Combined Statin and Niacin Therapy Remodels the High-Density Lipoprotein Proteome

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Background—Boosting low high-density lipoprotein (HDL) levels is a current strategy for preventing clinical events that result from cardiovascular disease. We previously showed that HDL₃ of subjects with coronary artery disease is enriched in apolipoprotein E and that the lipoprotein carries a distinct protein cargo. This observation suggests that altered protein composition might affect the antiatherogenic and antiinflammatory properties of HDL. We hypothesized that an intervention that increases HDL levels—combined statin and niacin therapy—might reverse these changes.

Methods and Results—HDL₃ isolated from 6 coronary artery disease subjects before and 1 year after combination therapy was analyzed by liquid chromatography–Fourier transform–mass spectrometry. Alterations in protein composition were detected by spectral counting and confirmed with extracted ion chromatograms. We found that combination therapy decreased the abundance of apolipoprotein E in HDL₃ while increasing the abundance of other macrophage proteins implicated in reverse cholesterol transport. Treatment-induced decreases in apolipoprotein E levels of HDL₃ were validated biochemically in a second group of 18 coronary artery disease subjects. Interestingly, the changes in HDL₃ proteome with niacin/statin treatment resulted in a protein composition that more closely resembled that of HDL₃ in healthy control subjects.

Conclusions—Combined statin and niacin therapy partially reverses the changes in the protein composition seen in HDL₃ in coronary artery disease subjects. Our observations raise the possibility that quantifying the HDL proteome could provide insights into the therapeutic efficacy of antiatherosclerotic interventions. (Circulation. 2008;118:1259-1267.)

Key Words: arteriosclerosis ▪ cardiovascular diseases ▪ drugs ▪ inflammation ▪ lipoproteins

Epidemiological and clinical studies demonstrate that low levels of high-density lipoprotein (HDL) cholesterol are an independent risk factor for premature coronary artery disease (CAD).1,2 A primary mechanism by which HDL protects against atherosclerosis is by removing cholesterol from artery wall macrophages through reverse cholesterol transport.3,4 However, HDL exhibits other biological activities that may contribute to its antiatherogenic properties, including the ability to reduce oxidative stress and combat inflammation.5,6 The protein component of HDL plays critical roles in mediating these biological activities.

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Apolipoprotein (apo) A-I accounts for ≈70% of HDL protein mass, and apoA-II accounts for ≈20%.7-10 However, HDL contains a wide array of other proteins and therefore exists as a family of distinct particles that vary in protein composition.7 Moreover, changes to these proteomes can alter both the functions and cardioprotective effects of HDL. For example, animal studies demonstrate that increasing the apoA-II content of HDL promotes atherosclerosis.11,12 In both humans and animals, acute and chronic inflammation changes HDL protein content,13-16 perhaps impairing its cardioprotective effects.17 It has been proposed, for example, that alterations in the balance between pro-oxidative and antioxidative enzymes in HDL play a key role in rendering the lipoprotein atherogenic.5,7,9-18,20

Recently, mass spectrometry (MS) has been used to elucidate the proteome of both HDL₁⁹-²¹ and HDL₃,²⁰ its dense subfraction. These studies revealed that HDL contains multiple proteins that regulate the complement system and a diverse array of serine-type endopeptidases.²⁰ Many acute-phase response proteins also were identified, supporting a central role for HDL in inflammation.²⁰ The protein composition of HDL also differs in normolipidemic and hyperlipidemic subjects.²² Moreover, HDL₃ in subjects with established CAD is enriched in several proteins, including apoE²⁰,

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indicating that these proteins may serve as markers—and perhaps mediators—of vascular disease.

Intense interest exists in pharmacological approaches to promote the antiatherogenic effects of HDL. Most clinical studies have focused on increasing HDL cholesterol levels, but studies indicate that HDL levels can be dissociated from the cardioprotective functions of lipoprotein.5,6,11,12 Indeed, a recent study was terminated prematurely because the rate of cardiovascular events increased when an agent that elevates HDL cholesterol was added to statin therapy in established CAD subjects.23–25 Collectively, these observations indicate that alterations in HDL cholesterol levels may not be the only determinant of the cardioprotective effects of HDL.

