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## HIV infection induces structural and functional changes in High Density Lipoproteins

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### Abstract

**Background and Aims**—Coronary artery disease is a growing clinical problem in HIV-infected subjects. The increased risk of coronary events in this population has been linked to low levels of HDL, but the effects of HIV infection and anti-retroviral treatment (ART) on HDL structure and function remain unknown. Here, we aimed to determine the composition and function of HDL particles isolated from ART-naïve and ART-positive HIV-infected patients.

**Methods and Results**—Proteomic profiling revealed decreased levels of paraoxonase (PON) 1 and PON 3 in HDL from HIV patients relative to HDL from uninfected controls ( $p < 0.0001$ ), and PON activity of HDL from control group ( $0.13 \pm 0.01$  U/ $\mu$ l) was significantly higher than PON activity of HDL from HIV-infected untreated subjects ( $0.12 \pm 0.01$  U/ $\mu$ l,  $p = 0.0035$ ), subjects treated with non-nucleoside reverse transcriptase inhibitor (NNRTI)-based therapy ( $0.11 \pm 0.01$  U/ $\mu$ l,  $p < 0.0001$ ), subjects treated with protease inhibitor (PI)-based therapy with detectable viral load

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Conflict of Interest

The authors declare no conflict of interest in this study.

( $0.11 \pm 0.01$  U/ $\mu$ l,  $p < 0.0001$ ), and PI-treated patients with undetectable viral load ( $0.12 \pm 0.01$  U/ $\mu$ l,  $p = 0.0164$ ). Lipidomic profiling uncovered a negative correlation between CD4 T cell counts and particle sphingomyelin, lyso-phosphatidylcholine and ether-linked phosphatidylserine content in the ART-naive ( $R^2 = 0.2611$ ,  $p < 0.05$ ;  $R^2 = 0.2722$ ,  $p < 0.05$ ; and  $R^2 = 0.3977$ ,  $p < 0.05$ , respectively) but not treated HIV-infected subjects. Functional analysis demonstrated a negative correlation between cholesterol efflux capacity of HDL and viral load in the ART-naive HIV-infected group ( $R^2 = 0.26$ ,  $p = 0.026$ ).

**Conclusions**—Taken together, these results indicate that HIV infection associates with a number of both protein and lipid compositional changes in HDL particles. Moreover, HIV infection affects cholesterol efflux function of HDL, thus contributing to an increased risk of atherosclerosis in this patient population.

### Keywords

HIV; anti-retroviral treatment; HDL; proteomics; lipidomics; cholesterol efflux; PON, atherosclerosis

### Introduction

Accelerated atherosclerosis and the resultant coronary artery disease have been recognized as a serious and growing problem in HIV-infected subjects<sup>1-3</sup>. Despite the fact that anti-retroviral treatment (ART) has resulted in a dramatic improvement in the morbidity and mortality of HIV-infected patients, conflicting data have been reported regarding the effects of ART on the risk of cardiovascular disease (CVD). Early publications suggested that ART reduces the risk of atherosclerosis<sup>4</sup>, although some drugs, in particular abacavir (nucleoside reverse transcriptase inhibitor or NRTI) and ritonavir (protease inhibitor or PI), were associated with increased risk of myocardial infarction and metabolic syndrome, respectively<sup>5-7</sup>. Later studies established that ART may reduce but does not eliminate the risk for developing CVD in HIV-infected individuals<sup>8</sup>. Our recent study comparing groups of HIV-infected subjects treated with PI, non-nucleoside reverse transcriptase inhibitors (NNRTI) or untreated demonstrated that drugs increase cholesterol efflux to apoB-depleted plasma, most likely due to increased abundance of high density lipoprotein (HDL) particles<sup>9</sup>.

The reason for the increased risk of CVD in HIV-infected patients is not clearly understood. Recent reports have demonstrated that atherosclerotic changes occur in drug-naïve patients and in patients undergoing treatment interruption<sup>10-13</sup>, indicating a direct contribution of HIV to CVD. Given that low level viral replication may occur even in well-controlled patients<sup>14</sup>, it appears likely that some risk of atherosclerosis in drug-treated subjects may be attributed to direct effects of the virus. Chronic low-level inflammation associated with HIV infection, and in particular monocyte activation, is also a likely contributing factor<sup>15</sup>.

A characteristic feature in both treated and untreated HIV-infected subjects is a disturbance in plasma lipoprotein metabolism characterized by low levels of HDL<sup>16,17</sup>. HDL has been established as a major anti-atherogenic lipoprotein, both because of its role in reverse cholesterol transport and its anti-inflammatory properties<sup>18</sup>. Low HDL levels are associated

with a high risk of atherosclerosis in animal models and in humans<sup>19</sup>. A defect in HDL maturation has been demonstrated in macaques infected with simian immunodeficiency virus<sup>20</sup>, suggesting that HDL particles in virally-infected subjects may be structurally and functionally altered compared to uninfected controls. This study was designed to test the hypothesis that HIV infection and/or HIV therapy induce qualitative and quantitative changes in HDL protein and lipid composition thereby impairing HDL functionality, which could account for an increased risk of atherosclerosis in infected subjects.

## Methods

### Study Participants

Samples from three distinct groups of HIV-infected subjects, stored in GW HIV Institute Biorepository or the MFA's HIV Specimen Bank, were used in this retrospective study (IRB approval #091034): HIV-infected subjects who were naïve to ART or had been off ART for over 1 year, HIV-infected subjects on PI-based antiretroviral therapy for over 1 year, and HIV-infected subjects on NNRTI-based ART for over 1 year. None of these subjects were on any lipid lowering medications including statins, which was the only exclusion criterion. Samples from these three groups of subjects were compared with HDL samples collected from HIV-negative subjects not treated with lipid lowering medications, who were used as the control group. The control group was a group of convenience composed of adults recruited at GWU MFA. Post-prandial plasma samples were collected from all groups under the George Washington University HIV Institute Biorepository protocol, and the study was approved by the George Washington University IRB. The samples were aliquoted and stored at  $-80^{\circ}\text{C}$ . We used plasma samples collected from 21 HIV-infected subjects not on ART therapy, 27 HIV-infected subjects on PI-based ART, 27 HIV-infected subjects on NNRTI-based ART, and 19 control uninfected subjects. The exact regimens were as follows: in the PI-treated group 10 subjects received emtricitabine/tenofovir with ritonavir-boosted atazanavir, 2 - emtricitabine/tenofovir with unboosted atazanavir, 2 - emtricitabine/tenofovir with ritonavir-boosted darunavir, 2 - abacavir/lamivudine with unboosted atazanavir, 2 - emtricitabine/tenofovir with ritonavir-boosted fosamprenavir, 1 - emtricitabine/tenofovir with lopinavir/ritonavir, 1 - lopinavir/lamivudine with lamivudine and raltegravir, 1 - abacavir/lamivudine with ritonavir-boosted fosamprenavir, 1 - abacavir/lamivudine with tenofovir and ritonavir-boosted atazanavir, 1 - abacavir/lamivudine with ritonavir-boosted atazanavir, 1 - emtricitabine/tenofovir with unboosted fosamprenavir, 1 - abacavir/lamivudine with unboosted fosamprenavir, 1 - abacavir/lamivudine with lopinavir/ritonavir and didanosine, and 1 - tenofovir and lamivudine and ritonavir-boosted darunavir (ritonavir was part of the treatment of 21 of 27 patients in this group); in the NNRTI-treated group 23 subjects received efavirenz/emtricitabine/tenofovir, 1 etravirine with raltegravir and ritonavir-boosted darunavir, 1 - abacavir/lamivudine with efavirenz, 1 - tenofovir with didanosine and efavirenz, and 1 - emtricitabine/tenofovir with nevirapine (efavirenz was part of the treatment of 25 of 27 patients in this group). The age, sex and BMI distribution for the groups are presented in Table 1. BMI data were not collected from control group, but all subjects in this group were non-obese. It should be noted that some subjects in treated groups exhibited elevated viral load at the time of testing (6 subjects on PI-based therapy and one on NNRTI-based therapy had viral load above 1,000 copies/ml, and 9 on PI therapy

and one on NNRTI therapy had viral loads between 50 and 1,000 copies/ml). In all cases the reason for high viral load was non-compliance, as viral loads were undetectable in follow-up measurements. Nevertheless, given that 15 out of 27 PI-treated patients had detectable viral load on the day of testing (only 1 such patient was in the NNRTI-treated group), we analyzed virus-positive and virus-negative PI-treated patients separately.

