

## Article

# Proinflammatory cytokines and chemokines are related to MRI measures of white and gray matter in HIV infection

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**Abstract: Background:** HIV is accompanied by production of proinflammatory cytokines which are regarded as critical in neuronal damage, leading to brain dysfunction, hence the need to identify cytokine biomarkers sensitive to brain damage. **Methods:** We applied MRI volumetric neuroimaging and high throughput Luminex based immunoassays to examine the relationship between cortical white matter, subcortical gray matter and total gray matter brain volumes and plasma cytokines in HIV individuals using generalised linear models and Partial least square regression model. **Results:** Higher plasma inflammatory cytokines CCL5/RANTES and MCP-1 were significantly associated with lower cortical white matter volume. Higher IL-6 was associated with both lower subcortical gray matter and lower total gray matter, whereas higher IL-8 and GM-CSF were associated with higher total gray matter only. Higher VEGF, PDGF-BB and IL-9 were associated with higher cortical white matter volumes. After standardisation and adjusting for clinical and demographic variables, IL-6, IL-8, MCP-1 remained associated with lower volumes of the three brain regions whereas IL-9, VEGF and PDGF-BB were associated with higher volumes. **Conclusions:** Association proinflammatory cytokines RANTES, MCP-1 and IL-6 with lower brain volumes could imply possible involvement in neurodegenerative processes in HIV infection while IL-9, VEGF and PDGF may have a neuroprotective or neurotrophic role.

**Keywords:** inflammation; Luminex; volumetric neuroimaging, cognitive impairment

## 1. Introduction

Despite the immense contribution by combination antiretroviral therapy (cART) to reduce morbidity in people infected with HIV, people living with HIV (PLWH) continue to develop a spectrum of cognitive, motor and psychological manifestations, clinically referred to as HIV associated neurocognitive disorders (HAND). This condition affects more than 50% of HIV infected people [1-3]. The pathology of HAND is not completely understood, but it is thought to be a complex interaction of virus, inflammation and the host immune response. Pathological evidence of brain invasion by HIV includes neural loss, dendritic damage, astrogliosis, microgliosis and nucleated giant cell formation [4]. HIV is thought to penetrate the central nervous system (CNS) through trafficking of cell-free virus by transcytosis across the blood brain barrier (BBB) and also through the disrupted BBB caused by HIV infection. It is believed that BBB disruption is enhanced by

peripheral proinflammatory environment, which is induced by HIV [5]. Transport of cytokines across the BBB has been demonstrated for IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and tumor necrosis alpha (TNF- $\alpha$ ) [6]. This passage of blood-born cytokines to the brain can potentially affect brain integrity and function, with cytokines altering the integrity of the BBB and compromising the ability to regulate trafficking of immune cells [7-8]. Therefore, peripheral inflammatory cytokines may be putative mediators of neuroinflammation in HIV infection. A link between peripheral inflammation and increased cytokine production in the CNS has been demonstrated in HIV infection [9-10]. It has been suggested that the major impact of peripheral inflammatory cytokines on the CNS may possibly be through cytokine-mediated production of prostaglandins in the brain endothelium [11].

Plasma cytokine markers are easy to measure and could be clinically relevant to assess HIV associated neuropathological changes [12]. In fact, systemic immune activation and inflammation has been linked to HAND in several studies [13-14]. Greater changes in inflammation-associated neuronal loss have been observed in HAND patients [15]. Multiplex analytic technologies in proteomics can be used to accelerate the identification of cytokine biomarkers associated with clinical outcomes in HIV associated neurocognitive impairment [16]. Luminex based high throughput assays can be used to identify biomarker signatures of immune activation in HIV infection [17].

