Dysfunctional High-Density Lipoprotein in Patients on Chronic Hemodialysis

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Objectives
This study examined the functionality of high-density lipoprotein (HDL) in individuals with end-stage renal disease on dialysis (ESRD-HD).

Background
The high rate of cardiovascular disease (CVD) in chronic kidney disease is not explained by standard risk factors, especially in patients with ESRD-HD who appear resistant to benefits of statin therapy. HDL is antiatherogenic because it extracts tissue cholesterol and reduces inflammation.

Methods
Cellular cholesterol efflux and inflammatory response were assessed in macrophages exposed to HDL of patients with ESRD-HD or controls.

Results
HDL from patients with ESRD-HD was dramatically less effective than normal HDL in accepting cholesterol from macrophages (median 6.9%; interquartile range [IQR]: 1.4% to 10.2%) versus control (median 14.9%; IQR: 9.8% to 17.8%; p < 0.001). The profound efflux impairment was also seen in patients with ESRD-HD and diabetes compared with patients with diabetes without renal disease (median 8.1%; IQR: 3.3% to 12.9%) versus control (median 13.6%; IQR: 11.0% to 15.9%; p = 0.009). In vitro activation of cellular cholesterol transporters increased cholesterol efflux to both normal and uremic HDL. HDL of patients with ESRD-HD had reduced anti-chemotactic ability and increased macrophage cytokine response (tumor necrosis factor-alpha, interleukin-6, and interleukin-1-beta). HDL of patients with ESRD-HD on statin therapy had reduced inflammatory response while maintaining impaired cholesterol acceptor function. Interestingly, impaired HDL-mediated efflux did not correlate with circulating C-reactive protein levels or cellular inflammatory response.

Conclusions
These findings suggest that abnormal HDL capacity to mediate cholesterol efflux is a key driver of excess CVD in patients on chronic hemodialysis and may explain why statins have limited effect to decrease CV events. The findings also suggest cellular cholesterol transporters as potential therapeutic targets to decrease CV risk in this population. (J Am Coll Cardiol 2012;60:2372–9) © 2012 by the American College of Cardiology Foundation

Chronic kidney disease (CKD) dramatically increases cardiovascular disease (CVD) risk, which escalates with declining kidney function and is maximal in patients with end-stage renal disease on chronic hemodialysis (ESRD-HD) (1–3). Although increased CV mortality in patients with ESRD-HD is attributable to many causes, death from atherosclerotic coronary artery disease is persistently higher than in the general population, even after adjustments for traditional CVD risk factors (2–6). Patients with ESRD-HD are also unique in their diminished benefit from statin interventions that are effective in reducing CVD risk in other high-risk patients (7–9). The recent SHARP (Study of Heart and Renal Protection) trial reported decreased numbers of CV events with aggressive cholesterol lowering using simvastatin and ezetimibe, in patients with CKD (9,10). Results were overall positive, but the difference in the ESRD-HD subgroup was too small to show statistical significance. The lack of benefit of aggressive lipid-lowering
therapy in ESRD suggests other mechanisms, such as low levels of high-density lipoprotein (HDL). HDL has generated considerable interest as a target for interventions to decrease atherosclerotic burden (11–13). One key function of HDL, namely macrophage cholesterol efflux capacity (14–18), was inversely associated with measures of carotid and coronary atherosclerosis in a non-CKD population, independent of HDL concentration (18).

Interestingly, Holzer et al. (14) recently reported reduced efflux ability of HDL in patients with ESRD-HD compared with HDL from controls without kidney disease. Although intriguing, these data did not examine whether coexisting comorbidities such as diabetes, obesity, underlying CVD, and certain demographic characteristics of patients with ESRD-HD (e.g., age, sex, race, underlying CKD) contributed to the observed differences. It is critically important to determine the mechanisms by which abnormalities in HDL function predispose patients with CKD to increased atherosclerotic burden to develop rational therapeutic strategies. In this study, we evaluated the ability of HDL from patients with ESRD-HD to elicit cholesterol efflux and modulate inflammatory responses in macrophages and compared this with HDL from individuals with normal kidney function with similar coexisting clinical and demographic characteristics that influence development of atherosclerotic CVD (19).

