Low Clusterin Levels in High-Density Lipoprotein Associate With Insulin Resistance, Obesity, and Dyslipoproteinemia

Andrew N. Hoofnagle, Mingyuan Wu, Albina K. Gosmanova, Jessica O. Becker, Ellen M. Wijsman, John D. Brunzell, Steven E. Kahn, Robert H. Knopp, Timothy J. Lyons, Jay W. Heinecke

Objective—To determine whether obesity and insulin resistance associate with changes in the protein content of high-density lipoprotein (HDL) in 2 different groups of men by using targeted proteomics.

Methods and Results—Insulin resistance and obesity are hallmarks of type 2 diabetes mellitus and the metabolic syndrome, which confer an increased risk of cardiovascular disease. Recent studies suggest that the protein cargo of HDL makes important contributions to the lipoprotein’s cardioprotective effects. In a discovery study, we used isotope dilution mass spectrometry to quantify the relative concentrations of 5 proteins previously implicated in HDL’s cardioprotective effects in 3 groups of healthy subjects: lean insulin-sensitive, lean insulin-resistant, and obese insulin-resistant individuals. We validated our findings in a different group of subjects. The clusterin concentration in HDL strongly and negatively associated with insulin resistance and body mass index in both populations. HDL clusterin levels were lower in subjects with low HDL and high triglycerides, key components of the metabolic syndrome. There was an inverse correlation between clusterin levels in HDL and very-low-density lipoprotein/low-density lipoprotein.

Conclusion—Clusterin levels in HDL are lower in men with reduced insulin sensitivity, higher body mass index, and an unfavorable lipid profile. Our observations raise the possibility that clusterin depletion contributes to the loss of HDL’s cardioprotective properties. (Arterioscler Thromb Vasc Biol. 2010;30:2528-2534.)

Key Words: high-density lipoprotein ▪ atherosclerosis ▪ apolipoprotein J ▪ clusterin ▪ intra-abdominal fat ▪ insulin resistance ▪ obesity

Many environmental and genetic factors contribute to the metabolic syndrome, whose hallmark is reduced insulin sensitivity accompanied by hypertension, hypertriglyceridemia, and obesity.1,2 Obesity and insulin resistance are also components of type 2 diabetes mellitus.3,4 These factors, in turn, contribute to the initiation and progression of atherosclerotic cardiovascular disease (CVD).3,4 Another cardinal feature of metabolic syndrome is a low level of high-density lipoprotein (HDL), which strongly associates with an increased risk of atherosclerotic vascular disease.

One important cardioprotective function of HDL is to remove cholesterol from cholesteryl ester–laden macrophages in the artery wall.5–7 The anti-inflammatory properties of HDL may also contribute to its antiatherogenic effects.8,9 HDL’s cardioprotective ability may depend on the types of particles generated metabolically and on the fact that HDL in humans with established CVD is dysfunctional.8–12 Indeed, animal studies13–15 convincingly demonstrate that changes in HDL’s protein composition can promote atherosclerosis, even when plasma levels of HDL cholesterol (HDL-C) are normal or elevated.

To explore the potential clinical relevance of protein composition in HDL’s cardioprotective effects, we used targeted proteomics to determine the relationship between the relative concentration of 5 different proteins in HDL and 2 key features of metabolic syndrome: obesity and insulin resistance. We quantified proteins that have previously been implicated in HDL’s cardioprotective effects: apolipoprotein (apo) A-I,5,6,7 clusterin,5,16–20 apoE,21 apoM,22,23 and complement component C3.20,24–26 Our observations indicate strong positive correlations between clusterin levels in HDL and insulin sensitivity and strong negative correlations with increased obesity and dyslipoproteinemia. In contrast, levels of the other proteins were not associated with these features of metabolic syndrome. These results raise the possibility that clusterin depletion indicates the presence of dysfunctional HDL.

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Methods

Study Populations
All studies were approved by the institutional review boards of the University of Washington, Seattle, and the University of Oklahoma Health Sciences Center, Oklahoma City, and subjects gave written informed consent. The discovery study (University of Washington) involved 30 healthy nonsmoking men with no history of atherosclerosis. Subjects were distributed into 3 groups: lean insulin-sensitive, lean insulin-resistant, and obese insulin-resistant individuals (n=10 per group).27 Body habitus was based on body mass index (BMI) (lean subject, BMI <27.5 kg/m²).28 Insulin sensitivity was quantified by the sensitivity index (SI), using the frequently sampled tolbutamide-modified intravenous glucose tolerance test (insulin-sensitive subject, SI = 4.2×10⁻⁴ min⁻¹ per μU/mL).27 Intraperitoneal and subcutaneous fat areas were determined by computed tomography.29 The validation study (University of Oklahoma) involved 23 healthy men age matched to 16 men with type 2 diabetes; 6 (15%) of the 39 subjects were smokers at the time of the study. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as previously described (fasting glucose [mmol/L]/fasting insulin [μU/mL]/22.5).30 Additional details on the 2 populations of subjects, including how subjects were diagnosed as having metabolic syndrome, are provided in the supplemental data (available online at http://atvb.ahajournals.org).