Recently, magnetic resonance imaging (MRI) techniques have shown greater potential in understanding the mechanisms underlying HIV associated neurocognitive impairment [18]. MRI imaging technologies which are non-invasive are able to generate objective brain measurements that are able to determine the extent of neurodamage associated with HIV infection. Fully automated MRI technologies have been developed for automated segmentation of the brain [19-21] in which specific brain regions of interest are assessed for abnormal structural changes in HIV infected people [22]. The advantage of segmentation-derived brain volumetric measures are non-invasiveness and high throughput, and both have led to widespread use to investigate brain changes in HIV infection [23-28]. It has been observed that volumetric changes correlate with neuropsychological testing and clinical measures in HIV associated cognitive impairment and brain gray and white matter atrophy has been reported in people living with HIV (PLWH) [29]. Primary brain regions that have been shown to be affected by neuroinflammation are the hippocampus and entorhinal and temporal cortices [30]. Structural imaging of HIV patients have shown impact on grey matter structures and subcortical regions [31] and cerebral atrophy with ventricular enlargement [32-33].

This study applies volumetric neuroimaging and high throughput Luminex based immunoassays to examine the relationship between specific brain regions and plasma cytokine levels in order to assess the inter-relatedness of brain changes in HIV and concomitant peripheral immune activation in treatment naïve patients. We hypothesized that higher levels of pro-inflammatory cytokines are associated with lower volumes of specific brain regions..

## 2. Results

Considering the three regions of interest (ROI), the unstandardised regression coefficients and associated p-values for cytokines significantly associated with brain volumes using generalised linear model (GLM) are shown in **Table 1**. Regarding cortical white matter, only RANTES and MCP-1 were related to lower white cortical volume. The rest, were related to higher cortical white matter volume including IL-7, IL-9 and VEGF. Considering subcortical gray matter, IL-1 $\alpha$  was significantly associated with higher volume, whereas IL-6 was associated with lower volume. Lower total gray matter volume was associated with higher levels of 9 cytokines/chemokines including, IL-6, IL-8, MCP-1 and RANTES. Only TNF- $\alpha$  and IL-1 $\alpha$  were associated with higher total gray matter volume.

Table 1: Relationship between cytokines levels and brain volumes

Cytokine	Coef.	SE.	Z	P-value	95% CI	
Cortical white matter volume						
IL-1 $\beta$	.0020477	.0009955	2.06	0.040	.0000966	.0039988
IL1 $\alpha$	4.44e-06	1.67e-06	2.66	0.008	1.17e-06	7.70e-06
IL7	.0010158	.000482	2.11	0.035	.000071	.0019606
IL9	1.42e-06	6.33e-07	2.24	0.025	1.80e-07	2.66e-06
MCP-1	-.0009288	.0003753	-2.47	0.013	-.0016643	-.0001933
PDGFBB	3.57e-07	1.42e-07	2.52	0.012	7.89e-08	6.36e-07
RANTES	-1.52e-06	4.82e-07	-3.16	0.002	-2.47e-06	-5.77e-07
VEGF	1.57e-06	4.93e-07	3.19	0.001	6.07e-07	2.54e-06
Subcortical gray volume						
IL1 $\alpha$	7.88e-06	2.47e-06	3.19	0.001	3.04e-06	.0000127
IL6	-.0021455	.000831	-2.58	0.010	-.0037743	-.0005167
Total gray volume						
IL1 $\alpha$	3.16e-06	1.57e-06	2.01	0.045	7.61e-08	6.25e-06
IL6	-.0020823	.0005579	-3.73	0.0001	-.0031758	-.0009888
IL8	-.0014577	.0006186	-2.36	0.018	-.0026701	-.0002453
IL10	-.0035407	.0013919	-2.54	0.011	-.0062688	-.0008126
IL12p70	-.0020964	.0008961	-2.34	0.019	-.0038527	-.0003402
IL15	-.0001525	.0000626	-2.44	0.015	-.0002751	-.0000298
GCSF	-.003109	.0010898	-2.85	0.004	-.0052449	-.0009732
GMCSF	-.0059812	.0031477	-1.90	0.057	-.0121505	.0001881
MCP1	-.0024353	.0008448	-2.88	0.004	-.0040912	-.0007795
RANTES	-2.54e-06	1.04e-06	-2.44	0.015	-4.57e-06	-5.02e-07
TNF- $\alpha$	5.11e-06	2.52e-06	2.03	0.042	1.75e-07	-.00001