## Laboratory Assays

**HDL isolation**—HDL was isolated from plasma samples using gradient purification as previously described <sup>21</sup>. Briefly, plasma density was adjusted to 1.085 g/ml by adding KBr. Samples were centrifuged at 100,000 rpm/18 h/4°C in Ti70 rotor, the bottom HDL fraction was collected, the density was adjusted to 1.21 g/ml by adding KBr, and samples were centrifuged again at 100,000 rpm/18 h/4°C in Ti70 rotor. The upper fraction of HDL was collected and analyzed on a 12% SDS gel to verify albumin depletion. HDL samples were dialyzed against PBS to remove KBr. Total protein was measured by Bradford assay, and apoA-I was measured by ELISA (Mabtech, Cincinnati, OH).

**Cholesterol efflux**—Cholesterol efflux (CE) was measured as described recently <sup>22</sup>. Briefly, human monocyte cell line THP-1 was differentiated by PMA and labeled by incubation with [<sup>3</sup>H]cholesterol for 48 h. Expression of ABC transporters was induced by incubation with LXR agonist TO-901317 (1 μmol/L, 18 h) and cells were incubated for 6 h with apoB-depleted plasma (2%) or isolated HDL (5 μg/ml). Cholesterol efflux was calculated as percentage of released cholesterol.

**PON activity**—PON activity of 5 μg isolated HDL was measured using EnzCheck Paraoxonase Assay kit (Invitrogen) following manufacturer's instructions.

**Mass spectrometry (MS) proteomic and lipidomic profiling**—MS was used to profile protein and lipid content of the purified HDL as follows. To determine the particle protein complement, 50 μg of total HDL protein (determined by BCA assay) was precipitated with 100 L of 100% trichloroacetic acid (w/v) (Sigma, biotech grade, redistilled, 99%) dissolved in H<sub>2</sub>O (Fluka, LC-MS grade), after mixing and a 15 minute 4°C incubation, samples were spun (14,000 rpm, 4°C, 30 min) and the resulting pellets were washed progressively with 1 mL 10% TCA (w/v) followed by 1 mL acetone (Sigma, HPLC grade) and solubilized in 50 mM NH<sub>4</sub>HCO<sub>3</sub> (Sigma, 99.5% purity) and run approximately 4 centimeters on a 12% SDS-PAGE gel. After staining with Coomassie Blue, each gel lane was separated into two individual samples: one containing apoA-I, which comprises approximately 75% of the protein mass of HDL, while the second sample contained the remainder of the gel lane containing the separated lower abundant proteins. After trypsin digestion of the gel bands the resulting peptides were identified using an LTQ Orbitrap Velos system mass spectrometer equipped with a Famos Autosampler, and Surveyor MS Pump (Thermo Electron) with split flow to deliver 200 nL/min to the in-house packed C18 column (75 cm, inner diameter - 18 cm). For each run, 10 μl of reconstituted sample was injected and separated using a gradient of 5–60% buffer B (acetonitrile with 0.1% formic acid) over the course of 90 min. In between each set of samples, standards (a mix of five angiotensin peptides, Michrom BioResources) were analyzed to ascertain column

performance and to determine any potential carryover. The LTQ was operated in the data-dependent mode with a top five configuration (one full MS scan followed by five MS/MS scans). Dynamic exclusion was set to 1 with a limit of 30 s. Peptides were identified using the Sequest software package through the Bioworks Browser 3.3.1. Sequential database searches were made using the NCBI RefSeqHuman Data base using differential carbamidomethyl-modified cysteines and oxidized methionines. Peptide score cutoff values were chosen at a cross-correlation of 1.8 for singly charged ions, 2.5 for doubly charged ions, and 3.0 for triply charged ions, along with CN values of 0.1 or greater, and RSP values of 1. The cross-correlation values chosen for each peptide assure a high confidence match for the different charge states, whereas the CN cutoff insures the uniqueness of the peptide hit. The rank score preliminary (RSP) value of 1 insures that the peptide matched the top hit in the preliminary scoring.

To profile the lipid species of the HDL particles, an electrospray ionization-MS/MS approach was used as reported previously<sup>23, 24</sup>. In brief, 50 µg of total HDL protein was extracted with 2.5 ml of chloroform/methanol/distilled water (1:1:0.5, v/v), and two 0.5-ml chloroform extractions. Combined organic phases were washed with 0.5 ml of KCl (1 M, 1 volume) and with 0.5 ml of dH<sub>2</sub>O (2 volumes) and dried with N<sub>2</sub> gas. Samples were resuspended in 1 ml of chloroform, mass of extracted lipids was determined by drying 10 ml of the extracted lipids and measuring the resulting mass in an electro-microbalance (Sartorius Cubis), and phospholipid scans were performed as described<sup>23, 24</sup>. To analyze ceramides (Cers) and glucosylceramides (GlcCers), additional scans were performed for precursors of m/z 264 in the positive mode (collision energy, 50 V) for GlcCers and high mass Cers and neutral loss of m/z 316 in the negative mode (collision energy, 50 V) for low mass Cers. Internal standards for quantitating these sphingolipids were d18:1/14:0Cer and d18:1/12:0GlcCer. To measure oxidized phospholipids within the HDL particles, we focused on phosphatidylcholine, the most abundant species and quantitated levels of the following oxidized acyl side chains: oxovaleric, oxo-nonanoic, hydroxy-octadecadienoic, hydroxy-octadecenoic, hydroperoxy-octadecadienoic, unknown structure). The amounts of the analyte lipids are indicated in units of normalized mass spectral signal with 1 unit representing the amount of lipid producing the same amount of signal as 1 nmol of the internal standard. These amounts are then expressed as nanomole lipid species per µg of total HDL lipid extract.

**Principal Components analysis**—Multivariate transformations, normality transformations, data imputation and principal components analysis (PCA) were performed using the Microsoft Excel implementation of the R statistical program, imDEV v1.4.2<sup>25</sup>. Data was transformed to normality prior to statistical analyses using the procedures of Box and Cox<sup>26</sup>. Imputation of missing values was performed using probabilistic PCA, if greater than 80% of the dataset was available. The PCA was calculated using the NIPALS model, univariate scaling, and mean centering.

**Statistical Analysis**—HIV viral load below the limit of detection (48 copies/ml) was imputed at 24. For group comparisons, variables were examined for evidence of non-normality and tested with parametric or non-parametric tests, accordingly. Parametric testing

of group differences was by one-way ANOVA, followed, when statistically significant ( $p < 0.05$ ), by t-tests of all group pairs. Non-parametric testing followed the same procedure using the Kruskal-Wallis and Wilcoxon Sum Rank tests. This two-step procedure was designed to balance type 1 error rates and statistical power.

## Results

Table 1 shows clinical characteristics of the study participants. Control (uninfected) subjects were younger and this group contained more females; however, the four HIV-infected groups were similar in terms of age, gender and BMI. The viral load in the untreated group was higher than in the PI-treated viral load positive ( $p < 0.001$ ) and the NNRTI-treated ( $p < 0.0001$ ) groups, but differences in CD4+ T cell counts were not statistically significant. Unexpectedly, LDL-C levels were higher and triglyceride (TG) levels were lower in subjects in HIV-infected groups compared to controls, although TG differences were not statistically significant. The PI subgroup with detectable viral load had lower LDL-C and higher TG levels than the subgroup with undetectable virus (Table 1), but not at a statistically significant level. In comparison to uninfected controls, a statistically significant decrease in HDL levels was observed in the untreated group of HIV-infected subjects ( $p < 0.001$ ), but, in contrast to previous reports<sup>16, 17</sup>, no statistically significant differences in HDL levels were seen between the control group and groups of HIV-infected subjects treated with PI- or NNRTI-based HAART (Table 1). As has been previously reported<sup>27, 28</sup>, HDL levels in both subgroups of PI-treated patients ( $56.2 \pm 26.8$  mg/dl for virus-negative patients and  $54.4 \pm 21.1$  mg/dl for virus-positive patients) and NNRTI group ( $54.1 \pm 22.6$  mg/dl) were higher than in untreated group ( $45.1 \pm 11.1$  mg/dl), but the differences did not reach statistical significance. When cholesterol efflux to apoB-depleted plasma was analyzed, efflux to plasma from each group of HIV-infected subjects was significantly lower than efflux to plasma from control subjects (Table 2). Both subgroups of PI-treated subjects, one with undetectable viral load (cholesterol efflux of  $6.9 \pm 1.9\%$ ), and another with detectable viral load (cholesterol efflux of  $7.3 \pm 2.7\%$ ), showed a trend towards increased cholesterol efflux relative to untreated HIV-infected group (cholesterol efflux  $6.4 \pm 1.4\%$ ). Lack of significant differences between the subgroups of PI-treated subjects in HDL content and cholesterol efflux is likely due to transitory nature of viral load increase. Results of this analysis are consistent with our previous report, which suggested that increase of cholesterol efflux in patients treated with anti-retroviral drugs is likely due to an increased abundance of HDL particles<sup>9</sup>. However, these results indicate that ART, while reversing the reduction of HDL in HIV patients, does not fully restore HDL functionality.

