Neurofilament light chain protein as a marker of neuronal injury: review of its use in HIV-1 infection and reference values for HIV-negative controls

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Abstract

Several CSF biomarkers of neuronal injury have been studied in people living with HIV. At this time, the most useful is the light subunit of the neurofilament protein (NFL). This major structural component of myelinated axons is essential to maintain axonal caliber and to facilitate effective nerve conduction. CSF concentrations of NFL provide a sensitive marker of CNS injury in a number of neurological diseases, including HIV-related neuronal injury.

Areas Covered: In this review, the authors describe CSF NFL concentrations across the spectrum of HIV-infection, from its early acute phase to severe immunosuppression, with and without neurological conditions, and with and without antiretroviral treatment (n = 516). Furthermore, in order to provide more precise estimates of age-related upper limits of CSF NFL concentrations, the authors present data from a large number (n = 359) of HIV-negative controls. Expert Commentary: Recently a new ultrasensitive diagnostic assay for quantification of NFL in plasma has been developed, providing a convenient way to assess neuronal damage without having to perform a lumbar puncture. This review also considers our current knowledge of plasma NFL in HIV CNS infection.
Introduction

Shortly after the first cases of AIDS were described in 1981, it became clear that the causative agent was not only destructive to the immune system, but that it also had the capability of entering and causing harm to the central nervous system (CNS) [1, 2]. A few years later it became evident that the virus causing this syndrome, HIV, can be detected in the cerebrospinal fluid (CSF) of virtually all infected individuals, beginning with primary infection and continuing throughout the entire infectious course [3, 4]. Despite the fact that viral entry into the CNS occurs as a very early event, it usually takes several years from infection to development of CNS manifestations.

Our knowledge about the effects of HIV on the CNS is based on clinical descriptions, CSF analyses, neuropsychological tests, pathological studies, and neuroimaging. Studies of CSF provide an easily accessible and useful window into CNS disease. Chronic HIV CNS infection can be asymptomatic, often with CSF pleocytosis, in one end to increasingly severe neurological complications, such as HIV-associated dementia (HAD) in the other end [5-7].

The majority of patients with HAD have low CD4+ T-cell counts and high CSF HIV RNA levels, but this is not enough for diagnosing HAD. Despite major diagnostic advances, diagnosis of HAD relies mainly on clinical recognition and exclusion of other diagnoses and is supplemented by neuropsychological testing [7]. It is also not possible to predict the risk for developing HAD in an individual patient on the basis of laboratory findings.

Antiretroviral therapy (ART) has been very effective in reducing the incidence of severe HAD [8, 9], but milder neurocognitive impairment has been noted in several studies in otherwise well-treated individuals and is now of particular concern as people living with HIV
(PLHIV) live longer [10-13]. Classification of these milder forms of HIV-associated neurocognitive disorders (HAND) are based on neurocognitive testing and clinical symptoms, if any, and they are divided into mild neurocognitive disorder (MND) and asymptomatic neurocognitive impairment (ANI) [7].

CSF studies have provided important information about the evolution of HIV inside the CNS from the earliest stages of infection to late stages with advanced HAD [14, 15]. They have also increased our knowledge about the intrathecal immune activation and brain cell injury, as well as about the effects of ART on HIV and the accompanying immune response and neurological damage in the CNS [16-21].

Several biomarkers have been investigated in CSF studies [22]. CSF biomarkers can be divided in the different components of the neuropathogenesis of HIV [22, 23]. First is the presence of HIV itself, which is the underlying drive of infection. HIV RNA levels in CSF can be quantified by polymerase chain reaction (PCR) with the same methods as used for blood. The second feature is the immune response. The state of cellular immune activation can be determined by quantifying various CSF biomarkers, for example the white blood cell count (WBC), neopterin (a marker of macrophage activation), β2-microglobulin (part of the major histocompatibility complex class I molecule), monocyte chemotactic protein (MCP)-1 (recruits and activates monocytes and macrophages), YKL-40 (a marker of glial activation), and cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-6, IL-10, and interferon (INF)-γ [22].

