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Cerebrospinal fluid extracellular vesicles and neurofilament light protein as biomarkers of central nervous system injury in HIV-infected patients on antiretroviral therapy

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Objective: The relationship of cerebrospinal fluid (CSF) extracellular vesicles to neurocognitive impairment (NCI) in HIV-infected individuals is unclear. Here, we characterize CSF extracellular vesicles and their association with central nervous system (CNS) injury related biomarkers [neurofilament light (NFL), S100B, neopterin] and NCI in HIV-positive individuals on combination antiretroviral therapy (cART).

Design: A cross-sectional and longitudinal study of CSF samples from HIV-positive individuals on cART.

Methods: NFL, S100B and neopterin were measured by ELISA in 190 CSF samples from 112 individuals (67 HIV-positive and 45 HIV-negative). CSF extracellular vesicles were isolated and characterized by electron microscopy, nanoparticle tracking analysis, immunoblotting for exosome markers (CD9, CD63, CD81, FLOT-1) and ELISA for HLA-DR.

Results: HIV-positive individuals had median age 52 years, 67% with suppressed plasma viral load (< 50 copies/ml), median $CD4^+$ nadir 66 cells/μl and $CD4^+$ cell count 313 cells/μl. CSF NFL, S100B and neopterin levels were higher in HIV-positive vs. HIV-negative individuals, and nonsuppressed vs. suppressed HIV-positive individuals. Although CSF NFL and S100B levels were higher in NCI vs. unimpaired HIV-positive individuals ($P < 0.05$), only NFL was associated with NCI in adjusted models ($P < 0.05$). CSF extracellular vesicles were increased in HIV-positive vs. HIV-negative individuals, and NCI vs. unimpaired HIV-positive individuals ($P < 0.05$), and correlated positively with NFL ($P < 0.001$). HLA-DR was enriched in CSF extracellular vesicles from HIV-positive individuals with NCI ($P < 0.05$), suggesting that myeloid cells are a potential source of CSF extracellular vesicles during HIV infection.

Conclusion: Increased CSF extracellular vesicles correlate with neuronal injury biomarker NFL in cART-treated HIV-positive individuals with neurocognitive impairment, suggesting potential applications as novel biomarkers of CNS injury.

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Keywords: biomarkers, cerebrospinal fluid, exosomes, extracellular vesicles, HIV, neurocognitive impairment

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Introduction

HIV-associated neurocognitive disorders (HAND), consisting of asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND) and HIV-associated dementia (HAD), affect 20–50% of HIV-positive individuals despite viral suppression on combination antiretroviral therapy (cART) [1–3]. Although the incidence of HAD has declined significantly, the prevalence of milder forms of HAND has increased [1,4]. The progression and severity of HAND are highly variable, and little is known about mechanisms underlying milder forms of HAND in virally suppressed individuals. Prognostic and/or diagnostic markers are needed to identify the onset of neurological disorders and predict progression in HIV-positive individuals on cART.

HIV-infected cells and immune activation can be detected in the central nervous system (CNS) even after long-term viral suppression on cART [5,6]. Biomarkers of CNS immune activation, such as elevated cerebrospinal fluid (CSF) neopterin, β2-microglobulin and soluble CD14, are frequently detected in HIV-positive individuals with suppressed plasma and CSF viral load [7–11]. Increased CSF inflammatory cytokines and chemokines have been associated with neurocognitive impairment in HIV-positive individuals on cART [12–14]. Neuronal injury and glial disease in cART-treated HIV-positive individuals can result from ongoing viral replication, immune activation, chronic inflammation, oxidative stress, comorbidities and other factors [1,3,15,16]. Neuronal injury in HIV-positive individuals can be detected by elevated CSF neurofilament light chain (NFL), a structural component of axons released by damaged neurons [17–23]. Glial responses in HIV-positive individuals can be detected by elevation of CSF S100B, a marker released by astrocytes and oligodendrocytes [24–27]. Thus, identification of biomarkers related to neuroinflammation, glial responses and CNS injury may be useful to elucidate mechanisms underlying neurocognitive impairment, and monitor disease progression and therapeutic responses in cART-treated HIV-positive individuals.

Extracellular vesicles (EVs) are a focus of growing interest as potential biomarkers for various diseases, including HIV infection and neuroinflammatory and neurodegenerative diseases [28–33]. EVs are generated from most cell types and released into body fluids, including blood and CSF. Depending on their subcellular origin and particle size, EVs are classified as exosomes (50–150 nm, originate from multivesicular bodies) and microvesicles (200 nm–1 μm, originate from plasma membrane). EVs carry proteins, lipids and nucleic acids from parental cells and can deliver their cargo to neighbouring or distant cells to mediate physiological functions, including cell proliferation, immune responses, inflammation and cell death [34,35]. EVs can also influence pathological processes during viral infection by transferring viral proteins [31,36,37] and delivering pro-inflammatory or anti-inflammatory factors to target cells [38,39].

EVs have been identified as putative biomarkers for several neurological disorders, including Alzheimer's disease, Parkinson's disease and multiple sclerosis [29,30,40,41]. Recent studies have begun to characterize plasma EVs abundance and cargo in HIV-infected individuals [33,42–44]. However, little is known about sources and functional roles of CSF EVs in the context of HIV-related neurocognitive disorders. Here, we examined the relationship of HIV infection and associated CNS injury to CSF EV concentration and cargo. We also investigated the potential role of CSF EVs as CNS injury biomarkers in HIV-positive individuals on cART.

