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ORIGINAL ARTICLE

The Association of Immune Markers with Cognitive Performance in South African HIV-Positive Patients

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Abstract

Dysregulated expression of neuro-immune markers has previously been linked to HIV-associated neurocognitive impairment. We undertook an exploratory approach in a HIV clade-C cohort, investigating the association between eight immune markers and neurocognitive performance in 99 HIV+ and 51 HIV- participants. Markers were selected on preliminary and putative evidence of their link to key neuro-immune functions. Cognitive performance was established using a battery of tests sensitive to HIV-associated neurocognitive impairment, with domain-based scores utilized in analysis. The markers Thymidine phosphorylase (TYMP) and Neutrophil gelatinase-associated lipocalin (NGAL) were significantly higher while Matrix Metalloproteinase (MMP)9 was significantly lower in HIV+ participants. Our results further showed that in the HIV+ group, worse psychomotor processing speed was associated with higher TYMP and NGAL levels and worse motor function was associated with higher NGAL levels. Future studies should explore the underlying mechanisms of these markers in HIV-associated neurocognitive impairment.

Keywords HIV · HIV-associated neurocognitive impairments · HAND · Cognition · Neuroinflammation and cytokines

Introduction

HIV-associated neurocognitive impairment occurs in up to 50% of the HIV-infected population (Heaton et al. 2010). HIV-1 invades the brain through a "Trojan Horse" method by crossing the blood–brain barrier (BBB) through infected monocytes that later differentiate into macrophages (González-Scarano and Martín-García 2005). This triggers an inflammatory response, which is considered to be a crucial contributor to the neuropathogenesis of HIV-associated neurocognitive impairment (Beck et al. 2015; Jones et al. 2016). In human studies, associations of increased pro-inflammatory markers in peripheral blood with cognitive impairments in HIV further support the involvement of a dysregulated immune system in cognitive impairment in HIV (Cohen et al. 2011; Correia et al. 2013; Yuan et al. 2013, 2015b)

Damage to the BBB is another common hallmark of HIV-associated neurocognitive impairments (Banks et al. 2006). BBB damage allows for the further recruitment of infected cells into the brain (Banks et al. 2006). Differential expression of BBB integrity markers in blood and CSF (e.g. MMP9 and s100-β) correlate with neurocognitive impairment in HIV participants (Li et al. 2013; Abassi et al. 2017; Xing et al. 2017). Therefore, a dysregulated expression of immune markers may contribute to the development of HIV-associated neurocognitive impairments. Efforts in elucidating the neuro-immune response of HIV-associated neurocognitive impairment have to date largely been centered on investigation of monocyte activation markers (Lyons et al. 2011; Kamat et al. 2012; Burdo et al. 2013; McGuire et al. 2015;

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Imp et al. 2017) and pro-inflammatory markers (e.g. TNF-α) (Wesselingh et al. 1997; Brabers and Nottet 2006).

To further build on this field it is of interest to explore additional markers that may be potentially associated with neurocognitive impairments in HIV. Studies present inconsistent findings for the associations of commonly investigated peripheral inflammatory markers (e.g. TNF, IP-10 and IL-10) with HIV associated neurocognitive impairment (Cohen et al. 2011; Correia et al. 2013; Yuan et al. 2015a; Krebs et al. 2016). It is therefore of interest to investigate immune markers that may be involved in the pathophysiology of HIV associated neurocognitive impairment. This approach can result in the identification of markers with a greater consistency in the association with cognitive impairments in HIV and potentially provide new insights into the neuropathophysiology of HIV associated neurocognitive impairments. Here, we aimed to explore the associations between several immune markers and neurocognitive performance in HIV (clade-C) participants that were treatment naïve or had only recently started combination antiretroviral therapy (cART) (< 1 month) to elucidate the inflammatory profile without the confounding effects of cART on neuronal health. Specifically, to investigate the association between these markers and neurocognitive domains commonly affected by HIV (Butters et al. 1990). Furthermore, a systematic review and meta-analysis indicate that globally, a large percentage of patients do not adhere to treatment (Uthman et al. 2014), making this population interesting for further investigation. Majority of longitudinal studies also report that with the introduction of cART, the first neuroimmune responses are noted at durations greater than 3 months (Hattab et al. 2014; Krebs et al. 2016; Richert et al. 2017). This population represents participants with a higher risk of developing dementia and therefore this study may provide an opportunity to investigate the immune response in these individuals.

