Anatomical and cellular reservoirs for HIV-1 during potent antiretroviral therapy
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Citation for published version (APA):
CHAPTER 5

Increasing cerebrospinal fluid chemokine concentrations despite undetectable cerebrospinal fluid HIV-RNA in HIV-1-infected patients receiving antiretroviral therapy

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Abstract

Only limited data on cerebrospinal fluid (CSF) HIV-1 RNA responses and markers of local inflammation in CSF during antiretroviral therapy are available. HIV-1 RNA, soluble tumor necrosis factor (TNF)-receptor (sTNFr)-II, monocyte chemoattractant protein (MCP)-1, and interferon-γ-inducible protein (IP)-10 were measured in the peripheral blood and CSF of 26 antiretroviral-naive HIV-1-positive patients, who were treated with ritonavir (RTV)/saquinavir (SQV; n=5), RTV/SQV/stavudine (d4T; n=8) or zidovudine/lamivudine/abacavir/nevirapine/indinavir (n=13). After 8 to 12 weeks of treatment, CSF HIV-1 RNA dropped to <400 copies/mL in 1 of 5 patients in the RTV/SQV group, 8 of 8 patients in the RTV/SQV/d4T group, and 9 of 10 patients in the five-drug group. CSF sTNFr-II and IP-10 levels increased in patients with detectable CSF HIV-1 RNA. However, increases in CSF chemokine and sTNFr-II concentrations were also observed in some patients with good CSF HIV-1 RNA responses. Moreover, CSF MCP-1 concentrations increased in the whole population after 2 months of treatment. Ongoing residual HIV replication in the central nervous system, which cannot be detected with CSF HIV-1 RNA measurements, may account for this phenomenon.
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Introduction

Antiretroviral combination therapy that includes at least one protease inhibitor (PI) reduces plasma HIV-1 RNA and thereby AIDS-related morbidity and mortality. The goal of antiretroviral treatment is to produce an as low as possible plasma HIV-1 RNA level and maintain that level for as long as possible. However, a low HIV-1 RNA in plasma during antiretroviral therapy does not necessarily mean that viral replication is suppressed in the whole body. HIV-1 RNA concentrations in other compartments of the body, such as the lymph nodes and the male genital tract, may be higher than the HIV-1 RNA level in plasma. The central nervous system (CNS) also acts as an anatomical reservoir for HIV-1. The blood-brain barrier (BBB) may prevent certain antiretroviral drugs from entering the CNS. Detectable HIV-1 RNA in the cerebrospinal fluid (CSF), even while HIV-1 RNA in the peripheral blood was below the lower limit of quantification (LLQ), has been described in patients who were treated with antiretroviral drugs that do not properly penetrate the BBB. In addition, viral resistance patterns may differ between blood and CSF.

CSF is the only CNS substrate that can be obtained repeatedly from a live patient; however, HIV replication in the brain may not be reflected directly in HIV-1 RNA concentration in CSF. Inflammatory markers in CSF may provide additional information on HIV-1 replication in the brain. We therefore measured soluble tumour necrosis factor (TNF)-receptor (sTNFr)-II, monocyte chemoattractant protein (MCP)-1 and interferon-γ-inducible protein (IP)-10 concentrations in the CSF and peripheral blood of patients on three different antiretroviral therapy regimens, which included different numbers of drugs that can penetrate the CNS.

Methods

Patients

The Prometheus study and the ERA study were approved by the Institutional Review Boards of the participating sites. All participants gave written informed consent.

One baseline and at least one follow-up paired CSF/blood sample was available of 26 HIV-1-infected patients. Thirteen antiretroviral-naive, neurologically asymptomatic patients participated in the Prometheus study, an open-label, randomised, controlled, multicentre trial. Patients received either ritonavir (RTV) 400 mg twice daily plus saquinavir (SQV) 400 mg twice daily (n=5) or RTV 400 mg twice daily plus SQV 400 mg twice daily plus stavudine
(d4T) 40 mg twice daily (n=8). As part of a neurological substudy participants volunteered to undergo lumbar punctures at day 0 (before start of study medication) and after 12 weeks of treatment. In four patients (RTV/SQV arm: n=1; RTV/SQV/d4T arm: n=3) a third lumbar puncture was performed after 48 weeks of treatment.