As is the case for CSF markers of immune activation, there is also an abundance of CSF biomarkers for the third component of HIV-related CNS disease, that is the neuronal injury.
HIV-induced brain changes can be determined by analysing CSF levels of a wide range of substances, such as total- and phosphorylated-tau (t- and p-tau), soluble amyloid precursor proteins α and β (sAPPα and -β), soluble amyloid-β 42 (Aβ1-42), and neurofilament light chain protein (NFL). We have found NFL to be most useful in studying neurodegeneration, particularly axonal degeneration. This is therefore the main focus of this review. We have several years of experience with determining and interpreting CSF NFL in both cross-sectional and longitudinal cohorts in PLHIV across the entire spectrum of disease, without and with ART. Recently, a new ultrasensitive technique was developed making it possible to quantify NFL in plasma despite 50 to 100 times lower concentrations than in CSF [24].

The aim of this review is to put together our current knowledge about CSF and plasma NFL in HIV CNS infection. We will start by giving a brief overview of the above-mentioned CSF biomarkers of CNS injury, including the neurofilament proteins, and then more in detail review CSF and plasma NFL in healthy controls from various cohorts and at different stages of HIV-infection, including the effects of ART on CSF and plasma NFL.

**CSF biomarkers of brain injury**

Biomarkers are objective measures of biological or pathogenic processes. They can be used to determine clinical diagnosis, to monitor response to therapeutic interventions, and to evaluate risk for disease, and/or prognosis. The CSF is in close contact with the extracellular space of the brain, and biochemical changes in the brain are therefore reflected in the CSF, at least to some extent. There are numerous markers of brain injury that have been used in CSF studies in PLHIV, studies that have provided us with insight about the pathogenesis of HIV. Many of these biomarkers have been analysed in other neurological conditions as well, Alzheimer disease probably being the best characterised one [25].
Amyloid metabolites and tau proteins

CSF biomarkers, other than NFL, that have been associated with neurodegenerative processes include t-tau and its hyperphosphorylated component p-tau, sAPPα and -β, and soluble Aβ1-42 (Figure 1) [18, 19, 26, 27]. T-tau is a microtubule-associated protein promoting axon stability expressed primarily in non-myelinated cortical axons. Increases on CSF t-tau also reflect axonal injury. Hyperphosphorylation of tau leads to detachment of tau from microtubules, destabilization of axons, and formation of neurofibrillary tangles. This pathological process is characteristic for a group of neurodegenerative disorders referred to as tauopathies, which includes Alzheimer’s disease [28]. APP is ubiquitously expressed in neurons and undergoes sequential proteolytic cleavage, resulting in production of soluble APPα and APPβ, which are both shed from the cell membrane and diffuse into the CSF. APPs have been linked to a variety of neuropathological conditions [29].

Typical CSF changes in Alzheimer’s disease include increases in t-tau and p-tau and a decrease in Aβ1-42 [30]. These markers reflect the core pathology of the Alzheimer’s disease, including cortical axonal degeneration and neurofibrillary tangle and senile plaque pathology (Blennow K et al., Lancet 2006). During the course of HIV-infection, CSF t-tau levels are normal in neuroasymptomatic individuals and high in those with HAD and CNS opportunistic infections, whereas CSF p-tau generally remains normal in individuals with HAND [19, 31]. Soluble APPα and particularly APPβ are markedly decreased in individuals with HAD and CNS opportunistic infections compared with neuroasymptomatic PLHIV. Both of the soluble APPs are also decreased in HIV-negative individuals with various CNS infections (bacterial and viral) [32]. Levels of CSF Aβ1-42 range from normal to low in individuals with HAND. Aβ1-42 is also reduced in acute bacterial meningitis [33].
Neurofilament proteins

Neurofilament (NF) proteins are the most abundant structural components of neuronal axons. The expression of these proteins is particularly high in large myelinated axons where they determine the conduction velocity [34]. Neurofilaments comprise about 85% of the cytoskeleton proteins and consist of three subunits with different molecular weights: light (NFL, 68 kDa), medium (NFM, 150 kDa), and heavy (NFH, 190–210 kDa). Of these three subunits, NFL is the most abundant and the most soluble one.

In conditions with loss of or damage to cortical neurons, such as HAD and degenerative dementias, the NP proteins can be used as specific biomarkers for axonal degeneration. Following neuronal death and axonal degeneration proteins from the neuro–axonal compartment will be released into interstitial fluid and diffuse into CSF, in which they can be measured by sandwich enzyme-linked immunosorbent assay (ELISA). NF proteins are thus markers of ongoing axonal injury, but they do not tell us anything about the cause.