Methods

Participants and study design. Adult patients with ESRD undergoing maintenance hemodialysis at Vanderbilt University Medical Center (VUMC) for more than 3 months were studied (n = 29). Control participants (n = 28) with normal renal function who did not differ from the patients in age, sex, race, presence of diabetes mellitus, history of CVD (angina, myocardial infarction, stroke/transient ischemic attack), and use of statin and angiotensin-converting enzyme inhibitors (ACEIs)/angiotensin receptors blockers (ARBs) were recruited from the general medicine outpatient clinic at VUMC. Exclusion criteria included pregnancy and current smoking. The study protocols were approved by the institutional review board at VUMC, and all participants gave informed consent.

Assessment of plasma lipid profile and C-reactive protein and isolation of lipoproteins. Blood was taken after an overnight fast (prior to hemodialysis) in K2 ethylenediamine tetraacetic acid–containing tubes and centrifuged at 1,700 g for 15 min. Plasma levels of total cholesterol, triglycerides, and HDL were measured enzymatically (Clinica, San Marcos, California). High-sensitivity C-reactive protein (hsCRP) was measured using a high-sensitivity immunoturbidimetric assay (Roche Modular Systems, Indianapolis, Indiana) (20). The apolipoprotein B (apoB)–containing fraction (d = 1.006 to 1.063 g/ml) and HDL fraction (d = 1.063 to 1.21 g/ml) were isolated from fresh plasma by sequential density ultracentrifugation after density adjustment with potassium bromide (21). The purity of the isolation was checked by the QuickGel lipoprotein electrophoresis system (Helena Laboratories, Beaumont, Texas).

Macrophase assays of cholesterol uptake with apoB fraction and cholesterol efflux with HDL. THP-1 cells (American Type Culture Collection, Manassas, Virginia) were plated onto 35-mm wells and differentiated into macrophages by RPMI containing 10% fetal bovine serum and 50 ng/ml phorbol 12-myristate 13-acetate. For uptake studies, THP-1 macrophages were exposed to the apoB fraction (50 μg/ml) from patients with ESRD-HD or controls for 24 h. For efflux studies, THP-1 macrophages were cholesterol enriched with acetylated low-density lipoprotein (LDL) (100 μg/ml, Intracel, Frederick, Maryland) and exposed to HDL (50 μg/ml) and lipopolysaccharide (LPS) (50 ng/ml, Sigma-Aldrich Co., St. Louis, Missouri) for 24 h. In some studies, liver X receptor (LXR) agonist T0901317 (1 μM, Sigma-Aldrich) was added to activate ATP-binding cassette (ABC) transporters. Cellular cholesterol content was measured by gas chromatography (21,22). Cholesterol efflux was determined as the percent decrease in cellular cholesterol content at baseline versus after incubation with HDL (15,23). Cell proteins were solubilized by addition of 0.1 N NaOH and protein content was measured by bicinchoninic acid assay.