Materials and methods

Study participants

One hundred and ninety CSF samples from 112 individuals ($n = 67$ HIV-positive on cART and $n = 45$ HIV-negative) were collected during 1999–2013. HIV-positive samples were collected from four sites in the National NeuroAIDS Tissue Consortium (NNTC) (Manhattan HIV Brain Bank, National Neurological AIDS Bank, California NeuroAIDS Tissue Network and Texas NeuroAIDS Research Center). Among 67 HIV-positive individuals, 34 had longitudinal CSF samples available for two to four visits within 42 months. All individuals were enrolled with written informed consent and institutional review board (IRB) approval at each study site. We included HIV-positive individuals at least age 35 on cART with CSF viral load less than 100 HIV RNA copies/ml or undetectable by clinical diagnostic assay (95% with lower limit of quantification of 50 copies/ml) at baseline. We also included two individuals with high CSF viral load (1824 and 75 000 HIV RNA copies/ml). CSF samples from HIV-negative control individuals without a diagnosed neurological disease (from Bioreclamation LLC, Westbury, New York, USA) were frequency-matched for age, race and sex.

Neurocognitive testing and classification of neurocognitive impairment

HIV-positive individuals were administered a comprehensive neuropsychological test battery designed to assess seven neurocognitive domains. Demographically corrected global T scores were generated from individual test *T* scores as described [45]. *T* scores correlate negatively with severity of neurocognitive impairment, with values below 40 (corresponding to 1 standard deviation of 10 below a normalized mean of 50) signifying impairment. HAND clinical diagnoses were determined using established criteria [46] based on neurocognitive testing and neurological evaluation. Neuropsychological

impairment due to other causes (NPI-O) was diagnosed when factors such as injury, trauma or illicit drug use [8,47] could contribute to neurocognitive impairment in addition to HIV infection. HIV-positive individuals were classified as neurocognitively impaired (NCI) if they had a clinical HAND diagnosis (ANI, MND, HAD) or NPI-O and/or global *T* score below 40.

Isolation and characterization of cerebrospinal fluid extracellular vesicles

To isolate CSF EVs, CSF samples (250 μl) were centrifuged at 3000*g* to remove cellular debris and supernatants were incubated overnight at 4°C with ExoQuick reagent (System Biosciences, Inc., Mountain View, California, USA) according to manufacturer's instructions. The suspensions were centrifuged at 1500*g* for 30 min and CSF EV pellets were suspended in 20 μl PBS for transmission electron microscopy (TEM) or RIPA buffer (Triton X-100 1%, NaCl 150 mmol/l, sodium deoxycholate 0.5%, Tris-HCL 50 mmol/l, SDS 0.1%, pH 7.4) for ELISA and western blotting. The supernatants (EV-depleted CSF) were stored at −80°C. EV concentrations and sizes were measured by nanoparticle tracking analysis (NTA) (Particle Metrix, Germany) either directly from CSF samples (diluted 1 : 500) or from isolated EV fractions (diluted 1 : 5000). TEM was performed using a Tecnai G^2 Spirit BioTWIN instrument (FEI company, Hillsboro, Oregon, USA) equipped with an AMT 2k CCD camera (Harvard University TEM core).

Western blotting

EV pellets were lysed in 20 μl RIPA buffer with protease inhibitors. Proteins in EVs and EV-depleted CSF were separated on SDS-polyacrylamide gels and transferred onto PVDF membranes. Blots were probed with primary antibodies against exosome markers, CD9, CD81, CD63, Flotillin-1 (FLOT-1) and HSP70 (EXOAB-KIT-1; System Bioscience, Palo Alto, California, USA), overnight at 4°C. Secondary antibodies were incubated 1.5 h at room temperature and signals were developed using enhanced chemiluminescence (ECL). Images were captured using Biorad ChemiDoc Imaging System (Bio-Rad Laboratories, Inc. Life Science Research, Hercules, California, USA), ImageJ software was used to quantitate western blot band intensities.

Quantification of soluble cerebrospinal fluid biomarkers

ELISA to quantify CSF NFL (Uman Diagnostics, Sweden), S100B (BioVendor, Asheville, North Carolina, USA) and neopterin (Geneway Biotech, San Diego, California, USA) was performed blinded to clinical information. NFL, S100B and HLA-DR (MyBiosource, San Diego, California, USA) were quantified in CSF EV lysates by ELISA. EV pellets were suspended in 20 μl RIPA buffer and diluted 1 : 3–1 : 4 for ELISAs. Concentrations were calculated using four parameter logistic standard curves. Undetected values were imputed using lowest detected values (LDVs) for each marker.

Statistical analysis

Differences between groups were compared by Mann–Whitney *U* test. Associations between baseline neurocognitive status and CSF biomarker levels were analysed using multivariable linear regression models adjusting for age and $\log_{10}$ plasma viral load as continuous variables and race as a binary variable (white, nonwhite). The association between baseline neurocognitive status and time-varying $\log_{10}$ CSF NFL was examined using mixed-effects models with baseline age, race, time-varying $\log_{10}$ plasma viral load and months in study as covariates. Mixed-effects models included random intercept and slope. Interaction terms for neurocognitive status with months in study were nonsignificant, and therefore not used in final models. Statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, North Carolina, USA). Graphical representation of regression lines for CSF biomarker concentrations and age were plotted in R version 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria). Correlation analyses were performed using GraphPad Prism version 7.0 (Graphpad Software, Inc., La Jolla, California, USA).

