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Cerebrospinal Fluid Ceruloplasmin, Haptoglobin, and Vascular Endothelial Growth Factor Are Associated with Neurocognitive Impairment in Adults with HIV Infection

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Abstract

Dysregulated iron transport and a compromised blood–brain barrier are implicated in HIV-associated neurocognitive disorders (HAND). We quantified the levels of proteins involved in iron transport and/or angiogenesis—ceruloplasmin, haptoglobin, and vascular endothelial growth factor (VEGF)—as well as biomarkers of neuroinflammation, in cerebrospinal fluid (CSF) from 405 individuals with HIV infection and comprehensive neuropsychiatric assessments. Associations with HAND [defined by a Global Deficit Score (GDS) ≥ 0.5, GDS as a continuous measure (cGDS), or by Frascati criteria] were evaluated for the highest versus lowest tertile of each biomarker, adjusting for potential confounders. Higher CSF VEGF was associated with GDS-defined impairment [odds ratio (OR) 2.17, $p = 0.006$] and cGDS in unadjusted analyses and remained associated with GDS impairment after adjustment ($p = 0.018$). GDS impairment was also associated with higher CSF ceruloplasmin ($p = 0.047$) and with higher ceruloplasmin and haptoglobin in persons with minimal comorbidities (ORs 2.37 and 2.13, respectively; both $p = 0.043$). In persons with minimal comorbidities, higher ceruloplasmin and haptoglobin were associated with HAND by Frascati criteria (both $p < 0.05$), and higher ceruloplasmin predicted worse impairment (higher cGDS values, $p < 0.01$). In the subgroup with undetectable viral load and minimal comorbidity, CSF ceruloplasmin and haptoglobin were strongly associated with GDS impairment (ORs 5.57 and 2.96, respectively; both $p < 0.01$) and HAND (both $p < 0.01$). Concurrently measured CSF IL-6 and TNF-α were only weakly correlated to these three biomarkers. Higher CSF ceruloplasmin, haptoglobin, and VEGF are associated with a significantly greater likelihood of HAND, suggesting that interventions aimed at disordered iron transport and angiogenesis may be beneficial in this disorder.

Keywords Ceruloplasmin · Haptoglobin · Vascular endothelial growth factor · Biomarker · HIV-associated neurocognitive disorder · Cerebrospinal fluid (CSF)

Abbreviations

HAND	HIV-associated neurocognitive disorder
NC	Neurocognitive
ART	Combination antiretroviral therapy
CSF	Cerebrospinal fluid
VEGF	Vascular endothelial growth factor
TNF-α	Tumor necrosis factor-alpha
IL-6	Interleukin 6
CXCL-10	C-X-C chemokine motif ligand 10
cGDS/GDS	(continuous) Global Deficit Score
BBB	Blood–brain barrier
CHARTER	CNS HIV antiretroviral therapy effects research (study)
HCV	Hepatitis C virus
PC	Principal components
OR	Odds ratio
IQR	Interquartile range
T (1–3)	Tertile (1–3)
WRAT	Wide-range achievement test

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Introduction

HIV-associated neurocognitive disorder (HAND) remains a common complication of HIV infection, despite the effectiveness of combination antiretroviral therapy (ART) in suppressing viral replication and reducing the frequency of HIV-associated dementia [1]. Milder forms of neurocognitive (NC) impairment are nevertheless responsible for a majority of HAND diagnoses that occur in up to half of unselected persons with HIV (HIV+ individuals) [2]. These forms of HAND are also clinically significant [3], if not always progressive, contributing to loss of employment, medication nonadherence, and reduced quality of life [4,5]. Although inflammation, mediated by activated monocyte–macrophages, is recognized to be central to HIV neuropathogenesis, clinical interventions for HAND remain elusive, and other mechanisms that promote NC decline in HIV+ persons continue to merit exploration.

Recent studies by our group and others increasingly implicate dysregulated iron transport and mitochondrial dysfunction in HAND [6, 7]. Iron is required for mitochondrial function, and iron transport influences immune activation and angiogenesis [8,9], both of which have in turn been linked to HAND and/or other neurodegenerative processes [10–12]. Glial and immune-cell activation and altered angiogenesis may compromise the integrity of the blood–brain barrier (BBB) in HIV+ persons, thereby facilitating the transmigration of activated immune cells into the brain and promoting infection of resident microglia, perivascular macrophages, and possibly other non-neuronal cells [13]. These secondarily infected cells establish an HIV reservoir early in the disease course that is not appreciably impacted by ART [1].

