein levels of HDL can be used to discriminate between control and ESRD subjects.

Diabetes, Age, and Gender Are Not Confounders in the Proteomics analysis of HDL Isolated from ESRD Subjects

All of the control subjects were apparently healthy, without known diabetes. In contrast, about half of the ESRD subjects were diabetic (Table 1). To investigate the effect of diabetes and other potential confounders on the alteration of HDL proteome, we plotted the fold change of relative HDL protein abundance in all 40 ESRD subjects for several clinically relevant variables (including age, sex, and diabetic status) together with the fold change of relative HDL protein abundance in all 60 subjects for ESRD status (Figure 8). Within the 40 ESRD subjects, the relative differences in HDL protein expression levels for age (<58 years old vs ≥58 years old), sex, and diabetic status varied from 0.5- to 2-fold. In contrast, ESRD status associated with >4-fold difference in relative abundance for eight HDL proteins (Figure 8). For example, for PTGDS the average level in ESRD cases was 29 times higher than the level in controls (Figure 8, black circle). Among ESRD patients, however, older subjects had protein levels that were 1.21 times that found in younger subjects (Figure 8, red triangle), males had protein levels that were 1.13 times the level in females (Figure 8, blue square), and DM subjects had protein levels that were 0.82 times the levels in non-DM subjects (Figure 8, olive star). We observed similar results for all seven other HDL proteins that were enriched in ESRD subjects (Figure 8). To further investigate the effect of age on HDL proteome, we matched 14 subjects with similar ages from control and ESRD group (45.2 ± 9.2 vs 44.9 ± 9.6 years; control vs ESRD, $P = 0.69$). The results of the proteomics analyses of HDLs isolated from these two groups were essentially the same as those we observed for the entire cohort (Figure 5). These data suggest that ESRD but not DM or other potential confounders remarkably alters HDL proteome.

DISCUSSION

In the current study, we investigated alterations in the HDL proteome of end-stage renal disease patients undergoing hemodialysis. Two approaches were used: shotgun proteomics by LC–ESI–MS/MS and targeted proteomics by isotope-dilution LC–ESI–SRM–MS/MS. Shotgun analysis of HDL isolated from 20 control and 40 ESRD subjects identified 63 proteins. Our analysis detected the majority of the proteins that have been reproducibly observed in multiple HDL proteomics studies.[27] Consistent with other studies, GO analysis revealed that about half of the proteins (28 of 63) were linked to lipid and lipoprotein metabolism. As previously reported, other major functional categories centered on the immune response, the acute phase response, complement regulation, and inhibitors of proteolysis. We also found proteins that are involved in vitamin binding and transport, platelet activation/coagulation, and regulation of oxidation.

Because shotgun proteomics is only semiquantitative, we next used targeted SRM with isotope dilution to quantify 37 proteins in HDL. All 37 proteins were detected in all HDL samples from both ESRD and control groups. This approach identified two cardioprotective proteins, apoA-I and PON1, as significantly decreased in ESRD patients. Importantly, hyper-cholesterolemic mice lacking PON1 or apoA-I are more susceptible to atherosclerosis,[57–60] suggesting that altered levels of apoA-I and PON1 might promote atherogenesis in ESRD subjects. We also identified 18 other proteins, including apoA-IV, apoC-II, and apoC-III, that were present at significantly greater levels in the HDL of ESRD patients. Elevated levels of apoC-III are implicated in increased triglyceride levels and increased CVD risk in subjects with normal renal function,[61] suggesting that apoC-III might similarly account in part for the hypertriglyceridemia and CVD risk associated with ESRD.

Importantly, we identified six proteins that are markedly enriched in the HDL of ESRD subjects: PTGDS (29-fold), CFD (14-fold), B2M (13-fold), AMBP (11-fold), CST3 (9.5-fold), and RBP4 (5-fold). It is noteworthy that the blood levels of five out of the six proteins are used as biomarkers for kidney disease. Cystatin C (CST3) is a 13 kDa protein and a member of a family of competitive inhibitors of lysosomal cysteine protease.[62] Cystatin C is mainly used as a biomarker of kidney function because this small protein can be freely filtered across the glomerular membrane.[63] Beta-2 microglobulin (B2M), which is also a low-molecular-weight protein, forms part of the major histocompatibility class I family.[64] B2M levels are elevated in hemodialysis patients[54] and its levels increase with progression of CKD from stage I to stage V.[65] Complement factor D (CFD) is a member of the alternative pathway for complement activation, where it cleaves complement factor B. Compared with healthy controls, the plasma concentration of CFD increases 10-fold in end-stage renal failure; the serum concentration of CFD correlated very well with that of creatinine.[55,66] Prostaglandin-H2 D-isomerase (PTGDS) is a glutathione-independent prostaglandin D synthase that catalyzes the conversion of prostaglandin H2 to prostaglandin D2. PTGDS, also called β-trace protein (BTP), is used in concert with cystatin C to monitor the progression of kidney disease.[53] Retinol binding protein 4 (RBP4) belongs to the lipocalin family and is the specific carrier for retinol in the blood. RBP4 levels are 4 times higher in hemodialysis patients compared with control subjects, and its levels are correlated

with serum creatinine,[56] suggesting that RBP4 concentrations are significantly increased in ESRD subjects.[67,68]

Correlation analysis demonstrated that the levels of the six proteins highly enriched in HDL of ESRD subjects were correlated with each other ($r > 0.90$), suggesting that those six proteins form a cluster of proteins that bound to HDL particles in ESRD subjects. Moreover, levels of those six proteins in HDL isolated from ESRD subjects did not overlap with those of HDL from control subjects. Indeed, cluster analysis resulted in clear separation of the HDLs isolated from control and ESRD subjects. In future studies, it will be of great interest to determine whether the levels of those six proteins in HDL associate with different stages of CKD and disease progression. Importantly, elevated plasma levels of cystatin C,[69–71] B2M,[65,72] PTGDS (β-trace protein),[73] and RBP4[74] all associate with CVD status and increased mortality in patients with renal dysfunction.

Three HDL proteomics studies[24,25,38] have been reported in the literature on ESRD or hemodialysis patients, but none applied targeted SRM analysis. Holzer et al.[24] used shotgun proteomics analysis to identify 35 proteins in HDL isolated from 27 HD patients and 19 healthy controls. They demonstrated elevated levels of SAA1 and apoC-III in HDL of ESRD subjects, consistent with our observations. Moreover, CST3 and RBP4 were identified in 5 and 17 of the 27 ESRD subjects, respectively, but in none of the 19 control subjects, suggesting that those two proteins are specifically enriched in ESRD status. In contrast, apoA-I, apoA-II, and apoM were significantly decreased in HDL isolated from HD patients. Weichhart et al.[25] identified 49 HDL-associated proteins in 10 ESRD patients and 10 control subjects using shotgun proteomics analysis. As in our study, they found that surfactant protein B (SP-B), apoC-II, SAA, and AMBP were enriched in HDL from patients with ESRD. They also observed increased levels of CFD in ESRD patients. Using iTRAQ labeling and nanoflow LC–MS analysis, Mangé et al.[38] identified a total of 122 proteins in HDL isolated from ESRD subjects; however, in that study, the enrichment of four proteins (B2M, CFD, CST3, and PTGDS) in ESRD patients was not significant. Moreover, apoA-I, apoA-II, apoL-I, apoM, PON1, LCAT, and so on were increased in ESRD patients. In contrast, we and Holzer et al.[24] found that those proteins were significantly decreased in HDL isolated from ESRD subjects compared with control subjects. Collectively, these observations support the proposal that MS/MS-based proteomics of HDL can be quantitative, that similar results can be obtained by MS analysis performed in different laboratories, and that the HDL of ESRD subjects contains a proteome that is markedly different from that of control subjects.

