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Blood-Based Inflammation Biomarkers of Neurocognitive Impairment in People Living with HIV

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Abstract

Inflammation in people living with HIV (PLWH) correlates with severity of HIV-associated neurocognitive disorders. The objective of this study is to identify blood-based markers of neurocognitive function in a demographic balanced cohort of PLWH. Seven neurocognitive domains were evaluated in 121 seropositive Black/African American, Non-Hispanic White and White Hispanic men and women using computerized assessments. Associations among standardized neurocognitive function and HIV-related parameters, relevant sociodemographic variables and inflammation-associated cytokines measured in plasma and cellular supernatants were examined using multivariate and univariate regression models. Outlier and covariate analyses were used to identify and normalize for education level, CD4 T cell count, viral load, CNS and drug abuse comorbidities, which could influence biomarker and neurocognitive function associations. Plasma levels of chemokine (C-C motif) ligand (CCL) 8 significantly associated with memory, complex attention, cognitive flexibility, psychomotor speed, executive function and processing speed. Plasma tissue inhibitor of metalloproteinases 1 associated with the aforementioned domains except memory and processing speed. In addition, plasma IL-23

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Author Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analyses were performed by Naomi Swanta, Kathleen Borgmann, Subhash Aryal, Sangeeta Shenoy and Anuja Ghorpade. The first draft of the manuscript was written by Naomi Swanta, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare that there is no conflict of interest.

significantly associated with processing speed and executive function. Analysis of blood cell culture supernatants revealed no significant markers for neurocognitive function. In this cohort, CD4 T cell count and education level also significantly associated with neurocognitive function. All identified inflammatory biomarkers demonstrated a negative correlation to neurocognitive function. These cytokines have known connections to HIV pathophysiology and are potential biomarkers for neurocognitive function in PLWH with promising clinical applications.

Keywords

HIV-associated neurocognitive disorders (HAND); chronic immune activation; CNS; health disparities

Introduction

HIV infection remains widespread with approximately 37.9 million individuals living with the disease worldwide (UNAIDS, 2019). The success of antiretroviral therapy (ART) has resulted in decreased incidence of the most severe form of HIV-associated neurocognitive disorders (HAND); conversely, prevalence of milder forms of HAND have increased. Sustained immune activation and chronic systemic inflammation remain a hallmark of HIV disease and are significant contributors to the development of HAND (Deeks et al, 2013; Harezlak et al, 2011; Montoya et al, 2019).

Diagnosis of HAND is heavily dependent on comprehensive neuropsychological evaluations, using symptom questionnaires, functional assessments and computer tests while simultaneously excluding other possible neurological disorders (Antinori et al, 2007; Clifford and Ances, 2013; Letendre, 2011; Mind Exchange Working, 2013). Identification of milder cases is challenging because often the subtle forms of impairment are not as overt as dementia (Antinori et al, 2007; Mind Exchange Working, 2013). As immune system responses are associated with HAND, it seems important to investigate biomarkers as a complement to neurocognitive testing.

Currently, 30-50% of people living with HIV (PLWH) experience some form of HAND, which is characterized by a spectrum of neurocognitive dysfunctions (Clifford and Ances, 2013; Gott et al, 2017; Heaton et al, 2011; Letendre, 2011). In contrast to PLWH without HAND, neurocognitive impairments in PLWH can significantly impact their ability to perform activities of daily living, resulting in unemployment, lower economic status, and an inability to afford their medication or adhere to ART regimens. (Benedict et al, 2000; Hinkin et al, 2002; Marquine et al, 2018).

Current evidence suggests that clinical measurements of HIV disease state are poor markers for HIV-related comorbidities in the ART era (Achhra et al, 2015; Burdo et al, 2013; Lyons et al, 2011; Veenstra et al, 2019). Elevated peripheral immune activation is sustained in PLWH even in otherwise healthy individuals; thus, measurements of immune activity could serve as markers of HAND. While several studies have investigated peripheral inflammation and its association to neurocognitive impairment in PLWH in the United States of America (USA) (Burdo et al, 2013; Kalayjian et al, 2019; Lyons et al, 2011), these studies reported

only two biomarkers or examined a majorly Non-Hispanic White men cohort. Furthermore, the prevailing race/ethnic and sex disparities, such as the disproportionate effect of HIV on Black/African Americans, in the context of HAND have not been examined and remain unclear. Biomarkers for HIV-associated neurocognitive impairment that are clinically relevant and are with race/ethnicity and sex implications are critical. It is vital to delineate whether the combined outcomes from biomarkers and neurocognitive testing have the potential to help identify HIV-associated neurocognitive impairment and measure treatment responsivity from particular demographic groups. To help answer this question we explored the relationships of inflammatory markers with neurocognitive domains as a measure of cognitive function in PLWH using a panel of 26 inflammation-associated cytokines assayed in two blood derived samples from a well characterized and demographic balanced cohort of PLWH.

