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## Persistent intrathecal immune activation in HIV-1-infected individuals on antiretroviral therapy

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### Abstract

**Background**—Neopterin is a well-established marker of macrophage activation. The cerebrospinal fluid (CSF) neopterin levels are elevated in most HIV-1-infected individuals, and decrease significantly after initiation of antiretroviral therapy (ART). Unexpectedly, CSF concentrations often remain mildly abnormal even in patients treated for a long time with suppressive ART. The aims of this study were to analyse if persistently elevated CSF neopterin levels were associated with either the type of antiretroviral regimen or with low-level CSF HIV-1 concentrations, and to evaluate if plasma HIV-1 RNA levels correlated to lingering CSF neopterin concentrations in patients with effective ART.

**Methods**—One hundred and fifty-seven chronically HIV-1-infected patients with stable ART for  $\geq 6$  months and no neurological symptoms were included, and 193 HIV-1-infected patients without ART served as controls. Neopterin was analysed with either a radio-immunoassay or an enzyme-linked immunosorbent assay. HIV-1 RNA quantification was performed with the Roche Amplicor assay, version 1.5. Two quantitative HIV-1 RNA assays with sensitivities  $\leq 2.5$  copies/ml were used in 40 samples.

**Results**—As anticipated, HIV-1 RNA and CSF neopterin levels were markedly lower in patients on ART compared to untreated controls. No significant difference in CSF neopterin concentrations was found between those treated with protease inhibitor- and non-nucleoside reverse transcriptase-based regimens in combination with 2 nucleoside analogues. Subjects with CSF HIV-1 RNA loads  $< 2.5$  copies/ml had the lowest CSF neopterin levels. Plasma viral load had no impact on intrathecal immune activation in cases with CSF viral loads  $< 50$  copies/ml.

**Conclusions**—The persistent intrathecal cell-mediated immune response was associated with CSF viral load, but not with treatment regimen in individuals on ART.

### Keywords

HIV-1 RNA; cerebrospinal fluid; neopterin; antiretroviral therapy

## Introduction

Neopterin is a low molecular weight pteridine, mainly produced by macrophages and dendritic cells stimulated by interferon-gamma (IFN- $\gamma$ ). Its concentrations in body fluids rise when the cellular immune response is activated, for example during infections, autoimmune disorders, tumors, and allograft rejections<sup>1</sup>. In cerebrospinal fluid (CSF), increased concentrations of neopterin have been found in several infections of the central nervous system (CNS), including aseptic meningitis, Lyme neuroborreliosis, herpes simplex virus type 1 encephalitis and HIV-1 infection, but also during conditions like multiple sclerosis and several neurodegenerative disorders<sup>2-4</sup>.

HIV-1 enters the CNS shortly after transmission, and HIV-1 RNA can be detected in the CSF of nearly all individuals infected by HIV-1, irrespective of disease stage and neurological symptoms. Elevated levels of CSF neopterin have also been found during all stages of infection, with the highest levels in patients with AIDS dementia complex (ADC) and opportunistic infections<sup>5</sup>. After initiation of highly active antiretroviral therapy (HAART), concentrations of CSF neopterin decrease rapidly, although nearly half of the patients have slightly elevated levels after two years of successful viral suppression in CSF<sup>6</sup>. The reason for this persistent intrathecal immune activation and its clinical significance are unknown. Low level virus replication, autoimmune phenomena, or a slow down-regulation of the immune activity are some pathogenic theories<sup>6</sup>.

For many years it was suggested that HIV-1 infection in the CNS might be more difficult to treat than systemic HIV-1, but several studies have indicated that this is not the case. One example is the dramatic decline in the incidence of ADC since the introduction of ART<sup>7</sup>. In addition, the CSF viral load decreases markedly in HIV-1-infected patients on effective ART<sup>8</sup>. Even patients with incomplete systemic viral suppression, have a pronounced decrease of CSF viral load, as well as of the intrathecal immune activation<sup>9</sup>.

Most nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and some ritonavir-boosted protease inhibitors (PIs) are considered CNS-penetrating<sup>10-14</sup>, and lead to suppression of CSF viral load. However, it is not known what impact various combinations of antiretroviral drugs have on the intrathecal immune response, or if suppressing the CSF viral load to levels less than 2.5 copies/ml leads to decreased immune activation in the CNS.