Strengths of our study include the use of two complementary MS-based proteomics methods to quantify HDL proteins, a relatively large number of subjects, and the marked differences in levels of certain proteins in HDL isolated from control and ESRD subjects. To further increase the power of our targeted proteomics technique, we used ^{15}N-labeled recombinant human apoA-I as an internal standard.[31,40,45] Moreover, adding [^{15}N]ApoA-I to HDL prior to digestion helps control for variations in digestion efficiency. Potential limitations include the cross-sectional study design and the large number of clinical variables that differ in control and ESRD subjects. However, subgroup analysis suggested that certain key factors, including age, sex, and diabetes, were unlikely to be contributing to the differences in

protein composition of HDL of control and ESRD subjects. In future studies it will be of great interest to determine if proteins with altered abundance contribute to the formation of dysfunctional HDL in ESRD subjects and to determine prospectively whether altered protein levels predict the progression of renal disease and the risk of CVD.

CONCLUSIONS

We have demonstrated that ESRD markedly remodels the HDL proteome. Moreover, our quantitative analysis of the HDL proteome identified a cluster of six proteins that are dramatically enriched in HDL from patients with ESRD. Our observations support the proposal that the protein cargo of HDL can serve as a marker–and perhaps mediator–of renal disease and serve as novel biomarkers for ESRD status. Importantly, alterations of protein composition in HDL may have significant impact on HDL's cardioprotective properties. Therefore, quantifying proteins in HDL might help diagnose and perhaps treat human cardiovascular disease in kidney disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AMBP	protein AMBP
ApoA-I	apolipoprotein A-I
apoA-II	apolipoprotein A-II
apoA-IV	apolipoprotein A-IV
apoC-II	apolipoprotein C–II
apoC-III	apolipoprotein C–III
AVG	arteriovenous grafts
AVF	arteriovenous fistula
B2M	beta-2-microglobulin
BHT	butylated hydroxytoluene
CE	cholesteryl esters
CFD	complement factor D

CKD	chronic kidney disease
CST3	cystatin-C
CVD	cardiovascular disease
DM	diabetes
ESRD	end-stage renal disease
GO	gene ontology
HDL	high-density lipoprotein
HDL-C	HDL cholesterol
LC-ESI-MS/MS	liquid chromatography electrospray ionization and tandem mass spectrometric analysis
LDL	low-density lipoprotein
OR	odds ratio
PCKD	polycystic kidney disease
PON1	serum paraoxonase/arylesterase 1
PTGDS	prostaglan-din-H2 D-isomerase
RBP4	retinol-binding protein 4
SAA1	serum amyloid A-1 protein
SAA2	serum amyloid A-2 protein
SRM	selective reaction monitoring
TG	triglycerides

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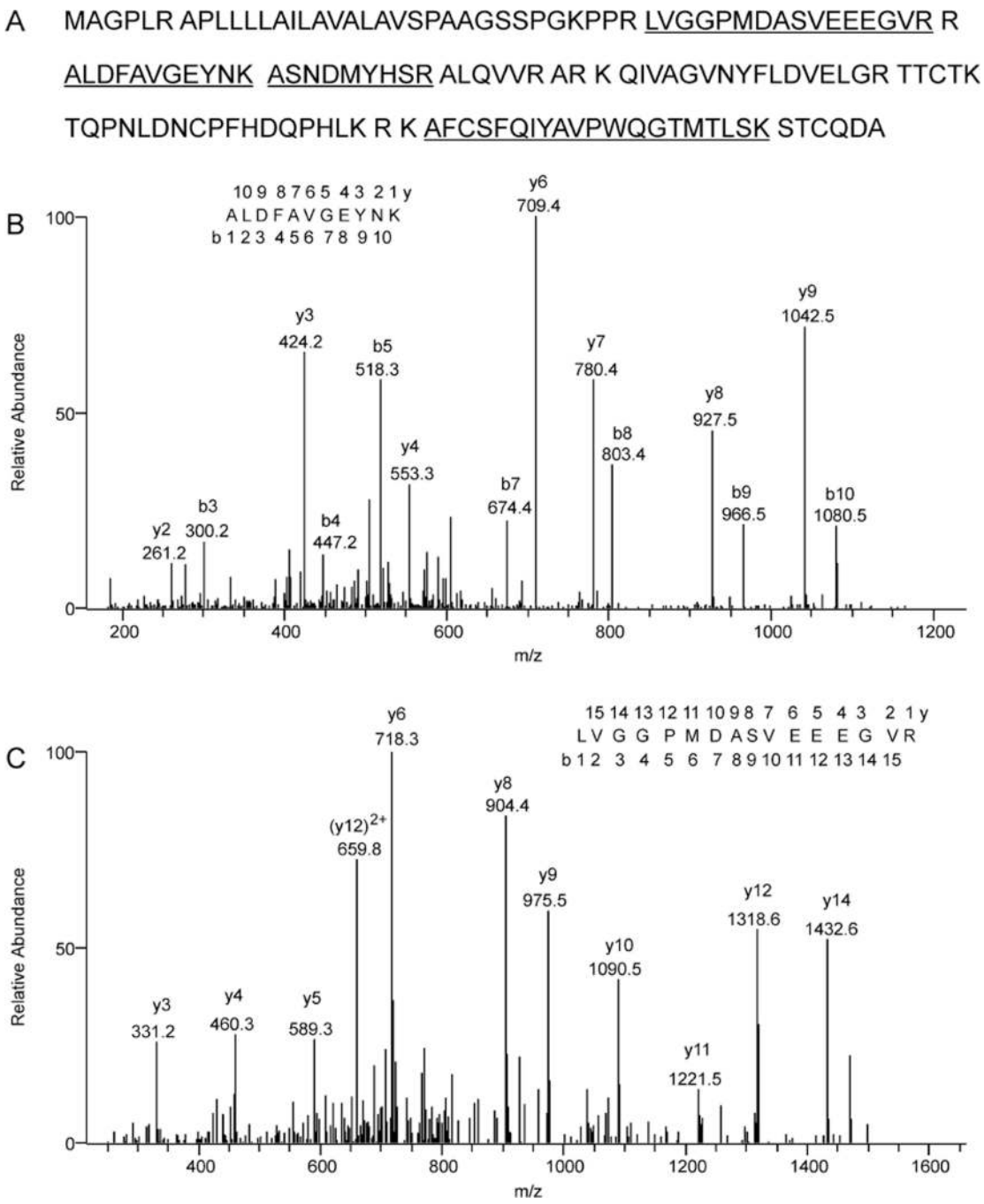

Figure 1.