The markers Monocyte chemoattractant protein-1/C-C motif ligand 2 (MCP-1/CCL2), Transforming growth factor (TGF)-β, Matrix metalloproteinase (MMP)9, Vascular endothelial growth factor (VEGF), Thymidine phosphorylase (TYMP) and Neutrophil gelatinase-associated lipocalin (NGAL) were selected for investigation based on their potential involvement in the pathophysiology of neurocognitive impairments in HIV, as identified from a review of the scientific literature. A full description of the markers investigated is described in the supplementary file (table 1).

Methods

Study Participants

Data from HIV seropositive participants from two independent studies were pooled for the analysis reported in this paper: cohort 1 represents a subsample of a previously published dataset (Joska et al. 2011), whilst cohort 2 are participants from an ongoing study that is investigating the effects of heavy drinking on HIV-associated neurocognitive impairments. 99 HIV+ participants were included in this study, which were previously recruited from primary health care clinics in Cape Town and the Western Cape region of South Africa. HIV+ participants completed at least one study visit, which included a detailed sociodemographic, medical, and neuropsychological assessment as well as the relevant laboratory measures (including genotyping, viral load and CD4 count). HIV serostatus was confirmed by two independent rapid tests and confirmed via ELISA analysis. In addition, 51 HIV- control participants recruited from local Voluntary Counseling and Testing Clinics as part of the ongoing study were included in this study for comparison purposes. Participants included in this study ranged from 18 through 65 years in age with at least 7 years formal education, across both cohorts. HIV+ participants were treatment naïve or had only recently started cART (< 1 month) prior to neurocognitive measurements and blood collection. Cases were excluded from this study if they had 1) severe psychiatric disorder or presented any other neurological disorder, 2) substance abuse (other than alcohol) and 3) moderate to severe head injury.

Participation was voluntary, and individuals were informed that they could withdraw from the study at any time. Written informed consent was obtained following a thorough explanation of the study procedures. This study was approved by the Human Research Ethics Committee of the Faculty of Health Sciences (University of Cape Town) (Sub-study HREC 213/2018 linked to primary studies: 003/2015, 023/2008 and 263/2007). Individuals received financial compensation for their time. Bloods were obtained at the same visit when measures for neurocognitive performance were evaluated.

Neurocognitive Measures

The presence of HIV-associated neurocognitive impairment was assessed with a detailed battery. All participants were tested in their home language. The test battery represents measures of domains typically affected by HIV (Butters et al. 1990) and encompassed several domains of cognitive function. The specific scores used for each domain were acquired from the following neuropsychological measures: Processing speed (WAIS-III Digit symbol, WAIS-III Symbol search, Colour trails I Stroop Colour) (Golden 1975), Verbal (Animal Fluency) (Acevedo et al. 2000), Learning (the Hopkins Verbal Learning, Brief Visuospatial Memory Test) (Benedict et al. 1998), Memory (HVLT Delayed recall, BVMT Delayed recall), Motor Functioning (Groove peg board Dominant, Groove peg board Non-dominant) (Klove

1963) and executive functioning (Colour trails II) (D'Elia et al. 1996). The raw neuropsychological test scores were standardized using data from the HIV- control participants to calculate *z*-scores. *Z*-scores were then averaged to create 6 composite indices (processing speed, verbal, learning, memory, motor and executive functioning).

Laboratory Assessment of Blood

Blood samples for all study participants were collected into tubes via venipuncture. Serum tubes were kept at room temperature for 30 min to allow for clotting. Plasma EDTA tubes were kept on ice for 30 min until centrifugation. Serum and EDTA tubes were subsequently centrifuged at 2500×g. Serum and plasma were then aliquoted into cryo vials and immediately stored at −80 °C until analyses. HIV viral load was determined by the CAP/Roche Cobas Ampliprep (sampled post-2015) and the Abbott M2000SP and M2000RT (sampled prior to 2015) methods. The CD4 count was determined by PanLeucogate (PLG) (Beckman Coulter FC500MPL) method.

All cytokines were measured using Enzyme-linked Immunosorbent Assays (ELISA) (R&D systems, DuoSet ELISA) according to the manufactures instructions. CCL2, VEGF and TYMP were measured in serum. TGF-β1, IL-1β, IFN-γ, MMP9 were measured in plasma. Samples were diluted as follows MCP-1/CCL2: 1:2, TGF-β1: 1:30, IL-1β: no dilution, IFN-γ: no dilution, MMP9: 1:150, VEGF: no dilution, TYMP: 1:10 and NGAL: 1:100. All samples were assayed in duplicate. The intra- and inter-assay coefficients of variation for all tests were within acceptable ranges of <8% and < 10% respectively.

