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Serum paraoxonase-3 concentration in HIV-infected patients.

Evidence for a protective role against oxidation

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Abbreviated title: Paraoxonase-3 in HIV-infection
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Abbreviations: AIDS, acquired immunodeficiency syndrome; ELISA, enzyme-linked immunosorbent assay; FPLC, fast-performance liquid chromatography; HCV, hepatitis C virus; HDL, high-density lipoproteins; HIV, human immunodeficiency virus; IMT, intima-media thickness; LDL, low-density lipoproteins; PON, paraoxonase; SNP, single nucleotide polymorphism; TBBL, 5-thiobutyl butyrolactone.
Abstract We investigated the influence of the HIV infection on serum paraoxonase-3 (PON3) concentration, and assessed the relationships with lipoprotein-associated abnormalities, immunological response, and accelerated atherosclerosis. We studied 207 HIV-infected patients and 385 healthy volunteers. Serum PON3 was determined by in-house ELISA and PON3 distribution in lipoproteins was investigated by FPLC. Polymorphisms of the PON3 promoter were analyzed by the Iplex Gold MassArray™ method. PON3 concentrations were increased (about three times) in HIV-infected patients with respect to controls ($P < 0.001$), and were inversely correlated with oxidized LDL levels ($P = 0.038$). Long-term use of non-nucleoside reverse transcriptase inhibitor (NNRTI)-based antiretroviral therapy was associated with a decrease of PON3 concentrations. In a multivariate linear regression analysis, these relationships were still strong when the main confounding covariates were taken into consideration. PON3 was mainly found in HDL in HIV-infected patients, but a substantial amount of the protein was detected in LDL particles. The present study reports for the first time an important increase in serum PON3 concentrations in HIV-infected patients which is associated with their oxidative status and their treatment with NNRTI. Long-term, prospective studies are needed to confirm the possible influence of this enzyme on the course of this disease and its possible utility as an analytical biomarker.

**Abstract word count**: 207

**Supplementary key words**: antioxidants; atherosclerosis; high-density lipoproteins; HIV-infection; oxidative stress; paraoxonases.
Human immunodeficiency virus (HIV)-infected patients often develop long-term metabolic alterations and concomitant atherosclerosis (1,2). This association has acquired clinical relevance since the introduction of effective therapeutic measures which have modified HIV infection to a chronic disease, and the consequences of metabolic derangements become more evident over time. In the course of HIV infection there are several key changes in lipoprotein metabolism including increased lipid peroxidation, hypertriglyceridemia and low high-density lipoprotein (HDL) concentration (3). Among them, changes in HDL are particularly relevant since HIV-infected patients with higher HDL-cholesterol concentrations appear to have a better HIV disease course than those with lower HDL concentrations (4). In addition, we have previously documented that serum paraoxonase-1 (PON1) activity and concentration are influenced by HIV infection (5), and that PON1 gene polymorphisms are related to the presence of sub-clinical atherosclerosis and CD4+ T cell recovery following treatment (6).

The PON enzyme family comprises 3 members, PON1, PON2 and PON3, whose genes are located adjacent to each other on chromosome 7q21-22 (7). In mammals, the PON1 and PON3 genes are expressed in many cell types (8), and their protein products are found in the bloodstream bound to HDL (9). Conversely, PON2 is an intracellular enzyme which is not, however, found in the bloodstream (10). All these enzymes are able to delay low-density lipoprotein (LDL) oxidation and cellular oxidative stress (11). In addition, data obtained from a variety of mouse models of atherosclerosis have consistently shown that human PON1, 2 or 3 expression inhibits, or reverses, the development of atherosclerosis via mechanisms involving the reduction of oxidative stress, the promotion of cholesterol efflux from macrophages, and the
normalization of vascular endothelium function (12-15). Moreover, recent studies showed that PON2 expression is increased in cultured hematopoietic cells and mouse thymocytes after HIV-1 infection (16). The PON family also plays a role in innate immunity and can prevent bacterial infection (17).

