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ORIGINAL COMMUNICATION

CSF neurofilament protein (NFL) – a marker of active HIV-related neurodegeneration

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Abstract *Background and methods* The light subunit of the neurofilament protein (NFL), a major structural component of myelinated axons, is a sensitive indicator of axonal injury in the central nervous system (CNS) in a variety of neurodegenerative disorders. Cerebrospinal fluid (CSF) NFL concentrations were measured by ELISA (normal < 250 ng/l) in archived samples from 210 HIV-infected patients not taking antiretroviral treatment: 55 with AIDS dementia complex (ADC), 44 with various CNS opportunistic infections/tumours (CNS OIs), 95 without neurological symptoms or signs, and 16 with primary HIV infection (PHI). The effect of highly active antiretroviral treatment (HAART) was studied by repeated CSF sampling in four of the ADC patients initiating treatment. *Results* CSF NFL concentrations were significantly higher in patients with ADC (median 2590 ng/l, IQR 780–7360) and CNS OIs (2315 ng/l, 985–7390 ng/l) than in neuroasymptomatic patients (<250 ng/l, <250–300) or PHI (<250 ng/l, <250–280), $p < 0.001$. Among patients with ADC, those with more severe disease (stage 2–4) had higher levels than those with milder disease (stage 0.5–1), $p < 0.01$. CSF NFL declined during HAART to the limit of detection in parallel with virological response and neurological improvement in ADC.CSF NFL concentrations were higher in neuroasymptomatic patients with lower CD4-cell strata than higher, $p < 0.001$. This increase was less marked than in the ADC patients and noted in 26/58 neuroasymptomatic patients with CD4 counts <200/μl compared to 1/37 with CD4-cells ≥200/μl. *Conclusions* The findings of this study support the value of CSF NFL as a useful marker of ongoing CNS damage in HIV infection. Markedly elevated CSF NFL concentrations in patients without CNS OIs are associated with ADC, follow the grade of severity, and decrease after initiation of effective antiretroviral treatment. Nearly all previously suggested CSF markers of ADC relate to immune activation or HIV viral load that do not directly indicate brain injury. By contrast NFL is a sensitive marker of such injury, and should prove useful in evaluating the presence and activity of ongoing CNS injury in HIV infection.

Key words HIV · neurofilament protein · cerebrospinal fluid · AIDS dementia complex

Introduction

AIDS dementia complex (ADC) developed in approximately 20% of patients with advanced HIV infection before the widespread use of highly active antiretroviral treatment (HAART) [1]. More subtle cognitive disturbances were even more frequent before treatment and may continue to impact on the quality of life in treated patients, with a higher prevalence related to improved longevity [2]. A major difficulty in management of this condition is the lack of clear objective methods for diagnosis and for judging disease activity. Thus, ADC remains a syndromic diagnosis that relies on clinical measures or neuropsychological test impairment [3]. This has proved to be a critical impediment to clinical trials where entry based on these clinical criteria fails to exclude subjects with confounding stable encephalopathies or residual injury and stable pathology once related to HIV encephalitis.

HIV invades the central nervous system (CNS) early, around the time of primary infection [4], and during the asymptomatic phase of the infection HIV-RNA can almost always be detected in the cerebrospinal fluid (CSF) along with evidence of chronic CNS immunoactivation [5–8]. Thus, while patients with ADC often have high CSF HIV-RNA levels and increased concentrations of intrathecal immunoactivation markers [9, 10], these markers may be elevated in patients without discernible neurological impairment, also in early stages of the disease when immune defense are still preserved. Consequently, more specific markers for HIV-related brain injury are needed to distinguish active brain injury from 'innocent' infection. Such markers may, alone or more likely in combination with viral and inflammatory markers, be useful as diagnostic and prognostic tools for ADC.

The light subunit of neurofilament protein (NFL) is a sensitive marker of axonal disruption. Increased CSF NFL concentrations are principally thought to reflect injury to myelinated axons. A clear association has been found between the presence of white matter changes and increased CSF NFL levels in patients with Alzheimer's disease and subcortical vascular dementia [11]. CSF NFL is also increased in several other neurodegenerative disorders linked to demyelination and/or axonal degeneration, including active multiple sclerosis and amyotrophic lateral sclerosis [12, 13]. In an earlier pilot study, we found CSF NFL increased in patients with AIDS, including in two patients with ADC [14]. We also reported increased CSF NFL in three patients after antiretroviral treatment interruption [15]. In the present study we explored the potential of NFL as a marker for neurodegeneration and ADC in a larger cohort of HIV infected patients at different stages of infection. We hypothesized that this marker which is sensitive to CNS injury in other neurodegenerative diseases, would detect ongoing HIV-related brain injury.