Methods

Participants

Volunteers were recruited from various HIV care clinics and support agencies throughout the Dallas/Fort Worth metroplex, USA. Inclusion criteria were the following: HIV seropositive, at least 20 years old and self-identified as Black/African American, Non-Hispanic White or White Hispanic, man or woman. Exclusion criteria were the following: current suicidal ideation, substance abuse, an inability or unwillingness to complete the neuropsychological battery and other questionnaires, current traumatic brain trauma, intellectual disability, or any acute medical condition unrelated to HIV-infection that may have a significant impact on the neurocognitive function and tested positive in pregnancy, breathalyzer, and urine drug screens (cocaine, opiates, methamphetamine/amphetamine and cannabis).

Recent medical charts were reviewed by physicians, and relevant clinical parameters were recorded. These included CD4 T cell count, plasma viral load, time since HIV diagnosis in years, ART medication from which the ART CNS penetration effectiveness (CPE) score was calculated as described by Letendre (2011). These and other verified health conditions classified based on diagnosis and current therapy/medication are shown in Table 1. A sociodemographic questionnaire was administered for self-identified factors such as race/ethnicity, sex, age and education level (lower than high school education, high school graduate and at least some college education - trade/business school, four-year college, or graduate school). The North Texas Institutional Review Board (UNTHSC) approved the study procedures which were in accordance with the Declaration of Helsinki for medical research involving human subjects (World Medical, 2001). Written informed consent was obtained prior to study enrollment, and participant information was protected according to HIPAA guidelines.

Neurocognitive Performance Measures

Neurocognitive function in seven domains was assessed using Central Nervous Systems Vital Signs (CNSVS, Morrisville, NC) software according to testing guidelines (Gualtieri and Johnson, 2006). CNSVS is a computerized comprehensive battery of neuropsychological tests that yields raw and normalized standard scores. Raw standardized

scores, *e.g.* normalized to a median raw score of age matched healthy cohort, were used as a measure function in each domain independent of validity scores. Subsets of domains were designed to eliminate repeated test measures under the guidance of CNSVS. Thus, standard scores for composite memory (verbal + visual), complex attention, cognitive flexibility, psychomotor speed and reaction time, were clustered in subset 1 and standard scores for executive function and processing speed in subset 2. Neurocognitive testing did not include HAND diagnoses.

Blood Collection and Cytokine Assays

Blood samples (30 to 40 mL) were collected from the antecubital vein into lavender K_2 EDTA 10 ml blood collection tubes by butterfly collection sets with tube holder (21 and 23 G, Vacutainer®, BD, Flanklin Lakes, NJ). Within 30 minutes of collection, 2 ml of blood was centrifuged at 2000 x g for 20 minutes to harvest plasma. Concurrently, 1:1 PBS diluted blood was layered on lymphocyte separation medium (Histopaque-1077, Sigma-Aldrich, St. Louis, MO) and centrifuged at 400 x g for 30 minutes, and the lymphocyte layer was collected to isolate peripheral blood mononuclear cells (PBMCs) from blood. Residual red blood cells were lysed with red blood cell lysis solution (Miltenyi Biotec, Germany). Isolated PBMCs were incubated at 37°C and 5% $C0_2$ for 24 hours in media [RPMI, 20% FBS, penicillin-streptomycin-neomycin (50 U-50 μg-100 μg/mL)] for collection of 24-hour culture supernatants. A panel of 25 inflammation-associated cytokines (Chemokine (C-C motif) (CCL) ligand 1 (I-309), CCL2 or monocyte chemoattractant protein (MCP)-1, CCL5 or regulated upon activation, normal T cell expressed, and secreted (RANTES), CCL8 or MCP2, CCL11 (eotaxin), chemokine (C-X-C motif) ligand (CXCL)1 or growth regulated oncogene (GROα), CXCL10 or γ-induced protein 10 (IP-10), interferon (IFN)-γ, interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-23, soluble CD40 ligand (sCD40L), tissue inhibitor of metalloproteinases 1 (TIMP-1), tumor necrosis factor (TNF)-α, TNF-β) were measured in plasma and 24-hour PBMC supernatant by Q-Plex™ Custom multiplexed ELISA (Quansys Biosciences, Logan, UT). Soluble CD40 ligand (sCD40L) was measured by a monoclonal sandwich ELISA (eBioscience, Inc. San Diego, CA).

Statistical Analysis

At least thirty participants in each race/ethnicity, balanced by sex, maintained > 80% statistical power and < 5% type I error rate in a one-way ANOVA model. The primary analysis was an omnibus test for associations to subsets of neurocognitive domains in order to maintain type I error rate at < 5%. Sociodemographic factors, HIV-related clinical parameters and the cytokine panel were analyzed for association to subset 1 and 2 in a multivariate regression model using SPSS Statistics (version 23, IBM, New York, NY). Significant associations in the multivariate analyses were considered prior to examination of univariate associations to specific neurocognitive domains. Prism 8 (version 8.2.0, GraphPad software, San Diego, CA) was utilized to perform Pearson's correlation for directional relationship (Pearson's r) of markers to neurocognitive domains. Baseline characteristics between race/ethnic and sex groups were compared by one-way ANOVA and t-tests (χ^2 for categorical variables), respectively.

