Profile of neuronal exosomes in HIV cognitive impairment exposes sex differences

Bing Sun[a], Nicole Fernandes[a] and Lynn Pulliam[a,b]

See related paper on page 1795

Objective: To use plasma neuron-derived exosomes (NDEs) to detect proteins that diagnose HIV-associated neurocognitive disorders (HAND). To compare NDE cargo from HAND with Alzheimer's disease.

Design: Eighty plasma samples were assayed including men ($n = 29$) and women ($n = 51$) with and without HAND.

Methods: Plasma NDEs were isolated by immunoadsorption with neuron specific L1 cell adhesion molecule antibody. NDE proteins were quantified by ELISA and proximity extension assays for 184 targets.

Results: Neuronal enrichment of NDE was confirmed with elevated synaptophysin and normalized to the exosomal marker, apoptosis-linked gene-2-interacting protein X (ALIX). NDE from men and women had significant divergent results. High mobility group box 1 and neurofilament light were significantly increased in NDE from cognitively impaired men and were unchanged in women. NDE from HIV+ men had decreased p-T181-tau, a marker increased in Alzheimer's disease, compared with no difference in women. NDE amyloid beta was not increased in cognitive impairment. Proximity extension assays analysis showed 25 proteins were differentially expressed in HIV infection alone. Seven proteins identified asymptomatic and mild cognitive impairment in HIV+ women. NDE from women had significantly decreased cathepsin S, total tau, neuronal cell adhesion molecule and contactin 5 in mild impairment. Twelve proteins were increased in NDE from cognitively impaired men, including carboxypeptidase M, cadherin 3, colony stimulating factor 2 receptor alpha subunit and mesencephalic astrocyte-derived neurotropic factor.

Conclusion: NDE proteins differ in HIV infection alone and cognitive impairment between men and women suggesting mechanistic sex differences associated with HAND. Several NDE targets are different from that reported for Alzheimer's disease.

AIDS 2019, **33**:1683–1692

Keywords: Alzheimer's disease, exosomes, HIV, HIV-associated neurocognitive disorders, neuron, women

Introduction

Cognitive impairment in HIV-infection continues to be present in spite of effective antiretroviral therapy (ART). Diagnosing and monitoring HIV-associated neurocognitive disorder (HAND) requires specialized neuropsychological testing. Finding an inexpensive, objective, noninvasive biomarker for HAND is useful

[a]Department of Laboratory Medicine, San Francisco Veterans Affairs Medical Center, and [b]Departments of Laboratory Medicine, and Medicine, University of California, San Francisco, San Francisco, California, USA.

Correspondence to Lynn Pulliam, PhD, Department of Laboratory Medicine, San Francisco Veterans Affairs Medical Center, 4150 Clement St., San Francisco, California, USA.

Tel: +1 415 221 4810 x 26490; e-mail: lynn.pulliam@ucsf.edu

Received: 21 March 2019; revised: 16 April 2019; accepted: 29 April 2019.

DOI:10.1097/QAD.0000000000002272

not only for diagnosis but monitoring potential recovery of neuronal health after treatment or cure. Although cerebrospinal fluid (CSF) is thought to be the closest to the neuropathology, it requires an invasive procedure and, like plasma, has a complex protein profile comprised of different cell types. To date, while no CSF marker correlates with HAND in chronically infected individuals on ART, neurofilament light (NF-L) has emerged as an indicator of a neurodegenerative process [1,2].

Neuroinflammation in the chronic phase of HIV infection is most likely shifted to the brain parenchyma. Exosomes are small extracellular vesicles shed from all cells under normal and pathologic conditions [3,4]. By virtue of the size and our characterization, we have chosen to name the extracellular vesicles we study exosomes. Exosomes selectively reflect the parent cell's state at the time of secretion and can selectively package host cell proteins, microRNA and nucleic acids [5]. Exosomes are sufficiently robust to diffuse across the blood–brain barrier (BBB) and into the periphery where they can be selectively captured using cell surface specific antibodies. Neuron-derived exosomes (NDEs) can be isolated from plasma using the neuron-specific cell surface antibody, L1 cell adhesion molecule (L1CAM) [6,7]. NDEs are an emerging field to study neurological disorders from plasma including Alzheimer's disease, Parkinson's disease and traumatic brain injury.

With the HIV-infected population aging and ongoing neuroinflammation associated with infection, there is not only the interest in biomarkers to diagnose cognitive impairment but differentiate cognitive impairment from early Alzheimer's disease. Studies on NDE from Alzheimer's disease individuals identified increased p-T181-tau and amyloid β-42 (Aβ-42) as NDE proteins predictive of Alzheimer's disease before clinical onset.

In the current study, we expanded the number of NDE targets for biomarker discovery using multiplex proximity extension assays (PEA) that included 184 neural-associated proteins [8,9] that span neurological processes, both normal and pathogenic mechanisms, such as neural development, synaptic function, axonal disruption, cellular function, immune response, cell death and metabolism. This technology is based on antibodies linked to DNA tags close enough to form a new reporter gene sequence that is detected by quantitative PCR. This gene sequence expression is proportional to the protein concentration and is read as a unique biomarker. We also assayed NDE for markers associated with Alzheimer's disease, including NF-L, Aβ-42 and p-T181-tau to assess similarities and differences between Alzheimer's disease and HAND [10]. Finally, we found that NDE cargo can differ between men and women, giving a divergent biomarker signature for cognitive impairment.

Methods

Participants

One milliliter of frozen plasma was obtained from the National NeuroAIDS Tissue Consortium and HIV Neurobehavioral Research Program cohorts, which included four groups of 20 persons, HIV positive and negative, 51 women and 29 men. The four groups were HIV-negative neuropsychologically normal (NPN), HIV-positive NPN, HIV-positive asymptomatic neurocognitive impairment (ANI) and HIV-positive mild neurocognitive disorder (MND). The samples included medical, epidemiological and cognitive information, assessment of comorbidities, and laboratory values for immunologic and virologic parameters. Evaluations included neuropsychological diagnosis based on Frascati guidelines [11] and global deficit score (GDS) [12]. Exclusion criteria included recent infection at blood draw, psychoactive drug use or history of head injury.