Although knowledge on PON1 and PON2 structure and function is rapidly expanding, data about the PON3 protein remain elusive. Its gene was identified in 1996 when Primo-Parmo et al. (7) detected a large number of cDNA sequences in the Genome Data Base with significant similarity to, but not identical with, human PON1. The percentage identity among human PON1, PON2, and PON3 genes is high (about 70%) and the genes are believed to derive from a common precursor (11). Clinical research on PON3 has been hampered by the lack of methods for measurement, but we recently described a high-throughput, reliable enzyme-linked immunosorbent assay (ELISA) to analyze PON3 concentration in human serum (18). The main objective of the present study was to investigate whether serum PON3 concentration may provide new information to improve our understanding of metabolic complications associated with HIV infection.

MATERIALS AND METHODS

Study participants

From among the HIV-infected patients attending our clinic, 207 (139 men, 68 women; mean age 38 years; range 22 to 66) accepted an invitation to participate in the present study. Of these patients, 122 were co-infected by the hepatitis C virus (HCV). All patients were undergoing antiretroviral therapy with protease inhibitors (PI) or with non-nucleoside reverse transcriptase inhibitors.
(NNRTI) based schemes. The antiretroviral adjuvant drugs were zidovudine, stavudine, didanosine or lamivudine. The exclusion criteria were age under 18 years, or renal function impairment defined as creatinine levels higher than 106 \( \mu \text{mol/l} \), or having an AIDS-related opportunistic disease at the time of the study. Twenty-five patients had subcutaneous lipoatrophy, defined as the presence of hollow cheeks, prominent superficial veins in the limbs, or flattening of the buttocks (19). Carotid and femoral ultrasound measurements were performed in 178 patients and the intima-media thickness (IMT) was measured as an estimate of the presence of sub-clinical atherosclerosis, as previously described (20). Patients were considered to have sub-clinical atherosclerosis when IMT was \( \geq 0.8 \) mm, or when an atheromatous plaque was seen in the analyzed areas of the arteries. The main clinical characteristics of these patients are summarized in Supplementary table I. The control group consisted of 385 healthy volunteers (153 men, 232 women; mean age 47 years; range 19 to 75) who participated in an ongoing epidemiological study being conducted in our geographical area, and the details of which have been previously reported (21). All the volunteers had been invited to attend a clinical examination and to provide a fasting blood sample. There was no clinical or analytical evidence of renal insufficiency, liver damage, neoplasia, or neurological disorders.

A fasting venous blood sample was obtained from all the participants. CD4+ T-cells and CD8+ T-cells were analyzed immediately, and serum, plasma and leukocytes were stored at \(-80^\circ\text{C}\) in our biological sample bank until the other measurements were performed. We employed independent aliquots that were never thawed before this investigation, although participants in this study partially coincided with those reported in previous investigations (5, 6). All the
participants provided fully-informed consent to participation in the study on the understanding that anonymity of all data is guaranteed. The study was approved by the Hospital Universitari de Sant Joan de Reus Institutional Review Board.

Biochemical and serological measurements

Serum PON3 concentrations were determined by in-house ELISA using rabbit polyclonal antibodies generated against a synthetic peptide with a sequence specific to mature PON3. Details of this method have been previously reported (18,22). Plasma viral load was measured with the COBAS® TaqMan® HIV-1 assay (Roche, Basel, Switzerland) and CD4+ T-cell and CD8+ T-cell counts by flow cytometry (Coulter Epics XL-MLC, Beckman Coulter, Fullerton, CA, USA). Antibodies against HCV, serum β-2-microglobulin [a marker of lymphocyte destruction and progression of HIV-infection (23)], and serum cholesterol, triglycerides, HDL-cholesterol, and apolipoprotein (apo) A-I were measured in an automated analyzer (UniCel™ DxI 800, Beckman Coulter, Fullerton, CA, USA). Oxidized LDL levels were measured by ELISA (Mercodia, Uppsala, Sweden).