Outliers were identified by a combination of leverage scores and Cook's distance. Leverage (h_i) was considered large if $h_i > 2(p + 1)/n$ where p = number of independent variables and n = sample size (Hoaglin and Welsch, 1978). A large leverage datapoint was considered an influential outlier of the univariate model if its Cook's distance was greater than 1 (Hair, 2014). A univariate regression model of biomarkers and neurocognitive domain scores with race/ethnicity, sex, education level, CD4 T cell count, viral load, past treatment for drug abuse and CNS comorbidity as covariates, was used to correct for their influence on the association of biomarkers and neurocognitive function using SPSS. A two-sided P value of <0.05 was considered statistically significant. Scatterplot matrix of standard scores from neurocognitive domains created by RStudio (Boston, MA).

Results

Participant Characteristics

Since many clinical studies fail to include minority race/ethnic and sex groups (Oh et al, 2015), our cohort was balanced across race/ethnic and sex groups most affected by HIV in the USA (Table 1). The 121 study participants were an average of 50 ± 10 years old, seropositive for an average of 14 ± 8 years, and majority (93%) were on ART regimens. Viral load was undetectable in 85%, and average CD4 T cell count was considered clinically healthy (648 ± 327 mm^3); 17% had CD4 T cell counts below 200 mm^3 (Table 1). These statistics were consistent with the typical health status of the PLWH in the post-ART era (Yoshimura, 2017).

The average age and distribution of education levels were comparable across race/ethnic groups. However, the average age of women was seven percent lower than men ($p = 0.046$) (Table 1). While comparisons of HIV-relevant clinical parameters revealed no significant differences between race/ethnic groups for CD4 T cell count, viral load and ART CPE scores, the average years since diagnosis of White Hispanics (10.7 ± 5.3) was 29% and 30% lower than that of Black/African Americans and Non-Hispanic Whites, respectively ($p = 0.0055$). In addition, average CD4 T cell count in women (728 ± 320) was 22% higher than men (648 ± 327) ($p = 0.0068$) (Table 1). Study participants had a variety of verified comorbidities; cardiac-related conditions such as high blood pressure (57%) were most prevalent followed by history and treatment of depression (44%), and then co-infections, 40% of which were hepatitis infections.

Certain comorbidities could have direct implications on performance during neurocognitive assessments and were assessed by multivariate regression analyses for associations with neurocognitive subsets. While none of the participants in this study had been diagnosed with neurocognitive disorders, some patients had conditions with CNS implications (Table 1) including history of mild stroke and neuropathy, which were not directly related to cognition. CNS comorbidities did not significantly associate with neurocognitive function in subset 1 ($p = 0.214$) or subset 2 ($p = 0.636$). Participants were also screened for current drug use prior to inclusion in the studies. However, some participants (Table 1) had undergone treatment for alcohol/drug abuse in the past, which associated with neurocognitive function for neither subset 1 (p=0.255) nor subset 2 (p=0.796).

Education Level and CD4 T Cell Count Significantly Correlate with Neurocognitive Function

Sociodemographic and HIV-relevant clinical parameters were tested for associations with CNSVS neurocognitive subsets using a multivariate regression model followed by univariate testing of correlations to individual domains. CD4 T cell count significantly associated with neurocognitive subset 1 and 2 (Table 2). A Pearson's correlation analysis revealed a positive relationship between CD4 T cell count and neurocognitive function in memory ($r = 0.20$) and processing speed ($r = 0.23$). Other HIV-related clinical parameters, years since diagnosis, viral load and ART CPE score, were not significantly associated with neurocognitive subsets. While most sociodemographic factors, age, race/ethnicity, and sex, did not significantly associate with neurocognitive subsets, education level significantly associated with both subsets and all domains except reaction time (Table 2).

Inflammatory Markers of Neurocognitive Function

An extensive panel of 26 inflammation-associated (pro- and anti- inflammatory) cytokines were measured in participant plasma and 24-hour PBMC supernatants by ELISA. A multivariate regression model was utilized to identify cytokines that significantly associated with neurocognitive function (Table 3). Since education level and CD4 T cell count significantly associated with neurocognitive function, their influence on the relationship between biomarkers and neurocognitive domains were taken into account. After factoring in CD4 T cell count, we did not observe a significant change in biomarker-domain relationship; therefore, we report inflammatory biomarkers that remained significantly associated with neurocognitive function after correcting for education level.