Isolation of neuron-derived exosomes from plasma

NDEs were isolated as previously described [13]. Briefly, insoluble particles in plasma were removed by centrifugation at $1000 \times g$ for 10 min, and plasma fibril and coagulation proteins were removed by adding 100 μl thromboplastin to 250 μl plasma for 1 h followed by centrifugation at $3000 \times g$ for 20 min. Total exosomes were isolated by adding 126 μl ExoQuick (Systems Biosciences, Palo Alto, California, USA) to each sample in the presence of protease and phosphatase inhibitors. Pellets of total exosomes were resuspended and incubated with biotinylated-L1CAM mAb (Thermo-Fisher Scientific, Inc., Waltham, Massachusetts, USA). Labeled exosomes were captured with streptavidin-labeled-resin (Thermo-Fisher). The exosome–resin complexes were precipitated and washed five times with PBS. Pure neuronal exosomes were released from the resin beads into solution with 100 μl of 50 mmol/l Glycine-HCl (pH3) and neutralized with 10 μl 1M Tris–HCl (pH8). Exosomes were lysed using mammalian protein extraction reagent (Thermo-Fisher) in the existence of protease and phosphatase inhibitors and bovine serum albumin. The lysates were stored in −80 °C until use.

Characterization of neuron-derived exosome and protein quantification by ELISA and proximity extension assays

Exosome size and count were determined by a nanoparticle tracking analysis (NTA) and NanoSight LM10 instrument (Malvern Instruments, Malvern, UK) with a 405-nm laser-equipped sample chamber, as described previously [14]. The samples were diluted in PBS to 10^8–10^9 particles/ml. Camera shutter, gain and software detection threshold were manually adjusted for optimal detection and kept consistent during all sample analyses. Each sample was analyzed three times with three recordings of 30 s each. The modes were reported for

particle sizes due to the highly skewed distributions. The mean of the triplicate recordings was reported for size and concentration.

Protein concentrations in NDE were analyzed using commercial ELISA kits, apoptosis-linked gene 2-interacting protein X (ALIX) (Lifeome-Cusabio, Oceanside, California, USA), synaptophysin (American Research Products, Inc., Waltham, Massachusetts, USA), high mobility group box 1 (HMGB1) (LifeSpanBioSciences, Inc., Seattle, Washington, USA), NF-L (MyBioSource, San Diego, California, USA), p-T181-tau (Fujirebio US, Malvern, Pennsylvania, USA) and Aβ-42 (ThermoFisher). ELISAs were performed per manufacturer's instructions. All standards and samples were assayed in duplicate. Plates were analyzed using a SpectraMax M5 plate reader and SoftMax v5 software (Molecular Devices, San Jose, California, USA).

The abundance of 184 unique proteins in NDE were analyzed using the Neurology ($N = 80$) and NeuroExploratory ($N = 32$) multiplex assay panels from Olink Proteomics (Uppsala, Sweden) using PEA. All samples were tested on the Neurology panel; due to the constraints of sample volume, only 32 samples were tested with the Neuro Exploratory panel. The protein targets included a mix of established markers related to neurobiological processes and neurological diseases as well as some more exploratory proteins with broader roles in processes, such as cellular regulation, immunology, development and metabolism. Proteins were quantified by real-time PCR using the Fluidigm BioMark HD real-time PCR platform.

Statistical analysis

Counts of sex, ethnicity, hepatitis C virus (HCV) infection status, current major depressive disorder and current substance dependence in each group were compared using Fisher's exact test. Group means of age, education, $CD4^+$ cell counts and GDS were compared with analysis of variance (ANOVA). Correlation between particle counts and ALIX were performed using Pearson's correlation. Synaptophysin group means were compared with a Mann–Whitney test. Group means of ELISA and PEA data were compared using ANOVA adjusted for age, sex, education and ethnicity. Tukey honestly significant difference post-hoc tests were performed for pairwise comparisons. P values were adjusted with Benjamini and Hochberg method [15]. Continuous variables were log transformed to improve normality and/or linearity between independent and dependent variables. For PEA data analysis, the limit of detection (LOD) and normalized protein expression (NPX) values were provided by the vendor [8] after correcting background noise for each dataset. Proteins were further selected for analysis only if they passed quality control (provided by vendor) and were present in more than 25% of samples from at least one sample group. NPX values below LOD were replaced with LOD values. NPX values were normalized to ALIX concentrations. All statistical analyses were performed in R version 3.5.2 (R Foundation, Vienna, Austria) [16].

Results

Participants

Epidemiology, clinical and neuropsychological testing data for the 80 participants are summarized in Table 1. There was no significant difference in age and ethnicity. There were significantly more women ($n = 51$) than men ($n = 29$). The participants from the MND group had

Table 1. Characteristics of individuals.

HIV status	HIV−	HIV+			*P* value
Diagnosis	NPN	NPN	ANI	MND	
N					0.0116
Women	13	14	17	7	
Men	7	6	3	13	
Ethnicity					0.159
Asian	2	0	0	1	
Black	3	2	5	6	
White	11	11	8	12	
Hispanic/other	4	7	7	1	
Age	49.7 (12.1)	46.1 (8.4)	48.2 (10.6)	47.2 (10.4)	0.733
Education	14.3 (2.4)	13.6 (2.6)	12.8 (3.6)	11.9 (2.0)	0.0428
Viral load	NA	UD	UD	UD	
$CD4^+$	NA	668.8 (240.5)	641.8 (314.7)	412.4 (173.9)	0.0226
GDS	0.16 (0.14)	0.092 (0.089)	0.65 (0.29)	0.91 (0.48)	<0.0001
HCV infection	0	2/2	1/0	0	0.0510
Current major depressive disorder	0	0/1	2/0	0/1	0.899
Current substance dependence	0	0	0	0/1	1.000

Number of individuals (*N*) and ethnicity are presented as counts, using Fisher's exact tests; HCV infection, current major depressive disorder and current substance dependence are presented as counts (women/men), using Fisher's exact tests; age, education, $CD4^+$ and GDS are presented as mean (SD), using analysis of variance. ANI, asymptomatic neurocognitive impairment; GDS, global deficit score; HCV, hepatitis C virus; MND, mild neurocognitive disorder; NA, not available; NPN, neuropsychologically normal; UD, undetectable.

