

# UC San Diego

## UC San Diego Previously Published Works

### Title

Cerebrospinal fluid cell-free mitochondrial DNA is associated with HIV replication, iron transport, and mild HIV-associated neurocognitive impairment

### Permalink

<https://escholarship.org/uc/item/0x24019k>

### Journal

Journal of Neuroinflammation, 14(1)

### ISSN

1742-2094

### Authors

Mehta, Sanjay R  
Pérez-Santiago, Josué  
Hulgan, Todd  
et al.

### Publication Date

2017-12-01

### DOI

10.1186/s12974-017-0848-z

Peer reviewed

RESEARCH

Open Access



# Cerebrospinal fluid cell-free mitochondrial DNA is associated with HIV replication, iron transport, and mild HIV-associated neurocognitive impairment

Sanjay R. Mehta<sup>1,2\*</sup>, Josué Pérez-Santiago<sup>3</sup>, Todd Hulgán<sup>4</sup>, Tyler R. C. Day<sup>5</sup>, Jill Barnholtz-Sloan<sup>6</sup>, Haley Gittleman<sup>6</sup>, Scott Letendre<sup>1</sup>, Ronald Ellis<sup>7</sup>, Robert Heaton<sup>3</sup>, Stephanie Patton<sup>8</sup>, Jesse D. Suben<sup>1</sup>, Donald Franklin<sup>3</sup>, Debralee Rosario<sup>3</sup>, David B. Clifford<sup>9</sup>, Ann C. Collier<sup>10</sup>, Christina M. Marra<sup>11</sup>, Benjamin B. Gelman<sup>12</sup>, Justin McArthur<sup>13</sup>, Allen McCutchan<sup>1</sup>, Susan Morgello<sup>14</sup>, David Simpson<sup>14</sup>, James Connor<sup>8</sup>, Igor Grant<sup>3</sup> and Asha Kallianpur<sup>15,16</sup>

## Abstract

**Background:** Mitochondria are abundant organelles critical for energy metabolism and brain function. Mitochondrial DNA (mtDNA), released during cellular injury and as part of the innate immune response to viral pathogens, contains CpG motifs that act as TLR-9 ligands. We investigated relationships between cerebrospinal fluid (CSF) cell-free mtDNA levels and HIV viral load (VL), biomarkers of inflammation and iron transport, and neurocognitive (NC) function in the CNS HIV Antiretroviral Therapy Effects Research (CHARTER) cohort.

**Methods:** We quantified cell-free mtDNA in CSF by droplet digital PCR in 332 CHARTER participants who underwent comprehensive neuropsychiatric evaluation. NC performance was assessed using the global deficit score (GDS) as either a continuous or a binary measure ( $GDS \geq 0.5$ , impaired vs.  $GDS < 0.5$ , unimpaired). CSF, clinical, and biomarker data from the earliest available time point were analyzed. Cell-free mtDNA associations with CSF inflammation and iron-related biomarkers [CXCL10, IL-6, IL-8, TNF- $\alpha$ , transferrin (TF), ceruloplasmin (CP), and vascular endothelial growth factor (VEGF)], VL, and GDS were evaluated by multivariable regression.

**Results:** CSF cell-free mtDNA levels were significantly lower in participants with undetectable (vs. detectable) VL in either plasma ( $p < 0.001$ ) or CSF ( $p < 0.001$ ) and in those on antiretroviral therapy (ART;  $p < 0.001$ ). Participants on ART with undetectable VL in both CSF and plasma had lower mtDNA levels than those with detectable VL in both compartments ( $p = 0.001$ ). Higher mtDNA levels were observed in participants in the highest vs. lowest tertile (T3 vs. T1) of CSF CXCL10 (T3 vs. T1,  $p < 0.001$ ) and TNF- $\alpha$  (T3 vs. T1,  $p < 0.05$ ) in unadjusted analyses. MtDNA levels also correlated with CSF leukocyte count. After adjusting for CSF leukocyte count and VL, mtDNA levels were also associated with other inflammation- and iron-related biomarkers in CSF, including TF (T3 vs. T1,  $p < 0.05$ ) and CP (T3 vs. T1,  $p < 0.05$ ). With additional correction for ART use, mtDNA was also negatively associated with CSF VEGF ( $p < 0.05$ ) and IL-6 ( $p = 0.05$ ). We observed no associations of CSF mtDNA levels with age or GDS-defined NC impairment.

(Continued on next page)

\* Correspondence: [srmehta@ucsd.edu](mailto:srmehta@ucsd.edu)

<sup>1</sup>Department of Medicine, University of California-San Diego, San Diego, CA, USA

<sup>2</sup>Department of Medicine, San Diego Veterans Affairs Medical Center, San Diego, CA, USA

Full list of author information is available at the end of the article



(Continued from previous page)

**Conclusions:** CSF cell-free mtDNA levels were associated with HIV RNA and ART status, as well as with biomarkers of iron transport and VEGF, a growth factor with known effects on mitochondrial integrity and autophagy. CSF mtDNA may be a biomarker of iron dysregulation and/or neuroinflammation during HIV infection.

**Keywords:** Mitochondrial DNA, HIV, Cerebrospinal fluid, Neurocognitive impairment, Iron, Inflammation

## Background

The pathogenesis of HIV-associated neurocognitive impairment (NCI) remains incompletely understood. Neuroinflammation due to chronic immune activation and viral replication in the central nervous system (CNS) is important in promoting HIV-associated NCI (reviewed in [1] and [2]). In individuals with effective viral suppression on antiretroviral therapy (ART), other risk factors may contribute, including metabolic disorders, cardiovascular risk factors, and older age [3]. Notably, mitochondrial dysfunction is associated with all of these factors [4].

Mitochondria are intracellular organelles, critical for energy production and a host of metabolic processes, which contain their own distinct DNA genome (mtDNA). Most mammalian cells contain multiple copies of mtDNA (between 100 and 10,000 copies, depending upon cell type and function) [5, 6]. Brain tissue, by virtue of its high bioenergetic needs, contains higher mtDNA copy numbers than most other tissues [6]. Given the endosymbiotic origins of mitochondria, mtDNA has similarities with bacterial genomes, including high numbers of CpG motifs. These motifs tag free mtDNA as a “damage-associated molecular pattern” molecule (DAMP), which triggers toll-like receptor (TLR)-9 activation and downstream inflammation [7].

