Dialysis Modalities and HDL Composition and Function

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ABSTRACT
Lipid abnormalities may have an effect on clinical outcomes of patients on dialysis. Recent studies have indicated that HDL dysfunction is a hallmark of ESRD. In this study, we compared HDL composition and metrics of HDL functionality in patients undergoing hemodialysis (HD) or peritoneal dialysis (PD) with those in healthy controls. We detected a marked suppression of several metrics of HDL functionality in patients on HD or PD. Compositional analysis revealed that HDL from both dialysis groups shifted toward a more proinflammatory phenotype with profound alterations in the lipid moiety and protein composition. With regard to function, cholesterol efflux and anti-inflammatory and antiapoptotic functions seemed to be more severely suppressed in patients on HD, whereas HDL-associated paraoxonase activity was lowest in patients on PD. Quantification of enzyme activities involved in HDL metabolism suggested that HDL particle maturation and remodeling are altered in patients on HD or PD. In summary, our study provides mechanistic insights into the formation of dysfunctional HDL in patients with ESRD who are on HD or PD.


ESRD is associated with accelerated cardiovascular disease and high mortality. Mortality rates remain above 20% per year with the use of dialysis, with more than one half of the deaths related to cardiovascular disease. The effect of dialysis on metabolism differs between modalities; whereas hemodialysis (HD) is associated with a higher prevalence of hypotension and infectious complications, patients on peritoneal dialysis (PD) face a continuous glucose load. However, the effect of those metabolic changes on HDL functionality remains largely unknown.

In the general population, HDL cholesterol is associated with reduced cardiovascular events. Recent studies provided clear evidence of a lack of association between higher HDL cholesterol levels and lower cardiovascular risk in patients with ESRD. These studies clearly support the concept of dysfunctional HDL formation in CKD and provide additional evidence that the functionality of HDL might be of crucial importance with regard to cardiovascular protection.

HDL itself is a complex particle with numerous potential atheroprotective activities, including reverse cholesterol transport, inhibition of LDL oxidation, cytokine secretion from macrophages, adhesion molecule expression on endothelial cells, and stimulation of endothelial nitric oxide synthase to promote vasodilation. Profound alterations in HDL composition have been reported in patients with ESRD on HD. We and others have provided evidence that uremic HDL is less effective in promoting cholesterol efflux and shows defective antioxidative
and anti-inflammatory capabilities.9–17 The composition of uremic HDL was found to shift toward a proinflammatory phenotype, which was observed to negatively affect cholesterol efflux properties, cytokine production, and adhesion molecule expression on monocytes and endothelial cells.7,9,10,18 Those observations led to the conclusion that specific uremia-induced compositional changes in the lipid and protein moiety render HDL dysfunctional or even proinflammatory. Here, we investigated alterations in HDL composition and metrics of HDL functionality in patients undergoing HD or PD.

RESULTS

Clinical Characteristics of Study Subjects

Patient characteristics were comparable between groups, and all study participants were not patients with diabetes and free of lipid-lowering therapy (Table 1, Supplemental Table 1). The lipid profiles of patients with ESRD displayed increased triglycerides and decreased HDL cholesterol levels, whereas total cholesterol was unaltered in patients on HD and moderately reduced in patients on PD (Table 1). Concomitant treatments were equal between patients on dialysis (Supplemental Table 1). C-reactive protein levels were increased in both dialysis groups and highest in patients on HD (Table 1).

HDL Composition Is Altered in Patients on HD or PD

HDL was isolated from study subjects by one-step density ultracentrifugation and subjected to compositional analysis. HDL from patients on HD or PD showed significantly lower levels of cholesterylester and phospholipids and higher levels of triglycerides (Table 2, lipids). The ratio between phospholipid and sphingomyelin, a marker of surface rigidity, was lower in HD HDL (Table 2, lipids). With regard to protein composition, our analysis indicates that HDL from both dialysis groups showed lower amounts of apoA-I, apoA-II, and paraoxonase 1 (Table 2, proteins). ApoC-II, apoC-III, lipoprotein-associated phospholipase A2 (Lp-PLA2), and serum amyloid A (SAA) levels seem to be most profoundly elevated in HD HDL, whereas moderate increases were observed in PD HDL (Table 2, proteins). Our compositional analysis suggested a shift toward a proinflammatory composition in both dialysis groups, with similar alterations in the lipid moiety and more profound alterations in the protein moiety of HD HDL. Moreover, native gel analysis revealed that HD HDL shifts toward the smaller HDL3 subclass, whereas the subclass distribution was unaltered in patients on PD (Figure 1).