FPLC lipoprotein fractionation

PON3 distribution in lipoproteins was assessed by FPLC (Bio-Rad BioLogic DuoFlow 10 system, Bio-Rad Laboratories, Inc. Hercules, CA). Sera from 3 HIV-infected patients and 3 non-infected participants were pooled separately. To maximize the possible differences between groups, sera from the HIV-infected patients were chosen to have a PON3 concentration > 20 mg/l.
Two-hundred µl from each pool were injected into a Superose 6/300 GL column (GE Healthcare Europe GmbH, Glattbrugg, Switzerland), and five-hundred µl fractions were collected. Cholesterol, triglycerides and PON3 in each fraction were measured as described.

**PON3 promoter genotyping**

Genomic DNA was obtained from leukocytes (Puregene DNA Isolation reagent set, Gentra Systems Inc., Minneapolis, MN, USA). Selected single nucleotide polymorphisms (SNPs) of the *PON3* promoter were analyzed by the Iplex Gold MassArray™ method (Sequenom Inc., San Diego, CA, USA) at the Spanish National Genotyping Center (*Centro Nacional de Genotipado, Universitat Pompeu Fabra*, Barcelona, Spain).

**Statistical analysis**

The normality of distributions was determined with the Kolmogorov-Smirnov test. Differences between two groups were assessed with the Student’s *t*-test (parametric) or the Mann-Whitney *U* test (non-parametric). Differences between multiple groups were analyzed by the Kruskal-Wallis test. Pearson or Spearman correlation coefficients were used to evaluate the degree of association between variables. Each SNP was tested for Hardy-Weinberg equilibrium using Haploview 4.0 software (24). Estimates of linkage disequilibrium between SNPs were calculated using Fisher’s test. Diagnostic accuracy for the measurement of serum PON3 concentration was calculated with ROC analysis (25). A multiple linear regression model was fitted to evaluate the factors that were independently associated with PON3
concentrations in HIV-infected patients. Results are shown as means and SD (parametric) or as medians and 95% confidence interval (CI; non-parametric). The SPSS 18.0 package was employed for all statistical calculations.

RESULTS

Relationships among serum PON3 concentrations and lipoprotein abnormalities

Serum PON3 concentrations were significantly increased in HIV-infected patients with respect to the control group [5.5 (1.2 – 10.8) vs. 1.8 (1.0 – 2.5) mg/l, respectively; \(P < 0.001\); Fig. 1A]. The results of the ROC analysis for serum PON3 concentration measurement are shown in Fig. 1B. The area-under-the-curve (AUC) was 0.94 (95% CI: 0.92 – 0.97; \(P < 0.001\)), which highlights the remarkable differences in serum PON3 concentrations between patients and controls. We observed a significant inverse relationship (\(r = -0.147\); \(P = 0.038\)) between serum PON3 concentration and oxidized LDL levels (Fig. 1C) in HIV-infected patients, but not in the control group (\(r = 0.024\); \(P = 0.786\)). HIV-infected patients were characterized by raised serum triglyceride concentration and decreased cholesterol values in HDL and LDL (Table 1), and there were no significant associations among serum PON3 concentrations, cholesterol and triglycerides (Supplementary Table 2).

FPLC lipoprotein fractionation

In non-infected participants, PON3 immunoreactivity was observed almost exclusively in HDL fractions. However, in the HIV-infected pool, a
substantial amount of this protein eluted with the smallest HDL, and with LDL particles (Fig. 2).

**Influence of genotype on serum PON3 concentrations**

The frequency distributions of the selected *PON3* promoter gene polymorphisms are shown in Table 2. There were no significant differences between control subjects and HIV-infected patients. These polymorphisms moderately influenced serum PON3 concentrations in the control subjects, but not in the patient group.