Mitochondrial damage and mtDNA copy number within brain tissue have been associated with several neurodegenerative diseases, as well as with aging [8–10]. Fewer copies of mtDNA and larger numbers of mtDNA mutations are found in the brains of individuals with Parkinson’s disease (PD) and Alzheimer’s disease (AD), suggesting a possible role for reduced mitochondrial function in causing or promoting PD and AD neuropathology [11]. More recently, investigators have begun exploring the clinical utility of measuring cell-free mtDNA in cerebrospinal fluid (CSF) as a biomarker of mitochondrial damage or dysfunction and associated inflammation in the brain. In children with traumatic brain injury, higher cell-free CSF mtDNA levels are associated with severe disability and death [12]. Furthermore, cell-free mtDNA levels are lower in individuals with AD and PD when compared to healthy controls or individuals with other neurodegenerative diseases [13, 14].

Disruption of iron homeostasis is also a characteristic feature of neurodegenerative disorders, including AD and PD [15–17], and iron transport and iron homeostasis are required for mitochondrial biogenesis and function [18].

Furthermore, either iron accumulation or iron depletion can promote free radical formation, which may then also induce inflammation, mitophagy, and neurodegeneration [19–22]. In this study, we examined the relationship between CSF cell-free mtDNA and CSF biomarkers of inflammation and iron transport, HIV disease-related factors, and NCI in participants enrolled in the CNS HIV Antiretroviral Therapy Effects Research (CHARTER) study. We hypothesized that CSF levels of iron biomarkers and cell-free mtDNA, both of which are related to mitochondrial integrity, could explain the presence and the degree of NCI in HIV-infected individuals.

## Methods

### Study population and design

CHARTER is a prospective, observational study that enrolled ambulatory, HIV-infected adults from 2003 to 2007 at six USA locations: Johns Hopkins University, Baltimore, MD; the Icahn School of Medicine at Mount Sinai, New York, NY; University of California, San Diego, CA; University of Texas Medical Branch, Galveston, TX; University of Washington, Seattle, WA; and Washington University, St. Louis, MO. Standardized, comprehensive neuromedical, neurobehavioral, and laboratory assessments were performed, as previously described [23, 24]. This study involved a cross-sectional analysis associating CSF biomarker measurements with clinical data in 332 CHARTER participants who had sufficient CSF from the baseline visit available for all measurements. The two most common antiretroviral regimens were a non-nucleoside reverse transcriptase inhibitor-based regimen ( $n = 107$ ), and a protease inhibitor-based regimen ( $n = 152$ ). The study was approved by institutional review boards at each site, and all participants provided written informed consent.

### Neurocognitive assessments

HIV-associated neurocognitive disorder (HAND) was diagnosed when NCI and functional impairment were determined to likely be due to HIV-related effects on the brain rather than to comorbid conditions [24]. Detailed review by a senior CHARTER neuropsychologist and neurologist, using published guidelines and the Frascati criteria [25], provided categorization of comorbid conditions for all CHARTER participants as incidental to, contributing to, or confounding their neurocognitive performance. Several conditions (e.g., brain trauma,

epilepsy or other seizure history, CNS opportunistic diseases) informed this categorization; detailed information on their frequencies are presented elsewhere [23]. Individuals with confounding neurocognitive comorbidities (15% of the total CHARTER cohort), which by definition precluded an assessment of the contribution of HIV infection to their neurocognitive impairment, were not eligible for a diagnosis of HAND according to Frascati criteria [23] and were excluded from the present study. The global deficit score (GDS), calculated by averaging deficit scores from a battery of neurocognitive tests, was also used as a measure of neurocognitive performance, with NCI defined as  $GDS \geq 0.5$  [26]. Follow up neurocognitive assessments were available on 216 of our 332 subjects. We categorized these 216 individuals as improved, stable, or declined based on comparison between the baseline and last follow-up assessment (See Additional file 1: Table S1).

#### Clinical laboratory assessment

HIV infection was diagnosed by ELISA with western blot confirmation. HIV viral load (viral RNA copies per ml) were measured in plasma and CSF by reverse transcriptase PCR (Roche Amplicor, v. 1.5, lower limit of quantitation 50 copies/mL) performed at a central lab [23].

#### Cell-free mtDNA analysis

CSF samples, obtained at the baseline visit, were analyzed for cell-free mtDNA content. Briefly, CSF was centrifuged at  $250\times g$  for 15 min to remove cells and cellular debris, and the supernatant was stored at  $-80\text{ }^{\circ}\text{C}$ . Total genomic DNA was extracted from CSF supernatant samples using QIAamp DNA Mini Kit (Qiagen, CA) according to the manufacturer's protocol, and was digested using the BamHI HF (New England Biolabs, Ipswich, MA) restriction enzyme. MtDNA was then quantified by droplet digital PCR (Bio-Rad, Hercules, CA) using a primer-probe combination targeting the NADH dehydrogenase 2 gene (MT-ND2, Applied Biosystems, Foster City, CA) found within the mitochondrial genome. A genomic DNA (gDNA) control was also measured by targeting the ribonuclease P protein subunit p30 gene (RPP30, Applied Biosystems, CA). Quantification was performed according to manufacturer's instructions and was carried out in triplicate in 20  $\mu\text{L}$  reactions, consisting of 10  $\mu\text{L}$  of  $2\times$  Bio-Rad supermix for probes, 1  $\mu\text{L}$  of either  $20\times$  Primer/FAM ND2 mix or  $20\times$  Primer/VIC RPP30 mix, 4  $\mu\text{L}$  of molecular grade water, and 5  $\mu\text{L}$  of DNA. Droplet generation and amplification were performed per protocol, and after analysis of the droplets, mtDNA copy number per mL of CSF was calculated using QuantaSoft (Bio-Rad, CA).

#### Measurement of other biomarkers

Concentrations of inflammation and angiogenesis biomarkers were measured in CSF according to manufacturer's protocol by commercial high-sensitivity multiplex (interleukin (IL)-6, IL-8, tumor necrosis factor- $\alpha$ ) or single-plex (CXC motif chemokine 10 (CXCL10), vascular endothelial growth factor (VEGF) bead-based immunoassay arrays (Luminex FlexMap 3D platform, Millipore, Billerica, MA). Ten percent of all assays were repeated to assess operator and batch consistency.

Quantification of CSF iron content was performed using the Quantichrom Iron Assay (BioAssay Systems, Hayward, CA), following the manufacturer's protocol. CSF transferrin content was determined by enzyme-linked immunoabsorbent assay (ELISA, Human Transferrin ELISA kit (ab108902), Abcam, Cambridge, MA), performed according to the manufacturer's protocol. CSF samples were centrifuged, and duplicate samples were diluted 1:1000 with mix diluent. The human heavy chain ferritin (H-ferritin) content of CSF samples was determined by ELISA assay (Human H-Ferritin ELISA kit, Abnova, Taipei, Taiwan) performed according to the manufacturer's protocol. Additional iron-transport-related biomarkers quantified in CSF included ceruloplasmin (CP) and haptoglobin (HP). Levels of these proteins were measured using commercially available bead-based immunoassays (Millipore) validated for CSF.