Results

Study cohort

Demographic and clinical characteristics of the study cohort are summarized in Table 1. The cohort consisted of 67 HIV-positive individuals on cART (70% on protease inhibitors) and 45 HIV-negative individuals matched for demographics. Plasma and CSF viral load were suppressed (<50 copies/ml) in 67 and 90% of HIV-positive individuals, respectively, while median plasma viral load in HIV-positive individuals with measurable HIV RNA copies was 76 copies/ml [interquartile range (IQR), 48–620]. HIV-positive individuals were predominantly men (89.5%), 50% white, 22% black, with median age 52 years (IQR, 47–60). At baseline, median duration of HIV infection was 14 years (IQR, 8–20 years), 88% had nadir $CD4^+$ cell count less than 200 cells/μl, median nadir $CD4^+$ cell count was 66 cells/μl and $CD4^+$ cell count 313 cells/μl. Sixty-two percent of HIV-positive individuals were classified as NCI, of which 17, 15, 12 and 56% had ANI, MND, HAD and NPI-O, respectively. Compared with HIV-positive individuals, HIV-negative controls were of similar age, race and sex (median age 53 years, 58% white, 73% male).

Cerebrospinal fluid biomarkers associated with HIV infection and neurocognitive impairment

HIV-related factors, including duration of infection, $CD4^+$ cell count, detectable viremia and ongoing CNS viral replication have been associated with neurocognitive decline in cART-treated HIV-positive individuals [48–

Table 1. Baseline characteristics of the study cohort.

	HIV-negative ($n=45$)	HIV-positive ($n=67$)
Age (years)	53 (48–63)	52 (47–60)
Sex (male, n, %)	33 (73.3)	60 (89.5)
Race (*n*, %)		
Black	7 (15.5)	15 (22.4)
White	26 (57.8)	34 (50.7)
Other	12 (26.7)	18 (26.9)
Duration of HIV infection (years)		14 (8–20)
Plasma HIV RNA (copies/ml)	–	
≤ 50 copies/ml (*n*, %)	–	45 (67.2)
$CD4^+$ cell count (cells/μl)	–	313 (174–538)
< 350 cells/μl (*n*, %)	–	43 (64.2)
Nadir $CD4^+$ cell count (cells/μl)	–	66 (17–105)
< 200 cells/μl (*n*, %)	–	59 (88.1)
CSF HIV RNA (copies/ml)	–	
≤ 50 copies/ml (*n*, %)	–	58 (90.6)
Neurocognitive status (n, %)[a]		
NCI	–	41 (62.2)
No NCI	–	25 (37.8)
cART use (*n*, %)		67 (100)
Protease inhibitors	–	47 (70.1)
Efavirenz	–	15 (23.1)
Integrase inhibitors		5 (7.5)

Median (interquartile range) are shown unless otherwise indicated. cART, combination antiretroviral therapy; NCI, neurocognitive impairment.
[a]Neurocognitive data were not available for one individual.

50]. To analyse cross-sectional relationships between HIV infection and related factors and CSF biomarkers, we compared baseline CSF NFL, S100B and neopterin levels in groups by HIV status, plasma viral load, $CD4^+$ cell count and NCI (Fig. 1a). Compared with HIV-negative controls, median CSF NFL, S100B and neopterin levels were higher in HIV-positive individuals ($P=0.01$, $P=0.036$ and $P=0.03$, respectively), particularly with nonsuppressed plasma viral load (>50 copies/ml) ($P=0.007$, $P=0.027$ and $P=0.005$, respectively). Median CSF NFL ($P=0.04$) and neopterin ($P=0.0008$) but not S100B were elevated in HIV-positive individuals with $CD4^+$ cell count less than 350 cells/μl vs. HIV-negative controls. When HIV-positive individuals were stratified by $CD4^+$ cell counts, CSF neopterin ($P=0.02$) but not NFL or S100B was elevated in HIV-positive individuals with $CD4^+$ cell count less than 350 vs. at least 350 cells/μl.

We next examined the association between baseline neurocognitive status and CSF biomarkers. CSF NFL and S100B were higher in NCI ($P=0.009$ and $P=0.023$, respectively) or HAND ($P=0.007$ and $P=0.002$, respectively) but not unimpaired HIV-positive individuals vs. HIV-negative controls (Fig. 1a and Supplemental Digital Content 1, http://links.lww.com/QAD/B419). CSF NFL was also higher in NCI ($P=0.026$) or HAND ($P=0.03$) vs. unimpaired HIV-positive individuals, while S100B and neopterin showed no significant difference in unadjusted analyses. To determine whether NFL was independently associated with baseline NCI, we used multivariable linear regression models adjusted for age, race and plasma viral load (Fig. 1b). In these models, higher CSF NFL was an independent biomarker of baseline NCI status ($P=0.049$); increasing age ($P=0.0008$) and higher plasma viral load ($P=0.001$) were additional factors associated with baseline NCI status (Fig. 1b and Supplemental Digital Content 2, http://links.lww.com/QAD/B419). CSF S100B or neopterin levels were not independent biomarkers of baseline NCI status in similar adjusted models. Figure 1b shows estimated CSF biomarker levels across ages 35–75 years and stratified by NCI status; regression lines for CSF NFL show increased levels with advancing age in addition to group differences by NCI status. These findings suggest that CSF NFL is a more sensitive biomarker of CNS injury in cART-treated HIV-positive individuals than CSF S100B or neopterin.