Thirteen antiretroviral-naive, neurologically asymptomatic patients participated in the ERA study, an open-label, controlled trial, studying the effect of a five-drug regimen in HIV-1-infected patients. All patients started with a five-drug regimen (zidovudine (ZDV) 300 mg twice daily/lamivudine (3TC) 150 mg twice daily/abacavir (ABC) 300 mg twice daily/indinavir (IDV) 1000 mg three times daily/nevirapine (NVP) 400 mg once daily). Ten patients switched from IDV 1000 mg three times daily to IDV 800 mg twice daily plus RTV 100 mg twice daily during the study. Seven patients used d4T 40 mg twice daily instead of ZDV 300 mg twice daily during (one part of) the study. Lumbar punctures were performed at day 0, weeks 8, 24, and in a subgroup (n=9) also at week 48.

Controls
The serum of 8 healthy laboratory technicians was used as control material. CSF that was used as control was obtained from 9 HIV-negative patients, who did not have a systemic or CNS infection.

HIV-1 RNA Quantification
In the Prometheus study, serum and CSF HIV-1 RNA levels were measured using a commercial assay based on polymerase chain reaction (PCR) with a variable LLQ (Amplicor HIV Monitor Test, Roche Diagnostic Systems Inc., Branchburg, New Jersey, USA). If HIV-1 RNA values were <LLQ, cut-off values were used as the individual’s HIV-1 RNA value in all analyses.

In the ERA study, HIV-1 RNA levels in plasma were measured using the NucliSens HIV-1 QT assay (Organon Teknika, Boxtel, The Netherlands). When RNA levels decreased to below 50 copies/mL, an initial input volume in the assay of 2 mL plasma was used combined with the ultrasensitive protocol adaptation, resulting in a LLQ of 5 copies/mL. HIV-1 RNA in CSF was measured using the NucliSens HIV-1 QT assay with an input volume of 2 mL, resulting in a lower cut-off level of 40 copies/mL.

Assays
sTNF-II and chemokine measurements
To determine sTNF-II levels in serum and CSF, an enzyme-linked immunological binding assay (ELIBA) was used as described previously. Reagents for sTNF-II measurements were kindly donated by Hoffmann-La Roche.
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(Basel, Switzerland). Chemokine concentrations in serum and CSF were measured by enzyme-linked immunosorbent assay (ELISA). MCP-1 was measured using purified monoclonal mouse anti-human MCP-1 (2 μg/mL; PharMingen, San Diego, CA, U.S.A.) as coating antibody, biotinylated rabbit anti-human MCP-1 (1 μg/mL; PharMingen) as detecting antibody and human recombinant MCP-1 (PharMingen) as standard. For determination of IP-10, purified monoclonal mouse anti-human IP-10 (4 μg/mL; R&D Systems, Abingdon, UK) was used as coating antibody, biotinylated goat anti-human IP-10 (50 ng/mL; R&D Systems) as detecting antibody, and recombinant human IP-10 (R&D Systems) as standard. Detection limits of the assays were 100 pg/mL (sTNFr-II), 25 pg/mL (MCP-1) and 75 pg/mL (IP-10), respectively.

Other measurements
CD4+ T lymphocytes were measured in peripheral blood using immunofluorescence flow cytometry. In the CSF of all ERA and in 8 of 13 Prometheus-study patients, the protein level and leukocyte cell count were measured.

Statistical analysis
A log₁₀ transformation was performed on all HIV-1 RNA concentration values. Results are reported as medians and interquartile ranges, if not specified otherwise. Kruskal-Wallis and Fisher's exact test were used to compare baseline characteristics between the three treatment groups. Median sTNF-II and chemokine concentrations of HIV-infected patients and HIV-negative controls were compared using Wilcoxon's two-sample test. Spearman's rank correlation was performed to explore correlations between baseline characteristics. The signed log-rank test was used to test whether significant changes occurred over time in concentrations of HIV-1 RNA, sTNF-II, MCP-1 and IP-10 concentrations. All P values were two-tailed and were considered statistically significant if P <0.05.

Data were analysed using the SAS software package (version 6.12, SAS Institute, Cary, North Carolina, USA).