We hypothesized that combination therapy with a statin and niacin, which increases HDL cholesterol levels and reduces CAD risk,26 would modify the proteome of HDL₃ in CAD subjects and that these modifications might provide insights into its antiatherogenic and antiinflammatory properties. To test this proposal, we used MS to investigate the impact of intensive lipid-lowering therapy with atorvastatin and extended-release niacin on the HDL proteome of CAD subjects.23–25 Collectively, these observations indicate that alterations in HDL cholesterol levels may not be the only determinant of the cardioprotective effects of HDL.

### Methods

#### Subjects

We investigated the HDL proteome in 2 groups of CAD subjects enrolled in the Carotid Plaque Composition Study and in a group of healthy control subjects (Table 1).20–27 The first group (n = 6) was used for proteomic analysis of HDL₃, and the second group (n = 18) was used to quantify apoE in HDL₃, biochemically. All subjects were men recently diagnosed with CAD documented by at least 1 stenotic lesion (>50%) on coronary angiography. All subjects were treated with extended-release niacin (2 g daily) and the statin atorvastatin (10 to 20 mg daily) for 12 months. Blood was drawn from each patient at baseline and after 12 months of treatment. All subjects were concurrently taking aspirin and antihypertensive medication (β-blockers, 5 of 6; angiotensin-converting enzyme inhibitors, 4 of 6; 4 of 6 subjects were also using clopidogrel. Concurrent medications did not change over the course of the study.

A healthy control group of age-matched men (n = 6) had no known history of CAD, were not hyperlipidemic or diabetic, had no family history of premature CAD, and were not receiving lipid-lowering therapy. All studies involving human material were approved by the Human Studies Committee at the University of Washington.

#### Preparation of HDL₃

Blood anticoagulated with EDTA was collected from subjects who had fasted overnight. HDL₃ (d = 1.110 to 1.210 g/mL) was isolated from EDTA plasma stored at −80°C by sequential density ultracentrifugation with KBr.28

#### Protein Digestion

HDL₃, digested with trypsin20 (1:20, wt/wt) was reconstituted with 0.1% acetic acid and desalted with an Agilent HP1100 high-performance liquid chromatography (LC) system (Agilent, Palo Alto, Calif) interfaced with a peptide macrotrap (Michrom BioSciences Inc, San Jose, Calif). Desalted samples were freeze-dried and stored at −80°C.

#### LC–Fourier Transform Ion Cyclotron Resonance–MS

Interleaved, randomly ordered samples were analyzed on a hybrid LTQ-FT mass spectrometer (Thermo Electron, Erlangen, Germany) with a nanoelectrospray source29 (Molecular Profiling Proteomics Group, Merck, Rahway, NJ). Each sample was resuspended in 10 μL 0.1% acetic acid, and 1 μL was injected onto the C₁₈ reverse-phase capillary column (New Objective, Woburn, Mass). Chromatographic separations were performed with an Agilent HP1100 high-performance liquid chromatography using a 50-minute linear gradient of 0.1 mol/L acetic acid and 0.1 mol/L acetic acid in 90% acetonitrile at 1 μL/min. One Fourier transform ion cyclotron resonance (FT)–MS scan, 1 linear ion trap MS scan, and 3 data-dependent MS/MS scans were acquired. For data-dependent MS/MS acquisition, the dynamic exclusion settings were as follows: repeat count, 2; repeat time, 30 seconds; exclusion list, set at 50; and exclusion time, 180 seconds.

MS/MS data were converted to .dta format with Extract_msn in Bioworks 3.0 (Thermo Inc) (15 ions minimum; each MS/MS spectrum exported individually; charge state not determined) and searched against the complete human International Protein Index database30 (version 3.01) with SEQUEST31 (version 2.7). Tryptic peptides (up to 2 missed cleavages), fixed carbamidomethyl on cysteine residues, and variable oxidation on methionine residues were allowed in the search. Precursor ion tolerance was ±2.8 m/z, and fragment ion tolerance was 0.8 m/z. SEQUEST results were validated with PeptideProphet32 and ProteinProphet33 with peptide probability of ≥0.90 and protein probability ≥0.95, resulting in maximum 0.4 false identifications. Each charge state of a peptide was considered a unique identification. Only protein identifications with at least 2 unique peptides detected in at least 2 samples were considered valid for further evaluation.