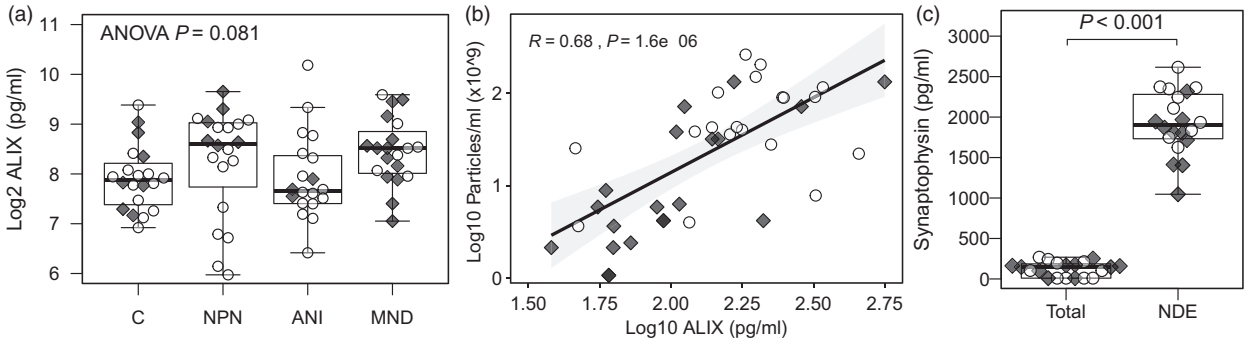

Fig. 1. Neuron-derived exosome (NDE) characterization. General exosome marker apoptosis-linked gene 2-interacting protein (ALIX) showed no significant difference among groups of HIV+ subjects and controls (a). C, HIV-negative neuropsychologically normal; NPN, HIV+ neuropsychologically normal; ANI, HIV+ asymptomatic neurological impairment; MND, HIV+ mild neurocognitive disorder. Analysis of variance was used. A correlation between exosome marker ALIX and neuron-derived exosome concentrations (b) with 95% confidence interval (in gray shade) using Pearson's correlation. NDE counts were determined by nanoparticle tracking analysis (NTA) and ALIX quantified by ELISA. NDE showed enriched synaptophysin compared with total exosomes (c) quantitated by ELISA using a Mann–Whitney test. The Box–Whisker plots indicate the 25th, 50th and 75th percentile with the Box, Whiskers extending from the boxes indicate extreme values within 1.5 interquartile ranges from the 25th and 75th percentile. Women (○), Men (◆).

2.4 years less education than HIV-negative controls. They also had lower blood $CD4^+$ cell counts than HIV-infected NPN participants. All participants were treated with ART and had undetectable plasma HIV viral RNA at the time of blood draw. Participants in the ANI and MND groups showed more severe neuropsychological impairment as shown by GDS. Too few samples from male individuals with ANI were available to give meaningful statistically significant comparisons, although they are reported here.

Characterization and normalization of neuron-derived exosome

To characterize NDE, we quantified ALIX protein concentrations from all four groups (Fig. 1a). ALIX is a member of the exosome proteome [17] and is used as a marker of exosomes. We evaluated the correlation of nanoparticle counts, considered the gold standard, with ALIX concentrations of NDE to find a more rapid and accurate surrogate for the tedious and labor intensive NDE counts which also consume 0.25 ml of plasma. The results show that NTA counts were highly correlated with ALIX concentrations ($R = 0.68$; $P < 0.0001$) (Fig. 1b). Thus, we used ALIX to normalize ELISA and PEA assays. To confirm the neural source of NDEs, we showed that neuron specific synaptophysin was highly enriched in NDE compared with total exosomes ($P < 0.001$) (Fig. 1c).

Biomarkers of neuronal damage and Alzheimer's disease in neuron-derived exosomes from HIV-infected subjects

A general marker of neuroinflammation and 'alarmin', HMGB1, was increased in NDE from impaired men ($P = 0.047$) but not women (Fig. 2a). Another neuronal injury marker NF-L was increased with HIV+ impaired men ($P = 0.003$) (Fig. 2b). Alternatively, NDE from women had elevated levels of NF-L from HIV-infected cognitively normal participants compared with controls ($P = 0.009$) and a decrease in NF-L from HIV-infected impaired women ($P = 0.028$). Phosphorylated tau (p-T181-tau), which is considered an Alzheimer triggering protein and possible biomarker for NDE in Alzheimer's disease diagnosis, was significantly decreased in NDE from both normal and impaired HIV-infected groups of men while women showed no difference in p-T181-tau levels (Fig. 2c). Amyloid β in NDE showed no differences between groups or across sex (Fig. 2d). For these analyses, ANI and MND were combined as neuropsychologically impaired.

HIV infection alone alters neuron-derived exosome cargo in men and women

ALIX normalized PEA analyses showed 25 proteins significantly decreased at P less than 0.05 in cognitively normal participants with and without HIV infection. Seven of the most significant proteins are shown (Fig. 3), including ezrin (EZR) ($P = 0.0002$), galectin 8 ($P = 0.0002$), LDL receptor related protein associated protein 1 ($P = 0.0002$), protogenin ($P = 0.0004$), repulsive guidance molecule A ($P = 0.0004$), scavenger receptor class A member 5 ($P = 0.0004$) and serine/threonine-protein kinase receptor R3 ($P = 0.0004$). The rest of the significantly differentially expressed proteins can be found in Supplemental Digital Content 1, http://links.lww.com/QAD/B485. Men and women were similar.