The aims of this study were to analyse if the persistent CSF neopterin levels in well-treated HIV-1-infected individuals were associated with antiretroviral regimen or with the CSF viral load < 50 copies/ml but detected by more sensitive assays. We also wanted to evaluate whether the plasma HIV-1 RNA levels correlated with elevated CSF neopterin concentrations in patients on HAART having CSF HIV-1 RNA <50 copies/ml, since it has been suggested that the increased intrathecal immune activation may reflect viral replication in the periphery and not in the CNS<sup>9</sup>.

## Materials and Methods

### Patients

Subjects were selected from cohorts in Göteborg, Sweden and San Francisco, USA. All were neurologically asymptomatic and chronically HIV-1-infected maintained on stable ART regimens for  $\geq 6$  months. In total, 157 HIV-1-infected patients with ART and 193 HIV-1-infected controls without treatment for  $\geq 6$  months (74 female, 276 male) were retrospectively included in the study (232 from Göteborg and 118 from San Francisco) between November 1985 and August 2006. The subjects were divided into groups according to the number of

antiretroviral drugs with which they were treated: (1) 1 NRTI, (2) 2 NRTIs, and (3) HAART, defined as  $\geq 3$  drugs. If more than one comparable lumbar puncture was available, a single interval was randomly selected. One hundred and thirty-four of the participants were treated with HAART, 11 with 2 NRTIs, and 12 with 1 NRTI. Patient characteristics are shown in Table 1. Among the controls not receiving ART, 151 (78 %) were naive to ART, and the 42 previously treated subjects had been off therapy for a median of 1.9 (IQR 0.7–4.1) years. Study participants were further analysed regarding the effect of CSF viral load on the intrathecal immune activation, and subjects with CSF viral loads  $< 50$  copies/ml were additionally evaluated regarding treatment regimens and the impact of plasma viral load on CSF neopterin levels. This study was approved by the ethics committees of Göteborg University and University of California San Francisco.

## Methods

HIV-1 RNA in CSF and plasma was measured using the Roche Amplicor assay (version 1.5, Hoffman-La Roche, Basel, Switzerland). The assay has a dynamic range down to 50 copies/ml ( $1.70 \log_{10}$  copies/ml) and a lower detection limit of 20 copies/ml ( $1.30 \log_{10}$  copies/ml) for both plasma and CSF. All HIV-1 RNA values  $< 20$  copies/ml were set at 19 copies/ml. Fourty patients (11 from Sweden and 29 from USA) had their plasma and CSF viral loads determined by modified quantification assays with detection limits of 2.0 and 2.5 copies/ml respectively, methods described elsewhere<sup>15, 16</sup>. Neopterin was measured by a commercially available radio-immunoassay (Henningtest Neopterin, BRAHMS, Berlin, Germany)<sup>17</sup>, or enzyme-linked immunosorbent assay (same manufacturer). The same antibodies are used in these two assays, and the results are interchangeable<sup>18</sup>. Normal reference values were  $\leq 8.8$  nmol/l in serum and  $\leq 5.8$  nmol/l in CSF<sup>19</sup>. The CD4+ T-cell count was analysed by flow cytometry.

## Statistical analysis

$\log_{10}$  transformation was applied to all HIV-1 RNA and neopterin data in figures and tables. Nonparametric methods were used for group descriptives (median and interquartile range, IQR). When two groups were being compared, comparisons were done using the Mann-Whitney U-test. Differences among more than two groups were detected using one-way analysis of variance, with multiple-group comparisons done using the Tukey post hoc test.

## Results

A larger proportion of patients on HAART (88 %,  $p < 0.001$ ) and 2 NRTIs (64 %,  $p < 0.001$ ) had CSF viral load  $< 50$  copies/ml than patients treated with 1 NRTI (0 %). Plasma HIV-1 RNA was  $< 50$  copies/ml in 70 % of individuals on HAART, 9 % of individuals on 2 NRTIs, 0 % among subjects treated with 1 NRTI, and 2 % for those without treatment. CSF neopterin levels were significantly lower for individuals on HAART ( $p < 0.001$ ) or 2 NRTIs ( $p < 0.01$ ) compared with untreated controls, while patients on 1 NRTI had levels in the same range as the controls (Figure 1). The patients on HAART had significantly higher CD4+ T-cell counts than individuals on 1 NRTI and subjects without ART. Subjects not receiving ART were more recently diagnosed with HIV-1 than treated subjects. It should be kept in mind that the number of subjects receiving 1 NRTI or 2 NRTIs is quite small.