### Table 1. Clinical Characteristics of Study Subjects

<table>
<thead>
<tr>
<th>Group and Status</th>
<th>Age, y</th>
<th>Cholesterol, mg/dL</th>
<th>TG, mg/dL</th>
<th>LDL-C, mg/dL</th>
<th>HDL-C, mg/dL</th>
<th>HDL-C₃, mg/dL</th>
<th>HDL-C₂, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1, proteomics study</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAD (n=6)</td>
<td>58 (5)</td>
<td>230 (93)</td>
<td>165 (103)</td>
<td>168 (27)</td>
<td>42 (12)</td>
<td>6.4 (3.8)</td>
<td>36 (10)</td>
</tr>
<tr>
<td>CAD + niacin/statin (n=6)</td>
<td>59 (5)</td>
<td>142 (29)*</td>
<td>68 (39)*</td>
<td>79 (16)*</td>
<td>56 (16)*</td>
<td>11.6 (9)*</td>
<td>45 (11)*</td>
</tr>
<tr>
<td>Healthy control (n=6)</td>
<td>54 (14)</td>
<td>183 (29)†</td>
<td>117 (61)†</td>
<td>121 (22)†</td>
<td>45 (12)</td>
<td>8.2 (4.3)</td>
<td>37 (10)</td>
</tr>
<tr>
<td>Group 2, apoE validation study</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAD (n=18)</td>
<td>54 (7)</td>
<td>227 (33)</td>
<td>159 (75)</td>
<td>160 (29)</td>
<td>41 (9)</td>
<td>6.6 (3)</td>
<td>36 (7)</td>
</tr>
<tr>
<td>CAD + niacin/statin (n=18)</td>
<td>55 (7)</td>
<td>145 (29)*</td>
<td>78 (41)*</td>
<td>83 (25)*</td>
<td>52 (15)*</td>
<td>10.6 (5.6)*</td>
<td>41 (10)*</td>
</tr>
</tbody>
</table>

TG indicates triglycerides; C, cholesterol. Results are given as means (SD). *P < 0.01 by a paired 2-tailed Student t test; †P < 0.01 vs untreated CAD subjects by 2-tailed Student t test.
Quantifying HDL3 Proteins by Extracted Ion Chromatograms

HDL3 proteins identified as potentially different in relative abundance by spectral counting were quantified by extracted ion chromatograms using 2 to 7 peptides for each protein. Extracted ion chromatograms were constructed from the FT-MS data, and peak area was determined with LCQuan software (Thermo Electron). Ion chromatograms were reconstructed for the peptide charge state identified by SEQUEST using accurate monoisotopic mass with 0.05-Da tolerance. Peptides selected for further analysis exhibited no evidence of interference from closely eluting isomeric or isobaric peptides. For apoC-II, apoE, apoA-I, phospholipid transfer protein (PLTP), and apoF, we quantified all peptides that met the criteria and were identified in at least 50% of the samples. Differences in protein levels were determined by analyzing peak areas for multiple peptides obtained from each peptide using a repeated-measures 2-way ANOVA with treatment and peptide as within-subject factors based on natural log-transformed values. We used this analytical strategy because spectral counting offers a rapid, simple mechanism of detecting differences in protein levels, whereas spectral peak intensity provides more quantitative estimates of such ratios.

Biochemical Quantification of ApoE Levels

ApoE levels in HDL3 were determined by nephelometry with Dade-Behring (Deerfield, Ill) reagents and normalized to protein concentration determined by the bicinchoninic acid method.

Statistical Analysis

Results are reported as means and SDs. Differences in lipoprotein values were determined by a paired 2-tailed t test, and differences in apoE values were determined with a Wilcoxon matched-pairs test. Pearson product-moment correlation coefficients were used to assess linear relationships between variables. Values of \( P < 0.05 \) were considered significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

HDL3 was isolated from 6 recently diagnosed CAD patients at baseline and after 1 year of treatment with extended-release niacin and atorvastatin. The lipoprotein was digested with trypsin and analyzed by LC-MS/MS, and the resulting spectra were searched against the International Protein Index database. We studied the HDL dense subfraction, HDL3, because total HDL and HDL2 from CAD subjects are contaminated with apoB100, an low-density lipoprotein (LDL) protein, likely reflecting the presence of small dense LDL. Furthermore, we previously established that changes in the HDL3 proteome associate with CAD.