#### Statistical analyses

Univariate linear regressions were performed to assess associations between mtDNA levels and clinical variables, including demographic characteristics, ART use, CSF HIV viral load and biomarkers of inflammation, angiogenesis, and iron transport in CSF. To fulfill the normality assumption of linear regression, mtDNA copy number was log-transformed. CSF biomarkers other than mtDNA, which were also continuous but non-normally distributed variables, were categorized as tertiles to improve sensitivity of statistical association tests. Student's *t* test was used to determine the difference in log mtDNA levels between participants who were on ART and those who were not on ART. Within the ART-treated subset, analysis of variance (ANOVA) was used to determine differences in mtDNA levels between the following four groups: (1) detectable plasma and CSF viral load ( $n = 27$ ), (2) detectable CSF viral load but undetectable plasma viral load ( $n = 5$ ), (3) detectable plasma viral load but undetectable CSF viral load ( $n = 56$ ), and (4) undetectable plasma and CSF viral load ( $n = 147$ ). The small number of subjects with detectable HIV in CSF but not plasma limited the power to compare this category with other groups. Pearson's correlations were calculated to evaluate

the relationship between mtDNA level and CSF leukocyte count. To evaluate the relationship between mtDNA and neurocognitive performance, Wilcoxon tests were performed to compare mtDNA levels between the individuals with no NCI, and those with asymptomatic NCI (ANI) and mild NCI or neurocognitive disorder (MND). Too few participants had HIV-associated dementia (HAD,  $n = 5$ ) to power comparisons with the other groups. In order to determine if associations with mtDNA were independent of other factors that may relate to neuroinflammation and NCI, associations between cell-free mtDNA levels and neurocognitive performance were assessed in multivariable linear regression models, stratified by ART status (on vs. off) and adjusted for nadir CD4<sup>+</sup> T-cell count, CSF leukocyte count, and CSF virus detectability, or adjusted for nadir CD4<sup>+</sup> T-cell count, CSF leukocyte count, and plasma virus detectability [27, 28]. Similar models were also used to assess associations of cell-free mtDNA levels with other CSF biomarkers (iron, ferritin, transferrin, CP, HP, IL-6, IL-8, CXCL-10, TNF- $\alpha$ , and VEGF) as independent outcomes, stratifying by ART status and adjusting for CSF WBC, in case differences in CSF mtDNA were driven by pleocytosis (WBC elevations in the CSF that occur as a result of CNS infections). These linear regression models were also run with CSF virus detectability included as a covariate. Statistical analyses were performed using R version 3.1.2 [29] and SAS 9.4. Two-sided  $p$  values  $< 0.05$  were considered statistically significant.

## Results

### Study population

The mean age of the study population was 43 years (standard deviation 8.2); 77% were men, and 54% reported non-white race/ethnicity. Sixty-nine percent of participants were on ART at the time of CSF sampling. The median HIV RNA concentrations were 1.9 in plasma (Interquartile range (IQR) 1.7–4.0)  $\log_{10}$  copies/ml and 1.7 in CSF (IQR 1.7–2.3)  $\log_{10}$  copies/ml. Thirty-four percent (plasma) and 60% (CSF) had undetectable HIV RNA. The median (IQR) current CD4<sup>+</sup> T-cell count was 455 (270–636) cells/mm<sup>3</sup>. Thirty percent of participants were classified as having NCI based on the GDS [26], and these impaired participants were slightly older than those without NCI (45 vs. 43 years,  $p = 0.02$ ). Unimpaired participants were also less likely to have a self-reported diagnosis of diabetes (Type I or II) (5 vs. 20%,  $p = 0.001$ ). Demographic and clinical characteristics of study participants are presented, overall and stratified by NCI status, in Table 1.

### Socio-demographic and clinical factors associated with cell-free CSF mtDNA levels

Overall median (IQR) CSF cell-free mtDNA was 3.92 (3.67–4.27)  $\log_{10}$  copies/ml. Individuals on ART at the

time of CSF sampling had significantly lower cell-free CSF mtDNA levels than those who were ART-naïve ( $p = 0.03$ ) or who had previously received ART ( $p = 0.02$ ). Additionally, participants with detectable HIV RNA in either plasma or CSF (grouped together) had significantly higher cell-free CSF mtDNA levels than those with undetectable HIV RNA in either plasma ( $p < 0.001$ ) or CSF ( $p < 0.001$ ) (*data not shown*). Higher cell-free CSF mtDNA was seen with lower nadir CD4<sup>+</sup> T-cell counts ( $p < 0.01$ ). Higher CSF WBC and CSF HIV RNA were also associated with higher mtDNA levels (both  $p < 0.001$ ), with a modest positive correlation between CSF WBC and cell-free CSF mtDNA ( $r = 0.36$ ,  $p < 0.001$ ; Fig. 1). This association also persisted in individuals both on and off ART. After adjusting for CSF HIV RNA and nadir CD4<sup>+</sup> T-cell count, women had significantly lower median cell-free CSF mtDNA levels than men ( $p = 0.01$ ). No associations between CSF cell-free mtDNA levels and age, self-reported race/ethnicity, body-mass index, lifetime diagnosis (self-report) of diabetes mellitus, or depression (Beck's Depression Inventory Score) were identified.

### Association of cell-free CSF mtDNA levels with neurocognitive performance

No association was observed between CSF mtDNA levels and GDS-defined NCI. However, CSF mtDNA was significantly higher in 25 participants with MND compared to those without NCI ( $n = 193$ , 4.17 vs. 3.97  $\log_{10}$  copies/ml;  $p = 0.04$ ) or to those with ANI ( $n = 110$ , 4.17 vs. 3.93  $\log_{10}$  copies/ml;  $p = 0.02$ ). No individuals with MND had a mtDNA level less than 3.69  $\log_{10}$  copies/ml (Fig. 2).

We did not observe any association between the CSF mtDNA level and the trajectory of neurocognitive status, as  $t$  tests comparing the improved/stable vs. declined, and improved vs. declined groups showed no significant differences ( $p$  values of 0.96 and 0.73, respectively).