Individuals on PI- or NNRTI-based HAART (defined as  $\geq 1$  PI or 1 NNRTI in combination with NRTIs) and with CSF viral loads  $< 50$  copies/ml had lower levels of CSF neopterin compared to untreated controls with CSF HIV-RNA  $< 50$  copies/ml ( $p < 0.001$  and 0.01, respectively) (Figure 2). The CSF HIV-1 RNA and neopterin for subjects on PI- or NNRTI-based regimens was in the same range. The median (IQR) plasma HIV-1 RNA levels did not differ between subjects on a PI-based regimen; 1.3 (1.3–1.8)  $\log_{10}$  copies/ml and a NNRTI-

based regimen; 1.3 (1.3–1.3)  $\log_{10}$  copies/ml, but was significantly higher in untreated controls; 3.2 (2.1–3.8)  $\log_{10}$  copies/ml ( $p < 0.001$ ). The patients had been on their PI- or NNRTI-based treatment for a median (IQR) of 1.9 (1.0–3.2) and 2.9 (1.1–4.0) years, respectively. Of the 70 patients on PI-based treatment, 21 were on single PI-treatment with indinavir ( $n = 11$ ), nelfinavir ( $n = 8$ ), or saquinavir ( $n = 2$ ); 8 had a regimen including saquinavir and nelfinavir; and 41 had a ritonavir-boosted PI with either lopinavir ( $n = 22$ ), atazanavir ( $n = 9$ ), saquinavir ( $n = 5$ ), and indinavir ( $n = 5$ ). No differences in CSF neopterin concentrations were found between those groups (data not shown). For the 43 subjects on NNRTI-based regimens, CSF neopterin did not differ between those on efavirenz ( $n = 24$ ) and those on nevirapine ( $n = 19$ ).

The individuals who had CSF HIV-1 RNA levels  $< 2.5$  copies/ml had significantly lower plasma viral loads than the other groups ( $p < 0.001$ ). The median (IQR) plasma HIV-1 RNA levels were 0.4 (0.4–0.9)  $\log_{10}$  copies/ml for subjects with CSF viral loads  $< 2.5$  copies/ml, 2.6 (1.6–3.5)  $\log_{10}$  copies/ml for subjects with CSF HIV-1 RNA 2.5–49 copies/ml, 4.1 (2.7–4.7)  $\log_{10}$  copies/ml for subjects with CSF HIV-1 RNA  $\geq 50$  copies/ml, and 3.3 (2.3–4.4)  $\log_{10}$  copies/ml for untreated controls. The subjects with CSF viral loads  $< 2.5$  copies/ml also had the lowest CSF neopterin concentrations ( $p < 0.01$ ) (Figure 3). Fifteen out of 35 subjects with CSF viral loads  $< 2.5$  copies/ml had CSF neopterin concentrations within the normal reference value, compared to two of the 29 participants with CSF HIV-1 RNA  $\geq 2.5$  copies/ml (13 with CSF HIV-1 RNA between 2.5 and 49, and 16 with CSF HIV-1 RNA  $\geq 50$  copies/ml).

For subjects on HAART and with CSF viral loads  $< 50$  copies/ml, there were no statistically significant differences in CSF and serum neopterin levels, for the 92 individuals with plasma viral loads  $< 50$  copies/ml, and the 26 with plasma viral loads  $\geq 50$  copies/ml, Figure 4. The median (IQR) plasma HIV-1 RNA level for individuals with detectable viremia was 3.1 (2.4–3.7)  $\log_{10}$  copies/ml.

