

Association between HIV replication and cholesterol in peripheral blood mononuclear cells in HIV-infected patients interrupting HAART

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Background: Cellular cholesterol is essential for HIV replication and may control HIV spread. HIV, in turn, appears to control cholesterol metabolism.

Objectives: To describe the relationships between serum lipids, cellular cholesterol and viral replication during highly active antiretroviral therapy (HAART) interruption.

Methods: We have correlated virological parameters with the level of circulating lipids in serum and the content of cellular cholesterol in peripheral blood mononuclear cells (PBMCs). The study included 33 patients interrupting HAART with ($n = 23$) or without ($n = 10$) atorvastatin treatment.

Results: Atorvastatin treatment did not modify PBMC cholesterol levels at week 4 after HAART interruption, although it significantly reduced serum cholesterol (total and LDL, where LDL stands for low density lipoprotein) ($P < 0.05$). Serum cholesterol or LDL marginally influenced PBMC cholesterol since no significant correlations were found between these parameters either at 0 or 4 weeks after HAART interruption. Analysis of virological data in all patients revealed a negative trend ($P = 0.07$) between baseline PBMC cholesterol and absolute CD4 T cell counts at baseline but a poor correlation ($P = 0.18$) with the viral load (VL) at week 4. Separate analysis of control patients showed a correlation between baseline PBMC cholesterol and VL at week 4 ($P = 0.01$). However, atorvastatin treatment abrogated this correlation by increasing viral replication in individuals with low cellular cholesterol.

Conclusions: Our data underscore the potential relevance of PBMC cholesterol in *in vivo* HIV replication and the complex effects of atorvastatin that seem to be unrelated to PBMC cholesterol.

Keywords: lipid, metabolism, membrane, replication, atorvastatin, PBMC

Introduction

The influence of the lipid composition of biological membranes on HIV replication *in vitro* has been widely described. Cellular and viral cholesterol are required for HIV binding to dendritic

cells,¹ HIV entry into target cells,² HIV budding and cell-to-cell HIV transmission.³ The relevance of the interplay between HIV and cholesterol is highlighted by the fact that HIV promotes both the synthesis and uptake of cholesterol into infected cells by a mechanism mediated by Nef.⁴ Despite extensive *in vitro*

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knowledge, little is known about the actual effects of cholesterol on HIV replication *in vivo*. Only a complete study on lipid metabolism during HIV primary infection described a decrease in serum cholesterol that was reverted by highly active antiretroviral therapy (HAART).⁵

Pharmacological control of cholesterol synthesis may be achieved using statins, a family of inhibitors that block the isoprenoid and cholesterol synthetic pathway.⁶ In addition to blocking cholesterol synthesis, statins display anti-HIV activity *in vitro*, acting through the blockade of the synthesis of isoprenoids that impairs HIV entry and budding,⁷ through the inhibition of adhesion molecules, thereby blocking HIV attachment to target cells⁸ or through the modulation of CCR5 function.⁹ Despite these potential mechanisms of action, all pilot clinical studies involving different statins in different clinical settings revealed the lack of anti-HIV activity of statins *in vivo*.^{10–12} In one of these studies, conducted in our unit, we reported the influence of serum cholesterol on *in vivo* HIV replication.¹²

Considering the relevance of cellular and serum cholesterol and the lack of *in vivo* information, we explored the relationships between HIV replication and different parameters of cholesterol metabolism, including the level of cholesterol in peripheral blood mononuclear cells (PBMCs), in a group of patients enrolled in a previous study designed to assess the effect of atorvastatin on HIV replication after HAART discontinuation.¹²

Patients and methods

Study design and patients

The study has been performed in patients enrolled in a previous open-label randomized pilot clinical study designed to evaluate the effect of different doses of atorvastatin on HIV rebound after HAART discontinuation.¹² Eligible subjects were chronically HIV-1-infected patients on HAART with documented off-therapy viral load (VL) $\geq 15\,000$ copies/mL, current stable (≥ 6 months) viral suppression (VL < 50 copies/mL) and CD4 cell count ≥ 500 cells/mm³ in two determinations over the last 6 months. Patients with a background of AIDS-defining pathologies, creatinine phosphokinase ≥ 500 U/L, transaminase levels (AST or ALT) ≥ 3 times higher than normal or concomitant treatment with other statins or fibrates within the last 3 months were excluded.