Lipoprotein Isolation
HDL (d=1.063 to 1.210 g/mL) and very-low-density lipoprotein (VLDL)/LDL (d<1.063 g/mL) particles were isolated by sequential density gradient ultracentrifugation from EDTA-anticoagulated plasma collected after overnight fasting.20 Plasma was brought to a density of 1.21 g/mL with solid potassium bromide, loaded into an ultracentrifuge tube, and gently overlaid with normal saline adjusted with potassium bromide to a density of 1.21 g/mL. After ultracentrifugation at 146 000 g for 41 hours, lipoproteins in the top fourth of the tube were collected; the density of the solution was adjusted to 1.21 g/mL and the samples were centrifuged at 146 000 g for 16 hours. HDL from the bottom two-thirds and VLDL/LDL from the top one-third of each tube were iteratively concentrated and diluted 7 times with 20-mmol/L potassium phosphate and 0.1-mmol/L diethylenetriamine pentacetic acid, pH 7, using a 50-kDa centrifuge filter. Protein concentrations were determined by the Bradford assay. Lipoproteins were stored at −80°C until analysis.

Liquid Chromatographic–Tandem Mass Spectrometric Quantification of Proteins in Lipoproteins
Complete details are provided in the supplemental data. Briefly, tryptic digests of reduced, alkylated HDL, or VLDL/LDL (10 μg protein) were supplemented with isotope-labeled internal standard peptides for 5 proteins (supplemental Table I). This approach controls for sample-specific suppression of ionization and variability in mass spectrometer performance.31 Peptides were desalted on a C18 trapping column and subsequently eluted and separated with a C18 analytic column using a linear gradient of acetonitrile. Peptides were detected by selected reaction monitoring on a triple quadrupole tandem mass spectrometer. Peptides were quantified by isotope dilution, using the ratio of endogenous peptide ion current to the corresponding internal standard peptide ion current. Relative concentrations for each protein were normalized across each population (discovery or validation) such that a relative protein concentration >1 indicates that the protein is enriched compared with the average of the population; a relative protein concentration <1 indicates that the protein is depleted. The method was validated by comparing liquid chromatographic–tandem mass spectrometric quantification of apoA-I with a nephelometric assay (supplemental Figure I). Absolute concentrations of clusterin in HDL were not calculated because a suitable matrix-matched calibrator was not available.

Statistical Analyses
BMI, triglycerides, intra-abdominal fat, subcutaneous fat, and insulin sensitivity (SI and HOMA-IR) were modeled using log-transformed data, based on the log-normal distribution of the larger population (n=186 to 275 observations for each variable) from whom the subjects of the discovery population were drawn.27 Two HDL samples in the discovery study with very-low apoA-I levels were excluded from the analysis. The means of groups were compared using 1-way ANOVA or the Student t test. For log-normal data, medians were compared using Kruskal-Wallis or Wilcoxon rank sum tests. All tests of significance were 2-sided.

Results
We used a 2-tiered strategy to determine whether the relative concentration of 5 important proteins in HDL associated with features of the metabolic syndrome and other risk factors for CVD. First, in the discovery study, we used isotope dilution mass spectrometry with selected reaction monitoring to measure the concentrations of the 5 proteins in HDL isolated from healthy subjects enrolled in a metabolic study (n=30). We validated our observations in a different group of subjects recruited from an outpatient medical clinic (n=39).

Characteristics of the Discovery Group Subjects
For the discovery study, we isolated HDL from 3 groups of age-matched healthy men: lean insulin-sensitive, lean insulin-resistant, and obese insulin-resistant men (n=10 per group; Table 1).27–29 None of the subjects were using lipid-lowering therapy46 or had diabetes. Insulin sensitivity was determined from the intravenous glucose tolerance test that was used to categorize the subjects in the original metabolic study. As expected, other measures of insulin sensitivity (HOMA-IR) and obesity (waist:hip ratio, intra-abdominal fat, and subcutaneous fat) were statistically different among the 3 groups. Plasma HDL-C levels were slightly, but not significantly, lower and plasma triglyceride levels were higher in the insulin-resistant groups. Mean arterial pressure was not different between the groups.

Clusterin Levels in HDL Correlate With Features of the Metabolic Syndrome
HDL was proteolytically digested, and the resulting peptides were analyzed by tandem mass spectrometry. The relative amounts of 5 HDL proteins (per total HDL protein) were quantified by isotope dilution, using 2 synthetic internal standard peptides. By ANOVA, the amount of clusterin in HDL differed significantly among the 3 groups of subjects (P=0.014; Table 1). However, the amounts of the other 4 proteins measured (apoA-I, apoE, apoM, and complement component C3) were not statistically different between the 3 groups (supplemental Table II).