## Discussion

As expected, HAART effectively reduces plasma and CSF HIV-1 RNA, as well as the degree of immune activation measured as decreasing concentrations of serum and CSF neopterin. However, despite of virologically successful ART, many patients demonstrate ongoing intrathecal immune responses, which is in concordance with previous studies<sup>6</sup>. The clinical significance of this finding is unknown. Although there have been reports about neurological deterioration in patients with virologically effective treatment<sup>20</sup>, it is a very uncommon clinical development. By analysing various treatment regimens and by using a very sensitive HIV-1 RNA quantification assay we have extended the characterisation of the persisting signs of intrathecal immune activation, but the question whether a low-grade intrathecal immune activation is harmful or not remains.

A weakness with the study is its retrospective approach. CSF analyses are difficult to include in prospective studies because of the reluctance of many to include lumbar punctures in the study protocols comparing treatments. Therefore retrospective studies in well categorised patient materials are, as yet, the main means of gathering more information.

Current treatment guidelines recommend a combination of 2 NRTIs plus either a NNRTI or a PI when initiating antiretroviral treatment<sup>21, 22</sup>. In this study, we compared patients treated with NRTIs + PI and NRTIs + NNRTI. The CSF neopterin levels were similar in these two groups which indicates that there are no large differences between these HAART regimens on the persistent low-level intrathecal immune activation. It should be noticed, however, that NNRTI-treated patients had been somewhat longer on their treatment than PI-treated patients. Another finding demonstrating that many treatment regimens are effective in the CSF is that

the nowadays non-accepted treatment with only 2 NRTIs leads to a much more pronounced reduction of viral load in CSF than in plasma<sup>23</sup>, which was confirmed in the present study.

There were previously concerns that the CNS could be a sanctuary site that is difficult to reach with antiretroviral drugs. Theoretically, the lower drug concentrations in CSF, and the large mass of infected cells in the CNS being long-lived macrophages and microglia, might lead to a slower and less impressive decline in CSF than in plasma. This type of viral kinetics has been observed in patients with ADC<sup>24</sup>, where virus in CSF mostly emanates from local production within the CNS, as opposed to a larger ratio of virus trafficking from the periphery in neurologically asymptomatic individuals, as in this study.

Beneficial virological treatment results in CSF in individuals without neurocognitive symptoms could, at least in part, be due to the fact that the plasma viral load in most cases exceeds the CSF viral load<sup>25</sup>, or that some ART regimens more effectively reduce viral replication in CSF than in blood. In vitro experiments have demonstrated a superior antiviral effect for NRTIs in macrophages than in activated lymphocytes, since cells like macrophages have much lower levels of 2'-deoxy-nucleoside triphosphates (dNTPs), thereby enhancing the chain-terminating activity of NRTIs<sup>26</sup>. Antiretroviral drugs are required to penetrate into the CNS in order to achieve a substantial decline of the CSF viral load, but the number of CSF-penetrating drugs needed to do this is not established. Monotherapy with zidovudine, reduces the viral burden and the cell-mediated immune response in CSF more than in plasma, and even led to a decrease in the incidence of ADC in the late 1980s<sup>27-29</sup>. Drugs that are undetectable or barely detectable in CSF, such as saquinavir/ritonavir, didanosine, or nelfinavir, do not lead to viral suppression in CSF when used on their own<sup>27, 30, 31</sup>. Lopinavir/ritonavir, on the other hand, reaches CSF concentrations exceeding the 50 % inhibitory concentration (IC<sub>50</sub>)<sup>13</sup>, and suppresses the CSF viral load successfully for a median of 38 weeks when used as monotherapy<sup>32</sup>. Thus one potent antiretroviral drug could suffice, as long as it has the capacity to cross the BBB. How long the effect of a single agent will last in the CSF, and whether this in the long run can lead to increased development of resistance needs further evaluation.

Among treated subjects, the group with CSF HIV-1 RNA < 2.5 copies/ml had significantly lower CSF neopterin levels than subjects with higher CSF viral loads. It has previously been observed that higher CSF viral loads are associated with higher CSF neopterin concentrations, but this has not been extended to CSF HIV-1 RNA levels < 50 copies/ml. We found that also a very low viral activity stimulates the immune activation albeit at much lower levels than in untreated patients. If the intrathecal immune activation is thought to be harmful a conclusion drawn from these results would be to include more CSF monitoring analysis and to lower the threshold for viral detection and goal for treatment.