Ceruloplasmin is a multifunctional copper ferroxidase involved in copper and iron transport with diverse roles in the brain [14–16]. Similarly, haptoglobin is a small but ubiquitous hemoglobin-binding protein in the circulation, which thereby serves as co-ligand for the macrophage–monocyte scavenger receptor CD163, a molecule recognized to be induced in neurocognitively impaired HIV+ individuals [17–19]. To our knowledge, neither ceruloplasmin nor haptoglobin has been quantified in the CSF of HIV+ persons. Vascular endothelial growth factor (VEGF) is an angiogenesis factor, whose effects in the CNS may be either neuroprotective or deleterious, depending on the setting [20]. Ceruloplasmin, haptoglobin, and VEGF are induced to varying degrees by acute inflammation [16]. In this study, conducted in a large observational cohort of HIV+ individuals with neuropsychiatric and neuromedical characterization, we investigated whether higher CSF levels of these multifunctional proteins are associated with an increased prevalence of HAND.

Patients and Methods

Study Population

The CNS HIV Antiretroviral Therapy Effects Research (CHARTER) study is a prospective, observational study based in the USA, which enrolled over 1500 ambulatory, HIV-seropositive adults between 2003 and 2007 at six medical centers. All participants underwent baseline assessments that included standardized, structured interviews and laboratory studies, to obtain basic demographic information, as well as other data, including complete blood counts, current and nadir $CD4^+$ T-cell counts, HIV RNA levels in plasma and CSF, hepatitis C virus (HCV) serology, history of ART, exposure to nucleoside reverse-transcriptase inhibitors, major depressive disorders, substance use/dependency, and comorbid conditions that might influence NC function. Further details of CHARTER study eligibility and assessment protocols have been previously published [21]. CSF samples were obtained by lumbar puncture in all consenting participants at baseline and at follow-up visits. Genetic ancestry for all individuals was determined previously, using the Affymetrix Genome-Wide Human SNP Array 6.0™ (Affymetrix, Inc., Santa Clara, CA, USA) and categorized using principal component (PC) analysis, as described elsewhere [22].

Assessment of NC Function and Comorbid Conditions

Comprehensive neuropsychiatric and NC testing was performed in all participants at baseline and follow-up visits and included 15 measures in 7 cognitive ability domains known to be affected by HIV infection [23, 24]. The effects of age, sex, educational level, race/ethnicity, and practice or learning effects in those repeatedly tested were accounted for in this test battery, which incorporates the best available normative standards. Composite test scores (e.g., the Global Deficit Score (GDS)) were derived from demographically corrected standardized scores (T scores) on individual tests. The GDS may be evaluated as a continuous measure (cGDS) that reflects the overall number and severity of impairments on the test battery, or dichotomized to define neurocognitive impairment (*impaired*, GDS ≥ 0.5 and *neurocognitively normal*, GDS < 0.5) [24]. While the binary GDS variable is the more clinically relevant measure of NC function, the cGDS improves power for evaluating impacts on NC function. NC impairment criteria incorporating functional assessments were also classified according to the well-accepted Frascati criteria [25]. Individuals with neuropsychiatric and severe comorbid conditions that were deemed likely by expert neurologists to confound the diagnosis of HAND (e.g., ongoing substance use, prior stroke or cardiovascular complications without return to normal cognitive function after the event, severe depression with suboptimal effort in cognitive testing,

decompensated liver disease, or history of traumatic brain injury with loss of consciousness of 30 min or more) were excluded from analysis [21, 24].

Quantification of CSF Biomarkers

Ceruloplasmin and haptoglobin were quantified in CSF from the baseline visit in 405 participants, using commercially available multiplex bead-based suspension array immunoassays validated for CSF (EMD Millipore™, Billerica, MA) on a FlexMAP3D platform (Luminex Corporation, Madison, WI); VEGF was quantified using the Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN). All assay results were reviewed for quality assurance, and 10% of all assays were repeated to assess operator and batch consistency. Biomarker precision was ensured by assaying specimens in duplicate and repeating measurements with coefficients of variation greater than 20% or outliers that were more than 3 standard deviations (SDs) from the mean. In addition, interleukin (IL)-6, CXCL-10, and tumor necrosis factor-alpha (TNF-α) were measured by high-sensitivity assays using the Millipore™ Luminex FlexMap3D platform.