Subject Characteristics

CAD patients had symptoms consistent with angina and were recently diagnosed as documented by the presence of at least 1 stenotic lesion (>50%) on coronary angiography. These subjects were clinically stable, and at least 3 months had elapsed since their acute coronary syndrome. None smoked cigarettes, had liver or renal disease, or had received lipid-lowering medication for at least 4 weeks before their blood collection.

The subjects’ clinical characteristics and lipid values before and during aggressive lipid-lowering therapy are shown in Table 1. Niacin/statin treatment significantly decreased both total plasma cholesterol and LDL cholesterol, with an average reduction of 53% (SD, 7.0%; \( P < 0.001 \)) and 40% (SD, 9.5%; \( P < 0.001 \)), respectively. HDL cholesterol increased by an average of 34% (SD, 12.3%; \( P = 0.001 \)) after 1 year of combination therapy, with a 26% increase in the HDL3 fraction (SD, 17.3%; \( P = 0.003 \)). A control group with no known history of CAD and not receiving lipid-lowering therapy also was analyzed (Table 1, group 1). These healthy subjects had lower levels of plasma total cholesterol, LDL, and triglycerides than CAD subjects at baseline. Importantly, subjects’ weight remained stable over the study (212 lb [SD, 30 lb] at baseline and 207 lb [SD, 30 lb] at 1 year). No significant difference was found between the healthy subjects’ and CAD subjects’ body weights (205 lb [SD, 30] and 212 lb [SD, 30], respectively; \( P = 0.67 \)) or body mass index (30.2 kg/m\(^2\) [SD, 3.3 kg/m\(^2\)] and 27.0 kg/m\(^2\) [SD, 4.7], respectively; \( P = 0.17 \)).

Mass Spectrometric Analysis of HDL3

Our LC-FT-MS/MS analysis of the HDL3 proteome detected 27 proteins in HDL3 (Table 1 in the online-only Data Supplement), including 8 of the 12 known HDL apolipoproteins. We identified proteins implicated in lipid metabolism (lecithin:cholesterol acyl transferase, PLTP, paraoxonase-1), serum amyloid proteins (SAA1 and SAA4), complement pathway regulatory proteins (complement C3, complement C4B, vitronectin), and endopeptidase inhibitors (\( \alpha \)-antitrypsin, kininogen-1). This analysis detected 25 of 32 proteins identified by SEQUEST using accurate monoisotopic mass with 0.05-Da tolerance. Peptides selected for further analysis exhibited no evidence of interference from closely eluting isomeric or isobaric peptides identified in CAD patients before and during niacin/statin treatment. For proteins found in only 1 group of subjects, a 1-sample t test was used to compare the number of total peptides to a theoretical mean of 0.

### Table 2. Differentially Expressed HDL3 Proteins Detected by Spectral Counting

<table>
<thead>
<tr>
<th>Protein Identification No.*</th>
<th>Protein Name</th>
<th>CAD†</th>
<th>CAD + Niacin/Statin† (on Therapy to off Therapy)‡</th>
<th>Average Ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPI00022733</td>
<td>PLTP</td>
<td>0</td>
<td>3.0 (2.7)</td>
<td>&gt;3</td>
<td>0.02</td>
</tr>
<tr>
<td>IPI00480119</td>
<td>ApoF</td>
<td>3.8 (1.1)</td>
<td>5.2 (0.75)</td>
<td>1.4</td>
<td>0.005</td>
</tr>
<tr>
<td>IPI00451262</td>
<td>Clusterin (ApoJ)</td>
<td>0.3 (0.8)</td>
<td>1.8 (2.2)</td>
<td>6.2</td>
<td>0.12</td>
</tr>
<tr>
<td>IPI00021842</td>
<td>ApoE</td>
<td>15 (3.5)</td>
<td>11 (4.2)</td>
<td>0.8</td>
<td>0.13</td>
</tr>
<tr>
<td>IPI00021858</td>
<td>ApoC-II</td>
<td>15 (5.5)</td>
<td>11 (4.2)</td>
<td>1.08</td>
<td>0.43</td>
</tr>
</tbody>
</table>