Briefly, plasma levels of CCL8 significantly associated with subset 1 and 2 (Table 3); further univariate analysis revealed significant correlation with complex attention, cognitive flexibility, memory, psychomotor speed, executive function, and processing speed (Figure 1a). Plasma levels of IL-10 significantly associated with subset 2 (Table 3) and significantly correlated with processing speed; however, the relationship was dependent upon a single outlier. Plasma levels of TIMP-1 significantly associated with subset 1 and 2 (Table 3) and significant correlated with complex attention, cognitive flexibility, psychomotor speed and executive function (Figure 1b). Plasma levels of IL-23 significantly associated with subset 2 (Table 3) and significantly correlated with executive function and processing speed (Figure 1c), which were not dependent upon outliers identified in either graph. In parallel, the cytokine panel measured in 24-hour PBMC supernatants identified significant associations between CCL2 and IL-17 to subset 1 (Table 3). CCL2 significantly correlated with complex attention; however, upon exclusion of a statistical outlier the association was lost. IL-17 did not significantly correlate with any specific domain, suggesting a multivariate relationship to the domains in subset 1.

Outlier analyses excluded plasma IL-10 and 24-hour PBMC supernatant CCL2 as biomarkers of neurocognitive function and strengthened the relationships between IL-23 and executive function and processing speed. The outliers were different individuals in each instance. Interestingly, each of the identified immune markers, plasma CCL8, TIMP-1 and IL-23, demonstrated an inverse relationship to standardized neurocognitive scores, in other

words, elevated cytokine levels correlated with lower neurocognitive function (Figure 1a, b and c, respectively).

Since viral load was detectable in 15% of the participants in our study (Table 1) and viral replication can increase systemic inflammation, we sought to determine if inadequate viral suppression influenced the associations between the inflammatory biomarkers and neurocognitive function identified above. Participants with detectable viral loads were distributed of across all concentrations of biomarkers and neurocognitive domain standard scores (Figure 1a-c, grey circles). Thus qualitatively, participants with detectable viral loads did appear to drive the biomarker-domain relationships. If participants with detectable viral loads (n=18) were excluded from statistical analyses, significant multivariate associations to neurocognitive subsets were maintained for plasma IL-23, and decreased for CCL8 and TIMP-1. However, plasma CCL8, TIMP-1 and IL-23 maintained all significant univariate correlations (Supplementary Table 1). Together these data indicate that incomplete viral suppression did not significantly influence plasma biomarker associations to individual neurocognitive domains, but could affect the strength of relationships within neurocognitive subsets.

A scatterplot matrix of the neurocognitive domains standard scores mapped to race/ethnic and sex categories (Supplementary Figure 1) identified differential patterns in ethnic and sex function in executive function and cognitive flexibility suggesting their potential influence on the relationship between biomarkers and those neurocognitive domains. A covariate analysis to identify race/ethnic and sex effects determined that neither significantly influenced the relationship between the neurocognitive domains and biomarkers (Supplementary Table 2) in this cohort.

Discussion

Although preliminary, our data provide compelling support that blood-based inflammatory biomarkers are associated with neurocognitive impairment in a demographic balanced cohort of PLWH. By creating subsets of neurocognitive domains, statistical methods and normalization were utilized to identify CCL8, TIMP-1 and IL-23 as plasma markers of neurocognitive function in PLWH. These cytokines may serve as easily accessible biomarkers that complement current methods by enhancing sensitivity of diagnosis resulting in timely therapeutic interventions. Since frequent blood draws are a regular occurrence in HIV primary care, blood-based markers have the potential for clinical implementations. In all the neurocognitive domains examined (except reaction time), lower functionality correlated with higher levels of blood-based markers.

None of the study participants had previously been evaluated for HAND and diagnosis thereof was not a goal of the study. However, our results are broadly consistent with previous studies that describe domain specific impairment in PLWH. PLWH performed worse in tests of processing speed, working memory and perspective memory, and executive function (Anderson et al, 2018; Burdo et al, 2013; Gott et al, 2017; Lyons et al, 2011). While HIV has reportedly been associated with reaction time (Hardy and Hinkin, 2002), our studies did not identify biomarkers for the domain.

As a diagnosis of exclusion, cognitive impairment can only be attributed to chronic HIV infection after exclusion of other possible causes or comorbidities. Further, many comorbidities including Alzheimer's disease, heart disease, and liver disease have been associated with localized and systemic inflammatory changes (Kaspar and Sterling, 2017; Morgan et al, 2019; Tan et al, 2007; Vos et al, 2016). A study of comorbidities in PLWH found that the duration of HIV infection associated with the comorbidity severity patterns of cardiovascular diseases, mental health problems, metabolic disorders and chest/other infections (De Francesco et al, 2019). It is intuitive that a current AIDS diagnosis (CDC, stage 3) would be associated with mortality due to opportunistic infections, the prevalence of non-AIDS associated mortality due to cardiovascular disease, liver disease and cancer is on the rise in developed nations (Croxford et al, 2017; Farahani et al, 2017; Taramasso et al, 2019). Therefore, we cannot exclude the possibility that the associations between biomarkers and neurocognitive domains in our cohort could be due to the variety of comorbidities inherent in our study population or in PLWH as a whole. This population is living longer, aging faster and burdened with a higher prevalence of comorbidities than the general population; however, it is yet clear if this is due to the infection, the treatment or other environmental and societal influences (Croxford et al, 2017; Veenstra et al, 2019).