After adjusting for age, sex, plasma viral load and CD4+T absolute count only IL-9 ( $p = 0,045$ ) remained significantly associated with higher cortical white matter volume. MCP-1 ( $p = 0.047$ ) and GCSF ( $p = 0.025$ ) were related to lower cortical white matter volume after adjusting for the same clinical and demographic parameters. With regards to subcortical gray matter two cytokines, IL-6 ( $p = 0.008$ ) and IL-8 ( $p = 0.042$ ) remained significantly associated lower subcortical. The same cytokines, including MCP-1 ( $p = 0.032$ ) remained associated with lower total gray matter volume.

### 2.1. White matter volume as a function of plasma cytokine volumes

Using PLS-R modelling, 13 cytokines/chemokines including RANTES, MCP-1, IP-10, IL-6, IFN- $\gamma$  Eotaxin, GM-CSF, IL-13 and IL-8 were associated with lower cortical white matter volume as shown as indicated by their relationship with standardised coefficients in Fig 1. Among these pro-inflammatory cytokine, MCP-1, RANTES, GM-CSF, and IL-6 had strongest negative association as shown by the magnitude and direction of bars on the graphs showing standardised coefficients of cortical volume as a function of cytokines in Fig 1.

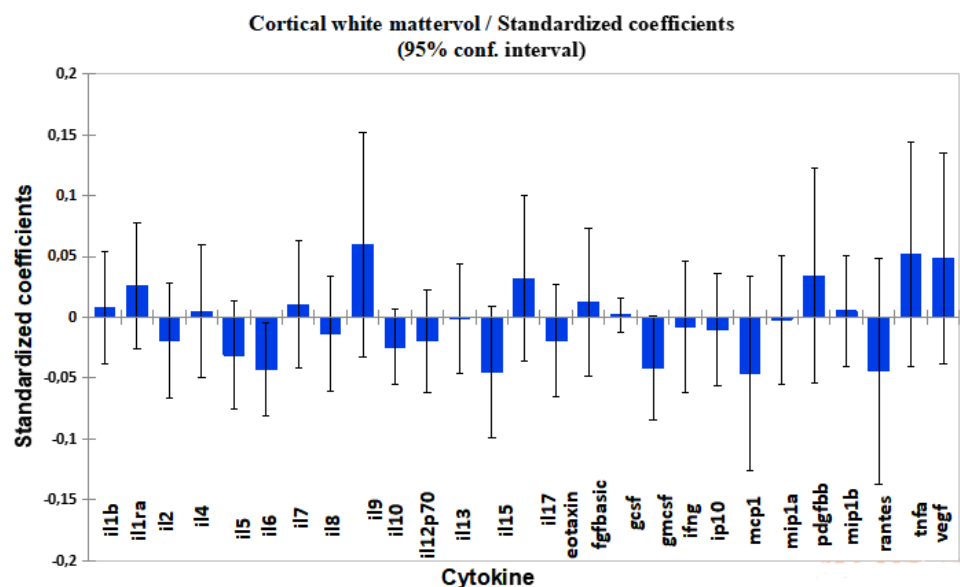


Fig 1. Graphical representation of the magnitude and direction of plasma cytokines on cortical white matter volume using PLS-R standardized coefficients and 95% confidence intervals. RANTES (-0.044; -0.137 – 0.049), MCP-1 (-0.046; -0.136 – 0.34), IL-6 (-0.043; -0.082 – (-0.005)) and

GM-CSF (- 0.042; - 0.082 – 0.001) were prominent cytokines in the reduction of cortex volume (table 3).