Elevated CSF levels of NF proteins are found in a variety of neurological disorders, for example vascular dementia, normal-pressure hydrocephalus, cerebral infarction, subarachnoid haemorrhage, frontotemporal dementia, neonatal asphyxia, multiple sclerosis, head trauma, and cardiac arrest [35-39]. In most cases of Alzheimer’s disease, however, CSF NFL levels are normal or only moderately elevated [40]. CSF NF proteins can therefore be of diagnostic value to discriminate between different types of dementias.

CSF NFL in HIV-negative individuals
CSF NFL increases with age. We have compiled CSF NFL data from 359 neurologically and psychiatrically healthy HIV-negative controls from different cohorts and studies, all derived using the commercial UmanDiagnostics NF-Light assay (currently the only commercially available ELISA for NFL): The Gothenburg cohort, Sweden [18], The San Francisco cohort, USA [18], the SEARCH study, Thailand (ref), and the COBRA study (Amsterdam, the Netherlands and London, UK) (ref). Median age was 42 years (interquartile range (IQR) 31–56). Log CSF NFL showed a strong linear correlation with age ($r = 0.77$, $p < 0.0001$).

Expressed in the original scale this relationship becomes CSF NFL = 97.48 $\times$ 1.031$^{\text{age}}$. The implication of the formula is thus a yearly increase of 3.1% in CSF NFL. The upper reference limit (+2SD) is 201.2 $\times$ 1.031$^{\text{age}}$ (figure 2).

**CSF NFL in untreated HIV-infected individuals**

*Individuals with HIV-associated dementia*

The highest levels of CSF NFL during the natural course of HIV are seen in patients with HAD. Elevated CSF NFL is a nearly universal finding in individuals with HAD, abnormal levels ranging from 88% to 100% of subjects [18, 26, 41, 42]. Median CSF NFL levels in HAD patients were more than ten-fold elevated than in HIV-negative controls [42]. In the same study, CSF NFL levels in subjects with HAD were significantly higher compared to all other subgroups of HIV patients, untreated and treated, except for neuroasymptomatic subjects in the lowest CD4-strata (< 50 cells/µL) and a small variable group of elite controllers [42]. Patients with severe HAD (old AIDS dementia complex stage 2–4) have been shown to have higher CSF NFL than patients with less severe HAD (old AIDS dementia complex stage < 2) [26].
Interestingly, a small proportion of patients with HAD, 7–12% [18, 26, 41], have CSF NFL levels within normal range. This indicates that this group of patients with distinct symptomatology does not have signs of active on-going brain injury, at least as measured by NFL. Instead they may suffer from static impairment related to earlier, but now inactive, neuronal damage that occurred before initiation of ART, so called inactive disease, or perhaps they were incorrectly diagnosed from the beginning. It is highly unlikely that untreated patients with active HAD and concomitant axonal injury would have normal CSF NFL levels.

**HIV-infected individuals with CNS opportunistic infections**

NFL is a specific marker of axonal damage, but as mentioned earlier, it is not specific for HIV. An increase in CSF NFL informs us that there is an active neurological insult, but it does not tell us about the reason behind it. It is therefore not unexpected that other CNS infections can cause elevated CSF NFL. Some individuals with opportunistic infections in CNS have high levels of CSF NFL, typically in the same range as patients with HAD [26, 27]. This means that NFL alone cannot distinguish between HAD and CNS opportunistic infections, and we have to rely on other diagnostics such as neuroimaging and CSF analysis to diagnose correctly. The highest CSF NFL concentrations among CNS opportunistic infections have been noted in patients with CMV encephalitis and the lowest in those with cryptococcal meningitis [26, 27]. This most likely has to do with the histopathological correlates in these two infections. CMV leads to extensive necrotising periventricular encephalitis whereas cryptococcal infection in the CNS usually only involves the meninges, although it occasionally can cause meningoencephalitis and cryptococcomas. A similar pattern with very high CSF NFL levels is found in HIV-negative patients with herpes simplex encephalitis [43].
**Acute and primary HIV-infection**

HIV can be detected in CSF almost immediately after transmission [44]. This early viral invasion is accompanied by an inflammatory response [45], but it is not known how early during the course of infection events associated with HIV in the CNS lead to neuronal injury. In 32 subjects with acute HIV-infection (median time since exposure, 18 days), only one (3%) had CSF NFL above the upper limit of normal, compared with 31% of persons with chronic infection [46]. In a study using an older less sensitive assay for NFL [26], 25% (4/16) of subjects with primary infection had modestly elevated CSF NFL levels, not significantly different from HIV-negative controls. More recent work, using a sensitive NFL assay [47], showed that 36/82 (44%) of individuals with primary HIV (median three months after transmission) had CSF NFL levels above the upper limit of normal for their age group and median CSF NFL levels were significantly elevated compared with healthy controls.

