Marked Increase of the Astrocytic Marker S100B in the Cerebrospinal Fluid of HIV-infected Patients on LPV/r-Monotherapy

Renaud A. du Pasquier[a], Samantha Jilek[b], Malela Kalubi[a], Sabine Yerly[c], Christoph A. Fux[d], Christine Gutmann[e], Alexia Cusini[d,f], Huldrych F. Günthard[f], Matthias Cavassini[b], and Pietro L. Vernazza[e] the Swiss HIV Cohort Study

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

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

Methods: 65 CSF samples (34 on CT and 31 on MT) from 49 HIV+ 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-beta 1–42 (Abeta), the latter three indicating neuronal damage. Controls: CSF samples of 29 HIV-negative patients with Alzheimer dementia (AD).

Results: In the CSF of MT, concentrations of S100B and neopterin were significantly higher than in CT ($p = 0.006$ and $p = 0.013$, respectively) and AD patients ($p < 0.0001$ and $p = 0.0005$, respectively). In AD, concentration of Abeta was lower than in MT ($p = 0.005$) and CT ($p = 0.016$) and concentrations of tTau were higher than in MT ($p = 0.019$) and CT ($p = 0.001$). 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 MT than in the two other groups.

Conclusions: Despite full VL-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

[a]Service of neurology, [b]Service of infectious diseases, University Hospital Lausanne, [c]Laboratory of Virology, Division of Laboratory Medicine and Division of Infectious Diseases, Geneva University Hospital, [d]University Clinic for infectious diseases, University Hospital Bern, [e]Service of infectious diseases, Hospital of St-Gall, and [f]Division of Infectious Diseases and Hospital Epidemiology, University Hospital of Zurich, University of Zurich.

Correspondence to Prof. Renaud Du Pasquier, MD, Service of Neurology, Department of clinical neurosciences, CHUV BH-10, Avenue du Bugnon, 46, 1011 Lausanne.

E-mail: Renaud.du-pasquier@chuv.ch

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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 psycho-motor 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 (MND) in HIV+ patients has paradoxically increased in the cART era [4]. The reasons of such a phenomenon are unclear. Aging of HIV+ 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 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 (MT) with ritonavir-boosted lopinavir (LPV/r) was compared to continuous cART (CT). The rationale for the evaluation of MT 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 MT presented HIV-RNA failure in blood after around 12 weeks [10] of MT. Out of these six patients, five had a LP which 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 between 450 and 500/mcl. These baseline data show that, prior to study entrance, HIV was well controlled in all patients, including those who were subsequently enrolled into the MT arm.

Concerns have been raised that MT with boosted protease inhibitors might have limited activity in the central nervous system (CNS) [11] but so far, no study has systematically evaluated the antiretroviral activity of MT in the CNS. In MOST, CSF samples were obtained prior to randomization, after one 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-beta 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 (AD), 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 AD [13]. In order to determine whether the profile of neuronal biomarkers in the CSF of HIV+ patients resembles those in AD, we included CSF samples from 29 HIV-negative patients with AD.

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+ 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

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

	S100-b (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

MT, HIV+ patients on lopinavir/ritonavir monotherapy; CT, HIV+ patients on continuous cART; AD, HIV- patients with Alzheimer disease. Results are expressed as median +/− interquartile range (IQR). Statistical analyses were performed with GraphPad Prism software (San Diego, CA, USA).

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

	S100-b (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

MT, HIV+ patients on lopinavir/ritonavir monotherapy; CT, HIV+ patients on continuous cART; AD, HIV- patients with Alzheimer disease. Results are expressed as median +/- interquartile range (IQR). Statistical analyses were performed with GraphPad Prism software (San Diego, CA, USA).

different excitatory mechanisms [16], thus decreasing the neuroprotective effect of astrocytes [17].

The current MOST-substudy evaluated potential antiviral differences of MT as compared to CT. We hypothesized that MT is associated with a disturbed profile of either a) neuronal, b) inflammatory and/or c) astrocytic markers in the CSF.