To characterize the effects of HIV infection and ART on HDL composition, we purified HDL particles from a subset of samples (10 control, 14 untreated, 15 NNRTI-treated, and 5 PI-treated using gradient centrifugation (Fig. 1A) and subjected them to proteomics analysis. Of note, a subset of samples for this analysis that came from ART-treated patients included four subjects with detectable viral load: two from NNRTI-treated group (viral load 120 and 35,020 copies/ml) and two from PI-treated group (viral load 389 and 2,070 copies/ml); other ART-treated patients had undetectable virus in the blood. Samples with detectable viral load did not stand out from other samples in the analysis. To profile the protein complement of the particles we used an approach described by Vaisar et al.<sup>29</sup>. During implementation of



this method we found that direct analysis of the crude trichloroacetic acid (TCA) precipitated protein derived from the particles did not show a strong positive linear correlation between the input HDL protein mass and the number of total or unique identified peptides. Hence, we added a one dimensional SDS-PAGE separation of the TCA precipitated and solubilized material into two fractions - a gel slice containing apoA-I, which constituted approximately 75% of the protein, and the remainder of sample lane, containing lower abundant proteins. With this step added, we obtained an acceptable dose response relation between input HDL protein mass and the total number of identified peptides (Fig. 1B). To characterize this data set, we initially used principal component analysis (PCA) to test if the various groups contained distinct protein cargos (Fig. 1C). This analysis showed a clear separation of the control HDLs from the HDLs of HIV-infected subjects, but did not further segregate the HIV treatment arms. The PCA loading values that were separating out the controls included a number of apolipoproteins, but interestingly also included paraoxonase (PON) 1 and 3 (Figs. 1D and E). Likewise, the computed peptide index for the data set derived as described by Vaisar et al.<sup>29</sup> showed significantly lower PON1 and 3 associated peptides in the HIV-positive samples ( $p < 1 \times 10^{-10}$  and  $p < 1 \times 10^{-6}$ , respectively, with Bonferroni correction for multiple tests, Fig. 2A). Another peptide underrepresented in HDL from HIV-infected subjects was prenylcysteine oxidase 1 (PCYOX1). To verify the correlation between the abundance of peptides identified by our MS method for PON1 and protein levels, we conducted an immunoblot analysis of PON1 in a subset of the HDLs from control and ART-naïve HIV-positive subjects, which confirmed the lower level of this protein in the HIV-positive samples (Fig. 2B). We further measured PON activity in HDL samples, using 5 µg of protein per assay. Again, PON activity of HDL from HIV-infected groups was significantly lower than the activity of control HDL (Table 3). It should be acknowledged that the differences in PON activity were not as dramatic as differences in protein levels. A possible reason for this discrepancy is an excess of PON substrates in HDL from all groups, resulting in close to saturation conditions of the assay<sup>30</sup>. It may also be related to differences in PON distribution between large and small HDL particles in HIV-infected and control samples, as PON activity depends very much on the HDL particles it is located on<sup>31</sup>.

To further characterize the effect of HIV infection on functional activity of HDL particles, we analyzed cholesterol efflux to isolated HDL. Surprisingly, and in seeming conflict with the efflux to apoB-depleted sera, cholesterol efflux to HDL from HIV-infected subjects trended to be higher than to HDL from control group, although the difference was not statistically significant (Table 4). However, a strong and statistically significant negative correlation was revealed between the viral load and cholesterol efflux to HDL from untreated group (Fig. 3). No such correlation was observed within ARV-treated groups, likely because of transient nature of virus replication in these subjects.

We next profiled particle lipid composition using an electrospray ionization-MS/MS approach, which measures over 400 individual lipid species. Using increasing amounts of input total HDL, we confirmed detection of both highly prevalent species (e.g., PC) as well as low abundant species (e.g., lysoPC) in a linear manner across an acceptable dynamic range (Fig. 4A). When the samples from different groups were analyzed, we found that the particles from ART-naïve HIV-positive subjects trended to have lower total PC levels, while

showing an increase in less abundant species, including the sphingomyelins and other potentially pro-inflammatory ether-linked glycerophospholipid species; significant differences were found in the levels of phosphatidic acid, which was increased in HIV-infected untreated group relative to controls, and returned to levels not significantly different from controls in NNRTI-treated group (Table 5). Moreover, a number of significant correlations were observed between the lipid levels in the ART naïve HIV-positive samples and viral load (Fig. 4B) and CD4+ T cell counts (Figs. 4C-E). Here, sphingomyelin and ether-linked glycerophospholipid species in the HDL particles correlated positively or negatively with viral load and CD4+ T cell counts, respectively. Given these correlations of lipid levels, we tested for correlations of phospholipid transfer protein (PLTP) to viral load and CD4+ T cell counts. Consistent with analysis of lipids, the level of PLTP showed a significant positive correlation with viral load and was negatively correlated with CD4+ T cell counts in ART-naïve HIV-positive samples (Fig. 5).

Given the low PON1 and PON3 levels in HDL from HIV-infected subjects, we tested for the level of oxidized phospholipids in the HDL particles from a subset of HIV+ ART-naïve and HIV+ NNRTI-treated subjects with completely suppressed viremia; these groups were well matched for age and gender. Interestingly, a significant increase in the level of 5 out of the 6 oxidized lipid species measured was detected in the NNRTI-treated samples (Fig. 6A). No significant differences in oxidized lipids were found between HDL from control and untreated or PI-treated subjects (not shown). To test whether the increased level of oxidized lipid seen in the HDL particles was potentially a more general effect of the NNRTI drug therapy, we also measured the level of oxidized LDL using an ELISA approach. Although the absolute level of oxLDL detected in this assay did not significantly differ between samples from HIV+ ART-naïve subjects and those from NNRTI-treated patients (n=10, 22.7±4.5 U/L versus n=11, 23.6±6.4 U/L, respectively), when these values were normalized to total LDL levels the ratio of oxLDL to LDL was significantly increased in the NNRTI-treated samples (Fig. 6B). These results suggest that reduced PON levels in HDL of HIV-infected subjects do not translate to increased levels of oxidized lipids, however, low PON may contribute to increased oxidation in NNRTI-treated patients.

## Discussion

In this study, we characterized functional and structural features of HDL from a cross-sectional group of HIV-infected subjects treated or not with anti-retroviral drugs. Cholesterol efflux to apoB-depleted plasma from all HIV-infected patients was reduced relative to efflux to plasma from control individuals. Interestingly, cholesterol efflux did not fully correlate with HDL concentration in the plasma, as HDL levels in PI- and NNRTI-treated groups were not reduced relative to control group in our study subjects, whereas cholesterol efflux was significantly reduced. This result indicates a functional defect in HDL from HIV-infected subjects, and suggests that HAART treatment does not fully reverse this defect. It also minimizes the bias introduced by a higher number of women in the control group: higher HDL levels in pre-menopause women would have increased the differences in cholesterol efflux to plasma. The HIV-induced defect in HDL functionality was supported by a strong negative correlation between cholesterol efflux to HDL isolated from plasma of ART-naïve HIV-infected subjects and viral load, indicating that HIV infection contributes to



the impairment of this function of HDL. However, no significant differences were observed between the groups in cholesterol efflux to isolated HDL; if anything, cholesterol efflux trended to be higher in HIV-infected groups. This observation suggests a compensatory reaction of the organism to infection that compensates for virus-induced impairment (see below). Such interpretation is supported by a slightly higher efflux to HDL from PI-treated subjects that had detectable viral load ( $6.24\% \pm 2.12\%$ ) compared to subjects in this group with undetectable viral load ( $6.07\% \pm 1.68\%$ ), although this difference did not reach statistical significance. Also, no correlation between cholesterol efflux to HDL and viral load was detected in ART-treated groups, despite detectable viral load in a number of subjects. A likely explanation to this inconsistency is a transient nature of viral replication in ART-treated subjects, due to non-compliance.