The main study was approved by the Ethics Committee of the hospital. All participants ($n = 41$) gave written informed consent and interrupted antiretrovirals, 15 of them without any treatment (control group), while 13 began atorvastatin 40 mg/day (Ator40 group) and 13 began atorvastatin 80 mg/day (Ator80 group) at the same time as antiretroviral interruption. Whole blood, plasma and PBMCs were obtained and processed as described previously¹³ on the randomization day and at weeks 4 and 12. A total of 16 participants discontinued the study for virological or safety reasons.¹² Available samples from all participants who achieved week 4 of follow-up were analysed in the current substudy; 10 corresponded to the control group, 10 to the Ator40 group and 13 to the Ator80 group.

To determine the influence of serum and cellular cholesterol on HIV replication, we monitored the following virological and lipid metabolism parameters at baseline and at week 4 after treatment interruption: CD4 T cells count, VL, serum levels of

total cholesterol, low density lipoprotein (LDL), triglycerides and high density lipoprotein (HDL), and cholesterol content in PBMCs.

Determination of cholesterol content in PBMC

Cholesterol content in PBMC samples was determined using the Amplex Red Cholesterol Assay Kit (Molecular Probes). Briefly, PBMCs were thawed and extensively washed in PBS. PBMC cholesterol was analysed after sequential washes. In these preliminary experiments, three washes were sufficient to yield identical results in two sequential measures. Routinely, four washes were performed to remove any trace of cholesterol or protein coming from serum used in the freezing process. To evaluate the reproducibility of the sample processing, we determined cholesterol levels in samples processed independently. The coefficient of variation was 14.3% and 17.1% in two samples tested in triplicate. After washes, the final cellular pellet was resuspended in lysis buffer (Molecular Probes) before being assayed for cholesterol following the manufacturer's instructions. The efficiency of this process was calculated by adding known amounts of cholesterol (from 0.3 to 10 $\mu\text{g/mL}$) to a sample containing 2.0 μg of cholesterol/mL. The recovery of added cholesterol was 87.7% with a range from 73% to 108%.

In parallel, samples were assayed for protein content using the BCA protein assay (Pierce). Results were given as ng of cholesterol/ μg of protein. All samples were assayed in triplicate (cholesterol data) or duplicate (protein data). Mean values were used for statistical analysis.

Statistical analysis

Continuous variables were described as medians (25 and 75 interquartile ranges) and compared using non-parametric tests (Mann–Whitney or Kruskal–Wallis). Linear regression analyses were performed using Pearson's correlation tests. Statistical analyses were performed using SPSS v.11.5 (SPSS Inc., Chicago, IL, USA), with univariate two-tailed significance levels of 5%. For most analyses, Ator40 and Ator80 patients were grouped and compared with untreated control patients.

Results

Serum and cellular cholesterol

By analogy to the reported influence of serum cholesterol on the viral rebound in our patients,¹² we explored the role of cellular cholesterol. First, we analysed serum and PBMC levels of cholesterol at baseline and at week 4. Our data revealed a significant decrease in serum cholesterol in atorvastatin-treated patients, without differences between the Ator40 and Ator80 groups, and a slight but not significant decrease in the control group (Figure 1a). The effect of atorvastatin was associated with a specific decrease in LDL without changes in HDL or triglycerides (data not shown). In contrast, PBMC cholesterol remained unchanged in all groups during the treatment period (Figure 1a). No significant differences were observed within (baseline versus week 4) or between groups (control versus Ator40 or Ator80), suggesting that PBMC cholesterol in whole PBMC samples is not significantly altered by either HIV replication (in the control

