

## Asymptomatic Cerebrospinal Fluid HIV-1 Viral Blips and Viral Escape During Antiretroviral Therapy: a Longitudinal Study

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### Abstract

### Background

We examined asymptomatic cerebrospinal fluid (CSF) viral escape in relation to CSF biomarkers of neuronal damage (NFL) and intrathecal immune activation (neopterin) in a longitudinal cohort of HIV-1 infected patients on effective antiretroviral therapy (ART).

## **Methods**

Neuroasymptomatic patients on ART (plasma HIV-1 RNA <50 copies/ml) with  $\geq 2$  available CSF samples were included. HIV-1 RNA was analyzed with real-time PCR (lower limit of quantification (LLQ) 20 copies/ml). CSF NFL and neopterin concentrations were measured by ELISA.

## **Results**

Seventy-five patients (median (IQR) 5 (3-8) CSF samples) were included. Median (IQR) treatment time was 93 (60-129) months. Twenty-seven (36%) patients had  $\geq 1$  CSF HIV-1 RNA >LLQ (median 50 copies/ml). Forty-two (56%) patients also had  $\geq 1$  plasma blip. CSF virus >LLQ was associated with a 19% higher geometric mean CSF neopterin ( $p=0.003$ ) and 3x higher CSF white blood cell count (WBC) ( $p=0.00001$ ), but no difference was seen in CSF NFL ( $p=0.2$ ). No patients had clinical symptomatic progression or selective CSF viral failure.

## **Conclusion**

A substantial minority (36%) of neuroasymptomatic patients had  $\geq 1$  CSF RNA >LLQ in longitudinal follow-up. Although intrathecal immune activation was increased, the lack of correlation to CSF NFL or clinical progression suggests that asymptomatic CSF viral escape is benign.

## **Introduction**

Despite the apparent success of antiretroviral therapy (ART) in preventing severe complications to HIV-1 infection in the central nervous system (CNS), the reported prevalence of milder forms of HIV-associated neurocognitive impairment (HAND) remains high [1, 2]. Although ART is usually as effective in suppressing virus in cerebrospinal fluid (CSF) as in plasma, cerebrospinal fluid (CSF) viral escape occurs infrequently in patients responding well to antiretroviral therapy (ART).

Neurosymptomatic CSF escape is a seemingly rare but clinically significant condition characterized by new or progressive CNS symptoms during ART. These symptoms often respond well to intensified or modified therapy [3, 4]. In contrast, asymptomatic CSF escape, where HIV-1 can be detected in CSF despite suppression of plasma virus to below detection limits of standard assays, lacks clinical signs of active CNS disease [5, 6].

In its most severe form, HIV-associated dementia (HAD), HIV-1 infection of the CNS initiates neuropathological inflammatory responses characterized by immune activation and neuronal damage, called HIV encephalitis (HIVE); processes that can be measured using CSF biomarkers. CSF neopterin is a marker of macrophage and microglial activation that decreases in response to ART. However, CSF neopterin is often not normalized despite apparently effective therapy, reflecting residual immune activation in the CNS that can be detected in many patients despite long-term therapy [7, 8]. The light chain of the neurofilament protein (NFL) is an important structural component of myelinated axons, and has been shown to be a sensitive marker of CNS injury in HIV-1 infection, as well as in other neurologic diseases [9-11]. In patients with HAD, CSF concentrations of NFL are generally high, but usually decrease to within normal levels after initiation of therapy [11].

We have previously shown that 10 % of patients on effective ART had asymptomatic CSF escape in a cross-sectional analysis, where patients with CSF escape had higher concentrations of CSF neopterin reflecting increased intrathecal immune activation [5]. It is unclear if asymptomatic escape represents an active CNS infection despite apparently effective ART, or reflects benign low level variations in release of virus into the CSF compartment similar to plasma blips. The aims of this study were to characterize the occurrence of asymptomatic CSF viral escape, and the relationship between CSF viral

escape and CSF biomarkers of intrathecal immune activation (neopterin) and axonal injury (NFL) in a longitudinal cohort of patients