MS/MS identification of CST3 protein in HDL isolated from ESRD subjects by shotgun analysis. HDL (10 μg) was reduced with dithiothreitol and alkylated with iodoacetamide. Following digestion of HDL with trypsin, the tryptic digests of HDL proteins were analyzed using an LC–ESI–MS/MS system. MS/MS spectra were searched against the UniProtKB human database, using the SEQUEST search engine. Four unique peptides to CST3 were identified by shotgun analysis. The MS/MS spectra were inspected manually to verify the results. (A) The sequence of CST3 protein with 4 identified peptides (underlined). (B) The

MS/MS spectra of peptide ALDFAVGEYNK. (C) MS/MS spectra of peptide LVGGPMDASVEEEGVR. A series of y and b ions confirmed the identification of both peptides.

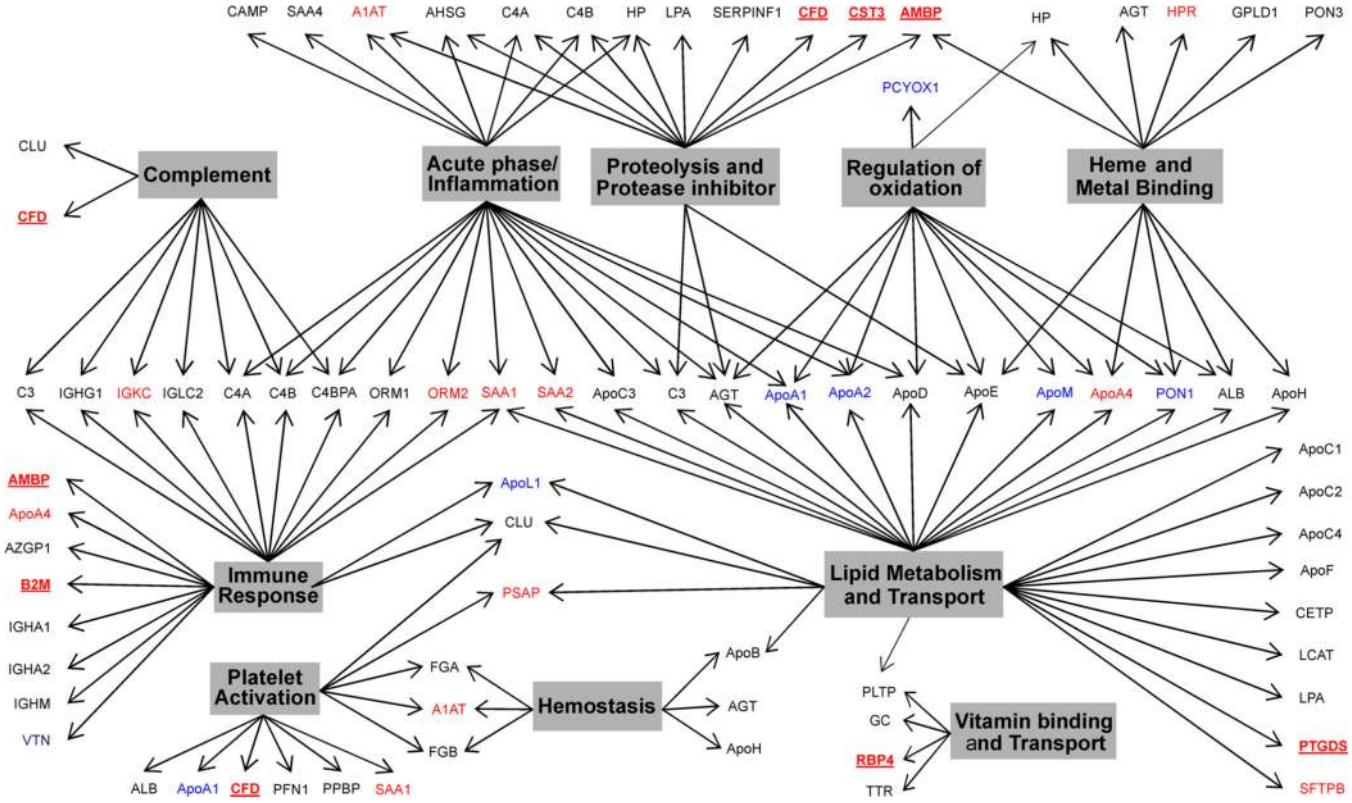

Figure 2.

Gene ontology analysis of biological processes and molecular functions of HDL proteins. Proteins in total HDL were identified using LC– ESI–MS/MS (Table 2). Proteins detected in HDL were associated with biological functions using GO process annotations. Proteins that are enriched in HDL isolated from ESRD group are shown in red and proteins that are decreased in HDL isolated from ESRD group are shown in blue. The six proteins that are dramatically increased in HDL isolated from ESRD group are shown in red and underlined.