All *PON3* promoter polymorphisms were strongly linked in a single haplotype, and we did not observe any significant differences between patients and controls (Supplementary Fig. 1).

**Relationships among serum PON3 concentrations and the immunological and virological outcomes**

Co-infection with HCV was associated with a significantly higher PON3 concentration [5.8 (2.6 – 11.1) vs. 4.5 (2.4 – 11.6) mg/l, respectively; *P* = 0.024]. There were not any significant associations between serum PON3 concentrations and CD4+T and CD8+T cell counts, the CD4+/CD8+ ratio or the plasma HIV-1 viral load (Supplementary Table 3). There was a significant direct linear relationship (*r* = 0.397; *P* < 0.001) between serum PON3 and β-2-microglobulin concentrations (Fig. 3A). There were no significant differences in serum PON3 concentrations between HIV-infected patients with or without lipoatrophy [5.5 (2.0 – 12.8) vs. 5.1 (2.4 – 10.3) mg/l, respectively; *P* = 0.644].
Influence of treatments on serum PON3 concentrations

We observed a significant inverse relationship between serum PON3 concentrations and the duration of the antiretroviral therapy in patients under NNRTI-based scheme ($r = -0.250; P = 0.035$; **Fig. 3B**), but not in patients receiving a PI-based scheme ($r = -0.059; P = 0.408$).

PON3 and sub-clinical atherosclerosis

When patients were classified according to the presence ($n = 137$) or absence ($n = 41$) of sub-clinical atherosclerosis, no significant differences in serum PON3 concentrations were found [5.2 (2.5 – 11.2) vs. 5.4 (2.5 – 11.1) mg/l, respectively; $P = 0.959$]. Furthermore, there was no significant association between serum PON3 concentration and the quantitative value of the IMT ($r = 0.047; P = 0.544$).

Multivariate analysis for PON3 concentration in HIV-infected patients

A multiple linear regression analysis showed significant and independent relationships of serum PON3 concentrations with oxidized LDL and with the use of NNRTI as the backbone of the antiretroviral therapy (**Table 3**). These results were confirmed when the use of antiretroviral treatment was substituted by its duration [NNRTI: $B = -0.186 (0.080), \beta = -0.249, t = -2.083, P = 0.040$; PI: $B = -0.023 (0.050), \beta = -0.055, t = -0.419, P = 0.677$].

DISCUSSION

Viral replication and some clinical manifestations of HIV infection involve a misbalance in reduction-oxidation (redox) status and free radical production
Moreover, oxidative stress may also be induced by antiretroviral treatments (27). PON3 is an enzyme with lactonase activity (28), the physiological function of which is not completely understood, but evidence suggests that it has an antioxidant role by hydrolyzing oxidized lipid peroxides, similarly to PON1 and PON2. Purified human and rabbit PON3 and recombinant PON3 have been shown to decrease macrophage oxidative stress and inhibit the in vitro oxidation of LDL (29-32). The present study revealed a remarkable increase (about three times) in serum PON3 concentration in HIV-infected patients that may be clinically relevant. ROC analysis showed an AUC very close to 1.0, demonstrating a high sensitivity and specificity of serum PON3 measurement in distinguishing between HIV-infected and non-infected subjects. Interestingly, oxidized LDL levels were not significantly increased, but a significant inverse relationship was observed between their serum levels and those of PON3. These data support the concept that PON3 plays a protective role against oxidative stress and increased lipid peroxidation in HIV infection. Whether this increase in circulating PON3 is related to a higher cellular expression is, at present, unknown. However, a recent study reported a similar increase (5 times) in Pon3 mRNA expression in late gestation, a physiological state with high oxidative stress (33). Contrarily to what we previously observed for PON1 in HIV-1 infection (5), serum PON3 concentrations were not significantly related with markers of lymphocyte recovery. We did not observe any association with CD4+ T-cell counts, and the correlation observed with β-2 microglobulin in the bivariate analysis, disappeared in the multiple regression analysis. An interesting observation was the association of HCV coinfection with higher PON3 concentrations. These results confirm a recent report from our
group describing increased serum PON3 concentrations in patients with chronic liver disease, mainly secondary to HCV infection (34).