The results from the abovementioned studies are a bit contradictory. In one of them [46] only 3% of those with acute infection had CSF NFL above the upper limit of normal and in the other [47] 44% of those with primary infection. One possible explanation could be that in the first mentioned study, median time since exposure was only 18 days and in the latter 92 days. These findings indicate that neuronal injury is initiated at some point after acute infection, presumably after several months of processes. The absence of elevated CSF NFL levels during acute infection may be related to a short duration and low level of immune activation at this early point, as suggested by lower CSF WBC and neopterin levels compared with those in acute HIV-infection [46]. One could speculate that HIV-related CNS degeneration is initiated during primary infection and continues during subsequent stages of infection, but
there are indirect evidence suggesting that CSF NFL levels normalise as the HIV-infection becomes chronic with higher CD4+ T-cell counts [42]. This will be discussed more in detail in the next section. In addition, elevated CSF NFL levels in primary infection are not accompanied by a corresponding elevation of CSF t-tau and decline of APPs as seen in subjects with HAD, indicating that this early neuronal injury is less severe and/or has a different mechanism than the one seen in HAD [42]. From a neurological point of view it would be valuable to identify subjects with on-going CNS injury during primary infection since they would most likely benefit from early ART. Current treatment guidelines, however, recommend initiating ART in all individuals with primary infection, regardless of symptoms (http://www.who.int/hiv/pub/guidelines/en), (http://www.eacsociety.org/guidelines/eacs-guidelines/eacs-guidelines/eacs-guidelines.html), (http://aidsinfo.nih.gov/guidelines/html/1/adult-and-adolescent-arv-guidelines/0/), (https://www.iasusa.org/guidelines) [48].

**Chronic infection in subjects without neurological symptoms**

Analysis of CSF NFL has demonstrated that a substantial number of untreated asymptomatic patients suffer subclinical CNS injury. The prevalence of injury increases as systemic disease progresses and the CD4+ T-cell count drops. Untreated PLHIV without neurological symptoms and with CD4+ T-cell count < 50 cells/µL have higher CSF NFL levels compared with individuals with higher CD4+ T-cell counts [18]. Sixty-nine per cent of neuroasymptomatic patients with CD4+ T-cell count < 50 cells/µL had CSF NFL above the age-related cut-off compared with 19–31% for subjects with CD4+ T-cell counts > 50 cells/µL [18]. This strong inverse correlation with CSF NFL and CD4+ T-cell count in neuroasymptomatic untreated PLHIV is not found in treated and suppressed individuals [18]. Even in subjects with relatively preserved immunity (CD4+ T-cell count > 350 cells/µL), however, almost one fifth had elevated CSF NFL.
Another study with approximately 20 neuroasymptomatic PLHIV in different CD4+ T-cell strata showed the same pattern with increasing prevalence of CNS injury as the CD4+ T-cell count declined; 75% of neuroasymptomatic individuals with CD4 < 50 cells/μL and 40% of those with CD4+ 50–199 cells/μL had substantial elevations of CSF NFL [42]. To further characterise this active axonal insult in untreated neuroasymptomatic patients with advanced systemic disease, we regrouped subjects with the lowest CD4+ T-cell count (< 200 cells/μL) into those with normal CSF NFL and those with CSF NFL levels above the age-related cut-off, separating individuals without and with neuronal injury. We then compared these new groups with the HAD-patients and found that the group with low CD4+ T-cells and high CSF NFL was similar to the HAD group in some respects, but not all. CSF NFL concentrations were lower in the group with low CD4+ T-cells and high CSF NFL but not significantly different from subjects with HAD subjects. Interestingly, CSF t-tau levels were significantly lower in subjects with low CD4+ T-cells and high CSF NFL compared with the HAD subjects, indicating again that CSF NFL is a very sensitive biomarker of CNS injury, in this study increasing prior to CSF t-tau [42]. Neuropsychological performance, measured with QNPZ-4 was markedly decreased in PLHIV with advanced immune suppression and elevated CSF NFL compared with those with normal NFL levels.

