Impaired Lipoprotein Processing in HIV Patients on Antiretroviral Therapy

Aberrant High-Density Lipoprotein Lipids, Stability, and Function


Objective—HIV patients on antiretroviral therapy (HIV/ART) exhibit a unique atherogenic dyslipidemic profile with hypertriglyceridemia (HTG) and low plasma concentrations of high-density lipoprotein (HDL) cholesterol. In the Heart Positive Study of HIV/ART patients, a hypolipidemic therapy of fenofibrate, niacin, diet, and exercise reduced HTG and plasma non–HDL cholesterol concentrations and raised plasma HDL cholesterol and adiponectin concentrations. We tested the hypothesis that HIV/ART HDL have abnormal structures and properties and are dysfunctional.

Approach and Results—Hypolipidemic therapy reduced the TG contents of low-density lipoprotein and HDL. At baseline, HIV/ART low-density lipoproteins were more triglyceride (TG)-rich and HDL were more TG- and cholesteryl ester-rich than the corresponding lipoproteins from normolipidemic (NL) subjects. Very-low-density lipoproteins, low-density lipoprotein, and HDL were larger than the corresponding lipoproteins from NL subjects; HIV/ART HDL were less stable than NL HDL. HDL-[3H]cholesteryl ester uptake by Huh7 hepatocytes was used to assess HDL functionality. HIV/ART plasma were found to contain significantly less competitive inhibition activity for hepatocyte HDL-cholesteryl ester uptake than NL plasma were found to contain (P<0.001).

Conclusions—Compared with NL subjects, lipoproteins from HIV/ART patients are larger and more neutral lipid-rich, and their HDL are less stable and less receptor-competent. On the basis of this work and previous studies of lipase activity in HIV, we present a model in which plasma lipolytic activities or hepatic cholesteryl ester uptake are impaired in HIV/ART patients. These findings provide a rationale to determine whether the distinctive lipoprotein structure, properties, and function of HIV/ART HDL predict atherosclerosis as assessed by carotid artery intimal medial thickness. (Arterioscler Thromb Vasc Biol. 2013;33:1714-1721.)

Key Words: hepatocyte cholesteryl ester uptake ■ high-density lipoprotein function ■ HIV dyslipidemia ■ lipoprotein composition

HIV–infected patients receiving antiretroviral therapy (ART) present with a dyslipidemia and insulin resistance, which places them at a high risk for accelerated cardiovascular disease. The increased cardiovascular disease among HIV/ART patients is a persistent public health challenge for which current therapies are not adequate. HIV/ART dyslipidemia comprises hypertriglyceridemia, low plasma high-density lipoprotein (HDL) cholesterol, and elevated plasma non–HDL-C concentrations. Other distinctive pathogenic features of HIV/ART dyslipidemia include hypoadiponectinemia, increased lipolysis, and hepatic free fatty acids flux, which increases very-low-density lipoprotein (VLDL) synthesis, in a way that is mechanistically linked to defects in HDL metabolism. Given that the low plasma HDL-C concentrations place HIV/ART patients at a high cardiovascular disease risk, we tested the hypothesis that the compositions and properties of HDL from these patients were distinct from control subjects in a way that impaired HDL metabolism. This hypothesis was tested by studying dyslipidemic HIV/ART patients with and without hypertriglyceridemia, and control non-HIV normolipidemic (NL) subjects.

Materials and Methods

Materials and Methods are available in the online-only Supplement.
Results

Altered Plasma Lipid Profiles in HIV/ART

Lipoprotein compositions were determined in a large subset of HIV patients from the Heart Positive Study (predominantly those who completed the 24-week trial). Subset plasma lipid profiles simulated those of the entire Heart Positive patient set (Table I in the online-only Data Supplement). Treatment groups were 1, placebo; 2, diet and exercise (D&E); 3, fibrate+D&E; 4, niacin+D&E; 5, fibrate+niacin+D&E. In all 5 treatment groups, patient plasma lipid profiles at baseline satisfied the lipid criteria for metabolic syndrome, that is, hypertriglyceridemia and a low plasma HDL-C concentration. Moreover, the HDL-C levels were lower and those for TG were higher than those of NL controls. Analysis of variance confirmed that the lipid profiles at baseline for the 5 groups, assigned at random on enrollment in the Heart Positive Study, were not significantly different. As reported for the entire Heart Positive study treatment with fenofibrate+D&E (group 3), niacin+D&E (group 4), and the combined therapy of fenofibrate+niacin+D&E (group 5) significantly reduced plasma TG, with median reductions of 35% to 42%. Treatments including niacin with or without fenofibrate increased median HDL-C by 21% to 24%. Treatments including fibrate with or without niacin reduced median non–HDL-C by 20% to 25%.

