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Cerebrospinal fluid metabolomics reveals altered waste clearance and accelerated aging in HIV patients with neurocognitive impairment

Edana Cassol^{a,b}, Vikas Misra^a, Anupriya Dutta^{a,b}, Susan Morgello^c and Dana Gabuzda^{a,b,d}

Objective(s): HIV-associated neurocognitive disorders (HAND) remain prevalent in HIV-infected patients on antiretroviral therapy (ART), but the underlying mechanisms are unclear. Some features of HAND resemble those of age-associated cognitive decline in the absence of HIV, suggesting that overlapping mechanisms may contribute to neurocognitive impairment.

Design: Cross-sectional analysis of cerebrospinal fluid (CSF) from 100 individuals (46 HIV-positive patients and 54 HIV-negative controls).

Methods: Untargeted CSF metabolite profiling was performed using liquid/gas chromatography followed by mass spectrometry. Cytokine profiling was performed by Bioplex. Bioinformatic analyses were performed in Metaboanalyst and R.

Results: Alterations in the CSF metabolome of HIV patients on ART mapped to pathways associated with neurotransmitter production, mitochondrial function, oxidative stress, and metabolic waste. Many CSF metabolites altered in HIV overlapped with those altered with advanced age in HIV-negative controls, suggesting a pattern indicative of accelerated aging. Machine learning models identified neurotransmitters (glutamate, N-acetylaspartate), markers of glial activation (myo-inositol), and ketone bodies (beta-hydroxybutyric acid, 1,2-propanediol) as top-ranked classifiers of HAND. These CSF metabolites correlated with worse neurocognitive test scores, plasma inflammatory biomarkers [interferon (IFN)- α , IFN- γ , interleukin (IL)-8, IL-1 β , IL-6, IL-2Ra], and intrathecal IFN responses (IFN- γ and kynurenine : tryptophan ratio), suggesting inter-relationships between systemic and intrathecal inflammation and metabolic alterations in CSF.

Conclusions: Alterations in the CSF metabolome of HIV patients on ART suggest that persistent inflammation, glial responses, glutamate neurotoxicity, and altered brain waste disposal systems contribute to mechanisms involved in HAND that may be augmented with aging. © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins

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Keywords: aging, antiretroviral therapy, HIV, HIV-associated neurocognitive disorders, inflammation, metabolomics

Introduction

Despite reduced incidence of severe forms of HIV-associated neurocognitive disorders (HAND) in HIV patients on combination antiretroviral therapy (ART),

mild forms including asymptomatic neurocognitive impairment (ANI) and minor neurocognitive disorder (MND) remain prevalent, affecting 20–50% [1–3]. Prior to the introduction of ART, factors associated with HAND included plasma and cerebrospinal fluid (CSF) HIV RNA

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and inflammation markers [e.g. chemokine (CC motif) ligand 2 (CCL2), tumor necrosis factor (TNF), interleukin (IL)-6, neopterin] [4–8]. In HIV patients on ART, HAND is associated with older age (> age 50), lower nadir CD4⁺, innate immune activation, chronic inflammation, hepatitis C virus (HCV) coinfection, cardiovascular risk factors, and metabolic disorders [9–16]. The multifactorial nature of factors contributing to HAND suggests these disorders consist of subtypes reflecting distinct pathophysiological mechanisms [3,8,17].

HIV patients on ART have a higher burden of neurological disorders with advancing age compared to HIV-negative controls, and these disorders occur at a younger age [18,19]. The increased prevalence of these disorders, as well as other age-associated comorbidities, including cardiovascular, kidney, liver, and bone disease, is thought to reflect accelerated aging [20–22]. Chronic inflammation plays an important role in this phenotype, termed ‘inflammaging’, and predicts age-associated comorbidities and mortality in HIV patients [23–25]. Markers of inflammation are detected in CSF and brain from HIV patients on ART [26–31]. Neuroinflammation is a prominent feature of age-associated neurodegenerative diseases including Alzheimer’s and Parkinson’s disease, and has been associated with altered synaptic connectivity and blood–brain barrier (BBB) function and neuronal injury [32–34]. Whether similar mechanisms contribute to HAND is unclear.

Chronic HIV infection is associated with metabolic changes in brain, even among patients on suppressive ART [27,35–37]. Whereas brain tissue is difficult to obtain, CSF is accessible and reflects the biochemical milieu of the central nervous system [38–40]. Early targeted studies of CSF metabolites identified alterations in several neurotoxic metabolites including those associated with the kynurenine (e.g. quinolinic acid) and nitric oxide pathways during HIV and simian immunodeficiency virus (SIV) infection [7,41,42]. CSF lipidomics identified alterations in lipid metabolism, including increased carnitine, acyl-carnitines, fatty acids, and phospholipids in SIV infection [43], and increased ceramides, sphingomyelins, and cholesterol in HIV patients on ART with HAND [44–47]. Here, we performed untargeted metabolomics of CSF from 100 HIV patients and HIV-negative controls to identify altered metabolic pathways associated with HAND. We also examined relationships between these metabolic alterations and those associated with advancing age in HIV-negative controls.

Methods

Study participants

Cerebrospinal fluid samples from HIV patients ($n = 46$; 36 on ART and 10 not on ART) collected between 1999 and 2009 were from the National NeuroAIDS Tissue

Consortium (NNTC) (Manhattan HIV Brain Bank, National Neurological AIDS Bank, California NeuroAIDS Tissue Network, Texas NeuroAIDS Research Center) and CNS HIV Anti-Retroviral Therapy Effects Research (CHARTER) study. Matched plasma metabolite profiles were available for 20 HIV patients. All HIV patients were enrolled with written informed consent and institutional review board (IRB) approval. Inclusion criteria were advanced disease (nadir CD4⁺ <300 cells/ μ l). Exclusion criteria were confounding neurological and psychiatric disorders, systemic opportunistic infection, severe hepatotoxicity (defined as grades 3 or 4 by AIDS Clinical Trials Group [48]), and moderate/severe renal insufficiency [49]. HAND clinical diagnoses were determined using established criteria [50]. Neuropsychological impairment due to other causes (NPI-O) was diagnosed when factors in addition to HIV made significant contributions to neurocognitive impairment (NCI). HIV and HCV-negative control CSF samples from young (<50 years old) and older (≥ 50 years old) patients collected between 2010 and 2011 were from Bioreclamation (Westbury, New York) and used with Dana-Farber Cancer Institute IRB approval. Samples were de-identified remnants from diagnostic testing, prescreened for sCD14 and CCL2, to exclude those with levels outside normal ranges reported in the literature (>0.25 μ g/ml and >1000 pg/ml, respectively).

