Marked increase of the astrocytic marker S100B in the cerebrospinal fluid of HIV-infected patients on LPV/r-monotherapy

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Objective: To determine changes of cerebrospinal fluid (CSF) biomarkers of patients on monotherapy with lopinavir/ritonavir.

Design: The Monotherapy Switzerland/Thailand study (MOST) trial compared monotherapy with ritonavir-boosted lopinavir with continued therapy. The trial was prematurely stopped due to virological failure in six patients on monotherapy. It, thus, offers a unique opportunity to assess brain markers in the early stage of HIV virological escape.

Methods: Sixty-five CSF samples (34 on continued therapy and 31 on monotherapy) from 49 HIV-positive patients enrolled in MOST. Using enzyme-linked immunosorbent assay, we determined the CSF concentration of S100B (astrocytosis), neopterin (inflammation), total Tau (tTau), phosphorylated Tau (pTau), and amyloid-β 1–42 (Aβ), the latter three indicating neuronal damage. Controls were CSF samples of 29 HIV-negative patients with Alzheimer dementia.

Results: In the CSF of monotherapy, concentrations of S100B and neopterin were significantly higher than in continued therapy ($P = 0.006$ and $P = 0.013$, respectively) and Alzheimer dementia patients ($P < 0.0001$ and $P = 0.0005$, respectively). In Alzheimer dementia, concentration of Aβ was lower than in monotherapy ($P = 0.005$) and continued therapy ($P = 0.016$) and concentrations of tTau were higher than in monotherapy ($P = 0.019$) and continued therapy ($P = 0.001$). There was no difference in pTau among the three groups. After removal of the 16 CSF with detectable viral load in the blood and/or CSF, only S100B remained significantly higher in monotherapy than in the two other groups.

Conclusion: Despite full viral load-suppression in blood and CSF, antiretroviral monotherapy with lopinavir/ritonavir can raise CSF levels of S100B, suggesting astrocytic damage.

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Keywords: astrocyte, central nervous system, HIV, lopinavir-ritonavir, monotherapy, neopterin, S100B

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Introduction

HIV can cause neuropathological abnormalities, which are located mainly in the basal ganglia and are characterized by microglial giant cells, reactive astrocytosis and perivascular monocytes [1]. Clinically, HIV encephalopathy is characterized by psychomotor slowing, memory loss, difficulties with complex tasks requiring executive functions, as well as motor disorders [2]. These cognitive deficits are grouped under the acronym of HIV-associated neurocognitive disorders (HAND) [3].

With the introduction of combined antiretroviral therapy (cART), the incidence of HIV-associated dementia has decreased, however, the prevalence of mild neurocognitive deficits (MNDs) in HIV-positive patients has paradoxically increased [4]. The reasons of such a phenomenon are unclear. Aging of HIV-positive patients [5], possibly in relation with the development of neurodegenerative diseases [6], cART toxicity [7], low grade inflammation resistant to cART [sanctuary effect of the central nervous system (CNS)] [8], or still an insufficient penetration-effectiveness of several antiretroviral compounds [9] have all been called upon to explain this phenomenon. One way to address this question is to determine the pathogenic events that occur in the early stages of HIV-related damages to the CNS. Precisely, here, we had the opportunity to observe putative dysregulation of brain cell biomarkers in the cerebrospinal fluid (CSF) of patients enrolled into the MOST trial [10].

In MOST, monotherapy with ritonavir-boosted lopinavir (LPV/r) was compared to continuous cART. The rationale for the evaluation of monotherapy was its potential of reduced toxicity and cost while maintaining full virological suppression [10]. However, as previously reported, the study had to be prematurely stopped because six out of 42 patients under monotherapy presented HIV-RNA failure in plasma after around 12 weeks [10] of monotherapy. Out of these six patients, five had a lumbar puncture that also showed HIV virological failure in the CSF (Table 2 of [10]) Importantly, prior to randomization, mean duration of conventional cART was more than 4 years and the mean $CD4^+$ T-cell count was between 450 and 500 cells/μl. These baseline data show that prior to study entrance, HIV was well controlled in all patients, including those who were subsequently enrolled into the monotherapy arm.