Decreased levels of PON1 and PON3 in HDL from HIV-infected subjects compared to the control samples is an intriguing finding of this study, and supports previous report of decreased PON1 activity in HIV-infected subjects, both treated and untreated<sup>32</sup>. While we cannot fully exclude the possibility that the observed result was affected by the gender and age differences between HIV-infected and control samples, sub-analysis of 6 samples from control females and 4 samples from HIV-infected females (3 from untreated group and 1 from NNRTI-treated group) matched by age (control:  $42.7 \pm 3.8$ ; HIV+:  $45.5 \pm 4.7$ ;  $p=0.65$ ) confirmed significant differences in PON1 (control:  $37.3 \pm 4.5$ ; HIV+:  $8.0 \pm 4.1$ ;  $p=0.0019$ ) and PON3 (control:  $22.0 \pm 2.0$ ; HIV+:  $3.3 \pm 2.5$ ;  $p=0.0006$ ). PON1 and PON3 are enzymatic proteins located on the surface of HDL particles that have the ability to prevent the oxidation of phospholipids in low density lipoproteins (LDL), as well as alter the oxidative state of macrophages<sup>33</sup>. In addition, these proteins have been shown to influence the cholesterol efflux activity of HDL, in part by catalyzing lysophosphatidylcholine (lysoPC) formation on target cells thus increasing HDL binding<sup>34</sup>. PON1 has been shown to be an important anti-atherosclerotic component of plasma HDL<sup>35</sup>, and low PON1 levels have been prospectively shown to predict new coronary events<sup>36</sup>. There have only been a few studies published to date looking at the association between HIV infection and PON levels, and these have produced conflicting results<sup>32, 37-39</sup>. Daminelli et al. demonstrated significantly decreased activity of PON1 in HIV-infected subjects compared to uninfected controls, and ART treatment did not reverse this deficiency<sup>32</sup>. Parra et al. also found significantly decreased PON1 activity, but saw increased PON1 concentrations in HIV-infected subjects compared to controls<sup>39</sup>. We measured both the activity and concentration of PON in HDL particles, and found decreased levels of both. In view of the findings by Parra et al., a potential explanation of our results is that a defect in incorporation of PON1 into HDL, rather than low PON abundance in these patients, was responsible for low PON levels we observed. PON1 activity measured in isolated HDL was also found to inversely correlate with age<sup>40</sup>, which, given that our control group was younger than HIV-infected subjects, could contribute to lower observed PON1 activity. Similar to PON1, the study by Aragonès et al. reported a 3-fold increase in PON3 concentrations in HIV-infected subjects relative to control subjects, and this difference was partially reversed in patients treated with NNRTI-based ART<sup>38</sup>. Again, incorporation of PON3 into HDL particles may be impaired in HIV-infected subjects. Interestingly, we also found decreased levels of prenylcysteine oxidase 1 (PCYOX1) corresponding peptides in HDL samples from HIV-infected subjects. This

enzyme cleaves the thioether bond of prenylcysteines in lipid-modified proteins to generate H<sub>2</sub>O<sub>2</sub>, and may contribute to pro-atherogenic activity of LDL<sup>41</sup>, however, its role in HDL has not been established.

Comparing HDL from HIV-infected patients to control samples, we found increased levels of oxidized phospholipids only in HDL from NNRTI-treated subjects, but not in untreated or PI-treated groups. This finding suggests that PON levels are not the bottleneck for lipid oxidation, but low PON may contribute to NNRTI-induced oxidation. Increased lipid oxidation in NNRTI-treated subjects is supported by our finding of a significant increase in the oxLDL to LDL ratio in this group relative to either HIV+ ART-naïve subjects or subjects in the PI-treated group. Thus, although a number of our findings indicated that viremia may be a significant factor in modifying the HDL particle composition and function, viremia is not correlated with an increase in oxidized lipid associated with either HDL or LDL. That a NNRTI regimen may cause a general increase in the level of oxidized lipids carried by HDL and LDL is suggested by these results but will need confirmation in larger longitudinal studies.

Another interesting finding of this study is a positive correlation between etherPS levels in the HDL particles from the ART-naïve subjects and the viral load. Moreover, when individuals on ART with detectable viral load were included in the linear regression analyses, the positive association between viral load and ePS was not changed (not shown). Phosphatidylserine is the key HDL phospholipid that determines the magnitude of efflux and other functional activities of HDL<sup>42</sup>, and an increase in ePS levels may be a compensatory reaction to HIV infection. Therefore, it appears that the functional status of cholesterol efflux activity of HDL depends on the balance between virus-induced damage to HDL, such as depletion of the PON factors, and compensatory reactions of the organism, such as increase in HDL phosphatidylserine. However, PON1 depletion is unlikely to be a major cause of changes in cholesterol efflux capacity of HDL in our subjects, as we did not observe correlation between cholesterol efflux and PON1 content in HDL, although previous study in diabetic patients demonstrated that enriching HDL with PON1 increased cholesterol efflux in vitro<sup>43</sup>. Additionally, and consistent with viral load correlations, in the ART-naïve subjects there was a negative correlation between CD4+ T cell counts and the levels of HDL sphingomyelins, etherPS, and lysoPC. When samples from ART patients with detectable viral load were included in this analysis, the negative correlation between CD4+ T cell levels and ePS or PLTP was preserved but not strengthened (not shown), likely because of a transient nature of viral load rise which does not significantly affect CD4+ T cells. Thus, our results indicate remodeling of lipid and protein content of the HDL particles in HIV-infected subjects. The increased level of sphingomyelin in the HDL of ART-naïve patients with low CD4+ T cell counts is of interest since HDL with excess sphingomyelin has been previously associated with impaired HDL biogenesis and maturation in Niemann-Pick disease<sup>44</sup>. Interestingly, in the ART-treated groups we did not find a correlation between sphingomyelin levels and CD4 counts. This difference may, in part, explain why we saw an efflux defect only in HDL particles from the ART-naïve samples and not the ART-treated samples.

There are several limitations of our study. The number of subjects in groups was small, in particular in subsets used for proteomics and lipidomics analyses, which could increase the possibility of false positive results. Our control group was a convenience group of adults recruited from clinical personnel and had a different gender ratio (more females) than HIV-infected groups, which may have impacted our comparisons. However, when male and female samples were compared separately the results did not change: cholesterol efflux to apoB-depleted plasma and PON activity were significantly lower in HIV-infected subjects than in controls, whereas cholesterol efflux to HDL did not significantly differ (Table 6). There was also a difference in age between control and infected groups. While the age of control and untreated groups was not significantly different by statistical analysis, there was statistically significant difference between control group, which was younger, and two drug-treated groups. However, correlation analyses performed in HIV-infected groups do not suffer from these limitations. In addition, most of our analyses were performed on isolated HDL, thus negating the bias in HDL levels introduced by age and gender differences, which, to published reports, mainly influence HDL levels<sup>45-47</sup>. Finally, PON1 haplotypes were shown to segregate with HIV infection, and certain haplotypes associated with higher HDL levels and better CD4+ T cell recovery after treatment<sup>48</sup>, whereas we did not consider the PON gene polymorphisms in our study.