Neurocognitive testing and neurocognitive impairment classification

All participants were administered a comprehensive test battery designed to assess seven domains of neurocognitive function (Supplemental Digital Content 1, <http://links.lww.com/QAD/A519>). Demographically corrected global T scores were generated from individual-domain T scores as described [51]. HIV patients were classified as impaired if they had a HAND clinical diagnosis (ANI, MND, HAD, or NPI-O) together with global T score less than 40 (or at least two domain T scores <40). Patients were classified as not impaired if they had no clinical diagnosis of HAND and global T score at least 40. Three patients with an ANI diagnosis, global T scores at least 40, and only one domain score less than 40 were classified as not impaired.

Quantification of soluble markers in cerebrospinal fluid and plasma

Interferon (IFN)- α , IFN- γ , IL-8, C-X-C motif chemokine ligand (CXCL)9, CXCL10, IL-1b, IL-6, TNF- α and IL-2 receptor alpha (Ra) were measured using Bioplex (Bio-Plex System; Bio-Rad Laboratories, Hercules, California, USA). Soluble CD14 (sCD14) and CCL2 were quantified by ELISA (R&D Systems, Minneapolis, Minnesota, USA).

Metabolomic profiling

Metabolomic profiling was performed by Metabolon (Durham, North Carolina, USA) using ultra high

performance liquid or gas chromatography and tandem mass spectrometry as described in the Supplemental Methods (Supplemental Digital Content 1, <http://links.lww.com/QAD/A519>) [52,53].

Data processing, bioinformatics, and statistical analysis

Metabolite data were normalized by median centering. Missing values were imputed with the lower limit of detection for a given metabolite. Significantly altered metabolites were defined by fold change greater than 1.2, a *P*-value less than 0.05, and false discovery rate (FDR) 10% or less. Classification analysis [principal component analysis (PCA), partial least-squares discriminant analysis (PLS-DA), random forest, support vector machine (SVM), and unsupervised hierarchical clustering] were performed in Metaboanalyst (<http://www.metaboanalyst.ca>). Quantitative enrichment analysis was performed in Metabolite Set Enrichment Analysis (MSEA) using the Metabolic Pathways library. Visualization of pathway mapping [Kyoto Encyclopedia of Genes and Genomes (KEGG) and Small Molecule Pathway Database (SMPDB) pathways] was performed in Cytoscape. Additional statistical analyses were performed on log-transformed data in R. Pearson correlations were used to evaluate relationships between metabolites ($P < 0.05$, $FDR \leq 0.1$). Spearman correlations were used to examine relationships between metabolites, global and domain T scores, and markers of intrathecal [sCD14, CCL2, IL-6, IFN- γ , and kynurenine to tryptophan ratio (K : T)] and systemic inflammation (IFN- α , IFN- γ , IL-8, CXCL9, CXCL10, IL-1b, IL-6, IL-2Ra, sCD14, and CCL2). Multiple hypothesis testing corrections were performed for fold change and correlation analyses by calculating FDR in *fdrtool*.

Results

HIV and aging cohorts

The HIV cohort was predominately male with late-stage disease (nadir CD4⁺ <300 cells/ μ l) and high prevalence of HCV coinfection (64%) (Supplemental Digital Content 2, <http://links.lww.com/QAD/A519>), and included young and older patients (50% <45 years and 50% \geq 45 years). Two patients had mild hyperlipidemia, one had lipodystrophy, and one had diabetes. Of those on ART [$n = 36$; median time on ART 26 months; interquartile range (IQR) 16–49], 72% were on protease inhibitors, 100% were on nucleoside reverse transcriptase inhibitors (NRTIs), and 31% were on drugs associated with mitochondrial toxicity (zidovudine, stavudine and didanosine) HIV patients on ART ($n = 36$) had lower plasma (mean log₁₀ 2.87 vs. 4.99 copies/ml; $P < 0.001$) and CSF viral loads (mean log₁₀ 1.68 vs. 2.87 copies/ml; $P = 0.009$) than those not on ART ($n = 10$). HIV patients had normal CSF protein levels and white blood cells

counts. The HIV cohort had a high prevalence of NCI (69%), the majority having ANI or MND. The aging cohort was composed of HIV-negative controls. Among them, 44% were young [$<$ age 50; median age 40 (33–44)] and 56% were older [\geq age 50; median age 57 (53–67)].

Characterization of cerebrospinal fluid metabolomes from HIV and aging cohorts

Untargeted metabolomic profiling of 100 CSF samples detected 199 (145 named and 54 unnamed) and 204 (149 named and 55 unnamed) metabolites in the HIV and aging cohorts, respectively (Supplemental Digital Content 3, <http://links.lww.com/QAD/A519>). To reduce noise in the analysis, preprocessing was performed to exclude xenobiotics and metabolites with more than 70% imputed values. One HIV-negative sample was excluded on the basis of outlier analysis in Metaboanalyst. One hundred and seven named metabolites detected across both cohorts met the acceptability criteria. The majority of the detected metabolites were amino acids (49%), followed by carbohydrates (19%), lipids (16%), and nucleotides (8%), and were mapped to biologically relevant pathways including neurotransmitters [glutamate, N-acetylaspartate (NAA), N-acetylaspartylglutamic acid (NAAG), glycine]; pathways associated with neurotransmitter production [phenylalanine and tyrosine metabolites (dopamine), tryptophan metabolites (serotonin), and homocarnosine (gamma-aminobutyric acid (GABA))]; markers of glial activation (choline, myo-inositol, arachidonate); markers of mitochondrial dysfunction (acyl-carnitines and Krebs cycle components); oxidation products (5-oxoproline and homocarnosine) and markers of oxidative stress (purine metabolites); and metabolic waste products (ketone bodies, lactate, creatinine, phenylacetylglutamine, p-cresol sulfate).