Statistical Analysis

The total of 405 participants included 176 with HAND. Baseline characteristics were compared between neurocognitively impaired and unimpaired participants using *t* tests (age and education), Wilcoxon signed-rank tests (WRAT, nadir $CD4^+$ T-cell count, cGDS, and all CSF biomarkers), or Fisher's exact tests [sex, race/ethnicity cluster, HCV status, detectable CSF virus, detectable plasma virus, ART status (on vs. off treatment at the time of sampling), comorbidity, HAND (Frascati definitions) [25], and GDS-defined NC impairment status]. To test associations of CSF biomarkers with the GDS, univariate logistic regression was performed with the binary GDS outcome and univariate linear regression was performed with the cGDS outcome. Multivariable logistic or linear regression analyses were then performed for analyses of GDS-defined impairment and cGDS, respectively, with adjustment for nadir CD4, ART status, the first two PCs of genetically determined ancestry (PC1, PC2), and comorbidity. These specific covariates were selected due to their known associations with HAND [23], to prevent potential confounding of biomarker effects. In addition, self-reported race/ethnicity was associated with CSF biomarkers of iron transport in our prior studies [6], and genetic ancestry is widely accepted as more representative of racial ancestry than racial/ethnic self-classification. Only the first two PCs of ancestry were included in order to optimize power and because these PCs contributed most of the ancestry-related variability and were statistically significant in univariate analyses, as published previously [26]. Because comorbidity was found to be a factor significantly associated with NC status in all analyses, multivariable analyses were repeated, stratifying by comorbidity severity, which was categorized as minimal (non-contributory to NC impairment), or mild-moderate severity (likely to contribute to NC impairment), as described elsewhere [21]. Participant age was not included in regression models, in order to optimize power for detection of biomarker effects, since the GDS measure in CHARTER already incorporates adjustment for age, and additional inclusion of age did not alter the observed associations [21]. In the subset of individuals on ART with undetectable virus in plasma, associations of each CSF biomarker with NC impairment were assessed using multivariable logistic regression modeling of GDS-defined impairment (vs. GDS-unimpaired), and HAND (binary variable, using the Frascati definitions of asymptomatic NC impairment, mild NC disorder, or HIV-associated dementia, vs. neuropsychiatrically unimpaired). These analyses were further stratified by comorbidity and adjusted for nadir CD4, PC1, and PC2. The potential impact of outlier biomarker values in a few individuals was also assessed in this analysis by excluding individuals with two or more biomarker values greater than 2 SDs from the mean, but overall results were unchanged; hence, only results for all individuals are presented. For all analyses, CSF biomarkers were divided into tertiles to optimize power for detection of small effects due to the non-normality of their distributions. Odds ratios and their 95% confidence intervals (95% CI) were then estimated for tertile (T3) compared to tertile 1 (T1) of each biomarker. Since this analysis was exploratory in nature, corrections for multiple testing were not employed. Finally, a supplementary analysis to determine the correlations between all of the CSF biomarkers was performed using pairwise Spearman's correlation coefficients. Two-sided p values < 0.05 were considered statistically significant for all analyses, which were conducted using SAS™ statistical software version 9.4.

Results

Study Population Characteristics

Cerebrospinal fluid biomarker measurements from a total of 405 CHARTER study participants were included in this analysis. Baseline demographic and HIV disease characteristics of this population are summarized in Table 1. The mean age of the sample was 43 years, and 79 (19%) were women; 43% were of European ancestry, 43% were of African ancestry, and 14% were of admixed Hispanic ancestry. Ninety-five individuals (24%) were co-infected with hepatitis C virus. The median CD4 nadir was 177 [interquartile range (IQR) 56–308], 73% were on ART at the time of NC assessment and CSF sampling, and 54% had detectable plasma HIV. One third of participants had mild-moderate (contributory) comorbid neuromedical conditions. The median GDS was 0.32 (IQR, 0.11–0.58), and 57% of individuals were NC normal, 34% had asymptomatic impairment, and 10% had symptomatic

Table 1 CHARTER study participant characteristics at baseline

Baseline variable	Entire population ($N = 405$)	Impaired ($N = 176$)	Not impaired ($N = 229$)	p value[a]
Age (years)	43 (8)	45 (8)	42 (8)	**< 0.01**
Sex (female)	79 (19)	33 (19)	46 (20)	0.83
Ancestry cluster[b]				0.17
European	171 (43)	84 (48)	87 (39)	
African	169 (43)	66 (38)	103 (47)	
Admixed Hispanic	55 (14)	24 (14)	31 (14)	
Education level (years)	12.9 (2.4)	13.1 (2.4)	12.7 (2.3)	0.12
CD4 nadir ($\times 10^3$ cells/mm^3)	177 (56, 308)	162 (50, 266)	193 (59, 333)	0.18
HCV co-infected	95 (24)	49 (28)	46 (20)	0.09
Detectable plasma virus	216 (54)	90 (51)	126 (56)	0.45
ART (on)	296 (73)	133 (76)	163 (71)	0.38
Comorbid conditions[c]				**< 0.01**
Mild-moderate	135 (33)	76 (43)	59 (26)	
Minimal	270 (67)	100 (57)	170 (74)	
HAND (Frascati category)				–
NP-normal	229 (57)	0 (0)	229 (100)	
ANI	137 (34)	137 (78)	0 (0)	
MND	32 (8)	32 (18)	0 (0)	
HAD	7 (2)	7 (4)	0 (0)	
Global Deficit Score (cGDS)	0.32 (0.11, 0.58)	0.63 (0.42, 1.0)	0.16 (0.05, 0.26)	–
CSF biomarker[d]				
Ceruloplasmin	1622 (1279, 2150)	1744 (1322, 2253)	1584 (1250, 2087)	**0.03**
Haptoglobin	2510 (928, 4551)	2530 (965, 5259)	2510 (925, 4229)	0.48
IL-6	3.4 (2.5, 4.7)	3.3 (2.5, 4.6)	3.6 (2.5, 4.7)	0.36
CXCL-10	1557 (1058, 2745)	1564.5 (1027, 2724)	1540 (1093, 2745)	0.66
TNF-α	0.44 (0.33, 0.63)	0.44 (0.33, 0.59)	0.45 (0.32, 0.67)	0.45
VEGF	7.10 (4.16, 9.47)	6.76 (4.15, 9.39)	7.50 (4.20, 9.50)	0.56