Next, we determined if the abundance of clusterin in HDL associated with obesity in the discovery population (Table 2). Univariate linear regression revealed that clusterin correlated negatively with BMI (r=−0.50, P<0.01) and with intra-abdominal and subcutaneous fat content, as assessed by computed tomography (r=−0.58 [P<0.01] and r=−0.48 [P=0.01], respectively). The correlation was higher for intra-abdominal fat area, suggesting that this specific depot might influence clusterin levels in HDL.
Table 1. Characteristics of the Discovery Population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Lean Insulin-Sensitive Group (n=10)</th>
<th>Lean Insulin-Resistant Group (n=10)</th>
<th>Obese Insulin-Resistant Group (n=10)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>44.6 (6.7)</td>
<td>46.6 (5.9)</td>
<td>46.9 (5.0)</td>
<td>0.61</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.8 (3.8)</td>
<td>25.4 (1.3)</td>
<td>30.5 (2.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sₐ, ×10⁻⁴ min⁻¹ per μU/mL</td>
<td>6.3 (3.6)</td>
<td>2.8 (1.5)</td>
<td>2.1 (1.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOMA-IR, mmol/L×μU/mL</td>
<td>1.50 (1.06)</td>
<td>1.89 (0.51)</td>
<td>3.17 (1.58)</td>
<td>0.012</td>
</tr>
<tr>
<td>Waist:hip ratio</td>
<td>0.83 (0.06)</td>
<td>0.89 (0.02)</td>
<td>0.96 (0.06)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Intra-abdominal fat area, cm²</td>
<td>56.1 (66.5)</td>
<td>92.9 (30.1)</td>
<td>200.9 (55.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Subcutaneous fat area, cm²</td>
<td>67.0 (71.3)</td>
<td>152.3 (78.3)</td>
<td>257.3 (137.2)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Cholesterol, mmol/L*

| Total                           | 4.32 (1.00)                         | 5.14 (0.77)                         | 4.93 (1.31)                         | 0.26    |
| HDL                             | 1.16 (0.18)                         | 1.03 (0.17)                         | 0.98 (0.16)                         | 0.076   |
| LDL                             | 2.75 (0.78)                         | 3.41 (0.70)                         | 3.13 (0.96)                         | 0.25    |
| Triglycerides, mmol/L†          | 0.77 (0.29)                         | 1.30 (1.15)                         | 1.16 (0.91)                         | 0.009   |
| Arterial blood pressure, mm Hg* | 86.9 (6.4)                          | 87.4 (8.0)                          | 93.9 (6.4)                          | 0.067   |
| HDL clusterin, arbitrary units*| 1.18 (0.20)                         | 0.97 (0.22)                         | 0.85 (0.27)                         | 0.014   |

*Data are given as mean±SD. Significance was assessed by ANOVA.
†Data are given as median (25th–75th interquartile range). Significance was assessed by the Kruskal-Wallis test.
‡Data are given as mean±SD. Significance was assessed by Student t test.

HDL clusterin also correlated with insulin sensitivity, as assessed by both the SI and HOMA-IR. The direction of change (and units) associated with insulin sensitivity, as assessed by the SI and HOMA-IR, are different; thus, increased insulin sensitivity increases the SI but decreases the HOMA-IR. For both measures of insulin sensitivity, the correlation with HDL clusterin was significant (r=0.52 [P=0.004] for SI and r=−0.54 [P<0.01] for HOMA-IR; Table 2).

More important, none of the other 4 quantified proteins, including apoA-I, correlated with obesity or insulin resistance (supplemental Table III).

Characteristics of the Validation Group Subjects

To validate the correlations between HDL clusterin concentration and BMI and insulin sensitivity, we analyzed a second population of apparently healthy controls (n=23) and type 2 diabetic men (n=16) who lived in a different city. We selected these subjects because the samples were collected and stored at a different institution, thus eliminating the possibility that factors related to sample collection at the first site could explain our observations. All diabetic subjects were receiving oral hypoglycemic agents (1 subject was also receiving insulin therapy ). More important, none of the subjects was receiving lipid-lowering therapy.16 The BMI of the validation group was significantly greater than that of the discovery group (median, 30.4 and 26.9, respectively; P=0.015). Within the validation population, the diabetic subjects were more obese and less insulin sensitive and had higher hemoglobin A₁c levels than healthy subjects (Table 3).

Table 2. Correlation of Subject Characteristics With HDL Clusterin Levels in the Discovery Population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Correlation With HDL Clusterin by LC-MS/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>−0.31</td>
</tr>
<tr>
<td>BMI∗</td>
<td>−0.50†</td>
</tr>
<tr>
<td>Sₐ∗</td>
<td>0.52†</td>
</tr>
<tr>
<td>HOMA-IR∗</td>
<td>−0.54†</td>
</tr>
<tr>
<td>Waist:hip ratio</td>
<td>−0.56†</td>
</tr>
<tr>
<td>Intra-abdominal fat area∗</td>
<td>−0.58†</td>
</tr>
<tr>
<td>Subcutaneous fat area∗</td>
<td>−0.48†</td>
</tr>
</tbody>
</table>

LC indicates liquid chromatography; MS, mass spectrometry.
*For these characteristics, logₑ-transformed data were used.
†P<0.01.