Results

Baseline characteristics
Patients were HIV-1 infected and antiretroviral naive. Their median CD4+ T cell count was 365/mm³ (range, 10-870 cells/mm³) at baseline. In the RTV/SQV and RTV/SQV/d4T groups, 7 of 13 patients had symptomatic HIV-disease (at levels B or C of the U.S. Centres for Disease Control and Prevention scale) compared with 1 of 13 in the five-drug group (Table 1). Patients
had not been diagnosed with any neurological disease and did not experience neurological symptoms before or during the study. Two patients in the five-drug group were treated within 1 to 2 months after their primary HIV-infection.

Table 1 Baseline characteristics

|                      | RTV/SQV | RTV/SQV/d4T | Five-drug group | P Value
|----------------------|---------|-------------|-----------------|----------
| n                    | 5       | 8           | 13              |          |
| Male gender, n (%)   | 5 (100) | 7 (88)      | 13 (100)        | 0.26     |
| Age (y)              | 42 (26-55) | 37 (31-51) | 41 (31-57)      | 0.81     |
| CDC classification, A/B/C (n) | 3/1/1 | 3/1/4 | 12/0/1 | 0.04         |
| CD4+ count (cells/mm³) | 250 (90-500) | 300 (10-870) | 450 (30-680) | 0.80     |
| Cells in CSF/3 (μL) | 26 (3-49) | 17 (1-66) | 5 (0-59) | 0.45     |
| Protein in CSF (g/L) | 0.33 (0.30-0.35) | 0.33 (0.29-0.50) | 0.44 (0.18-1.36) | 0.49 |

a Kruskal-Wallis or Fisher's exact test for differences between the three treatment groups; b median (range); c RTV/SQV group: n=2; RTV/SQV/d4T group: n=6; five-drug group: n=13.

RTV, ritonavir; SQV, saquinavir; d4T, stavudine; n, number of individuals; CSF, cerebrospinal fluid.

Median baseline peripheral blood HIV-1 RNA concentrations were 5.5, 4.5 and 4.9 log_{10} copies/mL for the RTV/SQV, RTV/SQV/d4T and five-drug group, respectively (P = 0.01). Median baseline CSF HIV-1 RNA levels were 3.5, 2.9 and 3.6 log_{10} copies/mL (P = 0.47), in the same groups, respectively. No significant correlation was found between baseline peripheral blood and CSF HIV-1 RNA concentrations (P = 0.15).

Serum sTNFr-II, MCP-1 and IP-10 concentrations at baseline were higher in the HIV-infected patients than HIV-negative controls (P= 0.0001, P= 0.0007, and P= 0.0006, respectively).

Baseline CSF sTNFr-II, MCP-1 and IP-10 concentrations in this study population were not significantly different from those found in HIV-negative controls, although they tended to be higher when compared with HIV-negative controls (P= 0.75, P= 0.06, and P= 0.06, respectively). Median baseline CSF IP-10 levels were higher in the five-drug group compared with the RTV/SQV group or with the RTV/SQV/d4T group (P= 0.01). There were significant correlations between baseline serum and CSF sTNFr-II and between serum and CSF MCP-1 levels (Spearman's ρ 0.48; P= 0.01 and 0.42; P= 0.03, respectively).
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Correlations between baseline HIV-1 RNA and markers of inflammation in serum and cerebrospinal fluid

In peripheral blood, a correlation was found only between baseline HIV-1 RNA and IP-10 levels (P = 0.009).

Higher baseline CSF HIV-1 RNA levels were significantly correlated with higher baseline CSF sTNFr-II, protein concentrations, and CSF cell counts. Correlations were also found between CSF sTNFr-II, IP-10 and protein concentrations. However, CSF MCP-1 concentrations did not correlate with CSF HIV-1 RNA, sTNFr-II, IP-10, CSF protein levels or CSF cell counts (Table 2).

Table 2 Correlations at baseline within cerebrospinal fluid (CSF)

<table>
<thead>
<tr>
<th>CSF</th>
<th>sTNFr-II</th>
<th>MCP-1</th>
<th>IP-10</th>
<th>Protein</th>
<th>Cells</th>
</tr>
</thead>
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<tr>
<td>HIV-1 RNA</td>
<td>0.46 a</td>
<td>-0.20</td>
<td>0.35</td>
<td>0.73 b</td>
<td>0.49 a</td>
</tr>
<tr>
<td>sTNFr-II</td>
<td>0.30</td>
<td>0.49 a</td>
<td>0.46 a</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.17</td>
<td></td>
<td>-0.19</td>
<td>-0.21</td>
<td></td>
</tr>
<tr>
<td>IP-10</td>
<td></td>
<td>0.46 a</td>
<td></td>
<td>0.28</td>
<td></td>
</tr>
</tbody>
</table>

a 0.05 < P ≤ 0.01 in Spearman’s rank correlation; b P < 0.01 in Spearman’s rank correlation.