Interestingly, in another study, subjects who exhibited systemic virological failure (defined as HIV-1 RNA > 500 copies/ml), still had beneficiary results of their ART with a decreased systemic immune activation, and a larger reduction of CSF than plasma viral load<sup>9</sup>. The failing patients in that study had lower CSF neopterin than untreated subjects, but higher than the successes. It was hypothesised that the reduced activity of the peripheral immune system might lead to fewer T-cells trafficking through the BBB, and reduced CSF viral load as a consequence, since short-lived cells from outside the CNS have been shown to sustain a large proportion of the CSF viral load in neurologically asymptomatic subjects<sup>33</sup>. Bearing this in mind, we expected the individuals in our study with systemic virological failures (plasma HIV-1 RNA ≥ 50 copies/ml) to have higher CSF neopterin levels than the treatment successes (plasma HIV-1 RNA < 50 copies/ml). However, this was only true for subjects who also had CSF viral loads ≥ 50 copies/ml. Individuals with plasma viral loads ≥ 50 copies/ml (failures), but with undetectable CSF viral loads did not have higher CSF neopterin levels than the group with



undetectable plasma HIV-1 RNA as well. This again points towards the viral burden in CSF as an important factor behind increased CSF neopterin concentrations. Furthermore, no difference in CSF neopterin was found between patients on HAART and CSF HIV-1 RNA  $\geq$  50 copies/ml and matched untreated controls. Our findings thus may argue against the prevalent role of T-cell trafficking through the BBB even in individuals without neurological symptoms.

Although ART reduces the CSF viral load dramatically, decreases the incidence of neurological complications, and even improves symptoms in patients with ADC, it does not have the same impressive effect on the intrathecal cell-mediated immune activation, as measured by neopterin. Even though the individuals with CSF viral loads  $<$  2.5 copies/ml had the lowest CSF neopterin levels, they were still slightly elevated in many (57%) of these virologically well-suppressed patients. We do not yet know the clinical consequences of having an active immune system in the CNS for a prolonged time, and if this could lead to neurological injuries in the future.