Concerns have been raised that monotherapy with boosted protease inhibitors might have limited activity in the CNS [11], but so far, no study has systematically evaluated the antiretroviral activity of monotherapy in the CNS. In MOST, CSF samples were obtained prior to randomization, after 1 year of treatment, and at termination of the study.

Several parameters in the spinal fluid have been described to evaluate immune activation or cellular damage in the brain. Among those, the neuronal markers encompass amyloid-β 1–42 (Aβ 1–42), which reflects amyloid deposits in the brain; the protein Tau in its total form (tTau), which is a marker of the intensitiy of neuronal degeneration; and the protein Tau in its phosphorylated form (pTau), which correlates with the amount of neurofibrillary tangles in the brain [12]. These three markers have been extensively studied in Alzheimer disease, which is an age-related neurodegenerative disease characterized clinically by cortical dementia and pathologically by the accumulation of amyloid plaques and neurofibrillary tangles. Yet, some authors have suggested that there are similarities between the effects of HIV-associated brain damages and Alzheimer dementia [13]. In order to determine whether the profile of neuronal biomarkers in the CSF of HIV-positive patients resembles those in Alzheimer dementia, we included CSF samples from 29 HIV-negative patients with Alzheimer dementia.

Neopterin, a product of the guanosine triphosphate pathway, is a marker of inflammation [14]. It is produced by activated monocytes/macrophages, in particular in the CNS compartment, and is thus recognized as a valuable marker of inflammation in the CNS of HIV-positive patients [15].

S100B is an acidic calcium-binding protein, secreted mostly by astrocytes [14]. This protein plays a role in neural development and neuronal maintenance, however, high levels of S100B lead to neuronal apoptosis through different excitatory mechanisms [16], thus decreasing the neuroprotective effect of astrocytes [17].

The current MOST-substudy evaluated potential antiviral differences of monotherapy as compared to continued therapy. We hypothesized that monotherapy is associated with a disturbed profile of either neuronal or inflammatory and/or astrocytic markers in the CSF.

Material and methods

HIV-infected study patients

All reported HIV-positive patients were participating in the Swiss HIV Cohort Study [18] and had been enrolled in MOST. [10]. The MOST study had been approved by the local ethics committees and all patients had signed a written informed consent. Patients had previously been treated with cART for 4.4 (± 3.3) and 5.4 (± 3.7) years in the continued therapy and monotherapy arm, respectively, a difference which was not significant. Prior to randomization, the HIV plasma viral load had been undetectable for at least 3 months. The median duration of uninterrupted complete HIV RNA suppression, as defined by a viral load less than 40 copies of HIV-1 RNA

per ml, before study enrollment was 50 months (range 9–63) in monotherapy and 25 months (range 6–121) in continued therapy group. All MOST patients had a lumbar puncture at the time of enrollment (CSF#1). The second lumbar puncture was planned at 48 weeks (CSF#2) and a third lumbar puncture at study termination (originally planned at week 96, CSF#3). However, the MOST study had to be prematurely terminated due to virological failure in plasma in six patients [10]. Thus, the majority of patients did not complete the planned 96-week study, and as a consequence the interval between CSF#2 and CSF#3 was shorter than 48 weeks. Some patients did not even reach week 48, thus, in those patients, the interval between CSF#1 and CSF#2 was less than 48 weeks. Nevertheless, for the present study, we were able to obtain a total of 85 samples, including 31 CSF#1, 41 CSF#2, and 13 CSF#3.

These CSF samples came from 49 of 60 patients including 27 who were enrolled into the monotherapy arm, 12 who were in the continuous highly antiretroviral therapy arm (continued therapy), and 10 who switched at week 48 from continued therapy to monotherapy.

Data analysis

We analyzed the CSF corresponding to the longest time spent on continued therapy, respectively, the longest time spent on monotherapy. In the case of a patient who was part of the continued therapy arm, we analyzed the CSF sample that corresponded to the longest time spent on continued therapy. For patients who were enrolled in the monotherapy arm from the beginning, we included CSF#1 in the continued therapy group (because all enrolled patients had been for years on continued cART) and we included the result of CSF#3 (or CSF#2 if CSF#3 was not available) in the monotherapy group. Finally, if a patient was part of the switch arm (change from continued therapy to monotherapy at week 48), then we included the CSF#2 in the continued therapy group and the CSF#3 in the monotherapy group (whenever this third lumbar puncture was performed between 48 and 96 weeks).