Correlations between HIV-1 RNA and markers of inflammation in peripheral blood and CSF, before start of antiretroviral therapy. Numbers indicate Spearman’s rank correlation coefficient.

sTNFr-II, soluble tumour necrosis factor receptor-II; MCP-1, monocyte chemoattractant protein-1; IP-10, interferon-γ inducible protein-10.

HIV-1 RNA during antiretroviral therapy

After 8 to 12 weeks of treatment, peripheral blood HIV-1 RNA levels were <400 copies/mL in 2 of 5 patients in the RTV/SQV group, in 6 of 8 patients in the RTV/SQV/d4T group, and in 10 of 13 patients in the five-drug group. In all patients with available week-48 samples, peripheral blood HIV-1 RNA was lower than LLQ at that time point (1 of 1 in the RTV/SQV group, 3 of 3 in the RTV/SQV/d4T group, 9 of 9 in the five-drug group).

At weeks 8 to 12, 9 of 10 patients in the five-drug group reached a CSF HIV-1 RNA level <400 copies/mL and/or lower than LLQ, compared with 8 of 8 in the RTV/SQV/d4T group and only 1 of 5 in the RTV/SQV group. In all patients with available week-48 samples, CSF HIV-1 RNA was lower than LLQ at that timepoint. Because of the different lower limits of quantification of the assays used in the two studies, HIV-1 RNA decline in the five-drug group was larger before reaching LLQ of the assay.
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sTNF-II, MCP-1 and IP-10 responses to antiretroviral therapy

Serum sTNF-II concentrations decreased in all three treatment groups to levels within the normal range by week 48 (medium sTNF-II concentration at week 48: 1903 pg/mL). Median serum MCP-1 and IP-10 levels declined during antiretroviral therapy, although the concentrations remained higher than in HIV-negative controls (median serum MCP-1 concentration 1612 versus 721 pg/mL for HIV-negative controls, P = 0.004 and median IP-10 concentration 289 versus 172 pg/mL for HIV-negative controls, P = 0.03; Figure 1).

![Graphs showing changes in HIV-1 RNA, sTNF-II, MCP-1, and IP-10 concentrations during antiretroviral therapy.](image)

Figure 1 Results of HIV-1 RNA, soluble tumour necrosis factor receptor (sTNF)-II, monocyte chemoattractant protein (MCP)-1 and interferon-γ inducible protein (IP)-10 concentrations in serum.

Lines represent median values (± interquartile ranges) of HIV-1 RNA, sTNF-II, MCP-1, and IP-10 concentrations during the study for HIV-1-infected patients receiving ritonavir (RTV)/saquinavir (SQV; dotted lines), RTV/SQV/stavudine (d4T; broken lines), or five drug combination therapy (solid lines). ARVT, antiretroviral therapy.

Results plotted at week -2 represent results of the HIV-negative controls.
In CSF, sTNFr-II levels were lower than LLQ in 15 of 26 patients at week 0 and in 11 of 13 at week 48. In 1 patient receiving RTV/SQV/d4T therapy, CSF sTNFr-II levels increased between weeks 12 and 48 from <100 pg/mL to 2543 pg/mL, although the patient's CSF HIV-1 remained lower than LLQ and serum sTNFr-II became undetectable.

Median CSF MCP-1 levels declined during the first 8 to 12 weeks. However, between weeks 8 and 48 a significant increase in CSF MCP-1 concentration was observed in the five-drug group (median increase, 1204 pg/mL; range, 577-2320 pg/mL; signed log rank test: P=0.004), which was much less pronounced in serum (median increase, 338 pg/mL; range, -146-958 pg/mL). In addition, the patient receiving RTV/SQV who was followed through 48 weeks, and 2 of 3 patients in the RTV/SQV/d4T group had increases in CSF MCP-1 levels between weeks 12 and 48. All these patients had CSF HIV-1 RNA lower than LLQ at week 48.