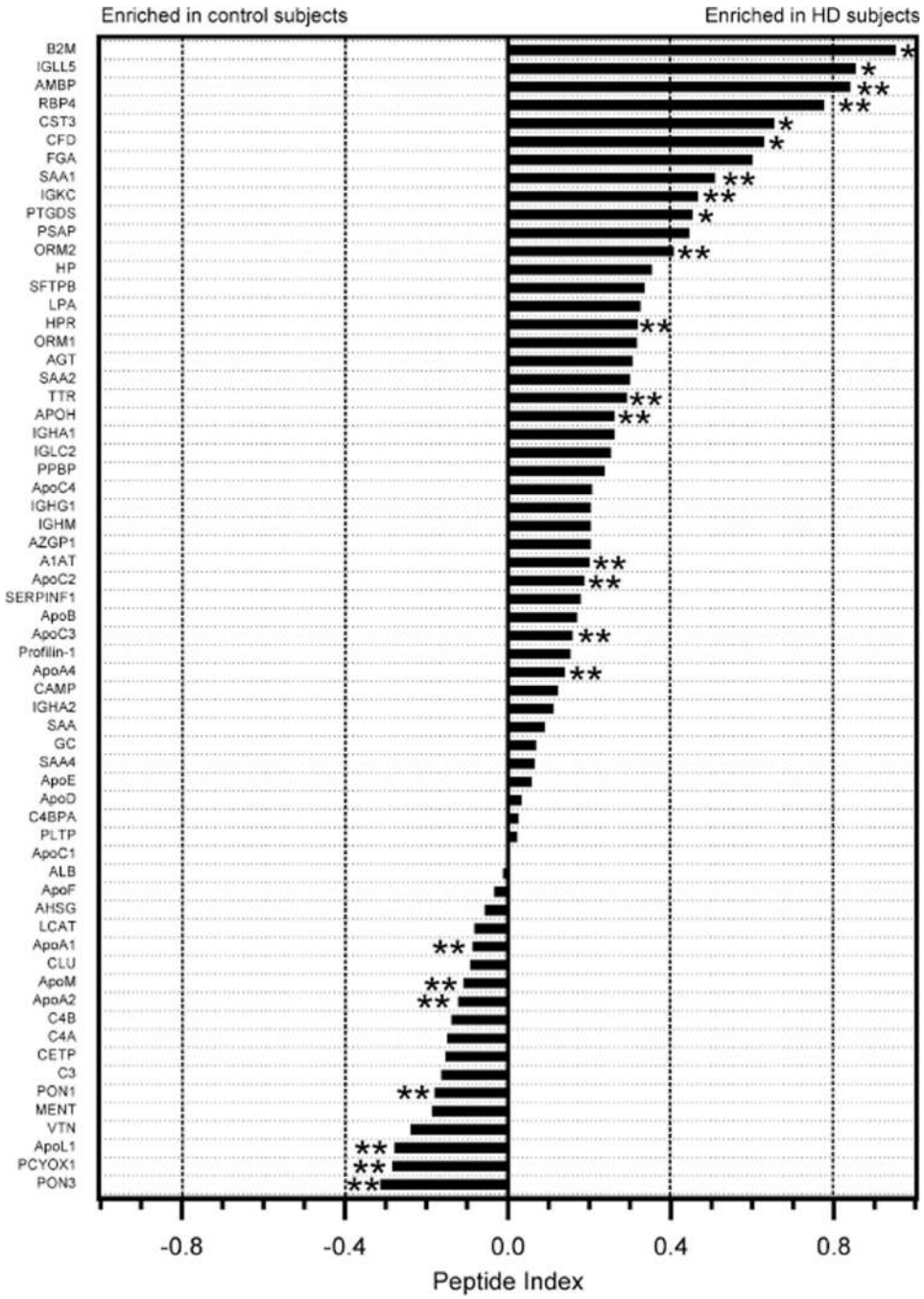

Figure 3.

Peptide index of HDL proteins by shotgun analysis. HDL was isolated from plasma of 20 control subjects and 40 subjects with ESRD on dialysis. The relative abundance of proteins in the ESRD subjects versus the control subjects was assessed by the peptide index, as described in Methods. *P value was not available because the protein was detected in none or one control subjects **$P < 0.01$ by Student's t test.

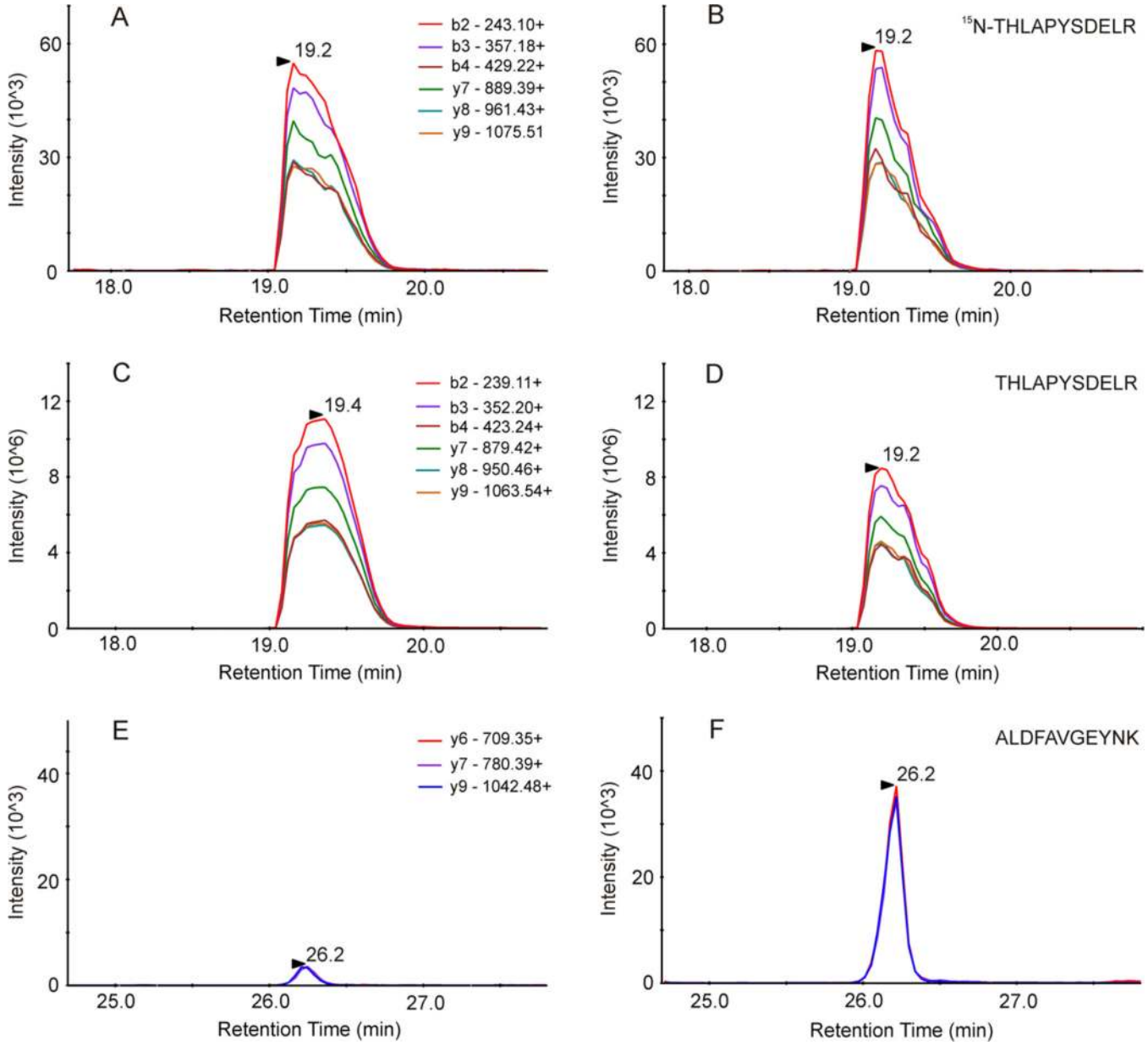

Figure 4.

Quantitative analysis of HDL proteins between control and ESRD subjects by SRM. Following the digestion of HDL with trypsin, peptide digests of HDL (0.5 μg) was injected into a Thermo TSQ Vantage system for nano-LC–SRM–MS/MS analysis. The SRM data were analyzed with Skyline to obtain the peak area of each transition, which will be used for quantitative calculations of relative levels of peptides and proteins. (See the Experimental Section.) The SRM data in (A, C, E) were from HDL of a control subject and the SRM data in (B, D, F) were from HDL of an ESRD patient. Six transitions (b_2, b_3, b_4, y_7, y_8, y_9) were selected for peptide [^{15}N]THLAPYSDELR derived from [^{15}N]apoA-I as the internal standard (A and B). The same six transitions were chosen for quantifying the levels of peptide THLAPYSDELR derived from endogenous apoA-I in HDL (C and D). Three SRM

transitions (y6, y7, y9) were chosen for quantifying the levels of peptide ALDFAVGEYNK derived from CST3 protein in HDL (E and F). Note: the chromatograms were plotted on the same scale for a peptide derived from the control subject and for the same peptide derived from the ESRD subject (A and B, C and D, and E and F).