In addition to the inverse correlation with CD4+ T-cell count, there is also a strong and independent correlation for CSF NFL with CSF neopterin and CSF WBC in untreated PLHIV [18]. This finding is in agreement with the theory that intrathecal immune activation is important in HIV’s neuropathogenesis. Impairment of the blood-brain barrier (BBB), as measured by CSF/plasma albumin ratio, has also been found to be an independent predictor of
neuronal injury (elevated CSF NFL levels) in untreated and treated neuroasymptomatic individuals [49]. This supports the hypothesis that increased BBB permeability may be associated with axonal injury in HIV-infection. An impaired BBB facilitates influx of proteins, viral particles, and other possibly neurotoxic substances into CNS. These molecules together with inflammation may cause axonal injury, and further enhance breakdown of the BBB, eventually leading to clinically significant neurocognitive deficits.

Interestingly, one study reported a correlation between plasma homocysteine and CSF NFL levels in neuroasymptomatic PLHIV, both untreated and those on ART [50]. Homocysteine levels increase with B₁₂ vitamin and/or folate deficiency, indicating that B₁₂/folate deficiency might contribute to axonal damage in PLHIV.

Subjects with milder forms of HIV-associated neurocognitive disorders

Individuals classified as having ANI according to the 2007 Frascati criteria have been suggested to have a higher risk of symptomatic progression compared with unimpaired subjects [51]. A recently published study investigated if mild HAND was associated with neuronal damage measured by CSF NFL in suppressed subjects on ART [52]. Twenty-nine PLHIV were classified as unimpaired based on neuropsychological (NP) testing at baseline and 70 as having neurocognitive impairment (NCI); 37 with ANI and 33 with MND. In the NCI group, 19% (13/70) had CSF NFL levels above the upper normal age-related reference in at least one sample, compared with only 1/29 (3%) in the unimpaired group (p = 0.06). Thirty-two participants within the ANI or MND groups experienced a decline in their NP performance. There were, however, no differences in changes of CSF NFL or neopterin in subjects with a decline in, compared with the 38 participants with stable neurocognitive
performance. CSF neopterin correlated significantly with NFL in individuals with neurocognitive impairment but not in those with normal neurocognitive performance, indicating that there may be an association between immune activation, neuronal damage, and neurocognitive impairment.

Both CSF NFL and phosphorylated NFH (pNFH) have been determined in a cohort of untreated HIV-infected individuals, some with normal neurocognitive function and some with HAND [53]. Of the 48 included participants, three were diagnosed with HAD, 15 with MND and ANI respectively, and 10 were neurocognitively normal as assessed by NP testing. Among individuals with a history of immunosuppression (CD4+ nadir < 200 cell/μL), CSF NFL levels were significantly elevated in those with HAD compared with those with MND and neurocognitively normal subjects (but not those with ANI). There was no significant difference in CSF NFL between groups when including all individuals irrespective of nadir CD4+ T-cell count. Levels of CSF pNFH were similar for all groups. Two out of three subjects with HAD, 11/15 with MND, 11/15 with ANI, and 10/15 neuroasymptomatic individuals had elevated CSF NFL levels, indicating subclinical neuronal injury regardless of neurocognitive performance. CSF NFL and pNFH correlated with each other and also with markers of monocyte/macrophage activation in the CSF (soluble CD14 and CD163).

**Elite controllers**

A small proportion of HIV-infected patients maintain undetectable plasma HIV RNA levels in the absence of ART. This group is termed elite controllers, and the character of CSF HIV infection and degree of immune activation is comparable to that of HIV-uninfected and ART-suppressed HIV-infected individuals, but distinct from that of untreated, viremic HIV-infected individuals (ref) [54]. We have only scarce knowledge about CSF NFL levels in this rare and
variable group of PLHIV. One of eight elite controllers had elevated CSF NFL after age correction [42].