However, using multiple generalised linear model to determine significance of the association only MIP-1 $\beta$  ( $\beta$  = - 0.005;  $p$  = 0.029) was significant. The other cytokines IL-1 $\alpha$ , IL-7, IL-9, PDGF-BB, TNF- $\alpha$ , FGF-basic. IL-9 had the strongest positive association with cortical white matter volume as shown in fig 1. However, multiple generalized regression models did not show statistical significance of the association as shown in **Table 2**.

**Table 2: Standardized coefficients for cortical white matter volume in multiple GLM**

Variable	Coefficient	Std. deviation	Lower bound (95%)	Upper bound (95%)	P value
IL-1 $\beta$	0.008	0.023	-0.038	0.054	0.147
IL-1 $\alpha$	0.026	0.026	-0.026	0.078	0.627
IL-2	-0.019	0.024	-0.067	0.028	0.545
IL-4	0.005	0.027	-0.049	0.059	0.815
IL-5	-0.031	0.022	-0.076	0.013	0.723
IL-6	-0.043	0.019	-0.082	0.005	0.281
IL-7	0.010	0.026	-0.042	0.063	0.794
IL-8	-0.014	0.024	-0.061	0.034	0.694
IL-9	0.059	0.047	-0.033	0.152	0.099
IL-10	-0.024	0.015	-0.055	0.006	0.24
IL-12p70	-0.020	0.021	-0.062	0.023	0.856
IL-13	-0.001	0.023	-0.047	0.044	0.955
IL-15	-0.045	0.027	-0.099	0.009	0.822
IL-17	0.032	0.034	-0.036	0.100	0.693
Eotaxin	-0.019	0.023	-0.066	0.027	0.429
FGF-basic	0.013	0.031	-0.048	0.073	0.648
GCSF	0.001	0.007	-0.013	0.014	0.255
GMCSF	-0.042	0.022	-0.084	0.001	0.633
IFN- $\gamma$	-0.008	0.027	-0.062	0.046	0.898
IP-10	-0.010	0.023	-0.056	0.036	0.967
MCP-1	-0.046	0.040	-0.126	0.034	0.13
MIP-1 $\alpha$	-0.001	0.027	-0.054	0.052	0.49
PDGF-BB	0.034	0.045	-0.055	0.123	0.688
MIP-1 $\beta$	0.005	0.023	-0.040	0.051	0.029
RANTES	-0.044	0.047	-0.137	0.049	0.694
TNF- $\alpha$	0.052	0.046	-0.041	0.144	0.381
VEGF	0.048	0.044	-0.038	0.135	0.155

### 2.1.2. Subcortical gray matter volume as a function of cytokine levels

The relationship of plasma cytokines with subcortical gray matter volume followed the same pattern as the one observed in cortical white matter. Although regression coefficients were smaller in subcortical gray matter than white matter as shown in **Fig 2**, they were statistically significant. For example proinflammatory cytokines IL-2 ( $\beta$  = -0.008;  $p$  = 0.012), IL-6 ( $\beta$  = 0.0017;  $p$  = 0.002) and GM-CSF ( $\beta$  = -0.016;  $p$  = 0.03) were significantly related lower volumes as shown in **table 2**. Cytokines showing positive with subcortical gray matter volume included IL-7, IL-9, FGF-basic and VEGF but the association was not significant.