Data are presented as mean (SD) for continuous, normally distributed variables; N (%) for count data; and median (interquartile range) for continuous, non-normally distributed variables. Statistically significant p-values (<0.05) are shown in bold font

SD standard deviation, *IQR* interquartile range, *WRAT* wide-range achievement test (an estimate of reading comprehension and education), *HCV* hepatitis C virus, *CSF* cerebrospinal fluid, *ART* combination antiretroviral therapy, *HAND* HIV-associated neurocognitive disorder, *NP-normal* neuropsychiatrically normal, *ANI* asymptomatic neurocognitive impairment, *MND* mild neurocognitive disorder, *HAD* HIV-associated neurocognitive disorder, *IL* interleukin, *CXCL-10* chemokine CXC motif ligand [also known as interferon-inducible protein (IP)-10], *TNF-α* tumor necrosis factor-alpha, *VEGF* vascular endothelial growth factor

[a] p values represent comparison of impaired to unimpaired participants

[b] Ancestry clusters were determined using principal component analysis of genome-wide genetic data, as described in "Patients and Methods" section

[c] Comorbid conditions are classified as minimal (non-contributory to neurocognitive impairment) or mild-moderate (contributing to neurocognitive impairment), based on previously established criteria [21]

[d] Raw biomarker values are summarized as the median (interquartile range)

(either mild or severe) impairment on combined NC testing and functional assessment. Neurocognitively impaired individuals were slightly older, had slightly lower WRAT scores (an estimate of education and reading ability), and were non-significantly less likely to have detectable virus in plasma than unimpaired individuals. Significant comorbid conditions were also more prevalent in the impaired group.

Biomarkers of Iron Metabolism and Inflammation

Levels of CSF ceruloplasmin ranged from 592 to 8028 pg/mL (median 1622, IQR 1279–2150) and were significantly higher among neurocognitively impaired persons in unadjusted analysis (Table 1; $p = 0.03$). Haptoglobin ranged from 2.68 to 12,500 pg/mL (median 2510, IQR, 928–4551), and median

VEGF levels in CSF were 7.10 pg/mL (IQR, 4.16, 9.47; range 0.037 to 31.2). Interleukin (IL)-6 levels, CXCL10, and TNF-α levels were 3.4 pg/mL (IQR 2.5, 4.7), 1557 (IQR, 1058, 2745), and 0.44 (0.33, 0.63), respectively. No statistically significant differences by NC impairment status were observed for levels of CSF biomarkers other than ceruloplasmin.

Measures of Association of CSF Biomarkers with NC Outcomes

Unadjusted logistic regression analyses of GDS-defined NC impairment (binary GDS variable) and linear regression analyses of cGDS were performed for each CSF biomarker separately, with results as shown in Table 2. Ceruloplasmin and haptoglobin levels were not significantly associated with either GDS variable, nor were associations with any of the inflammation biomarkers (IL-6, CXCL-10, and TNF-α) observed. Higher CSF VEGF, however, showed a borderline association with GDS-defined impairment [crude OR for T3 vs. T1, 2.17 (95% CI 1.25–3.74)], as well as with higher values of the cGDS ($p = 0.04$).

As shown in Table 3, adjustment of multivariable regression models for the nadir CD4, ART, the first two ancestry PCs, and severity of other medical comorbidities revealed a modestly increased likelihood of GDS impairment with higher levels (T3 vs. T1) of both ceruloplasmin and VEGF [OR 1.77 (95% CI 1.01–3.10) and 2.00 (95% CI 1.13–3.54), respectively, both p values < 0.05]. The point estimate for the association of comorbidity (significant vs. minimal) in all regression models was consistently 2.70 to 2.87 (all p values < 0.0001). Since we felt it unlikely that any single biomarker's association would be equal to or greater than that of chronic comorbidities with NC status, we also performed analyses stratified by comorbidity severity

Table 2 Crude biomarker associations with the Global Deficit Score (GDS)

Biomarker	GDS (binary)		GDS (continuous)	
	OR	95% CI	β-coefficient	p value
Ceruloplasmin (T3 vs. T1)	1.58	(0.94, 2.64)	0.054	0.33
Haptoglobin (T3 vs. T1)	1.07	(0.64, 1.79)	− 0.002	0.98
IL-6 (T3 vs. T1)	0.96	(0.57, 1.64)	− 0.055	0.32
CXCL-10 (T3 vs. T1)	0.93	(0.56, 1.57)	− 0.028	0.61
TNF-α (T3 vs. T1)	0.77	(0.45, 1.32)	− 0.065	0.24
VEGF (T3 vs. T1)	**2.17**[a]	**(1.25, 3.74)**	**0.117**[a]	**0.04**