Other Potential Covariates

Years of completed formal education and age were included as covariates in models of the association of immune system markers and cognitive function. In addition, given the presence of heavy drinkers in cohort 2, and the observation that chronic excessive alcohol consumption may affect both the immune system and cognitive function in people with HIV (Meyerhoff 2001), problematic drinking was also included as a covariate. Hazardous drinking was operationalised as more than 5 standard drinks per occasion over a 2-week period for 3 months prior to the marker assays, as assessed using the timeline follow-back calendar questionnaire. A further description of the cohort is described in the supplementary file (table 2).

Statistical Analyses

All analyses were conducted using SPSS (version 25, IBM, USA). *P* values were considered statistically significant for all analyses at a value of less than .05. Data distribution for markers NGAL, CCL2 and TGF- β1 were found to be skewed, therefore the data was log transformed prior to statistical analyses. MMP9, TYMP and VEGF demonstrated normal data distribution with acceptable skewness and kurtosis. IL-1β and INF-γ were below the detectable ranges in serum for all samples and omitted from further analyses. Unpaired t-test analyses were performed to investigate differences in the levels of plasma/serum markers between HIV+ and HIV- study participants. Analyses of covariance (ANCOVA) with levels of inflammatory markers as dependent variables were subsequently performed to analyze the levels of the inflammatory markers between HIV+ and HIV- study participants, adjusted for age, sex, education, cohort and alcohol use. Exploratory analyses were done with Pearson correlations to investigate associations between the inflammatory markers with the cognitive domains in HIV+ and HIV- respectively. A Bonferroni correction was accounted for the number of cognitive domains tested ($\alpha/n = .05/6 = .008$). Subsequently, separate regression models were conducted to examine the associations between cognitive functions and marker concentrations, after adjusting for the following covariates individually: demographics (age, sex and education), cohort (cohort, heavy drinkers) and HIV+ disease parameters (nadir CD4 count, and viral load).

Results

Sample Characteristics

The study population included a total of $n = 150$ participants which comprised of $n = 99$ HIV+ participants and $n = 51$ HIV- controls. HIV+ participants had a mean age of 33.88 years and 36% were female. The HIV+ group had a mean nadir CD4 count of 306.13 cells/μl and viral load of 3.74 log copies/ml. From the HIV+ participants, $n = 19$ had been on treatment for less than one month upon sample collection, with the remainder cART-naïve. The two cohorts differed with respect to mean age, gender composition and current CD4 count. Differences between mean age and gender were controlled for upon statistical analysis. Sample characteristics are summarized in table 1. Information on the sample characteristic of the separate cohorts is presented in the supplementary file (table 2). The effect of drinking habits on cognitive performance in either HIV- controls or HIV+ participants in the second cohort was evaluated. No significant differences between the heavy drinkers vs. non/light drinkers in either the HIV- control group or the HIV+ participants were found for any of the cognitive domains tested (all *p* values were greater than 0.1) (results not shown).

Table 1 Characteristics of HIV- and HIV+ study participants

	HIV– ($n = 51$) Controls	HIV+ ($n = 99$) HIV+	p-value
Age years mean (SD)	37.51 (9.07)	33.88 (6.91)	.01
Sex, female N (%)	27 (52.9)	36 (36.4)	.051
Education, years (SD)	10.28 (1.55)	10.50 (1.30)	.38
CD4 + nadir, mean (SD)	–	306.13 (184.723)	–
Viral load log copies/mL mean (SD)	–	3.74 (1.07)	–
Heavy drinkers, N (%)	26 (51.0)	18 (18.2)	<.001

Inflammatory Markers in HIV+ Versus HIV- Participants

Bivariate analyses revealed that TYMP ($p < .001$) and CCL2 ($p = .027$) were significantly higher whereas MMP9 ($p = .007$) was lower in HIV+ participants relative to controls (Fig. 1). TYMP remained significantly higher ($p < .015$) and MMP9 significantly lower ($p = .005$) in HIV+ participants after controlling for age, sex, education and alcohol use with ANCOVA. CCL2 was no longer significantly higher ($p = .78$) in HIV+ participants after controlling for these covariates. NGAL gained significance ($p = .046$) after controlling for these variables.