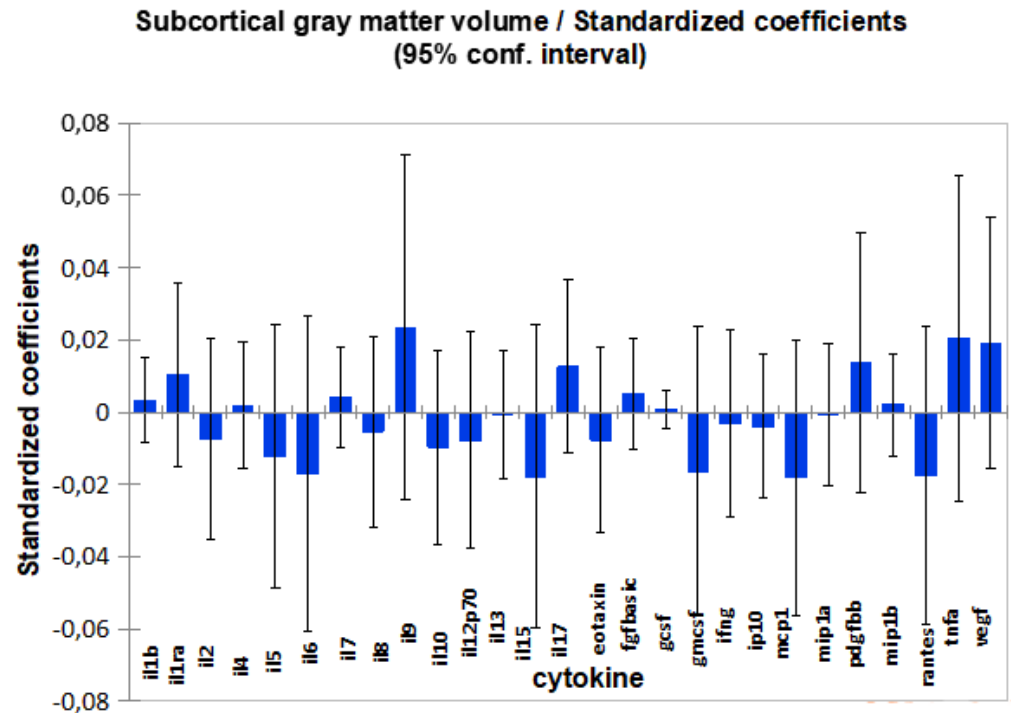


Fig 2. Graphical representation of the magnitude and direction of plasma cytokines impact on subcortical gray matter volume using PLS-R standardized coefficients and 95% confidence intervals. RANTES (-0.17; -0.059 - 0.024), MCP-1 (-0.018; -0.056 - 0.020), IL-6 (-0.017; -0.061 - 0.026) and GM-CSF (-0.016; -0.057 - 0.024) were prominent cytokines in the reduction of cortex volume (table 4)

Table 3: Standardized coefficients for subcortical gray matter volume in multiple GLM

Variable	Coefficient	Std. deviation	Lower bound (95%)	Upperbound (95%)	P value
IL-1 $\beta$	0.003	0.006	-0.009	0.015	0.046
IL-1 $\alpha$	0.010	0.013	-0.015	0.036	0.304
IL-2	-0.008	0.014	-0.035	0.020	0.012
IL-4	0.002	0.009	-0.016	0.019	0.543
IL-5	-0.012	0.018	-0.049	0.024	0.216
IL-6	-0.017	0.022	-0.061	0.026	0.002
IL-7	0.004	0.007	-0.010	0.018	0.014
IL-8	-0.005	0.013	-0.032	0.021	0.018
IL-9	0.023	0.024	-0.024	0.071	0.419
IL-10	-0.010	0.014	-0.037	0.017	0.89
IL-12p70	-0.008	0.015	-0.038	0.022	0.81
IL-13	-0.001	0.009	-0.018	0.017	0.05
IL-15	-0.018	0.021	-0.060	0.024	0.821
IL-17	0.013	0.012	-0.011	0.037	0.87
Eotaxin	-0.008	0.013	-0.034	0.018	0.064
FGFbasic	0.005	0.008	-0.010	0.020	0.039
GCSF	0.000	0.003	-0.005	0.006	0.388
GMCSF	-0.016	0.020	-0.057	0.024	0.03
IFN- $\gamma$	-0.003	0.013	-0.029	0.023	0.927
IP-10	-0.004	0.010	-0.024	0.016	0.072
MCP-1	-0.018	0.019	-0.056	0.020	0.481
MIP-1 $\alpha$	0.000	0.010	-0.020	0.019	0.249
PDGF-BB	0.014	0.018	-0.022	0.050	0.988
MIP-1 $\beta$	0.002	0.007	-0.012	0.016	0.761
RANTES	-0.017	0.021	-0.059	0.024	0.655
TNF- $\alpha$	0.020	0.023	-0.025	0.065	0.357
VEGF	0.019	0.017	-0.016	0.054	0.705