Altered Lipoprotein Composition in HIV/ART

Compared with NL controls, low-density lipoprotein (LDL)-%TG values of Heart Positive HIV/ART patients at baseline were a median 2-fold higher, whereas other constituents were not significantly different (Table 1). HDL compositions were also significantly different, with higher %TG, % cholesterol ester (CE), and % free cholesterol (core components), and lower % phospholipid and % protein (surface components) in Heart Positive HIV/ART patients versus NL controls. The fraction of HDL composed of surface and core components, respectively, were lower and higher in HIV/ART patients with significant elevations of both %CE and %TG. The elevation of neutral lipid core components suggested that hypertriglyceridemic HIV/ART HDL is larger than NL HDL.

Table 1. Heart Positive HIV and NL Control HDL and LDL Compositions at Baseline*

<table>
<thead>
<tr>
<th>Donor</th>
<th>n</th>
<th>% Protein</th>
<th>% PL</th>
<th>% FC</th>
<th>% CE</th>
<th>% TG</th>
<th>% (CE+TG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV</td>
<td>114</td>
<td>42.0 (36.6–45.7)</td>
<td>19.3 (18.4–20.7)</td>
<td>4.08 (3.36–5.25)</td>
<td>27.2 (20.2–30.0)</td>
<td>7.41 (5.68–10.17)</td>
<td>35.3 (31.2–38.3)</td>
</tr>
<tr>
<td>NL</td>
<td>12</td>
<td>47.7 (45.3–50.7)</td>
<td>23.1 (21.6–24.6)</td>
<td>2.08 (1.92–2.41)</td>
<td>21.8 (18.9–23.7)</td>
<td>4.56 (3.21–6.19)</td>
<td>26.6 (24.7–29.0)</td>
</tr>
<tr>
<td>P†</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Ratio</td>
<td>0.9</td>
<td>0.8</td>
<td>2.0</td>
<td>1.3</td>
<td>1.6</td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

| HIV   | 113 | 24.2 (22.4–26.1) | 18.7 (17.8–19.7) | 7.56 (6.86–8.33) | 36.5 (33.4–39.1) | 11.97 (9.27–16.3) | 49.2 (47.6–50.9) |
| NL‡   | 22  | 22        | 8     | 42    | 6     | 48    |
| Ratio | 1.1  | 1.1       | 1.1   | 1.1   | 1.1   | 1.1   |

CE indicates cholesteryl ester; FC, free cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NL, normolipidemic; PL, phospholipid; and TG, triglyceride.

*Values are median and (25%–75%) range.
†Mann–Whitney rank-sum test; P value for comparison of Heart Positive HIV with NL values.
‡NL values for LDL composition are from Havel et al.38

Effect of Treatment on Lipoprotein Composition

The 5 treatment arms differentially affected HDL and LDL neutral lipid compositions. As expected, the placebo group showed no change in HDL and LDL composition (Figure 1; group 1). The various treatments did not have much effect on lipoprotein CE content (Figure 1A and 1C). Although increased for all treatments, median LDL %CE (average increase of 4%) was not significantly different except for group 2 (D&E). Similarly, although all treatments trended toward decreased HDL %CE (average 6%), this was not significant. In contrast, all 4 interventions reduced the median LDL %TG contents (Figure 1B) and HDL %TG (Figure 1D). Because of the limited sample size and large individual variability in %TG values, the differences in paired entry versus after-therapy values for any single treatment group only approached P<0.05 significance. However, for all treatments combined there was a 24% decrease in LDL %TG (P=0.003) and a 25% decrease in HDL %TG (P=0.004). These results indicate that whereas the elevated TG contents of both LDL and HDL were responsive to treatment, the elevated CE content of HTG-HIV/ART HDL was more resistant to these treatment protocols.

Hypertriglyceridemic HIV/ART HDL Are not Stable on Freezing

As the neutral core to surface lipid ratio of lipoproteins increases, the particle size increases. Assessment of lipoprotein particle size by size exclusion chromatography (SEC) revealed that Heart Positive HIV/ART HDL were unstable on freezing and exhibited other SEC peaks eluting earlier (larger particle size), which were absent from the chromatograms of normal HDL (Figure 2A). We assigned these large species to HDL fusion. As shown in Figure 2B, SEC uncovers freezing-dependent differences in the profile of NL control HDL after multiple freeze–thaw cycles. However, the effect of freeze–thaw is much more profound for the HDL from 4 Heart Positive HIV/ART patients. Thus, the frozen-while-stored samples could not be used for studies of HDL sizing or functionality.