### Inflammation, angiogenesis, and iron biomarker associations with cell-free CSF mtDNA

In univariate analyses ( $n = 332$ ) comparing CSF mtDNA levels in the third (T3) and first (T1) tertiles of each biomarker, mtDNA levels were positively associated with CXCL-10 (T3 vs. T1,  $p < 0.001$ ) and TNF- $\alpha$  (T3 vs. T1,  $p = 0.04$ ) (Additional file 1: Table S2). No significant associations of mtDNA levels were seen with CSF iron, ferritin, transferrin, CP, HP, or other biomarkers, including VEGF, in unadjusted analyses. To better understand the independent contribution of cell-free mtDNA to inflammation and iron transport in the CNS, analyses of mtDNA were adjusted for the potentially confounding effects of HIV RNA, and CSF WBC. After adjustment for these covariates ( $n = 329$ ), lower cell-free CSF mtDNA levels were associated with higher levels of CSF transferrin



**Table 1** Study participant characteristics broken down by the presence or absence of HIV-associated neurocognitive impairment as defined by global deficit score

Variable (n, %, or median, IQR)	Overall (n = 332)	NP-unimpaired (n = 234)	NP-impaired (n = 98)	p value
Age (years)	43.0 (38.8–48.0)	43.0 (38.0–48.0)	45.0 (40.3–49.8)	0.019
Education (years)	12 (11–14)	12 (11–14)	13 (12–16)	0.030
Self-reported race/ethnicity (n, %)				
Black	138 (42%)	107 (46%)	31 (32%)	
Hispanic	30 (8%)	21 (9%)	12 (12%)	
White	152 (46%)	99 (42%)	53 (54%)	
Other	9 (3%)	7 (3%)	2 (2%)	0.095
Sex (n, % male)	262 (77%)	184 (79%)	78 (80%)	0.883
Diabetes mellitus (n, %)	31 (10%)	12 (5%)	19 (20%)	0.001
BMI	25.8 (23.2–29.0)	25.5 (23.1–29.3)	26.3 (24.1–28.6)	0.437
Plasma HIV RNA (log <sub>10</sub> copies/ml)	1.9 (1.7–4.0)	2.2 (1.7–4.1)	1.7 (1.7–3.5)	0.087
Undetectable HIV RNA in plasma (n, %)	113 (34%)	79 (34%)	34 (34%)	0.899
CSF HIV RNA (log <sub>10</sub> copies/ml)	1.7 (1.7–2.3)	1.7 (1.7–2.4)	1.7 (1.7–1.9)	0.164
Undetectable HIV RNA in CSF (n, %)	198 (60%)	132 (56%)	64 (65%)	0.144
Absolute CD4+ T-cell count	455 (270–636)	441 (264–645)	461(308–632)	0.639
CD4+ T-cell nadir	193 (60–315)	204 (69–325)	174(57–281)	0.224
HCV status negative (n, %)	78 (24%)	54 (23%)	24 (24%)	0.778
ART status (n, %)				
ART-naïve	51 (15%)	38 (16%)	13 (13%)	
ART	230 (69%)	158 (67%)	72 (74%)	
Non-ART	44 (13%)	34 (15%)	10 (10%)	
No ARVs	7 (2%)	4 (2%)	3 (3%)	0.508
WRAT	96 (85–105)	97(85–107)	94(83–102)	0.143
Beck's Depression Inventory Score	11(5–22)	11(5–20)	12(6–24)	0.399

Diabetes mellitus was self-reported and could be either type I or II  
 IQR interquartile range, ART highly active antiretroviral therapy, BMI body mass index, HCV hepatitis C virus, ARVs antiretroviral drugs, WRAT Wide Range Achievement Test, CSF cerebrospinal fluid, BMI body-mass index, NP neurocognitive performance

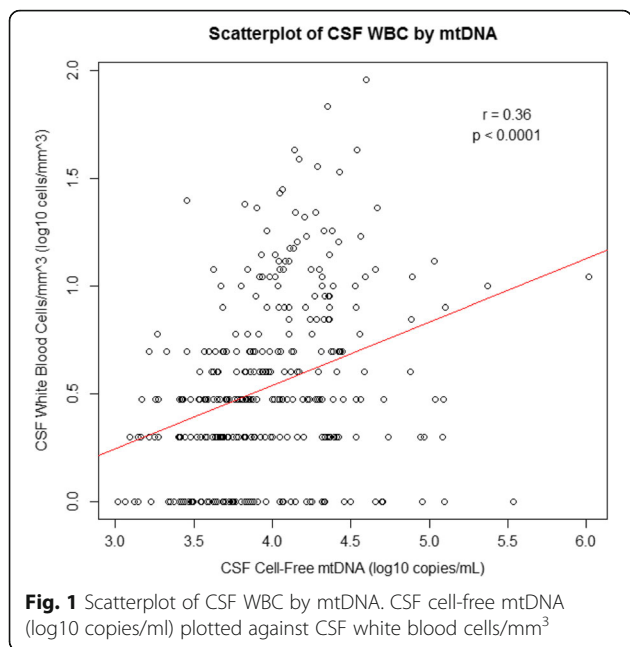
(T3 vs. T1; OR 0.69,  $p = 0.01$ ), and CP (T3 vs. T1; OR 0.71,  $p = 0.02$ ). Age did not contribute to any of these models. Associations with CXCL-10 and TNF- $\alpha$  did not persist after adjustment for CSF WBC and HIV RNA.

We also addressed potential confounding due to non-suppressed HIV infection by analysis restricted to individuals on ART. In this subgroup ( $n = 294$ ), CSF mtDNA was again negatively associated with CSF transferrin ( $p < 0.01$ ), CP ( $p = 0.06$ ), VEGF ( $p = 0.03$ ), and IL-6 ( $p = 0.05$ ). Since comorbid conditions can influence neurocognitive performance, we also stratified by the severity of comorbidities present. Among participants with comorbidities that were assessed to adversely affect neurocognitive performance, cell-free CSF mtDNA levels continued to be negatively associated with CSF transferrin ( $p = 0.02$ ), CP ( $p < 0.01$ ), VEGF ( $p < 0.05$ ), and IL-6 ( $p < 0.05$ ) ( $n = 134$  except for iron biomarker levels ( $n = 80$ )), even after adjusting for CSF WBC. Among individuals who only had

comorbidities deemed to be incidental, no associations with CSF mtDNA were identified.