The median CSF IP-10 concentration increased during the first 12 weeks in the RTV/SQV group. However, in the RTV/SQV/d4T and the five-drug groups, CSF IP-10 levels declined during the first 8 to 12 weeks and remained stable afterward. This difference in CSF IP-10 response was also observed when only asymptomatic (CDC stage A) patients were considered. In one patient who was receiving RTV/SQV, who reached CSF HIV-1 RNA lower than LLQ by week 48, the CSF IP-10 concentration increased threefold between weeks 12 and 48. In one patient receiving RTV/SQV/d4T, CSF IP-10 levels increased during the first 12 weeks, although CSF HIV-1 RNA became lower than LLQ. In another patient receiving RTV/SQV/d4T, CSF IP-10 levels increased between weeks 12 and 48 from 177 pg/mL to 653 pg/mL, although CSF HIV-1 RNA remained lower than LLQ (Figure 2). In peripheral blood, both HIV-1 RNA and IP-10 levels declined during therapy in these patients with increasing CSF IP-10 concentrations.

Discussion

Many data favour the contention that the CNS should be considered as a separate compartment in which HIV can replicate independent of peripheral blood. In untreated patients, evidence for compartmentalisation of the infection is found in the lack of association between CSF HIV-1 RNA levels or CSF abnormalities and plasma HIV-1 RNA. In this study as well, we did not find any correlation between baseline CSF and peripheral blood HIV-1 RNA levels.

In treated patients, penetration of drugs into the CNS may be sub-optimal, resulting in varying virological response in CSF compared with findings in the rest of the body. To explore the differential effects of antiretroviral
Figure 2 Individual results of HIV-1 RNA, soluble tumour necrosis factor receptor (sTNFr)-II, monocyte chemoattractant protein (MCP)-1, and interferon-γ inducible protein (IP)-10 concentrations in cerebrospinal fluid.

Lines represent individual results for HIV-1 infected patients on ritonavir (RTV)/saquinavir (SQV; left), RTV/SQV/stavudine (d4T; middle) or five-drug therapy (right). Results plotted at week –2 represent the results of the HIV-negative controls. ARVT, antiretroviral therapy.
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therapy in CNS versus peripheral blood, we measured inflammatory markers in CSF and peripheral blood of patients on antiretroviral therapies that differ in their ability to penetrate the CNS.

We specifically measured MCP-1 and IP-10 because these are the most sensitive markers of inflammation in children with non-HIV viral meningitis.\textsuperscript{20} In untreated asymptomatic HIV-1 infected patients, CSF MCP-1 concentrations are elevated, which reflects macrophage activation.\textsuperscript{21-23} This is probably a result of viral replication, given that MCP-1 in CSF is correlated with CSF HIV-1 RNA levels.\textsuperscript{16-21} Elevated expression of IP-10 has been shown in the brain of macaque monkeys with simian immunodeficiency virus-related encephalitis.\textsuperscript{24} Although the relative immune activation in the brain can also be demonstrated by immunostaining for TNF-\(\alpha\) and other cytokines,\textsuperscript{25,26} CSF concentrations of TNF-\(\alpha\), interleukin (IL)-6, and the chemokines macrophage-inflammatory (MIP)-1\(\alpha\), MIP-1\(\beta\), and regulated-on-activation normal T-expressed and secreted (RANTES) are lower than the detection limit in most asymptomatic patients.\textsuperscript{18,21,22,27}

We also measured sTNFr-II concentrations, inasmuch as persistent TNF activation potentially plays a role in the pathogenesis of the HIV infection and can predict progression to AIDS.\textsuperscript{28} Upregulation of sTNFr expression on macrophages and microglia is demonstrated in the brains of AIDS patients without encephalitis.\textsuperscript{26}

We have shown that therapy with RTV/SQV without nucleoside analogue reverse-transcriptase inhibitors results in rapid declines of peripheral blood HIV-1 RNA concentrations to lower than LLQ in most patients. However, CSF HIV-1 RNA remained detectable in most of these neurologically asymptomatic patients at week 12.\textsuperscript{8} CSF IP-10 and sTNFr-II concentrations increased in those patients who did not reach CSF HIV-1 RNA lower than LLQ.