Association of Cytokine Levels and Cognitive Performance in HIV- Participants

After applying Bonferroni corrections of $p = .05/6$, higher levels of TGF-B1 were associated with better performance in processing speed in HIV- participants ($p = .008$) (table 2) (unadjusted). Linear regression analyses with covariates; 1) alcohol use and 2) demographics (age, sex and education) were controlled for. After controlling for 1) alcohol use only ($B = .367$, SE = .149, β = .391, $p = .019$) and 2) demographics ($B = .172$, SE = .148, β = .171, $p = .25$) the association was attenuated.

Association of Cytokine Levels and Cognitive Performance in HIV+ Participants

After applying Bonferroni corrections of p = .05/6, higher TYMP ($p = .001$) and NGAL ($p = .007$) levels were associated with worse processing speed and higher NGAL levels with worse motor functioning ($p = .002$) in HIV+ participants (table 3). Further analyses were performed for these markers after adjusting for the covariates, age, sex, education, alcohol use, nadir CD4 count, cohort and HIV viral load (table 4). The association between TYMP and NGAL with neurocognitive performance remained after correcting

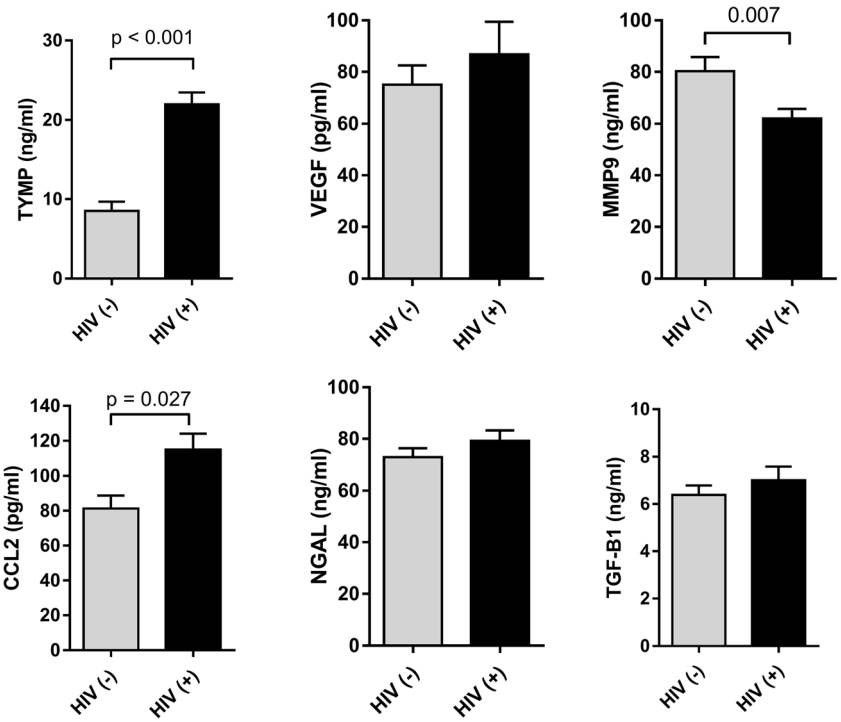

Fig. 1 Concentrations of inflammatory markers between HIV- and HIV+ study participants. NGAL, CCL2 and TGF-β1 were ln-transformed and for interpretation purposes presented means were back-transformed. Bars indicate the mean protein concentrations in the different study groups and are expressed as mean ± standard error of the mean (s.e.m.)

Table 2 Pearson correlations between neuroinflammatory markers and composite cognitive domain scores (through averaging Z scores of specific within-domain tests) in HIV- participants

Cognitive domain	TYMP	VEGF	MMP9	CCL2	NGAL	TGF-B1
Processing speed	−.118	.053	.101	.047	−.189	**.425**[a]
Verbal	−.086	−.250	−.154	.041	−.159	−.007
Learning	−.211	−.048	−.027	.002	−.163	.021
Memory	−.122	−.072	.123	−.142	−.167	.166
Motor Functioning	.187	.119	.085	.210	.117	−.227
Executive function	.098	−.061	.028	.042	.061	−.078

Data with significant *p* values are presented in bold

[a] $p = .008$

for demographics, cohort, alcohol use and HIV disease parameters (table 4).

Discussion

In this exploratory study, we investigated the serum and plasma levels of several immune markers and their associations with domain-based neurocognitive performance. We found significantly higher levels of TYMP and NGAL, and significantly lower levels of MMP9 in HIV+ participants compared to HIV- controls, after controlling for age, sex, education, and alcohol use. Furthermore, in HIV+ participants we found that after controlling for age, sex, education, alcohol use, CD4 count, cohort and viral load, higher TYMP levels were associated with worse processing speed and higher NGAL levels were associated with worse processing speed and motor functioning.