## Discussion

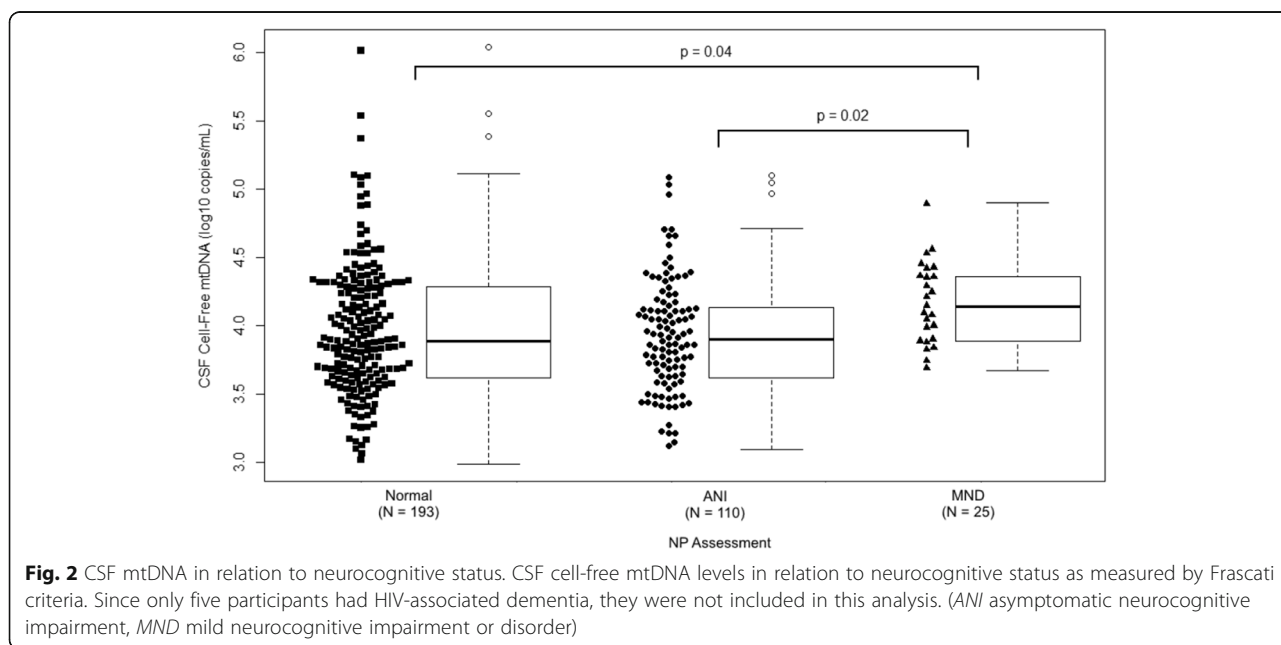
Cell-free mtDNA is a novel biomarker being explored in inflammation [30, 31], cancer [32, 33], heart disease [34, 35], diabetes [31], and neurodegenerative processes [13, 14, 36]. These published data support a complex relationship in which higher cell-free mtDNA levels were associated with neuroinflammation, while lower levels were associated with neurocognitive impairment [13, 14]. In this CHARTER analysis, we determined associations of cell-free CSF mtDNA with CSF biomarkers of inflammation, angiogenesis, and iron transport, and with HIV-associated neurocognitive impairment, a phenotype likely driven by both inflammatory and neurodegenerative processes and in which iron transport may also be abnormal [37, 38]. We observed cell-free mtDNA levels in the range reported by other groups [13], and these levels were higher in individuals with detectable CSF HIV RNA and



in the absence of ART. CSF mtDNA also increased with the levels of some (e.g., CXCL-10, TNF- $\alpha$ ) but not all (e.g., IL-6) biomarkers of inflammation in univariate analyses. However, only the association with IL-6 persisted after adjusting for HIV RNA and CSF WBC, suggesting that these covariates may at least partially mediate the relationship between CSF mtDNA and inflammation. Finally, although mtDNA levels did not distinguish impaired from unimpaired individuals, they were higher in those with mild NCI as compared to asymptotically impaired and

unimpaired individuals, suggesting a link between CSF mtDNA, neuroinflammation, and NCI in HIV infection.

Interestingly, after adjusting for the presence of HIV RNA and WBCs in CSF, which are possible drivers of the release of, or the source of, cell-free mtDNA, we found that higher mtDNA levels were associated with lower levels of two proteins involved in iron metabolism and transport, CP and transferrin, and the angiogenesis marker (VEGF), supporting novel mechanisms of CNS injury in HAND. CP plays a critical role through its ferroxidase activity which facilitates binding of iron to transferrin. In addition, both CP and VEGF have neuroprotective as well as strong angiogenic properties [39, 40]. CP and VEGF are also acute-phase proteins and may themselves reflect levels of neuroinflammation. However, the absence of positive associations of cell-free mtDNA levels with other measured inflammation markers in multivariable-adjusted analyses suggests that, in the absence of HIV replication in the CSF and CSF pleocytosis, increased mtDNA levels primarily reflect disrupted CNS iron transport in HIV-infected adults. For example, CP is expressed primarily in a membrane-bound form by astrocytes, pericytes, and brain endothelial cells to provide the ferroxidase activity needed for oxidation of ferrous iron that is released into the brain and bound to transferrin [41, 42]. Downregulation of CP can therefore lead to cellular iron accumulation, mitochondrial injury, and oxidative stress [42]. Likewise, lower levels of VEGF may have adverse effects, since this molecule has beneficial effects on mitochondrial integrity, and it inhibits autophagy [43]. Consistent with this interpretation, transferrin, which tends to



decline in the serum during inflammation but is essential for iron transport across the blood-brain barrier [44], was also negatively associated in CSF with mtDNA (when controlling for CSF HIV RNA and WBC). Altered CSF transferrin levels may indicate disrupted iron transport in the CNS, perhaps leading to mitochondrial degeneration, as cellular iron is an important co-factor for mitochondrial biogenesis [18]. In contrast to other biomarkers of inflammation, lower CSF IL-6 levels were consistently associated with higher mtDNA in individuals on ART. Although IL-6 is a well-known pro-inflammatory cytokine, it is also important in regulation of iron metabolism and macrophage iron content via the hepcidin-ferroportin axis [38]; hence, this association may relate mainly to its iron-regulatory functions. Inverse associations of all of these biomarkers with cell-free mtDNA therefore point to a disruption of iron homeostasis and induction of neuroprotective mechanisms in HIV-infected individuals, which may in turn be linked to altered mtDNA biogenesis and/or mitophagy in HAND [19, 45].

In persons with HIV RNA in the CSF, pleocytosis was more common than in those with suppressed HIV replication on ART [46–48], and in the present study, cell-free mtDNA levels correlated with CSF WBC as well as with CSF HIV RNA. The source of this mtDNA remains unclear, as mtDNA may be released by dying cells, or in neutrophil extracellular traps [49]. There may also be reduced mtDNA degradation as neurons depend on astrocytes for mitochondrial degradation (a process termed transmitophagy) [50]. Individuals chronically infected with HIV have impaired astrocyte function, leading to impaired clearance of neurotransmitters and other molecules through disrupted CNS proteostasis (reviewed in [51, 52]). In addition, the reduced CP association with higher CSF mtDNA may reflect sequestration of iron by astrocytes [53]. On the other hand, antiretroviral drugs, particularly those drugs with good CNS penetration, generally reduce CNS inflammation, and therefore, the association between ART and reduced CSF cell-free mtDNA is expected. However, some ART drugs (e.g., certain nucleoside analogues) can reduce cellular levels of mtDNA due to interference with mtDNA synthesis, and this observation could provide an alternative explanation for reduced CSF mtDNA levels in persons on ART.