Material and methods

HIV-infected study patients

All reported HIV+ patients were participating in the Swiss HIV Cohort Study (SHCS) [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 CT and MT arm, respectively, a difference which was not significant. Prior to randomization the HIV plasma viral load (pVL) had been undetectable for at least three months. The median duration of uninterrupted complete HIV RNA suppression, as defined by a viral load <40 copies of HIV-1 RNA per ml, before study enrollment was 50 month (range 9–63) in MT and 25 month (range 6–121) in CT group. All MOST patients had an LP at the time of enrollment (CSF#1). The second LP was planned at 48 weeks (CSF#2) and a third LP 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 49/60 patients including 27 who were enrolled into the monotherapy (MT) arm, 12 who were in the continuous highly antiretroviral therapy arm (CT), and 10 who switched at week 48 from CT to MT.

Data analysis:

We analyzed the CSF corresponding to the longest time spent on CT, respectively the longest time spent on MT. In the case of a patient who was part of the CT arm, we analyzed the CSF sample that corresponded to the longest time spent on CT. For patients who were enrolled in the MT arm from start, we included CSF#1 in the CT group (since 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 MT group. Finally, if a patient was part of the switch arm (change from CT to MT at week 48), then we included the CSF#2 in the CT group and the CSF#3 in the MT group (whenever this third LP was performed between 48 weeks 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 CT and one while on MT). There were, 34 CSF samples of patients on CT and 31 CSF samples of patients on MT, with a mean time of exposure to MT of 48 +/− 15 (median +/− IQR) weeks (Fig. 1).

HIV-negative Alzheimer study patients

The diagnosis of AD was definite in 6 patients and probable in the remaining 23, according to the NINCDS-ADRDA criteria [19]. HIV was formally tested in 10 AD 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.

CSF 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 2007and 2008 whereas the CSF of AD patients was collected between 1999 and 2008 in the

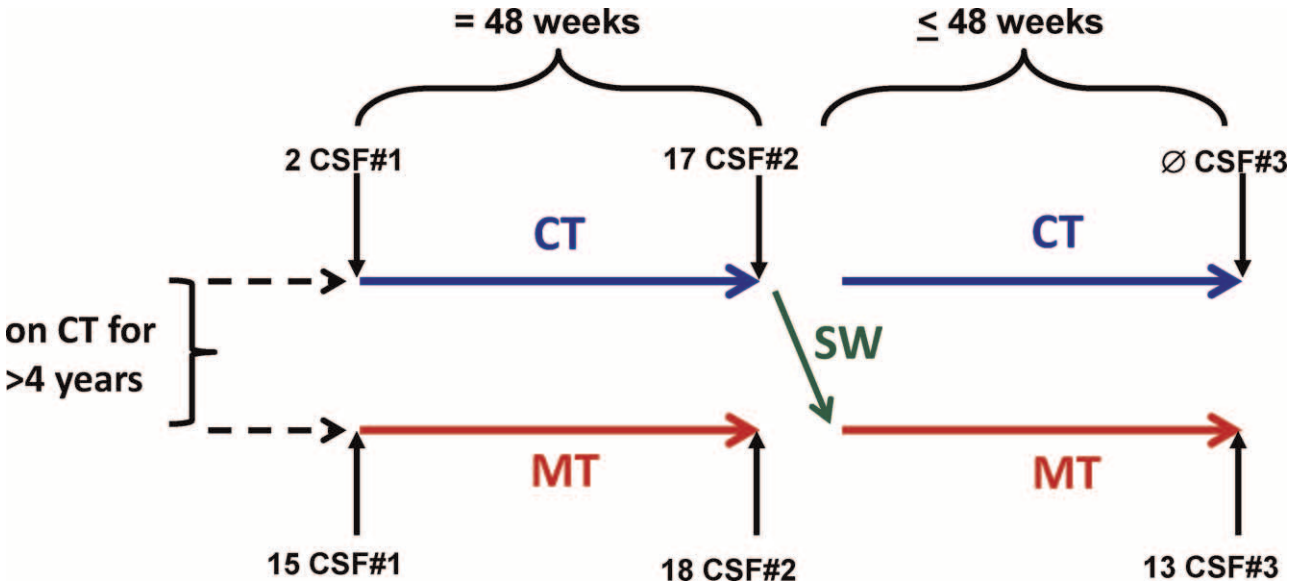

Fig. 1. Design of the study. The cartoon indicates how the 65 CSF samples (from the 49 HIV+ study patients) were distributed. The 17 CSF#1 (15+2) as well as the 17 CSF#2 in the CT arm were analyzed in the CT arm. The 18 CSF#2 and 13 CSF#3 in the MT arm were analyzed in the MT arm. CT, continuous therapy: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

Service of neurology of Lausanne University Hospital and had never been thawed until the current study.