*Protein identification numbers are from the International Protein Index database, version 3.01.
†Numbers shown are total number of peptides identified (SD) for spectral counting.
‡Average ratios were calculated from the individual ratio for each protein for each subject at baseline and after 1 year of niacin/statin therapy.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscipt as written.
Figure 1. Niacin/statin treatment remolds the HDL₃ proteome in 6 subjects with CAD. HDL₃ was isolated from 6 CAD subjects before and after 1 year of therapy with sustained-release niacin and atorvastatin and tryptic digests of HDL₃ analyzed by LC-MS/MS. Proteins identified as differentially expressed before (solid bars) and during (open bars) treatment by spectral counting were quantified by extracted ion chromatograms using the area under the curve of the chromatograms. Average areas under the curve for 2 representative peptides from each protein are shown (left), along with a sample chromatograph for a peptide from each protein (right). The peptides analyzed were as follows: PLTP, AGA (AGALQQLLVGDK<sup>3</sup>); and SVV (SSVDELVGIDYSLM<sup>3</sup>) (A and B); apoF, SGV (GGVQQLQYYQDQK<sup>3</sup>), and SYD (SYDLDPAGAEL<sup>3</sup>) (C and D); apoJ, LFD (LFDSDPITTVPVEVSR<sup>3</sup>), and VTT (VTTVASHTSDSDPSGVTEVVK<sup>3</sup>) (E and F); and apoE, AAT (AATVGSLAGQPLQER<sup>2</sup>), and GEV (GEVQAMLQSTEELRVR<sup>3</sup>) (G and H). The effect of treatment on protein levels was determined using all peptides quantified for each protein by repeated-measures 2-way ANOVA. P<0.05 for all proteins other than apoA-I (P=0.44) and apoC-II (P=0.06). Shown are mean±SEM.
The lower number of proteins identified in this analysis compared with a previous analysis likely reflects differences in chromatography, data acquisition, and mass spectrometers used for the studies. In addition, we identified kininogen and vitamin D-binding protein, previously found in HDL but not specifically in the HDL₃ subfraction.

Impact of Combination Therapy on the Proteome of HDL₃ Isolated From CAD Subjects

Spectral counting identified apoE, apoF, and PLTP as differentially expressed (Table 2) in HDL₃ isolated from 6 recently diagnosed CAD patients at baseline and after 1 year of treatment (Table 1, group 1). ApoE levels decreased, whereas apoF and PLTP levels increased. Two additional proteins, apoC-II and apoJ (P = 0.13 and P = 0.12, respectively), had borderline significant differences. Spectral counting did not detect a significant change in apoA-I levels (P = 0.49). It is important to note that proteins with low spectral counts exhibit greater relative variability. Consistent with this proposal, we found an average coefficient of variation of 3% for apoA-I, 4% to 6% for apoC-II and apoE, and 9% to 12% for apoF, apoJ, and PLTP in our analyses.

We used extracted ion chromatograms to confirm changes in relative abundance of HDL₃ proteins in CAD subjects on combination therapy. ApoF, PLTP, and apoJ levels in HDL₃ increased by 85%, 247%, and 198%, respectively, as a result of therapy (Figure 1A through 1F and online-only Data Supplement Table II; F = 42.6, 7.2, 17.2, respectively; P = 0.001, 0.02, 0.009, respectively). In contrast, levels of apoE in HDL₃ were significantly reduced an average of 41% after niacin/statin treatment (Figure 1G and 1H and supplementary Table 2; F = 11.35, P = 0.02) for all 7 apoE peptides. Although levels of 3 peptides from apoC-II decreased by an average of 38%, the change was of borderline significance (F = 5.96, P = 0.059; online-only Data Supplement Table II).