Table 3. Characteristics of the Validation Population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy Subjects</th>
<th>Diabetic Subjects</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>45.8±13.0</td>
<td>49.8±9.2</td>
<td>0.28</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.4±8.0</td>
<td>32.1±9.3</td>
<td>0.004</td>
</tr>
<tr>
<td>HOMA-IR, mmol/L×μU/mL†</td>
<td>1.02±1.38</td>
<td>2.77±4.40</td>
<td>0.037</td>
</tr>
<tr>
<td>Hemoglobin A₁c, %</td>
<td>5.2±0.3</td>
<td>7.5±1.5</td>
<td>0.008</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.65±0.96</td>
<td>5.12±0.71</td>
<td>0.09</td>
</tr>
<tr>
<td>HDL</td>
<td>1.06±0.26</td>
<td>0.97±0.21</td>
<td>0.28</td>
</tr>
<tr>
<td>LDL</td>
<td>3.03±0.94</td>
<td>3.40±0.64</td>
<td>0.16</td>
</tr>
<tr>
<td>Triglycerides, mmol/L†</td>
<td>0.99±0.65</td>
<td>1.25±1.50</td>
<td>0.23</td>
</tr>
<tr>
<td>Arterial blood pressure, mm Hg*</td>
<td>92.5±21.9</td>
<td>89.5±26.3</td>
<td>0.70</td>
</tr>
</tbody>
</table>

*Data are given as mean±SD. Significance was assessed by Student t test.
†Data are given as median (25th–75th interquartile range). Significance was assessed by Wilcoxon rank sum test.
HDL Clusterin Level Correlates Negatively With BMI and Insulin Resistance in Control and Type 2 Diabetic Subjects

HDL clusterin content was quantified in the validation population by isotope dilution mass spectrometry. Again, clusterin levels in HDL correlated negatively with BMI and reduced insulin sensitivity (Figure 1A and B), and the correlations remained significant after smokers were removed from the analysis (15% of the validation population). In contrast, HDL clusterin did not correlate with mean arterial blood pressure (Figure 1C), another risk factor for CVD and an important component of the metabolic syndrome. The correlations of clusterin with BMI and HOMA-IR were similar in the validation and discovery populations (r=−0.55 and r=−0.50, respectively, for loge-transformed BMI; and r=−0.61 and r=−0.54 for loge-transformed HOMA-IR, respectively); both were significant (P<0.001). More important, the slopes of the linear regression analysis of HDL clusterin and HOMA-IR were also similar, despite the significantly different median BMIs of the validation and discovery populations (standardized coefficients=−0.63 and −0.54, respectively), suggesting that the relationship is valid over a wide range of body sizes (Figure 1A and B).

To further investigate the relationships among BMI, insulin resistance, and the relative concentration of clusterin in HDL, we combined the discovery and validation populations (Figure 1). The correlations and slopes between HDL clusterin and BMI (r=−0.51) or HOMA-IR (r=−0.57) were similar to those for the discovery and validation populations. These observations indicate that in 2 distinct groups of male subjects, clusterin levels in HDL correlate negatively with insulin resistance and BMI.

HDL Clusterin Is Depleted in Subjects With Metabolic Syndrome

By immunoassay, plasma concentrations of clusterin are elevated in subjects with diabetes.32,33 By using isotope dilution mass spectrometry, we also found an increased plasma concentration of clusterin in diabetic subjects, which was statistically significant even after removing 3 outlying data points (supplemental Figure II). However, when quantifying the clusterin in HDL, we observed no significant differences between diabetic and control subjects, although there was a trend toward a lower level in diabetic subjects (Figure 2A; P=0.14).

Because our observations suggested that HDL clusterin levels associate with insulin resistance and obesity, we next determined if subjects with metabolic syndrome had lower HDL clusterin levels. We found that subjects with metabolic syndrome had significantly lower HDL clusterin levels than control subjects (P=0.004; Figure 2B).

HDL Clusterin Is Depleted in Subjects With Dyslipoproteinemia

Because high triglyceride and low HDL-C levels are characteristic features of metabolic syndrome, we investigated the relationships between dyslipoproteinemia and the concentration of clusterin in HDL. When the data from the discovery and validation populations were combined (Figure 2C–E), levels of HDL clusterin were significantly lower in subjects with low levels of HDL-C (<1.03 mmol/L; P<0.001) (Figure 2C), high levels of triglycerides (≥1.69 mmol/L; P<0.01) (Figure 2D), or elevated levels of LDL cholesterol (≥3.33 mmol/L; P=0.015) (Figure 2E).