Total gray matter volume as a function of plasma cytokines

Seven proinflammatory cytokines, chemokines namely, RANTES, MCP-1, GM-CSF, IL-6, IL-8, IL-15 and were associated with lower gray matter volume, shown in Fig 3. However, only IL-6 ( $\beta = -0.041$ ;  $p = 0.019$ ), IL-8 ( $\beta = -0.013$ ;  $p = 0.023$ ) and Eotaxin ( $\beta = 0.019$ ;  $p = 0.035$ ) were statistically significant as shown in **Table 3**.

### Total gray matter volume / Standardized coefficients (95% conf. interval)

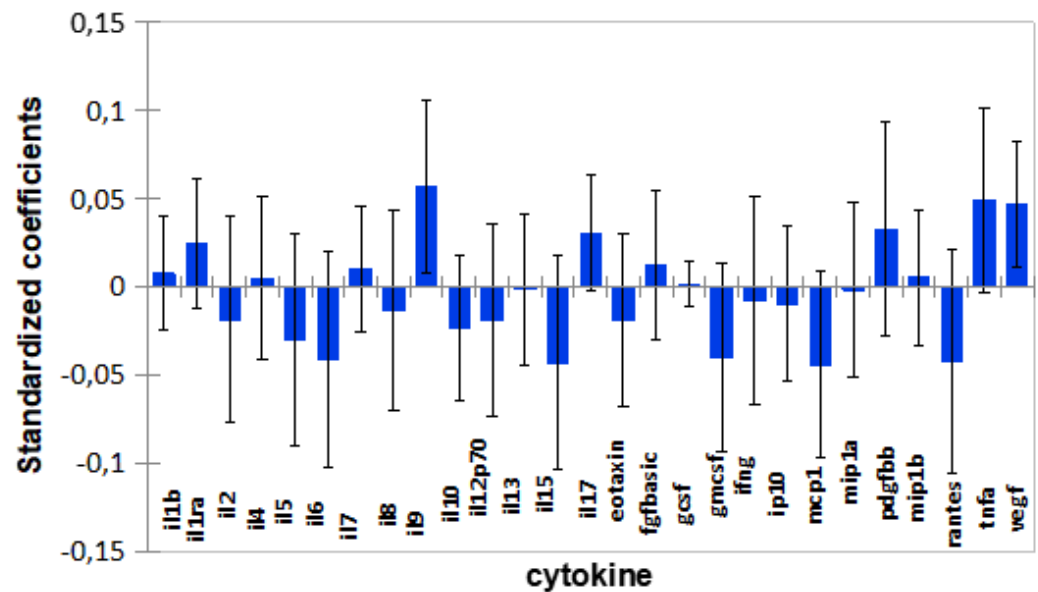


Fig 3. Graphical representation of the magnitude and direction of plasma cytokines on total gray matter volume using PLS-R standardized coefficients. RANTES (-0,042; -0,106 – 0,021), MCP1 (-0,044; -0,097 – 0,009), IL-6 (-0,041; -0,103 – 0,020) and gmcsf (-0,040; -0,093 – 0,013) were outstanding cytokines related to lower total gray matter volume