The inflammatory markers of neurocognitive function identified in this study play significant roles in the pathology of HAND and other neurological diseases. CCL8 is an agonist for the C-C chemokine receptor (CCR)5, which is a co-receptor for macrophage-tropic strains of HIV. CCL8 is upregulated in HIV-infected brain cell cultures and microglia (Rom et al, 2010; Wang and Gabuzda, 2006) and promotes migration of activated monocytes and T cell (Gouwy et al, 2011). CCL8 may contribute to neurocognitive impairment by stimulating infiltration of CNS by activated immune cells. Moreover, CCL8 secretion is upregulated in monocytes, epithelial cells and macrophages in response to IL-1β and TNF-α; both cytokines are overexpressed during HIV infection (Brabers and Nottet, 2006; Yang et al, 2002). While there are a limited number of reports on the contribution of CCL8 to neurocognitive impairment, CCL8 shares 62% amino acid sequence similarity with CCL2, which is a significant contributor to HAD pathogenesis (Dhillon et al, 2008; Kelder et al, 1998). Based on this evidence we can speculate that higher CCL8 promotes biological processes that contribute to neurocognitive impairment in PLWH. On the contrary, studies by Rom *et al.* show that immunohistochemical staining of CCL8 was diminished in HIV encephalitic brains compared to uninfected controls (Rom et al, 2010). They speculated that active HIV infection, which is often the case in HIV encephalitis, causes an explosive expression of a variety of cytokines. This may result in increased expression of other factors that may be inhibitory to CCL8. Thus, CCL8 pathogenic activity may differ in highly active versus chronic HIV.

During HIV infection, the systematic loss of Th17 cells in the gut leads to the upregulation of IL-23 in an attempt to activate the unresponsive Th17 cells. The subsequent stimulation of other immune cells, such as neutrophils, leads to a microenvironment of immune activation (Fernandes et al, 2017). HIV-mediated breakdown of the gut mucosal line of defense promotes the systemic dissemination of bacterial products such as LPS into the blood; aggravating systemic inflammation (Louis et al, 2010; Maek et al, 2007). Recent studies uncovered gut-brain communication as pathogenic element in neurological diseases

(Vujkovic-Cvijin and Somsouk, 2019). Disruption of the gut mucosal surfaces directly alters neurocognitive function in PLWH. Gut derived LPS and sCD14 in the plasma negatively impacts processing speed in PLWH (Monnig et al, 2017). HIV Tat induced diarrhea correlated with activation of glial cells and neurocognitive impairment in a mouse model (Esposito et al, 2017). These studies suggest that IL-23 could negatively impact neurocognitive function through the gut-CNS axis. There are limited studies demonstrating the direct contribution of IL-23 to HAND; however, in an autoimmune encephalomyelitis model, IL-23 was upregulated by microglia, induced T cell mediated inflammation and correlated with increased CNS lesions (Becher et al, 2003; Cua et al, 2003; Yannam et al, 2012).

TIMP-1 is an inhibitor of matrix metalloproteinases (MMP), enzymes that affect blood-brain barrier (BBB) integrity. Upregulated in response to HIV, MMPs degrade BBB tight junction proteins and promote monocyte infiltration into the CNS (Ju et al, 2009; Liuzzi et al, 2000). In the brains of HAD patients, the MMP/TIMP-1 balance is tilted towards the excessive accumulation of MMPs, contributing to BBB breakdown and CNS immune invasion (Ghorpade et al, 2001; Suryadevara et al, 2003; Vos et al, 2000). TIMPs are extracellular inhibitors of MMP activity. In contrast to CCL8 and IL-23, TIMP-1 plays a protective role during chronic HIV. TIMP-1 plasma levels are upregulated in HIV patients, possibly in response to increased MMPs activity (Mastroianni et al, 2002). Interestingly, IL-23 upregulated MMP-9 secretion in serum of autoimmune disease patients and cancer cells (Li et al, 2012; Plee et al, 2015). TIMP-1 attenuates MMP- mediated BBB permeability and protects neurons from HIV induced apoptosis, suggesting its therapeutic potential alleviating HIV-related CNS dysregulation (Ashutosh et al, 2012; Chaturvedi et al, 2014; Chen et al, 2013). These studies suggest that plasma TIMP-1 is increased as an attempt to mitigate MMP driven insults.

Essentially, our study corroborates reports of immune dysregulation in HIV disease (Brockman et al, 2009), resulting in the imbalance of anti-inflammatory proteins such as TIMP-1 and pro-inflammatory proteins such as CCL8. Furthermore, marker levels in the two blood-based samples, plasma *vs* culture supernatants, were different. This indicates that they have distinctly separate inflammatory profiles and suggests that plasma inflammation is not exclusively influenced by blood cells. It is more likely representative of systemic inflammation, including the brain. Moreover, systemic inflammation has been shown to affect the brain. (Corlier et al, 2018; Wang et al, 2018). Chronic, persistent inflammation in PLWH has been attributed to low grade HIV replication (Canestri et al, 2010; Palmer et al, 2008), HIV proteins produced by latently infected cells (King et al, 2006), as well as immune dysregulation in virus concentrated areas such as the gut (Brenchley et al, 2004; Merlini et al, 2011).