Logistic regression models were not adjusted for clinical or demographic factors. Global Deficit Score [binary—GDS ≥ 0.5 (impaired) vs. < 0.5 (unimpaired); continuous—higher values generally indicate more impairment)]

OR (unadjusted) odds ratio, *95% CI* 95% confidence interval, *T (3 vs. 1)* biomarker tertile 3 compared to tertile 1

[a] Statistically significant estimates at α = 0.05 level (bolded)

Table 3 Multivariable-adjusted biomarker associations with GDS in the CHARTER study

Biomarker	GDS (binary)		GDS (continuous)	
	OR	95% CI	β-coefficient	p value
Ceruloplasmin (T3 vs. T1)	**1.77**[a]	**(1.01, 3.10)**	0.062	0.262
Haptoglobin (T3 vs. T1)	1.20	(0.69, 2.07)	0.014	0.791
IL-6 (T3 vs. T1)	1.05	(0.60, 1.83)	− 0.051	0.347
CXCL-10 (T3 vs. T1)	1.20	(0.67, 2.16)	0.000645	0.991
TNF-α (T3 vs. T1)	0.87	(0.48, 1.56)	− 0.052	0.361
VEGF (T3 vs. T1)	**2.00**[a]	**(1.13, 3.54)**	0.087	0.126

All analyses are adjusted for nadir $CD4^+$ T-cell count, ART (on vs. off treatment), PC1, PC2, and severity of comorbidity (mild-moderate/contributory vs. minimal/non-contributory to neurocognitive impairment)

[a] Statistically significant at α = 0.05 level (bolded)

(i.e., comorbid conditions deemed to be either minimal or mild-moderate, as described in the "Patients and Methods" section), adjusting for all of the same covariates mentioned above except comorbidity (Table 4). In 270 individuals with minimal comorbidity, significant ceruloplasmin and haptoglobin associations with binary GDS were detected. Adjusted ORs were 2.37 for ceruloplasmin (95% CI 1.17–4.78) and 2.13 for haptoglobin (95% CI 1.02–4.42), for T3 compared with T1. CSF ceruloplasmin was also associated with higher cGDS (beta estimate 0.178, $p = 0.001$). Significant associations with VEGF were not observed in this subset. When we tested the sensitivity of these findings to exclusion of extreme outlier values, we found the results to be robust: exclusion of 18 individuals in whom two or more biomarkers had values > 2 SD from the mean strengthened these associations (data not shown). In the subset of 135 individuals with mild-moderate (neuro-influential) comorbidities, interestingly, significant associations for both biomarkers were observed in the opposite direction for ceruloplasmin with cGDS (beta estimate = − 0.257, $p = 0.027$) and for haptoglobin with GDS impairment (adjusted OR 0.41, 95% CI 0.17–0.99). Interleukin-6 levels were also associated with reduced likelihood of GDS impairment and lower (better) values of cGDS in the presence of mild-moderate comorbidities (adjusted OR for GDS impairment 0.40, 95% CI 0.16–0.98). No association with VEGF was observed for either GDS outcome.

Analyses stratified by comorbidity were also conducted in 185 study participants with undetectable viral load on ART (Table 5). Among 69 individuals with mild-moderate comorbidities, only CXCL-10 levels in CSF were significantly associated with GDS impairment (OR 6.5 for T3 vs. T1, 95% CI 1.29–33.28, adjusted for nadir CD4, PC1, and PC2). Individuals with neurologically insignificant comorbidities ($N = 116$) and CSF ceruloplasmin levels in the highest versus lowest tertile were significantly more likely to have GDS impairment (adjusted OR 5.57, 95% CI 1.64–18.94) and higher cGDS values

Table 4 Multivariable-adjusted biomarker associations with the GDS, stratified by comorbidity

Mild-moderate comorbidity ($N = 135$)				
	GDS (binary)		GDS (continuous)	
Biomarker	OR	95% CI	β-coefficient	p value
Ceruloplasmin (T3 vs. T1)	0.70	(0.29, 1.71)	**−0.257**[a]	**0.027**
Haptoglobin (T3 vs.T1)	**0.41**[a]	**(0.17, 0.99)**	−0.142	0.213
IL-6 (T3 vs. T1)	**0.40**[a]	**(0.16, 0.98)**	**−0.300**[a]	**0.007**
CXCL-10 (T3 vs. T1)	1.85	(0.73, 4.73)	0.010	0.933
TNF-α (T3 vs. T1)	1.09	(0.45, 2.62)	−0.101	0.379
VEGF (T3 vs. T1)	1.62	(0.68, 3.85)	0.026	0.820
Minimal comorbidity ($N = 270$)				
	GDS (binary)		GDS (continuous)	
Biomarker	OR	95% CI	β-coefficient	p value
Ceruloplasmin (T3 vs. T1)	**2.37**[a]	**(1.17, 4.78)**	**0.178**[a]	**0.001**
Haptoglobin (T3 vs. T1)	**2.13**[a]	**(1.02, 4.42)**	0.089	0.113
IL-6 (T3 vs. T1)	0.89	(0.424, 1.856)	−0.018	0.753
CXCL-10 (T3 vs. T1)	1.45	(0.701, 2.987)	0.092	0.110
TNF-α (T3 vs. T1)	0.53	(0.26, 1.10)	−0.065	0.246
VEGF (T3 vs. T1)	1.11	(0.50, 2.47)	−0.012	0.849