Determination of biomarkers in the CSF

In order to determine the CSF concentrations of the five biomarkers, we used Enzyme- Linked Immunosorbent Assay (ELISA) according to manufacturer instructions.

Amyloid-β 1–42 (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 phosphorylated Tau (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 AD 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.

Total Tau (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) was used. 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, CA, USA). One way 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 exact test was used to compare the proportion of MT versus CT patients with high S100B CSF levels.

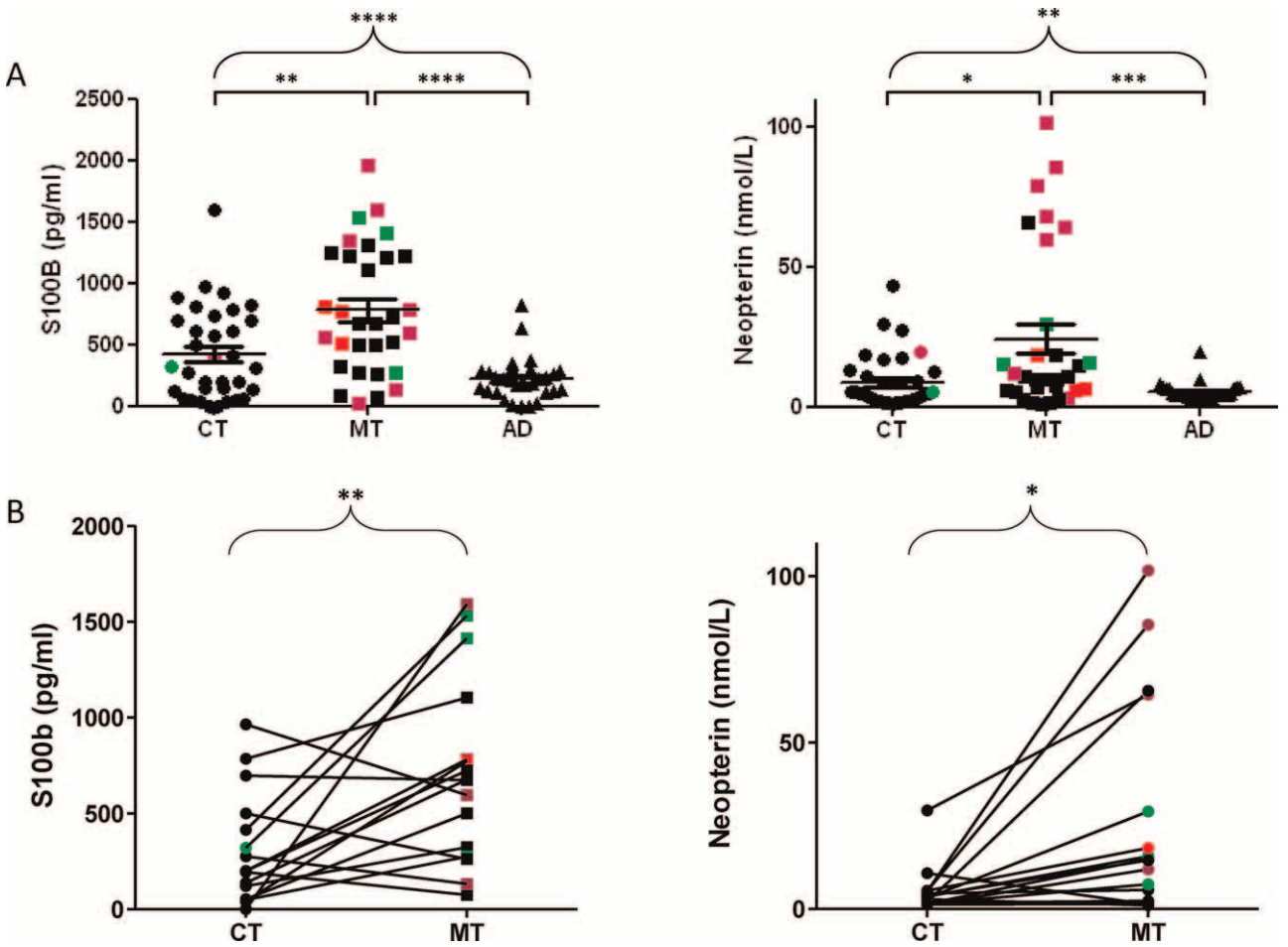

Fig. 2. Increase of S100B and neopterin in the CSF of MT patients. A) CSF levels of S100B and neopterin in all three categories of study subjects. B) Only study subjects who had two CSF samples available, the first one while on CT, the second one while on MT, are displayed. Color dots: black, HIV viral load <40 copies/ml in the blood and the CSF at the time of CSF sampling; red, >40 copies/ml in the blood only; green, >40 copies/ml in the CSF only; magenta, >40 copies/ml in both blood and CSF. CT, continuous therapy; MT, LPV/r monotherapy; AD, Alzheimer disease. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

Results

Analysis of all 65 CSF samples of MOST patients

We found a significant increase in neopterin and S100B in MT as compared to CT and as compared to AD patients (Fig. 2A). When the analysis was restricted to the 16 HIV+ study subjects for whom paired CSF samples were available, one while on CT and the other while on MT, neopterin and S100B levels were significantly higher in the CSF taken while on MT (Fig. 2B). However, there were no difference between these two categories of HIV+ patients in terms of neuronal markers, i.e. tTau, pTau and Aβ 1–42 (Table 1).

In the CSF of AD patients, tTau and Aβ 1–42 were significantly different from the CSF of MOST study subjects (either CT or MT, Table 1). In this sense, neuronal markers in the CSF of MOST study subjects were at the same levels as expected in healthy controls [13].

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