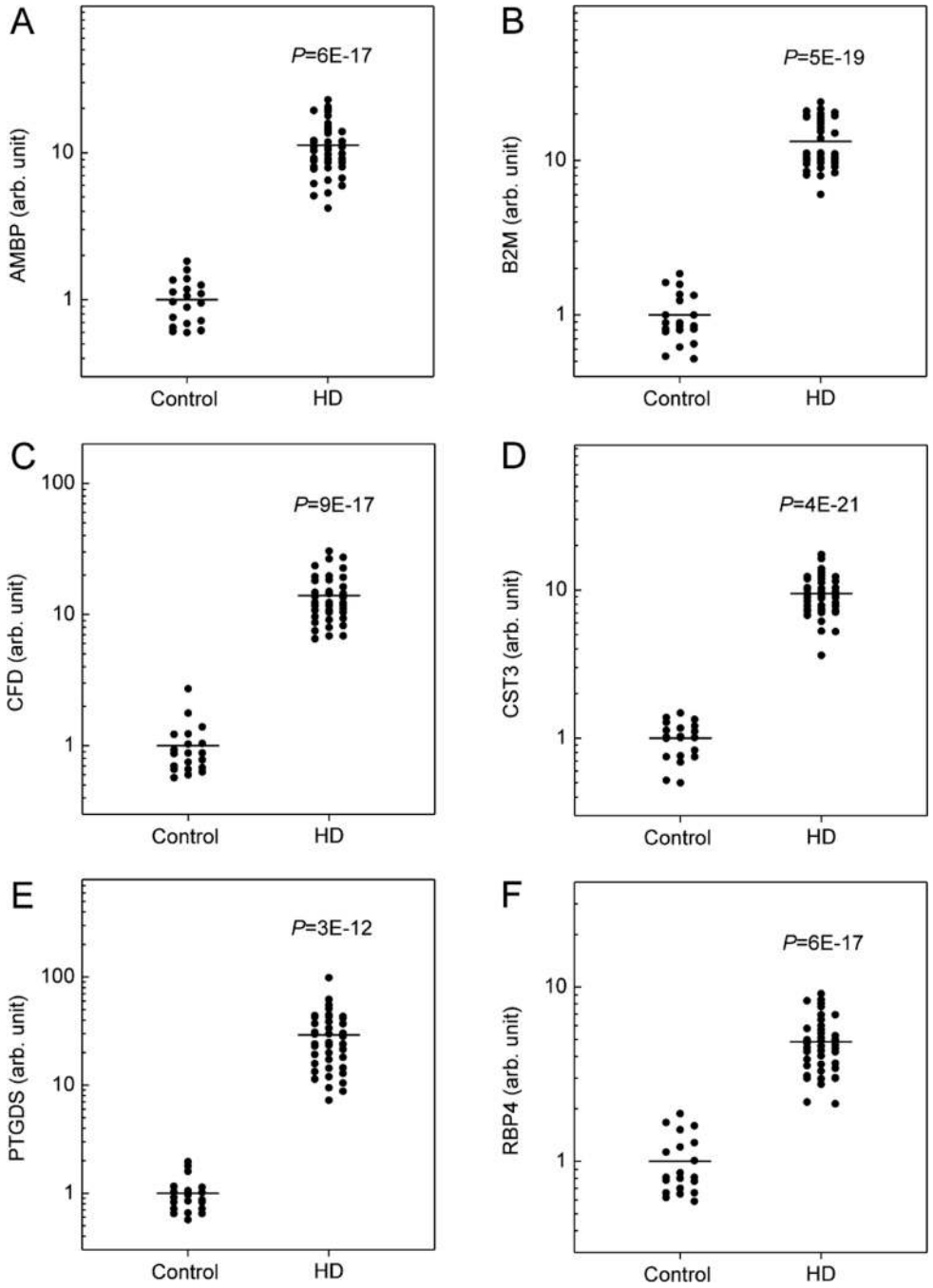

Figure 5.

A cluster of proteins implicated in kidney disease are dramatically increased in HDL isolated from hemodialysis subjects. The relative levels of proteins in HDL were determined by isotope-dilution SRM analysis. (See the Experimental Section.) The average levels of proteins in HDL isolated from control subjects were set as an arbitrary unit of one. AMBP, protein AMBP; B2M, beta-2-micro-globulin; CFD, complement factor D; CST3, cystatin-C; PTGDS, prostaglandin-H2 D-isomerase; RBP4, retinol-binding protein 4. Values were

logarithmically transformed to achieve normal distribution. *P* values were obtained by two-tailed Student's *t* test.