Alterations in the cerebrospinal fluid metabolome of HIV patients on antiretroviral therapy map to pathways associated with neurotransmitter production, mitochondrial dysfunction, oxidative stress, and metabolic waste

Fifteen named and 12 unnamed metabolites distinguished between HIV patients on ART ($n = 36$) and age and sex-matched HIV-negative controls (fold change > 1.2 , $P < 0.01$, $FDR < 10\%$; Fig. 1a, Supplemental Digital Content 4 and 5, <http://links.lww.com/QAD/A519>). The 15 named metabolites classified HIV vs. control individuals with more than 90% predictive accuracy in random forest. Mapping altered named metabolites to KEGG and SMPDB pathways identified alterations in aspartate and glutamate metabolism, phenylalanine and tyrosine metabolism, GABA synthesis, Krebs cycle, mitochondrial electron transport chain, carnitine metabolism, glutathione metabolism, and ketone body metabolism, corresponding to metabolic alterations associated with altered neurotransmitter production, mitochondrial

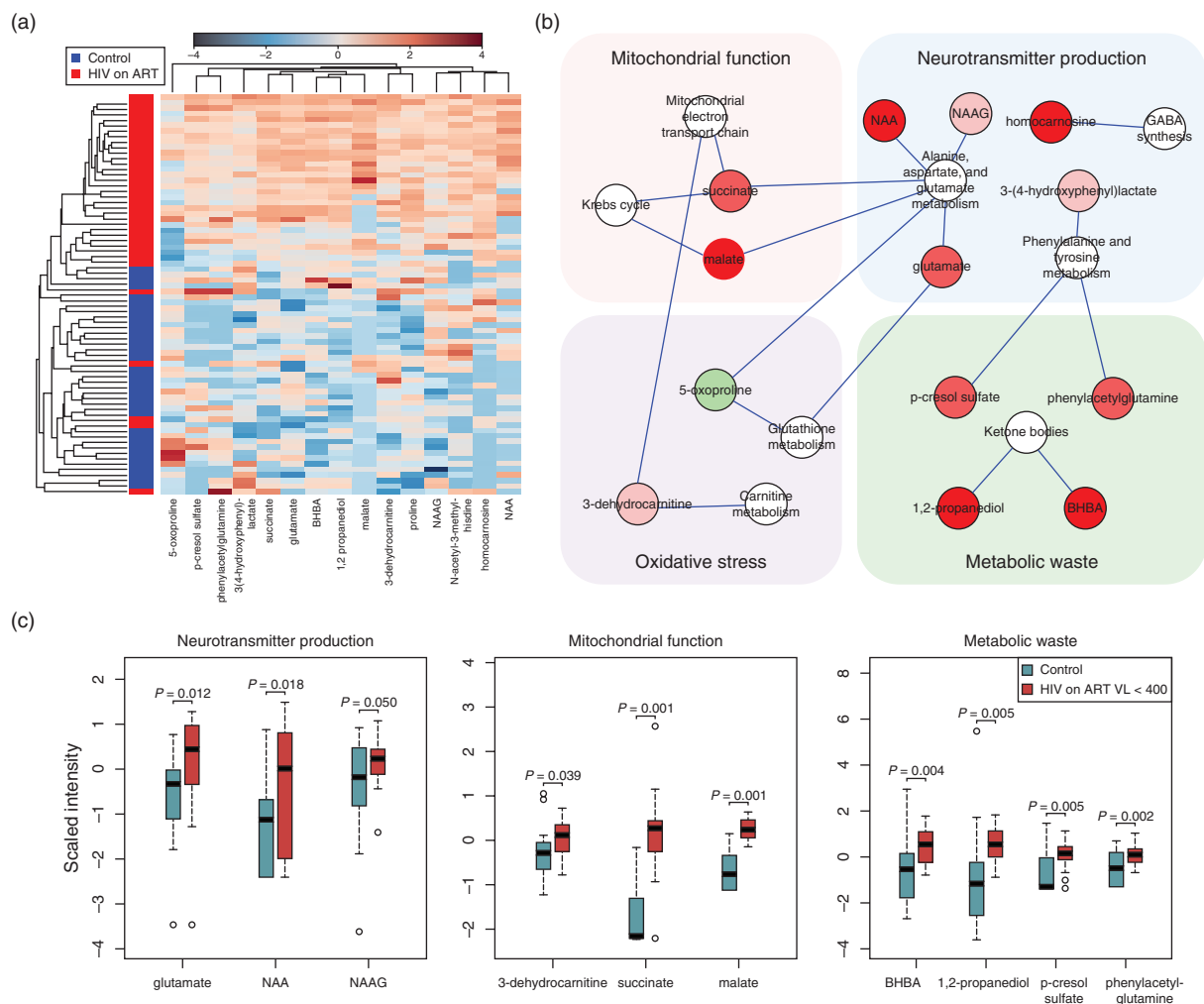


Fig. 1. Cerebrospinal fluid metabolomics identifies metabolites that distinguish HIV patients on antiretroviral therapy from HIV-negative controls. (a) Unsupervised hierarchical clustering of signature metabolites ($n = 15$; FC > 1.2, $P < 0.01$, FDR < 10%) altered in HIV patients on ART ($n = 36$, red) compared to age and sex-matched HIV-negative controls ($n = 36$, blue). Red and blue indicate increased and decreased metabolite levels, respectively. FDR was used to correct for multiple hypothesis testing. (b) Metabolites altered in HIV patients on ART compared to HIV-negative controls mapped to biosynthetic pathways linked to production of neurotransmitters, mitochondrial dysfunction, oxidative stress, and metabolic waste products. Altered metabolites (FC > 1.2, $P < 0.01$, FDR < 10%) were mapped to metabolite pathways and interaction networks were generated in Cytoscape. Green and red nodes represent metabolites with increased and decreased levels, respectively. White nodes represent pathways. (c) Box plots of metabolites altered in HIV patients on ART with low plasma viral loads ($n = 20$, plasma VL < 400 copies/ml, CSF VL < 50 copies/ml) compared to age and sex-matched HIV-negative controls ($n = 20$) that could be mapped to biological processes associated with NCI. Medians are represented by horizontal bars, boxes span the interquartile range (IQR) and whiskers extend to extreme data points within 1.5 times IQR. Outliers plotted as open circles lie outside 1.5 times the IQR. Blue and red represent controls and HIV patients, respectively. The P -values were calculated using Welch's t -tests. ART, antiretroviral therapy; BHBA, beta-hydroxybutyric acid; CSF, cerebrospinal fluid; FC, fold change; FDR, false discovery rate; GABA, gamma-aminobutyric acid; NAA, N-acetylaspartate; NAAG, N-acetylaspartylglutamate; NCL, neurocognitive impairment; VL, viral load.

dysfunction, oxidative stress, and accumulation of metabolic waste products (Fig. 1b). Fourteen of these 15 named metabolites were altered in the subgroup of HIV patients on ART with suppressed viral replication ($n = 20$, plasma viral load < 400 copies/ml, CSF viral load < 50 copies/ml) compared to age and sex-matched HIV-negative controls ($n = 20$; Fig. 1c). Eleven were altered in the subgroup of HIV patients on stable ART

(> 2 years) with maximally suppressed plasma viral loads (< 50 copies/ml) ($n = 10$ per group; Supplemental Digital Content 6, <http://links.lww.com/QAD/A519>) and 13 were altered in HIV patients not on ART ($n = 10$ patients per group, median CSF viral load 328 copies/ml, median plasma viral load 76 936 copies/ml) (Supplemental Digital Content 5, <http://links.lww.com/QAD/A519>). An additional 12 metabolites were altered only in HIV

patients not on ART, including kynurenine and markers of glial cell activation (choline and myo-inositol) (Supplemental Digital Content 7, <http://links.lww.com/QAD/A519>). Therefore, alterations in these CSF metabolites were detected irrespective of viral replication in CSF and plasma or current ART.