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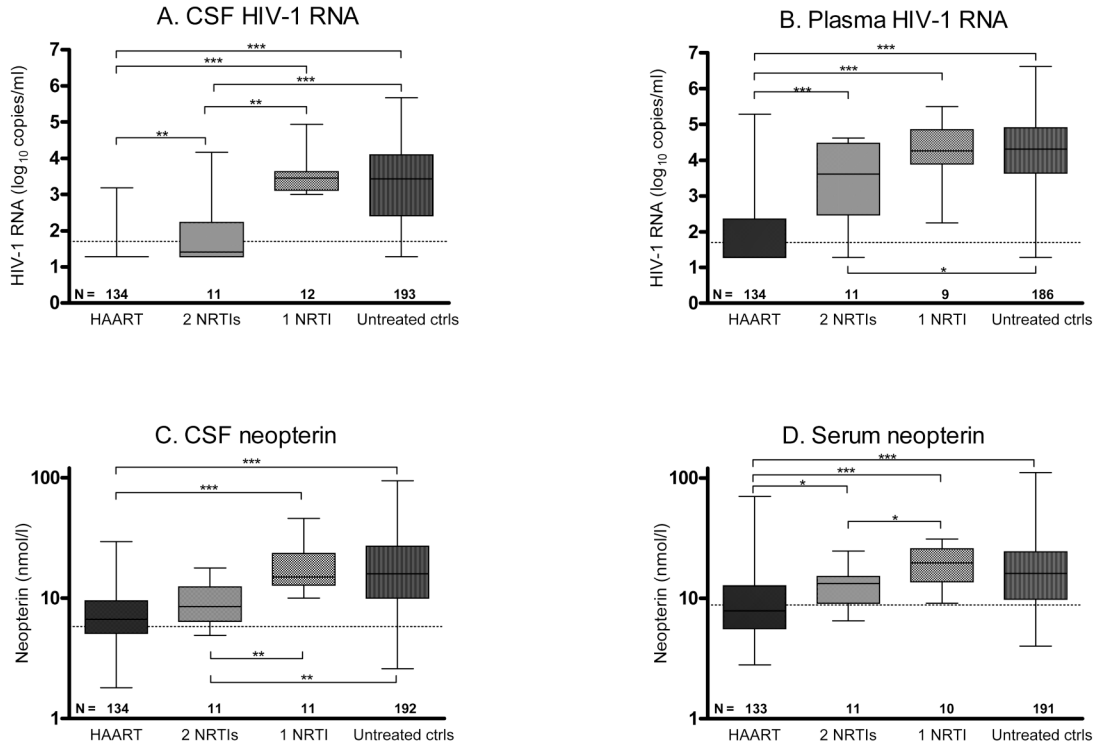
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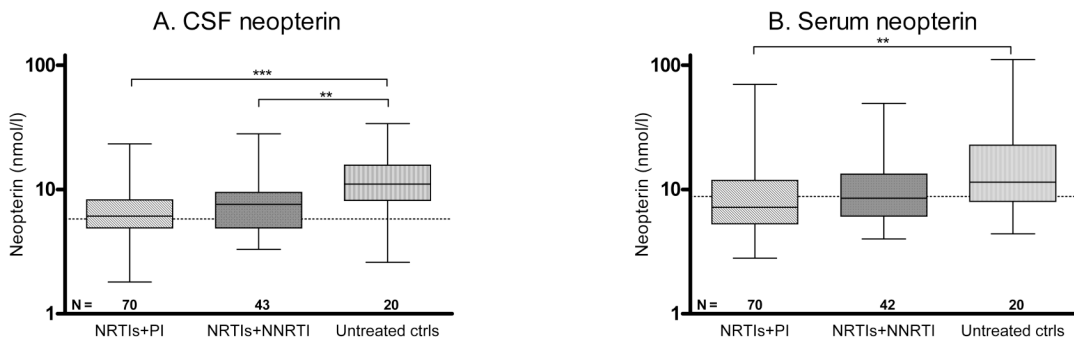
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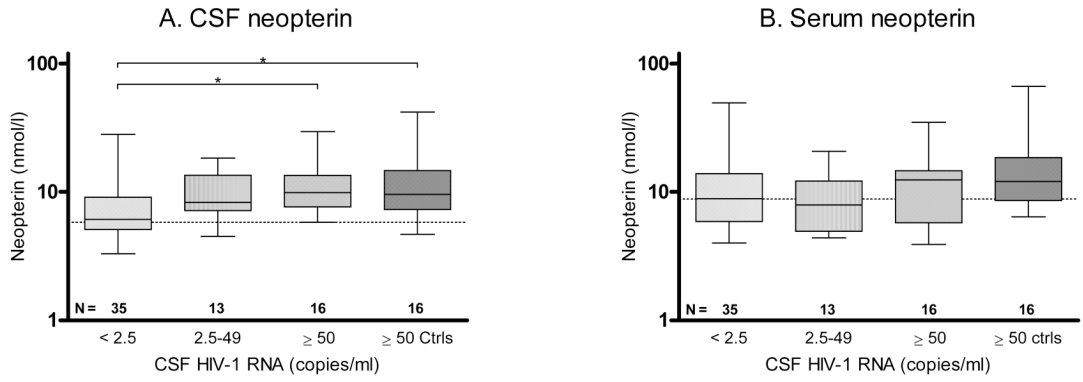