Using this type of data analysis, we used 65 CSF samples (thus, out of 49 patients, there were 16 patients who had two CSF samples tested, one while on continued therapy and one while on monotherapy). There were 34 CSF samples of patients on continued therapy and 31 CSF samples of patients on monotherapy, with a mean time of exposure to monotherapy of 48 ± 15 (median $\pm$ IQR) weeks (Fig. 1).

HIV-negative Alzheimer study patients

The diagnosis of Alzheimer dementia was definite in six patients and probable in the remaining 23, according to the NINCDS-Alzheimer dementiaRDA criteria [19]. HIV was formally tested in 10 Alzheimer dementia patients and was negative in all. In the remaining 19 patients, HIV was not tested as there was not any hint of such infection in these patients.

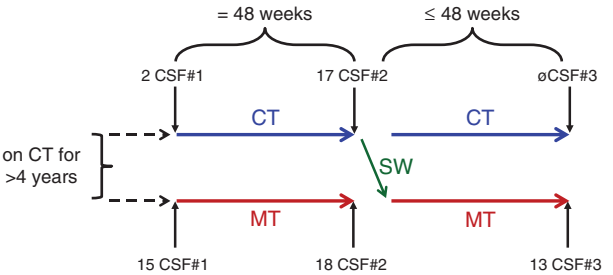

Fig. 1. Design of the study. The cartoon indicates how the 65 cerebrospinal fluid (CSF) samples (from the 49 HIV-positive study patients) were distributed. The 17 CSF#1 (15 + 2) as well as the 17 CSF#2 in the continued therapy (CT) arm were analyzed in the CT arm. The 18 CSF#2 and 13 CSF#3 in the lopinavir/r monotherapy (MT) arm were analyzed in the MT arm. SW, switch from CT to MT arm at week 48.

Cerebrospinal fluid processing

CSF was collected and processed in polypropylenes tubes and stored at −80°C until the time of the assay. The CSF of MOST patients had been collected between 2007 and 2008, whereas the CSF of Alzheimer dementia patients was collected between 1999 and 2008 in the Service of neurology of Lausanne University Hospital and had never been thawed until the current study.

Determination of biomarkers in the cerebrospinal fluid

In order to determine the CSF concentrations of the five biomarkers, we used ELISA according to manufacturer instructions.

Aβ 1–42 was detected with the Aβ42 Human ELISA kit (Invitrogen, Zug, Switzerland). This assay is specific for the COOH-terminus of the Aβ 1–42 sequence, a sequence which is created upon cleavage of the analyzed precursor. The minimum detectable dose of Hu Aβ 1–42 was 10 pg/ml.

For pTau, we used the Invitrogen Human Tau [pT181] ELISA kit (Invitrogen) to assess 39 CSF samples all from MOST study patients and the Innotest Phospho-Tau (181p) ELISA from Innogenetics (Gent, Belgium) to assess the remaining 56 samples including 26 from MOST study patients and the 29 Alzheimer dementia patients. The minimum detectable dose of pTau was 10 pg/ml for the Invitrogen Human Tau [pT181] ELISA kit and 15.6 pg/ml for the Innotest Phospho-Tau (181p) ELISA.

tTau was assessed with the Invitrogen Human Tau (Total) ELISA kit (Invitrogen). The minimum detectable dose of tTau was 12 pg/ml.

S100B was measured with the Human S100B ELISA Kit (Abnova, Heidelberg, Germany). The detection limit of this kit was 5 pg/ml.

For the neopterin, we used the Neopterin ELISA (RE59321) kit (IBL International, Hamburg, Germany). The sample limit detection was 0.7 nmol/l.

For all ELISA kits, absorbance was read with a spectrophotometer at 450 nm. In all cases, samples were diluted for the assay according to manufacturer instructions and measured values multiplied by the appropriate sample dilution factor before analysis.