Because the combined statin/niacin therapy increased HDL levels, we were interested in whether this change also involved changes in apoA-I levels of HDL₃. Five peptides from apoA-I were monitored (supplementary Table II) and increased with niacin/statin therapy by an average of 26%; however, this change was not statistically significant (F = 0.71, P = 0.44). As an additional control, we analyzed peptides from 3 additional proteins with an abundance that did not change by spectral counting: apoA-II, apoC-III, and albumin. None of these proteins showed significant changes in peak area (F = 0.37, 0.48, and 0.073, respectively; P = 0.57, 0.52, and 0.80, respectively; online-only Data Supplement Table II).

The Protein Composition of HDL From Subjects on Combination Therapy Resembles That of Control Subjects

To determine whether combination therapy might reverse CAD-associated changes in HDL₃ protein composition, we analyzed HDL₃ from apparently healthy male subjects (n = 6) who were age matched with the CAD subjects. As assessed by extracted ion chromatograms, mean levels of apoE and apoC-II were 60% and 51% lower, respectively, in HDL₃ of control subjects than in CAD subjects (Figure 2). In contrast, levels of apoJ, apoF, and PLTP were significantly higher in healthy control HDL₃ (by 81%, 45%, and 76%, respectively). The 2 groups had comparable levels of apoA-I. Taken together, these observations indicate that statin/niacin combination therapy remodels the HDL₃ proteome, resulting in a protein composition that resembles that of apparently healthy subjects.

Biochemical and Mass Spectrometric Analyses of ApoE Levels in HDL

Model system studies indicate that mass spectral peak intensities correlate well with independent measures of protein concentration in biological materials. To validate the utility of our approach, we compared the extracted ion chromatogram peak area for 7 different apoE peptides in HDL₃ with apoE as quantified by nephelometry (Figure 3). The MS and immunochemical analyses were performed on 2 independent HDL₃ preparations. The 7 peptides showed significant correlations with nephelometric values (r² < 0.01 for 6 of the 7 peptides), with an overall correlation coefficient of r² = 0.62 (SD, 0.17). Moreover, the levels of apoC-II in HDL₃ determined biochemically correlated with those of 2 apoC-II peptides as quantified by MS (r² = 0.48 and 0.55). These observations provide strong evidence that extracted ion chromatograms afford quantitative assessments of protein abundance in HDL₃.

Biochemical Validation That Statin/Niacin Treatment Alters ApoE Levels of HDL₃ in an Independent Group of Subjects

To extend and confirm our observations biochemically, we used an antibody-based approach to quantify apoE in HDL₃ isolated from a second set of subjects: 18 men with established CAD (age, 54 years; SD, 7 years) before and during niacin/statin therapy. As expected, combination therapy significantly lowered LDL cholesterol and triglyceride levels and elevated HDL cholesterol levels (Table 1, group 2). Niacin/statin treatment lowered HDL₃-associated apoE in 15 of 19 subjects (P = 0.02), with an average reduction of 17 μg apoE per 1 mg HDL₃ protein (Figure 4A). Importantly, changes in the protein composition of HDL₃ isolated from subjects before and during therapy, as assessed by MS and biochemical assays, were similar, supporting the proposal that extracted ion chromatograms quantitatively assess relative protein abundance in biological material. Thus, niacin/statin therapy reduced apoE levels in HDL₃ of male subjects with established CAD using 3 independent methods.

We next examined the relationship between the levels of apoE in HDL₃ and plasma. Combination therapy reduced apoE levels in plasma (Figure 4B) and HDL₃ (Figure 4A). Plasma levels of apoE before therapy correlated negatively with HDL₃ levels (Figure 4C; n = 13; r² = 0.45, P = 0.01), and treatment-related decreases in plasma apoE did not correlate significantly with decreases in HDL₃-associated apoE (Figure 4D; r² = 0.23, P = 0.07). These data suggest that the alterations of HDL₃ apoE induced by lipid-lowering therapy were not directly linked to changes in plasma levels of apoE.
Discussion

We used MS to test the hypothesis that aggressive lipid therapy with atorvastatin and niacin modifies the HDL proteome in humans with established CAD. To quantify changes in protein abundance, we used 2 complementary label-free quantification methods, spectral counting and extracted ion chromatograms, which are well suited for analyzing HDL obtained in clinical studies. Spectral counting is based on the observation that tryptic peptides derived from proteins that are more abundant in a sample have a higher probability of being identified during data-dependent MS/MS scanning.34–36,38 For extracted ion chromatograms, the ion current for a given peptide and charge state are extracted from the full-scan mass spectrum and used to construct a chromatogram.39 The area under the curve then provides a quantitative measure of relative peptide abundance.