Macrophase chemotaxis assay and inflammatory reaction with HDL. Macrophage migrating activity used THP-1 cells exposed to HDL from patients with ESRD-HD or controls. The studies were performed in a microchemotaxis chamber in which the upper and lower compartments are separated by an uncoated polycarbonate filter (Neuroprobe, Gaithersburg, Maryland). THP-1 cells (5 × 10^5/well) were exposed to HDL (50 μg/ml) for 1 h and then added to the upper compartment. Monocyte chemotactic protein (MCP)-1 (0.1 μg/ml, Preprotech, Rocky Hill, New Jersey) was added to the lower compartment. Filters were fixed in methanol and stained with 1% crystal violet, and the migrated cells were counted under the microscope. Duplicate wells were used for each experimental condition, and 5 fields (×40) were counted for each well. The anti-inflammatory function of HDL was measured as the cytokine response in LPS-activated macrophages. THP-1 macrophages were exposed to HDL (50 μg/ml) and LPS (50 ng/ml) for 4 to 24 h. Total RNA was extracted from cells with the RNeasy mini kit (Qiagen, Valencia, California). Quantification of human interleukin (IL)-1-beta, IL-6,
tumor necrosis factor (TNF) alpha, and endogenous control human Euk 18S rRNA levels was performed by real-time reverse transcriptase polymerase chain reaction (PCR) using a Prism 7700 sequence detection system (Applied Biosystems, Inc., Foster City, California). Probes for IL-1-beta (Hs99999029_m1), IL-6 (Hs99999032_m1), TNF-alpha (Hs99999043_m1), and 18S rRNA were obtained from Applied Biosystems.

**Statistical analysis.** The sample size was calculated using published results showing 11 ± 3% as mean value and standard deviation for cholesterol efflux to normal HDL (18). To detect a 25% difference, we estimated 26 participants per arm to achieve 90% statistical power with a 2-sided significance level of 5%. Results are expressed as mean of triplicate assays. Descriptive statistics are presented as frequencies and percentages for categorical variables and mean ± SD or median (interquartile range [IQR]) according to the distribution of the continuous variables. Demographic and clinical factors were compared between patients with ESRD-HD and controls using the Wilcoxon rank sum test or Pearson chi-square test, as appropriate. Correlation analysis was performed using R version 2.10.0. A 2-sided significance level of 5% was required for consideration as statistically significant.

### Results

**Characteristics of study participants.** There were no statistically significant differences between patients with ESRD-HD and controls in any of the demographic or comorbidity variables, except that patients with ESRD-HD had significantly elevated plasma hsCRP levels (p = 0.001) (Table 1). There were no statistically significant differences in total cholesterol, triglyceride, and HDL levels between patients with ESRD-HD and controls.

**Macrophage uptake of apoB lipoproteins.** Because foam cell formation reflects an imbalance between lipoprotein uptake and cholesterol efflux, we evaluated the macrophage cholesterol-loading capacity of apoB-containing lipoprotein fractions. ApoB fractions isolated from patients with ESRD-HD and controls caused similar degrees of cholesterol accumulation in THP-1 macrophages (38.0 ± 5.0 μg/mg vs. 34.4 ± 5.5 μg/mg protein in controls and patients with ESRD-HD, respectively; p = 0.19).

| Table 1 Characteristics of Study Participants |

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Controls (n = 28)</th>
<th>Patients With ESRD-HD (n = 29)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>57</td>
<td>46 ± 11</td>
<td>48 ± 10</td>
<td>0.51</td>
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<tr>
<td>Sex, % male</td>
<td>57</td>
<td>36</td>
<td>52</td>
<td>0.22</td>
</tr>
<tr>
<td>Race, % white</td>
<td>57</td>
<td>46</td>
<td>34</td>
<td>0.36</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>57</td>
<td>31.2 ± 7.2</td>
<td>31.6 ± 7.9</td>
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<td>Diabetes mellitus, %</td>
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<td>50</td>
<td>48</td>
<td>0.90</td>
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<td>Cardiovascular disease, %</td>
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<td>39</td>
<td>34</td>
<td>0.71</td>
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<td>Statin usage, %</td>
<td>57</td>
<td>29</td>
<td>31</td>
<td>0.84</td>
</tr>
<tr>
<td>ACEI/ARB, %</td>
<td>57</td>
<td>32</td>
<td>34</td>
<td>0.85</td>
</tr>
<tr>
<td>hsCRP, mg/dl</td>
<td>56</td>
<td>1.1 (0.6,6.5)</td>
<td>6.2 (3.1,17.7)</td>
<td>0.001</td>
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<tr>
<td>Total cholesterol, mg/dl</td>
<td>56</td>
<td>198 ± 66</td>
<td>170 ± 35</td>
<td>0.12</td>
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<tr>
<td>Triglycerides, mg/dl</td>
<td>56</td>
<td>95 (73,124)</td>
<td>102 (75,135)</td>
<td>0.84</td>
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<tr>
<td>HDL, mg/dl</td>
<td>56</td>
<td>40.6 ± 9.9</td>
<td>36.9 ± 17.2</td>
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<td>LDL, mg/dl</td>
<td>56</td>
<td>139 ± 64</td>
<td>110 ± 31</td>
<td>0.24</td>
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</tbody>
</table>