Alterations in the HIV cerebrospinal fluid metabolome overlap with those associated with normal aging in HIV-negative individuals

Given recent studies suggesting accelerated aging in HIV patients on ART [23,24], we compared alterations in the CSF metabolome of young HIV patients on ART ($n = 16$, age < 50 , plasma viral load < 1000 copies/ml and CSF viral load < 50 copies/ml) to the profile altered with aging in HIV-negative controls ($n = 23$ per group, young vs. older patients) (Supplemental Digital Content 8, <http://links.lww.com/QAD/A519>). Thirty-four named and 12 unnamed metabolites were altered in older (age ≥ 50) compared to sex and race-matched young HIV-negative controls (age < 50), including metabolites associated with neurotransmitter production [glutamate, homocarnosine, 3-(4-hydroxyphenyl)lactate], markers of glial activation (choline and arachidonate), oxidative products (5-oxoproline) and markers of oxidative stress (urate, hypoxanthine), and metabolic waste products [3-hydroxybutyrate (BHBA), 1,2-propanediol, phenylacetylglutamine, lactate] (Fig. 2a). Many of these metabolites correlated positively with advancing age ($P < 0.05$; Fig. 2b). Ten named and four unnamed metabolites altered in young HIV patients on ART vs. age and sex-matched HIV-negative controls overlapped with those associated with normal aging in HIV-negative individuals including glutamate, phenylacetylglutamine, succinate, and ketone bodies (BHBA and 1,2 propanediol) (Fig. 3), suggesting a pattern indicative of accelerated aging.

Neurocognitive impairment is associated with alterations in cerebrospinal fluid neurotransmitters/neuropeptides, markers of glial activation, and accumulation of metabolic waste in HIV patients on antiretroviral therapy

To identify metabolic pathways associated with HANDs, we compared CSF metabolite profiles between HIV patients on ART with ($n = 12$) and without ($n = 14$) NCI for groups matched by age, sex, race, and current and nadir CD4⁺ (plasma viral load < 1000 copies/ml, CSF viral load < 50 copies/ml). Seven metabolites differentiated between HIV patients with and without NCI, but these differences did not reach statistical significance following multiple testing correction (Supplemental Digital Content 9, <http://links.lww.com/QAD/A519>). Next, we performed fold-change analysis on HIV patients with or without NCI vs. age and sex-matched HIV-negative controls ($n = 14$). Ten metabolites distinguished HIV patients with NCI, but not HIV patients without NCI, from HIV-negative controls including

neurotransmitters (glutamate, NAA); markers of glial cell activation (myo-inositol, arachidonate); markers of mitochondrial dysfunction (propionylcarnitine, 3-dehydrocarnitine); and metabolic waste products (BHBA, lactate, phenylacetylglutamine) (fold change > 1.2 , $P < 0.05$, FDR $< 5\%$). Twelve metabolite sets were enriched in HIV patients with NCI compared to HIV-negative controls. Five of these sets were enriched only in HIV patients with NCI including ketone body metabolism, aspartate metabolism, and phenylalanine and tyrosine metabolism ($P < 0.05$, FDR $< 5\%$; Supplemental Digital Content 10, <http://links.lww.com/QAD/A519>). Recursive SVM classification models identified eight metabolites as top-ranked classifiers of HANDs; these included neurotransmitters (glutamate, NAA); markers of glial activation (myo-inositol); and ketone bodies (beta-hydroxybutyric acid, 1,2-propanediol) (Fig. 4a and c). These eight metabolites distinguished HIV patients with vs. without HAND with greater than 85% predictive accuracy (Fig. 4b). Further validation in a cohort of HIV patients with plasma viral loads greater than 10 000 copies/ml ($n = 15$; 9 with NCI and 6 without NCI) showed that six of these metabolites also distinguished HIV patients with vs. without HAND in a separate test cohort (glutamate, NAA, BHBA, 1,2 propanediol, isobutyrylcarnitine, and N-acetylvaline) (Fig. 4d). These CSF metabolite profiles suggest that altered neurotransmitter production, glial cell activation, mitochondrial dysfunction, and accumulation of metabolic waste products are processes that characterize HAND, irrespective of HIV replication.

Inter-relationships between cerebrospinal fluid and plasma metabolites, neurocognitive test scores, and inflammation in HIV patients on antiretroviral therapy

Next, we examined inter-relationships between CSF metabolites associated with NCI, plasma metabolites, and markers of systemic and intrathecal inflammation. Two distinct subclusters of CSF metabolites were identified by correlation analysis ($P < 0.05$, FDR < 0.1) (Fig. 5). In subcluster 1, isobutyrylcarnitine, myo-inositol, and N-acetylvaline correlated negatively with global and several domain T scores (motor, memory-encoding, memory-retrieval and executive function) and positively with intrathecal IFN responses (CSF IFN- γ and K : T ratio). In subcluster 2, succinate, glutamate, NAA, BHBA, and 1,2 propanediol correlated positively with systemic markers of inflammation (IFN- α , IFN- γ , IL-8, IL-1 β , IL-6, IL-2Ra) and negatively with plasma lysophosphocholines (LPCs) and steroids. Plasma LPCs and steroids correlated positively with T scores (global, motor, memory-encoding, memory-retrieval and executive function) and negatively with systemic inflammation. These results suggest inter-relationships between systemic and intrathecal inflammation and plasma or CSF metabolite alterations associated with NCI.