Longitudinal relationship between neurocognitive impairment and cerebrospinal fluid neurofilament light

To examine the association between CSF NFL and NCI status over time in cART-treated HIV-positive individuals, we performed longitudinal analyses. Given that CSF NFL correlated with S100B ($P=0.018$) and CSF protein ($P=0.004$) in HIV-positive individuals at baseline (Supplemental Digital Content 3, http://links.lww.com/QAD/B419), we also included S100B in these analyses. Among 34 HIV-positive individuals with longitudinal CSF samples, three to four CSF samples were available for 24 individuals and the remaining 10 had two CSF samples; duration of follow-up ranged from 8 to 42 months. For individuals with longitudinal CSF samples, trajectories of CSF NFL levels [increasing ($n=5$), declining ($n=5$) and stable ($n=14$) over time] were compared with corresponding S100B levels and global *T* scores, which reflect HAND severity in similar cohorts [51]. Within individual participants, CSF NFL tended to increase over time in individuals with worsening neurocognitive status, and decrease over time in individuals with improving neurocognitive status (Fig. 1c and Supplemental Digital Content 4, http://links.lww.com/QAD/B419). CSF S100B levels followed similar patterns as NFL in most individuals. In mixed-effects models, time-varying NFL remained independently associated with baseline NCI status (beta = 0.205; $P=0.027$) after adjusting for baseline age ($P<0.001$), time-varying plasma viral load ($P<0.001$), white race ($P=0.265$) and months in study ($P=0.641$). These findings suggest inter-relationships between markers of axonal injury, glial responses and NCI status in HIV-positive individuals on cART.

Characterization of cerebrospinal fluid extracellular vesicles

CSF EVs were isolated and examined for morphological and molecular characteristics. TEM revealed both round and cup-shaped EVs with diameter between 50 and