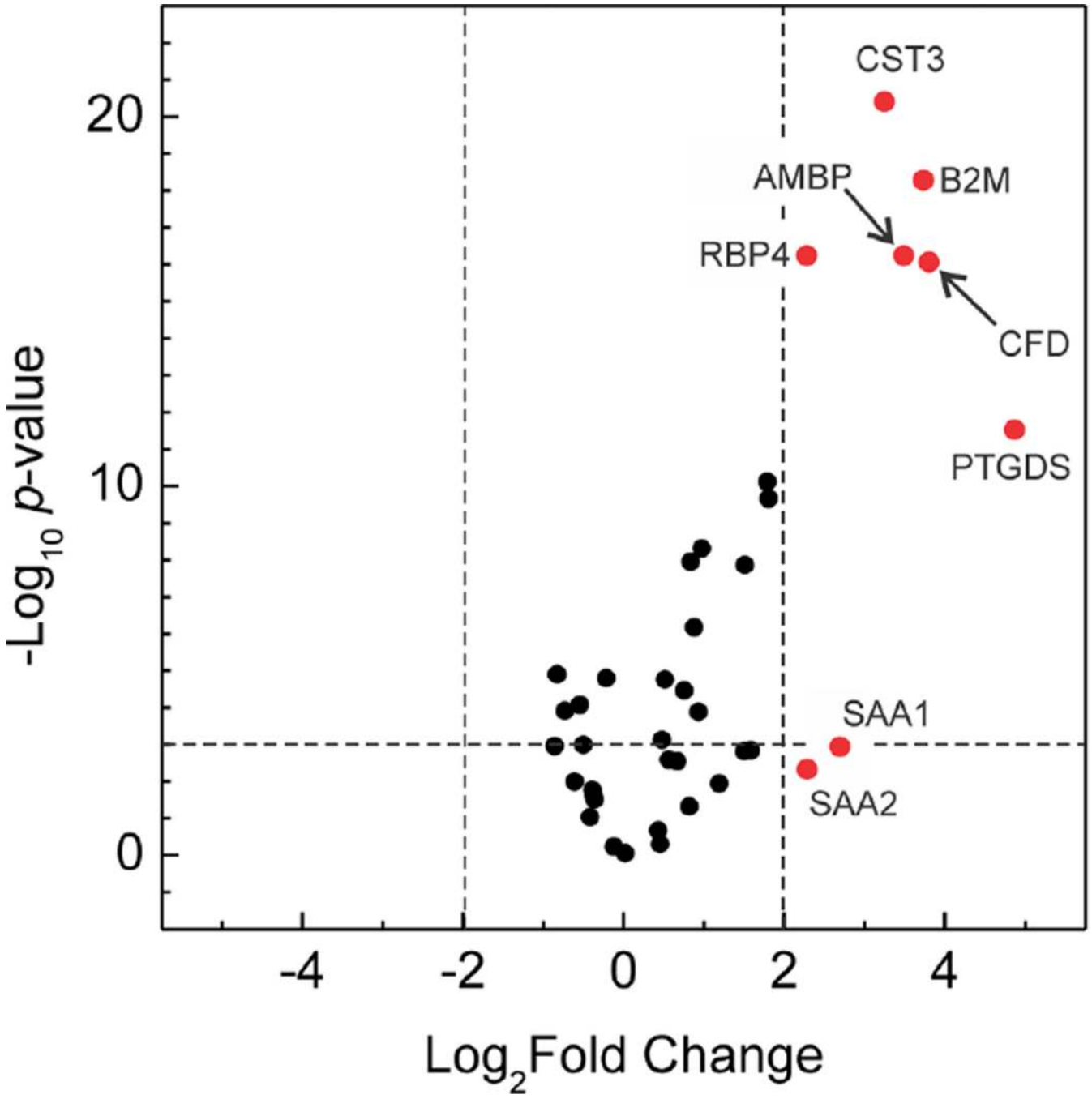

Figure 6.

Volcano plot of HDL proteins: *P* value versus fold changes (ESRD to control). The relative levels of proteins in HDL were determined by isotope-dilution SRM analysis. (See the Experimental Section.) The *y* axis is negative base 10 logarithm of the *P* values from unpaired Student *t* test for 37 proteins quantified with SRM. The *x* axis is base 2 logarithm of the fold change of proteins (ratio of ESRD to control). The horizontal dotted line marks the threshold of *P* value less than 0.001 (to the top). The right vertical dotted line marks the threshold of greater than 4-fold increases in ESRD subjects than in control subjects (to the

right). The left vertical dotted line marks the threshold of greater than 4-fold decreases in ESRD subjects than in control subjects (to the left). The eight proteins that were increased greater than 4 times in ESRD subjects than in control subjects were shown in red. AMBP, protein AMBP; B2M, beta-2-microglobulin; CFD, complement factor D; CST3, cystatin-C; PTGDS, prostaglan-din-H2 D-isomerase; RBP4, retinol-binding protein 4; SAA1, serum amyloid A-1 protein; SAA2, serum amyloid A-2 protein.

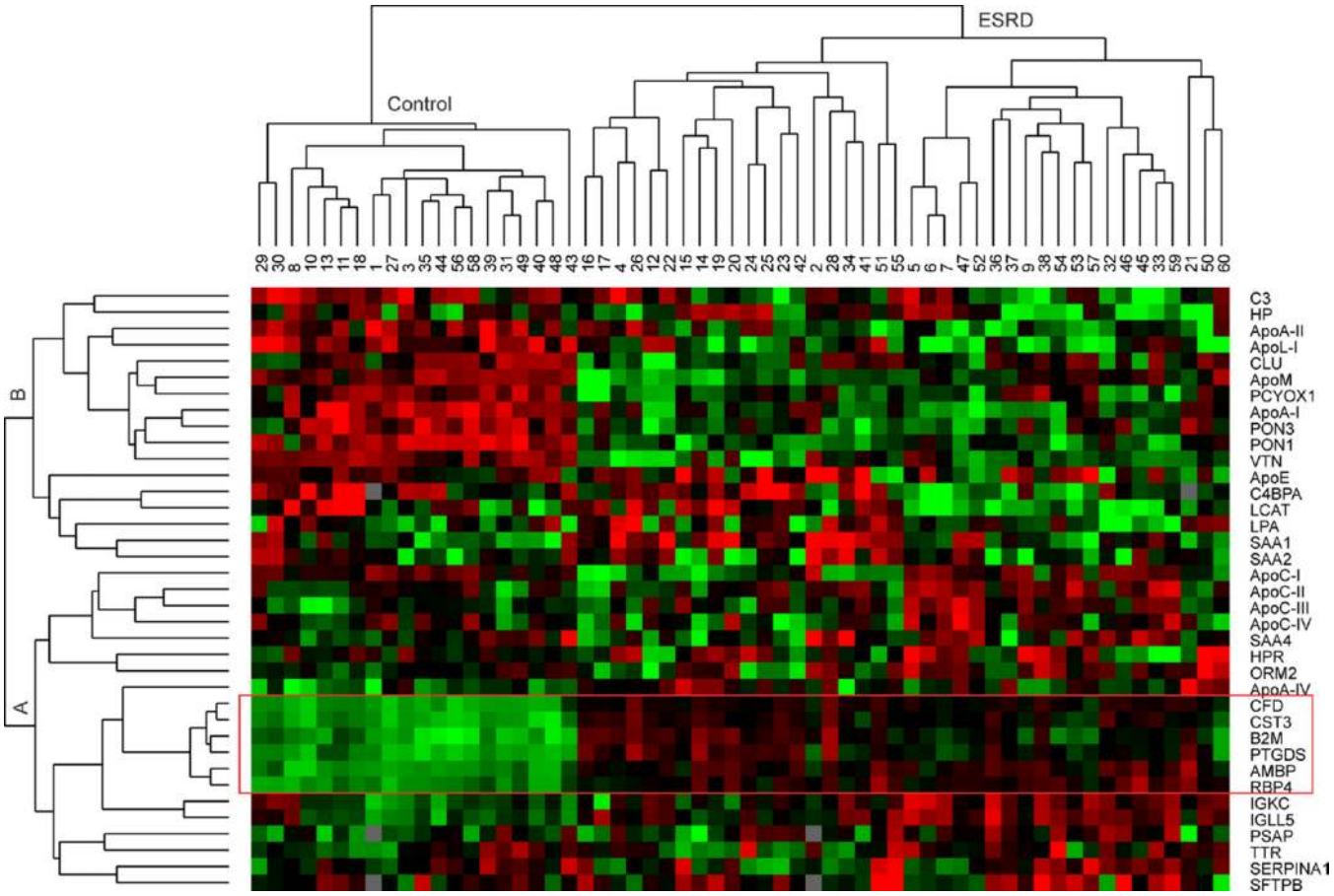

Figure 7.