Functional Metrics of HDL from Patients on HD or PD

Major determinants of HDL-mediated reverse cholesterol transport are the capabilities of HDL to mediate cholesterol efflux

Table 1. Clinical characteristics of study subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control</th>
<th>HD</th>
<th>PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>24</td>
<td>14</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>65±7.2</td>
<td>59±16.2</td>
<td>57±17.5</td>
</tr>
<tr>
<td>Men, n (%)</td>
<td>10 (50)</td>
<td>12 (50)</td>
<td>7 (50)</td>
</tr>
<tr>
<td>Dialysis vintage (yr)</td>
<td>—</td>
<td>4.4±4.0</td>
<td>2.7±2.4</td>
</tr>
<tr>
<td>Kt/V</td>
<td>—</td>
<td>1.51±0.26</td>
<td>2.29±0.59</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.9±0.2</td>
<td>7.6±2.2a</td>
<td>8.2±2.9a</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>27±8</td>
<td>99±27b</td>
<td>113±38b</td>
</tr>
<tr>
<td>Unine volume (ml/24 h)</td>
<td>—</td>
<td>503±596</td>
<td>979±658</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>1.08±2.11</td>
<td>10.4±6.5a</td>
<td>4.5±8.9</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>219±33</td>
<td>191±72b</td>
<td>178±38b</td>
</tr>
<tr>
<td>Free cholesterol (mg/dl)</td>
<td>63±9</td>
<td>63±22</td>
<td>56±13</td>
</tr>
<tr>
<td>Cholesterylester (mg/dl)</td>
<td>156±25</td>
<td>129±52b</td>
<td>123±27b</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>120±46</td>
<td>196±13b</td>
<td>170±92</td>
</tr>
<tr>
<td>Phospholipids (mg/dl)</td>
<td>249±30</td>
<td>218±52b</td>
<td>209±32a</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>57±15</td>
<td>38±13a</td>
<td>42±15a</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>139±32</td>
<td>114±55</td>
<td>103±38</td>
</tr>
</tbody>
</table>

Results are given as means±SDs. Kt/V values represent single pool for HD and weekly values for PD.

*Significance from the Kruskal-Wallis with Dunn post hoc test was accepted at the level of 0.01 versus control.