Differential neuron-derived exosome protein cargo in HIV-infected women and men

ALIX normalized PEA analysis showed significant differences in seven NDE proteins from HIV-infected women. These proteins were significantly differentially

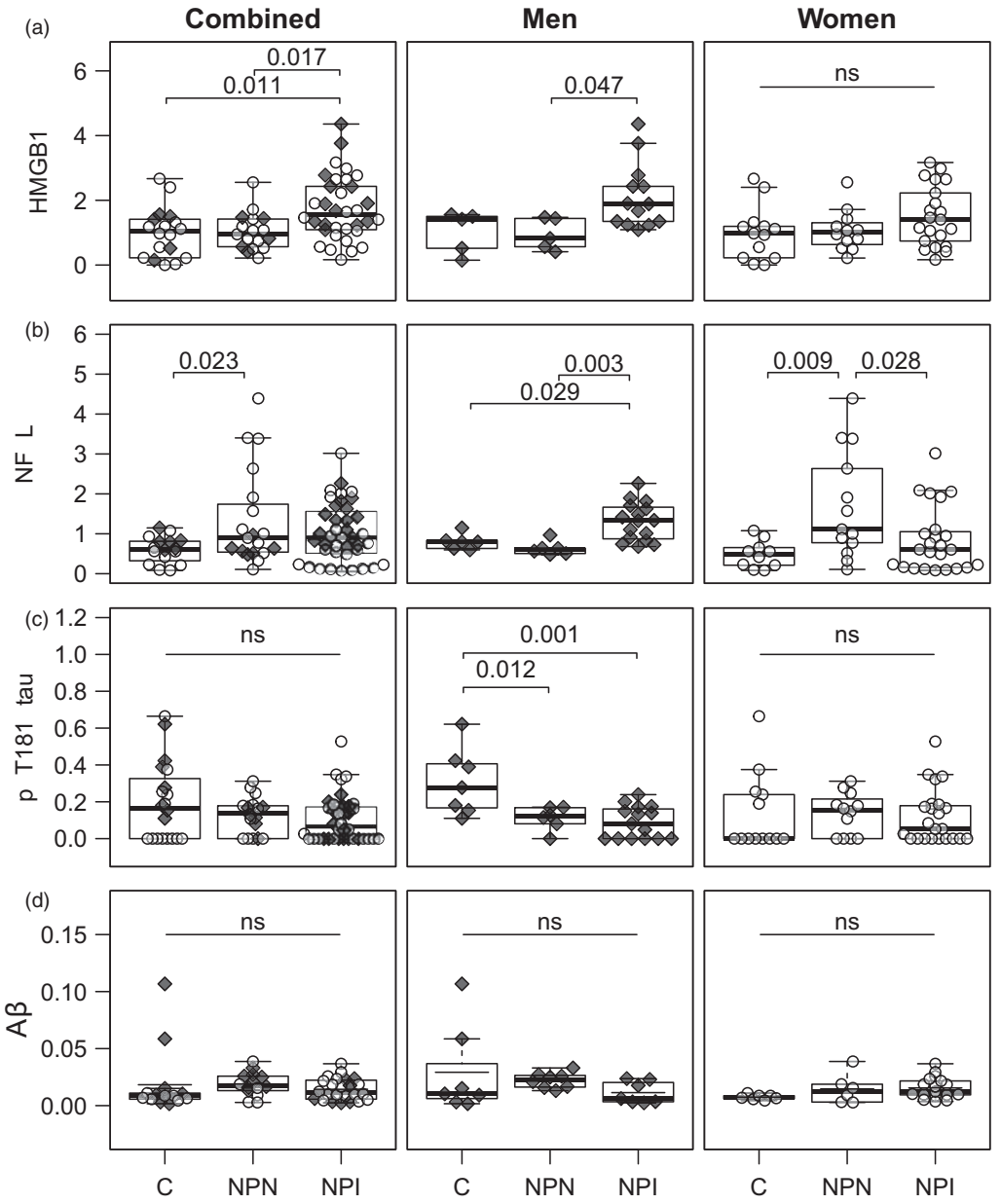

Fig. 2. Markers of neuronal damage and Alzheimer's disease in neuron-derived exosome. Neuron-derived exosomes were isolated and analyzed by ELISA for (a) high mobility group box 1, (b) neurofilament light, (c) p-T181-tau and (d) amyloid β. Data were normalized to apoptosis-linked gene 2-interacting protein (ALIX) and *Y*-axis indicates pg/ml over ALIX (pg/ml). C, control, HIV-negative normal cognition; NPN, neuropsychologically normal, HIV+ normal cognition; NPI, HIV+ neuropsychologically impaired cognition including asymptomatic neurological impairment and mild neurocognitive disorder. Analysis of variance, *P* values were adjusted with Tukey post-hoc analysis. Women (○), Men (◆).

expressed between NPN and/or ANI and/or MND in women. All significantly expressed brain-related proteins are shown (Fig. 4a). Three showed significant decreases in MND compared with NPN and ANI in HIV-infected women (Fig. 4a, top panel) including cathepsin S, total tau (microtubule-associated protein tau, MAPT) and neuronal cell adhesion molecule (NRCAM). Contactin 5, scavenger receptor class F member 2 and IL12 were higher in ANI compared with MND ($P = 0.019$, 0.049 and 0.022, respectively). EZR was increased in ANI compared with cognitively normal HIV+ women ($P = 0.043$). Men with MND showed no differences for these proteins (Fig. 4a, lower panel).

In NDE from men, 12 proteins were found significant when comparing MND with NPN (Fig. 4b). Although data comparing ANI and NPN are shown, the significance is dampened based on the very small numbers

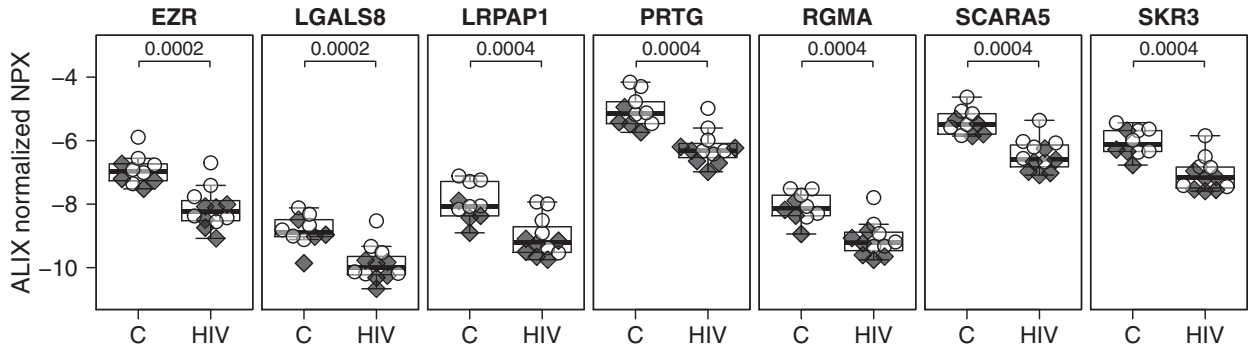

Fig. 3. HIV infection alone changes neuron-derived exosome cargo. Each dot is a single individual and all are neurocognitively normal. $N = 12$ for women; $N = 10$ for men. C, HIV-negative normal cognition; HIV, HIV+ normal cognition. Linear model adjusted for age, sex, education and ethnicity, P values adjusted for multiple comparison using Benjamini and Hochberg method [15], showing seven of 25 significantly expressed proteins. See figure, Supplemental Digital Content 1, http://links.lww.com/QAD/B485, which included all 25 proteins. Women (○), Men (◆).