Despite these limitations, the findings of our study are provocative and warrant further investigation. A particularly important question to be addressed in future studies is the mechanism of PON reduction in HDL. We did not detect a significant correlation between PON levels and viral load or CD4 counts, and there was no significant difference in PON levels between ART-treated and untreated groups, suggesting that some features of chronic infection, such as persistent inflammation, rather than HIV itself, may be responsible for PON disbalance in HDL. The functional effect of PON reduction remains unclear, as the only group with increased oxidation levels was NNRTI-treated group. The oxidative effect of NNRTI treatment and the role of reduced PON in this phenomenon remain to be confirmed in larger studies. We also did not find a correlation between PON levels and cholesterol efflux, suggesting that PON deficiency may not be the major contributor to virus-induced impairment of this HDL function. In summary, our results indicate substantial remodeling and functional impairment of HDL in HIV-infected subjects, which are likely responsible for increased risk of atherosclerosis in HIV infection, even in ART-treated individuals with higher levels of HDL.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>ART</b>	anti-retroviral treatment
<b>CE</b>	cholesterol ester
<b>Cer</b>	ceramide
<b>DSM</b>	dihydrosphingomyelin
<b>ePC</b>	etherphosphatidylcholine
<b>ePS</b>	etherphosphatidylserine
<b>ePE</b>	etherphosphatidylethanolamine
<b>Hex-Cer</b>	hexylceramide
<b>HIV</b>	human immunodeficiency virus
<b>LPE</b>	lysophosphatidylethanolamine
<b>LPC</b>	lysophosphatidylcholine
<b>LXR</b>	liver X receptor
<b>NNRTI</b>	non-nucleoside reverse transcriptase inhibitor
<b>PA</b>	phosphatidic acid
<b>PC</b>	phosphatidylcholine
<b>PE</b>	phosphatidylethanolamine
<b>PCYOX1</b>	Prenylcysteine oxidase 1
<b>PI</b>	protease inhibitor
<b>PI<sub>n</sub></b>	phosphatidylinositol
<b>PLTP</b>	phospholipid transfer protein
<b>PON</b>	paraoxonase
<b>PS</b>	phosphatidylserine
<b>VCAM</b>	vascular cell adhesion molecule
<b>VL</b>	viral load
<b>TCA</b>	trichloroacetic acid

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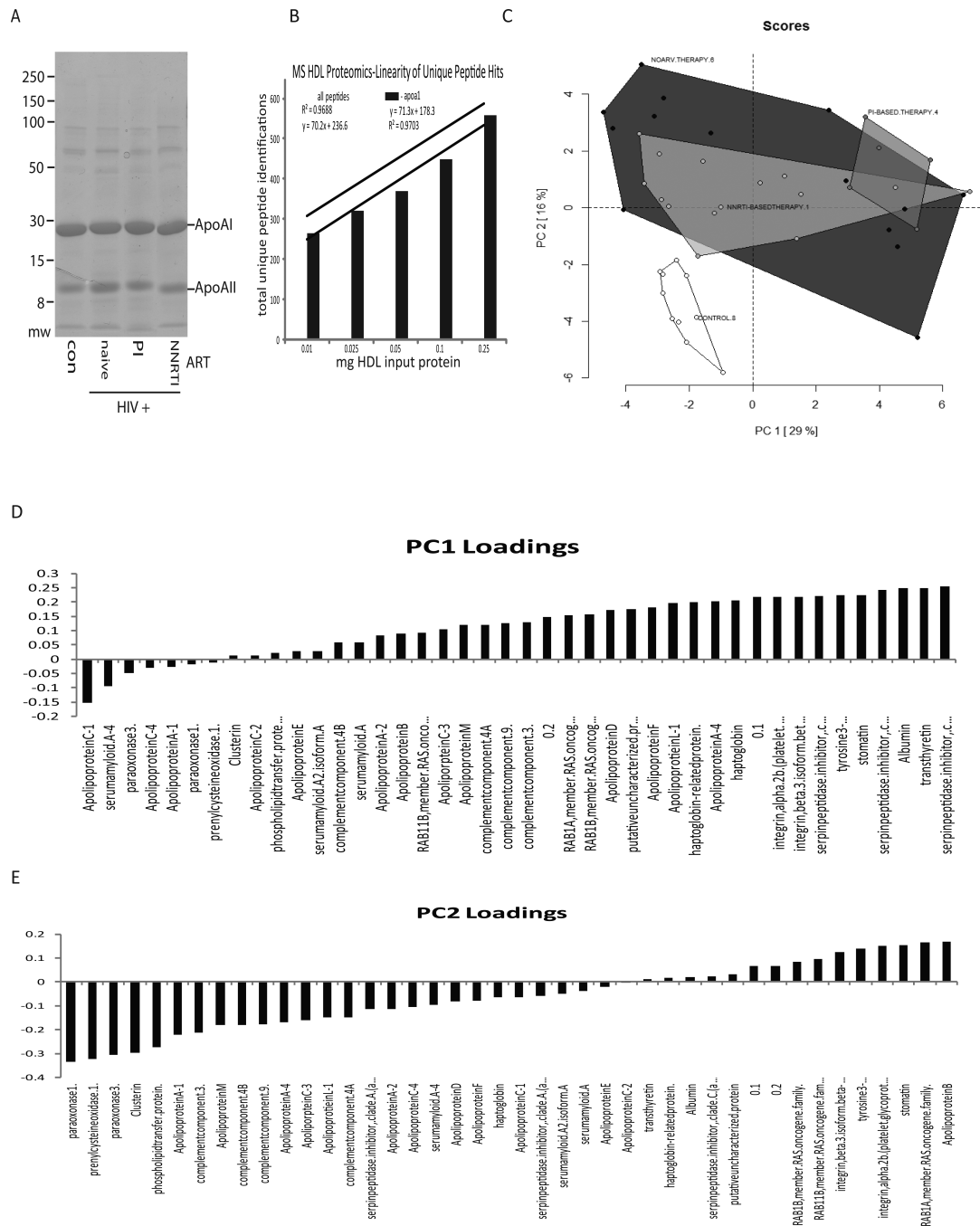
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### Highlights

- HDL particles from HIV-infected subjects have reduced PON levels and PON activity
- EtherPS levels in HDL from ART-naïve HIV patients correlated with viral load
- Cholesterol efflux to HDL from ART-naïve subjects negatively correlated with HIV load
- Oxidized LDL were increased in NNRTI-treated patients
- Our results indicate remodeling of lipid and protein HDL content in HIV patients



**Figure 1. Proteomic profiling of HDL particles**

A - Purified HDLs from the indicated groups were acetone precipitated and analyzed by SDS-PAGE. Panel shows representative lanes from the sample run. B - Graph shows a linear response of total and ApoA-I peptide identifications with increasing amounts of input total HDL protein. C - Principal component analysis of all analyzed samples (n=45) indicates a significant separation in the proteomic content of the control HDL particles versus the HDL particles isolated from HIV-positive individuals. Scores from PC1 and PC2 explained 45% of the total variance within the data set. Loading plots from both PC1 and

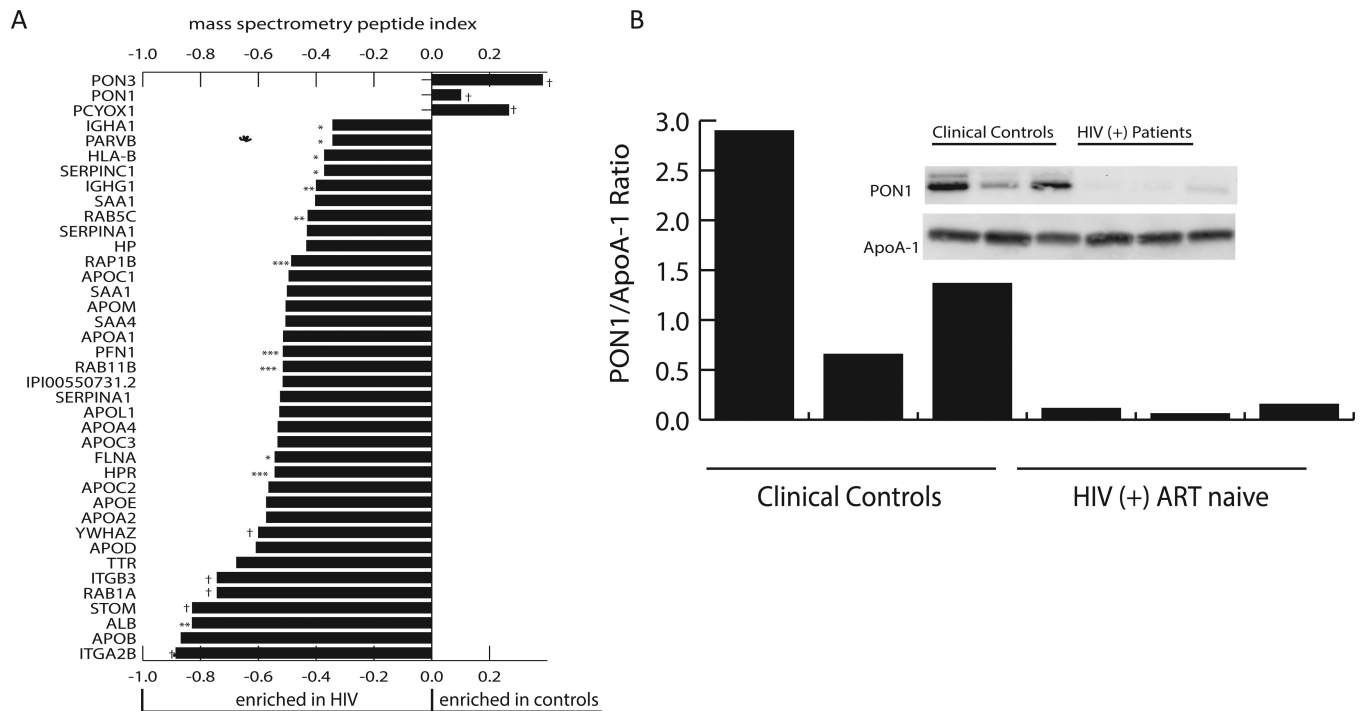
PC2 are also presented to illustrate variables driving separation of experimental groups. The PCA plot shown was used as a tool for data exploration. D and E - Proteins associated with the two principal components affecting the separation of the groups.