In the present study, treatment of HIV-infected patients with NNRTI, but not with PI, was associated with a significant decrease of serum PON3 concentrations. The use of some NRTI and PI is associated with an atherogenic lipoprotein profile (35), but NNRTI, such as efavirenz, promote anti-atherogenic changes in HDL particles and function, including normalization of size and lipid composition, enhancement of the reverse cholesterol transport, and improvement of the antioxidant capacity (36). A previous study from our group showed that NNRTI treatment was associated with a relative normalization of serum apolipoproteins A-I and A-II, and HDL-cholesterol concentrations in HIV-infected patients (37), which is consistent with the reported beneficial effects of this compound on HDL composition and function. However, an alternative explanation is that the influence of NNRTI fades with time. Similar effects have been described for some antiretroviral drugs. Shlay et al (38) reported that NRTI are associated with positive changes in subcutaneous tissue distribution during the early periods, and negative changes in the late periods of treatment.

We did not find any significant differences in genotype or haplotype of PON3 promoter gene polymorphisms between patients and controls, suggesting that genotype does not influence the course of the disease. In our previous report, we observed a moderate influence of some polymorphisms on serum PON3 concentration in the general population (18), but this is not the case in HIV-infected patients. Possibly, the up-regulation of PON3 expression secondary to the infection masks the small effect of these polymorphisms. Unlike PON1 (6), PON3 seems not to be associated with the presence of sub-
clinical atherosclerosis is HIV-infected patients. Although lipid peroxidation and atherosclerosis are known to be strongly linked phenomena (39), the pathophysiology of atherosclerosis is complex, and our results suggest that the protective effects of these enzymes differs under certain situations. Perhaps PON1 is more efficient in protecting against the alterations leading to atherosclerosis and PON3 is in some way involved in protection against infection. This is, to the best of our knowledge, the first in vivo evidence suggesting such a hypothesis, and warrants further investigation. Another interesting point is the wider lipoprotein distribution of PON3 in HIV-infected patients. They are observed in substantial amounts in the smallest HDL and in LDL particles. The accepted concept to-date is that PON1 and PON3 are exclusively transported in the bloodstream by HDL (12), and are only associated with other lipoproteins in exceptional circumstances (40). Perhaps the excess PON3 produced in HIV infection cannot be properly packed in the HDL particles, as the conformation of HDL-associated apolipoprotein A-I leaves little free surface area for other proteins to bind (41). This may have resulted in some PON3 redistributing to LDL. The physiological implications of this observation require further investigation.

In conclusion, the present study reports for the first time an important increase in serum PON3 concentrations in HIV-infected patients which is associated with their oxidative status and that could be partially attenuated by the treatment with some antiretroviral agents. Long-term, prospective studies are needed to further confirm the possible influence of this enzyme on the course of this disease and its possible utility as an analytical biomarker.
Acknowledgments

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TABLE 1. Selected biochemical variables in the control group and in HIV-infected patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group (n = 356)</th>
<th>HIV-infected patients (n = 207)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.28 (0.98)</td>
<td>4.89 (1.23)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.1 (0.5 – 2.6)</td>
<td>1.5 (0.6 – 8.5)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.48 (0.39)</td>
<td>1.18 (0.45)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>3.20 (0.95)</td>
<td>2.75 (0.96)</td>
<td>&lt; 0.001</td>
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<tr>
<td>Apolipoprotein A-I (g/l)</td>
<td>1.69 (0.28)</td>
<td>1.38 (0.31)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Oxidized LDL (U/l)</td>
<td>84.5 (81.8 – 88.7)</td>
<td>81.5 (40.5 – 145.9)</td>
<td>0.951</td>
</tr>
</tbody>
</table>