Methods

Archived samples from 210 HIV-1-infected patients followed at four centres: Göteborg, Sweden; Milan, Italy; San Francisco, CA, USA; and Sydney, Australia, were studied. Fifty-five were diagnosed with ADC, 44 had CNS opportunistic disease (eight with cytomegalovirus encephalitis (CMV-E), five with primary CNS lymphoma, 10 with cryptococcal meningitis, nine with progressive multifocal leukoencephalopathy (PML) and 12 with cerebral toxoplasmosis), 95 had chronic HIV infection without neurological symptoms or signs ('neuroasymptomatics'), and 16 were sampled during primary HIV infection (PHI, defined as within 2 months of onset of acute retroviral syndrome or within 3 months of exposure to HIV infection followed by documented seroconversion). Subject characteristics are shown in Table 1. Four patients presenting with ADC were additionally followed with repeated CSF sampling after antiretroviral treatment initiation. One of these ADC patients was switching from a failing regimen while all other subjects in this study were antiretroviral naïve or had been off HAART for at least 6 months at the time of sampling.

Diagnosis of ADC and each of the opportunistic infections (OIs) was established according to CDC and American Academy of Neurology AIDS Task Force criteria's [16, 17] In addition, PML and CMV-E were also diagnosed on the basis of clinical neurological findings and the detection of JC virus or CMV DNA, respectively, in CSF [18, 19]. The Memorial Sloan-Kettering scale was used to rate the severity of the ADC [20]. In patients with ADC or CNS OIs, samples were collected at the time of diagnosis, and for the OIs, within 1 week of initiating specific treatment. In all of the subjects included in the cross-sectional analysis, samples were drawn before initiating any antiretroviral therapy.

CSF samples were obtained within the context of research studies approved by ethics committees at each site, and all subjects gave their informed consent. Aliquots of CSF were after centrifugation stored at −70°C until the time of the study.

CSF concentrations of NFL were analysed using a previously described enzyme-linked immunoassay [13]. In brief, capturing antibody (hen anti-NFL IgG) was absorbed to microtest plates, and CSF samples or reference NFL were then incubated. Rabbit anti-NFL was used as secondary antibody. Bound secondary antibody was detected using peroxidase conjugated donkey anti-rabbit IgG. The upper normal reference value at the laboratory is 250 ng/l below the age of 60 years.

HIV-1 RNA was quantified in cell-free CSF and plasma by the Roche Amplicor Monitor assay (version 1.0 and 1.5, Hoffman La-Roche, Basel, Switzerland). Neopterin was measured by a commercially available radio-immunoassay (Henningtest Neopterin, BRAHMS, Berlin, Germany) with a normal reference value of ≤4.3 nmol/l in CSF [21]. Routine assessments also included CSF white blood cell (WBC) count and peripheral blood CD4+ T-lymphocyte (CD4) cell determination.

The four ADC subjects studied by serial CSF sampling also underwent standardized quantitative neurological performance testing incorporating four tasks (timed gait, grooved pegboard with the dominant hand, finger tapping with the nondominant hand, and the Digit Symbol test of the WAIS-R), yielding an aggregate scaled *z*-score termed the QNPZ-4 score [22]. This was used to track changes in neurological performance after treatment initiation.

Table 1 CD4 cell counts, CSF and plasma viral loads, CSF Neopterin, CSF white blood cells and CSF NFL levels among the study participants