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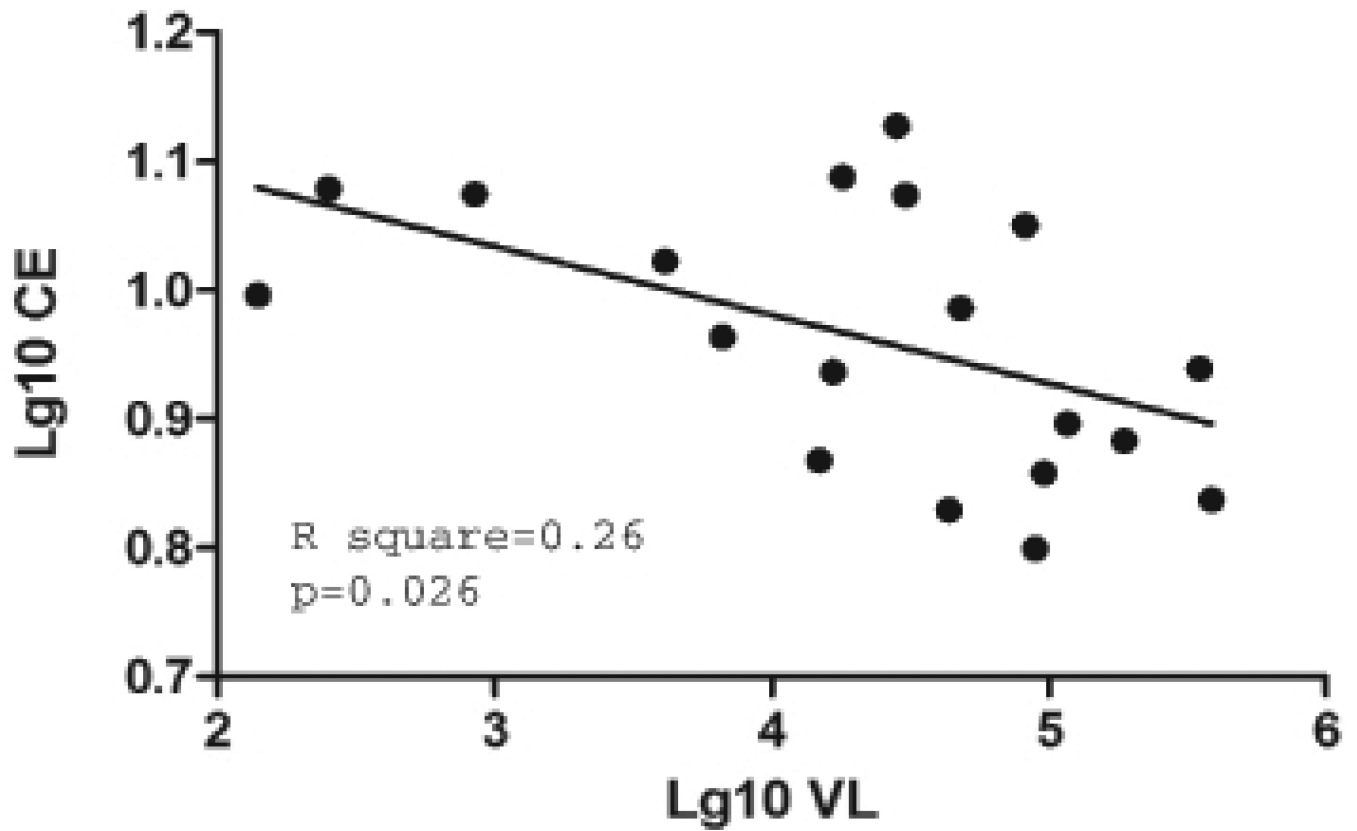
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**Figure 2. Analysis of PON in HDL particles**

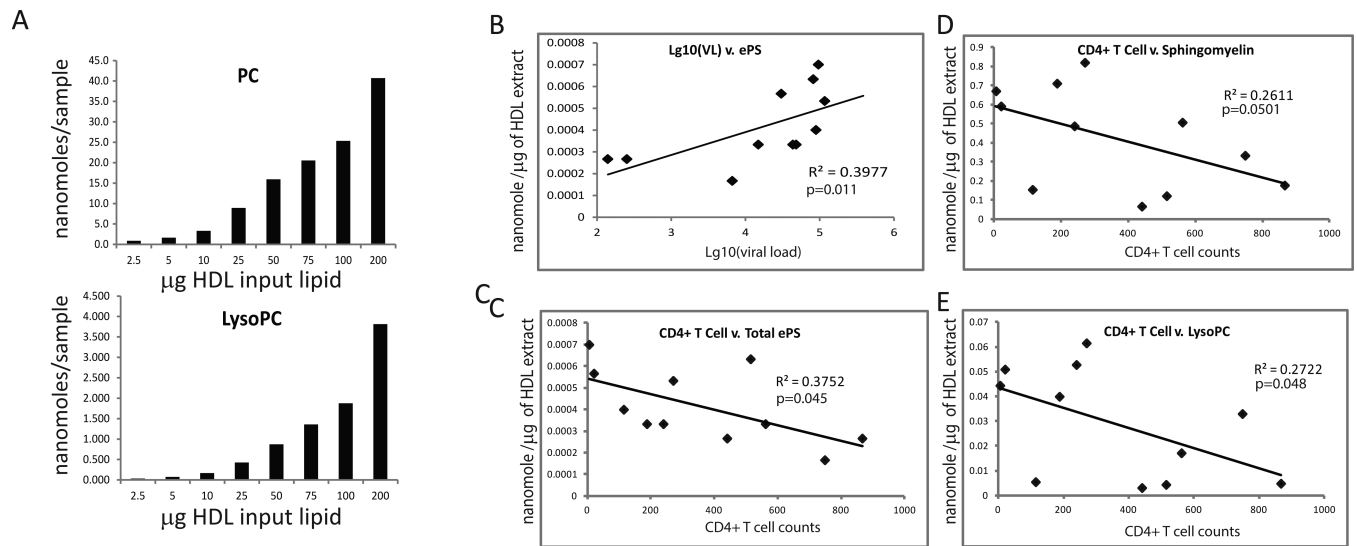
A - Peptide indices for HDL associated proteins that showed marked differences between control and HIV infected samples. Positive scores indicate a greater abundance of the protein in the HDL from control samples, while negative scores indicate a greater abundance of the protein in the HDL from HIV-positive subjects. Peptide indices of the uninfected controls and HIV-positive samples were compared via a Student's t-test. \* indicates significance at the  $p=0.05$  level, \*\* indicates significance at the  $p=0.01$  level, \*\*\* indicates significance at the  $p=0.001$  level, and † indicates significance with  $p<0.0001$ . All  $p$  values were Bonferroni corrected for multiple tests ( $n=163$  comparisons). B - Analysis of PON1 and apoA-1 levels by Western blotting in HDL from 3 controls and 3 HIV-positive ART-naïve subjects.