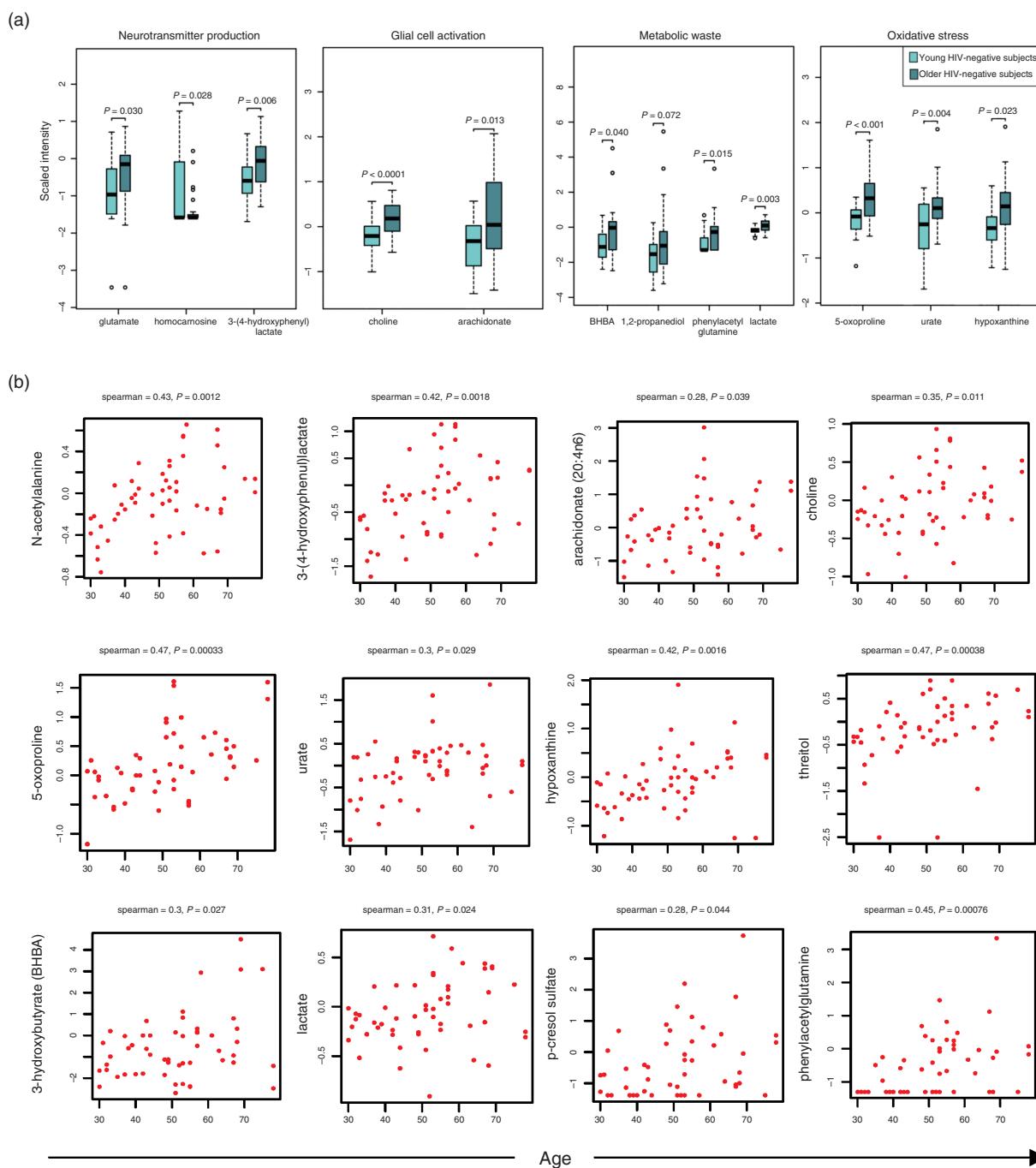


Fig. 2. Alterations in the cerebrospinal fluid metabolome associated with aging in HIV-negative controls. (a) Box plots of metabolites altered in older ($n = 23$; ≥ 50 years old) compared to young HIV-negative controls ($n = 23$; < 50 years old). Light and dark blue represent young and older controls, respectively. Medians are represented by horizontal bars, boxes span the interquartile range (IQR) and whiskers extend to extreme data points. The P -values were calculated using Welch's t -tests. (b) Correlation plots of biologically relevant metabolites (\log_2 scaled intensity) vs. age in HIV-negative controls ($n = 53$). Spearman correlations were used to examine relationships between metabolite levels and age. The correlation coefficient R and P -value are shown above each plot. False discovery rate below 10% was used to correct for multiple hypothesis testing.

Discussion

Here, we characterized the CSF metabolome in a cohort of HIV patients on ART with advanced disease and identified eight metabolites as top-ranked classifiers of

HAND, including neurotransmitters (glutamate, NAA); markers of glial cell activation (myo-inositol); mitochondrial function (succinate); and ketone bodies, suggesting that glutamate excitotoxicity, astrocyte activation, mitochondrial dysfunction, and accumulation of metabolic