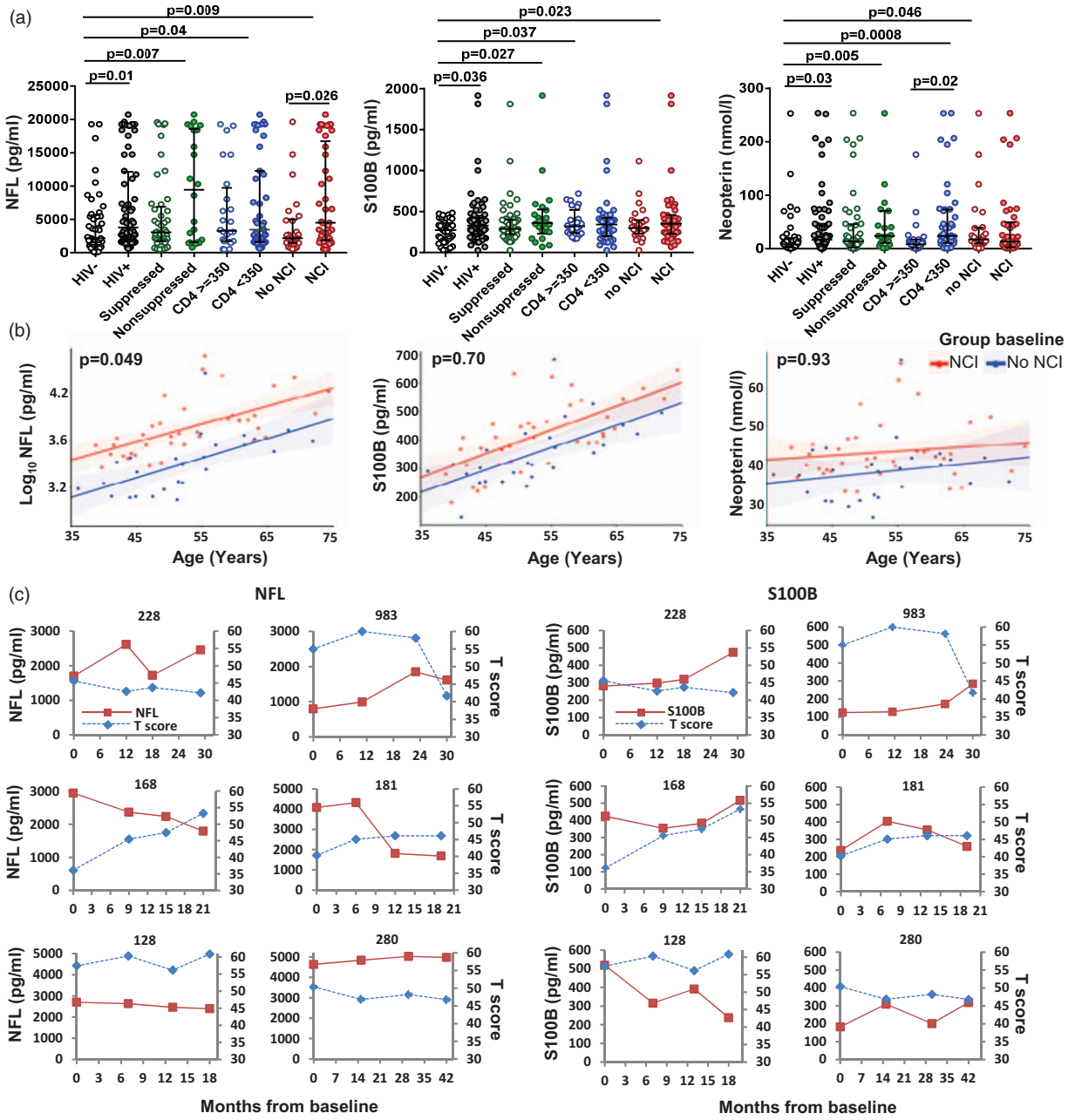

Fig. 1. HIV infection and related factors are associated with biomarkers of central nervous system injury. (a) Cross-sectional association of CSF NFL, S100B and neopterin concentrations with HIV ($n = 67$ HIV-positive vs. n = 45 HIV-negative), plasma VL ($n = 45$ suppressed vs. $n = 22$ nonsuppressed), $CD4^+$ cell count ($n = 24$ with ≥ 350 cells/μl vs. $n = 43$ with <350 cells/μl) and NCI status ($n = 25$ no NCI vs. $n = 41$ NCI) at baseline. Medians and IQRs are indicated as horizontal and vertical lines, respectively. Statistical significance was calculated using Mann–Whitney *U* test; significant differences ($P < 0.05$) are indicated. (b) NCI status is associated with increased CSF NFL. Multivariate linear regression models of estimated CSF $\log_{10}$ NFL, S100B and neopterin levels adjusted for baseline age, race and plasma viral load; individuals were categorized by NCI status at baseline. An HIV-positive individual with NCI (red line) is estimated to have CSF NFL concentrations equivalent to an unimpaired (blue line) individual of similar race and plasma VL who is 11.7 years older. Predicted regression lines and 95% confidence intervals for CSF biomarker levels by NCI status; significant differences by NCI status ($P < 0.05$) from fully adjusted models. (c) Longitudinal changes in CSF NFL and S100B (red lines) in relation to changes in neurocognitive test scores (global *T* scores) (blue dotted lines) of HIV-positive individuals. Red lines in upper, middle and lower panels show trajectories (increasing, decreasing and stable NFL levels over time, respectively) of CSF NFL (left) and S100B (right) from baseline over time.

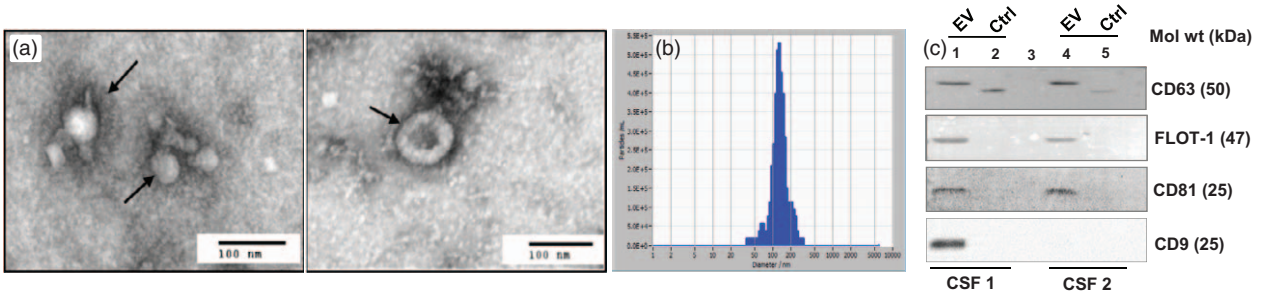

Fig. 2. Characterization of cerebrospinal fluid extracellular vesicles. (a) Transmission electron microscope images of whole-mounted CSF EVs isolated from a representative HIV-negative individual. Arrows indicate round (left) and cup-shaped (right) EVs. Scale bars = 100 nm. (b) Size distribution histogram of CSF EVs by nanoparticle tracking analysis of a representative HIV-negative individual. (c) Immunoblotting of CSF EVs for CD9, CD81, FLOT-1 and CD63 from two representative HIV-negative individual. EV-depleted CSF (from corresponding 250 μl CSF sample) was used as a negative control. Lanes 1 and 4 are CSF EV fractions, lanes 2 and 5 are corresponding EV-depleted CSF (Ctrl), lane 3 is blank.

150 nm, corresponding to the size of exosomes (Fig. 2a). Most particles were 50–150 nm in diameter (58.3%), with a peak at 100–150 nm (Fig. 2b) and concentrations ranging from 10^9 to 10^{12} per ml in CSF. Some smaller particles (<50 nm) as well as larger particles (>150 nm) were also detected; hence, particles were termed EVs rather than exosomes. Isolated EVs were further characterized by immunoblotting for exosome markers CD9, CD81, CD63 and FLOT-1 (Fig. 2c). CD9, CD81, CD63 and FLOT-1 were enriched in EV fractions compared with EV-depleted CSF samples. Low levels of CD63 were also detected in EV-depleted CSF. These results suggest that CSF EV fractions are enriched in exosomes.