Results are presented as means and SD in parentheses (parametric) or as medians and 95% CI in parenthesis (nonparametric).
TABLE 2. Distribution of PON3 genotypes in the control group and HIV-infected patients

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype frequency (%)</th>
<th>PON3, mg/l</th>
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<td></td>
<td>Control</td>
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<td><strong>PON3-567</strong></td>
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<td></td>
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<tr>
<td>CC</td>
<td>59.8</td>
<td>61.7</td>
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<tr>
<td>CT</td>
<td>36.4</td>
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<td>TT</td>
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<td>AA</td>
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<td>61.7</td>
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<tr>
<td>AG</td>
<td>36.6</td>
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<td>3.8</td>
<td>6.3</td>
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<td><strong>PON3-746</strong></td>
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<tr>
<td>CC</td>
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<tr>
<td>CT</td>
<td>36.5</td>
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<td><strong>PON3-4105</strong></td>
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<td>GA</td>
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</tr>
<tr>
<td>GG</td>
<td>2.8</td>
<td>4.9</td>
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TABLE 3. Multiple regression analysis of the determinants of serum PON3 concentration in HIV-infected patients

BMI: body-mass index; NNRTI: non-nucleoside analogues reverse transcriptase inhibitors; PI: protease inhibitors.

<table>
<thead>
<tr>
<th></th>
<th>Unstandardized coefficients</th>
<th>Standardized coefficients</th>
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<td></td>
<td>B</td>
<td>Std. Error</td>
</tr>
<tr>
<td>Age, years</td>
<td>-0.0.36</td>
<td>0.047</td>
</tr>
<tr>
<td>Gender, male</td>
<td>0.528</td>
<td>0.772</td>
</tr>
<tr>
<td>BMI, kg/m^2</td>
<td>0.098</td>
<td>0.105</td>
</tr>
<tr>
<td>Use of NNRTI</td>
<td>-2.276</td>
<td>0.875</td>
</tr>
<tr>
<td>Use of PI</td>
<td>-0.815</td>
<td>0.447</td>
</tr>
<tr>
<td>Lipoatrophy</td>
<td>1.035</td>
<td>0.870</td>
</tr>
<tr>
<td>Hepatitis C virus co-infection</td>
<td>0.486</td>
<td>0.798</td>
</tr>
<tr>
<td>HIV-1 viral load &lt; 200 copies/ml</td>
<td>0.932</td>
<td>0.821</td>
</tr>
<tr>
<td>CD4+ T-cell count, cells/mm^3</td>
<td>-0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>0.457</td>
<td>0.322</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/l</td>
<td>-0.529</td>
<td>0.831</td>
</tr>
<tr>
<td>Oxidized LDL, mmol/l</td>
<td>-0.037</td>
<td>0.013</td>
</tr>
<tr>
<td>β-2-microglobulin, mg/l</td>
<td>0.176</td>
<td>0.292</td>
</tr>
</tbody>
</table>

Dependent variable: PON3 (mg/l)
FIGURE LEGENDS

**Fig. 1.** (A) Serum PON3 concentrations in control subjects and HIV-infected patients. (B) ROC plot for serum PON3 concentration measurement in HIV-infected and non-infected subjects. AUC: Area-under-the curve; CI: Confidence interval. (C) Relationship between serum PON3 concentrations and oxidized LDL levels (ox-LDL) in HIV-infected patients.

**Fig. 2.** FPLC lipoprotein fractionation and PON3 lipoprotein distribution in the control and the HIV-infected pools.

**Fig. 3.** Relationships among serum PON3 concentrations, β-2-microglobulin concentrations, and the duration of non-nucleoside reverse transcriptase inhibitors (NNRTI) in HIV-infected patients.
Figure 1

A

B

C
Figure 2

Control

HIV infection
Figure 3