**CSF NFL in subjects on suppressive antiretroviral therapy**

The effects of ART on CSF NFL have been documented in several case reports and studies, some using the old CSF NFL assay and some using the new more sensitive assay [18, 41, 55]. In one study, four patients with HAD were followed for at least one year after initiation of ART. All four showed a decline in CSF NFL in parallel with reduction of plasma and CSF HIV RNA and better performance on neurocognitive testing [26]. In a multicentre study including archived CSF samples from 53 individuals, CSF NFL at baseline was increased in 21 (40%) [41]. Among those with increased CSF NFL levels there were 18 patients with an AIDS-defining illness, out of whom nine had HAD. All 21 patients had a decrease in CSF NFL, with 48% (10/21) having normal concentrations after three months of treatment. After 12 months on ART only four HAD-patients still had elevated NFL levels. Eight subjects were followed two to ten years. All eight had CSF NFL within the normal range.

In a more recent longitudinal cohort study with 78 neuroasymptomatic participants using the new highly sensitive NFL assay, 26 (33%) had elevated CSF NFL prior to initiation of ART. Follow-up time on ART was in median 15 weeks (IQR 14–23). Eighty-one per cent (21/26) had a reduction in CSF NFL levels and 35% (9/26) had normalised levels at the end of the study [18].

CSF NFL is known to increase with age in HIV-negative individuals, even though the underlying mechanism for this is not known. In a large cross-sectional study of 252 PLHIV and 204 HIV-negative controls, 200 of the HIV-infected neuroasymptomatic individuals were
untreated and 85 had been on suppressive ART with plasma HIV RNA < 50 copies/mL for > 12 months (46 patients were included in both the untreated and treated group at different time points) [18]. When analysing the differences between treated and untreated PLHIV and HIV-negative controls with age as covariate (using a linear mixed effects model), we found that CSF NFL concentrations in the untreated neuroasymptomatic group were equivalent to HIV-negative individuals who were 18.5 years older. Not unexpected, ART substantially reduced CSF NFL levels and CSF NFL concentrations in the treated group were equivalent to those of HIV-negative controls who were 3.9 years older (Figure 4).

All these studies show that the HIV-driven neurological process can be halted and, at least, partly reversed as indicated by reductions in CSF NFL as well as HIV RNA and inflammatory markers, and clinical improvements in parallel. But, as this last cited study shows, treated and virologically suppressed PLHIV still have slightly higher levels of CSF NFL than HIV-negative individuals. This finding might reflect a mild on-going immune activation and axonal degradation caused by continuous low-level viral replication with discrete neuropathological effect.

**CSF NFL in subjects with asymptomatic CSF viral escape**

The phenomenon of CSF (or CNS) viral escape from ART has been defined in different ways, but the prevailing definition is CSF HIV RNA greater than 50 copies/mL in CSF and less than 50 copies/mL in plasma in an individual on ART [21, 56, 57]. CSF viral escape has been described in PLHIV with and without neurological complications, but CSF NFL has not always been analysed [21, 58, 59]. To assess whether HIV persistence in CSF is associated with active HIV-related neurologic damage, 75 neuroasymptomatic PLHIV with suppressed plasma viral load were longitudinally followed [60]. Of these participants, 23% (17/75) had at
least one CSF HIV RNA measurement > 50 copies/mL and only one patient had a repeated
isolated increase in virus in CSF (> 50 copies/mL). The latter could indicate persistent
replication within the CNS, constituting possible CSF viral escape whereas intermittent
detection of HIV in CSF probably more likely is equivalent to plasma viral blips. Although
individuals with detectable CSF HIV RNA had higher CSF neopterin levels, indicating
intrathecal immune activation, compared with subjects with undetectable CSF HIV RNA,
they did not have higher CSF NFL. This suggests that presence of virus in the CNS is coupled
to immune activation in treated suppressed patients, but that CNS viral persistence, at least by
this measure, may not be a cause of neuronal injury detected during ART in those with
asymptomatic CSF escape.

Switching to monotherapy with darunavir/ritonavir after achieving plasma viral suppression
with combination ART has been demonstrated to maintain viral suppression in the periphery
[61, 62], but the efficacy in preventing HIV replication in the CNS is not as certain. CSF viral
escape and new neurological symptoms have been described in patients on darunavir/ritonavir
monotherapy [62], and in two longitudinally followed subjects on this regimen with viral
breakthrough in CSF, there were also signs of intrathecal immune activation with pleocytosis
and increased levels of CSF neopterin, and signs of neuronal injury with elevated CSF NFL
levels [63]. None of the patients developed neurological symptoms and both initiated
treatment with two nucleoside analogues again in addition to darunavir/ritonavir. We do not
know what would have happened if they would have continued with the protease inhibitor
monotherapy.