We used spectral counting as an initial screen to identify proteins that appeared to be differentially expressed and then used extracted ion chromatograms to quantify the relative

![Figure 2. The HDL3 proteomes of healthy subjects and subjects with CAD differ. The peptides listed in online-only Data Supplement Table II were quantified by extracted ion chromatogram using areas under the curve of the chromatograms from HDL3 isolated from 6 healthy subjects and 6 CAD subjects. The average normalized change for all peptides examined for each protein is shown. Values are mean±SEM. *P<0.01 for all proteins other than apoF (P=0.08) and apoA-I (P=0.39).]
abundance of these proteins in HDL₃ isolated from CAD subjects before and during treatment. This approach offers 2 important advantages. First, extracted ion chromatograms estimate protein ratios more accurately than spectral counting.35 Second, it is possible to compare the extracted ion chromatogram ratios of multiple peptides detected from the same protein, which should increase confidence in the results.

Spectral counting identified 3 HDL₃ proteins with a relative abundance that appeared to change as a result of treatment: apoE, apoF, and PLTP. Levels of apoE fell, whereas levels of apoF and PLTP rose. Spectral counting also detected trends toward lower apoC-II and higher apoJ levels with borderline statistical significance. The extracted ion chromatogram results confirmed that therapy with atorvastatin and niacin significantly lowered apoE and increased apoJ, apoF, and PLTP levels in HDL₃ isolated from CAD subjects. We previously showed biochemically and by MS that apoE levels are elevated in HDL₃ isolated from CAD subjects, indicating that therapy reverses this change in the HDL₃ proteome. Although elevation of plasma apoA-I levels is a well-established effect of niacin, no significant change was found in the apoA-I content of HDL₃, which is consistent with the proposal that niacin increases the number of HDL particles but not the amount of apoA-I per particle. It is also possible that niacin increases apoA-I levels in HDL species distinct from HDL₃.

We used 2 approaches to confirm that our MS techniques quantify changes in the HDL proteome. First, we observed a strong linear correlation between apoE levels \( r^2 = 0.62 \) assessed by extracted ion chromatograms and nephelometry when we analyzed HDL₃ isolated from a different set of 13 subjects. Second, we used a biochemical approach to confirm that combination therapy with niacin and statin reduced levels of apoE in HDL₃ in an independent group of subjects. The validity of our approach for assessing global changes in the protein composition of HDL is further supported by reports of decreased apoE levels in both plasma and HDL during atorvastatin treatment of hypertriglyceridemic subjects or hypertriglyceridemic subjects with type 2 diabetes mellitus.

Our analysis detected 27 HDL₃-associated proteins. We previously identified 25 of these in HDL₃ and 7 additional proteins.20 The increased protein coverage of our earlier study likely reflects significant differences in the conditions used to separate peptides, which centered on 2-dimensional LC. This approach increases peptide separation and improves the

**Figure 3.** ApoE levels measured by nephelometry correlate with apoE levels quantified by extracted ion chromatogram (XIC). The peptides used for the XICs were AAT (AATVGSLAGQPLQER₂, \( m/z \) [M+2H]² 749.405) (A) and LKS (LKSWFEPLVEDMQR₃, \( m/z \) [M+3H]³ 593.303) (B). Nephelometry and MS were performed on 2 different preparations of HDL₃ from the same plasma sample. The solid line represents the fit; dashed lines, 95% confidence intervals in linear regression analysis.