Causes for heightened inflammation in PLWH are a complex combination of several factors. Incomplete viral load suppression or relapses viral expression have been linked with increased immune activation, inflammation and cell-associated viral DNA as compared to HIV+ patients with undetectable viral loads (Falasca et al, 2017). A randomized ART cessation study demonstrated that increased viral load associated with decreased IL-10 levels and increased soluble vascular cell adhesion molecule-1 and CCL2 as measures of

endothelial and immune activation (Calmy et al, 2009). This study was conducted in recently diagnosed individuals who had maintained viral suppression for an average of only eight months and viral rebound was significant in the absence of ART. In PLWH with long-term viral load suppression, inflammation has consistently been shown to be independent of viral persistence (Falasca et al, 2017; Malhotra et al, 2019). In fact studies have shown that sustained inflammation and HIV persistence strongly associated with pre-ART viral load and not cell-associated viral RNA and DNA measures post-ART (Gandhi et al, 2017). Research has also shown peripheral inflammation as markers of neurocognitive impairment, plasma levels of sCD163 (Burdo et al, 2013), sCD14 (Lyons et al, 2011) and CCR2 expression on monocytes (Veenstra et al, 2019) and CD4 nadir (Ellis et al, 2011) were identified to associate with neurocognitive function. Gene expression studies in peripheral immune cells of HIV elect controllers, *e.g.* individuals whose suppress HIV replication in the absence of ART, showed lower inflammation and a strong killing capacity for HIV+ cells compared to PLWH on ART (Hocini et al, 2019). These studies indicate that immune responses early in the course of HIV infection may directly influence the sustained levels of immune activation, inflammation and viral persistence, independent of successful viral suppression or relapse.

While few of these studies examined neurocognitive function in association with measures of inflammation, our study examined participants with detectable viral loads that were not congregated at the high end of the distribution (Figure 1). As such, the relationship between decreased neurocognitive function and elevated plasma biomarker levels was not dependent upon participants with detectable viral loads. The validation of peripheral biomarkers for neurocognitive impairment will require a complex analysis of long-term associations to disease in consideration of acute changes in immune responses. This is especially true for PLWH since the disease directly affects the immune system and the force driving sustained inflammation remains resistant to therapy.

It is well-documented that disparities of minority inclusion in clinical studies exists, and when included, the special patterns and responses of these groups are often not investigated (McCarthy, 1994; Oh et al, 2015). Here, we found race/ethnic and sex differences in HIV-related clinical parameters. Sex differences were identified in CD4 T count, where counts in women were 22% higher than males. Favorable HIV-related clinical patterns have been documented in women (Collazos et al, 2007; Ruel et al, 2011) and attributed to immunomodulatory effects of sex hormones, although these did not lower risk for HIV-related complications (Fish, 2008). Race/ethnic differences were detected in years since diagnosis, where Hispanic whites were diagnosed an average of four years later than other ethnic groups. This may be indicative of low rates of HIV testing resulting in diagnosis at later stages of infection in the Hispanic population (Arya et al, 2013).

The disparities in incidence of HIV in the US warrant the investigation of the effect of race/ethnicity and sex on markers of HIV-associated neurocognitive impairment. Our studies identified no unique contributions of race/ethnicity and sex to the relationship between inflammatory biomarkers and neurocognitive scores, in contrast to studies by Burlacu *et al.,* which demonstrate plasma CXCL10 as a marker for HAND in women (Burlacu et al, 2019). This could be due to the small sample size of the cohort, which might have reduced the

statistical power to detect significant associations. Nevertheless, this is an important step toward consideration of minorities in HAND studies.

This study has important limitations. Firstly, the relatively large number of biomarkers combined with the sample size required stringent analysis approaches and restricted the number of comparisons performed. This restriction resulted in an inability to investigate the effect of comorbidities in this cohort. Secondly, greater than 50% of the study participants suffered from at least one comorbid disease or infection, and these may impact inflammatory biomarkers in relation to neurocognitive function. Lastly, we recognize that the biomarker panel measured in the study, though substantial and diverse, did not encompass the entire immune system or its relationship to neurocognitive function in PLWH. Other inflammatory markers, which could have stronger associations to neurocognitive function in PLWH, were not examined.