HDL Composition of Non-HTG HIV/ART

HIV/ART HDL from a second HIV/ART patient group were stored at 4°C. The plasma lipid values for HIV/ART...
patients are compared with those of NL controls and the Heart Positive HIV/ART patients in Table 2. Unlike the Heart Positive Clinical Trial, which had completed by this time, the second HIV/ART patient group was not selected for hypertriglyceridemia. According to their plasma lipid concentrations, these patients presented with isolated low plasma HDL-C ($P=0.004$ versus NL controls) and reduced total plasma cholesterol ($P=0.025$ versus NL controls) but no hypertriglyceridemia. The HDL compositions for this group (Table 3) show that HDL-CE contents were lower than those of NL controls, the opposite of what was observed for the HTG HIV/ART patients. However, similar to the hypertriglyceridemic Heart Positive patients, this group of normotriglyceridemic HIV/ART patients also had HDL neutral lipid cores enriched in TG relative to CE compared with NL control HDL. HDL samples from this group were studied further for stability, size, and functionality.

**HIV/ART HDL Are Less Stable to Chaotropic Perturbation Than NL Control HDL**

Incubation of HDL with guanidinium chloride (GdmCl) induces the release of lipid-free apolipoprotein (apoAI) and the formation of a larger fused HDL (Figure 3). Comparison of the effects of GdmCl on the lipoprotein profiles of HDL from NL controls and HIV/ART patients revealed notable differences in the amounts of the released products (Figure 3A and 3B). Although GdmCl converted the HDL from both NL controls and HIV/ART patients to the expected products, the relative amounts of fused HDL and lipid-free apoAI formed were different (Figure 3C and 3D). Post-GdmCl, the relative amount of HDL remaining was lower and the amount of lipid-free apoAI was higher in HIV/ART HDL versus NL control HDL. Accordingly, even non-HTG HIV/ART HDL is less stable than NL HDL.

**HIV/ART Lipoproteins Are Larger Than NL Control Lipoproteins**

The high neutral lipid content of LDL and HDL of Heart Positive patients suggested these might be larger lipoproteins.

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**Figure 1.** Comparison of the effects of 4 different antilipidemic therapies vs placebo control on low-density lipoprotein (LDL) and high-density lipoprotein (HDL) composition. LDL and HDL % cholesteryl ester (CE) and % triglyceride (TG) weight composition are shown as median, box plot of (25%–75%) range and bar (10%–90%) range at entry (white bars) and after therapy (gray bars). Therapy groups 1 to 5 are denoted on the abscissa. Wilcoxon signed rank-sum analysis of paired entry and after-therapy values are indicated for $P<0.06$. For all treatments combined, both LDL and HDL %TG decrease significantly: $P=0.003$ and 0.004, respectively.

**Figure 2.** Effects of freezing on high-density lipoprotein (HDL) as assessed by size exclusion chromatography. Top, A–D, Four different HIV/antiretroviral therapy (ART) HDL samples after 3 freeze–thaw cycles. Bottom, A–D, a single normolipidemic (NL) control HDL sample analyzed before freezing and after 1 to 3 freeze–thaw cycles.
The sizes of lipoproteins in the total lipoproteins (TLP) from non-HTG HIV patients and controls were compared by SEC (Figure II in the online-only Data Supplement). The means±SE of these data, calculated and plotted as shown in Figure 4, reveal profound differences between HIV/ART and NL control TLP. NL control TLP contains prominent peaks for VLDL, LDL, and HDL with relatively small SEs (Figure 4A). SEC profiles for HIV/ART TLP differ (Figure 4B). First, peaks for the 3 major lipoproteins are shifted to earlier elution volumes corresponding to larger particle sizes (compare gray vertical lines). Second (Figure 4B, ×10 insert), the SEC of HIV/ART TLP contains a peak between LDL and VLDL, which we assign to intermediate density lipoprotein (IDL). Last, the SEs at each elution volume of the HIV/ART TLP chromatogram are much larger than those of control TLP. The relative amounts of the lipoproteins are also altered. On the basis of the ratios of the peak heights, HIV/ART lipoproteins are altered relative to NL controls, which we assign to intermediate density lipoprotein (IDL). Last, the SEs at each elution volume of the HIV/ART TLP chromatogram are much larger than those of control TLP. The relative amounts of the lipoproteins are also altered. On the basis of the ratios of the peak heights, HIV/ART lipoproteins are altered relative to NL controls, with higher amounts of VLDL and IDL relative to LDL and HDL (Figure 5). The ratios relative to LDL for NL controls and HIV/ART patients are as follows: VLDL/LDL=1.10 versus 2.10 ($P=0.032$); IDL/LDL=0.160 versus 0.436 ($P=0.001$). These larger sizes, and the greater amounts of VLDL and IDL relative to LDL and HDL, suggest delayed processing of apoB lipoproteins in HIV plasma.