Statistics

Results of CSF biomarkers are expressed as median ± interquartile range (IQR). Statistical analyses were performed with GraphPad Prism software (San Diego, California, USA). One way analysis of variance (ANOVA), using the non-parametric Kruskal–Wallis test, was performed to assess differences between the three categories of study patients. The non-parametric Mann–Whitney ranked test was used to look for differences between two given categories of study patients. The paired t test was used in study subjects with paired CSF samples. Fisher's exact test was used to compare the proportion of monotherapy vs. continued therapy patients with high S100B CSF levels.

Results

Analysis of all 65 cerebrospinal fluid samples of MOST patients

We found a significant increase in neopterin and S100B in monotherapy as compared to continued therapy and as compared to Alzheimer dementia patients (Fig. 2a). When the analysis was restricted to the 16 HIV-positive study participants for whom paired CSF samples were available, one while on continued therapy and the other while on monotherapy, neopterin and S100B levels were significantly higher in the CSF taken while on monotherapy (Fig. 2b). However, there were no difference between these two categories of HIV-positive patients in terms of neuronal markers, that is, tTau, pTau, and Aβ 1–42 (Table 1).

In the CSF of Alzheimer dementia patients, tTau and Aβ 1–42 were significantly different from the CSF of MOST study participants (either continued therapy or

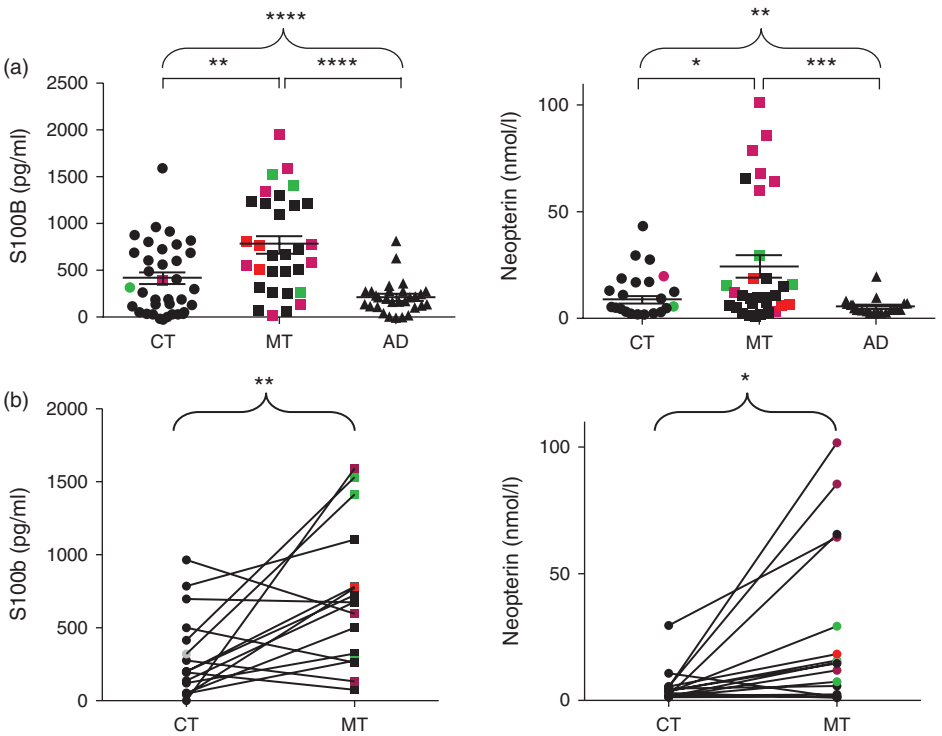

Fig. 2. Increase of S100B and neopterin in the cerebrospinal fluid of lopinavir/r monotherapy patients. (a) Cerebrospinal fluid (CSF) levels of S100B and neopterin in all three categories of study participants. (b) Only study participants who had two CSF samples available, the first one while on continued therapy (CT), the second one while on lopinavir/r monotherapy (MT), are displayed. Color dots: black, HIV viral load less than 40 copies/ml in the blood and the CSF at the time of CSF sampling; red, more than 40 copies/ml in the blood only; green, more than 40 copies/ml in the CSF only; magenta, more than 40 copies/ml in both blood and CSF. AD, Alzheimer disease. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Table 1. Results of the five biomarkers in the cerebrospinal fluid of all three categories of study participants, including the 16 CSF samples taken at a time when HIV-1 RNA was detectable in plasma and/or in the CSF.