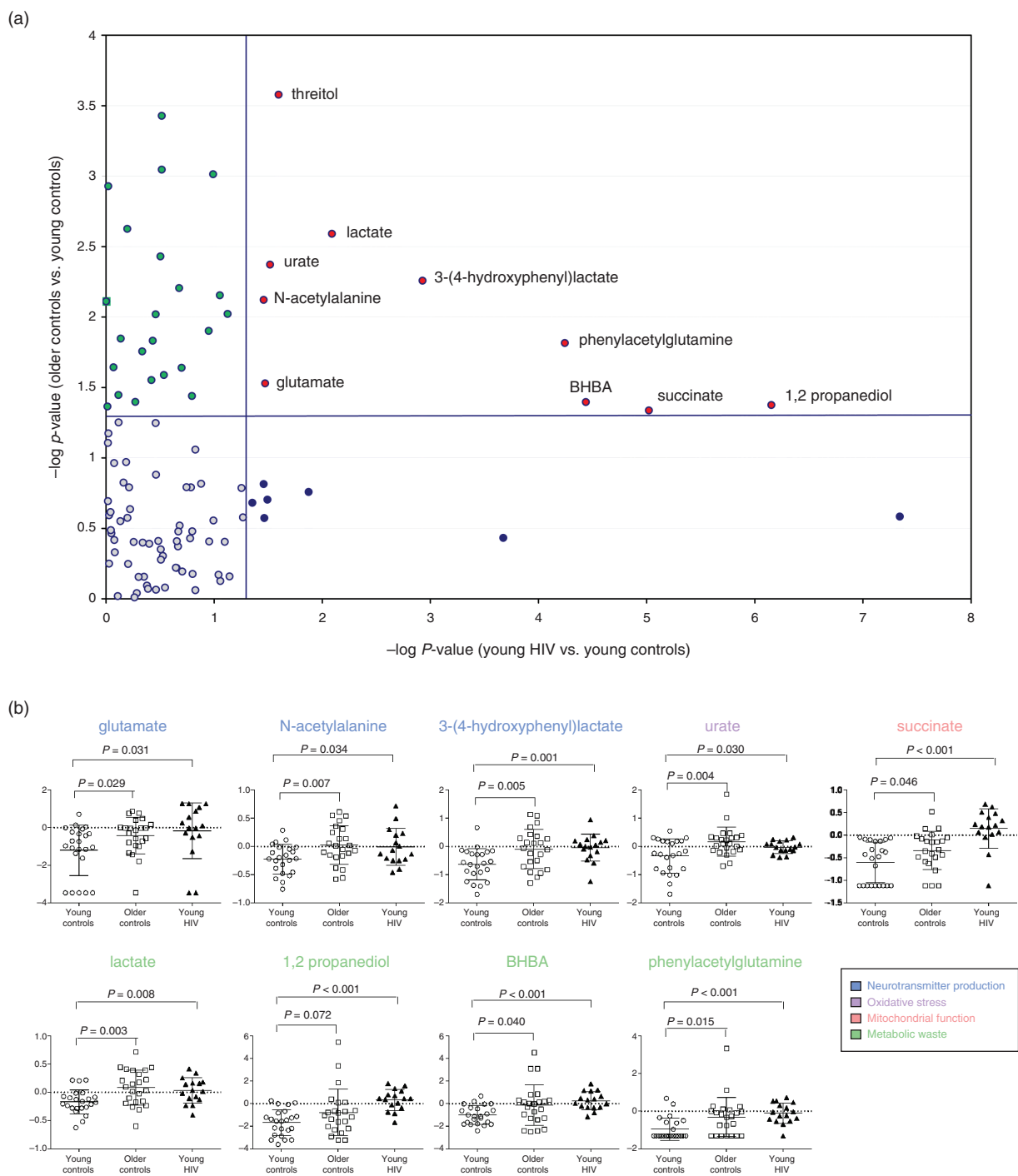


Fig. 3. Metabolites altered in the cerebrospinal fluid metabolome of young HIV patients on antiretroviral therapy overlap with those altered in older HIV-negative controls. (a) P -value scatter plot of metabolites altered in the HIV CSF metabolome (16 young HIV patients on ART vs. 23 young HIV-negative controls) vs. metabolites altered during normal aging in HIV-negative controls (23 older vs. 23 young HIV-negative controls). The Y-axis shows $-\log_{10} P$ -values of metabolites altered in older (≥ 50 years old) vs. young HIV-negative controls (< 50 years old) matched for sex and race. The X-axis shows $-\log_{10} P$ -values of metabolites altered in young HIV patients on ART (< 50 years old) with viral loads (plasma VL < 1000 copies/ml, CSF VL < 50 copies/ml) vs. young HIV-negative controls (≥ 50 years old) matched for age and sex. (b) Beeswarm plots of metabolites altered in young HIV patients on ART (< 50 years old, $n = 16$) compared to young (< 50 years old, $n = 23$) and older HIV-negative controls (≥ 50 years old, $n = 23$). Medians are represented by horizontal bars. The P -values were calculated using Welch's t -tests. ART, antiretroviral therapy; BHBA, beta-hydroxybutyric acid; CSF, cerebrospinal fluid; VL, viral load.

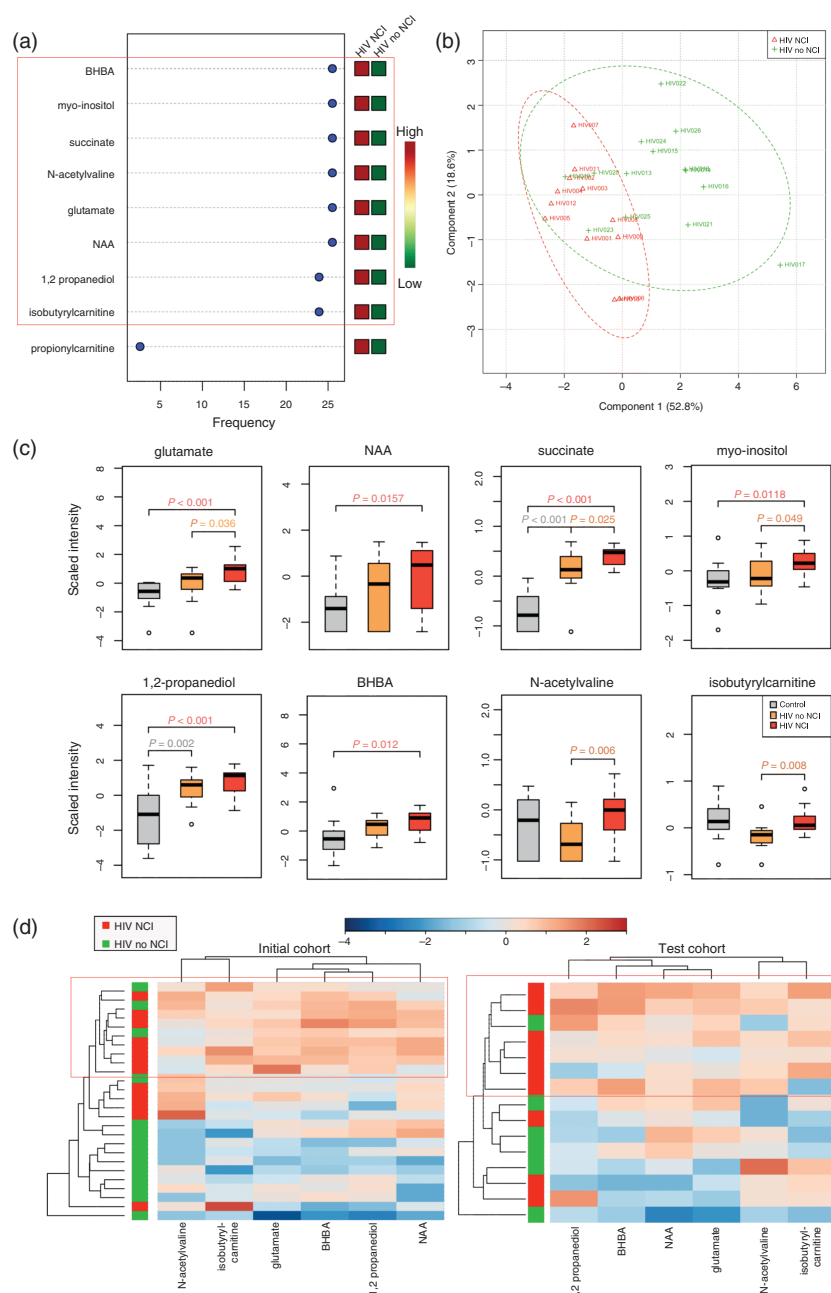


Fig. 4. Cerebrospinal fluid metabolites associated with neurocognitive impairment in HIV patients on antiretroviral therapy.