Table 2. HDL composition

<table>
<thead>
<tr>
<th>Composition</th>
<th>Controls</th>
<th>HD</th>
<th>PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC (µg/mg protein)</td>
<td>216±35</td>
<td>192±37</td>
<td>192±39</td>
</tr>
<tr>
<td>CE (µg/mg protein)</td>
<td>164±27</td>
<td>144±24a</td>
<td>144±26a</td>
</tr>
<tr>
<td>TC (µg/mg protein)</td>
<td>52.9±10.1</td>
<td>48.5±15.1</td>
<td>47.9±13.6</td>
</tr>
<tr>
<td>TG (µg/mg protein)</td>
<td>39.4±5.0</td>
<td>65.9±11.8b</td>
<td>69.2±11.5b</td>
</tr>
<tr>
<td>PL (µg/mg protein)</td>
<td>423±58</td>
<td>366±50b</td>
<td>359±51b</td>
</tr>
<tr>
<td>SM (µg/mg protein)</td>
<td>52.2±9.6</td>
<td>52.9±12.2</td>
<td>50.5±13.0</td>
</tr>
<tr>
<td>S1P (mg/mg protein)</td>
<td>249±16</td>
<td>247±44</td>
<td>265±32</td>
</tr>
<tr>
<td>PL/SM ratio</td>
<td>8.5±2.7</td>
<td>7.1±1.2a</td>
<td>7.5±2.0</td>
</tr>
<tr>
<td>Proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-I (µg/mg protein)</td>
<td>560±48</td>
<td>442±34b</td>
<td>461±38b</td>
</tr>
<tr>
<td>ApoA-II (µg/mg protein)</td>
<td>158±21</td>
<td>125±27b</td>
<td>136±30b</td>
</tr>
<tr>
<td>ApoC-II (µg/mg protein)</td>
<td>5.2±2.6</td>
<td>9.3±3.5a</td>
<td>6.5±2.7</td>
</tr>
<tr>
<td>ApoC-III (µg/mg protein)</td>
<td>23.9±8.0</td>
<td>41.2±13.6b</td>
<td>32.7±11.3a</td>
</tr>
<tr>
<td>ApoE (µg/mg protein)</td>
<td>12.3±4.7</td>
<td>13.6±7.1</td>
<td>9.3±5.0</td>
</tr>
<tr>
<td>SAA (µg/mg protein)</td>
<td>6.8±8.4</td>
<td>17.6±12.7b</td>
<td>9.9±11.0</td>
</tr>
<tr>
<td>PON (relative index)</td>
<td>1.00±0.50</td>
<td>0.44±0.29b</td>
<td>0.37±0.22b</td>
</tr>
<tr>
<td>Lp-PLA2 (relative index)</td>
<td>1.00±1.16</td>
<td>1.95±1.83a</td>
<td>1.10±1.02a</td>
</tr>
</tbody>
</table>

Results are given as means±SDs. TC, total cholesterol; CE, cholesterylester; FC, free cholesterol; TG, triglyceride; PL, phospholipid; SM, sphingomyelin; S1P, sphingosine-1-phosphate; PON, paraoxonase.

*Significance from one-way ANOVA and the least significant difference post hoc test was accepted at the level of 0.05 versus control.

**Significance from one-way ANOVA and the least significant difference post hoc test was accepted at the level of 0.01 versus control.
from macrophages and other cells and promote cholesterol delivery to the liver. We observed that HDL from both dialysis groups showed a substantially impaired capability to promote cholesterol efflux from macrophages, with the most profound alterations in patients on HD (Figure 2A). Correlation analysis revealed that compositional variations in HDL of patients on dialysis were associated with impaired cholesterol efflux capacity, with HDL phospholipids being the strongest positive predictor in each dialysis subgroup (Supplemental Table 2). To assess whether HDL lipid delivery toward liver cells is altered in patients on dialysis, we labeled the lipid moiety of HDL with the lipophilic dye 1,1’-Dioctadecyl-3,3,3’-tetramethylindocarbocyanine (DiI) which is a reliable surrogate to determine HDL lipid delivery to cells.19,20 Interestingly, we observed that HDL lipids from all studied groups were taken up equally well by liver cells (Figure 2B).

Other than the important role of HDL in reverse cholesterol transport, HDL has additional properties that are thought to be antiatherogenic. The major proteins of HDL, apoA-I, and apoA-II as well as other proteins, such as paraoxonase, that cotransport with HDL in plasma are well known to have antioxidant properties.21,22 As a consequence, HDL has the ability to inhibit the oxidative modification of LDL in a process that reduces the atherogenicity of these lipoproteins. We assessed the antioxidative activity of HDL particles by measuring the inhibition of free radical-induced oxidation of dihydrorhodamine (DHR),23 a dye that fluorescence highly increases on oxidation. We observed that HDL from patients on HD or PD was less potent in inhibiting this process (Figure 3A). HDL also possesses anti-inflammatory properties by its ability to inhibit the activation of monocytes, thereby reducing the recruitment of blood monocytes into the artery wall. To assess the anti-inflammatory capability of HDL, we used a human monocyte cell line with a reporter cassette for the NF-κB, which induces green fluorescent protein (GFP) expression on NF-κB translocation into the nucleus.24 We stimulated NF-κB activation with LPS in the presence of HDL to counteract this effect. Notably, HD HDL was less efficient in reducing NF-κB activation, whereas PD HDL was as efficient as control HDL (Figure 3B).