**Figure 4.** Niacin/statin treatment reduces apoE levels in HDL₃. ApoE levels from 18 subjects (Table 1, group 2) were measured by nephelometry in HDL₃ (A) and plasma (B) before and after 1 year of combined niacin/statin therapy. Shown is the correlation between baseline (C) and changes with niacin/statin therapy (D) in apoE between plasma and HDL₃. Values are mean±SEM. *P=0.02.
detection of low-abundance peptides by minimizing interference from more abundant peptides. Consistent with this proposal, the proteins we did not detect in the present studies were of low abundance.\textsuperscript{20} Gel electrophoresis with proteomic analysis also has been used to elucidate the HDL proteome. This approach identified 12 proteins associated with HDL\textsubscript{3}.\textsuperscript{19} and 14 proteins with total HDL\textsubscript{3},\textsuperscript{21} all of which we have identified in both our previous\textsuperscript{20} and present (supplementary Table II) analyses of HDL\textsubscript{3}. Collectively, these results suggest that LC-MS/MS methods are more sensitive than gel-based proteomic methods for detecting HDL-associated proteins.

Changes in lipid metabolism or in the lipid composition of HDL are likely to contribute to the alterations in the HDL proteome induced by statin/niacin therapy. For example, both apoE and apoC-II are exchangeable apolipoproteins. It is therefore possible that therapy with niacin and atorvastatin might lower their levels in HDL\textsubscript{3}, by redistributing them to other lipoproteins. It is of interest that the reduction of apoE in HDL previously reported with atorvastatin treatment in humans was associated with an increased level of apoE in very-low-density lipoprotein particles.\textsuperscript{39,40} Interestingly, apoE-rich HDL binds with high affinity to the LDL receptor.\textsuperscript{41} Combined statin/niacin therapy may help determine the protein composition of total HDL by upregulating LDL receptors in the liver, thereby promoting the removal of apoE-rich HDL.

Our observations also indicate that combined statin/niacin therapy increases levels of apoA, apoF, and PLTP in HDL\textsubscript{3}. It is noteworthy that those levels were lower in CAD subjects than in control subjects. Interestingly, both apoA and PLTP can contribute to reverse cholesterol transport by macrophages.\textsuperscript{42,43} ApoF, also known as lipid transfer inhibitor protein because it inhibits cholesterol ester transfer protein, has been proposed to cause redistribution of cholesterol between HDL and LDL.\textsuperscript{44} Increased apoF levels could plausibly contribute to treatment-associated increases in plasma HDL-C. A limitation of our study is the lack of statin-only and niacin-only groups. Taken together, these observations indicate that it will be important to extend our observations to monotherapy and suggest that alterations produced in the HDL proteome with combined therapy may have functional significance.

Conclusions

We found that combined niacin and statin treatment remodelled the HDL\textsubscript{3} proteome in subjects with established CAD, yielding a protein composition that resembled that in apparently healthy age- and sex-matched control subjects. Our observations indicate that MS is a useful tool for assessing global changes in HDL protein composition with pharmacotherapy. This demonstration raises the possibility that quantifying the HDL proteome could provide insights into the efficacy of lipid therapy and help identify agents with cardioprotective actions.

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Disclosures

Drs Pennathur and Heinecke are on the speakers’ bureau at Merck. Dr Heinecke consults for Merck, Novartis, and Insilicos. Dr Knopp has grant support from Merck and AstraZeneca, is on the speakers’ bureau at Abbott and AstraZeneca, and consults for Abbott. The other authors report no conflicts.

References

Intense interest exists in high-density lipoprotein (HDL) as a target for therapies designed to prevent coronary artery disease (CAD). Although most studies of HDL have focused on HDL cholesterol levels, recent work has indicated that HDL carries a wide range of previously unsuspected components. These include families of proteins implicated in inhibiting proteolysis or activating the complement system, and both factors are thought to be involved in the pathogenesis of CAD. Moreover, control and CAD subjects appear to carry different protein cargos on their HDL. In the present studies, we used mass spectrometry to examine the protein composition of HDL isolated from subjects with established CAD before and during treatment with extended-release niacin and atorvastatin, which elevate HDL cholesterol and reduce the risk of CAD. Our observations indicate that this combination therapy remodels the HDL proteome so that it resembles the proteome of apparently healthy age- and sex-matched control subjects. This finding raises the possibility that quantifying the HDL proteome of CAD patients could provide insights into the therapeutic efficacy of antiatherosclerotic interventions.
Combined Statin and Niacin Therapy Remodels the High-Density Lipoprotein Proteome

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