Table 4: Standardized coefficients for total gray matter vol in a multiple GLM

Variable	Coefficient	Std. deviation	Lower bound (95%)	Upper bound (95%)	P value
IL-1 $\beta$	0.008	0.016	-0.025	0.040	0.176
IL-1 $\alpha$	0.025	0.019	-0.012	0.062	0.894
IL-2	-0.018	0.029	-0.077	0.040	0.605
IL-4	0.005	0.023	-0.042	0.051	0.730
IL-5	-0.030	0.030	-0.091	0.031	0.444
IL-6	-0.041	0.031	-0.103	0.020	0.019
IL-7	0.010	0.018	-0.025	0.045	0.144
IL-8	-0.013	0.028	-0.070	0.044	0.023
IL-9	0.057	0.025	0.008	0.106	0.826
IL-10	-0.023	0.021	-0.065	0.018	0.349
IL-12p70	-0.019	0.027	-0.073	0.035	0.778
IL-13	-0.001	0.021	-0.044	0.041	0.425
IL-15	-0.043	0.031	-0.104	0.017	0.592
IL-17	0.031	0.016	-0.002	0.063	0.979
Eotaxin	-0.019	0.025	-0.068	0.031	0.035
FGF-basic	0.012	0.021	-0.030	0.054	0.651
GCSF	0.001	0.007	-0.012	0.014	0.297
GM-CSF	-0.040	0.027	-0.093	0.013	0.054
IFN- $\gamma$	-0.008	0.029	-0.066	0.051	0.626
IP-10	-0.009	0.022	-0.053	0.035	0.255
MCP-1	-0.044	0.027	-0.097	0.009	0.075
MIP-1a	-0.001	0.025	-0.050	0.049	0.672
PDGF-BB	0.033	0.030	-0.028	0.093	0.532
MIP-1b	0.005	0.019	-0.033	0.044	0.216
RANTES	-0.042	0.032	-0.106	0.021	0.860
TNF-a	0.050	0.026	-0.003	0.102	0.347
VEGF	0.046	0.018	0.011	0.082	0.147

### 3. Discussion

Brain volume loss is widespread in neurological disease, hence it can be a useful measure of CNS damage and a marker of clinical disease progression and cortical



volume loss has been used as a marker to detect overall structural changes during disease process. We examined the relationship between plasma cytokine concentration and cortical white matter and gray matter volumes in untreated HIV infection. Our findings showed that RANTES was the strongest predictor of low white matter and gray matter volumes followed by IL-2, eotaxin, IL-8, MCP-1 and IL-6 and GMSCF respectively. All the cytokines are proinflammatory cytokines and chemokines. Brain volume reduction is thought to be enhanced by proinflammatory environment, which is partly caused by blood derived activated monocytes/macrophages and their associated inflammatory cytokines [34]. Structural brain imaging of patients suspected of HIV associated neurocognitive impairment has revealed a profound impact on grey matter, subcortical regions, and cerebral atrophy [34]

HIV-infected patients have elevated levels of systemic inflammatory markers, due to persistent viral replication, release of progeny virus and viral proteins from infected cells with clear effects on white matter microstructure [35-36]. Brain structural studies in HIV infection have shown decreased cortical white matter volume [37] and subcortical gray matter with executive deficits [38]. Results of this study clearly demonstrated inverse association on the brain volumes and peripheral inflammation implying that there is a link between systemic levels and HIV associated brain volume alterations. Chemokine-mediated inflammation is known to cause gliosis and dendritic damage in HIV [39] and cortical thinning on MRI was shown to be a sensitive index of declining neurological and immune function in AIDS patients [40]. Even mild dendritic loss may lead to behavioral alterations in HIV-associated minor cognitive motor disorder. Our previous studies have shown that plasma proinflammatory cytokines/chemokines, particularly RANTES, IP-10 and IL-2 were strong correlates of HIV-associated cognitive impairment [41].