Values are mean ± SD or %, The Wilcoxon rank sum test was used for comparing continuous variables, and categorical variables were compared using the chi-square test.

ACEI = angiotensin-converting enzyme inhibitor; ARB = angiotensin receptor blocker; ESRD-HD = end-stage renal disease on chronic hemodialysis; HDL = high-density lipoprotein; hsCRP = high-sensitivity C-reactive protein; LDL = low-density lipoprotein.
Efflux capacity of HDL. Cholesterol acceptor function from cholesterol-loaded THP-1 cells was significantly lower for HDL of patients with ESRD-HD (median 6.9%; IQR: 1.4% to 10.2%) versus control (median 14.9%; IQR: 9.8% to 17.8%; p < 0.001) and remained significantly lower in patients with ESRD-HD after adjustment for age, sex, race, BMI, diabetes, hsCRP, total cholesterol, triglycerides, HDL, LDL, CVD, and use of statins or ACEIs/ARBs (difference 10.2%; 95% confidence interval [CI]: 5.8% to 14.5%; p < 0.0001) (Fig. 1). Even within the diabetic subgroup, the HDL of patients with ESRD-HD and diabetes had reduced efflux capacity compared with HDL efflux capacity of controls with normal renal function and diabetes (median 8.1%; IQR: 3.3% to 12.9%) versus control (median 13.6%; IQR: 11.0% to 15.9%; p = 0.009) (Online Fig. 1). Statin usage did not improve HDL efflux capacity in patients with ESRD-HD (median 6.8%; IQR: 1.8% to 8.8% versus median 6.8%; IQR: 1.4% to 10.5% for statin users and nonusers, respectively).

To assess whether activation of cellular transporters can affect the acceptor capacity of HDL, we exposed THP-1 cells to an LXR agonist (T0901317), which increased expression of ABCA1 by 21.4-fold and ABCG1 by 2.7-fold (p < 0.001 for each). Up-regulation in cellular ABCA1/G1 levels significantly increased cholesterol efflux to HDL of both controls and patients with ESRD-HD to a similar extent (Fig. 2).

Inflammatory capacity of HDL. Compared with the cytokine response observed in LPS-exposed THP-1 cells treated with HDL of controls, HDL isolated from patients with ESRD-HD caused a greater inflammatory response (Fig. 3A). However, the cytokine response to HDL from the subgroup of patients with ESRD-HD on statin therapy was not different than that of controls taking statins (Fig. 3B). In addition, although HDL from controls reduced MCP1-induced migration of THP-1 macrophages, HDL from patients with ESRD-HD provided no anti-chemotactic effect (Online Fig. 2).

Macrophage cholesterol efflux correlations with systemic and cellular inflammation. In view of the link between cholesterol handling and inflammation in atherogenesis, we evaluated the correlation between individual macrophage cholesterol efflux capacity of HDL and markers of systemic inflammation and inflammatory response of macrophages. Efflux and circulating levels of hsCRP or macrophage inflammatory response did not show a statistically significant correlation within either controls or patients with ESRD-HD (Table 2).