**Figure 3. Analysis of cholesterol efflux to isolated HDL**

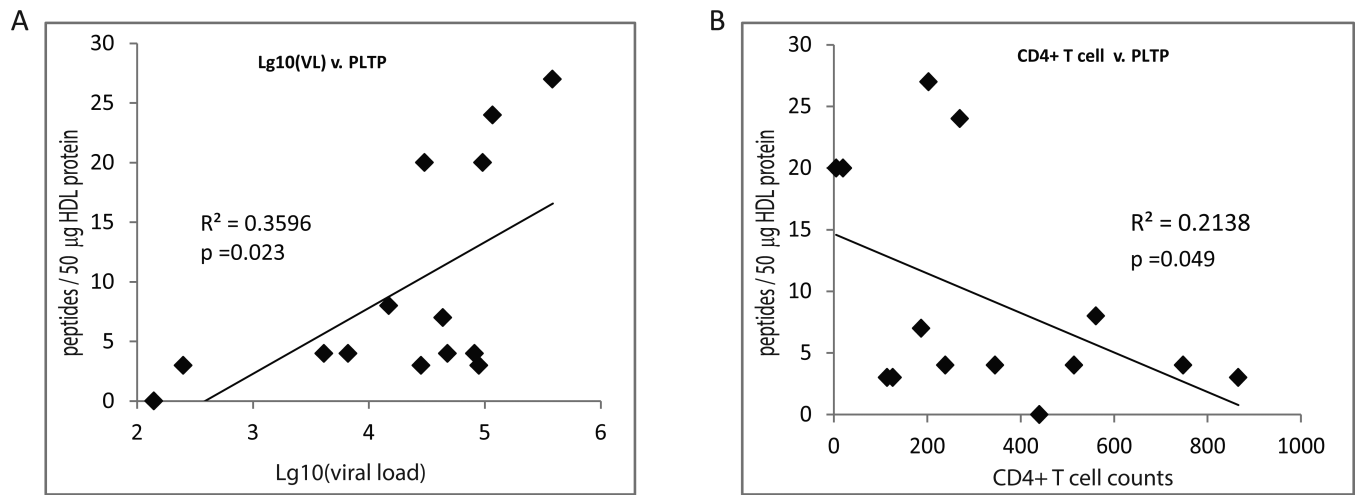
Spearman correlation analysis between viral load and cholesterol efflux to isolated HDL was performed on samples from ART-naïve group. Cholesterol efflux was measured in THP-1 cells incubated with HDL (20 µg/ml) as described in Materials and Methods.



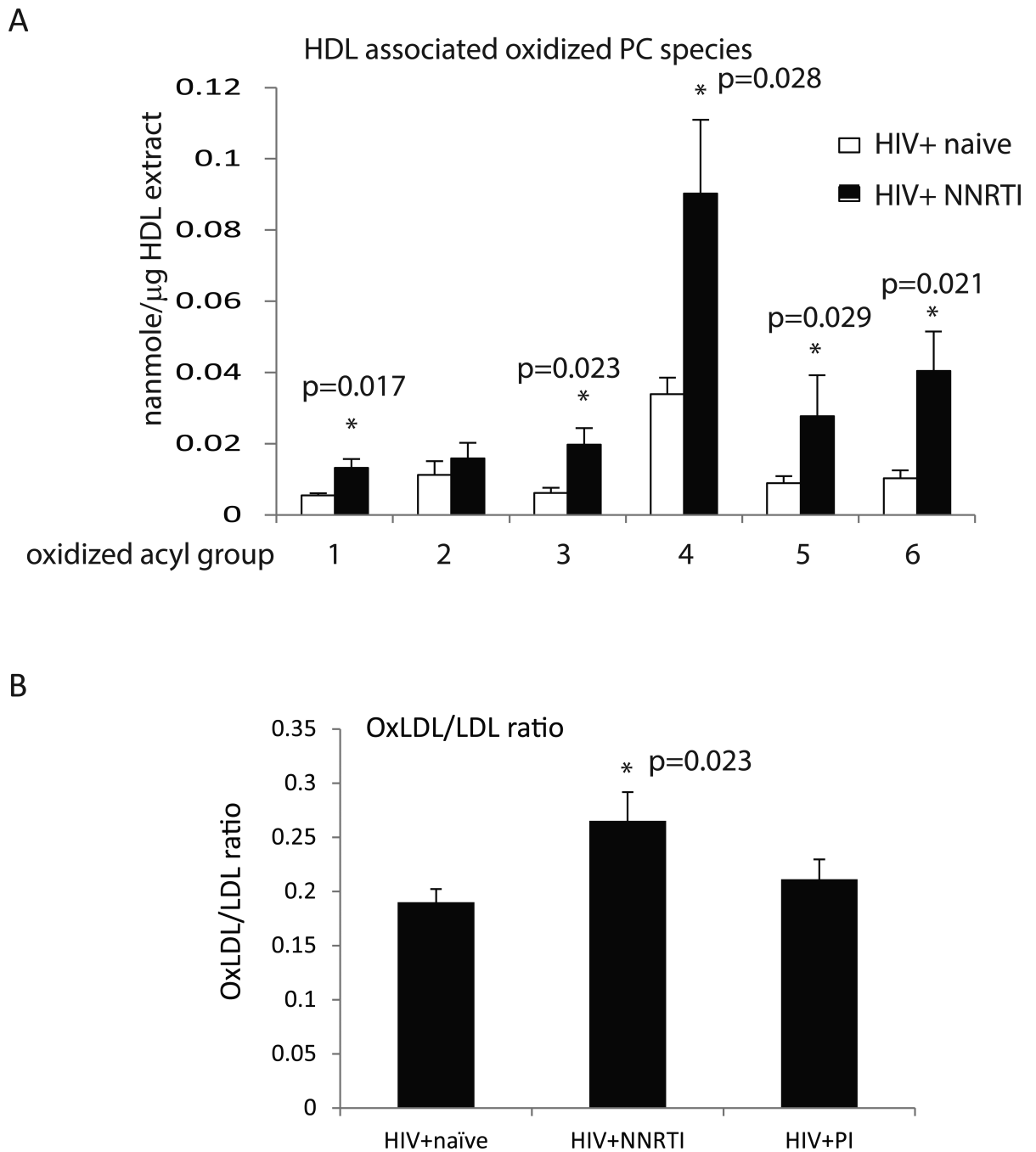


**Figure 4. Analysis of lipids in HDL particles**

A - Graphs show that both PC and lysoPC levels are detected in a linear manner with increasing amount of input total HDL lipid. B-E - Spearman correlation analyses of the indicated lipid species relative to viral load and CD4+ T cell counts in the HIV-positive ART-naïve samples.



**Figure 5. Spearman correlation analysis of HDL PLTP**  
Graphs show significant correlations between the level of HDL-associated PLTP peptides and viral load (A) or CD4+ T cell counts (B) in the HIV-positive ART-naïve subjects.



**Figure 6. Analysis of oxidized lipid in HDL and LDL**

A – Graph shows levels of oxidized PC species in HIV+ ART-naïve (n=5) and HIV+ NNRTI-treated subjects with completely suppressed viremia (n=5). Data are presented as mean  $\pm$  SEM, \* - significant p values are shown above the bars. Lipid acyl side chains: 1 - oxovaleric, 2 - oxo-nonanoic, 3 - hydroxy-octadecadienoic, 4 - hydroxy-octadecenoic, 5 - hydroperoxy-octadecadienoic, 6 - unknown. B - Graph shows the ratio of oxLDL to LDL in samples from HIV+ ART-naïve (n=10), HIV+ NNRTI-treated (n=11), and HIV+ PI-treated

(10). Data are presented as mean  $\pm$  SEM, the difference between untreated and NNRTI-treated groups is significant ( $p=0.023$ ).