Among the 65 analyzed CSF samples, 16 were obtained at a time when HIV viral load was detectable (>40 copies/ml) either in the blood (3) or the CSF (4) or in both compartments (9). Of note, CSF samples of 4/6 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 CT and on MT, even when only CSF samples corresponding to timepoints when HIV viral load was undetectable both in the blood 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 MT and CT patients disappeared ($p = 0.456$). The CSF levels of neopterin tended to be higher in MT as compared to AD patients ($p = 0.084$). However, the CSF levels of S100B

remained significantly higher in patients on MT than on CT ($p = 0.037$), and AD 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 AD patients than in the one of HIV+ 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 (MT) as compared to those who stayed on continuous cART therapy (CT). By contrast, no differences were noted for the CSF levels of neuronal markers Aβ 1–42, tTau and pTau between both categories of HIV+ patients. However, in the CSF of AD patients, as compared to HIV+ subjects, 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 AD, MOST patients behaved as healthy control subjects [12]. These findings suggest that a short period of potentially sub-optimal treatment can impact on astrocytes and microglia/macrophages, but not neurons, at least not on the pathogenic pathways leading to amyloidosis and taupathy.

Since our primary goal was to identify early markers of sub-optimal therapy in the CNS, we then analyzed the data by removing the 16 CSF samples that had been collected 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 VL 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 MT as compared to CT, a finding that contrasted with CSF neopterin levels which were no longer different between both groups. Interestingly 6/17 (35%) of non failing MT patients, but only 1/32 (3%) CT patient had a CSF S100B value higher than 1000 pg/ml ($p = 0.005$). Whether this cutoff may serve to identify patients with sub-optimal anti-retroviral 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. showed that approximately 20% of astrocytes of HAD patients were infected by HIV, suggesting that these cells may play a more important role for HIV neuropathogenesis than previously acknowledged [22]. 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 SIV encephalitis in non human primates, which warrants similar studies in humans [25]. Other authors have found that the higher the executive dysfunction in HIV+ 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 MT.