Extracellular vesicle concentrations and size in relation to HIV status and neurocognitive impairment

EV concentrations and size were compared between groups by HIV and NCI status. The majority of CSF EVs were in the 50–150 nm size range of exosomes in both HIV-positive and HIV-negative individuals (58.4 and 57.8%, respectively) (Fig. 3a). CSF EVs were more abundant in HIV-positive than in HIV-negative individuals, regardless of NCI status ($P < 0.0001$ and $P = 0.0008$ for EVs and CSF, respectively) (Fig. 3b and c), and were more abundant in NCI ($P = 0.04$ and $P = 0.011$ for EVs and CSF, respectively) or HAND ($P < 0.0001$ and $P = 0.02$ for EVs and CSF, respectively) than in unimpaired HIV-positive individuals (Fig. 3b, c and Supplemental Digital Content 5, http://links.lww.com/QAD/B419). Median CSF EV size was smaller in HIV-positive vs. HIV-negative individuals ($P = 0.011$) (Fig. 3b). There was no significant correlation between CSF EV concentrations and plasma HIV RNA or $CD4^+$ cell counts ($P > 0.1$) (data not shown). To assess whether exosomes contribute to increased abundance of CSF EVs in HIV-positive individuals, we evaluated two exosome markers by immunoblotting (Fig. 3d). CD9 and Hsp70 were detected at higher levels in CSF EV fractions of HIV-positive vs. HIV-negative individuals ($P < 0.0001$) regardless of neurocognitive status (Fig. 3e). To account for increased EV loading in HIV-positive samples, CD9 and HSP70 band intensities were normalized to corresponding EV numbers; after normalization, CD9 levels were lower in CSF EV immunoblots from HIV-positive ($P = 0.008$) and NCI ($P = 0.02$) individuals compared with HIV-negative controls when normalized for EV numbers, and Hsp70 showed a similar trend (Supplemental Digital Content 6, http://links.lww.com/QAD/B419). Thus, higher CSF exosome marker levels reflect increased CSF EV abundance in HIV-positive and NCI individuals.

Myeloid cells are a potential cellular source of cerebrospinal extracellular vesicles

Cellular sources of CSF EVs in cART-treated HIV-positive individuals are unknown. To address this question, we measured NFL (neuronal marker) and S100B (glial cell marker) by ELISA in CSF EVs isolated from four HIV-positive individuals in a pilot study. NFL and S100B were not detected, or detected at only very low levels (data not shown), suggesting that these markers are not significantly enriched in CSF EVs. Detection of HLA-DR, a myeloid cell marker, in plasma and CSF exosomes has been reported [52–54]. Therefore, we next examined whether CSF EVs contain HLA-DR by ELISA ($n = 48$ HIV-positive and $n = 16$ HIV-negative individuals). HLA-DR was detected in CSF EVs from the majority of HIV-positive and HIV-negative− individuals. HLA-DR levels were higher in CSF EVs from HIV-positive ($P = 0.024$) and NCI ($P = 0.035$) but not unimpaired than in HIV-negative individuals (Fig. 3f), suggesting myeloid cells are a potential source of CSF EVs during HIV infection.

Cerebrospinal fluid extracellular vesicle abundance correlates with cerebrospinal fluid biomarkers of central nervous system injury

Next, we performed correlation analyses of EV concentrations vs. CNS injury related biomarkers in HIV-positive individuals. CSF NFL levels correlated