CSF NFL in subjects stopping antiretroviral therapy
Some years ago, it was theorised that standardised treatment interruptions could have several beneficial effects, among other things on HIV-specific immunity and reducing drug exposure and drug costs. Since it has been clearly demonstrated that treatment interruptions are associated with increased morbidity and mortality, they are no longer recommended [64], but sometimes people stop therapy for different reasons anyway. A small retrospective study of eight subjects on ART with CSF HIV RNA < 50 copies/ml and normal CSF NFL levels sought to find out whether treatment interruptions could have any negative effects on the brain [65]. None of the participants developed any clinical neurological symptoms after stopping ART, but three individuals experienced considerable increases in CSF NFL indicating axonal insult. All eight subjects had a rapid increase of plasma HIV RNA and a somewhat slower increase of CSF HIV RNA, followed by elevations of plasma and CSF neopterin, and after that by CSF NFL with the earliest documented increase 58–80 days post treatment interruption. It could be hypothesised that reappearance of virus in blood and CSF after pausing ART leads to inflammation and subsequent neuronal damage. The association with increased CSF neopterin and NFL concentrations in untreated individuals has been found in several studies and is consistent with the theory that intrathecal immune activation is important for neuronal damage [26, 27, 41]. To further complicate things, all eight patients in the treatment interruption study developed elevations in CSF neopterin, but only three had increased CSF NFL. The link between inflammation and axonal insult is thus not entirely straightforward, at least not during the timeframe of this study.

**Can CSF NFL levels be predictive for development of HAD?**

In addition to determining clinical diagnosis, monitoring response to therapeutic interventions, and evaluating prognosis, it would be very useful if a biomarker could predict the development of a certain disease. The possibility to utilise CSF NFL concentrations for
predicting the future development of HAD has been explored [66]. One retrospective study included nine patients who had developed HAD and who had undergone a research lumbar puncture one-two years prior to onset of symptoms. Elevated CSF NFL levels were found in seven out of these nine neuroasymptomatic patients (78%) who later developed HAD, compared with only 9/27 (33%) CD4+ T-cell count–matched HIV-infected controls. There were no differences in CSF HIV RNA or neopterin concentrations between the two groups. Although this study is small, it shows that CSF NFL could be a useful predictive marker for HIV-related CNS injury.

**Plasma NFL and HIV CNS infection**

The need for lumbar punctures has limited the use of CSF NFL measurements in some settings. However, a new ultra-sensitive Single molecule array (Simoa) immunoassay for measuring plasma NFL levels has recently been developed [24, 67]. In a cross-sectional study on archived material, NFL was quantified in paired CSF and plasma samples from 121 PLHIV and 19 HIV-negative controls. PLHIV were divided into groups according to stage of systemic disease, presence of HAD, and on ART or not. NFL was more than 50-fold lower in plasma than in CSF, but the levels were quantifiable with the new assay in all subjects, including HIV-negative healthy volunteers and PLHIV with normal CSF NFL concentrations. There was a strong correlation between plasma and CSF NFL concentrations (r = 0.89) with plasma NFL exhibiting a similar pattern as CSF NFL with age-related increases in NFL, highest values in HAD-patients, and significant elevations in untreated neuroasymptomatic subjects with low CD4+ T-cells [24].

Other examples of neurodegenerative conditions where plasma NFL has been investigated are
frontotemporal dementia, multiple sclerosis, and Creutzfeldt-Jakob disease. Frontotemporal dementia is characterised by progressive neuronal loss in the frontal and/or temporal lobes and accounts for approximately one fifth of early-onset dementia [68]. Serum concentrations of NFL are raised in frontotemporal dementia and higher concentrations have been associated with faster rates of brain atrophy [69]. In Creutzfeldt-Jakob disease, serum NFL was increased before the clinical diagnosis [70], and in multiple sclerosis, baseline NFL concentrations predicted progression of neurodegeneration and disability [71].

Plasma NFL, thus, seems to have the potential to serve as a biomarker of neurodegeneration, although of limited differential diagnostic value. In HIV-infection, it could be used to screen or evaluate individuals with neurocognitive problems, predict progression of or quantify severity of disease in those with HAND. It could also be of value in clinical trials where CSF sampling may be difficult to perform, or in patients refusing lumbar punctures.