Validation of a Functional Assessment of NL Control Versus HIV/ART HDL
Because of the altered CE content and the lower stability of the HIV/ART HDL samples, we hypothesized that HIV/ART HDL are dysfunctional in the final step of reverse cholesterol transport, transfer of HDL CE to hepatocytes. The most straightforward method for measuring hepatic HDL-CE uptake requires preparation of HDL-[3H]CE, a procedure that can take 10 days and requires significant quantities of starting HDL20 to yield HDL-[3H]CE with >99% radiochemical purity,21,22 followed by uptake assays according to Acton et al.23 This assay is impractical in the context of large patient numbers and limited plasma volumes, so we modified and validated a competitive assay of hepatic CE uptake that we have used to compare the HDL-[3H]CE uptake before and after remodeling by streptococcal serum-opacity factor.20 This assay uses NL controls and HIV/ART patient HDL to compete with the uptake of a standard stock HDL-[3H]CE (Figure III in the online-only Data Supplement). Inhibition constants, ID50%, were calculated from the inhibitor HDL dose–response curves. In this assay, a large ID50% corresponds to HDL that interacts poorly with hepatic HDL receptors and vice versa. Our assay is reproducible; each panel in Figure III in the online-only Data Supplement contains assays on the same HDL sample repeated on different days, with ±17 weeks between assays. Assays in triplicate showed a within-day variability of 10.6% (n=28) and day-to-day variability of 10.4% (n=4).

### Table 2. Plasma Lipid Values for HIV Patients With Isolated Low HDL-C (non-HTG HIV) Compared With Heart Positive HIV and NL Controls*

<table>
<thead>
<tr>
<th>Donor</th>
<th>n</th>
<th>Cholesterol, mg/dL</th>
<th>Triglyceride, mg/dL</th>
<th>HDL-C, mg/dL</th>
<th>Calculated LDL-C, mg/dL</th>
<th>Non–HDL-C, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P^*$</td>
<td></td>
<td>0.914</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.755</td>
<td>0.010</td>
</tr>
<tr>
<td>Non-HTG HIV</td>
<td>10</td>
<td>125 (72–208)</td>
<td>109 (91–228)</td>
<td>33 (20–58)</td>
<td>44 (34–128)</td>
<td>80 (53–159)</td>
</tr>
<tr>
<td>$P^*$</td>
<td></td>
<td>0.025</td>
<td>0.668</td>
<td>0.004</td>
<td>0.052</td>
<td>0.106</td>
</tr>
<tr>
<td>NL control</td>
<td>12</td>
<td>196 (166–233)</td>
<td>118 (90–142)</td>
<td>67 (52–92)</td>
<td>103 (90–120)</td>
<td>124 (111–153)</td>
</tr>
</tbody>
</table>

HDL-C indicates high-density lipoprotein cholesterol; HTG, hypertriglyceridemic; LDL-C, low-density lipoprotein cholesterol; and NL, normolipidemic.

*Values are median and (25%–75%) range.

$P^*$ Mann–Whitney rank-sum test $P$ value for comparison of HIV values with NL values.

Figure 3. Comparison of the stability of high-density lipoprotein (HDL) from HIV/antiretroviral therapy (ART) and normolipidemic (NL) control subjects. A and B, Examples of size exclusion chromatography profiles of NL and HIV/ART HDL before (gray shaded curve) and after (line) treatment with 2 mol/L guanidinium chloride (GdmCl). Stability was assessed on the basis of the HDL peak height after treatment (C) and the lipid-free apolipoprotein (apo)A1/HDL$_{fused}$ ratio (D). Less fused (lipidated) HDL and more lipid-free apoA1 are formed from the HDL of the HIV/ART patients than from that of the NL controls.
HIV HDL Is a Poor Competitor for Hepatocyte HDL-CE Uptake

Using this competitive inhibition assay, we compared the ID50% of HDL from HIV/ART patients with those from NL controls. As shown in Figure 6A, ID50% for individual NL and HIV/ART HDL distributed over a wide range, 72 to 211 and 75 to 191 µg/mL HDL protein for NL and HIV/ART, respectively. These ranges, which are far greater than the assay variability, illustrate the individual heterogeneity of donor HDL with respect to selective hepatic CE uptake and the power of this assay to distinguish HDL functionality on the basis of hepatic CE uptake. Our data show that the mean ID50% for the NL group was lower than that of the HIV/ART subjects (rank-sum P=0.052), indicating the HIV/ART HDL was a poorer competitor, that is, required more HDL to reach its ID50%.