The current study is the first to our best knowledge to investigate peripheral circulating TYMP levels in HIV+ participants. Changes to the neuroimmmunity in HIV+ participants may contribute to the higher serum levels of TYMP demonstrated in this study. A microarray study of gene expression of TYMP across tissues reported that its expression is particularly high in $CD14^+$ monocytes (Su et al. 2002). The TYMP gene expression was reported to be increased in primary monocytes of HIV patients with viremia (Wu et al. 2013). Moreover, $CD14^+$ monocytes are increased in HIV+ patients (Bowers et al. 2014) regardless of treatment status (Mendez-Lagares et al. 2013; Bowers et al. 2014). $CD14^+$ monocytes may account for the higher TYMP levels in patients with HIV.

Evidence from basic science research may provide insights into the association of TYMP with neurocognitive performance, specifically in the domains of processing speed. Generally, in a treatment naïve cohort, HIV replication is not controlled and a typical sub-cortical pattern of pathology is seen, with resulting impairment of processing speed and executive functions (Bell 2004; Moore et al. 2006). A study on post mortem brain tissues from patients with HIV/Tuberculosis Meningitis (TBM) demonstrated that the TYMP protein expression was further increased when HIV was present in patients with TBM (Kumar et al. 2012). It was further reported that TYMP co-localized with microglia (Kumar et al. 2012), indicating that activated microglia may produce TYMP in the HIV brain. In a study conducted by Chapouly and colleagues, it was demonstrated that TYMP was an important contributor to BBB disruption in brain cell cultures and in an experimental autoimmune encephalitis mouse model (Chapouly et al. 2015). Further studies are needed to elucidate the potential involvement of TYMP in BBB dysregulation in HIV. This is of interest since BBB damage

Table 3 Pearson correlations between neuroinflammatory markers and composite cognitive domain scores (through averaging Z scores of specific within-domain tests) in HIV+ participants

Cognitive domain	TYMP	VEGF	MMP9	CCL2	NGAL	TGF-B1
Processing speed	**-.340**[a]	−.136	−.025	−.101	**-.285**[b]	.049
Verbal	−.100	−.101	−.046	−.134	−.061	−.034
Learning	−.012	-.257[c]	.208[d]	.176	−.049	−.012
Memory	.014	−.186	.025	.120	−.016	−.015
Motor Functioning	.178	.061	.007	.130	**.329**[e]	−.026
Executive function	.132	.180	.106	.091	.146	.089

Data with significant *p* values are presented in bold

[a] $p = .001$, [b] $p = .007$, [c] $p = .011$, [d] $p = .043$, [e] $p = .002$

Table 4 Association of TYMP and NGAL with cognitive performance, including covariates in HIV+ study participants

	TYMP and Processing speed			NGAL and Processing speed			NGAL and motor functioning		
	B (SE)	β	p	B (SE)	β	p	B (SE)	β	p
Unadjusted	−.013 (.004)	−.34	.001	−.37 (.134)	−.285	.007	.602 (.182)	.329	.001
Model 1	−.014 (.004)	−.37	.001	−.463 (.128)	−.358	.001	.611 (.180)	.350	.001
Model 2	−.013 (.004)	−.33	.003	−.362 (.142)	−.279	.012	.690 (.190)	.377	<.001
Model 3	−.014 (.005)	−.319	.010	−.533 (.172)	−.375	.003	.615 (.269)	.279	.025

Model 1: adjusted for demographics; age, sex and education

Model 2: adjusted for cohort; cohort, heavy drinkers

Model 3: adjusted for HIV+ disease parameters; nadir CD4 count, and viral load

may play an important function in the pathophysiology of neurocognitive impairment in HIV (Banks et al. 2006; Maubert et al. 2017).