	Number of subjects	Blood CD4+ cells (cells/μl)	CSF HIV-1 RNA (log10 copies/ml)	Plasma HIV-1 RNA (log10 copies/ml)	CSF Neopterin (nmol/l)	CSF WBCs (cells/μl)	CSF NFL (ng/l)
PHI	16	413 (247–784)	3.67 (<1.70–4.88)	4.71 (3.51–5.88)	29.1 (6.20–82.0)	10 (1–34)	<250 (<250–1920)
Neuroasymptomatic	95	134 (0–1264)	3.59 (<1.70–5.67)	4.62 (<1.70–5.88)	18.2 (4.00–92.0)	3 (0–50)	<250 (<250–23390)
CD4 < 50	27	16 (0–48)	3.11 (1.90–5.12)	4.84 (3.06–5.88)	23.8 (6.70–92.0)	2 (0–12)	290 (<250–1960)
CD4 50–199	31	120 (52–186)	3.66 (<1.70–5.67)	4.71 (3.56–5.88)	22.8 (7.60–92.0)	3 (0–50)	<250 (<250–23390)
CD4 200-349	20	252 (219–330)	3.52 (<1.70–4.85)	4.37 (3.47–5.67)	16.2 (6.10–28.0)	4 (1–26)	<250 (<250–9410)
CD4 ≥ 350	17	500 (369–1264)	3.83 (<1.70–4.88)	4.36 (<1.70–5.57)	13.8 (4.00–39.4)	4 (0–26)	<250 (<250–<250)
ADC	55	76 (0–435)	4.45 (1.70–6.26)	4.95 (2.58–6.00)	58.0 (3.00–304)	8 (0–52)	2590 (<250–105000)
Stage 0.5–1	21	66 (0–435)	3.90 (1.81–5.56)	5.24 (2.58–5.88)	45.0 (18.6–304)	2 (0–20)	860 (<250–15540)
Stage 2–4	34	85 (0–378)	4.89 (1.70–6.26)	4.70 (3.03–6.00)	79.5 (3.00–270)	10 (0–52)	3590 (<250–105000)
CNS OIs	44	20 (0–314)	3.77 (2.00–5.91)	4.92 (3.76–5.78)	41.0 (13.6–97.8)	6 (1–18)	2315 (<250–36500)

Values given are medians (range)
Abbreviations: WBCs, white blood cells; PHI, primary HIV infection; neuroasymptomatic, neurologically asymptomatic HIV-1 infection; ADC, AIDS dementia complex; CNS OIs, opportunistic CNS disease

Statistics

Nonparametric methods were used for group descriptives (median and intraquartile range, IQR) and comparisons. Independent samples were analysed by means of Mann–Whitney U-test. The Kruskal–Wallis test was used when comparing more than two unpaired groups. Spearman's rank correlation coefficient was used for evaluations of correlations.

Results

CSF NFL was markedly increased in patients with ADC (Table 1), and 91% (50/55) had NFL levels above the upper normal reference value of 250 ng/l (Fig. 1A). CSF NFL levels were significantly higher in ADC patients than in neuroasymptomatic patients ($p < 0.001$), of which 28% had concentrations ≥250 ng/l. Patients with more severe ADC (stage 2–4) had higher CSF NFL concentrations than patients with ADC stage <2 ($p < 0.01$) (Fig. 1B).

Increased CSF NFL concentrations were also common among the patients with CNS OIs (Fig. 1A), with elevated concentrations in the same range as the ADC patients. High CSF NFL was noted in each of the five OIs assessed with highest levels in those with CMV encephalitis and lowest in those with cryptococcal meningitis (Fig. 1C).

Among the neuroasymptomatics, elevated CSF NFL was most common in those with lower blood CD4 counts (Fig. 1D). Thus, 16 of 27 (59%) subjects with blood CD4 counts <50 cells/μl and 10 of 31 (32%) with CD4 counts of 50–199 cells/μl had elevated CSF concentrations, although these elevations were generally in a lower range than in the ADC subjects. By contrast only 1 of 20 (5%) with CD4 counts of 200–349 cells/μl, and none of the 17 subjects with higher CD4 counts had abnormal values. Of 16 patients with PHI, four had increased CSF NFL, though in three of these the elevations were below 400 ng/l.

A positive correlation was found between CSF NFL concentrations and CSF neopterin ($r = 0.54$, $p < 0.001$, $n = 155$), and a weak correlation also between CSF NFL and CSF viral load ($r = 0.17$, $p < 0.05$, $n = 191$). No significant correlations were found between CSF NFL and plasma viral load ($n = 185$) or CSF WBC count ($n = 157$). CSF HIV RNA and CSF neopterin were significantly higher in patients with ADC compared to neuroasymptomatic patients and patients with PHI (Table 1).