Limitations of this study include the cross-sectional design, some degree of heterogeneity of treatment regimens in cohort participants on ART, and inability to extrapolate from cell-free mtDNA levels in CSF to intracellular mtDNA content in the brain. Nevertheless, this study provides new insights into the complex pathophysiology of neuroinflammation and mitochondrial damage during HIV infection.

## Conclusions

In conclusion, cell-free CSF mtDNA was inversely associated with biomarkers of iron transport in HIV-infected persons when controlling for CSF HIV RNA and WBCs. Given the importance of iron homeostasis in neuronal function [54] and also in regulating mitochondrial biogenesis, the relationship between mtDNA and markers of iron homeostasis [18] may reflect HIV-associated dysregulation of iron transport in the brain, including the possibility of iron deficiency in neurons and iron overload in astrocytes. This study is the first to report these specific associations in HIV-infected persons. These findings may have implications for the etiology of persistent neuroinflammation and neurodegeneration in HIV-infected persons and merit further investigation.

## Additional file

**Additional file 1: Table S1.** Categorization of the 216 individuals with follow-up neurocognitive assessments. Baseline assessments were compared to the assessment at the last follow-up visit, and individuals were categorized as improved, stable, or declined. **Table S2.** Univariate analyses of log CSF-free mtDNA and CSF biomarkers of inflammation and iron transport. (DOCX 12 kb)

## Abbreviations

AD: Alzheimer's disease; ANI: Asymptomatic neurocognitive improvement; ANOVA: Analysis of variance; ART: Antiretroviral therapy; CHARTER: CNS HIV Antiretroviral Therapy Effects Research; CNS: Central nervous system; CP: Ceruloplasmin; CSF: Cerebrospinal fluid; CXCL10: CXC motif chemokine 10; DAMP: Damage-associated molecular pattern; gDNA: Genomic DNA; GDS: Global deficit score; HAD: HIV-associated dementia; HAND: HIV-associated neurocognitive disorder; H-ferritin: Human heavy chain ferritin; HP: Haptoglobin; IQR: Interquartile range; MND: Mild neurocognitive disorder; mtDNA: Mitochondrial DNA; NC: Neurocognitive; NCI: Neurocognitive impairment; PD: Parkinson's disease; TF: Transferrin; TLR: Toll-like receptor; VEGF: Vascular endothelial growth factor; VL: HIV viral load

## Acknowledgements

The authors are grateful to the CHARTER study participants and for the efforts of all CHARTER site investigators, as follows: Director: Igor Grant, M.D.; Co-Directors: Scott L. Letendre, M.D., Ronald J. Ellis, M.D., Ph.D., Thomas D. Marcotte, Ph.D.; Center Manager: Donald Franklin, Jr.; Neuromedical Component: Ronald J. Ellis, M.D., Ph.D. (P.I.), J. Allen McCutchan, M.D.; Laboratory and Virology Component: Scott Letendre, M.D. (Co-P.I.), Davey M. Smith, M.D. (Co-P.I.); Neurobehavioral Component: Robert K. Heaton, Ph.D. (P.I.), J. Hampton Atkinson, M.D., Matthew Dawson; Imaging Component: Christine Fennema-Notestine, Ph.D. (P.I.), Michael J Taylor, Ph.D., Rebecca Theilmann, Ph.D.; Data Management Component: Anthony C. Gamst, Ph.D. (P.I.), Clint Cushman; Statistics Component: Ian Abramson, Ph.D. (P.I.), Florin Vaida, Ph.D., Reena Deutsch, Ph.D.; Johns Hopkins University site: Ned Sacktor (P.I.), Vincent Rogalski; Icahn School of Medicine at Mount Sinai site: Susan Morgello, M.D. (Co-P.I.) and David Simpson, M.D. (Co-P.I.), Letty Mintz, N.P.; University of California, San Diego site: J. Allen McCutchan, M.D. (P.I.), Kaori Phillips, B.S.N.; University of Washington, Seattle site: Ann Collier, M.D. (Co-P.I.) and Christina Marra, M.D. (Co-P.I.), Sher Storey, PA-C; University of Texas, Galveston site: Benjamin Gelman, M.D., Ph.D. (P.I.), Eleanor Head, R.N., B.S.N.; and Washington University, St. Louis Site: David Clifford, M.D. (P.I.), Muhammad Al-Lozi, M.D., Mengesha Teshome, M.D.

## Funding

We wish to acknowledge the following sources of funding: National Institutes of Health N01 MH22005, HHSN271201000036C, and HHSN271201000030C (to CHARTER); R01 MH095621 and Supplemental Award NIH MH095621 04S1 (to AK and TH); K23 AI093163 (to SRM); NS077384, AI69495, NR012907, NR014449, NR012657, UL1 TR000448, and



Alzheimer Association (to DC); Interdisciplinary Research Fellowship in NeuroAIDS (JPS).

#### Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the CHARTER repository on reasonable request.

#### Authors' contributions

SRM helped design the study and wrote the manuscript. JPS, TRCD, JDS performed the cell-free CSF mtDNA analysis. TH helped design and coordinate the study and edited the manuscript. JSB and HG performed the statistical analysis. SLL, RJL, JAM, RH, SM, DS, CCM, BBG, DBC, ACC, JM, DR, DF, and IG are all investigators or administrators for the CHARTER study, and assisted with the collection of data. JC and SP performed the iron biomarker analysis. AK helped design and coordinate the study, and edited the manuscript. All authors read and approved the final manuscript.

#### Competing interests

The authors declare that they have no competing interests.

#### Consent for publication

All subjects provided written consent to participate in the study. No individual protected health information is included in this analysis.

#### Ethics approval and consent to participate

The study was approved by institutional review boards at each site, and all participants provided written informed consent.

#### Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

#### Author details

<sup>1</sup>Department of Medicine, University of California-San Diego, San Diego, CA, USA. <sup>2</sup>Department of Medicine, San Diego Veterans Affairs Medical Center, San Diego, CA, USA. <sup>3</sup>Department of Psychiatry, University of California, San Diego, CA, USA. <sup>4</sup>Department of Medicine, Division of Infectious Diseases, Vanderbilt University, Nashville, TN, USA. <sup>5</sup>Division of Biostatistics, Washington University, St. Louis, MO, USA. <sup>6</sup>Case Comprehensive Cancer Center, Case Western Reserve University School of Medicine, Cleveland, OH, USA. <sup>7</sup>Department of Neurology, University of California-San Diego, San Diego, CA, USA. <sup>8</sup>Department of Neurosurgery, Pennsylvania State/Hershey College of Medicine, Hershey, PA, USA. <sup>9</sup>Department of Neurology, Washington University, St. Louis, MO, USA. <sup>10</sup>Department of Medicine, University of Washington, Seattle, WA, USA. <sup>11</sup>Department of Neurology, University of Washington, Seattle, WA, USA. <sup>12</sup>Department of Pathology, University of Texas Medical Branch, Galveston, TX, USA. <sup>13</sup>Department of Neurology, Johns Hopkins University, Baltimore, MD, USA. <sup>14</sup>Department of Neurology, Icahn School of Medicine at Mount Sinai, New York, NY, USA. <sup>15</sup>Genomic Medicine Institute/Lerner Research Institute Cleveland Clinic, Cleveland, OH, USA. <sup>16</sup>Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, OH, USA.