## **Methods**

### Study design and subjects

Since 1985, HIV-infected patients in Gothenburg, Sweden have been included in a longitudinal study including serial sampling of CSF, plasma and serum. Lumbar puncture (LP) is performed in a standardized manner at least annually and more frequently on initiation or cessation of ART. Asymptomatic as well as symptomatic patients are included, and as of May 2016, the cohort included 539 subjects who had undergone 1984 LPs. From this cohort, we retrospectively identified neuroasymptomatic subjects who had been followed longitudinally and who had undergone repeated ( $\geq 2$ ) LPs for research purposes. Subjects who were selected for inclusion had been on continuous ART  $\geq 6$  months and had achieved viral suppression in plasma (HIV-1 RNA  $< 50$  copies/ml) prior to inclusion. All treatment regimens were included, and change of ART combinations during follow up was allowed. Viral blips in plasma, defined as a transient HIV-1 RNA  $< 500$  copies/ml preceded and followed by HIV-1 RNA  $< 50$  copies/ml during ongoing therapy were allowed. If a patient had a treatment history of more than one time period fulfilling inclusion criteria, the most recent was included in the study. Subjects with ongoing or recent CNS opportunistic infections or disease (e.g. stroke) were excluded from the analysis. All included subjects were neurologically asymptomatic in clinical examination at inclusion, and without subjective neurological complaints. Neurocognitive performance testing was performed with CogState from 2011 [12]. Prior to that, not all subjects were

consistently evaluated with neurocognitive testing. The research protocol followed in the present study was approved by the Research Ethics Committee of the University of Gothenburg, and all subjects provided written informed consent to participate.

### CSF and blood measurements

At each study visit, subjects underwent clinical evaluation and venous and lumbar puncture. Clinical assessment of CSF white blood cell count (WBC) and CD4<sup>+</sup> T-lymphocyte count were performed using routine methods. Paired samples of blood and CSF were centrifuged and cell-free plasma and CSF was aliquoted and stored in -80°C. A subset of cell-free CSF and plasma samples (add number) used only for HIV-1 RNA analysis was stored at -20°C. HIV-1 RNA in plasma and CSF was measured with real-time RT-PCR using the commercial Cobas TaqMan HIV-1 version 2 assay (Roche) with a lower limit of quantification (LLQ) of 20 c/ml and a dynamic range of 20 to 1x10<sup>7</sup> c/ml. CSF NFL concentration was measured using a commercially available sandwich ELISA method (NF-light® ELISA kit, UmanDiagnostics AB, Umeå, Sweden) with a lower limit of quantification of 50 ng/l. CSF NFL increases with normal ageing and previously established upper normal reference limits were <380 ng/l (18-29 years), <560 (30-39 years), <890 (40-59 years), <1850 (>59 years) in healthy control individuals [11]. Neopterin concentrations in plasma and CSF were measured using a commercially available immunoassay (BRAHMS, Berlin, Germany), with an upper normal reference value of 5.8 nmol/l in CSF and 8.8 in plasma [13].

### Statistical analysis

Descriptive statistics were performed using Prism (version 6, Graph-Pad) or SPSS (IBM SPSS version 20) software. Continuous variables were log<sub>10</sub> transformed where appropriate for the tests used. Tests were two-sided and p<0.05 was considered statistically significant. Analyses involving repeated measurements of log<sub>10</sub> CSF NFL, log<sub>10</sub> CSF and plasma neopterin, and CD4<sup>+</sup> T-lymphocyte count

were performed with linear mixed effects models. A generalized linear model assuming Poisson distribution was used for analysis of WBC in relation to CSF HIV-1 RNA. Correlations were explored using Spearman's rank correlation.

## Results

Seventy-five (52 male) neuroasymptomatic subjects were included in the analysis. Subject characteristics are shown in Table 1. The study included samples collected between 1997 and 2015, and median (IQR) number of LPs in the study population was 5 (3-8). An overview of per-patient CSF samples and frequency of CSF HIV-1 RNA >LLQ is shown in Figure 1A. Median (IQR) number of LPs in subjects with suppressed CSF virus was 5 (3-6), compared to 5 (4-8) in subjects with  $\geq 1$  CSF HIV-1 RNA >LLQ, with the higher IQR representing an increased likelihood of having quantifiable CSF HIV-1 RNA with a larger number of available samples. Twenty-seven subjects (36%) had  $\geq 1$  CSF HIV-1 RNA >LLQ, in median (IQR) 50 (32-77) copies/ml. Seven subjects (9%) had CSF HIV-1 RNA >LLQ in 2 consecutive samples. When applying the commonly used cut-off of 50 copies/ml, 17 subjects (23%) had elevated CSF HIV-1 RNA at least once. In plasma, 42 subjects (52%) had  $\geq 1$  viral blip with a median (IQR) viral load of 44 (29-71) copies/ml. The corresponding number using the higher cut-off was 22 subjects (29%) with HIV-1 RNA >50 copies/ml. Six subjects had 1, and one subject 2, simultaneous plasma and CSF HIV-1 RNA >LLQ. In total, 418 CSF and plasma samples were analyzed. In CSF, 40 (9%) had HIV-1 RNA >LLQ. Using the higher cut-off, 20 (5%) had elevated CSF RNA. Corresponding numbers in plasma were 78 (19%) >LLQ and 33 (8%) >50 copies/ml.