Recent evidence has highlighted the importance of associated enzymes on HDL functionalities.25-26 In particular, the activity of paraoxonase has been related to the antioxidative activity of HDL,27 whereas Lp-PLA2 activity has been hypothesized to be involved in atherogenesis through pathways related to inflammation.28,29 We observed that HDL of both dialysis groups showed reduced paraoxonase-mediated atheroelastase activity; however, PD HDL exhibited the lowest activity (Figure 3C). This is in good agreement with protein mass measurements (Table 2, proteins) and previous reports on HDL-associated paraoxonase activity in patients on dialysis.13,30 In contrast to paraoxonase activity, Lp-PLA2 activity was increased in HD HDL and PD HDL (Figure 3D).

Apoptotic cell death after injury of the vascular endothelium is assumed to play an important role in the pathogenesis of atherosclerosis. There is clear evidence that HDL effectively suppresses apoptosis of endothelial cells.31 Serum starvation significantly induced caspase 3/7, an early marker of apoptosis in primary human coronary artery endothelial cells (Figure 3E). This activation was almost completely abolished by treatment with control HDL, whereas PD HDL and HD HDL seemed to be less effective. We observed a borderline negative correlation between the time spent on dialysis and cholesterol efflux capability (r = –0.305, P=0.07), whereas it was not associated with other functional parameters of HDL.
Activities of Serum Enzymes Involved in HDL Metabolism

Given that HDL composition and function are significantly altered in patients on dialysis, we assessed key regulators in HDL metabolism, such as phospholipid transfer protein (PLTP), lecithin-cholesterylester transferase (LCAT), and cholesterylester transfer protein (CETP). In agreement with previous reports, we found that LCAT activity was significantly reduced in patients on HD (Figure 4A). Recent studies have provided conflicting data on PLTP activity in patients on HD,34,36 and no experimental data are available for patients on PD. We observed a marked increase in PLTP activity in patients on HD or PD, almost doubling the activity of control samples (Figure 4B). PLTP activity was associated with a reduction in phospholipids, apoA-I (r = -0.614, P < 0.001), and apoA-II (r = -0.485, P < 0.001) and an increase in apoC-II (r = 0.332, P = 0.01) and apoC-III (r = 0.473, P < 0.001). Moreover, correlation analysis indicated that PLTP activity was negatively correlated with paraoxonase activity of HDL (Supplemental Table 3), suggesting that PLTP plays an important role in remodeling HDL composition in disease. We also assessed CETP activity and found it to be unaltered (Figure 4C).

We further sought to analyze serum lipolytic activities releasing acyl chains in stereospecific numbering 1 (SN1) and stereospecific numbering 2 (SN2) positions, because activities of lipases are altered under inflammatory conditions and can profoundly affect HDL composition.37 We observed that, in both dialysis groups, the SN1 lipolytic activity in serum was significantly reduced (Figure 4D), whereas SN2 lipolytic activity was significantly decreased in patients on PD (Figure 4E).

DISCUSSION

The latest studies provide evidence that higher HDL cholesterol levels are not associated with lower mortality risk and coronary artery disease severity in patients with reduced kidney function.5,38 Dysfunctional HDL might confound the outcome of HDL-targeted therapies in these patients.9,11,13,30,39 In this study, we observed that HDL from patients on HD or PD showed a shift toward a more inflammatory phenotype, with alterations in the lipid moiety and distinct alterations in the protein composition. Our comprehensive analysis revealed a profound suppression of several metrics of HDL functionality in patients on HD or PD, which is shown in the graphic overview in Figure 5.