Importantly, adjustment of these data for each donor’s HDL-C level, which were lower for the HIV/ART patients (Figure 6B), revealed the dysfunctionality of HIV/ART HDL. The difference between HIV/ART and NL plasma to affect HDL-CE uptake was profound—CE uptake inhibition by HIV/ART HDL, expressed as plasma volume-equivalents, was much lower. NL plasma contains 23.6±1.7 ID50% units/mL versus 14.0±1.8 ID50% units/mL for HIV/ART plasma (P<0.001). The mean

<table>
<thead>
<tr>
<th>Donor</th>
<th>n</th>
<th>% Protein</th>
<th>% PL</th>
<th>% FC</th>
<th>% CE</th>
<th>% TG</th>
<th>% (CE+TG)</th>
<th>TG/(TG+CE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Positive HIV</td>
<td>114</td>
<td>42.0 (36.6–45.7)</td>
<td>19.3 (18.4–20.7)</td>
<td>4.08 (3.36–5.25)</td>
<td>27.2 (24.2–30.0)</td>
<td>7.41 (5.68–10.17)</td>
<td>35.3 (31.2–38.3)</td>
<td>0.22 (0.16–0.29)</td>
</tr>
<tr>
<td>P†</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>Non-HTG HIV</td>
<td>10</td>
<td>49.3 (47.1–59.0)</td>
<td>24.8 (18.8–27.4)</td>
<td>2.22 (2.03–2.70)</td>
<td>15.2 (10.8–18.7)</td>
<td>5.34 (3.39–6.70)</td>
<td>20.3 (16.6–24.2)</td>
<td>0.25 (0.17–0.35)</td>
</tr>
<tr>
<td>P†</td>
<td></td>
<td>0.156</td>
<td>0.817</td>
<td>0.448</td>
<td>0.006</td>
<td>0.621</td>
<td>0.008</td>
<td>0.049</td>
</tr>
<tr>
<td>NL</td>
<td>12</td>
<td>47.7 (45.3–50.7)</td>
<td>23.1 (21.6–24.6)</td>
<td>2.08 (1.92–2.41)</td>
<td>21.8 (18.9–23.7)</td>
<td>4.56 (3.21–6.19)</td>
<td>26.6 (24.7–29.0)</td>
<td>0.17 (0.12–0.25)</td>
</tr>
</tbody>
</table>

CE indicates cholesteryl ester; FC free cholesterol; HDL-C, high-density lipoprotein cholesterol; HTG, hypertriglyceridemic; NL, normolipidemic; PL, phospholipid; and TG, triglyceride.

*Values are median and (25%–75%) range.

†Mann–Whitney rank-sum or Student t test P value for comparison of HIV values with NL values.
Metabolism of HIV/ART Lipoproteins Is Impaired

According to the total lipoprotein (TLP) SEC profiles, the dyslipidemia in HIV/ART patients is highly heterogeneous (large SEs), and is associated with the appearance of lipoproteins that are larger than those of NL controls (Figure 4). Consistent with this, the data of Aragonés et al\textsuperscript{25} showed greater heterogeneity in HIV HDL and increased CE in the larger HDL subfractions. Two observations suggest that increased lipoprotein heterogeneity is a result of impaired lipoprotein hydrolysis. First, the SEC profiles (Figure 4) showed that all HIV/ART lipoproteins elute earlier than their NL control counterparts; second, these data also show that unlike control TLP, HIV/ART TLP contains a prominent peak for IDL (Figure 4). Increased amounts of VLDL and IDL in HIV/ART fasting plasma indicate delayed processing of these lipoproteins (Figure 5). Given that both IDL- and HDL-TG are substrates for hepatic lipase (HL), which reduces their size by hydrolysis, we hypothesize that HL activity is impaired under the conditions of HIV/ART dyslipidemia, a hypothesis that is supported by reports that HL and to a lesser extent lipoprotein lipase activities are lower in HIV/ART-associated dyslipidemia\textsuperscript{26}; lower lipoprotein lipase activity would also explain the larger HIV/ART versus NL control VLDL. Although endothelial lipase activity has not been reported in HIV samples, low endothelial lipase activity could also cause larger HDL as well as reduced HDL phospholipid,\textsuperscript{27} but would be expected to cause higher rather than lower plasma HDL-C.\textsuperscript{28}

Interaction of HIV/ART HDL With Hepatocytes Is Impaired

According to our competitive assay of HDL-CE uptake by Huh7 cells, differences in the ID\textsubscript{50\%} of NL control versus HIV/ART HDL were nearly significant (P=0.052; Figure 6A). Moreover, when the ID\textsubscript{50\%} data were normalized to the plasma HDL-C levels of each individual, HIV/ART plasma contained significantly less competitive inhibition activity for hepatocyte HDL-CE uptake than NL plasma did (P<0.001). Decreased interaction of HIV/ART HDL with hepatic receptors may contribute to the observed elevated HDL %CE in the Heart Positive patient population (Table 1). It would suggest that the higher HDL-CE may result from an inability of HIV/ART HDL to deliver its CE to hepatocyte receptors for uptake and clearance. The molecular basis for this is not known but may be a result of altered surface protein structure or configuration in response to an altered core composition.