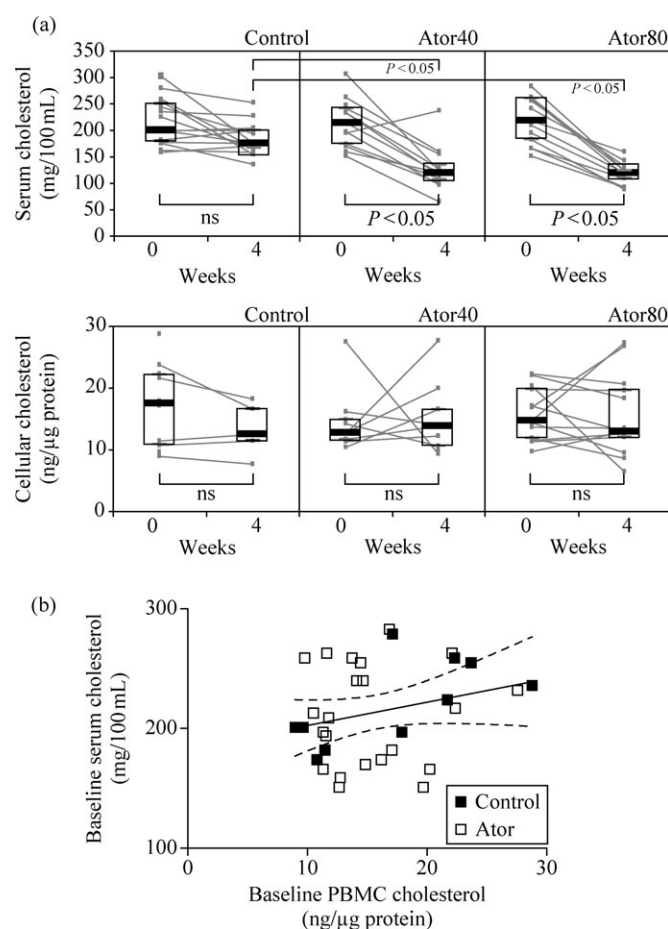


Figure 1. Relationship between serum and cellular cholesterol levels in control and atorvastatin-treated groups. (a) Total serum (upper panels) and cellular (lower panels) cholesterol levels at weeks 0 and 4 after HAART interruption. Thick horizontal lines represent median values; boxes indicate 25 and 75 percentiles. Non-significant (ns) and significant differences ($P < 0.05$) assessed by the Mann–Whitney test are indicated. (b) Cholesterol content in cellular pellets plotted against the level of circulating serum cholesterol for all patients at baseline before HAART interruption. Filled and open squares correspond to control and atorvastatin-treated patients, respectively.

group) or atorvastatin treatment. The discordance between PBMC and serum cholesterol was further confirmed by the lack of significant correlation between both parameters at baseline (Figure 1b; $r = 0.261$, $P = 0.15$) or at week 4 (data not shown). Taken together, these data confirm that serum and cellular cholesterol are differentially regulated in HIV-infected patients.

Cellular cholesterol and viral replication

Next, we evaluated the relationship between PBMC cholesterol and different virological parameters. The analysis of the correlation between PBMC cholesterol and CD4 T cell count at baseline showed a trend ($r = 0.405$, $P = 0.07$) but not a significant relationship (Figure 2a). Consistent with our previous observations,¹² a correlation was found between serum cholesterol at baseline and VL at week 4 ($r = 0.377$, $P = 0.02$). In contrast, baseline PBMC cholesterol did not significantly correlate ($r = 0.249$, $P = 0.18$) to VL at week 4 (Figure 2b) when all patients were analysed. A detailed analysis, separating control and

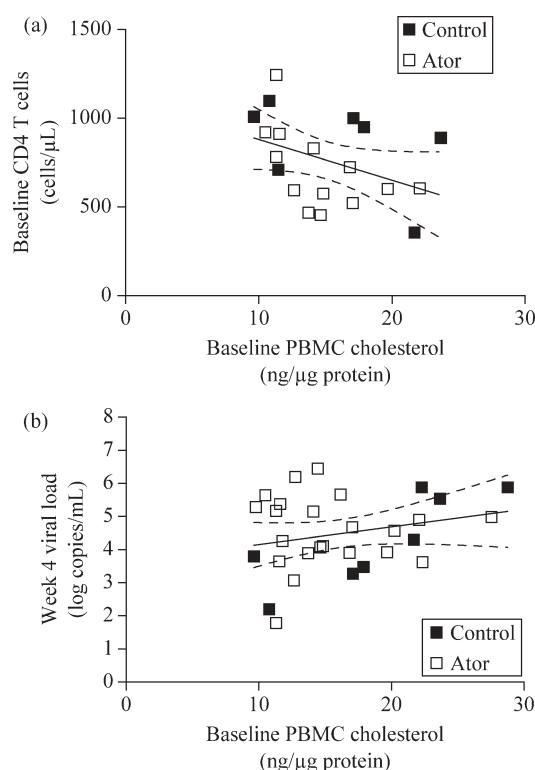


Figure 2. Effect of cellular cholesterol on virological parameters. (a) Cellular cholesterol plotted against absolute CD4 T cells at baseline. Filled and open squares correspond to control and atorvastatin-treated patients, respectively. (b) Lack of correlation between cellular cholesterol and VL rebound at week 4 after HAART interruption. Filled and open squares correspond to control and atorvastatin-treated patients, respectively. Analysis of control patients showed a significant correlation (see text).