Figure 2 shows the decline in CSF NFL in four ADC patients studied intensively after starting HAART. All four exhibited a prompt NFL response that paralleled the decrease in CSF HIV RNA and was accompanied by clinical improvement reflected in improved QNPZ-4 scores. This experience clearly documents a therapeutic effect of HAART on the process of brain injury related to HIV infection and shows the HIV-related specificity of the elevated NFL in these patients.

Discussion

NFL was substantially increased in the CSF in the majority of patients with a clinical diagnosis of ADC, with higher levels in those more severely affected. Antiretroviral treatment induced a decrease in NFL in association with reduction in CSF and plasma HIV RNA concentrations and with clinical improvement. These findings suggest that measuring CSF NFL may be useful for ADC diagnosis and, indeed, for management of CNS HIV infection more broadly, and might also be useful in clinical trials.

The neuropathological hallmark of ADC is HIV encephalitis [23, 24], and neuropathogenesis is largely driven by 'indirect' mechanisms in which infection

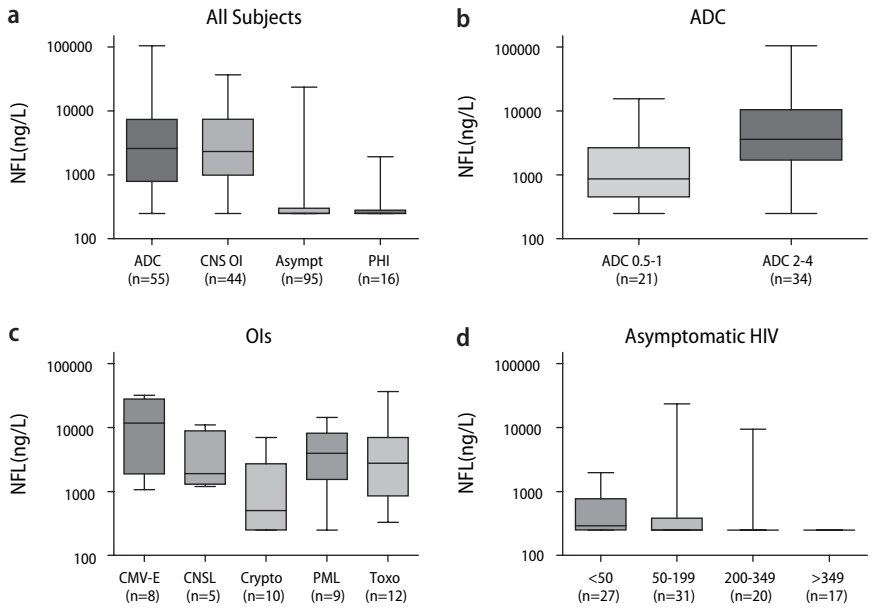
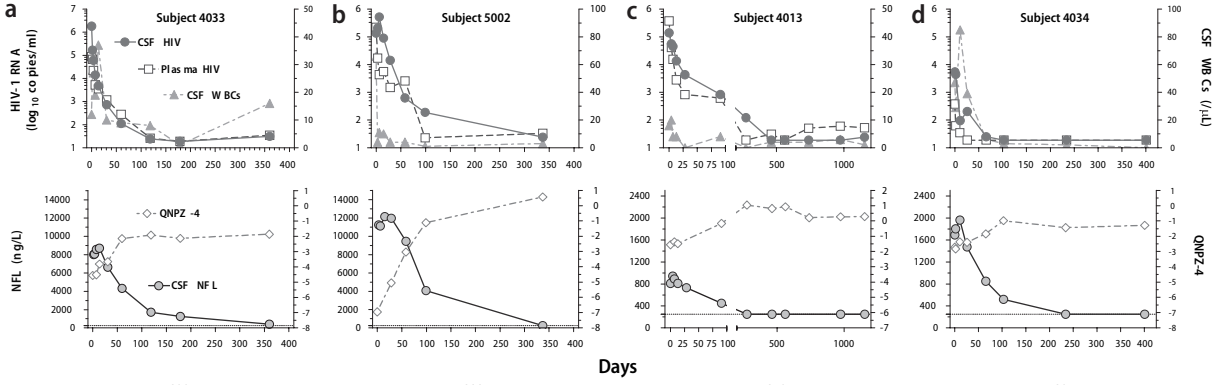