HDL Stability

HIV/ART HDL were found to be less stable than NL HDL both on freezing and on chaotropic perturbation. Importantly, HDL instability has been uncovered by lecithin:cholesterol acyltransferase, CE transfer protein, phospholipid transfer...
protein, and serum-opacity factor, all of which induce fusion or the release of lipid-free apoAI.22-30 Although there have been many studies of the effects of GdmCl on HDL,18-19,33,34 this study is the first to show that the effects of GdmCl on HDL vary among dyslipidemic patients and NL controls, and that the NL control and HIV/ART HDL respond differently to this denaturant. According to several studies, HDL resides in a kinetic trap from which it can escape via both physico-chemical16,19 and biological perturbations—lecithin:cholesterol acyltransferase, CE transfer protein, phospholipid transfer protein, and serum-opacity factor.29-31,35 Thus, in the context of the kinetic model of Gursky,18 HIV/ART HDL is less stable than NL control HDL. Given that a major marker of instability is the release of lipid-free apoAI, one could speculate that the plasma factors cited above mediate production of more lipid-free apoAI in HIV/ART patients compared with NL controls. A negative consequence of this would be increased renal clearance of apoAI, which would have the effect of lowering plasma HDL-C levels; this hypothesis needs more rigorous testing.

A Metabolic Model of Dysfunctional Lipoprotein Processing in HIV/ART Dyslipidemia

According to our data, HIV/ART dyslipidemia is associated with larger HDL, LDL, and VLDL, and impaired HDL hepatocyte binding, which likely affects hepatocyte HDL-CE uptake. Other studies in HIV patients have demonstrated peripheral tissue hyperlipolytic activity11 and lower lipoprotein and HL activities.26,28 In contrast, the initial step in reverse cholesterol transport, macrophage-cholesterol efflux to HDL, is unaltered by HIV and its treatment. Rose et al found similar rates of activated RAW 264.7 macrophage-cholesterol efflux to plasma of HIV-infected patients, currently treated HIV-infected patients, and HIV-negative subjects. Taken together, these data support a metabolic model for HIV dyslipidemia (Figure 7) that begins with peripheral tissue hyperlipolytic activity11 that results in the release of higher amounts of free fatty acids that are extracted by the liver and used for the production of more VLDL, thereby producing a hypertriglyceridemic state. In the presence of high VLDL-TG concentrations, CE transfer protein mediates the exchange of VLDL TG for HDL and LDL-CE, thereby producing TG-rich HDL and LDL. CE transfer protein activity has been reported to be elevated5 or within normal limits37 in HIV/ART subjects. Lipoprotein-TG hydrolysis by HL reduces their sizes by removing core lipids, and when this process is inhibited larger TG-rich lipoprotein species are observed. In our study, there is even some residual IDL, which under normolipemic conditions is converted to LDL by HL. All lipoprotein classes from HIV/ART are larger than those from NL subjects suggesting resistance to the activities of hepatic or lipoprotein lipases. As a consequence of the high TG content of the HDL particles, we speculate that they are less stable and that the HIV/ART apoAI is more labile than that of NL HDL. Whether the instability influences function remains to be investigated. Nevertheless, the CE of HIV/ART HDL are hepatically extracted at a lower rate than those of NL HDL because of their intrinsically lower uptake and the lower plasma HDL-C levels in HIV/ART patients.

Figure 7. Metabolic model for the production of dysfunctional lipoproteins in HIV/ART dyslipidemia. See text for discussion. ATGL indicates adipose triglyceride lipase; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; HL, hepatic lipase; HSL, hormone sensitive lipase; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LPL, low-density lipoprotein receptor; LPL, lipoprotein lipase; NEFA, non esterified fatty acids; SRBD, scavenger receptor class B type I; TG, triglyceride; and VLDL, very-low-density lipoprotein.

Acknowledgments

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Disclosures

None.