All estimates are adjusted for nadir CD4, ART, PC1, and PC2

[a] Estimates are considered statistically significant at $\alpha = 0.05$ level (bolded)

(beta-estimate 0.288, $p = 0.005$). Higher CSF haptoglobin levels in this subset were likewise associated with an increased risk of GDS impairment (adjusted OR 2.96, 95% CI 1.07–8.23).

Since the GDS-based NC outcomes we evaluated did not include a functional assessment, we also included the Frascati definition of HAND as a clinical outcome (Table 5). No associations were detected in the subset with mild-moderate neuromedical comorbidities, but among participants with minimal comorbidities and undetectable viral load on ART, those with ceruloplasmin and haptoglobin levels in T3 versus T1 were considerably more likely to have a diagnosis of HAND (adjusted ORs 4.61, 95% CI 1.62–13.12 for ceruloplasmin and 2.51, 95% CI 1.01–6.22 for haptoglobin). While point estimates for associations of VEGF levels with HAND were in the same direction, they were not statistically significant.

Correlations with Neuro-Inflammation

All three of the biomarkers evaluated in this study are multifunctional proteins and are also induced during periods of acute inflammation (acute-phase reactants). We therefore simultaneously measured biomarkers of inflammation in the same CSF samples to determine their correlation with iron-related biomarkers. A correlation matrix for the CSF biomarkers (Supplementary Table 1) showed that while ceruloplasmin and haptoglobin were moderately correlated with one another (rho = 0.45, $p < 0.01$), they are only very weakly correlated with IL-6, CXCL-10, and TNF-α. VEGF was only weakly correlated with ceruloplasmin in CSF and not at all correlated with haptoglobin or with biomarkers of inflammation.

Discussion

This study, conducted in over 400 clinically well-characterized individuals with HIV, is the first published CSF study to identify associations of two proteins involved in iron metabolism and angiogenesis, ceruloplasmin, and haptoglobin, with HAND, and also to suggest a possible link between HAND and higher CSF levels of another pro-angiogenic factor, VEGF. We previously showed that CSF levels of iron, transferrin, and H-ferritin (also an important plasma ferroxidase) are associated with other established risk factors for HAND, including demographic factors and viral load in either CSF or plasma, even after correction for variations in BBB integrity [6]. By simultaneously measuring biomarkers which directly reflect CSF inflammation (IL-6, TNF-α) or immune activation (CXCL-10), we were able to evaluate the extent to which the biomarkers of interest reflected neuroinflammation, rather than iron metabolism or angiogenesis.

Ceruloplasmin is one of the most important proteins involved in maintenance of iron and copper homeostasis systemically and in the brain [27]. Once released into the brain interstitium, iron must be loaded onto transferrin for transport

Table 5 Stratified multivariable regression analyses of GDS and HAND by comorbidity severity in individuals with undetectable plasma virus on ART

Mild-moderate comorbidity ($N = 69$)						
	GDS (binary)		GDS (continuous)		HAND	
Biomarker	OR	95% CI	β-coefficient	p value	OR	95% CI
Ceruloplasmin (T3 vs. T1)	0.47	(0.129, 1.677)	− 0.247	0.090	0.57	(0.162, 2.012)
Haptoglobin (T3 vs. T1)	0.36	(0.106, 1.219)	− 0.149	0.287	0.57	(0.169, 1.929)
IL-6 (T3 vs. T1)	0.85	(0.23, 3.08)	− 0.188	0.213	0.66	(0.18, 2.40)
CXCL-10 (T3 vs. T1)	**6.54**[a]	**(1.29, 33.28)**	0.216	0.199	1.36	(0.31, 5.93)
TNF-α (T3 vs. T1)	2.44	(0.59, 10.14)	0.014	0.930	0.96	(0.24, 3.76)
VEGF (T3 vs. T1)	0.83	(0.23, 2.96)	− 0.045	0.759	0.36	(0.10, 1.34)
Minimal comorbidity ($N = 116$)						
	GDS (binary)		GDS (continuous)		HAND	
Biomarker	OR	95% CI	β-coefficient	p value	OR	95% CI
Ceruloplasmin (T3 vs. T1)	**5.57**[a]	**(1.64, 18.94)**	**0.288**[a]	**0.005**	**4.61**[a]	**(1.62, 13.12)**
Haptoglobin (T3 vs. T1)	**2.96**[a]	**(1.07, 8.23)**	0.136	0.161	**2.51**[a]	**(1.01, 6.22)**
IL-6 (T3 vs. T1)	1.52	(0.52, 4.44)	− 0.005	0.962	0.72	(0.27, 1.93)
CXCL-10 (T3 vs. T1)	0.57	(0.16, 2.00)	− 0.154	0.174	0.99	(0.35, 2.83)
TNF-α (T3 vs. T1)	0.095[a]	(0.011, 0.81)	− 0.218	0.068	0.53	(0.16, 1.76)
VEGF (T3 vs. T1)	2.88	(0.92, 9.00)	0.037	0.720	1.40	(0.53, 3.71)