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+ 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 a 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+ patients with HAND (HAD and MND), similar to results in AD patients [13,29]. Gisslen et al. found that soluble amyloid precursor proteins alpha and beta CSF levels were even lower in HAD than in AD patients [30], 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 Gisslen et al.'s patients.

Studies that have examined the level of Tau in the CSF of HIV+ patients have yielded contradictory results. Some authors found similarly elevated levels of pTau and tTau in the CSF of HAD and AD patients [29], whereas others reported higher levels in AD than in HAD patients [13,30]. These data suggest that Tau may not be a sensitive marker of HAND. In our cohort of HIV+ patients without significant cognitive impairment, this marker was logically within normal ranges, contrary to the situation in AD patients who had high tTau levels. Despite the fact

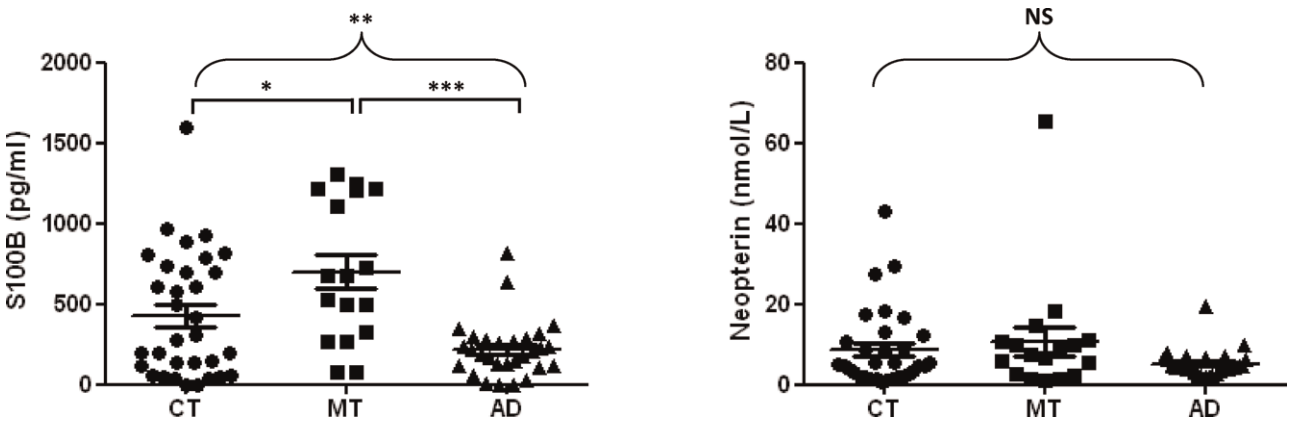

Fig. 3. Increase of S100B in the CSF of MT patients without detectable HIV viral load in any compartment. CSF levels of S100B and neopterin by patient category after removal of the 16 CSF samples corresponding to time point of HIV viral load detectable (>40 copies/ml) either in the blood or in the CSF or both. CT, continuous therapy; MT, LPV/r monotherapy; AD, Alzheimer disease. NS, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

that phospho-Tau was elevated in the CSF of AD as compared to MT (but not CT) patients, we consider that this difference was not relevant since the ANOVA test was not significant (Table 1). These data contrast with what is usually reported in the literature, i.e. that pTau is elevated in the CSF of AD 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 did not find a correlation between HIV viral load in the CSF and in the brain in non-demented HIV+ patients [32]. 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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Conflicts of interest

None declared.

Authors contribution to the paper: Renaud Du Pasquier led the study, supervised the acquisition and analysis of data and wrote the manuscript.

Samantha Jilek performed and coordinated the experiments, analyzed the data, and helped in writing the paper.

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

Sabine Yerly provided the CSF of HIV+ patients and edited the manuscript.

Christoph Fux enrolled patients into MOST study and edited the manuscript.

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

Alexia Cusini enrolled patients into MOST study and edited the manuscript.

Huldrych Günthard enrolled patients into MOST study and edited the manuscript.

Matthias Cavassini enrolled patients into MOST study and edited the manuscript.

Pietro Vernazza 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.

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