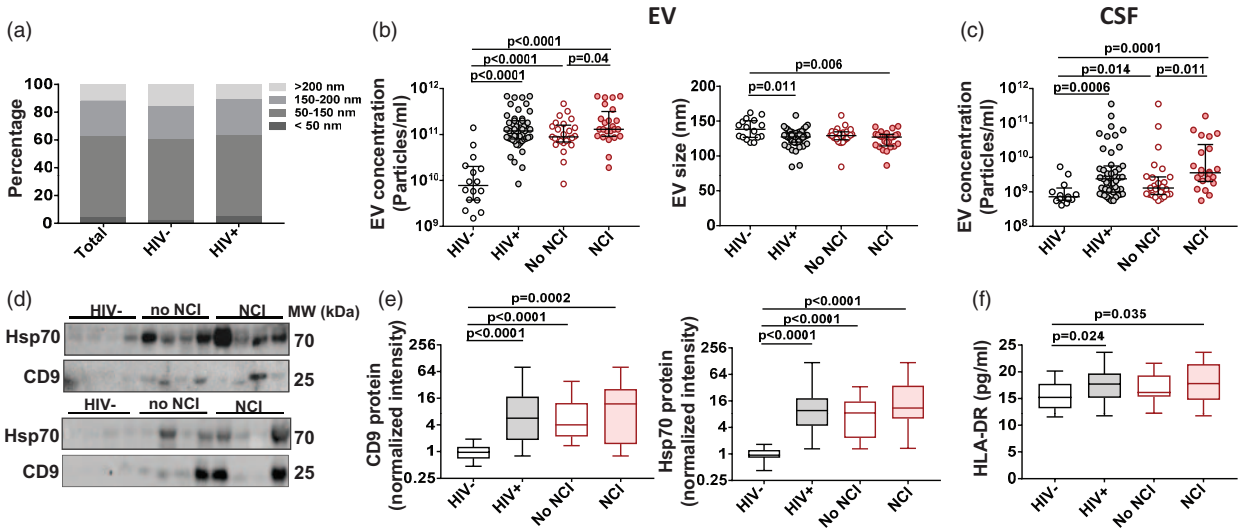

Fig. 3. HIV infection and neurocognitive impairment are associated with increased abundance of cerebrospinal fluid extracellular vesicles. (a) Size distribution of CSF vesicles in EV fractions from total ($n=65$), HIV-negative ($n=16$) and HIV-positive ($n=49$) individuals. EV concentration and size measured in EV fractions (b) or directly in CSF (c) are associated with HIV infection and NCI status. (d) Detection of exosome markers CD9 and Hsp70 in CSF EV fractions by immunoblotting. Eight representative individuals are shown in each group. (e) Densitometric quantification of CD9 and Hsp70 bands from 16 HIV-negative and 32 HIV-positive individuals with NCI ($n=16$) or no NCI ($n=16$). Bands in each lane were normalized to mean band intensities from eight HIV-negative controls. (f) HLA-DR levels in isolated EVs from equivalent volumes of CSF measured by ELISA in HIV-negative, HIV-positive, no NCI and NCI groups. Medians and IQRs are indicated as horizontal and vertical lines, respectively, in b, c, e and f. Significant differences between groups are indicated (Mann–Whitney U test; $P<0.05$).

positively with plasma viral load and age, and inversely with global T scores (Spearman $r=0.462$; $P=0.009$, $r=0.318$; $P=0.009$, and $r=-0.282$; $P=0.03$, respectively) (Fig. 4a), while S100B and neopterin levels showed no significant correlations in these analyses. CSF EV concentrations correlated positively with NFL levels (Spearman $r=0.753$; $P<0.0001$), but showed no significant correlations with S100B and neopterin ($P=0.377$ and $P=.097$, respectively) (Fig. 4b). These results show that CSF NFL correlates not only with predictors of neurocognitive impairment but also with increasing CSF EV abundance in cART-treated HIV-positive individuals, suggesting that NFL and CSF EVs are promising biomarkers of CNS injury in cART-treated HIV-positive individuals.

Discussion

In this study, we demonstrate that HIV infection and neurocognitive impairment in HIV-positive individuals on cART with virological suppression and advanced disease (67% with plasma viral load < 50 copies/ml, 88% with nadir $CD4^+$ cell count <200 cells/μl) are associated with increased abundance of CSF EVs enriched with exosome markers. Increased CSF EV abundance in cART-treated HIV-positive individuals correlates positively with CSF NFL, suggesting a relationship between CSF EVs and an established biomarker of neuronal injury. HLA-DR was increased in CSF EVs of HIV-positive individuals with NCI compared with controls, indicating myeloid cells are a potential source of these vesicles. These results suggest that evaluating CSF EVs may provide new insights into mechanisms impacting neurocognitive impairment in cART-treated HIV-positive individuals.

NFL is a sensitive biomarker for axonal injury in several neurological disorders including HAND [20–23,55,56]. Increased CSF NFL correlates with HAND severity [19,20,22], and is reduced by cART [18,57]. Mild elevation of CSF NFL has also been detected in virally suppressed individuals [18,19,57]. Consistent with prior studies [17,19–23], we detected higher CSF NFL in NCI individuals and positive correlations between CSF NFL and plasma viral load or advancing age. The correlation between NFL and duration of HIV infection was not statistically significant ($P>0.1$). S100B, a marker of glial responses, was elevated in CSF of HIV-positive individuals, particularly those with plasma viral load more than 50 copies/ml. In contrast to a study of cART-nakive individuals [24], we did not find a significant association between low $CD4^+$ cell count and S100B. Our finding that NCI status is associated with increased CSF S100B in HIV-positive individuals is consistent with a study by Pemberton and Brew [25], which reported high CSF S100B in HIV-positive individuals with AIDS dementia complex. Studies on the natural history of HAND suggest that most virally suppressed individuals remain stable, some improve and a small number deteriorate over time