All analyses are adjusted for nadir CD4, PC1, and PC2

[a] Estimates are considered statistically significant at $\alpha = 0.05$ level (bolded)

to neurons and other types of brain cells, a process for which readily available ferroxidase activity is essential [28]. Ceruloplasmin is produced by astrocytes in soluble and membrane-bound, glycophosphatidylinol (GPI)-anchored forms, as well as by pericytes and brain vascular endothelial cells, and provides ferroxidase activity for oxidation of ferrous iron in brain interstitial fluid to the ferric form which binds transferrin [14, 16]. Astrocyte-derived soluble ceruloplasmin (or possibly another extracytoplasmic ferroxidase such as H-ferritin) is probably also required for ferroportin-mediated iron efflux from brain vascular endothelial cells into the brain [29]. Indeed, astrocytes are believed to play a crucial role in regulating the efflux of iron from endothelial cells into the brain. Ceruloplasmin also serves other functions, including copper transport, coagulation, angiogenesis, and defense against oxidant stress; it is expressed on cells of the macrophage–monocyte lineage, which have a central role in HAND pathogenesis [30, 31]. A deficiency of ceruloplasmin has been associated with neurobehavioral phenotypes involving brain iron deficits, although *low* levels of ceruloplasmin have also been linked to elevated levels of brain iron and lipid peroxidation in neurodegenerative disorders like Alzheimer's disease (AD). [28, 32] Rozek et al. observed higher serum ceruloplasmin levels in individuals with HAD and speculated that this protein might simply reflect chronic inflammation [28]. In our study, however, the absence of strong correlations with biomarkers of inflammation in the CSF, or associations of IL-6 or TNF-α with HAND, argues against this possibility and suggests that higher levels of ceruloplasmin in individuals with HAND are unlikely to be due mainly to inflammation. Elevated levels of ceruloplasmin may either be a physiologic response to other changes that promote HAND, or part of the pathophysiology of HAND; these mechanisms can only be differentiated in prospective human studies or, potentially, in animal models of HAND. The former scenario might occur due to functional iron deficiency in the brain in HIV+ persons, a concept supported by the finding in ART-era "-omics" studies of HAND [33] that the clathrin-mediated endocytosis pathway (required for entry of iron-bound transferrin into cells) is downregulated, and by evidence from our prior unpublished studies that transferrin-receptor expression, an indicator of increased cellular iron demand, is upregulated [34, 35]. Increased need for ceruloplasmin in HIV infection would also be consistent with the loss of brain white matter in this disease, as the copper chelator and neurotoxin cuprizone induces demyelination in mice [36]. A relative deficiency of ceruloplasmin in the brain could occur as a response to increased brain iron in HIV+ individuals, as some studies indicate that ceruloplasmin prevents iron-mediated oxidant injury to neurons in the setting of brain iron loading [37]. However, evidence of increased brain iron in HIV+ individuals is scant in the ART era [38]. The latter scenario, in which ceruloplasmin has direct pathogenic effects in HAND, could also explain our observation that higher levels of this biomarker are associated with HAND. One older study suggested that ceruloplasmin may

activate microglia, playing a pro-inflammatory role [39]. Finally, this protein has been linked to glutamatergic neurotoxicity, which has been reported in HAND [35, 40, 41].

Haptoglobin is an immunomodulatory circulating glycoprotein with strong antioxidant properties, whose primary function is to bind free hemoglobin released from lysed or phagocytosed red blood cells. The resulting hemoglobin–haptoglobin complex is internalized via scavenger receptor CD163-mediated endocytosis on the macrophage–monocyte membrane, leading to metabolism of the heme by cytosolic heme oxygenase 1 (HO-1). Iron-binding proteins like haptoglobin are integral to the innate immune response, reducing iron availability to microbial pathogens; haptoglobin is also a powerful suppressor of lymphocyte function, promoting T helper-1 responses and reducing pro-inflammatory cytokine secretion [42]. Specific haptoglobin phenotypes with differing affinities for hemoglobin have been linked to higher mortality in HIV infection, and the Hp2-2 form has been associated with higher serum ceruloplasmin ferroxidase activity [42, 43]. CD163, haptoglobin, and HO-1 are all positive acute-phase proteins and might be expected to play an antioxidant, anti-inflammatory role in the brain as well [44]. Recently, HIV viral load was found to be positively correlated to macrophage–monocyte expression of haptoglobin–hemoglobin scavenger receptor CD163, and the productivity of HIV infection in macrophages has been associated with higher levels of CD163 expression [45, 46]. Soluble CD163 is shed by activated monocytes and macrophages and may remain elevated in chronic HIV infection despite ART, indicating ongoing efforts by the immune system to resolve immune activation and inflammation [19, 47]. It is therefore likely that increased CSF haptoglobin in HIV+ individuals represents a neuroprotective response to HIV-related neuroimmune activation, and further studies are needed to clarify its role in HAND.