Discussion

Our data indicated that HDL isolated from patients with ESRD on maintenance hemodialysis had markedly impaired capacity to elicit cholesterol efflux while potentiating inflammation in macrophages compared with HDL isolated from matched individuals with similar clinical and demographic characteristics but with normal kidney function. Within ESRD, HDL of statin-taking individuals did not have improved cholesterol acceptor capacity, although the proinflammatory effects were less, thus suggesting that different HDL functions do not respond similarly to ther-
apeutic interventions. Our data further revealed that cholesterol handling and regulation of vascular inflammation, both atheroprotective markers of HDL function, do not track together within a given individual with advanced uremia. This impaired cholesterol acceptor capacity of dysfunctional HDL is a potential novel mechanism to explain the exponentially increased CVD risk and lack of response to conventional treatment strategies in patients with advanced uremia. Instead, our findings that pharmacological up-regulation of cellular expression of ABCA1 and ABCG1 significantly increased efflux to control HDL as well as to the dysfunctional HDL of patients with ESRD-HD suggested the potential for cholesterol transporters as a novel target to lessen CVD in this population.

Although accelerated atherosclerosis and increased numbers of CV events have been extensively documented in CKD, the pathophysiological mechanisms are complex and not explained by traditional risk factors, especially in ESRD-HD (2,4–6). Although many of our patients with ESRD-HD had diabetes, the extreme impairment in efflux

Figure 3  Proinflammatory Effects of HDL From Patients With ESRD-HD and Controls

(A) Cytokine response of lipopolysaccharide (LPS)-exposed THP-1 macrophages to HDL from patients with ESRD-HD and controls not using statins expressed relative to cells exposed to LPS alone. (B) Cytokine response to HDL from statin-using patients with ESRD-HD and controls expressed relative to response of cells exposed to LPS alone. p value from the Wilcoxon rank sum test. IL = interleukin; TNF = tumor necrosis factor; other abbreviations as in Figure 1.
capacity of HDL was attributable to uremia and not affected by diabetes mellitus per se. These findings echo previous studies that showed that advanced uremia is the single most important amplifier of CVD in various populations, including those with diabetes mellitus (25–27). In a recent study, HDL from patients on chronic hemodialysis had reduced cholesterol efflux capacity compared with HDL from a control group (14). However, whereas the study group contained patients with diabetes and individuals on statin therapy, the control group was composed of healthy participants without diabetes, hyperlipidemia, or statin therapy. Thus, potential effects of these confounders on HDL dysfunction could not be teased out. In the current study, by considering coexisting comorbidities such as diabetes, obesity, underlying CVD, and relevant demographic characteristics of patients with ESRD-HD such as age, sex, and race, our results demonstrated the overarching impact of the uremic milieu on HDL’s decreased capacity for cholesterol efflux.

The relevance of HDL efflux capacity to the pathogenesis of atherosclerosis relates to the central role of HDL in reverse cholesterol transport, which removes excess cholesterol from peripheral cells and transports it in plasma for hepatic delivery, excretion in bile, and intestinal loss (16,17). HDL can adversely impact CVD risk in ESRD through several mechanisms. In our study, the trend for lower HDL level in our patients with ESRD-HD did not reach significance. However, circulating levels of HDL do not predict all-cause or CV mortality in patients with ESRD-HD (28). In our study, equal amounts of HDL were used to determine cholesterol efflux capacity. We showed that patients on hemodialysis have dysfunctional HDL with impaired macrophage cholesterol efflux. Our data showed that statin treatment of patients with ESRD-HD did not improve the cholesterol efflux capacity of their HDL. Indeed, cholesterol efflux capacity was also not affected in a clinical study of HDL function of patients without CKD treated with statins (18). Together, these observations parallel the recent appreciation that lipid-lowering therapies provide only limited benefits against CVD and that HDL function may explain the substantial residual risk in patients receiving standard of care (29,30).

Our data also made clear the important new observation that functionality of uremic HDL can be improved. Upregulation of ABCA1 and ABCG1 transporters by LXR activation significantly increased efflux not only to control HDL but also to the dysfunctional HDL of patients with ESRD-HD (Fig. 2). These findings raise the interesting possibility that, even in the face of dysfunctional HDL, activation of cellular transporters can abrogate a proatherogenic pathway and cause CVD risk reduction in this population. The biological and therapeutic relevance of these findings is underscored by our preliminary data that monocytes of patients with ESRD-HD have reduced expression of cellular ABCA1 and scavenger receptor BI compared with cells from controls (V. Kon, unpublished data, 2012). These observations complement findings that in scavenger receptor BI–expressing Chinese hamster ovary cells, efflux to uremic HDL was significantly reduced compared with control HDL (14).