We used linear regression analysis to examine the overall relationships between HDL clusterin and triglycerides or HDL-C in our combined study population. HDL clusterin concentration strongly correlated with plasma HDL-C (r=0.64, P<0.001; Figure 3A) and negatively correlated with loge-transformed triglycerides (r=−0.59, P<0.001; Figure 3B), LDL-C (r=−0.30, P=0.015; data not shown), and total cholesterol (r=−0.37; P=0.002; data not shown). These observations suggest that plasma lipid composition is an important factor that affects the concentration of clusterin in HDL. More important, HDL triglyceride concentration (per gram of HDL protein) had no relationship with HDL clusterin levels (supplemental Figure III).

HDL Clusterin Is Negatively Associated With VLDL/LDL Clusterin Levels

Although plasma concentrations of clusterin are elevated in subjects with insulin resistance and diabetes,32,33 to our kno-
edge, the relative distribution of clusterin between HDL and the apoB-containing lipoproteins has not been investigated. Therefore, we isolated total VLDL/LDL from plasma of the validation subjects and quantified clusterin by isotope dilution mass spectrometry. In contrast to HDL, clusterin in the non-HDL lipoprotein fraction was directly proportional to BMI ($r=0.61$, $P<0.001$; data not shown) and HOMA-IR ($r=0.46$, $P=0.009$; data not shown). In addition, there was a strong inverse relationship between clusterin in HDL and clusterin in LDL/VLDL ($r=-0.52$, $P=0.003$; Figure 3C).

**Discussion**

Insulin resistance, obesity, and dyslipidemia are hallmarks of the metabolic syndrome and type 2 diabetes, which increase the risk of death from CVD. We demonstrated a strong negative correlation between the concentration of clusterin in HDL and both insulin sensitivity and BMI in 2 different populations of male subjects. HDL clusterin was unrelated to elevated blood pressure, another component of metabolic syndrome; however, there was a significant decrease in HDL clusterin levels in subjects with metabolic syndrome. None of the other 4 HDL proteins that we investigated correlated with obesity or reduced insulin sensitivity. Collectively, our observations indicate that insulin resistance and the metabolic syndrome are associated with low clusterin levels in HDL.

Circulating clusterin is generally believed to be predominantly associated with HDL and is enriched in the dense HDL$_3$ subfraction. Based on peptide peak areas in this

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**Figure 2.** Relative concentration of HDL clusterin in control subjects and in subjects with diabetes mellitus, metabolic syndrome, or dyslipoproteinemia. A, Diabetic subjects were compared with age-matched healthy subjects in the validation population. B, Subjects with metabolic syndrome were compared with healthy subjects in the discovery population, as described in the supplemental data. C through E, Subjects were also categorized by plasma HDL-C (C; normal $\geq 1.03$ mmol/L), plasma triglycerides (D; normal $<1.69$ mmol/L), and plasma LDL cholesterol (E; normal $<3.33$ mmol/L). Results and error bars are mean±SEM. The number of subjects in each group is indicated.

**Figure 3.** Correlations of lipid parameters with relative HDL clusterin concentration. A through C, Linear regression analysis is shown for HDL clusterin and plasma HDL-C (A), plasma triglycerides (B), and clusterin concentration in VLDL/LDL particles (C). Results represent the discovery population (open circles) and validation population (closed circles). Lines represent linear regression analysis of the relationship between HDL clusterin and clinical parameters for the discovery (dashed lines), validation (dotted lines), and combined (solid lines) populations. Pearson correlation coefficients ($r$) and associated probability values are presented for the combined population.
study, approximately 22% of clusterin is present on HDL and approximately 9% is present on LDL/VLDL. Previous studies have found that clusterin levels increase in the serum of patients with diabetes or CVD, leading to the suggestion that levels of clusterin in HDL increase in subjects with an increased risk of vascular disease. However, none of these studies reported the concentration of clusterin in apoB-containing lipoproteins. In contrast, mass spectrometry-based studies suggest that the concentrations of clusterin in HDL₃ are apparently decreased in subjects with established CVD. Moreover, clusterin levels in HDL₃ increase when subjects with newly diagnosed CVD are treated with statin and niacin therapy, raising the possibility that there is interplay between the levels of clusterin in HDL, VLDL, and LDL.

To explore the factors that affect the levels of clusterin in HDL in metabolic syndrome and other disorders, we determined the relationship between levels of clusterin in HDL and triglycerides, total cholesterol, LDL-C, and HDL-C. Clusterin in HDL correlated negatively with total cholesterol, LDL-C, and triglycerides but was positively associated with HDL-C.