This study is novel as it investigates sociodemographic balanced cohort of PLWH and focuses on different components of blood, *e.g.* the plasma and blood cell secretions, while similar studies had evaluated inflammation in only one or the other. Our study contributes additional evidence to demonstrate the negative relationship between peripheral inflammation and neurocognitive function in PLWH. While the markers identified offer insight on inflammation and neurocognition at a single time point in a small cohort. Future studies should be performed in an expanded longitudinal cohort and consider the ability of biomarkers, or a set of biomarkers, to predict the likelihood of neurocognitive impairment in the future. If successful, identification of peripheral biomarkers for HIV-associated neurocognitive impairment will enable earlier interventions to improve outcomes in PLWH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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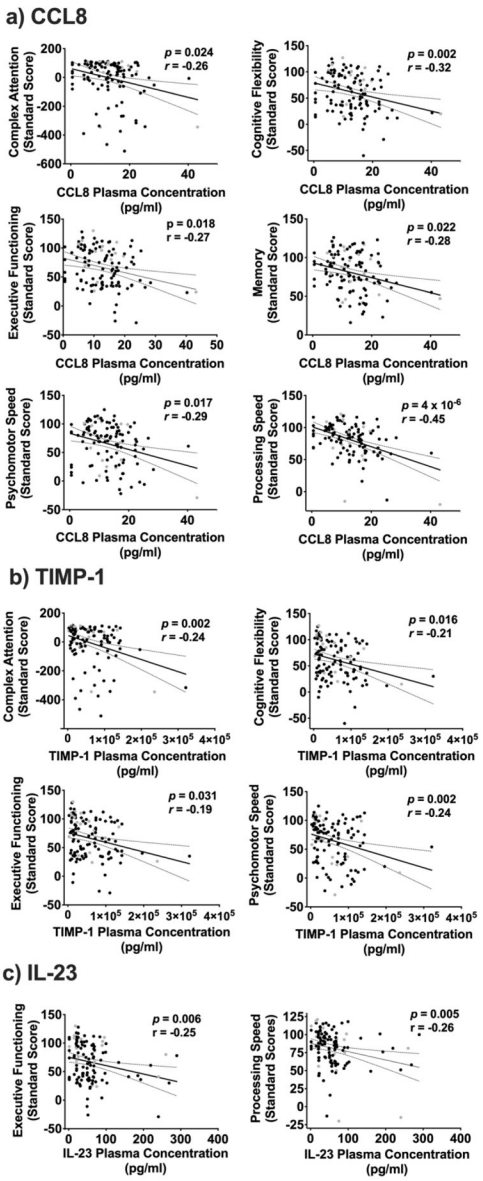

Figure 1. Correlation of Plasma CCL8, TIMP-1 and IL-23.

(a) Plasma concentration of CCL8 plotted against complex attention, cognitive flexibility, executive function, memory, psychomotor speed and processing speed, (b) plasma concentration of TIMP-1 plotted against complex attention, cognitive flexibility, executive function and psychomotor speed, (c) plasma concentration of IL-23 plotted against executive function and processing speed, after the removal of one outlier (n=120). dotted lines = standard error, grey = participants with detectable viral loads, p = univariate p-value, r = Pearson's correlation.

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Table 1.

Cohort Demographic and Clinical Characteristics (n=121)

	Black/African American			Non-Hispanic White			White Hispanic			Overall		
	Total (n=41)	**Men (n=20)**	**Women (n=21)**	***Total (n=41)***	**Men (n=21)**	**Women (n=20)**	***Total (n=39)***	**Men (n=20)**	**Women (n=19)**	***Total (n=121)***	**Total Men (n=61)**	**Total Women (n=60)**
Age												
Mean ± S.D. (Range)	*49.8 ±10.6 (23-68)*	51.6 ±7.12 (34-62)	48.1 ±13.04 (23-68)	*51.0 ±9.4 (26-67)*	52.8 ±7.70 (40-67)	49.1 ±10.65 (26-64)	*47.8 ±8.6 (29-66)*	49.4 ±7.88 (37-66)	46.1 ±9.22 (29-59)	*49.5 ±9.6 (23-68)*	51.3 ±7.6 (34-67)	47.8 * ±11.0 (23-68)
Education Level												
Less than HS	*15*	4	11	*6*	2	4	*15*	5	10	*36*	11	25
HS grad	*7*	4	3	*8*	1	7	*10*	6	4	*25*	11	14
At least some college	*18*	11	7	*27*	18	9	*14*	9	5	*59*	38 *	21
Years since Diagnosis												
Mean ± S.D. (Range)	*15.2 ±8.1 (1-29)*	15.1 ±7.81 (1-22)	15.3 ±8.47 (1-29)	*15.5 ±8.9 (2-31)*	17.3 ±9.63 (3-31)	13.8 ±7.91 (2-26)	*10.7 ¶ ±5.3 (1-20)*	10.7 ±5.55 (1-20)	10.7 ±5.10 (1-20)	*13.8 ±7.8 (1-31)*	14.3 ±8 (1-31)	13.3 ±7 (1-29)
CD4 T Cell Count												
Mean/mm^3 ± S.D. (Range)	*662.6 ±270.4 (97-1254)*	601.8 ±300.4 (97-1254)	720.6 ±230.8 (315-1166)	*638.0 ±372.9 (145-1799)*	531.76 ±353.3 (145-1799)	749.45 ±368.5 (183-1381)	*643.7 ±337.7 (97-1399)*	573.05 ±304.1 (97-1275)	714.36 ±362.6 (218-1399)	*648.2 ±327.0 (97-1799)*	568.2 ±316.9 (97-1799) (97-1799)	728.3 * ±319.6 (183-1399)
Viral Load, copies/mL												
<20	*35*	14	21	*33*	18	15	*33*	15	18	*101*	47	54
>20	*6*	6	0	*7*	3	4	*5*	4	1	*18*	13	5
ART CPE score												
Mean ± S.D. (Range)	*6.4 ±3.7 (0-14)*	6.4 ±3.7 (0-12)	6.5 ±3.7 (0-14)	7.4 ±*3.3 (0.16)*	8.1 ±3.0 (3-16)	6.5 ±3.4 (0-11)	*7.3 ±3.2 (0-16)*	7.6 ±2.3 (0-13)	6.5 ±3.9 (0-16)	*7.0 ±3.4 (0-16)*	7.4 ±3.1 (0-16)	6.6 ±3.6 (0-16)
Comorbidities												
CNS	*9*	5	4	*11*	5	6	*6*	1	5	*26*	11	15
Coin-fections	*14*	8	6	*25*	14	11	*13*	8	5	*52*	30	22
Psychiatric	*20*	6	14	*11*	3	8	*23*	9	14	*54*	18	35
Respiratory	*19*	9	10	*16*	6	10	*12*	4	8	*47*	19	28