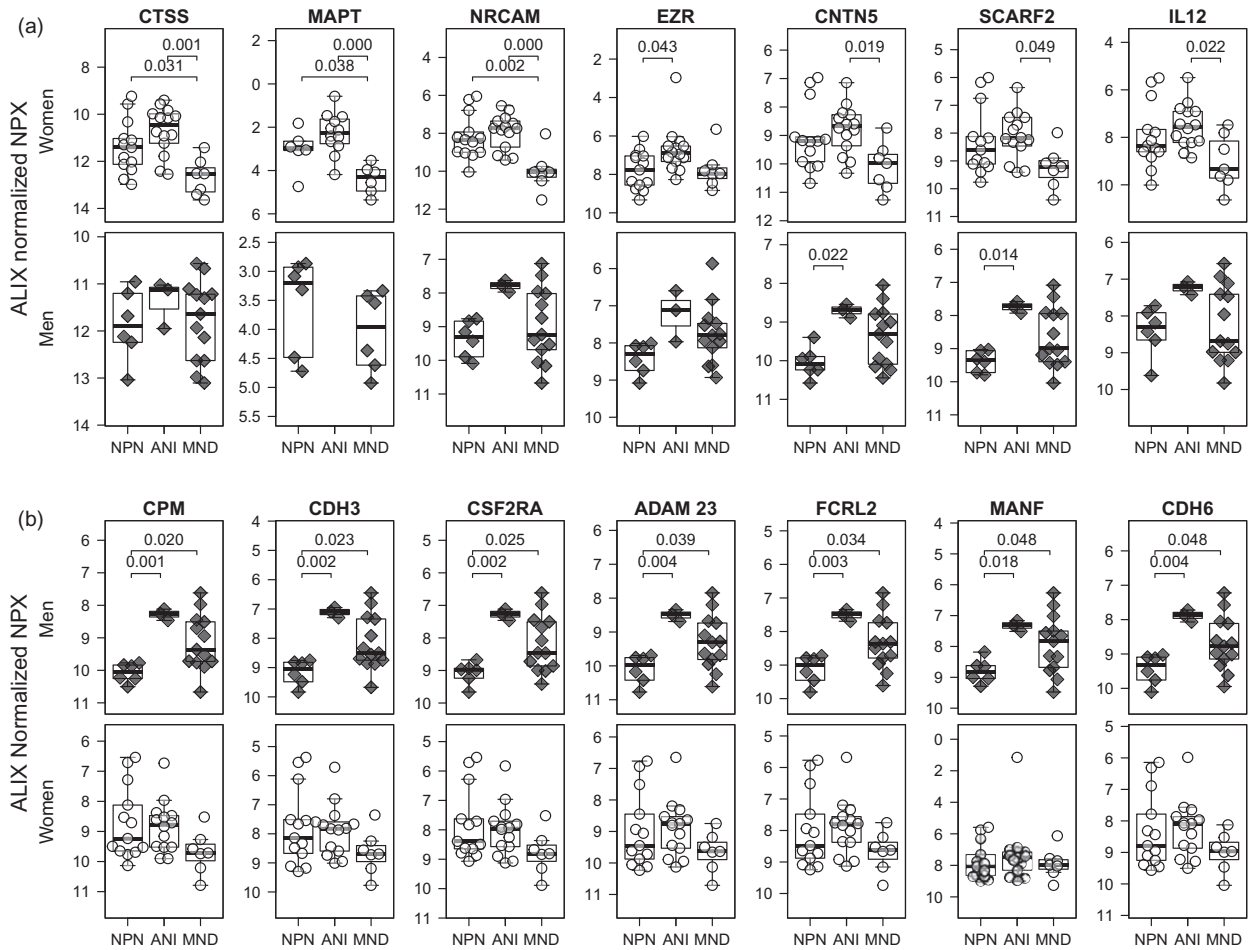

Fig. 4. Neuron-derived exosome proteins showing divergence patterns in neurocognitively impaired HIV+ individuals. (a) Seven proteins significantly decreased in women with cognitive impairment (upper panel) and not seen in men (lower panel). (b) Seven of 12 significantly expressed proteins increased in men with mild neurocognitive disorder (upper panel) and no changes in women for the same proteins (lower panel). ANI, asymptomatic neuropsychologically impaired; MND, mild neurocognitive disorder; NPN, neuropsychologically normal. All HIV+, $N = 34$ for women; $N = 22$ for men. Analysis of variance with Tukey post-hoc analysis.

of ANI persons. The seven most significant brain-related proteins shown were based on MND compared with normal cognition. These included carboxypeptidase M (CPM), cadherin 3 (CDH3), colony stimulating factor 2 receptor alpha subunit (CSF2RA), disintegrin and metalloproteinase domain-containing protein 23, Fc Receptor Like 2 (FCRL2), mesencephalic astrocyte-derived neurotrophic factor (MANF) and cadherin 6 (CDH6). Other proteins that were also higher in MND than NPN included C-type lectin domain containing 10A (CLEC10A), plexin B3 (PLXNB3), CD200 receptor 1 (CD200R1), netrin receptor UNC5C and hydroxyacylglutathione hydrolase (HAGH) (data can be found in Supplemental Digital Content 2, http://links.lww.com/QAD/B485). In comparison, women showed no differences in any of the 12 proteins (Fig. 4b, lower panel). The trends in comparisons of normal cognition (NPN) versus mild impairment (MND) in men and women are quite different. All the differentially expressed proteins in women with MND go down (Fig. 4a, top panel) and the significant proteins for men with MND go up (Fig. 4b, top panel).