Fig. 1 CSF NFL concentrations (normal < 250 ng/l) in 210 HIV-1 infected individuals in different stages of the disease. Median values, 25th and 75th percentiles, and ranges are shown in the box plots. Panel **A** shows all subjects divided into different groups, panel **B** subjects with AIDS dementia complex (ADC) staged according to the Memorial Sloan-Kettering Scale, panel **C** patients with CNS opportunistic infections (OIs); CMV encephalitis (CMV-E), CNS lymphomas (CNSL), cryptococcal meningitis (Crypto), progressive multifocal leucoencephalopathy (PML) and toxoplasmosis (Toxo), and panel **D** neurological asymptomatic subjects grouped according to their CD4 cell counts



	4033	5002	4013	4034
Clinical Presentation	38 yo male, Stage 2 ADC	36 yo male, Stage 3 ADC	36 yo male, Stage 1 ADC	45 yo male, Stage 1 ADC
Prior ART Exposure	Naïve	Delavirdine, briefly, otherwise naive	Naïve	On didanosine, ritonavir, saquinavir at presentation
New ART Regimen	Abacavir, nevirapine, indinavir/ritonavir, lamivudine	Nelfinavir, abacavir, lamivudine, nevirapine	Nelfinavir, stavudine, nevirapine, lamivudine	Changed to abacavir, nevirapine, indinavir/ritonavir
Clinical Outcome	Improved to Stage 1 ADC, residual spastic gait	Improved to Stage 0.5 ADC, resumed computer work	Remains Stage 1 ADC, residual spastic gait	Stage 1 ADC, residual spastic gait

Fig. 2 Treatment responses in four ADC patients. The upper panel of each pair shows the plasma and CSF HIV RNA responses along with CSF WBC changes, and the lower panels show treatment effects on the CSF NFL (normal < 250 ng/l; dotted lines) and the QNPZ-4 scores (normal = 0). The key to the symbols for all graphs are shown in the left panel set (A). Clinical characteristics of these subjects have been described in detail elsewhere [8]

and activation of macrophages and microglia play a prominent role [25]. The incidence of ADC has markedly decreased in regions where HAART is available [26], though there remains the concern that milder ADC remains common, and indeed is present in a larger number of patients related to the life-prolonging effects of HAART [2]. HAART can also ameliorate established ADC, though neurological injury in patients presenting with this condition may not fully reverse [8, 27, 28]. Additionally, it has been suggested that HAART may alter the clinical character of HIV-related CNS injury [29].

The diagnosis of ADC is currently made on the basis of its clinical presentation after exclusion of alternative diagnoses [17]. Unfortunately, there is no confirmatory diagnostic laboratory test to enhance

the certainty of this diagnosis. In group studies, also confirmed by the present study, both virological markers, such as CSF HIV-1 RNA levels, and immunological markers, such as CSF beta-2-microglobulin, neopterin and monocyte chemotactic protein 1 (MCP-1), are in mean higher in patients with ADC compared to neuroasymptomatic patients [7, 9, 10, 30]. However, there is a substantial overlap in the concentrations of these markers with other diagnoses and with neurologically asymptomatic HIV-infected patients [6, 31]. None specifically indicates that the nervous system is being injured. For these reasons, a marker that clearly indicates brain injury would represent a substantial advance for clinical diagnosis and management of ADC. Moreover, in HIV-infected patients with neurological impairment, it is often critical to establish whether the CNS is being injured at the time of clinical encounter rather than related to some prior, but static, disease either treated ADC or some other insult. In other words, it is important to establish whether the disease process is *active*.

The neurofilament is a major structural element of the neuron, found most conspicuously in larger neurons and their myelinated axons. It is composed of a triplet protein of which the light subunit (NFL) is an essential component of the neurofilament core. It maintains the axonal calibre and has a crucial role in the structural and functional integrity of axons and in their capacity to rapidly conduct nerve impulses [32]. NFL measured in CSF is a sensitive marker of axonal damage in a number of conditions. Both focal and systemic ischemia cause neuronal damage detectable as leakage of NFL into the CSF proportional to the severity of the injury [13, 33, 34]. In chronic disorders, the release of NFL is often less pronounced but still raised to several times normal in the presence of active degeneration of white matter or myelinated spinal tracts, for example in multiple sclerosis and in amyotrophic lateral sclerosis [12, 13, 35]. Dementia of vascular etiology with subcortical white matter pathology causes moderately increased CSF NFL levels [11, 36]. CSF NFL determination has thus been shown to be a versatile tool to detect neuronal damage of any cause and is currently used for diagnostic purposes in clinical practice in some European countries [37–39].