A finding of particular interest was that HDL from both dialysis groups showed a remarkable impaired capability to...
promote cholesterol efflux. Correlation analysis revealed that compositional alterations in patients on dialysis were associated with impaired cholesterol efflux capability. In line with our previous findings, HDL phospholipids and apoa-I were the strongest positive predictors (Supplemental Table 2), whereas apoC-III and SAA levels were negatively associated with HDL cholesterol efflux capability. We observed that HDL from patients on HD showed a clear trend toward the smaller HDL₃ subclass, suggesting that particle maturation is impaired. A crucial enzyme involved in this maturation step is LCAT. In agreement with previous reports, we observed a reduction in LCAT activity in patients on HD. The importance of LCAT in HDL maturation was underlined by an inverse correlation with the phospholipid content, which was strongest in patients on HD (r = -0.704, P < 0.001) and absent in patients on PD (r = -0.328, P = 0.27). These data suggest that reduced LCAT activity contributes to delayed HDL maturation in patients on HD, which might explain the observed shift in HDL₂/HDL₃ distribution. Our finding agrees with previous reports showing that the conversion of pre-β-HDL into spherical HDL is severely delayed in patients on HD. Importantly, the ratio of HDL₂ to HDL₃ significantly correlated with cholesterol efflux capability (r = 0.550, P = 0.005). Therefore, both increased levels of SAA and apoC-III and low LCAT activity might explain differences in cholesterol efflux properties of HDL from patients on HD or PD. A finding of particular interest was that PLTP activity was highly increased and that SN1 lipolytic activity was significantly decreased in both dialysis groups. A major source for SN1 lipolytic activity is lipoprotein lipase, with activity that has been found to be low in patients on HD. Lipoprotein lipase activity alters HDL composition, leading to higher phospholipid and lower triglycerides content. Therefore, a reduction in lipoprotein lipase activity in patients on HD or PD is expected to reduce HDL phospholipid content associated with a decreased cholesterol efflux capability. PLTP transfers phospholipids from triglyceride-rich lipoproteins to HDL, regulating the size of HDL particles. Given that, in healthy adults, a significant negative relationship between HDL size and PLTP serum activity was observed, PLTP seems to be another important uremia-associated factor altering HDL composition and cholesterol efflux capability.

A previous study has reported that elevation of PLTP in mice is associated with augmented atherosclerosis, despite the lowering effect on apoB-containing lipoproteins. PLTP overexpression in mice reduced HDL-associated paraoxonase activity. This might be of particular relevancy, given that there is evidence of a mechanistic link between HDL-associated paraoxonase activity, systemic oxidative stress, and prospective cardiovascular risk. In contrast to PLTP-overexpressing

SN1 lipases, and (E) SN2 lipases. Values shown represent means of two independent experiments.
within the small sample size of the subgroups, that functional and compositional alterations of HDL showed very similar trends for the different types of vascular access (Supplemental Table 4).

Furthermore, we observed that the time spent on dialysis tended to correlate with the cholesterol efflux capability of HDL, whereas it was not associated with other functional parameters of HDL. Subgroup analysis showed that this trend was only present in patients on HD ($r = -0.382, \ P=0.07$). However, it has to be noted that average dialysis vintage was 4.4 years for patients on HD and <3 years for patients on PD, precluding a statement on cause-effect relationship.

Limitations of this explorative study are the cross-sectional design and the small sizes of the patient cohorts, which were from a single institution. Therefore, our study is limited by the correlative nature, not permitting causal inference.

In summary, we provide novel mechanistic insights into the formation of dysfunctional HDL in patients with ESRD on HD and PD. Given that both the ability of HDL to promote cholesterol efflux and HDL-associated paraoxonase activity predict cardiovascular outcome,27,56 our results might provide valuable information to develop rational HDL-raising therapeutic strategies.

CONCISE METHODS

Study Subjects and Blood Collection

Stable patients with ESRD undergoing PD or HD/hemodiafiltration three times per week could be included in the study when they passed the exclusion criteria of being under 18 years of age, having diabetes, being on lipid-lowering therapy, having active malignancies, having liver cirrhosis, having coagulopathy, and having acute inflammation or systemic infection. Demographic data, medical history, concomitant medication, current dialysis treatment parameters, such as Kt/V, and residual renal function were recorded at the time of enrollment.