Hierarchical clustering of proteins identified in HDL discriminates between ESRD patients and control subjects. Relative protein abundance in HDL between ESRD and control individuals was quantified by isotope-dilution SRM analysis. (See the Experimental Section.) Hierarchical clustering was performed using the average linkage clustering method and centered correlation metric with Cluster and TreeView, two open-access programs. (See the Experimental Section.) The numbers at the top are randomized assay numbers for all 60 ESRD and control subjects. The gene names of 37 proteins quantified by SRM were shown on the right. The green and red colors represent low and high relative abundance of proteins, respectively. The labels A and B on the left indicate two major groups of proteins that were enriched in ESRD patients and control subjects, respectively. The red rectangle indicates six proteins that were markedly enriched in the ESRD subjects and were clustered together.

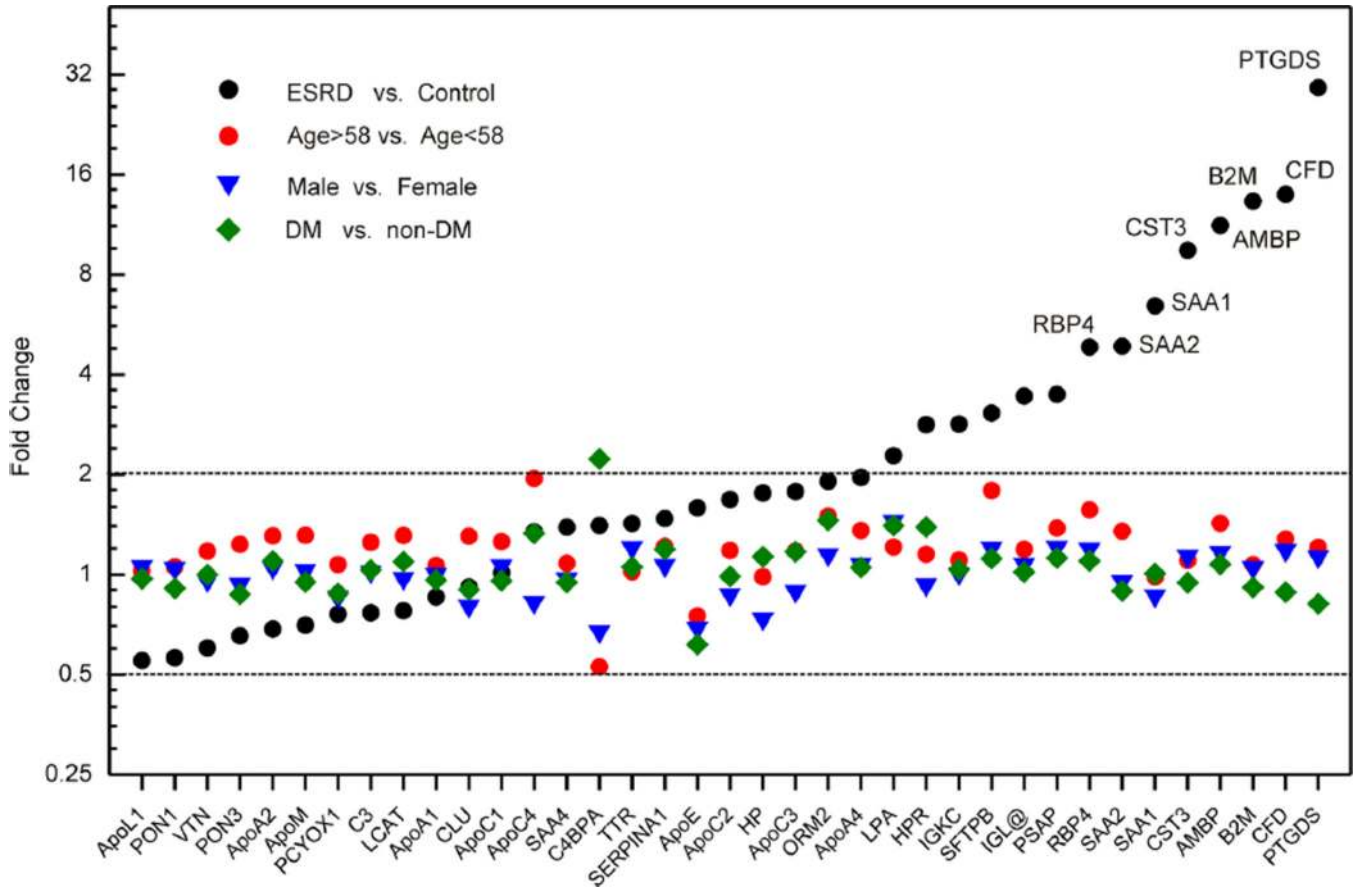

Figure 8.

Effect of potential confounders on the HDL proteins within the ESRD population. Relative protein abundance in HDL between ESRD and control individuals was quantified by isotope-dilution SRM analysis. The fold changes of 37 proteins quantified by SRM are plotted as ESRD versus control subjects (black circle). The analysis is also shown only for ESRD subjects for (i) age older than 58 years old versus younger than 58 years old (red triangle), (ii) males versus females (blue square), and (iii) DM versus non-DM (olive star) to examine the role of potential confounders. The two dotted horizontal lines indicate fold changes between 0.5 and 2. AMBP, protein AMBP; B2M, beta-2-microglobulin; CFD, complement factor D; CST3, cystatin-C; PTGDS, prostaglandin-H2 D-isomerase; RBP4, retinol-binding protein 4; SAA1, serum amyloid A-1 protein; SAA2, serum amyloid A-2 protein.

Table 1

Clinical Characteristics of Study Subjects[a,b]

	control (n = 20)	ESRD (n = 40)	P values
age (years)	40 ± 11 (32, 47)	58 ± 14 (49, 66)	$P < 0.0001$
male (%)	10 (50%)	20 (50%)	
white	19 (95%)	18 (45%)	
hypertensive patients (%)	0 (0%)	30 (75%)	
prevalent CVD	0 (0%)	15 (37.5%)	
diabetes (%)	0 (0%)	22 (55%)	
smokers (%)	0 (0%)	22 (55%)	
smoker			
never	20 (100%)	18 (45%)	
past	0 (0%)	10 (25%)	
current	0 (0%)	12 (30%)	
total cholesterol (mg/dL)	218 ± 34	168 ± 32	$P < 0.0001$
HDL cholesterol (mg/dL)	57 ± 15	41 ± 13	$P = 0.0003$
LDL cholesterol (mg/dL)	136 ± 33	93 ± 31	$P < 0.0001$
triglycerides (mg/dL)	126 ± 81	175 ± 96	$P = 0.049$
vascular access			
AVF		20 (50%)	
AVG		15 (38%)	
catheter		5 (12%)	
dialysis (years)		2.2 (1.6, 3.5)	
ESRD etiology			
diabetes		22 (55%)	
hypertension		11 (28%)	
PCKD		2 (5%)	
other		5 (12%)	
medications			
statin		8 (20%)	
fibrate		1 (2%)	
ezetimibe		3 (8%)	
loop diuretic		12 (30%)	
beta blocker		16 (40%)	
ACEI/ARB		22 (55%)	

[a] Plus-minus values are means ± SDs.

[b] Abbreviations: AVG, arteriovenous grafts; AVF, arteriovenous fistula; CVD, cardiovascular disease; ESRD, end-stage renal disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PCKD, polycystic kidney disease; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker.