Discussion

The primary goal of this study was to find biomarkers of cognitive impairment in HAND using neuronal exosomes in plasma. We found that plasma NDE can identify neuronal dysfunction in HIV infection and differentiate cognitive impairment between virally suppressed men and women. Plasma biomarkers of cognitive impairment offer a more rapid, inexpensive, noninvasive method to assess impairment over time and monitor changes with treatment and in the case of HIV, potential cure.

Plasma exosomes derived from a particular cell type of interest need to be isolated and the parent cell confirmed. In the case of neurons, we found synaptophysin to be an excellent marker of neurons as total plasma exosomes lack this protein. Just as important, a method of normalization needs to be determined. Although exosome counts are the gold standard for normalization, this procedure is lengthy and requires instrumental and technical capabilities. It also consumes a substantial amount of valuable sample volumes which makes it undesirable for biomarker discovery studies. To find a relatively fast and cost-effective method as a surrogate for exosome counts, we tested a number of exosome markers including CD63, CD81, CD9, epithelial cell adhesion molecule (EpCAM), Flotillin 1, Annexin A5 and tumor susceptibility gene 101 (TSG101). Most are either low expressed or change between groups. Protein concentrations are also frequently used to normalize exosome data. However, in preparation of lysates for ELISA, albumin and protease and phosphatase inhibitors are required to protect proteins from degradation, which interfere with most common protein estimation methods. Plasma exosomes also tend to have a large amount of contaminants carried-over, which makes it difficult to obtain accurate exosome estimates. We found a highly significant correlation between number of NDEs and NDE ALIX concentrations in this study, making it an excellent surrogate for counts. It is important to confirm this when studying other diseases and different exosomes. Neuronal exosomes from Alzheimer's patients were normalized to CD81 [7,18,19]; however, we found CD81 was variant in HIV cognitive impairment [13]. Details of normalization will be important for study comparisons across different diseases including HAND and Alzheimer's disease.

It was not our primary hypothesis to find sex differences. In our previous study on NDE in HAND, all participants were men [13]. The current study had a majority of women and the results showed significant differences in a number of proteins. These differences may be associated with the hypothalamic-pituitary-adrenal axis or stress, estrogen levels, depression, substance abuse and other comorbidities [20–22] and may be consistent with sex differences seen in Alzheimer's disease (review [23]). One recent study using a large number of women with HIV reported that HAND was more prevalent in women and it was associated with race, particularly black women and educational quality [24]. This current study had eight black women who were all impaired and had less education, one of them was over 50 years old, had current major depressive disorder, and was HCV positive with ANI.

Our initial studies looked at proteins associated with neuronal damage. We previously reported that men had increased HMGB1, NF-L and amyloid β in plasma NDE [13]. Here we again show that HMGB1 and NF-L increased in cognitively impaired men; however, we do not see an increase in amyloid β with increased numbers of men although there were several men with HIV viral loads in the previous report. NDE from women show a different pattern with no differences in HIV or cognitive impairment in HMGB1, amyloid β or NF-L levels suggesting that these three proteins are not differential in cognitively impaired women.

We were interested in differentiating HAND from Alzheimer's disease. Numerous reports show that NDE protein cargo is differentially expressed in mild cognitive impairment and Alzheimer's disease, specifically total tau, p-T181-tau and amyloid β [7,18,19]. We found that p-T181-tau decreased in HIV-infected patients overall, but not significantly different between cognitively normal and impaired in either men or women and importantly no increase in p-T181-tau like Alzheimer's disease. NDE amyloid β showed no difference in men or women unlike Alzheimer's disease which had increased amyloid β levels and in many cases increased in the progression from mild

impairment to Alzheimer's disease [7,18]. These divergent results reflect possibly different mechanisms in the cognitive impairment associated with Alzheimer's disease and HAND and may help differentiate the two conditions as HIV-infected persons age. NF-L is increased in plasma and serum of both men and women with Alzheimer's disease [25,26] which is consistent with our finding in NDE from HIV-infected cognitively impaired men. However, we found no difference in HIV-infected cognitively impaired women compared with women without HIV infection and even a decrease from normal cognition with HIV infection. Plasma NF-L is a general neuronal damage marker and is increased in a number of other neurodegenerative diseases, including frontotemporal dementia [27] and amyotrophic lateral sclerosis [28], suggesting that while NF-L is sensitive, it is not disease specific. Distinct properties in the cause and disease progression of men and women with Alzheimer's disease have been found [23] while similar studies lack in HAND.

In seeking new biomarkers and after observing sex differences in selected proteins, we used multiplex analysis to further differentiate impairment and sex mechanistic differences. PEA is a recent technology which requires very small amount of input sample and is highly sensitive with a broad range of detection. The multiplex of 92 targets/panel made it appealing for biomarker discovery and feasible to end up with a combination of proteins as a biomarker panel. An advantage of PEA is that it contains esoteric targets, which are not necessarily disease specific but may shed light on the pathways involved and other basic central nervous system functions not previously reported. We show a panel of seven proteins in NDE that were significantly decreased ($P \leq 0.001$) out of 25 proteins that were decreased ($P < 0.05$), indicating that HIV infection alone has a profound effect on the neuronal proteome, in spite of peripheral viral suppression. We speculate that ART may contribute to this pattern.

The protein pattern of NDE in women showed similar levels between HIV normal cognition and ANI with a decrease in proteins in MND except EZR, although in this cohort we had a disproportionate number of women with ANI ($n = 14$) compared with MND ($n = 7$). This pattern was not observed with men. An example of a new target for cognitive impairment is NRCAM. This protein regulates dendritic spines and excitatory synapses. When NRCAM is deficient, excitatory spine synapses are increased [29] and this is associated with autism spectrum disorders [30]. In men, the pattern was quite different with significant differences in normal cognition and MND and with limited samples, in most cases comparable protein levels with ANI and MND. In a recent neuroimaging study of virally suppressed men with normal cognition, ANI and MND, the authors were able to differentiate HIV positive from HIV-negative subjects and ANI and MND from normal subjects by brain volume [31]. Future studies to compare plasma biomarkers with neuroimaging parameters to identify ANI and MND are warranted.