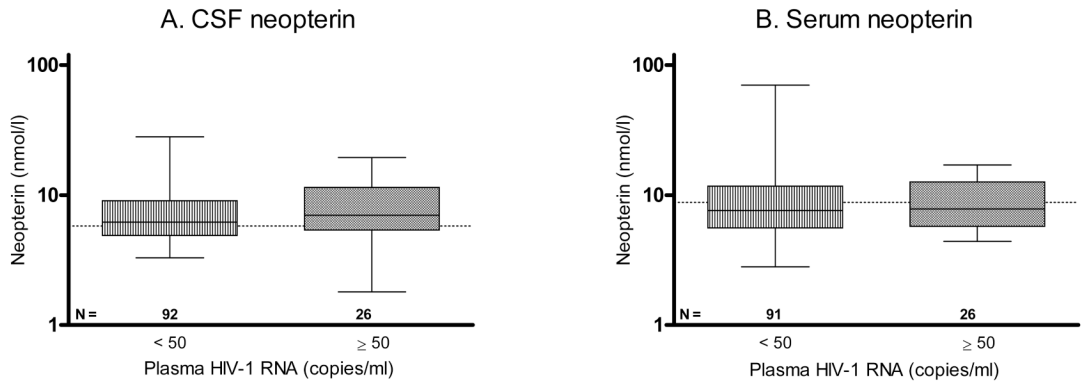
**Figure 1.** HIV-1 RNA and neopterin concentrations for the whole study population according to antiretroviral regimen. (A) CSF HIV-1 RNA, (B) plasma HIV-1 RNA, (C) CSF neopterin, and (D) serum neopterin. N denotes the number of patients in each group. Dotted lines indicate upper reference values. Median values, 25<sup>th</sup> and 75<sup>th</sup> percentiles, and ranges are shown in the box plots. Ctrls: controls. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , and \*\*\* =  $p < 0.001$ .



**Figure 2.** Subjects on PI- or NNRTI-based HAART and untreated controls (ctrls) with CSF viral loads < 50 copies/ml. (A) CSF neopterin, (B) serum neopterin. N denotes the number of patients in each group. Dotted lines indicate upper reference values. Median values, 25<sup>th</sup> and 75<sup>th</sup> percentiles, and ranges are shown in the box plots. \*\* =  $p < 0.01$  and \*\*\* =  $p < 0.001$ .



**Figure 3.** CSF and serum neopterin levels in HIV-1-infected individuals on HAART, and with different CSF HIV-1 RNA levels. The CSF viral loads for untreated controls (ctrls) were matched to subjects on HAART and with CSF viral loads  $\geq 50$  copies/ml. (A) CSF neopterin, (B) serum neopterin. N denotes the number of patients in each group. Dotted lines indicate upper reference values. Median values, 25<sup>th</sup> and 75<sup>th</sup> percentiles, and ranges are shown in the box plots. \* =  $p < 0.05$  and \*\* =  $p < 0.01$ .



**Figure 4.** Subjects on HAART and with CSF viral loads < 50 copies/ml grouped by level of plasma viral load (< 50 or ≥ 50 copies/ml). No significant differences were found in (A) CSF neopterin, or (B) serum neopterin concentrations between groups. N denotes the number of patients in each group. Dotted lines indicate upper reference values. Median values, 25<sup>th</sup> and 75<sup>th</sup> percentiles, and ranges are shown in the box plots.

## Tables

Characteristics of all HIV-1-infected subjects included in the study divided into groups based on antiretroviral treatment.

Characteristic	HAART (n = 134)	2 NRTIs (n = 11)	1 NRTI (n = 12)	Untreated controls (n = 193)	P-value
Sex (F:M)	31:103	2:9	1:11	40:153	Non-significant
Age	42.1 (36.6–50.2)	42.0 (35.2–52.0)	39.1 (33.5–49.6)	36.8 (30.5–43.7)	< 0.05 <sup>a</sup>
Years since diagnosis	7.0 (3.4–12.8)	10.0 (5.9–15.6)	3.4 (1.6–5.5)	2.5 (0.4–8.1)	< 0.05 <sup>a,b,c,d</sup>
Years on ART	2.0 (1.0–3.5)	2.6 (1.6–4.2)	1.3 (0.8–2.2)	—	Non-significant
CD4+ T-cell count ( $\times 10^6$ cells/l)	370 (249–581)	328 (170–598)	150 (76–256)	308 (120–502)	< 0.05 <sup>a,b</sup>

Values shown are median (interquartile range). Controls were untreated for  $\geq 6$  months. HAART: highly active antiretroviral therapy; NRTI: nucleoside reverse transcriptase inhibitor; F: female; M: male; ART: antiretroviral therapy. Comparisons were made using analysis of variance followed by Tukey test.

<sup>a</sup> HAART vs. untreated controls

<sup>b</sup> HAART vs. 1 NRTI

<sup>c</sup> 2 NRTI vs. 1 NRTI

<sup>d</sup> 2 NRTI vs. untreated controls