Our observations suggest a model in which alterations in lipid metabolism that are secondary to insulin resistance and metabolic syndrome diminish HDL clusterin levels. Thus, we observed an inverse association between clusterin in HDL and clusterin in non-HDL lipoproteins (VLDL and LDL). This finding, combined with the direct correlation between clusterin in apoB-containing lipoproteins and insulin resistance, raises the possibility that insulin resistance might shift proteins from HDL to VLDL and LDL. It is well established that certain lipoprotein-associated proteins can move from one particle type to another, which is related, in part, to the lipid composition of HDL and the apoB-containing lipoproteins. Insulin resistance, obesity, and diabetes alter the lipid content and composition of lipoproteins, raising the possibility that such alterations modulate the distribution of clusterin between different classes of lipoprotein particles. However, there was no association between HDL clusterin levels and HDL triglyceride levels (supplemental Figure III), indicating that another class of lipids or that particular triglycerides with specific fatty acyl side chains might be involved.

Decreased levels of clusterin might contribute to the loss of HDL’s cardioprotective properties in subjects with insulin resistance and metabolic syndrome. For example, synthetic peptides based on clusterin are potently atheroprotective in animal models. Clusterin is also an inhibitor of complement activation, which is implicated in atherogenesis. Recent studies indicate that complement activation plays a key role in promoting tissue injury in a rodent model of myocardial infarction, raising the possibility that clusterin in HDL modulates tissue damage. Indeed, mice deficient in clusterin exhibit increased tissue damage in a model of cerebral ischemia and clusterin prevents an age-related progressive glomerulopathy in mice.

The major strengths of our study include the use of isotope dilution mass spectrometry (an intrinsically precise, quantitative, and multiplex approach that is transferable across laboratories) to investigate 2 diverse clinical populations that included both healthy subjects and subjects with type 2 diabetes. Important limitations include the relatively few subjects, the cross-sectional design, and the focus on male subjects. In future studies, it will be important to extend our observations to larger groups of subjects, women, and younger and older subjects at increased risk of CVD. In summary, we showed that clusterin levels in HDL correlate negatively with obesity and insulin resistance and that they are lower in subjects with metabolic syndrome. Because clusterin is protective in mouse models of tissue injury, and because obesity and insulin resistance are important risk factors for CVD, our results raise the possibility that insufficient clusterin in HDL might impair the lipoprotein’s cardioprotective functions.

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Disclosures
Dr Hoofnagle has been a consultant for Thermo Fisher; and Dr Heinecke is on the speaker’s bureau at Merck/Schering Plough and serves as a consultant for Merck, Corcept, and Insilicos.

References


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Supplement Material

Supplemental Methods

Study populations
Each subject in the discovery study was age-matched to within 4 years to one member in each of
the other two discovery population groups (range 35–55 years). Lean body habitus was defined
as subjects with a BMI < 27.5 kg/m² [this cutoff was used in the original clinical research study
and was based on a healthy Seattle population before the redefinition of obesity based on
NHANES II data]. The insulin sensitivity index (SI; insulin sensitive ≥ 4.2 x 10⁻⁴ min⁻¹ per
µU/mL) was determined using the frequently sampled tolbutamide-modified intravenous-glucose
tolerance test. Fasting insulin, fasting glucose, and lipid measurements (total cholesterol, HDL
cholesterol, triglycerides) were performed as described. LDL cholesterol was estimated using
the Friedwald equation.

In the validation population, fasting insulin, glucose, and lipid levels were available for 32 of the
men, and lipid data and BMI were available for 37. Lipid, glucose, and insulin measurements
were performed on a Beckman Synchron automated analyzer. One subject was receiving insulin
therapy at the time of the study.

In the discovery population, we defined the metabolic syndrome by the presence of at least 3 of 5
CVD risk factors: HDL cholesterol <1.03 mmol/L (<40 mg/dL), triglycerides >1.69 mmol/L
(>150 mg/dL), waist circumference >102 cm, blood pressure >130/>85 mm Hg, and/or fasting
glucose >5.56 mmol/L (>100 mg/dL). In the validation population, waist circumference was
unavailable: therefore, we excluded these individuals from the analysis.

Protein quantification in lipoprotein particles
HDL (10 µg protein) was reduced in 100 µL of buffer B [0.1% Rapigest (Waters), 100 mM
ammonium bicarbonate] supplemented with 5 mM dithiothreitol at 37°C for 1 h with constant
agitation and alkylated with 15 mM iodoacetamide. Alkylated proteins were digested at 37°C
for 2 h with 0.5 µg trypsin and for another 16 h following the addition of 0.5 µg of trypsin.
Rapigest was cleaved with 0.5% TFA, and the peptides were lyophilized and suspended in 5%
acetonitrile, 0.1% formic acid.