	S100B (pg/ml)	Neopterin (nmol/l)	tTau (pg/ml)	pTau (pg/ml)	Ab 1–42 (pg/ml)
MT ($n=31$)	677 ± 607	10.8 ± 13.6	199 ± 147	30 ± 27	391 ± 333
CT ($n=34$)	313 ± 430	4.8 ± 7.3	131 ± 124	34 ± 30	466 ± 367
AD ($n=29$)	208 ± 122	4.2 ± 2.3	299 ± 274	54 ± 41	212 ± 242
One way ANOVA	<0.0001	0.002	0.002	0.116	0.011
p1 (MT vs. CT)	0.006	0.013	0.2	0.397	0.818
p2 (MT vs. AD)	<0.0001	0.0005	0.019	0.0377	0.005
p3 (CT vs. AD)	0.068	0.504	0.001	0.222	0.016

AD, HIV-negative patients with Alzheimer disease; ANOVA, analysis of variance; CT, HIV-positive patients on continued combined antiretroviral therapy; MT, HIV-positive patients on lopinavir/ritonavir monotherapy. Results are expressed as median ± interquartile range. Statistical analyses were performed with GraphPad Prism software (San Diego, California, USA).

monotherapy, Table 1). In this sense, neuronal markers in the CSF of MOST study participants were at the same levels as expected in healthy controls [13].

Analysis of cerebrospinal fluid samples after removal of samples corresponding to timepoints with detectable HIV viral load in the blood and/or in the cerebrospinal fluid

Among the 65 analyzed CSF samples, 16 were obtained at a time when HIV viral load was detectable (>40 copies/ml) either in the plasma (three) or the CSF (four) or in both compartments (nine). Of note, CSF samples of four of six of the patients with treatment failure (>400 copies/ml) who had led to premature study termination were included in these 16 samples [10]. To determine whether our CSF biomarkers were sensitive enough to show difference between patients on continued therapy and on monotherapy, even when only CSF samples corresponding to timepoints when HIV viral load was undetectable both in the plasma and the CSF were taken into account, we chose to remove those 16 CSF samples from the analysis. By doing so, we found that the difference in CSF neopterin levels between monotherapy and continued therapy patients disappeared ($P=0.456$). The CSF levels of neopterin tended to be higher in monotherapy as compared to Alzheimer dementia patients ($P=0.084$). However, the CSF levels of S100B remained significantly higher in patients on monotherapy than on continued therapy ($P=0.037$), and Alzheimer dementia patients ($P=0.0001$, Table 2 and Fig. 3).

The levels of tTau and Aβ 1–42 remained higher, or lower, respectively, in the CSF of Alzheimer dementia patients than in the one of HIV-positive patients (Table 2).

Discussion

We found that markers of astrocytes (S100B) and microglia/macrophages (neopterin) were significantly elevated in the CSF of patients who had been on LPV/r monotherapy as compared to those who stayed on cART. By contrast, no differences were noted for the CSF levels of neuronal markers Aβ 1–42, tTau, and pTau between both categories of HIV-positive patients. However, in the CSF of Alzheimer dementia patients, as compared to HIV-positive patients, Aβ 1–42 was decreased [20] (which is explained by retention of Aβ in the plaques and, thus, decreased release in the CSF [12]), whereas tTau was increased. In this sense, as compared to Alzheimer dementia, MOST patients behaved as healthy control participants [12]. These findings suggest that a short period of potentially suboptimal antiretroviral treatment can impact on astrocytes and microglia/macrophages, but not neurons, at least not on the pathogenic pathways leading to amyloidosis and taupathy.

Because our primary goal was to identify early markers of suboptimal therapy in the CNS, we then analyzed the data by removing the 16 CSF samples that had been collected

Table 2. Results of the five biomarkers in the cerebrospinal fluid of all three categories of study participants, after removal of the 16 cerebrospinal fluid samples that had been drawn at a time when HIV-1RNA was detectable in plasma and/or cerebrospinal fluid.