References

Patients with HIV receiving antiretroviral therapy (ART) present with a unique dyslipidemia that places them at risk for cardiovascular disease. We compared HIV/ART patients with normolipidemic subjects. All HIV/ART lipoproteins were larger than the corresponding normolipidemic lipoproteins; the HIV/ART high-density lipoprotein are richer in neutral lipids, less stable, and bind poorly to hepatocytes. These findings were based on new assays of high-density lipoprotein stability, and functionality; this study was the first to apply these assays to a clinical population. These findings are unprecedented in HIV/ART research and show that the underlying mechanisms for the unique HIV/ART dyslipidemia are impaired lipoprotein clearance by plasma lipases or hepatic receptors. This research brings a new perspective on the pathogenesis of HIV/ART-associated dyslipidemia, with high relevance to some other metabolic diseases—Cushing syndrome, polycystic ovarian disease, and obesity-linked diabetes mellitus.
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Materials and Methods

Study Populations: Our study was based on two HIV/ART patient groups. Initial results were obtained from stored baseline and final plasma from the Heart Positive Study (www.ClinicalTrials.gov ID NCT00246376), a 24-week, randomized, placebo-controlled, double blind test of additive mono and combination therapy for dyslipidemia in HIV patients on stable ART. The Heart Positive inclusion criterion was hypertriglyceridemia (fasting plasma TG > 150 mg/dL). Participants were randomized to a placebo (Group 1) and four different therapeutic groups—diet and exercise (D & E) (Group 2), D & E + fenofibrate (Group 3), D & E + niacin (Group 4), and D & E + fibrate + niacin (Group 5). The design of the D&E program has been reported. From the total population of 114 patients, 110 completed the 24-week trial and four participated for 12 to 20 weeks. We also recruited NL control patients and a second group of HIV/ART patients with near normal plasma TG levels but low plasma HDL-C levels to study HDL size, stability, and functionality without sample freezing. Plasma lipids were determined in the Atherosclerosis Clinical Research Laboratory. Liver function tests, glucose, and insulin were determined in The Methodist Hospital Pathology Laboratory or by Quest/Labcorp. The study was approved by the Institutional Review Boards of Baylor College of Medicine and Legacy Community Health Center.

Lipoprotein isolation: Lipoproteins were isolated from EDTA plasma by sequential flotation at d=1.006 g/mL (VLDL), d =1.02 g/mL (IDL), d=1.063 g/mL (LDL), and d=1.21 g/mL (HDL); the lipoprotein-deficient fraction (d> 1.21 g/mL) was also collected in the final flotation step. Fractions were stored at -80°C until analyzed for lipoprotein composition. Lipoproteins from the second group of HIV patients and normal controls were similarly isolated and stored at 4 °C.

Lipoprotein Compositions: The major lipoprotein-lipids, cholesteryl ester, phospholipids, free cholesterol, and triglyceride were assayed using commercial enzyme-based kits (Wako). Phospholipid assay mixtures contained 10 mM CaCl2 to correct for calcium depletion in the EDTA plasma. Protein was determined by RC DC Protein Assay (Biorad), a valid protein assay even in the presence of KBr.

Total Lipoprotein (TLP) Profiles: Lipoproteins sizes were assessed by size exclusion chromatography (SEC) using two Superose HR6 columns (GE Health Care-Life Sciences) in tandem. After flotation at d = 1.21 g/mL at 40,000 rpm (Beckman Ti 50.2) for three days, aliquots (200 µL) were injected and eluted at 0.5 mL/min. The column effluent was monitored by measuring absorbance at 280 nm. The mean ± SE TLP profiles for NL Control and HIV/ART were calculated and plotted in Sigma Plot 10 (Systat Software, Inc.). Based on 14 consecutive injections of a cholesteryl ester-rich microemulsion, the reproducibility of the elution volume was the mean ± 25 µL.

HDL Stability: HDL was collected from the bottom fraction after flotation of TLP at d = 1.063 g/mL. Guanidinium chloride (GdmCl) (MP Biomedicals, LLC) and HDL were combined to produce final concentrations of 2 M and 0.5 mg/mL respectively. After three days at room temperature, the samples were analyzed by SEC and the relative amounts of lipid-free apo A-I and fused HDL determined. The effects of freezing on HDL were similarly assessed.

Hepatic HDL-CE Uptake: Cellular HDL-CE uptake was studied in the human hepatoma cell line, Huh7, which express the major lipoprotein receptors—scavenger receptor class B Type I (SR-BI), the low density lipoprotein receptor family members LDLR, LRP-1 and VLDLR, and heparan sulfate proteoglycan syndecan-1 (SDC1) (Supplemental Figure I). Cholesterol ester uptake was determined as described. HDL functionality was based on a competitive assay of the inhibition of the cellular uptake of a standard HDL-[3H]CE by patient HDL. Huh7 cells were grown to confluence in 12-well plates, washed and transferred to DMEM + 0.5% fatty acid-free BSA. CE uptake assay was initiated by incubation of HDL-[3H]CE (20 µg HDL protein/mL) with

1
patient HDL in triplicate at 0, 20, 50 and 120 µg HDL protein/mL for 3 hr after which cells were washed, extracted with 2-propanol and the extract assayed for $^3$H-CE by $\beta$-counting. The data were fitted to a hyperbolic decay equation, i.e., \%CE Uptake = $aID_{50%}/(ID_{50%} + [I])$, where $[I]$ is the concentration of the added inhibitor protein, $a$ is the y-intercept, and $ID_{50%}$ is the added inhibitor concentration that produces 50% inhibition.