As expected, not only patients with ADC but also those with different CNS OIs had elevated CSF NFL, as it is a marker of axonal damage irrespective of cause. In itself, then, elevated NFL cannot be used to discriminate between ADC and CNS OI or other active neurodegenerative process. However, most CNS OIs are recognized by magnetic resonance imaging (MRI) scan and CSF analyses, including specific viral, fungal and bacterial assays. When these alternative CNS diseases have been ruled out, CSF NFL can serve as a valuable diagnostic tool to indicate ongoing CNS injury related to CNS HIV infection. Using CSF NFL in combination with complementary CSF markers might provide a more specific diagnostic pattern but needs to be studied further.

In 5/55 (9%) patients with ADC, the CSF NFL levels were not increased. Two of those had been on treatment earlier which might have halted the progress of CNS damage. Whether ADC patients with low CSF NFL concentrations were misdiagnosed and had a disease that differed neuropathologically from the others could not be established from this study of archived material, but it is tempting to hypothesize that there were differences in disease activity and CNS damage in these patients, at least at the time of sampling. On the other hand, 28% of the neuroasymptomatic patients had elevated CSF NFL concentration, of which 8/95 (8%) had high levels (>1000 ng/l). This indicates an ongoing brain injury, but we did not find any obvious difference between these individuals and the other asymptomatic patients. The proportion of these 'false positives' was even higher when subjects with lower CD4 cell counts were segregated, and a correlation was found between CD4 cell count and CSF NFL levels. Only 1/37 patients with CD4 count $\geq 200/\mu$l had elevated CSF NFL compared to 26/58 with CD4 $<200/\mu$l. Thus, with more advanced HIV infection, when CD4 counts are in the range defining AIDS, subclinical brain injury may be relatively common. It is in this same CD4 range that ADC characteristically develops [1]. Further studies will be needed to establish the significance of this apparent subclinical injury and the prognostic meaning of increased CSF NFL without overt neurological symptoms. The findings also support that treatment should be initiated before the CD4 cell count drop to $<200/\mu$l, not only for the risk of opportunistic infections, but also for protecting the brain from HIV injury.

Four of 16 patients with PHI also had modestly increased CSF NFL. Neurological injury in these individuals with an initial surge in viremia may parallel what was found in subjects undergoing treatment interruption. In a previous small study we found that three of eight subjects stopping effective treatment exhibited increased CSF NFL in association with renewed viremia and elevated CSF HIV RNA [15]. In these patients we noted a delay between viral rebound and the rise in CSF NFL, with the earliest documented increase at 58–86 days after treatment interruption [15]. Thus, some PHI patients might have been sampled too early to detect an increase in CSF NFL concentration.

A positive correlation was found between CSF NFL and CSF neopterin, a marker of intrathecal immunoactivation that is largely derived from activated macrophages and microglia [40], while no robust

correlations was found with either CSF viral load or CSF WBC count. This is in line with the findings in our earlier smaller pilot study [14] and is consistent with the hypothesis that the neuropathogenesis of ADC largely is driven by CNS immune activation. In turn, the immunoactivation is most probably driven by the CNS HIV infection which is imperfectly reflected in CSF HIV RNA measurements [41, 42]. In patients interrupting their treatment, the CSF NFL increase was preceded by increased CSF viral load but developed in parallel with an increase in CSF neopterin [15]. In this sense, CSF neopterin appears to better reflect the pathogenic potential of CNS infection than CSF HIV RNA levels.

The findings of this study support the value of CSF NFL as a marker of CNS damage in HIV infection. In patients without CNS OIs, elevated CSF NFL concentrations are associated with ADC, follow the grade of severity, and decrease after initiation of effective antiretroviral treatment. While previously reported CSF markers of ADC relate to immune activation or HIV viral load, NFL is a marker that directly indicates brain injury. As such, CSF NFL measurement warrants further study to assess its utility as an objective marker for ongoing or incipient ADC and active HIV-related brain injury in clinical practice and in clinical trial settings.

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