Received: 18 January 2017 Accepted: 21 March 2017

Published online: 31 March 2017

#### References

- Chen MF, Gill AJ, Kolson DL. Neuropathogenesis of HIV-associated neurocognitive disorders: roles for immune activation, HIV blipping and viral tropism. *Curr Opin HIV AIDS*. 2014;9:559–64.
- Williams DW, Veenstra M, Gaskill PJ, Morgello S, Calderon TM, Berman JW. Monocytes mediate HIV neuropathogenesis: mechanisms that contribute to HIV associated neurocognitive disorders. *Curr HIV Res*. 2014;12:85–96.
- McCutchan JA, Marquie-Beck JA, Fitzsimons CA, Letendre SL, Ellis RJ, Heaton RK, et al. Role of obesity, metabolic variables, and diabetes in HIV-associated neurocognitive disorder. *Neurology*. 2012;78:485–92.
- Lane RK, Hilsabeck T, Rea SL. The role of mitochondrial dysfunction in age-related diseases. *Biochim Biophys Acta*. 1847;2015:1387–400.
- Berdanier CD, Everts HB. Mitochondrial DNA in aging and degenerative disease. *Mutat Res*. 2001;475:169–83.
- Xie YM, Jin L, Chen XJ, He MN, Wang Y, Liu R, et al. Quantitative changes in mitochondrial DNA copy number in various tissues of pigs during growth. *Genet Mol Res GMR*. 2015;14:1662–70.
- West AP, Koblansky AA, Ghosh S. Recognition and signaling by toll-like receptors. *Annu Rev Cell Dev Biol*. 2006;22:409–37.
- Pinto M, Moraes CT. Mitochondrial genome changes and neurodegenerative diseases. *Biochim Biophys Acta*. 1842;2014:1198–207.
- Pinto M, Moraes CT. Mechanisms linking mtDNA damage and aging. *Free Radic Biol Med*. 2015;85:250–8.
- Var SR, Day TRC, Vitomirov A, Smith DM, Soontornniyomkij V, Moore DJ, et al. Mitochondrial injury and cognitive function in HIV infection and methamphetamine use. *AIDS Lond Engl*. 2016;30:839–48.
- Coskun P, Wyrembak J, Schriener SE, Chen H-W, Marciniack C, LaFerla F, et al. A mitochondrial etiology of Alzheimer and Parkinson disease. *Biochim Biophys Acta BBA - Gen Subj*. 1820;2012:553–64.
- Walko TD, Bola RA, Hong JD, Au AK, Bell MJ, Kochanek PM, et al. Cerebrospinal fluid mitochondrial DNA: a novel DAMP in pediatric traumatic brain injury. *Shock*. 2014;41:499–503.
- Podlesniy P, Figueiro-Silva J, Llado A, Antonell A, Sanchez-Valle R, Alcolea D, et al. Low cerebrospinal fluid concentration of mitochondrial DNA in preclinical Alzheimer disease. *Ann Neurol*. 2013;74:655–68.
- Pyle A, Brennan R, Kurzawa-Akanbi M, Yarnall A, Thouin A, Mollenhauer B, et al. Reduced cerebrospinal fluid mitochondrial DNA is a biomarker for early-stage Parkinson's disease. *Ann Neurol*. 2015;78:1000–4.
- Crespo AC, Silva B, Marques L, Marcelino E, Maruta C, Costa S, et al. Genetic and biochemical markers in patients with Alzheimer's disease support a concerted systemic iron homeostasis dysregulation. *Neurobiol Aging*. 2014;35:777–85.
- Medeiros MS, Schumacher-Schuh A, Cardoso AM, Bochi GV, Baldissarelli J, Kegler A, et al. Iron and oxidative stress in Parkinson's disease: an observational study of injury biomarkers. *PLoS ONE*. 2016;11:e0146129.
- Ringman JM, Schulman H, Becker C, Jones T, Bai Y, Immermann F, et al. Proteomic changes in cerebrospinal fluid of presymptomatic and affected persons carrying familial Alzheimer disease mutations. *Arch Neurol*. 2012;69:96–104.
- Rensvold JW, Ong S-E, Jeevananthan A, Carr SA, Mootha VK, Pagliarini DJ. Complementary RNA and protein profiling identifies iron as a key regulator of mitochondrial biogenesis. *Cell Rep*. 2013;3:237–45.
- Allen GFG, Toth R, James J, Ganley IG. Loss of iron triggers PINK1/Parkin-independent mitophagy. *EMBO Rep*. 2013;14:1127–35.
- Andersen HH, Johnsen KB, Moos T. Iron deposits in the chronically inflamed central nervous system and contributes to neurodegeneration. *Cell Mol Life Sci CMLS*. 2014;71:1607–22.
- Wen X, Wu J, Wang F, Liu B, Huang C, Wei Y. Deconvoluting the role of reactive oxygen species and autophagy in human diseases. *Free Radic Biol Med*. 2013;65:402–10.
- Schiavi A, Maglioni S, Palikaras K, Shaik A, Strappazon F, Brinkmann V, et al. Iron-starvation-induced mitophagy mediates lifespan extension upon mitochondrial stress in *C. elegans*. *Curr Biol CB*. 2015;25:1810–22.
- Heaton RK, Clifford DB, Franklin DR, Woods SP, Ake C, Vaida F, et al. HIV-associated neurocognitive disorders persist in the era of potent antiretroviral therapy: CHARTER Study. *Neurology*. 2010;75:2087–96.
- Heaton RK, Franklin DR, Deusch R, Letendre S, Ellis RJ, Casaletto K, et al. Neurocognitive change in the era of HIV combination antiretroviral therapy: the longitudinal CHARTER study. *Clin Infect Dis Off Publ Infect Dis Soc Am*. 2015;60:473–80.
- Antinori A, Arendt G, Becker JT, Brew BJ, Byrd DA, Cherner M, et al. Updated research nosology for HIV-associated neurocognitive disorders. *Neurology*. 2007;69:1789–99.
- Blackstone K, Moore DJ, Franklin DR, Clifford DB, Collier AC, Marra CM, et al. Defining neurocognitive impairment in HIV: deficit scores versus clinical ratings. *Clin Neuropsychol*. 2012;26:894–908.
- Ellis RJ, Hsia K, Spector SA, Nelson JA, Heaton RK, Wallace MR, et al. Cerebrospinal fluid human immunodeficiency virus type 1 RNA levels are elevated in neurocognitively impaired individuals with acquired immunodeficiency syndrome. HIV Neurobehavioral Research Center Group. *Ann Neurol*. 1997;42:679–88.
- Ellis RJ, Badie J, Vaida F, Letendre S, Heaton RK, Clifford D, et al. CD4 nadir is a predictor of HIV neurocognitive impairment in the era of combination antiretroviral therapy. *AIDS Lond Engl*. 2011;25:1747–51.
- R Core Team. R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2014. Available from: URL <http://www.R-project.org/>.