Tryptic digests were mixed with an equal volume of buffer C (100 mM ammonium bicarbonate)
containing two isotopically labeled peptides (synthesized with ¹⁵N-labeled and ¹³C-labeled
arginine [R*, ¹³C₆H₁₄O₂¹⁵N₄] and/or lysine [K*, ¹³C₆H₁₄O₂¹⁵N₂]) for each peptide that was
quantified. The peptides used were: clusterin, LFSDSDPITVTPVEVSR and ASSIIDELFQDR;
apoA-I, DLATVYVDVLK and VQPYLDDFQK; apoE, LAVYQAGAR and SELEEQLTPVAEETR; complement C3, TGLQEVK and IPIEDGSVEVLSR, and apoM,
SLTSDLK and DGLCVPR. Peptides were desalted on a C18 trapping column (Dionex,
Acclaim Pepmap100 100Å 5µm, 5 x 1.0 mm i.d.), using a Tempo 1D Plus autosampler-liquid
chromatography system (Applied Biosystems), eluted onto a C18 analytical column (Michrom,
Magic 200Å 5µm, 150 x 0.15mm i.d.), separated with a linear gradient of acetonitrile (5%–40% 
over 8 min), and ionized with a Microionspray II ion source (1 µL/min flow rate). Precursor-
product ion transitions (Supplemental Table I) were monitored simultaneously on multiple

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channels by selected reaction monitoring with an API 4000 QTRAP mass spectrometer (Applied Biosystems).

Peptides were quantified by selected reaction monitoring using the ratio of endogenous peptide ion current to the corresponding internal standard peptide ion current. Each peptide ratio was normalized to the mean ratio of that peptide across all analyses. Relative protein abundance of each protein was determined by dividing the mean ratio of the two protein-specific peptides by the mean ratio for all ten peptides in each sample. With this approach, a sample in which a protein has a value >1 is enriched in that protein relative to the population average, while values <1 represent depletion.

**Quantification of apoA-I in HDL**

The validity of the liquid chromatography-tandem mass spectrometric approach to the quantification of proteins in HDL was demonstrated using a clinical nephelometric immunoassay (Siemens, BN-II; Deerfield, IL) adapted for the quantification of apoA-I in isolated HDL. Briefly, HDL samples (20 µL) were diluted 1:10 with dilution buffer (Siemens) and the nephelometric signal was calibrated using the regular, clinical calibration curve (Siemens; serum-based standards). The apoA-I concentration was divided by the protein concentration of the isolated HDL as measured by the Bradford assay (Pierce). The results are shown in Supplemental Figure I. For many samples, the concentration of apoA-I is inappropriately higher than 1,000 µg/mg protein. This is likely due to the calibration of the nephelometric and Bradford assays with non-analyte-specific and/or non-matrix-matched calibrators.

**Clusterin concentration in plasma**

Plasma clusterin concentration was determined by isotope dilution mass spectrometry with an external calibration curve. Calibrators were prepared in fetal bovine serum (protein concentration ~60 mg/mL) at 30, 75, 125, and 200 µg/mL clusterin (Prospec). To make trypsin digestion consistent across samples and calibrators, we diluted samples and calibrators into the same fetal bovine serum background matrix (2 µL sample/calibrator diluted with 6 µL fetal bovine serum). Each sample (1 µl of sample:FBS dilution) was reduced at 37°C with constant agitation with 5 mM DTT, 0.2% Rapigest, 100 mM ammonium bicarbonate in a volume of 25 µL. Proteins were alkylated with 15 mM iodoacetamide for 2 h in the dark. Proteins were then digested with 0.125 µg trypsin for 2 h at 37°C with constant agitation. The trypsin digestion was repeated (final protein:trypsin ratio, 240:1) and the Rapigest was cleaved with 0.5% trifluoroacetic acid at 37°C for 45 min. After lyophilization the samples were reconstituted in 7.5 µL 5% acetonitrile/0.1% FA in water for 3 h with constant agitation. Samples were mixed with 7.5 µL of the internal standard peptide mix and 3 µL were injected onto the LC-MS/MS system in duplicate. To calculate the absolute concentration of clusterin in plasma, the ratio of the endogenous peptide peak area to the internal standard peptide peak area for each sample was compared with the same ratio for the calibration curve.