Blood sampling was performed before a mid-week dialysis session (patients on HD/hemodiafiltration) or during the monthly visit (patients on PD) using standard serum tubes (Greiner Bio-one, Kremsmünster, Austria). Healthy blood donors were recruited and included in the study when they passed the same exclusion criteria as patients on dialysis and were free of any sign of kidney disease. The control group was matched for age and sex. Patients on HD had different types of vascular access (Supplemental Table 1). We performed correlation analysis to examine the composition and several metrics of HDL function of patients on HD or PD. Functional and compositional alterations of HDL showed similar trends in patients on HD or PD (Supplemental Table 4). Furthermore, iron indices and intravenous iron administration were not significantly associated with HDL function in our study cohort (Supplemental Tables 5 and 6). The study protocol and all study procedures were reviewed and
approved by the local ethics committee (21–523 ex 09/10). All participants gave written informed consent before being enrolled into the study.

Materials
Radiochemicals were purchased from Hartman Analytic (Braunschweig, Germany). All other reagents were obtained from Sigma-Aldrich (Vienna, Austria).

Preparation of Lipoproteins
Serum density was adjusted with potassium bromide (Sigma-Aldrich) to 1.24 g/ml, and a two-step density gradient was generated in centrifuge tubes (16×76 mm; Beckman Instruments, Krefeld, Germany) by layering the density-adjusted plasma underneath an NaCl density solution (1.063 g/ml) as described with minor modifications.57 Tubes were sealed and centrifuged at 90,000 rpm for 4 hours in a 90Ti fixed angle rotor (Beckman). After centrifugation, the HDL- and LDL-containing bands were separately collected, desalted through PD10 columns (GE Healthcare, Vienna, Austria), and immediately used for experiments or stored at −70°C. Carbamylated LDL was generated by incubation with 20 mg/ml potassium cyanate for 4 hours. Afterward, LDL was desalted through PD10 columns to remove excess potassium cyanate. To evaluate the effect of the isolation method on HDL function, we compared the arylesterase activity of HDL-containing fractions derived from polyethylene glycol precipitation28 with one-step density gradient centrifugation as described above. Our analysis suggests that both isolation methods are comparable in terms of HDL-associated arylesterase activity (Supplemental Figure 1).

Determination of Plasma and HDL Lipid Composition
Levels of total cholesterol, nonesterified cholesterol, triglycerides, and phospholipids (DiaS ys, Holzheim, Germany) were measured enzymatically. Sphingomyelin and sphingosine-1-phosphate were measured with commercially available ELISA (Cayman Europe, Talinn, Estonia). LDL cholesterol was calculated according to the Friedewald equation using HDL cholesterol values measured in the supernatant of the phosphotungstic precipitation.

Apo Determination by Immunoturbidimetry
ApoA-I, apoA-II, apoB, apoC-II, apoC-III, and apoE (Greiner, Flacht, Germany) were determined by immunoturbidimetry. All lipoprotein analyses were performed on an Olympus AU640 analyzer (Olympus Diagnostika, Hamburg, Germany).

Cellular Cholesterol Efflux Assays
Cholesterol efflux capacity was quantified as described previously.9 RAW 264.7 macrophages were maintained in DMEM plus 10% FBS and 1× penicillin-streptomycin. Cells were plated at 3×10⁶ on 48-well plates and grown overnight. Then, cells were labeled with 1 μCi/ml [³H]-cholesterol in medium supplemented with 5% FBS and 50 μg/ml carbamylated LDL for 24 hours. For the last 14 hours of incubation, cells were additionally stimulated with the LXR agonist TO-901317 (2 μmol/L). Cells were washed two times and equilibrated with serum-free medium containing 0.2% BSA for 2 hours at 37°C. Afterward, cells were washed two times, and 200 μl cholesterol acceptor (50 μg/ml HDL) was added in serum-free media for 2 hours at 37°C. Supernatants and lysed cells (lysed with 0.3 M sodium hydroxide containing 0.1% SDS) were separately collected, and radioactivity was measured by liquid scintillations counting. Cholesterol efflux to the acceptors is expressed as the radioactivity in the medium relative to total radioactivity in medium and cells. Specific cholesterol efflux was calculated by subtracting cholesterol efflux in the absence of HDL from efflux in the presence of HDL.