None of the study subjects had signs of progressive CSF escape indicated by increasing CSF HIV-1 RNA despite control of plasma viremia, and all remained clinically neuroasymptomatic. One subject had CSF HIV-1 RNA >LLQ in both available samples, but although declining additional LPs continued to be effectively suppressed in plasma for several years with no neurological complaints.

The distribution of ART regimen type in relation to CSF HIV-1 RNA is shown in Table 1. We found no significant differences in type of antiretroviral regimen and CSF HIV-1 RNA >LLQ. As expected, the composition of individual drugs in ART regimens varied over time and 47 (63%) subjects changed drugs during the study period. The considerable number of individual combinations used by the study subjects did not allow for a conclusive analysis of the impact of individual drugs on CSF RNA or biomarkers. However, it is notable that CSF HIV-1 RNA was >LLQ in 2/3 samples drawn during monotherapy with a boosted protease inhibitor, as described previously [14].

Median (IQR) averaged CSF neopterin in the study population was 6.5 (5.2-7.7) nmol/l. In linear mixed model analysis of log<sub>10</sub> CSF neopterin, samples with CSF HIV-1 >LLQ had a 19% higher geometric mean CSF neopterin than samples with suppressed CSF virus (p=0.003). The distribution of CSF neopterin in relation to CSF HIV-1 RNA is shown in Figure 1B. No significant difference was found in plasma neopterin in subjects with or without CSF HIV-1 RNA >LLQ (p=0.3).

The previously described relation between CSF NFL and age was confirmed in this longitudinal analysis, with a highly significant correlation ( $p=3 \times 10^{-18}$ ) found (Figure 1C).

Median (IQR) average CSF NFL in the study population was 532 (314-781) ng/l. The distribution of CSF NFL in relation to CSF HIV-1 RNA is shown in Figure 1D. In a linear mixed model adjusted for age, no significant difference in CSF NFL was found in relation to CSF HIV-1 RNA above or below LLQ (p=0.2). However, CSF NFL was significantly correlated to CSF neopterin in the whole study population ( $r=0.16$ ;  $p=0.001$ ).

Although frequently low, median (range) CSF WBC was higher in samples with CSF HIV-1 RNA >LLQ, 2 (0-30)  $\times 10^6$  cells/l than in samples with suppressed CSF virus, 1 (0-12)  $\times 10^6$  cells/l. Using a generalized linear model, the difference corresponded to 3 times higher WBC in samples with CSF HIV-1 RNA >LLQ ( $p=0.00001$ ). Moreover, WBC was significantly correlated to CSF neopterin ( $r=0.13$ ;  $p=0.01$ ) but not to CSF NFL ( $r=0$ ;  $p=1$ ) in the whole study population.

## **Discussion**

In this analysis of neuroasymptomatic patients on ART with well-controlled plasma viremia, we found that a substantial minority (36%) of subjects had  $\geq 1$  CSF HIV-1 RNA above assay quantification limit (20 copies/ml) in longitudinal follow up. In 7/27 subjects, quantifiable CSF virus coincided with a plasma viral blip. Although RNA levels were low (in median [IQR] 50 [32-77] copies/ml), quantifiable CSF HIV-1 was associated with a significant increase in intrathecal immune activation, measured by CSF neopterin. These longitudinal observations confirm our previous cross-sectional findings, where 10% of subjects with controlled plasma virus had asymptomatic CSF escape and a concurrent increase in CSF neopterin [5].