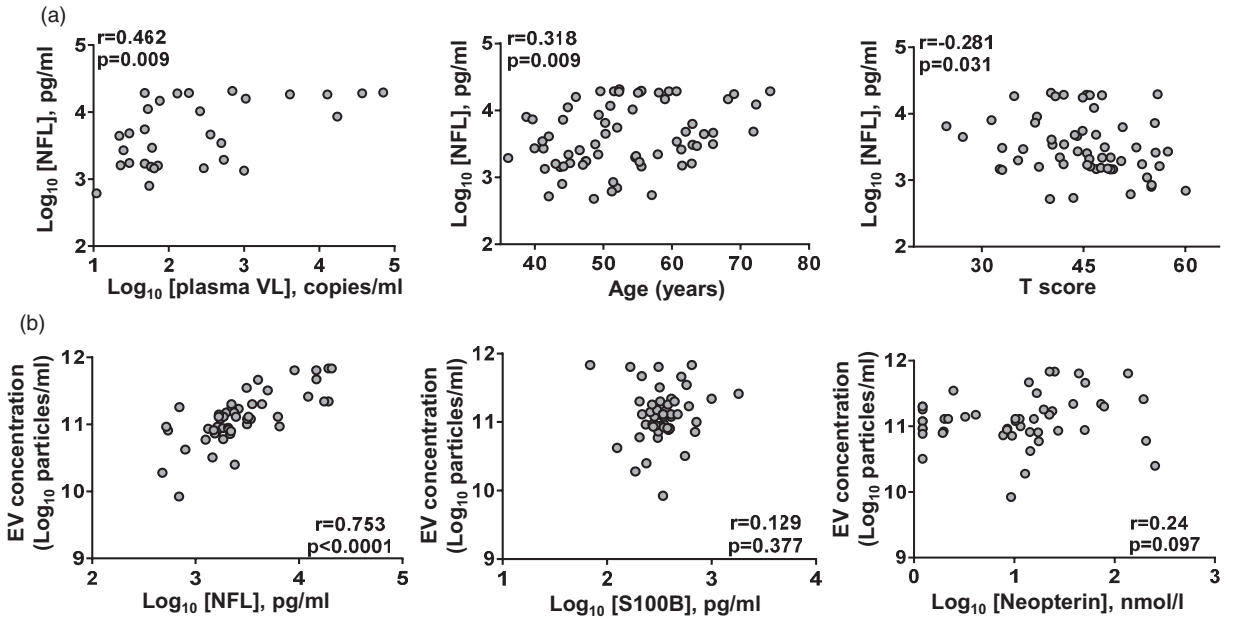

Fig. 4. Relationship of cerebrospinal fluid extracellular vesicle concentrations to biomarker levels in HIV-positive individuals on combination antiretroviral therapy. (a) CSF NFL levels correlate positively with plasma VL ($n = 31$ with measurable HIV RNA copies/ml) (left) and age ($n = 67$) (middle), and inversely with global *T* scores ($n = 60$ with available *T* scores) (right). (b) CSF EV concentrations correlate positively with NFL but show no significant correlation with S100B or neopterin levels ($n = 49$). Relationships between continuous variables were analysed by Spearman correlation (significant correlations $P < 0.05$).

[58], as was the case in our cohort. CSF NFL, and to some extent S100B, remained elevated or increased over time in HIV-positive individuals with baseline NCI, suggesting this population is vulnerable to ongoing neuronal injury and glial activation [7,22]. We found no significant relationship between CSF neopterin and NCI in our cohort. However, CSF neopterin was higher in subgroups of HIV-positive individuals with nonsuppressed viral load and low $CD4^+$ cell count, indicating CNS immune activation; it may be a relevant biomarker in specific contexts [10]. These findings suggest that CSF NFL and to some extent S100B are biomarkers of CNS injury and neurocognitive impairment in cART-treated HIV-positive individuals and highlight importance of maintaining viral suppression to reduce HIV-related brain injury.

CSF EVs and exosome markers were increased in HIV-positive compared with HIV-negative individuals in our study, consistent with previous studies that detected increased abundance of plasma exosomes in cART-naive and cART-treated HIV-positive individuals [33,43,44]. CSF EV concentrations were further increased in NCI and HAND compared with unimpaired HIV-positive individuals, consistent with previous studies demonstrating associations between other neurological disorders and CSF EVs [29,59,60]. Increased CSF EV concentrations in HIV-positive individuals correlated with increased NFL, likely reflecting severity or progression of CNS injury. A relationship between EVs and CNS injury was also reported by Sun *et al.* [42], who identified biomarkers (NFL, amyloid β, high mobility group box 1) related to cognitive impairment in neuron-derived plasma exosomes from NCI HIV-positive individuals. CSF EVs were enriched with HLA-DR in HIV-positive individuals, suggesting possible association with innate immune responses [34,61]. Given that myeloid cells release exosomes/microvesicles [36,60,62] and express higher HLA-DR in the CNS compared with astrocytes and endothelial cells [63,64], myeloid lineage cells represent a potential source of CSF EVs. Other possible sources of CSF EVs include choroid plexus epithelium [65] and activated astrocytes [54,66]. A mouse model study reported that neuroinflammation enhances extracellular vesicle abundance in CSF, which in turn activates astrocytes [62] and worsens neurological disorders. Thus, CSF EVs may play a role in shuttling neuroinflammatory signals between cells within the CNS of HIV-positive individuals.

We acknowledge some limitations of the study. One limitation relates to purity of isolated CSF EVs. The optimal method for isolating EVs from small volumes of CSF is to precipitate vesicles using an EV precipitating agent to prevent particle loss. However, this method also precipitates other nonmembranous particles and protein aggregates [67]. NTA does not distinguish these aggregates from membrane-bound EVs, which may result in some false-positive data [68]. CSF processing and storage conditions could also impact EV recovery and

size. Our study was also limited by the relatively small sample size and volume for CSF EV isolation, which limits the ability to detect markers present at low levels. Lastly, given limited numbers of HIV-positive individuals with CSF EV analyses, we were not able to determine longitudinal associations between NCI status and CSF EVs. Future studies of larger cohorts may overcome these shortcomings and determine whether progression of NCI in virally suppressed HIV-positive individuals has a significant association with CSF EVs.

In summary, this study demonstrates increased abundance of CSF EVs enriched with HLA-DR in cART-treated HIV-positive individuals with and without neurocognitive impairment. Increased CSF EV concentrations correlate with an established neuronal injury biomarker related to onset and progression of HAND. Understanding the source of CSF EVs and their relationship to neurocognitive impairment in cART-treated HIV-positive individuals may contribute to novel biomarker discovery.

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Conflicts of interest

There are no conflicts of interest.

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