**Statistical Analysis.** Statistical analysis and curve fitting were preformed in SigmaPlot 10 or 11. Multiple groups were compared using Kruskal-Wallis One Way Analysis of Variance on Ranks. Paired samples before and after treatment were compared using the Wilcoxon signed rank sum test. Other data were analyzed using the rank sum test, or when data were normally distributed, the Student t-test, as indicated in legends to Tables and Figures.

**References for Materials and Methods**


Table I: Heart Positive Plasma Lipid Values at Baseline and after 24 weeks of Treatment *

<table>
<thead>
<tr>
<th>Plasma Lipid</th>
<th>All HP patients</th>
<th>Treatment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=114</td>
<td>2. D&amp;E</td>
</tr>
<tr>
<td></td>
<td>n=23</td>
<td>3. D&amp;E+fibrate</td>
</tr>
<tr>
<td></td>
<td>n=28</td>
<td>4. D&amp;E+niacin</td>
</tr>
<tr>
<td></td>
<td>n=17</td>
<td>5. D&amp;E+fibrate+ niacin</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Baseline</td>
<td>Treated</td>
</tr>
<tr>
<td>mg/dL</td>
<td>195 (172-230)</td>
<td>205 (181-235)</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>Baseline</td>
<td>Treated</td>
</tr>
<tr>
<td>mg/dL</td>
<td>248 (171-329)</td>
<td>247 (182-321)</td>
</tr>
<tr>
<td>HDL-C</td>
<td>Baseline</td>
<td>Treated</td>
</tr>
<tr>
<td>mg/dL</td>
<td>37 (31-42)</td>
<td>38 (33-46)</td>
</tr>
<tr>
<td>Calc LDL-C</td>
<td>Baseline</td>
<td>Treated</td>
</tr>
<tr>
<td>mg/dL</td>
<td>108 (77-137)</td>
<td>113 (81-146)</td>
</tr>
<tr>
<td>Non-HDL-C</td>
<td>Baseline</td>
<td>Treated</td>
</tr>
<tr>
<td>mg/dL</td>
<td>158 (135-187)</td>
<td>175 (141-192)</td>
</tr>
</tbody>
</table>

a. Plasma lipid values are given as median and (25%-75%) range. Normal values are Cholesterol <200 mg/dL; Triglycerides <150 mg/dL; HDL-C > 45 – 55 mg/dL; Calculated LDL-C <130 mg/dL and Non-HDL-C <160 mg/dL.

b. Heart Positive patients were randomized to 5 groups. Kruskal-Wallis One Way Analysis of Variance on Ranks of the lipid values for the five groups at baseline confirmed that the lipid values at baseline did not differ among the groups ( p values of 0.088 to 0.812).

c. Wilcoxon Signed Rank Test comparing paired values at baseline and after treatment.
Supplemental Figure I:  A, Phase contrast image for Huh7 cells; note lipid droplets and confluent cobblestone monolayer architecture compared to HepG2 cells which tend to grow in overlaying clusters.  B, mRNA expression for major lipoprotein receptors in Huh7 cells compared to expression in HepG2 cells and primary human hepatocytes at days 2 and 5 of culture.  Expression levels of the low density lipoprotein receptor family members LDLR, LRP1, and VLDLR, Scavenger Receptor Class B Type I SR-BI (SCARB1) and heparan sulfate proteoglycan Syndecan-1 (SDC1) were determined by quantitative reverse transcription PCR (qRTPCR) and normalized to 18S for each cell type.
Supplemental Figure II. Comparison of Lipoprotein Sizes by SEC of TLP from HIV/ART and NL Control Subjects. Ten individual donor HIV/ART TLP chromatograms are shown in Panel A, and 12 individual donor NL Control TLP chromatograms are shown in Panel B. These chromatograms were used to calculate the mean and SEM profiles shown in Figure 4.
Supplemental Figure III: Reproducibility of assays of the inhibition of stock HDL-[³H]CE uptake by individual donor HDL. Intervals between assays were 1, 16, 17, and 15 wks for A to D respectively, with results from the two assays plotted as grey and black curves. For all curves, $r^2 > 0.96$. The ID$_{50\%}$ values for grey and black curves respectively were as follows: A, $86.9 \pm 4.2$ and $71.8 \pm 3.5 \mu g/mL$; B, $80.1 \pm 21.1$ and $71.0 \pm 9.0 \mu g/mL$; C, $82.5 \pm 16.7$ and $94.1 \pm 7.1 \mu g/mL$; and D, $104.5 \pm 2.0$ and $114.9 \pm 11.8 \mu g/mL$. 