Higher plasma NGAL levels in HIV+ participants have recently been reported in a clinical investigation (Morieri et al. 2018) and is supported by the significantly higher levels in HIV+ participants reported in our study. Elevated serum and plasma NGAL has been associated with neurocognitive impairment in patients with mild cognitive impairment in Alzheimer disease (Choi et al. 2011) as well as in patients acute ischemic cerebrovascular diseases (Elneihoum et al. 1996). This study further contributes to literature indicating that NGAL is an inflammatory marker that is associated with cognitive impairment (Choi et al. 2011). Our findings show that higher NGAL levels are associated with worse processing speed. In this regard, it was previously demonstrated that higher plasma NGAL levels were associated with impaired verbal memory and processing speed in females with late-life depression (Naudé et al. 2014). Mounting evidence from investigations in cell cultures and animal models suggest that NGAL may contribute to neurobiological mechanisms that are involved in the neuropathology of neurocognitive impairments. NGAL may act on neuronal health by in inhibiting remyelination (Al Nimer et al. 2016) and neuronal survival (Bi et al. 2013). Demyelination or change in myelin structure, in turn could contribute towards oligodendrocyte damage, resulting in desynchronized neural circuits and impaired information processing speed (Pajevic et al. 2014), which are common pathologies in HIV (Liu et al. 2016). NGAL may also be involved in motor functioning. Animal studies suggest that increased levels of NGAL may interfere with motor functioning (Jang et al. 2013) and BBB integrity (Egashira et al. 2016). However, the neurobiological mechanisms of NGAL in HIV requires further investigation.

Contradictory to previous findings (Lorenzl et al. 2008), here MMP9 was significantly lower in HIV+ participants. This may be due to clinical studies largely investigated MMP9 in extended duration treatment cohorts and cART may contribute to increased inflammatory levels. A HIV cART treatment cohort demonstrated higher MMP9 levels compared to that of the treatment naïve cohort (Li et al. 2013). In addition, the study by Li and colleagues also found that MMP9 expression levels were very similar for HIV- participants (33,385 pg/ml ± 16,230) and HIV+ treatment naïve participants (35,073 pg.ml ± 43,245) (Li et al. 2013). This may suggest that MMP9 levels may be strongly affected by the extended use of cART.

In addition to the above, Southern Africa is largely a HIV clade-C population (Wainberg 2004), which has been linked to lower levels of neurotoxicity (Rao et al. 2013). MMP9 investigations were largely done in clade-B HIV cohorts (Sporer et al. 1998; Li et al. 2013; Kim et al. 2014), which has been linked to increased prevalence of neurocognitive impairments (Langford et al. 2003) and increased neuroinflammatory levels (Gandhi et al. 2009; Wong et al. 2010). This has been largely attributed to the presence of a dicysteine motif in that TAT protein of clade-B cohorts, which potentiates increased monocyte chemotaxis and increased neurotoxicity (Rao et al. 2013). It is therefore possible that MMP9 levels are affected by clade differences as well as extended use of cART, potentially explaining the lower MMP9 levels in the HIV+ population from which our sample was drawn.

A limitation of the present study is its relatively small sample size, which warrants replication in a larger sample size. Other important confounding factors, i.e. medication use, and co-morbid diseases may influence the outcomes of the findings presented here. The cohorts pooled for investigation in this study may have influenced the results. However, we adjusted for various covariates such as cohort and alcohol use to minimize the effects of these differences. In addition, we performed a sub-analysis to determine the effects of heavy drinking on inflammatory profiles in our HIV+ population. We demonstrated that there were no significant differences in inflammatory profiles for the HIV+ non/light drinkers vs. HIV+ heavy drinkers (supplementary file, table 3). Participants included in this study were either treatment naïve or only recently initiated treatment (<30 days). cART is also a contributor to

dysregulated inflammatory levels in HIV+ participants (Shah et al. 2016). However, long-term treatment studies reported that in most cases, the first neuro-immune responses are noted at durations greater than 3 months (Hattab et al. 2014; Krebs et al. 2016; Richert et al. 2017). Serum IL-1β and IFN-γ were below the detectable ranges of the assays used in this study and therefore excluded from further statistical analyses. A number of the cognitive domains were only measured with a single neuropsychological test, whereas a larger number of neuropsychological tests per cognitive domain may increase the reliability and external validity of estimates presented here. The findings presented here may not apply to other HIV-1 clades (e.g. HIV-1 clade-B), as inflammatory markers may be affected by the HIV subtype (Gandhi et al. 2009).

Conclusion

This exploratory study shows that the immune markers TYMP and NGAL may be associated with neurocognitive impairment in HIV+ participants. This is the first study to our knowledge to investigate the associations of TYMP and NGAL with in neurocognitive impairments in patients with HIV. The results from this study indicate that TYMP and NGAL potentially are interesting targets for future fundamental and clinical investigations in HIV-associated neurocognitive impairments.

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Compliance with Ethical Standards

Conflict of Interest None.

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