Dil HDL Uptake
HDL was labeled with Dil as described previously.60 Briefly, Dil was added to HDL from a stock solution (30 mg/ml in DMSO) to yield a final ratio of 300 μg Dil per 1 mg HDL protein. The mixture was incubated for 20 hours at 37°C, and excess dye was removed by gel filtration with the PD Spin-Trap G-25 (GE Healthcare). The specific fluorescence at 565 nm for each individual HDL preparation was determined, expressed as fluorescence per 1 mg protein, and included in the uptake calculation.

HepG2 cells were cultivated in DMEM containing 10% FBS and 1× penicillin-streptomycin. Cells were seeded at 2.5×10⁶ on 48-well plates, grown overnight, and incubated with 20 μg/ml Dil HDL in serum-free DMEM with 2 mg/ml BSA for 3 hours. Cells were washed two times with 2 mg/ml BSA in PBS and one time in PBS. Cells were detached with accutase (Sigma-Aldrich), washed with PBS, and fixed with BD CellFIX solution (BD Biosciences). Uptake of the fluorescence lipid tracer Dil was analyzed by flow cytometry in the FL-2 channel, and 5,000 cells were counted for each condition.

Arylesterase and Lp-PLA₂ Activity
Ca²⁺-dependent arylesterase activity was determined with a photometric assay using phenylacetate as previously described.83 HDL (0.5 μg protein) was added to 200 μl buffer containing 100 mmol/L Tris, 2 mmol/L CaCl₂ (pH 8.0), and 1 mmol/L phenylacetate in a 96-well quartz glass plate (Hellma, Baden, Germany). The rate of hydrolysis of phenylacetate was monitored by the increase of absorbance at 270 nm, and readings were taken every 15 seconds at room temperature to generate a kinetic plot. The slope from the kinetic chart was used to determine the increase in fluorescence per minute. Enzymatic activity was calculated with the Beer–Lambert Law from the molar extinction coefficient of 1310 mol×L⁻¹×cm⁻¹ for phenylacetate. Lp-PLA₂ activity was measured with a commercially available photometric assay (Cayman Europe) with 2-thio platelet activating factor as substrate.

Determination of the Antioxidative Capacity of HDL
The antioxidative activity of HDL was determined as previously described.23 Briefly, 15 μg HDL protein was placed in a 384-well plate, and 100 μl 17 μmol/L DHR reagent containing 1 mmol/L 2,2‘-azobis-2-methyl-propanimidamide-dihydrochloride was added. The increase in fluorescence was monitored using an xMark plate reader (Biorad, Vienna, Austria) over time at 538 nm. After an initial lag phase of about 20 minutes, the rate of oxidation was linear. The increase in fluorescence was calculated from the linear range and used for comparison. The increase in fluorescence per minute of DHR in the absence of
HDL was set to 100%, and individual HDL samples were calculated as percentage of inhibition of DHR oxidation.

Determination of the Anti-Inflammatory Capacity of HDL
U937 cells, a monocyte cell line containing a 5 × NF-κB–GFP reporter cassette24 (obtained from Herbert Strobl, Medical University of Graz, Graz, Austria), were cultivated in RPMI 1640 containing 10% FBS and 1 × penicillin-streptomycin. For experiments, 25,000 cells were placed in 1.1-ml microtubes (Bioquote) in 100 μl RPMI 1640 with 7.5% FBS. Cells were pretreated for 90 minutes with 50 μg/ml HDL and stimulated for 24 hours with 50 ng/ml LPS. After the stimulation, cells were centrifuged at 400 × g for 7 minutes, the supernatant was discarded, and cells were fixed in 100 μl BD CellFIX solution (BD Biosciences). The expression of NF-κB GFP was assessed by flow cytometry.