In addition to cholesterol handling, HDL has anti-inflammatory properties (31). Among patients with ESRD-HD, individuals with proinflammatory HDL showed more comorbidities and increased risk of CV and all-cause mortalities than individuals with anti-inflammatory HDL (19). Our results showed that macrophage expression of IL-1-beta, IL-6, and TNF-alpha was greater with uremic HDL compared with HDL from controls. A tendency for greater cytokine response in statin-using controls than those without statin treatment is consistent with the fact that most participants within the statin-using control subgroup had a previous history of CVD, known to enhance inflammation in the non-CKD population (32). We also found that uremic HDL did not reduce MCP-1–induced chemotaxis. This is consistent with observations that HDL interacts with macrophage cholesterol transporters to modulate expression of MCP1 and chemotaxis (33,34). This disrupted inflammatory response of uremic HDL (35) may enhance recruitment of monocytes into the subendothelial space. Whether specific cellular phenotypes or cytokines underlie accelerated atherogenesis and acute CVD events in ESRD-HD remains to be clarified. Also unclear is the contribution of ESRD-specific metabolic abnormalities, such as those in calcium and phosphate transport. Interestingly, sevelamer, a resin that binds dietary phosphate and lessens experimental atherosclerosis (36), decreased mortality in patients on hemodialysis in association with improved arterial stiffness and increased levels of HDL (37).

We next evaluated the association between anti-inflammatory and lipid-handling properties of a given HDL sample (Table 2). We found that these functionalities did not track together in individual patients. Although unexpected, the results do not negate the importance of either inflammation or abnormal lipid handling in ESRD. Instead, the findings raise the intriguing possibility of separate and distinct mechanistic pathways for different functions of HDL that may involve compositional changes in specific HDLs. For example, reactive lipid peroxidation products such as malondialdehyde (MDA) modify and cross-link specific residues in apolipoprotein A1, causing distinct functional consequences (38,39). Interestingly, although progressive de-
cline in renal function increases plasma MDA levels (40), it is currently not known if MDA or other peroxidation products contribute to impairment in HDL’s anti-inflammatory function or influence levels or activity of antioxidant enzymes such as paraoxonase (41). These findings also suggest that reduced HDL ability to mediate cholesterol efflux may be the key driver for excess CVD among patients with ESRD and that correction of both cholesterol handling and inflammation must be remedied to achieve a therapeutic benefit in this population.

Study limitations. The sample size was small and from a single institution, which limits generalizability. On the other hand, patients with ESRD-HD and controls were well matched, which is a unique strength. Nevertheless, we were not able to account for all confounders that might be present in these patients. The results are associative and do not prove a cause and effect relationship, which should be tested in future studies. We did not relate cholesterol efflux to direct CVD events and view this as an important next step. Finally, ESRD alters several lipid and protein components of HDL, including enrichment in the acute phase reactant, serum amyloid A (14,42,43). Thus, the specific functional impact of uremia-associated HDL compositional changes on particular functions remains an important area for future investigations.

Conclusions

Patients on maintenance hemodialysis have abnormal HDL with impaired cholesterol acceptor function and proinflammatory effects. HDL from individuals in dialysis taking statins becomes anti-inflammatory but maintains a reduced capacity for cholesterol efflux. Improved cholesterol efflux to HDL of patients with ESRD-HD is instead achieved by activation of cellular cholesterol transporters via LXR, which suggests a novel target for therapeutic interventions in this population.

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REFERENCES


Key Words: atherosclerosis • kidney • lipoprotein • statin.

APPENDIX

For supplemental figures, please see the online version of this article.