Our results suggest that we may differentiate cognitively impaired persons, including ANI and MND, from cognitively normal based on a few proteins in men and similarly differentiate MND from ANI and normal in women. As an example, MANF is a neurotrophic factor maintaining endoplasmic reticulum homeostasis [32] and is essential for neurite extension [33]. It has been reported that MANF protects neurons from apoptosis in traumatic brain injury models [34,35], defends BBB integrity [36,37] and protects neurodegeneration in a Parkinson's model [38]. In this study, MANF was increased in ANI and MND in men and may suggest a compensatory response in neurons. We speculate that an increase in NDE protein content may indicate the neurons' effort in eliminating excess or deleterious proteins or leakage from dead or dying neurons. On the other hand, exosomes with decreased protein may signal homeostasis or a mechanism to contain a beneficial protein. It could also mean leakage into CSF.

There are several limitations of this research. The cohort is small with a majority of women and a deficiency of men with ANI so that comparisons with identify asymptomatic impairment in men is not possible. Our samples came from AIDS banks and were only 1-ml plasma for exosome isolation and protein analyses making further analyses impossible. Although plasma is not difficult to obtain, banked samples with full epidemiological, clinical and psychological analyses are limited and in the future longitudinal studies of these persons will be essential. PEA signatures are beneficial to illuminate new mechanisms and protein targets, but biomarkers must be confirmed with definitive protein assays and the limited plasma made that impossible for this study.

This report identifies a number of new targets as well as differences in response to cognitive impairment between sexes. As more reports come out showing sex differences in response to HIV, more studies to address treatment options based on sex should be considered. A recent study reported how HIV disease progression and response to treatment differed in activation soluble factors between men and women [39]. Biomarker discovery for HIV cognitive impairment for prognosis, diagnosis, treatment and recovery should reflect both men and women. Comingling of the data may mask these differences. NDE are a different approach to study the health of the neuron in real time and to gauge cognition over time.

Acknowledgements

The current study was funded by NIMH (LP R21MH112483). Participant plasma samples were funded

by shared resources from NIMH and NINDS by the following grants: Manhatten HIV Brain Bank (MHBB): U24MH10093, Texas NeuroAIDS Research Center (TNRC): U24MH100930, National Neurological AIDS Bank (NNAB): U24MHw100929, California NeuroAIDS Tissue Network (CNTN): U24MH100928. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of the NNTC or NIH. The authors also acknowledge the HIV Neurobehavioral Research Center (HNRC) supported by public funding from the National Institutes of Health (NIH), the State of California and other sources (NIMH/CSPAR Award Number P30MH062512) for samples used in this study. The authors also thank Linda Jung (Olink proteomics) for helpful discussions. L.P. and B.S. conceived the study and wrote the article. B.S. and N.F. acquired the data and B.S. performed the statistical analyses.

Conflicts of interest

There are no conflicts of interest.