(a) Top classifiers of NCI with expression heatmap from recursive SVM classification models. SVM models were constructed using metabolites identified in FC analysis ($FC > 1.2$, $P < 0.05$, $FDR < 10\%$) comparing HIV patients on ART with NCI (plasma VL $< 10\,000$ copies/ml), HIV patients on ART without NCI (plasma VL $< 10\,000$ copies/ml), and age and sex-matched HIV-negative controls. Red and green indicate increased and decreased levels, respectively. (b) PLS-DA analysis shows separation of metabolomes from HIV patients on ART with (red, $n = 12$) and without NCI (green, $n = 14$). (c) Box plots of top classifiers of NCI identified by SVM in HIV-negative controls ($n = 14$, gray), HIV patients on ART without NCI ($n = 14$, orange), and HIV patients on ART with NCI ($n = 12$, red). Medians are represented by horizontal bars, boxes span the interquartile range (IQR) and whiskers extend to extreme data points. The P -values were calculated using Welch's t -tests. (d) Unsupervised hierarchical clustering of six top-ranked metabolites that distinguish between HIV patients on ART with and without NCI in two independent cohorts. The initial cohort was composed of HIV patients on ART with low viral loads ($n = 26$, plasma VL $< 10\,000$ copies/ml, CSF VL < 50 copies/ml). The independent test cohort was composed of HIV patients with high plasma viral loads ($n = 15$, VL $> 10\,000$ copies/ml). Red and blue indicate increased and decreased levels, respectively. ART, antiretroviral therapy; BHBA, beta-hydroxybutyric acid; CSF, cerebrospinal fluid; FC, fold change; FDR, false discovery rate; NAA, N-acetylaspartate; NCL, neurocognitive impairment; PLS-DA, partial least-squares discriminant analysis; SVM, support vector machine; VL, viral load.

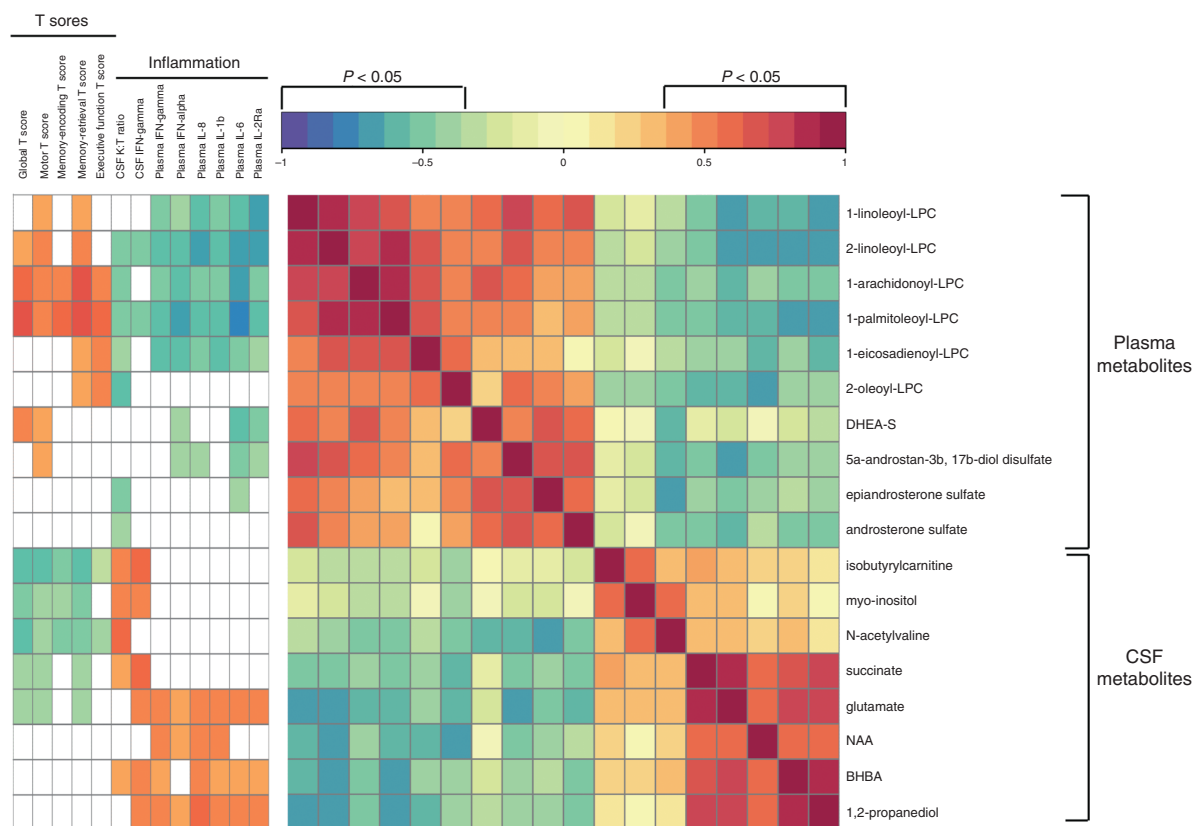


Fig. 5. Integrative analysis and correlation matrix visualization reveals relationships between cerebrospinal fluid classifiers of neurocognitive impairment, plasma lysophosphocholines and steroids, global and domain T scores, and markers of systemic and intrathecal inflammation in HIV patients on antiretroviral therapy. The correlation matrix and clinical covariate bar were constructed in R using the heatmap.2 function. Pearson correlations were used to evaluate relationships between metabolites ($P < 0.05$, $FDR \leq 0.1$). Spearman correlations were used to examine relationships between metabolites, global and domain T scores, and markers of inflammation. Significant correlations had a $-0.35 > r > 0.35$ ($P < 0.05$), and FDR below 10%. FDR was used to correct for multiple hypothesis testing. Red and blue indicate positive and negative correlations, respectively (see Color Key). BHBA, beta-hydroxybutyric acid; DHEA-S, dehydroepiandrosterone sulfate; FDR, false discovery rate; LPC, lysophosphocholine; NAA, N-acetylaspate.

waste may contribute to NCI. This HAND signature overlapped with a CSF metabolite profile associated with aging in HIV-negative controls, suggesting a pattern indicative of accelerated aging. Correlation analysis identified inter-relationships between plasma inflammation markers and intrathecal IFN responses and metabolic alterations associated with NCI, suggesting that ongoing systemic and intrathecal inflammation may contribute to this accelerated aging phenotype. These results suggest that therapeutic strategies targeting ‘inflammaging’ and associated metabolic abnormalities may be beneficial for treatment of HAND.

Alterations in CSF metabolites identified in our study provide insights into mechanisms that may contribute to HAND. In particular, alterations in neurotransmitters (glutamate and NAA) and markers of glial cell activation (myo-inositol) and mitochondrial dysfunction (succinate) were associated with NCI. Glutamate is neurotoxic at high concentrations and increased levels have been shown in HIV-associated dementia (HAD) and other

neurological disorders [54–56]. Decreased NAA, a marker of neuronal density and integrity, has been reported in brain from HIV patients with HAND [27,35–37,57,58]. Here, CSF NAA was increased in patients with HAND, possibly reflecting differences in the sample tested (brain vs. CSF), and may represent leakage into the CSF associated with neuronal damage. Alterations in N-acetylated alpha-linked acidic dipeptidase activity, which converts NAAG to glutamate and NAA, have been shown to correlate with neuronal loss in Alzheimer’s disease [59]. Myo-inositol, a marker of astrocyte activation, was also increased in HIV patients, consistent with magnetic resonance spectroscopy studies, showing increased myo-inositol in the brain of HIV patients with HAND, including those on ART [27,35–37,58]. This may be relevant to HAND pathophysiology because astrocyte activation can impair their neuroprotective functions (e.g. BBB integrity and glutamate reuptake) [60]. Another important finding was increased CSF succinate. Increased succinate, a component of the Krebs cycle, can reflect mitochondrial dysfunction [52].