Table 2

Proteins Detected in HDL of Control and/or ESRD Subjects[a,b]

		subjects with detectable proteins		average peptide number			
Gene	**description**	**control**	**ESRD**	**control**	**ESRD**	**peptide index**	***P* value**
A1at	alpha-1-antitrypsin	20	40	21.8	32.5	0.20	0.001
Alb	serum albumin	20	40	143.5	141.2	–0.01	0.83
Apoa1	apolipoprotein A-I	20	40	1041.7	881.4	–0.08	0.002
Apoa2	apolipoprotein A-Ii	20	40	344.4	271.4	–0.12	0.003
Apoa4	apolipoprotein A-Iv	20	40	61.3	80.5	0.14	0.0005
Apob	apolipoprotein B-100	20	40	264.8	370.6	0.17	0.04
Apoc1	apolipoprotein C–i	20	40	56.8	57.0	0.00	0.95
Apoc2	apolipoprotein C–ii	20	40	61.1	88.6	0.18	3×10^{-5}
Apoc3	apolipoprotein C–iii	20	40	106.4	145.3	0.15	2×10^{-6}
Apod	apolipoprotein D	20	40	56.7	60.2	0.03	0.21
Apoe	apolipoprotein E	20	40	117.7	131.2	0.05	0.09
Apof	apolipoprotein F	20	40	19.0	17.9	–0.03	0.40
Apom	apolipoprotein M	20	40	61.7	49.9	–0.11	9×10^{-5}
Lcat	phosphatidylcholine-sterol acyltransferase	20	40	15.1	12.9	–0.08	0.03
Pon1	serum paraoxonase/arylesterase 1	20	40	108.2	75.8	–0.18	6×10^{-6}
Saa4	serum amyloid A-4 protein	20	40	37.4	42.3	0.06	0.04
Ambp	protein Ambp	13	40	3.0	26.9	0.76	2×10^{-18}
Apol1	apolipoprotein L-I	20	39	35.4	20.5	–0.26	0.0007
C3	complement C3	20	39	30.1	22.3	–0.15	0.04
Clu	clusterin	20	39	16.8	14.4	–0.08	0.23
Hpr	haptoglobin-related protein	20	39	10.9	21.6	0.32	0.0009
Lpa	apolipoprotein (A)	19	39	46.6	90.9	0.31	0.02
Saa1	serum amyloid A-1 protein	16	39	12.2	33.8	0.43	0.002
B2m	beta-2-microglobulin	1	39	0.3	11.7	0.63	
Ttr	transthyretin	17	38	4.7	8.0	0.24	0.0002
Rbp4	retinol-binding protein 4	9	37	1.8	14.5	0.65	8×10^{-9}

Gene	description	subjects with detectable proteins		average peptide number		peptide index	*P* value
		control	ESRD	control	ESRD		
Ahsg	alpha-2-Hs-glycoprotein	20	36	12.0	11.9	0.00	0.38
Apoh	beta-2-glycoprotein 1	16	36	5.2	8.6	0.21	0.01
Apoc4	apolipoprotein C–iv	15	36	5.4	7.3	0.12	0.46
Pon3	serum paraoxonase/lactonase 3	20	35	12.1	7.1	–0.25	0.01
C4a	complement C4-A	19	35	12.3	9.7	–0.11	0.26
Pltp	phospholipid transfer protein	19	35	11.2	12.7	0.06	0.23
Igkc	Ig kappa chain C region	13	34	3.1	8.9	0.38	0.0002
Igll5	immunoglobulin lambda-like polypeptide 5	-	34	-	5.3	0.85	
Pcyox1	prenylcysteine oxidase 1	18	32	8.5	4.9	–0.23	0.004
Fga	fibrinogen alpha chain	2	31	1.2	4.7	0.19	0.18
Ppbp	platelet basic protein	11	27	2.6	4.6	0.17	0.11
Cst3	cystatin-C	-	26	-	2.5	0.65	
Cfd	complement factor D	-	25	-	3.2	0.63	
Ighg1	Ig gamma-1 chain C region	9	24	2.8	4.5	0.12	0.48
Psap	proactivator polypeptide	3	23	0.7	3.1	0.24	0.26
Gc	vitamin D-binding protein	11	22	1.8	2.2	0.06	0.23
Vtn	vitronectin	14	20	3.6	2.3	–0.13	0.36
Orm2	alpha-1-acid glycoprotein 2	2	18	0.2	2.2	0.29	0.0004
Ptgds	prostaglandin-H2 D-isomerase	-	18	-	1.8	0.45	
Agt	angiotensinogen	2	16	0.4	1.5	0.14	0.87
Igha1	Ig alpha-1 chain C region	4	15	0.6	2.4	0.19	0.03
Orm1	alpha-1-acid glycoprotein 1	2	15	0.3	1.7	0.19	0.05
Sftpb	pulmonary surfactant-associated protein B	1	14	0.1	2.1	0.25	
Hp	haptoglobin beta chain	-	14	-	1.8	0.35	
Saa2	serum amyloid A-2 protein	1	13	0.2	2.4	0.19	
C4b	complement C4–b	8	12	1.6	1.0	–0.08	0.29
Iglc2	Ig lambda-2 chain C regions	1	12	0.2	1.2	0.13	
C4bpa	complement component 4 binding protein, alpha, isoform Cra C	5	9	1.9	2.6	0.04	0.38
Camp	cathelicidin antimicrobial peptide	2	8	0.3	0.7	0.06	0.30

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Gene	description	subjects with detectable proteins		average peptide number		peptide index	*P* value
		control	ESRD	control	ESRD		
Azgp1	zinc-alpha-2-glycoprotein	-	8	-	0.8	0.20	
Ighm	Ig Mu chain C region	-	8	-	0.7	0.20	
Ment	protein Ment	7	7	1.4	0.6	–0.11	0.66
Igha2	Ig alpha-2 chain C region	2	7	0.2	0.6	0.07	0.02
Serpinf1	pigment epithelium-derived factor	-	7	-	0.5	0.18	
Pfn1	profilin-1	-	6	-	0.4	0.15	
Fgb	fibrinogen beta chain	-	5	-	0.2	0.13	
Cetp	cholesteryl ester transfer protein	4	2	0.6	0.2	–0.06	1.00

[a] HDL (d = 1.063–1.210 g/mL) was isolated from EDTA plasma by ultracentrifugation from 20 control subjects and 40 ESRD patients. Tryptic digests were subjected to reverse-phase separation followed by nano-ESI–MS/MS on a Thermo LTQ ion trap instrument. MS/MS spectra were searched against the UniProtKB human database (uniprot.sptr.human.20130503) using the SEQUEST search engine. The SEQUEST results were further processed using PeptideProphet and ProteinProphet, using an adjusted probability of greater than 0.90 and greater than 0.95 for peptides and proteins, respectively. For HDL, all protein identifications required detection of at least two unique peptides for each protein from at least five different individuals.

[b] –, not detected.

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