References

1. Peterson J, Gisslen M, Zetterberg H, Fuchs D, Shacklett BL, Hagberg L, *et al*. **Cerebrospinal fluid (CSF) neuronal biomarkers across the spectrum of HIV infection: hierarchy of injury and detection.** *PLoS One* 2014; **9**:e116081.
2. Gisslen M, Price RW, Andreasson U, Norgren N, Nilsson S, Hagberg L, *et al*. **Plasma concentration of the neurofilament light protein (NFL) is a biomarker of CNS injury in HIV infection: a cross-sectional study.** *EBioMedicine* 2016; **3**:135–140.
3. Gupta A, Pulliam L. **Exosomes as mediators of neuroinflammation.** *J Neuroinflam* 2014; **11**:68.
4. Hu G, Yang L, Cai Y, Niu F, Mezzacappa F, Callen S, *et al*. **Emerging roles of extracellular vesicles in neurodegenerative disorders: focus on HIV-associated neurological complications.** *Cell Death Dis* 2016; **7**:e2481.
5. Dalvi P, Sun B, Tang N, Pulliam L. **Immune activated monocyte exosomes alter microRNAs in brain endothelial cells and initiate an inflammatory response through the TLR4/MyD88 pathway.** *Sci Rep* 2017; **7**:9954.
6. Goetzl EJ, Boxer A, Schwartz JB, Abner EL, Petersen RC, Miller BL, *et al*. **Altered lysosomal proteins in neural-derived plasma exosomes in preclinical Alzheimer disease.** *Neurology* 2015; **85**:40–47.
7. Fiandaca MS, Kapogiannis D, Mapstone M, Boxer A, Eitan E, Schwartz JB, *et al*. **Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: a case–control study.** *Alzheimers Dement* 2015; **11**:600–607.e1.
8. Assarsson E, Lundberg M, Holmquist G, Bjorkesten J, Thorsen SB, Ekman D, *et al*. **Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability.** *PLoS One* 2014; **9**:e95192.
9. Solier C, Langen H. **Antibody-based proteomics and biomarker research – current status and limitations.** *Proteomics* 2014; **14**:774–783.
10. Pulliam L, Sun B, Mustapic M, Chawla S, Kapogiannis D. **Plasma neuronal exosomes serve as biomarkers of cognitive impairment in HIV infection and Alzheimer's disease.** *J Neurovirol* 2019. [Epub ahead of print].
11. Antinori A, Arendt G, Becker JT, Brew BJ, Byrd DA, Cherner M, *et al*. **Updated research nosology for HIV-associated neurocognitive disorders.** *Neurology* 2007; **69**:1789–1799.
12. Carey CL, Woods SP, Gonzalez R, Conover E, Marcotte TD, Grant I, *et al*. **Predictive validity of global deficit scores in detecting neuropsychological impairment in HIV infection.** *J Clin Exp Neuropsychol* 2004; **26**:307–319.
13. Sun B, Dalvi P, Abadjian L, Tang N, Pulliam L. **Blood neuron-derived exosomes as biomarkers of cognitive impairment in HIV.** *AIDS* 2017; **31**:F9–F17.
14. Tang N, Sun B, Gupta A, Rempel H, Pulliam L. **Monocyte exosomes induce adhesion molecules and cytokines via activation of NF-kappaB in endothelial cells.** *FASEB J* 2016; **30**:3097–3106.
15. Benjamini Y, Hochberg Y. **Controlling the false discovery rate – a practical and powerful approach to multiple testing.** *J R Stat Soc Ser B Stat Methodol* 1995; **57**:289–300.
16. R Core Team. *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing; 2018.
17. Hurley JH, Odorizzi G. **Get on the exosome bus with ALIX.** *Nat Cell Biol* 2012; **14**:654–655.
18. Winston CN, Goetzl EJ, Akers JC, Carter BS, Rockenstein EM, Galasko D, *et al*. **Prediction of conversion from mild cognitive impairment to dementia with neuronally derived blood exosome protein profile.** *Alzheimers Dement* 2016; **3**:63–72.
19. Abner EL, Jicha GA, Shaw LM, Trojanowski JQ, Goetzl EJ. **Plasma neuronal exosomal levels of Alzheimer's disease biomarkers in normal aging.** *Ann Clin Transl Neurol* 2016; **3**:399–403.
20. Rubin LH, Cook JA, Weber KM, Cohen MH, Martin E, Valcour V, *et al*. **The association of perceived stress and verbal memory is greater in HIV-infected versus HIV-uninfected women.** *J Neurovirol* 2015; **21**:422–432.
21. Rubin LH, Pyra M, Cook JA, Weber KM, Cohen MH, Martin E, *et al*. **Posttraumatic stress is associated with verbal learning, memory, and psychomotor speed in HIV-infected and HIV-uninfected women.** *J Neurovirol* 2016; **22**:159–169.
22. Rubin LH, Phan KL, Keating SM, Maki PM. **A single low dose of hydrocortisone enhances cognitive functioning in HIV-infected women.** *AIDS* 2018; **32**:1983–1993.
23. Nebel RA, Aggarwal NT, Barnes LL, Gallagher A, Goldstein JM, Kantarci K, *et al*. **Understanding the impact of sex and gender in Alzheimer's disease: a call to action.** *Alzheimers Dement* 2018; **14**:1171–1183.
24. Sundermann EE, Heaton RK, Pasipanodya E, Moore RC, Paolillo EW, Rubin LH, *et al*. **Sex differences in HIV-associated cognitive impairment.** *AIDS* 2018; **32**:2719–2726.
25. Lin YS, Lee WJ, Wang SJ, Fuh JL. **Levels of plasma neurofilament light chain and cognitive function in patients with Alzheimer or Parkinson disease.** *Sci Rep* 2018; **8**:17368.
26. Preische O, Schultz SA, Apel A, Kuhle J, Kaeser SA, Barro C, *et al*. **Serum neurofilament dynamics predicts neurodegeneration and clinical progression in presymptomatic Alzheimer's disease.** *Nat Med* 2019; **25**:277–283.
27. Steinacker P, Anderl-Straub S, Diehl-Schmid J, Semler E, Uttner I, von Arnim CAF, *et al*. **Serum neurofilament light chain in behavioral variant frontotemporal dementia.** *Neurology* 2018; **91**:e1390–e1401.
28. Verde F, Steinacker P, Weishaupt JH, Kassubek J, Oeckl P, Halbgebauer S, *et al*. **Neurofilament light chain in serum for the diagnosis of amyotrophic lateral sclerosis.** *J Neurol Neurosurg Psychiatry* 2019; **90**:157–164.
29. Demyanenko GP, Mohan V, Zhang X, Brennaman LH, Dharbal KE, Tran TS, *et al*. **Neural cell adhesion molecule NrCAM regulates semaphorin 3F-induced dendritic spine remodeling.** *J Neurosci* 2014; **34**:11274–11287.
30. Hutsler JJ, Zhang H. **Increased dendritic spine densities on cortical projection neurons in autism spectrum disorders.** *Brain Res* 2010; **1309**:83–94.
31. Nichols MJ, Gates TM, Soares JR, Moffat KJ, Rae CD, Brew BJ, *et al*. **Atrophic brain signatures of mild forms of neurocognitive impairment in virally suppressed HIV infection.** *AIDS* 2019; **33**:55–66.
32. Lindahl M, Saarma M, Lindholm P. **Unconventional neurotrophic factors CDNF and MANF: structure, physiological functions and therapeutic potential.** *Neurobiol Dis* 2017; **97 (Pt B)**:90–102.
33. Tseng KY, Danilova T, Domanskyi A, Saarma M, Lindahl M, Airavaara M. **MANF is essential for neurite extension and neuronal migration in the developing cortex.** *eNeuro* 2017; **4**: Available at https://doi.org/10.1523/ENEURO.0214-17.2017.

34. Gao L, Xu W, Fan S, Li T, Zhao T, Ying G, *et al*. **MANF attenuates neuronal apoptosis and promotes behavioral recovery via Akt/MDM-2/p53 pathway after traumatic spinal cord injury in rats.** *Biofactors* 2018; **44**:369–386.
35. Xu W, Gao L, Li T, Zheng J, Shao A, Zhang J. **Mesencephalic astrocyte-derived neurotrophic factor (MANF) protects against neuronal apoptosis via activation of Akt/MDM2/p53 signaling pathway in a rat model of intracerebral hemorrhage.** *Front Mol Neurosci* 2018; **11**:176.
36. Li QX, Shen YX, Ahmad A, Shen YJ, Zhang YQ, Xu PK, *et al*. **Mesencephalic astrocyte-derived neurotrophic factor prevents traumatic brain injury in rats by inhibiting inflammatory activation and protecting the blood–brain barrier.** *World Neurosurg* 2018; **117**:e117–e129.
37. Li T, Xu W, Gao L, Guan G, Zhang Z, He P, *et al*. **Mesencephalic astrocyte-derived neurotrophic factor affords neuroprotection to early brain injury induced by subarachnoid hemorrhage via activating Akt-dependent prosurvival pathway and defending blood–brain barrier integrity.** *FASEB J* 2019; **33**:1727–1741.
38. Zhang Z, Shen Y, Luo H, Zhang F, Peng D, Jing L, *et al*. **MANF protects dopamine neurons and locomotion defects from a human alpha-synuclein induced Parkinson's disease model in *C. elegans* by regulating ER stress and autophagy pathways.** *Exp Neurol* 2018; **308**:59–71.
39. Krebs SJ, Slike BM, Sithinamsuwan P, Allen IE, Chalermchai T, Tipsuk S, *et al*. **Sex differences in soluble markers vary before and after the initiation of antiretroviral therapy in chronically HIV-infected individuals.** *AIDS* 2016; **30**:1533–1542.