Succinate may also act as a danger signal stabilizing hypoxia-inducible factor (HIF)-1 α expression and enhancing IL-1 β production during inflammation [61]. More than 80% of the CSF metabolites which were altered in HIV patients on ART were also altered in HIV patients not on ART (Supplemental Digital Content 7, <http://links.lww.com/QAD/A519>), suggesting that these alterations are not directly associated with ART or ongoing HIV replication in plasma or CSF. These results suggest that glutamate excitotoxicity, astrocyte activation, and mitochondrial dysfunction may contribute to HAND.

Another important finding was increased accumulation of metabolic waste, including ketone bodies, phenylacetylglutamine, and p-cresol sulfate. Accumulation of metabolic waste, such as protein aggregates, is a hallmark of Alzheimer's disease and other age-associated neurodegenerative diseases [62–64]. In HIV patients, accumulation of hyperphosphorylated tau, amyloid, and alpha-synuclein has been reported in older patients [65–68]. CSF circulates nutrients filtered from the blood to the brain and removes metabolic waste by active transport or bulk flow. CSF is absorbed into the blood through arachnoid villi or exchanged with brain interstitial fluid via aquaporin 4 (AQP4) channels [69–71]. In reactive astrogliosis, mislocalization of AQP4 results in a loss of interstitial flow and accumulation of extracellular waste products [69–71]. Whereas astrogliosis is common in HIV [30,72] and AQP4 expression is increased in HIV patients with HAND [73], further studies are required to determine if loss of interstitial flow and detrimental effects on the glymphatic system [64,69–71] contribute to accumulation of metabolic waste and development of HAND.

Ketone bodies (BHBA and 1,2 propanediol) were identified as top-ranked CSF classifiers of HAND. Ketone bodies are an energy source for metabolically active tissues under conditions of glucose deficiency [74]. Whereas ketogenic diets have shown therapeutic potential in some neurological diseases [75,76], ketone bodies are toxic at high concentrations and can stimulate insulin release, generate oxygen radicals, and cause lipid peroxidation, contributing to oxidative stress [77,78]. Increased ketone bodies have been reported in metabolic disorders (i.e. diabetes and obesity), inflammatory diseases (i.e. multiple sclerosis and rheumatoid arthritis), and are associated with neurological complications in diabetic ketoacidosis [79–82]. In view of these observations, we predict that increased ketone bodies in CSF from patients with HAND reflect both increased production and decreased clearance. Given that ketone bodies are signaling molecules that play an important role regulating lipid metabolism and mitochondrial function (reviewed in [83]), further studies are warranted to examine their potential role in HAND via neurotoxic or metabolic effects.

Antiretroviral therapy is associated with increased age-associated comorbidities including cardiovascular, liver, kidney, and bone diseases [20,22]. These age-related comorbidities often occur at younger ages than would be expected among HIV-negative individuals, possibly reflecting 'inflammaging' along with other mechanisms [23,24]. Here, alterations in the HIV CSF metabolome, including those associated with NCI, showed significant overlap with metabolites altered in aging HIV-negative controls (e.g. glutamate, succinate, BHBA, and 1,2 propanediol), suggesting a pattern of accelerated aging. Consistent with these findings, increased glutamate and succinate were detected in plasma from HIV-negative individuals with advancing age [84,85], and increased glutamate and ketone bodies were detected in CSF from HIV-negative individuals with cognitive impairment and Alzheimer's disease [86]. These alterations likely reflect increased metabolite production, together with decreased metabolite clearance and reduced CSF turnover, which has been reported in aging populations and Alzheimer's disease [87,88]. In the present study, alterations in these CSF metabolites were associated with systemic inflammation (IFN- γ , IFN- α , IL-8, IL-1 β , IL-6, and IL-2Ra) and intrathecal IFN responses (IFN- γ and K : T ratio), suggesting ongoing systemic and intrathecal inflammation both contribute to this accelerated aging phenotype. Plasma LPC and steroids correlated positively with neurocognitive test scores, and negatively with markers of systemic inflammation. These findings, together with previous studies, suggest these LPC and steroids may have beneficial roles, such as anti-inflammatory or neuroprotective effects. Phosphatidylcholines represent a class of lipids altered in Alzheimer's and other neurodegenerative diseases [89,90]. Depletion of dehydroepiandrosterone sulfate and related steroids have been implicated in aging, age-related comorbidities, and immune dysfunction [91–93]. These results suggest that metabolic abnormalities and 'inflammaging' mechanisms both contribute to HAND.

We acknowledge certain limitations of our study. We selected HIV patients with advanced disease and high prevalence of HCV coinfection. Although we cannot exclude the possibility that HCV contributed to the results, matched analyses of HIV patients with and without HCV coinfection did not identify significant differences in CSF metabolites or inflammation markers. HIV samples were collected from 1999 to 2009 and therefore may not reflect contemporary populations due to differences in ART regimens. We cannot exclude the possibility that prolonged sample storage could affect the stability of some metabolites. However, recent studies suggest prolonged storage has minimal effects on the majority of CSF metabolites [94,95]. Small sample volumes limited our ability to detect certain lipids in CSF that were detected by others [43,44,46]. Further studies are required to define alterations in larger, recent cohorts with less advanced disease, treated with specific

ART regimens, and their possible association with HAND subtypes.

In summary, untargeted metabolomic profiling identified alterations in the CSF metabolome of HIV patients on ART that suggest persistent inflammation, glial responses, glutamate neurotoxicity, and age-dependent effects on brain waste disposal systems contribute to mechanisms involved in HAND that may be augmented by aging. These alterations were not directly associated with ART or ongoing HIV replication in CSF or plasma. This study provides insights into disease mechanisms associated with HAND and represents progress toward identifying biomarkers to predict and monitor neurocognitive outcomes and therapeutic responses.

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E.C. performed the experiments and data analysis, drafted the manuscript, and prepared figures. V.M. and A.D. performed bioinformatics and statistical analysis and prepared figures. S.M. participated in study design, data analysis, and manuscript editing. D.G. conceived the study, supervised its design, coordination, and data analysis, and helped write and edit the manuscript. All authors read, participated in editing the manuscript, and approved the final manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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