**Supplemental References**


Supplemental Table I. Peptides and precursor-product ion transitions. †

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide</th>
<th>Precursor</th>
<th>Product 1</th>
<th>Product 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clusterin</td>
<td>LFDSDPITVTVPEVSR</td>
<td>938.1</td>
<td>686.8 (y6)</td>
<td>578.6 (b5)</td>
</tr>
<tr>
<td></td>
<td>ASSIIDELFQDR</td>
<td>697.8</td>
<td>923.0 (y7)</td>
<td>1036.1 (y8)</td>
</tr>
<tr>
<td></td>
<td>DLATVYVVDVLK</td>
<td>618.7</td>
<td>744.9 (b7-H2O)</td>
<td>937.1 (y8)</td>
</tr>
<tr>
<td>ApoA-I</td>
<td>VQPYLDDFQK</td>
<td>627.2</td>
<td>422.5 (y3)</td>
<td>1025.49 (y8)</td>
</tr>
<tr>
<td></td>
<td>LAVYQAGAR</td>
<td>475.1</td>
<td>665.7 (y6)</td>
<td>774.9 (b8)</td>
</tr>
<tr>
<td>ApoE</td>
<td>SELEEQLTPVAEETR</td>
<td>866.4</td>
<td>913.0 (b8-H2O)</td>
<td>811.9 (b7-H2O)</td>
</tr>
<tr>
<td></td>
<td>SLTSC*LDSK</td>
<td>506.1</td>
<td>709.7 (y6)</td>
<td>810.9 (y7)</td>
</tr>
<tr>
<td>ApoM</td>
<td>DGLC*VPR</td>
<td>409.0</td>
<td>371.5 (y3)</td>
<td>531.6 (y4)</td>
</tr>
<tr>
<td></td>
<td>TGLQEVEVK</td>
<td>502.1</td>
<td>731.8 (y6)</td>
<td>611.7 (b6-NH3)</td>
</tr>
<tr>
<td>Complement C3</td>
<td>IPIEDGSGEVVLSR</td>
<td>736.3</td>
<td>1261.4 (y12)</td>
<td>-</td>
</tr>
</tbody>
</table>

† Peptides analyzed in the clusterin assay. Mass-to-charge ratios (m/z) are shown for unlabeled precursor peptide and product ions monitored by tandem mass spectrometry. C* Reduction and alkylation of cysteine residues increases peptide mass by 57 Da.
Supplemental Table II. Relative concentrations of five proteins in HDL measured in three groups of healthy males.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Average Peak Area Ratio&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Lean insulin-sensitive (N=9)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Lean insulin-resistant (N=9)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Obese insulin-resistant (N=10)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clusterin</td>
<td>0.11</td>
<td>1.18 (0.20)</td>
<td>0.97 (0.22)</td>
<td>0.85 (0.27)</td>
<td>0.014</td>
</tr>
<tr>
<td>ApoA-I</td>
<td>126</td>
<td>0.99 (0.11)</td>
<td>1.02 (0.18)</td>
<td>1.02 (0.12)</td>
<td>0.88</td>
</tr>
<tr>
<td>ApoE</td>
<td>0.64</td>
<td>0.98 (0.27)</td>
<td>0.87 (0.33)</td>
<td>1.16 (0.24)</td>
<td>0.086</td>
</tr>
<tr>
<td>ApoM</td>
<td>1.7</td>
<td>0.93 (0.15)</td>
<td>1.02 (0.13)</td>
<td>1.07 (0.17)</td>
<td>0.17</td>
</tr>
<tr>
<td>Complement C3</td>
<td>0.03</td>
<td>0.94 (0.20)</td>
<td>1.12 (0.26)</td>
<td>0.95 (0.28)</td>
<td>0.24</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average peak area ratio reports the approximate relative concentrations of each protein in HDL as measured by this method. Identical amounts of isotope labeled peptide were spiked for each peptide and as a result, the molar amount of each protein in HDL relative to one another can be estimated by the average peak area ratio for the population.

<sup>b</sup> To control for the actual amount of protein loaded onto the mass spectrometer, the relative concentrations of each protein were normalized across the whole discovery population and are reported for each group. As a result of normalization, the average concentrations for each group are all near one. For comparison of proteins to one another, see the average peak area ratio.
Supplemental Table III. Correlations of the relative concentrations of five HDL proteins by LC-MS/MS with BMI and insulin sensitivity in the discovery population.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Correlation with log_{e}-transformed BMI</th>
<th>Correlation with log_{e}-transformed SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clusterin</td>
<td>-0.50 *</td>
<td>0.52 *</td>
</tr>
<tr>
<td>ApoA-I</td>
<td>0.01</td>
<td>-0.10</td>
</tr>
<tr>
<td>ApoE</td>
<td>0.26</td>
<td>-0.04</td>
</tr>
<tr>
<td>ApoM</td>
<td>0.28</td>
<td>-0.33</td>
</tr>
<tr>
<td>Complement C3</td>
<td>0.05</td>
<td>-0.18</td>
</tr>
</tbody>
</table>

*p < 0.01
Comparison of liquid chromatography-tandem mass spectrometric and nephelometric quantification of apoA-I concentration in HDL. The relative concentration of apoA-I in HDL was determined as described in the Methods section and compared with the concentration determined by nephelometry as described in the Supplemental methods section. The linear regression line is shown with the Pearson correlation coefficient ($r^2$).
**Plasma clusterin concentrations in normal and diabetic subjects.** Using isotope dilution mass spectrometry, plasma concentrations of clusterin were determined for subjects in the validation population. N=23 for control subjects and N=16 for diabetic subjects (distributions were compared with the Wilcoxon rank sum test).
Supplemental Figure III

Correlation of HDL clusterin levels and loge-transformed triglycerides in HDL. Triglyceride concentrations in HDL were measured (Cayman Chemical) in 16 of the subjects in the validation population, which were normalized to the protein concentration. The linear regression line is shown with the Pearson correlation coefficient (r).