Current research on HIV eradication mainly focuses on resting CD4+ T-cells, but other cells such as macrophages in the CNS have the potential to act as latent cell reservoirs. Latency reversing agents used in eradication attempts can lead to rebound of virus rebound in the CNS and cerebral symptoms [72]. In macaques suppressed on ART for a long time, administration of latency reversing agents lead to not only to rebound of CSF viral load, but also increases of CSF immune activation markers (neopterin and CCL2) and NFL as a marker of neuronal damage [73]. Determination of plasma NFL has the potential to become a valuable tool in eradication studies in humans (as well as animal models) when it comes to detecting possible harmful activation of viral replication in the brain.

Conclusions
CSF NFL has proven to be a sensitive and independent biomarker of neuronal injury at different stages of HIV-infection. It can be used to discriminate between continuous active disease and inactive old irreversible damages. It has taught us that a substantial proportion of untreated PLHIV have subclinical brain injury, indicated by elevated CSF concentrations of NFL. We know that ART has a significant effect on neurodegeneration, but that even virologically suppressed patients have higher levels of CSF NFL than HIV-negative controls, suggesting on-going low-grade injury that is not fully reversed by treatment. NFL can, together with other CSF biomarkers, also be used for differential diagnostic purposes in PLHIV with neurological and neuropsychological symptoms.

CSF NFL strongly correlates with age and we have here presented new reference values for individuals between 20 and 80 years of age. The reference values were derived using the NF-Light assay from UmanDiagnostics (Umeå, Sweden), which is currently the only commercially available kit for CSF NFL; new assays may produce different absolute concentrations and it may be important to develop reference methods and materials for this marker to harmonize the test results of different assays when such exist.

Now, we also have the possibility of quantifying NFL in plasma. Plasma NFL correlates well with CSF NFL levels at all stages of HIV-infection. This opens up new possibilities to study neuronal injury in HIV-infected individuals in trials and in the clinical setting.

**Figure legends**
Figure 1. Biomarkers of neurodegeneration in HIV-1 CNS infection in cerebrospinal fluid and blood. NFL is a biomarker of injury to large-calibre myelinated axons. Total tau is a biomarker of injury to thin non-myelinated axons. APP and amyloid-β are produced in axon terminals and might be involved in synaptic activity and plasticity. Abbreviations: APP; amyloid precursor protein, NFL; neurofilament light chain protein.

Figure 2. CSF NFL versus age in HIV-negative controls. Linear regression of log CSF NFL on age reveals a yearly increase of 3.1% in CSF NFL. The regression line and an upper reference of +2 standard deviations are shown.

Figure 3. CSF NFL in various stages of HIV-infection and in HIV-negative controls. The number of individuals in each category is given within parenthesis. Median age of all individuals (HIV-infected and uninfected) is 42 years. CSF NFL was age-corrected to 42 years. The dotted line shows the upper reference value for CSF NFL 42 years = (773 ng/L). Boxes show median and interquartile range, whiskers 5–95 percentiles, and ’+’ designates the means. Abbreviations: PHI; primary HIV-infection, NA; neuroasymptomatic, HAD; HIV-associated dementia, ART; antiretroviral treatment, elites; elite controllers.

Figure 4. CSF NFL in relation to age and antiretroviral treatment. CSF levels of NFL in untreated individuals is equivalent to individuals 18.5 years older than HIV-negative controls. While treatment reduced these concentrations, CSF NFL concentration in the population on antiretroviral treatment was equivalent to controls 3.9 years older. The 95% prediction interval of CSF NFL levels of HIV-negative controls is demonstrated as dotted lines (Neg 95% PI). Figure from Jessen Krut et al, PLoS One 2014 Feb 11;9(2):e88591. Reprinted with

**Acknowledgements**

This project was supported by the Swedish Research Council (K2011-58P-20931-01-4,2013-2546), the Sahlgrenska University Hospital (ALFGBG-430271, ALFGBG-441051), the Knut and Alice Wallenberg Foundation, the National Institutes of Health (R01MH62701, R21MH096619, R21NS069219 and UL1 TR000004), and the European Union’s Seventh Framework Programme for research, technological development and demonstration under grant agreement no 305522.

**Tables**

**Table 1.** Age-related upper normal CSF NFL levels (CSF NFL = 201.2 \( \cdot \) 1.031\(^{\text{age}} \)).

<table>
<thead>
<tr>
<th>Age</th>
<th>CSF NFL upper reference values (ng/L)</th>
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<tr>
<td>20</td>
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References