Neurologically asymptomatic patients using antiretroviral combination therapies including at least one drug that penetrates well into the CSF (d4T in the RTV/SQV/d4T group, all drugs in the ZDV/3TC/ABC/NVP/IDV group\textsuperscript{7}), showed declines of HIV-1 RNA concentrations in both their peripheral blood and CSF. A decline in peripheral blood HIV-1 RNA was accompanied by declines of markers of inflammation in the peripheral blood. Surprisingly, virological responses and sTNFr-II, MCP-1, and IP-10 responses to antiretroviral therapy in CSF were not concordant in all patients. Some patients with good virological response to therapy in CSF showed an increase in CSF sTNFr-II, MCP-1, and/or IP-10 concentrations. There was no clear pattern of clinical stage or baseline CD4\(^+\) T cell count in patients with dyscongruent
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CSF responses. In addition, we observed a significant increase in CSF MCP-1 concentrations between weeks 8 to 12 and week 48 in the whole study population, although CSF HIV-1 RNA was lower than LLQ at week 48 in all patients.

The visit schedule differed between the ERA and Prometheus studies (8 versus 12 weeks, respectively). This could have caused less pronounced changes from baseline in the ERA patients. However, changes from baseline to week 8 in the ERA patients were comparable with, if not more pronounced than, the changes from baseline to week 12 in the Prometheus study.

Tests used for HIV-1 RNA measurements were different for the two studies. In the Prometheus study, the Roche Amplicor test was used for serum and CSF. In the ERA study, the NucliSens assay was used for plasma and CSF. Although there are differences between the tests and the test fluids used, results are reported elsewhere to be strongly correlated.

The observed decrease of plasma sTNFr-II and MCP-1 levels in patients with a good virological response is confirmed by other reports. During therapy with ZDV, decreased CSF neopterin and $\beta_2$-microglobulin levels have been reported, but no data are available on markers of inflammation in CSF during more potent antiretroviral therapy.

The important question is how to explain increasing CSF chemokine levels in neurologically asymptomatic patients who have a good virological response. Monocytes, macrophages and endothelial cells are the main cellular source of MCP-1 and IP-10. In our study, serum levels of these chemokines were lower than CSF levels. Moreover, serum chemokine concentrations did not increase during follow-up. Therefore, the MCP-1 and IP-10 found in CSF were most likely produced locally. The primary brain cells that are infected by HIV-1 are cells of the monocyte lineage. HIV-1 replication in cultured human monocytes results in an upregulation of MCP-1 secretion. HIV-TAT also increases MCP-1 release by astrocytes in vitro. Therefore, the most likely explanation for the increase in chemokine levels observed in our study is HIV-1 replication. We could not document such local HIV-1 replication, because we did not see an increase in CSF HIV-1 RNA. However, in plasma, markers of inflammation can increase long before virological failure is evident. A longer follow-up is required to rule out virological failure definitively as the cause of increasing chemokine levels.

Whatever the cause, increasing CSF chemokine levels are unwanted. MCP-1 is a major mediator of CSF chemotactic activity on monocytes. MCP-1 and IP-10 both attract activated T cells. When activated T cells are attracted
to the CNS and chemokines are secreted as a result of HIV replication by the activated T cells, a positive feedback loop may occur.

In conclusion, potent antiretroviral therapy including CSF-penetrating drugs reduced CSF HIV-1 RNA levels to lower than LLQ. However, in some patients, increasing CSF chemokine levels were observed during therapy. Residual HIV replication in the CNS, which could not be detected with CSF HIV-1 RNA measurements, may account for this phenomenon. Further studies are needed to explore whether increases in CSF chemokine concentrations, despite declines in CSF HIV-1 RNA levels to lower than LLQ, indeed reflect local HIV-1 replication.

Acknowledgements

The Prometheus study is financially supported by Abbott Laboratories, Abbott Park, Illionois, USA, and Hoffmann-La Roche, Basel, Switzerland. The ERA study is financially supported by a private foundation, which does not wish to be named. We thank T. van der Poll and P. Hollak for critical reading of the manuscript and the patients for participating in the studies. For the ERA study, Glaxo Wellcome kindly provided abacavir, and Boehringer-Ingelheim kindly provided nevirapine.
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