CSF neopterin has previously been shown to correlate closely to CSF viral load, and although ART reduces the level of immune activation, CSF neopterin remains elevated in many patients on suppressive therapy [7, 8]. It is not known whether this residual immune activation is generated by persistent viral replication within the CNS, or is a result of other causes. Interestingly, we found that quantifiable CSF virus was associated with WBC, and CSF WBC was also correlated to CSF neopterin. The increase seen in CSF HIV-1 RNA may result from an amplification of brain-derived CSF virus through recruitment of peripheral CD4<sup>+</sup> T-cells to the CSF compartment. Alternatively,



release of virus to the CSF from previously infected trafficking CD4<sup>+</sup> T-cells from the peripheral circulation may initiate macrophage activation and a subsequent increase in CSF neopterin [15-17].

None of the subjects included in the analysis developed progressive CSF escape with selective CSF treatment failure, and all remained clinically neuroasymptomatic, suggesting that asymptomatic CSF escape with a low-level increase in CSF HIV-1 RNA during systemically suppressive therapy represents the equivalent of a plasma viral blip in a majority of cases. We found no evidence in the study linking the presence of CSF virus to axonal injury indicated by the lack of correlation between CSF virus >LLQ and age-adjusted CSF NFL, which suggests that asymptomatic CSF escape is usually clinically benign. However, a cause for concern is the correlation found between CSF NFL and neopterin in the study population, indicating a mechanistic association between CNS inflammation and neuronal damage during ongoing therapy that needs to be characterized further. In addition, CD4 nadir in the study population was low (median 145 cells/mm<sup>3</sup>), indicating more advanced immunosuppression and possibly a more established compartmentalized CNS infection [18]. It is possible that more advanced immunosuppression prior to ART initiation may influence the subsequent frequency of CSF viral blips, as well as the risk of developing neurosymptomatic CSF viral escape that may be prevented by earlier ART initiation.

Antiretroviral regimens varied in our study population, in drug composition as well as due to change of therapy over time preventing a conclusive analysis of the impact of individual drugs on CSF viral blips. Interestingly, two patients had quantifiable CSF virus during monotherapy with boosted darunavir, and was subsequently suppressed after reintroducing NRTIs to the regimen [14]. Although darunavir has been shown to achieve high CSF exposure [19], it is possible that protease inhibitor monotherapy may be unreliable in controlling viral replication in the CNS. However, our results do not support any specific modification of ART in patients with asymptomatic CSF escape (CSF blips) as long as therapy is effective in achieving systemic viral suppression. In the more uncommon cases

with neurosymptomatic escape described in previous series, modification of therapy is likely of greater importance [3, 4]. In the present analysis, patients with incomplete plasma viral suppression as a reflection of non-adherence or less efficient ART were excluded. It is likely however, that patients on less successful therapy may have a higher incidence of asymptomatic, as well as neurosymptomatic CSF viral escape although future studies are need to investigate this issue further.

A subset of samples (n=xx) used for analysis of CSF and plasma HIV-1 RNA were stored at -20°C, while all other analyses were either performed directly or used samples stored at -80°C. Previous reports have shown minimal or no difference in nucleic acid detection after storage in -20°C compared to -80°C [20, 21]. However, we cannot completely rule out that sample storage may have had an impact on the results. In addition, neurocognitive testing was not consistently performed in all patients. All included subject were clinically neuroasymptomatic and without subjective complaints, excluding cases of symptomatic HAND – minor neurocognitive disorder (MND) and HAD. However, although neurocognitive testing (CogState) has more recently been included in the protocol, we cannot rule out that asymptomatic neurocognitive impairment (ANI) may have been present in some cases.

In conclusion, in this longitudinal analysis of highly adherent, successfully treated neuroasymptomatic patients on effective ART, we found that a substantial minority (36%) of subjects still had occasional low-level HIV-1 RNA measurable in the CSF. Although the presence of quantifiable virus was correlated to increased intrathecal immune activation, no correlation was found to CSF NFL representing axonal damage and none of the cases progressed to clinical CSF viral escape suggesting that CSF HIV-1 RNA in this setting is benign and represents the equivalent of plasma viral blips. However, additional studies are needed to separate seemingly benign CSF viral blips from the more significant, but more uncommon cases of clinically significant, neurosymptomatic CSF viral escape.

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