	S100B (pg/ml)	Neopterin (nmol/l)	tTau (pg/ml)	pTau (pg/ml)	Ab 1–42 (pg/ml)
MT ($n=17$)	673 ± 666	7.4 ± 6.1	179 ± 119	26 ± 31	582 ± 310
CT ($n=32$)	290 ± 452	4.8 ± 6.9	123 ± 123	33 ± 26	456 ± 389
AD ($n=29$)	208 ± 122	4.2 ± 2.3	299 ± 274	54 ± 41	212 ± 242
One way ANOVA	0.0012	0.302	0.002	0.178	0.017
p1 (MT vs. CT)	0.037	0.456	0.578	0.652	0.698
p2 (MT vs. AD)	0.0001	0.084	0.02	0.139	0.012
p3 (CT vs. AD)	0.114	0.549	0.0007	0.111	0.021

AD, HIV-negative patients with Alzheimer disease; ANOVA, ; CT, HIV-positive patients on continued combined antiretroviral therapy; MT, HIV-positive patients on lopinavir/ritonavir monotherapy. Results are expressed as median ± interquartile range. Statistical analyses were performed with GraphPad Prism software (San Diego, California, USA).

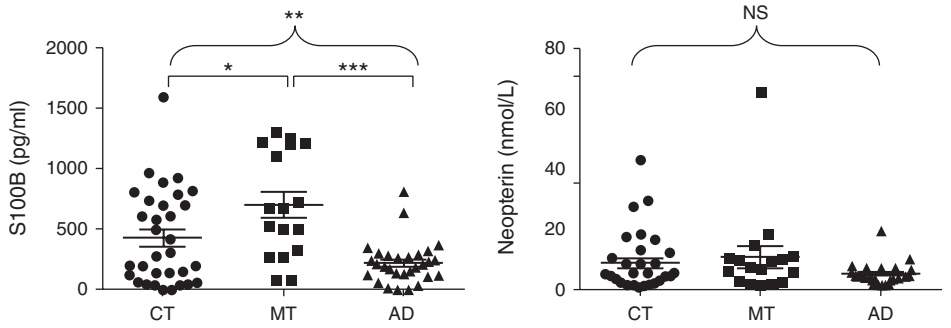

Fig. 3. Increase of S100B in the cerebrospinal fluid of lopinavir/r monotherapy patients without detectable HIV viral load in any compartment. Cerebrospinal fluid (CSF) levels of S100B and neopterin by patient category after removal of the 16 CSF samples corresponding to time point of HIV viral load detectable (>40 copies/ml) either in the plasma or in the CSF or both. CT, continued therapy; MT, lopinavir/r monotherapy; AD, Alzheimer disease. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

at a time when MOST patients showed evidence of incomplete virological control in the blood and/or the CSF. Indeed, in these 16 patients, the mere determination of HIV viral load was sufficient to demonstrate that LPV/r failed to keep HIV under control, and, thus, other biomarkers were not needed. In this analysis, S100B remained significantly higher in monotherapy as compared to continued therapy, a finding that contrasted with CSF neopterin levels, which were no longer different between both groups. Interestingly, six of 17 (35%) of nonfailing monotherapy patients, but only one of 32 (3%) continued therapy patient had a CSF S100B value higher than 1000 pg/ml ($P = 0.005$). Whether this cutoff may serve to identify patients with suboptimal antiretroviral treatment needs to be confirmed in prospective studies.

Astrocytes have traditionally been thought to play a minor role in models of HIV neuropathogenesis, owing to the fact that only about 1% of astrocytes in AIDS patients with HIV encephalitis exhibited HIV DNA [21]. However, in recent work, using combined double immunohistochemistry, laser capture microdissection, and highly sensitive PCR, Churchill *et al.* [22] showed that approximately 20% of astrocytes of HIV-associated dementia (HAD) patients were infected by HIV, suggesting that these cells may play a more important role for HIV neuropathogenesis than previously acknowledged. Furthermore, others have recently shown that even a small percentage (less than 10%) of HIV-infected astrocytes was sufficient to disrupt the blood–brain barrier by a gap–junction-dependent mechanism [23]. It has been shown that high levels of S100B in the CSF predicted a rapid progression to death in patients with HIV-associated dementia [24]. Interestingly, the serum level of S100B has recently been shown to correlate with the presence of simian immunodeficiency virus (SIV) encephalitis in nonhuman primates, which warrants similar studies in humans [25]. Other authors have found that the higher the executive dysfunction in HIV-positive patients, the higher the level of S100B in the CSF, suggesting that astrocytosis may account for this aspect of HAND [26]. Thus, together with previously published findings, our data suggest an important role of astrocytes in HIV neuropathogenesis. Increased levels of S100B in the CSF may be an early marker of HIV-related damage to the brain, particularly in monotherapy.