30. Arnalich F, Codoceo R, López-Collazo E, Montiel C. Circulating cell-free mitochondrial DNA: a better early prognostic marker in patients with out-of-hospital cardiac arrest. Resuscitation. 2012;83:e162–3.
31. Alvarado-Vásquez N. Circulating cell-free mitochondrial DNA as the probable inducer of early endothelial dysfunction in the prediabetic patient. *Exp Gerontol*. 2015;69:70–8.
32. Lu H, Busch J, Jung M, Rabenhorst S, Ralla B, Kilic E, et al. Diagnostic and prognostic potential of circulating cell-free genomic and mitochondrial DNA fragments in clear cell renal cell carcinoma patients. *Clin Chim Acta Int J Clin Chem*. 2016;452:109–19.
33. Yu M. Circulating cell-free mitochondrial DNA as a novel cancer biomarker: opportunities and challenges. *Mitochondrial DNA*. 2012;23:329–32.
34. Liu J, Cai X, Xie L, Tang Y, Cheng J, Wang J, et al. Circulating cell free mitochondrial DNA is a biomarker in the development of coronary heart disease in the patients with type 2 diabetes. *Clin Lab*. 2015;61:661–7.
35. Sudakov NP, Popkova TP, Katyshev AI, Goldberg OA, Nikiforov SB, Pushkarev BG, et al. Level of Blood Cell-Free Circulating Mitochondrial DNA as a Novel Biomarker of Acute Myocardial Ischemia. *Biochem*. 2015;80:1387–92.
36. Pérez-Santiago J, Schrier RD, de Oliveira MF, Gianella S, Var SR, Day TRC, et al. Cell-free mitochondrial DNA in CSF is associated with early viral rebound, inflammation, and severity of neurocognitive deficits in HIV infection. *J Neurovirol*. 2015;22(2):191–200.
37. Kallianpur AR, Levine AJ. Host genetic factors predisposing to HIV-associated neurocognitive disorder. *Curr HIV/AIDS Rep*. 2014;11:336–52.
38. Kallianpur AR, Wang Q, Jia P, Hulgan T, Zhao Z, Letendre SL, et al. Anemia and red blood cell indices predict HIV-associated neurocognitive impairment in the highly active antiretroviral therapy era. *J Infect Dis*. 2016; 213:1065–73.
39. Jayson GC, Kerbel R, Ellis LM, Harris AL. Antiangiogenic therapy in oncology: current status and future directions. *Lancet Lond Engl*. 2016;388(10043):518–29.
40. Lowndes SA, Harris AL. Copper chelation as an antiangiogenic therapy. *Oncol Res*. 2004;14:529–39.
41. Burkhart A, Skjørringe T, Johnsen KB, Siupka P, Thomsen LB, Nielsen MS, et al. Expression of iron-related proteins at the neurovascular unit supports reduction and reoxidation of iron for transport through the blood-brain barrier. *Mol Neurobiol*. 2016;53(10):7237–53.
42. Arber CE, Li A, Houlden H, Wray S. Insights into molecular mechanisms of disease in neurodegeneration with brain iron accumulation: unifying theories. *Neuropathol Appl Neurobiol*. 2015;42(3):220–41.
43. Kumar S, Guru SK, Pathania AS, Kumar A, Bhushan S, Malik F. Autophagy triggered by magnolol derivative negatively regulates angiogenesis. *Cell Death Dis*. 2013;4:e889.
44. Simpson IA, Ponnuru P, Klinger ME, Myers RL, Devraj K, Coe CL, et al. A novel model for brain iron uptake: introducing the concept of regulation. *J Cereb Blood Flow Metab Off J Int Soc Cereb Blood Flow Metab*. 2015;35:48–57.
45. Ivatt RM, Whitworth AJ. The many faces of mitophagy. *EMBO Rep*. 2014;15:5–6.
46. Ho EL, Ronquillo R, Altmeyden H, Spudich SS, Price RW, Sinclair E. Cellular composition of cerebrospinal fluid in HIV-1 infected and uninfected subjects. *PLoS ONE*. 2013;8:e66188.
47. Tiraboschi JM, Muñoz-Moreno JA, Puertas MC, Alonso-Villaverde C, Prats A, Ferrer E, et al. Viral and inflammatory markers in cerebrospinal fluid of patients with HIV-1-associated neurocognitive impairment during antiretroviral treatment switch. *HIV Med*. 2015;16:388–92.
48. Marra CM, Maxwell CL, Collier AC, Robertson KR, Imrie A. Interpreting cerebrospinal fluid pleocytosis in HIV in the era of potent antiretroviral therapy. *BMC Infect Dis*. 2007;7:37.
49. McIlroy DJ, Jarnicki AG, Au GG, Lott N, Smith DW, Hansbro PM, et al. Mitochondrial DNA neutrophil extracellular traps are formed after trauma and subsequent surgery. *J Crit Care*. 2014;29:1133.e1–5.
50. Davis CO, Kim K-Y, Bushong EA, Mills EA, Boassa D, Shih T, et al. Transcellular degradation of axonal mitochondria. *Proc Natl Acad Sci U S A*. 2014;111: 9633–8.
51. Borgmann K, Ghorpade A. HIV-1, methamphetamine and astrocytes at neuroinflammatory Crossroads. *Front Microbiol*. 2015;6:1143.
52. Dickens AM, Anthony DC, Deutsch R, Mielke MM, Claridge TDW, Grant I, et al. Cerebrospinal fluid metabolomics implicate bioenergetic adaptation as a neural mechanism regulating shifts in cognitive states of HIV-infected patients. *AIDS Lond Engl*. 2015;29:559–69.
53. Zhao L, Hadziahmetovic M, Wang C, Xu X, Song Y, Jinnah HA, et al. Cp/ Heph mutant mice have iron-induced neurodegeneration diminished by deferiprone. *J Neurochem*. 2015;135:958–74.
54. Singh N, Haldar S, Tripathi AK, Horback K, Wong J, Sharma D, et al. Brain iron homeostasis: from molecular mechanisms to clinical significance and therapeutic opportunities. *Antioxid Redox Signal*. 2014;20:1324–63.

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at  
[www.biomedcentral.com/submit](http://www.biomedcentral.com/submit)