Caspase 3/7 Activation Assay
Human coronary artery endothelial cells (Lonza, Basel, Switzerland) were cultured in EBM-2 Bullet Kit Media (Lonza) and used between passages 4 and 8. Cells were seeded on culture dishes with glass bottoms and grown overnight. Then, cells were starved in serum-free media in the absence or presence of 100 μg/ml HDL for 24 hours. Cells were stained with CellEvent Caspase 3/7 Green Detection Reagent (Life Technologies, Vienna, Austria) and imaged by confocal microscopy.

HDL Particle Size Analysis
HDL (5 μg protein per lane) was separated by native gradient gel electrophoresis (4%–16% NativePage; Life Technologies). Gels were run for 120 minutes at a constant voltage of 150 V in NativePage running buffer (Life Technologies). Afterward, gels were fixed with 25% isopropanol/10% acetic acid for 10 minutes and stained overnight with Coomassie Brilliant Blue G-250 (Thermo Fisher Scientific). To determine the size distribution of isolated HDL, an image of the gel was analyzed with ImageJ software. Intensity blots of individual samples separately were calculated with the help of the standard proteins (NativeMark; Life Technologies) containing BSA (7.1 nm), lactate dehydrogenase (8.2 nm), B-phycoerythrin (10.5 nm), apoferritin band 1 (12.2 nm), and apoferritin band 2 (18.0 nm). To compare individual samples, the percentage of HDL2 and HDL3 peak area on the total peak area was calculated and used for analysis.

PLTP and CETP Activity Assays
PLTP and CETP were measured in serum with assay kits from Abnova (Eubio, Vienna, Austria). Both activity assays use a specific donor molecule containing a fluorescent self-quenched neutral lipid (for CETP) or phospholipid (for PLTP) that is transferred to an acceptor molecule in the presence of CETP or PLTP. Enzyme-mediated transfer of the fluorescent neutral lipid to the acceptor molecule results in an increase in fluorescence (excitation=465 nm; emission=535 nm).

SN1 and SN2 Lipase Activity Assays
SN1 and SN2 lipase activity was measured in serum with commercially available kits (Cayman Europe). In the SN1 lipase assay, lipases hydrolyze arachidonoyl-1-thioglycerol to AA and thioglycerol, which react with a thiol fluorometric detector to yield a highly fluorescent product that results in an increase in fluorescence (excitation=385 nm; emission=515 nm).

The SN2 lipase assay uses the 1,2-dithio analog of diheptanoylphosphatidylcholine as a substrate. On hydrolyses of the thio ester bond at the SN2 position, free thiols are detected using 5,5-dithio-bis(2-nitrobenzoic acid), which results in an increase in absorbance at 414 nm.

LCAT Activity Assay
LCAT was measured in serum with a commercially available kit from Merck (Darmstadt, Germany). Samples were incubated with LCAT substrate for 5 hours at 37°C. The fluorescent substrate emits fluorescence at 470 nm. When the substrate is hydrolyzed by LCAT, a monomer is released that emits fluorescence at 390 nm. The LCAT activity is assessed over time and expressed in change of 470/390-nm emission intensity.

SDS-PAGE and Western Blotting
To assess HDL-associated Lp-PLA2 and paraoxonase mass, 10 μg HDL was separated by SDS-PAGE, blotted, and probed with Lp-PLA2 antibody (10279; Cayman Europe) and paraoxonase 1 antibody (clone 17A12; Abcam, Inc., Cambridge, UK) as described previously. Western blots were densitometrically analyzed, and the Lp-PLA2 and paraoxonase content was calculated as the relative index to the average of the control population.

Statistical Analyses
Results are expressed as means and SDs or numbers and percentages of the total study group. Study groups were compared using ANOVA with the least significant difference post hoc test. For non-Gaussian data, the Mann–Whitney U test was used for two-group comparisons, and the Kruskal–Wallis test with Dunn’s post hoc test was used for three-group comparisons. The association between HDL functionalities and enzymatic activities of plasma enzymes with clinical and compositional data was assessed with the use of Pearson correlation coefficients, with partial correlation coefficients after adjustment for C-reactive protein levels and dialysis vintage. All analyses were performed with GraphPad Prism, version 4 or SPSS, version 21.

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DISCLOSURES
None.
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