The involvement of microglia/macrophages has been recognized as an important contributor to the HIV-associated brain damage [8]. Increased CSF level of neopterin is a good marker of inflammation in the CNS of HIV-positive patients [27]. Others have shown that high concentrations of neopterin in the CSF correlated with the risk of HAD [14,15]. Here, we found that neopterin was increased in patients with incomplete control of HIV viral load in the blood and/or the CSF (Fig. 2), confirming previous observations [28], and suggesting that this biomarker may increase in response to HIV replication in the CSF.

Some authors have reported low Aβ 1–42 concentrations in the CSF of HIV-positive patients with HAND (HAD and MND), similar to results in Alzheimer dementia patients [13,29]. Gisslen *et al.* [30] found that soluble amyloid precursor proteins α and β CSF levels were even lower in HAD than in Alzheimer dementia patients, suggesting that HIV infection of the CNS impacts on amyloid synthesis or processing [31]. Somewhat contrasting with these data, we did not find decreased CSF levels of Aβ 1–42. However, although extensive neuropsychological battery was not performed here, MOST patients certainly did not present HAD and, thus, were at a much less advanced stage than patients of Gisslen *et al.*

Studies that have examined the level of Tau in the CSF of HIV-positive patients have yielded contradictory results. Some authors found similarly elevated levels of pTau and tTau in the CSF of HAD and Alzheimer dementia patients [29], whereas others reported higher levels in Alzheimer dementia than in HAD patients [13,30]. These data suggest that Tau may not be a sensitive marker of

HAND. In our cohort of HIV-positive patients without significant cognitive impairment, this marker was logically within normal ranges, contrary to the situation in Alzheimer dementia patients who had high tTau levels. Despite the fact that phospho-Tau was elevated in the CSF of Alzheimer dementia as compared to monotherapy (but not continued therapy) patients, we consider that this difference was not relevant because the ANOVA test was not significant (Table 1). These data contrast with what is usually reported in the literature, that is, pTau is elevated in the CSF of Alzheimer dementia patients [12]; however, we cannot completely rule out that methodological reasons may account for this absence of difference (see Methods); therefore, in our study, data regarding pTau have to be taken with caution.

In conclusion, our findings suggest that even short-term monotherapy with lopinavir/ritonavir may elicit not only a macrophage/microglia reaction, but especially an astrocytic response. These data also suggest that undetectable HIV viral load in the plasma and in the CSF do not necessarily rule out ongoing inflammation in the brain. In fact, McArthur *et al.* [32] did not find a correlation between HIV viral load in the CSF and in the brain in nondemented HIV-positive patients. Others, using the SIV model, showed that some monkeys exhibited a continued CNS inflammation despite suppressed plasma and CSF HIV viral load [33]. We propose that the value of S100B as an early indicator of incomplete virological control in the CNS should be examined in future studies.

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R.A.D.P. led the study, supervised the acquisition and analysis of data and wrote the manuscript.

S.J. performed and coordinated the experiments, analyzed the data, and helped in writing the article.

M.K. performed literature research on the different biomarkers, performed the experiments, analyzed the data and helped in writing the manuscript.

S.Y. provided the CSF of HIV-positive patients and edited the manuscript.

C.A.F. enrolled patients into MOST study and edited the manuscript.

C.G. was the first author on MOST study (AIDS 2010), enrolled patients into MOST study, and edited the manuscript.

A.C. enrolled patients into MOST study and edited the manuscript.

H.F.G. enrolled patients into MOST study and edited the manuscript.

M.C. enrolled patients into MOST study and edited the manuscript.

P.L.V. was the leading author of MOST study and co-led the current study: he provided the CSF samples, contributed financially, critically reviewed the data and edited the manuscript.

Conflicts of interest

There are no conflicts of interest.

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