atorvastatin-treated patients, revealed a significant correlation between PBMC cholesterol and VL at week 4 restricted to the control group ($r = 0.829$, $P = 0.01$). A similar analysis in atorvastatin-treated patients revealed the lack of correlation between cellular cholesterol and viral rebound ($r = 0.018$, $P = 0.893$). The disruption of the relationship between PBMC cholesterol and viral rebound by atorvastatin appears to be associated with increased viral rebound in atorvastatin-treated patients having low levels of cellular cholesterol (Figure 2b).

Discussion

The interplay between HIV and cholesterol is complex. HIV appears to increase cholesterol synthesis and uptake in productively infected cells to ensure HIV infectivity.⁴ Since cellular cholesterol content may also be influenced by serum lipids¹⁴ and our previous data suggested that individuals with high cholesterol have high viral rebound,¹² we have explored whether PBMC cholesterol content may influence HIV replication *in vivo*. The effect of atorvastatin treatment was also analysed.

Our data indicate a lack of correlation between cellular cholesterol and serum cholesterol or other parameters of lipid metabolism. Indeed, atorvastatin induced a significant decrease in both LDL and cholesterol in serum but did not modify cellular cholesterol in PBMCs (Figure 1). In contrast, other authors have found reduced levels of cholesterol in red blood cells

concomitant to statin-induced reduction of serum cholesterol.^{15,16} Statins are known to selectively inhibit cholesterol synthesis and increase cholesterol uptake in the liver.¹⁷ However, relatively little information exists on the effect of statins on lymphoid cells *in vivo*, although *in vitro* the inhibition of cholesterol synthesis has been reported to increase cholesterol uptake and to inhibit proliferation in cholesterol-poor environments.^{18,19} Interestingly, atorvastatin has many effects on gene expression in PBMCs,²⁰ including the up-regulation of the expression of LDL receptors, which is in turn up-regulated by HIV infection.⁴ Our data confirms that in HIV-infected patients, different mechanisms control serum cholesterol and cholesterol content in PBMCs, which may have a slow cholesterol turnover or may compensate the inhibition of cholesterol synthesis by increasing cholesterol uptake.

Analysis of the relationship between HIV replication and cellular cholesterol showed that HIV infection did not modify cellular cholesterol levels in total PBMC of control patients, probably due to the low level of productively infected cells in peripheral blood samples. On the other hand, cellular cholesterol appeared to influence HIV replication. First, CD4 T cell counts showed a negative but not significant trend at baseline. Moreover, a significant correlation was found between baseline cellular cholesterol and HIV rebound in control patients. By analogy to *in vitro* reported data, the simplest explanation for the effect of membrane cholesterol is that cholesterol-rich CD4 T cells may produce more infectious HIV particles that may, in turn, more easily infect new target cells.

Surprisingly, this relationship is disrupted by atorvastatin, which seems to favour viral replication in patients bearing low cholesterol cells. The reasons for this effect are unclear due to the complex action of statins on immune cells. Several hypotheses have been evaluated. One potential explanation may be related to the previously reported ability of atorvastatin to increase HIV production by PBMC *in vitro*.¹² However, this effect is expected to be general and not to only affect patients with low PBMC cholesterol. Another explanation might be associated with the compensatory mechanisms that regulate PBMC cholesterol through up-regulation of cholesterol uptake. Patients with low PBMC cholesterol may have a strong dependence of cholesterol uptake, which would be consistent with the reported relationship between serum cholesterol and HIV replication.¹² However, no significant increases in PBMC cholesterol were observed in those patients. Finally, atorvastatin may affect CD8 T cell function, reducing immune pressure on HIV replication. Although the absolute numbers of CD8 T cells and the expression of CD38 in these cells were not significantly modified by atorvastatin treatment,¹² an effect on the functionality of T cells cannot be ruled out.^{21–23} Further work is necessary to evaluate this possibility.

In conclusion, although several factors may affect lipid metabolism, including HAART, our data suggest a role for cellular cholesterol in HIV replication *in vivo* and define the lack of effect of atorvastatin on cellular cholesterol as a potential cause for the failure of statins to control HIV replication *in vivo*.

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Transparency declarations

None to declare.

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