	Black/African American			Non-Hispanic White			White Hispanic			Overall		
	Total (n=41)	**Men (n=20)**	**Women (n=21)**	***Total (n=41)***	**Men (n=21)**	**Women (n=20)**	***Total (n=39)***	**Men (n=20)**	**Women (n=19)**	***Total (n=121)***	**Total Men (n=61)**	**Total Women (n=60)**
Cardiac	*23*	16	17	*24*	14	10	*22*	12	10	*69*	42	37
Endo-crine, Liver & Kidney	*20*	8	12	*18*	5	13	7	3	4	*45*	16	29
Cancer	*1*	1	0	*9*	4	5	*1*	1	0	*11*	6	9
Tx for Drug & Alcohol	*10*	5	5	*12*	2	10	*5*	2	3	*27*	9	18

Abbreviations: ART, antiretroviral therapy; CPE, CNS penetration effectiveness; HS, high school, Tx for Drug and alcohol, Past treatment for drug and alcohol abuse.

¶Significance for sex comparisons by t-test, and

*race/ethnic comparisons by one-way ANOVA or Chi χ^2 for categorical variables. Missing data: Education level, one Black/African American man; one viral load, Non-Hispanic White woman, and one White Hispanic man.

Table 2.

Association of Sociodemographic and HIV-relevant Clinical Parameters with Neurocognitive Domains

	Subset 1 Multivariate Model (*p*-value)	**Subset 2 Multivariate Model (*p*-value)**	**Domains (univariate significance at *p <0.05 and **p <0.01, respectively)**
Race/Ethnicity	0.057	0.670	ns
Sex	0.189	0.059	ns
Age	0.836	0.339	ns
Education	**1.1×10^{-8}**	**3.3×10^{-6}**	Complex Attention**, Cognitive Flexibility**, Memory**, Psychomotor Speed**, Executive Function**, Processing Speed**
CD 4 T Cell Count	**0.029**	**0.002**	Memory*, Processing Speed**
Viral Load	0.591	0.119	ns
Years since diagnosis	0.992	0.879	ns
ART CPE Score	0.915	0.628	ns

Subset 1: complex attention, cognitive flexibility, memory, reaction time and psychomotor speed neurocognitive domains. Subset 2: executive function and processing speed. **Bolded *p*-values** is indicative of significant association observed with multivariate model. ns = no significance.

Univariate correlations were not considered if multivariate associations were not significant.

Table 3.

Association of Plasma and 24-hour PBMC Supernatant Inflammatory Biomarkers with Neurocognitive Domains

	Mean (pg/ml) Concentrations (±S.D.)	Subset 1 Multivariate Model (*p*-value)	Subset 2 Multivariate Model (*p*-value)	Domains (univariate significance at *p <0.05 and **p <0.01 respectively)
Plasma Biomarkers				
CCL8	13.04 (±7.32)	**0.014**	**1.6×10^{-6}**	Complex Attention*, Cognitive Flexibility*, Memory*, Psychomotor Speed*, Executive Function*, Processing Speed**
IL-10	3.03 (±3.68)	0.230	**0.005**	Processing Speed**
IL-23	66.37 (±91.23)	0.081	**0.035**	Executive Function*, Processing Speed*
TIMP1	58470.27 (±51668.42)	**0.010**	**0.011**	Complex Attention**, Cognitive Flexibility*, Psychomotor Speed*, Executive Function*
24-hour PBMC Supernatant Biomarkers				
CCL2	591.92 (±0.57)	**0.026**	0.385	Complex Attention**
IL-17	0.57 (±0.79)	**0.034**	0.725	ns

Subset 1: complex attention, cognitive flexibility, memory, reaction time and psychomotor speed neurocognitive domains. Subset 2: executive function and processing speed. **Bolded *p*-values** is indicative of significant association observed with multivariate model. ns = no significance. Univariate correlations were not considered if multivariate associations were not significant.