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**Table 1**Clinical characteristics of study participants<sup>1</sup>

	Control (n=19)	No ART (n=21)	PI VL+ (n=15)	PI VL- (n=12)	NNRTI (n=27)
Age (years)	38.4±10.2	45.0±12.9 <sup>a</sup>	47.7±7.2 <sup>a</sup>	47.9±9.7 <sup>a</sup>	51.4±9.7 <sup>a,b</sup>
Gender (% male)	42%	75%	73%	75%	85% <sup>c</sup>
BMI	ND <sup>2</sup>	25.2±2.6 (n=15)	28.6±9.2 (n=14)	29.7±6.5 (n=10)	25.6±5.8 (n=24)
Log VL <sup>3</sup>	ND <sup>2</sup>	4.4±1.0	2.9±1.1 <sup>d</sup>	Neg <sup>4</sup>	1.5±0.6 <sup>e</sup>
CD4 (cells/mm <sup>3</sup> )	ND <sup>2</sup>	357±257	337±250	555±354	537±365
LDL (mg/dl)	74.8±23.2 (n=8)	110.3±30.0 <sup>a</sup> (n=20)	84.1±28.2 <sup>b</sup> (n=14)	101.3±38.3 <sup>a</sup>	85.6±24.6 <sup>b</sup>
TG (mg/dl)	143±70.5 (n=8)	118.4±62.3	88.0±37.2 (n=14)	125.3±41.4	126.8±82.9
HDL (mg/dl)	56.0±7.5	45.1±11.1 <sup>f</sup>	54.4±21.1	56.2±26.8	54.1±22.6

<sup>1</sup> Means±SD are shown; table cell n shown when there are missing values<sup>2</sup> ND – not determined<sup>3</sup> base 10, below limit of detection imputed as 24 copies/ml<sup>4</sup> Neg - negative.<sup>a</sup> p<0.05 relative to Control<sup>b</sup> p<0.05 relative to No ART<sup>c</sup> p<0.004 relative to Control<sup>d</sup> p<0.001 relative to No ART<sup>e</sup> p<0.0001 relative to No ART and PI VL+<sup>f</sup> p<0.001 relative to Control

**Table 2**Cholesterol efflux to apoB-depleted plasma<sup>1</sup>

	Control (n=8)	No ART (n=19)	PI VL+ (n=15)	PI VL- (n=10)	NNRTI (n=25)
CE (%)	10.5±0.9	6.4±1.4 <sup>a</sup>	7.3±2.7 <sup>a</sup>	6.9±1.9 <sup>a</sup>	7.5±3.0 <sup>a</sup>

<sup>1</sup> Only a subset of samples was used for this assay due to limited sample availability. Means ± SD are shown.

<sup>a</sup> p<0.05 relative to Control.

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**Table 3**HDL PON activity<sup>1</sup>

	Control (n=19)	No ART (n=20)	PI VL+ (n=9)	PI VL- (n=10)	NNRTI (n=21)
PON activity (U/ $\mu$ l) <sup>2</sup>	0.13 $\pm$ 0.01	0.12 $\pm$ 0.01 <sup>a</sup>	0.11 $\pm$ 0.01 <sup>b</sup>	0.12 $\pm$ 0.01 <sup>c</sup>	0.11 $\pm$ 0.01 <sup>b</sup>

<sup>1</sup> Only a subset of samples was used for this assay due to limited sample availability.

<sup>2</sup> Shown are means  $\pm$  SD measured on 5  $\mu$ g of isolated HDL.

<sup>a</sup> p=0.0035 relative to control group

<sup>b</sup> p<0.0001 relative to control group

<sup>c</sup> p=0.0164 relative to control group.

**Table 4**Cholesterol efflux to HDL<sup>1</sup>

	Control (n=19)	No ART (n=18)	PI VL+ (n=10)	PI VL- (n=11)	NNRTI (n=25)
CE (%)	5.3±1.04	6.43±1.8	6.24±2.12	6.07±1.68	6.28±1.65

<sup>1</sup> Only a subset of samples was used for this assay due to limited sample availability. Means ± SD are shown. The differences between the groups were not statistically significant.

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**Table 5**Lipidomic changes in HDL particles as assessed by mass spectrometry<sup>1</sup>

Lipids (nanomole/ $\mu$ l)	Controls (n=10)	HIV+, ART naive (n=11)	HIV+, NNRTI (n=15)
lysoPC	0.025 $\pm$ 0.13 [0.020]	0.029 $\pm$ 0.022 [0.033]	0.022 $\pm$ 0.12 [0.022]
PC	3.36 $\pm$ 0.95	2.78 $\pm$ 1.34	2.56 $\pm$ 1.053
SM & DSM	0.34 $\pm$ 0.16	0.42 $\pm$ 0.26	0.33 $\pm$ 0.18
ePC	0.31 $\pm$ 0.12	0.30 $\pm$ 0.15	0.25 $\pm$ 0.11
lysoPE	0.00058 $\pm$ 0.00035	0.00094 $\pm$ 0.00076	0.00084 $\pm$ 0.0010
PE	0.0079 $\pm$ 0.0063 [0.0059]	0.019 $\pm$ 0.028 [0.0098]	0.014 $\pm$ 0.019 [0.0035]
ePE	0.0013 $\pm$ 0.0017 [0.00097]	0.0031 $\pm$ 0.0039 [0.0016]	0.0032 $\pm$ 0.0051 [0.00063]
PI	0.073 $\pm$ 0.036	0.086 $\pm$ 0.061	0.062 $\pm$ 0.034
PS	0.0032 $\pm$ 0.0028 [0.0028]	0.0072 $\pm$ 0.0072 [0.0046]	0.0036 $\pm$ 0.0023 [0.0035]
ePS	0.00028 $\pm$ 0.00017	0.00041 $\pm$ 0.00017	0.00031 $\pm$ 0.00021
PA	0.00049 $\pm$ 0.00028 [0.00048]	0.0011 $\pm$ 0.0053 <sup>a</sup> [0.0011]	0.00074 $\pm$ 0.00019 <sup>b</sup> [0.00070]
CE	18.65 $\pm$ 8.00	19.41 $\pm$ 12.70	17.29 $\pm$ 8.17
Cer	0.016 $\pm$ 0.0048	0.017 $\pm$ 0.0088	0.013 $\pm$ 0.013 $\pm$ 0.0045
Hex-Cer	0.010 $\pm$ 0.0077	0.011 $\pm$ 0.0094	0.0069 $\pm$ 0.0032

<sup>1</sup> Only a subset of samples was used for this assay due to limited sample availability. Means  $\pm$  SD are shown and also medians (in square brackets) for lipid classes with skewed distributions.

<sup>a</sup> p=0.006 relative to control group.

<sup>b</sup> p=0.03 relative to control group; p=0.01 relative to no ART group.

**Table 6**

Separate analysis of male and female samples.

	Cont. male	Cont. female	no ART male	no ART female	PI, VL+ male	PI, VL+ female	PI, VL- male	PI, VL- female	NNRTI male	NNRTI female
CE to apoB-depleted plasma (%)	10.2±0.8 (n=3)	10.6±1.1 (n=5)	6.1±1.1 <sup>1</sup> (n=13)	7.1±1.8 <sup>2</sup> (n=6)	7.0±2.6 (n=11)	8.5±2.8 (n=5)	6.1±1.7 <sup>3</sup> (n=7)	8.9±0.9 (n=3)	7.7±3.2 (n=21)	6.8±1.5 <sup>4</sup> (n=4)
CE to HDL (%)	5.5±1.1 (n=8)	5.2±1.0 (n=11)	6.5±2.0 (n=14)	6.3±1.2 (n=4)	6.3±2.4 (n=7)	6.1±1.9 (n=3)	6.2±1.8 (n=9)	5.6±1.1 (n=2)	6.5±1.7 (n=22)	5.0±1.1 (n=3)
PON activity of HDL (U/μl)	0.13±0.001 (n=8)	0.13±0.013 (n=11)	0.121±0.007 <sup>5</sup> (n=15)	0.117±0.007 (n=5)	0.112±0.009 <sup>6</sup> (n=6)	0.116±0.007 (n=3)	0.114±0.01 <sup>7</sup> (n=8)	0.124±0.003 (n=2)	0.113±0.012 <sup>8</sup> (n=19)	0.123±0.006 (n=2)

<sup>1</sup> p<0.0001 relative to control male  
<sup>2</sup> p=0.0036 relative to control male  
<sup>3</sup> p=0.0033 relative to control female  
<sup>4</sup> p=0.0029 relative to control female  
<sup>5</sup> p=0.0063 relative to control male  
<sup>6</sup> p=0.0019 relative to control male  
<sup>7</sup> p=0.0024 relative to control male  
<sup>8</sup> p=0.0008 relative to control male.