Weakly significant associations of ceruloplasmin with cGDS, and of haptoglobin with binary GDS in the opposite direction (i.e., reduced impairment) in individuals with mild-moderate comorbidity, as compared to the minimal comorbidity group, are intriguing and may provide clues to the underlying pathophysiology of HAND. However, these findings did not appear to be as robust as the results in the larger subset of individuals with insignificant comorbidities, and we believe they may be spurious, due to insufficient power and/or greater background inflammation masking true biomarker effects in individuals with significant comorbid disease. Comorbidities that contribute to NC impairment (mild-to-moderate severity subset, in this case) are more likely to involve systemic inflammation, which we have found to easily overwhelm individual biomarker effects and reduce the ability to detect CSF biomarker associations with HAND. In support of this argument, the positive associations of ceruloplasmin and haptoglobin observed with HAND in the study sample overall were stronger in the minimal-comorbidity subset and stronger still when the analysis was confined to individuals in whom virus was undetectable.

Our observation of associations between higher CSF VEGF and GDS-defined NC impairment in adjusted as well as unadjusted analyses suggests a possible role for angiogenesis in HAND. Furthermore, haptoglobin and ceruloplasmin are also distinctly pro-angiogenic proteins. While VEGF is neuroprotective via direct effects on neurons and vascular perfusion, increased levels of this factor may further disrupt an already compromised BBB in HIV+ persons, and this may be particularly true of disorders characterized by demyelination [20, 48, 49]. Loss of integrity of the neurovascular unit that makes up the BBB has been documented in several neurodegenerative disorders, including AD, HIV, and multiple sclerosis [50]. In addition, HIV Tat has structural similarity to VEGF, and both Tat and its derived peptides can induce angiogenesis via activation of VEGF receptors expressed on endothelial cells [51]. Human brain microvascular endothelial cells of the BBB have also been shown to upregulate VEGF receptors after exposure to interferon-γ, which, along with HIV Tat, is present in the CSF of HIV+ persons [52]. One prior study reported significantly higher VEGF levels in serum in 8 HIV+ individuals with associated neurological diseases such as HIV encephalitis, compared to 19 HIV+ persons without CNS disease, but this was not true of CSF VEGF, and VEGF levels decreased during ART in two individuals in whom levels were available [49]. It is unclear from our study whether the observed VEGF association with HAND represents a pathological induction or a compensatory response due to HIV infection and neuroinflammation, and either scenario is possible.

Limitations of this study include its cross-sectional design and the inability to confirm the source of CSF VEGF and proteins of iron metabolism such as ceruloplasmin and haptoglobin since plasma levels of these proteins were not measured. While ceruloplasmin has a molecular weight over twice that of albumin and does not generally cross an intact BBB, studies in HIV+ persons suggest significant variability in BBB integrity, as reflected by elevated age-adjusted CSF/serum albumin ratios [53, 54]. Similarly, haptoglobin levels in CSF may be partly related to damage to the BBB induced by HIV, which does not appear to be eliminated by ART; however, glial production of this protein may also be increased, owing to oligodendrocyte and neuronal injury [55, 56].

Conclusions

In conclusion, levels of ceruloplasmin, haptoglobin, and VEGF, measured for the first time in HIV+ CSF in the ART era, were found to be associated with HAND, using several metrics of NC outcome. Despite their biological plausibility, the associations we identified were not adjusted for multiple

testing and require replication. These observations are novel and lend support to the concept that dysregulated iron metabolism and angiogenesis are intimately linked to NC impairment in HIV+ persons, but the underlying mechanisms deserve further investigation.

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Authors' Contributions AK and TH designed, coordinated, and funded this study, and AK wrote the manuscript. HRG performed the analysis under the direction of JBS and AK and assisted in writing the statistical methods. DRF and DRC coordinated the selection of CSF samples, and DRC and SLL oversaw the laboratory assays. TH helped edit the manuscript. RJE, TH, SM, and JRC provided helpful comments on the manuscript. All remaining co-authors are CHARTER study investigators and/or site PIs, who assisted in the enrollment of participants and collection of primary data. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Ethics Approval and Consent to Participate The CHARTER study abides by the principles set forth in the Declaration of Helsinki. All study participants provided written informed consent, and only de-identified data was used in the present analysis. The Institutional Review Boards of all participating institutions approved the study.

Consent for Publication All subjects provided written informed consent to participate in the study. No individual's protected health information is included in this report.

Conflicts of Interest The authors declare that they have no potential conflicts of interest.

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