We have demonstrated that the detected changes in cortical white matter and total gray matter volumes of HIV-infected patients was associated plasma RANTES concentration, but with variation between the brain centres. HIV-related neurocognitive impairment is associated with cerebral atrophy [42]. Therefore, neuroimaging and measures of systemic inflammation like RANTES has a potential be used to diagnose HIV-related CNS injury. In fact higher levels of RANTES have been observed in brain lesions of HIV patients and have been expressed in several inflammatory diseases of the CNS[43-44].

The monocyte chemoattractant protein-1 (MCP-1/CCL2) showed a negative correlation with both cortical white matter and total grey matter volumes but had greater impact in cortical white matter than total grey matter. The link between MCP-1 and neuro-damage was shown in HIV encephalitis (HIVE) and increased in brains of AIDS patients with HIV associated dementia (HAD) [45-46]. It has been shown to play a major role in blood brain barrier disruption (Stamatovic et al., 2005)] and higher levels in CSF were associated with glial dysfunction [47]. This study which has combined quantitative neuroimaging and proteomic technologies has shown that plasma MCP-1 has impact on the degree of brain injury in HIV infection. Higher plasma MCP-1 level has been associated with greater severity and faster cognitive decline in HIV[48]. Therefore, plasma MCP-1 might reflect the risk and progression HIV associated neurodamage. Ongoing brain injury may be subclinical for long periods in HIV infection but the use of inflammatory biomarkers like MCP-1 and MRI technologies can quantify the degree of neurological involvement. The advantage of such approach is that MRI is non-invasive and plasma samples for cytokines are easy to access and the biomarker is to measure.

IL-6 mediated inflammation is known to cause higher incidence of gliosis and dendritic damage to patients with neurological diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) [49-50]. The observed association between elevated plasma IL-6 levels and significantly lower cortical white matter, and grey matter volumes in HIV

infection suggests that related processes in PD and AD pathology are involved. Using PLS-R, IL-6 had the greatest impact on cortical white volume reduction followed by total gray matter and subcortical grey matter was the least. However, only the IL-6 associated reduction of subcortical grey matter and total grey matter were significant. This implies that cortical grey matter and white matter loss may be partly linked to IL-6 mediated inflammation but the magnitude is different. Volumes of cortical grey matter had also been shown to be significantly negatively correlated with IL-6 in Schizophrenia [51]. Higher levels of IL-6 in older adults have been cross-sectionally and longitudinally associated with cortical thinning, cognitive impairment, as well as increased dementia risk [52-53]. These findings vindicates our observations on the link between the cytokine and neurological conditions. However, the consequence of the increase IL-6 on neuronal and glial health and the integrity of cortical volume in the context of HAND need to be further investigated to accumulate sufficient data for use in clinical assessments.

Contrary to the relationship between IL-6, RANTES, MCP-1, IL-2, eotaxin, IL-8, and GM-CSF other cytokines such as IL-9, IL-1 $\alpha$ , vascular endothelial growth factor (VEGF) were significantly associated with higher brain volumes. IL-9 is pleiotropic cytokine produced by a variety of cells [54]. It also preferentially promotes CD4<sup>+</sup> T cell proliferation and affects both B cell development and function. It has the capacity to actively induce resolution of inflammation [55]. We suggest that association between higher IL-9 and larger brain volumes could be related to the anti-inflammatory role of the cytokine which might have interfered with or inhibited or reduced inflammation-induced neuro-damage. It could also have influenced humoral immunity through its effects on B cell development and function. This cytokine might play an important in brain tissue regeneration and repair in the brain in HIV infection [56]. IL-9 mediated interference with inflammation and tissue repair could be further investigated to elucidate the neuroprotective mechanisms in HIV infection and exploring strategies of IL-9 immunotherapy in HIV associated neurocognitive impairment.

The VEGF, produced by many cells is both a potent angiogenic factor and mitogen which has been demonstrated to directly stimulate tumor cells to induce apoptosis resistance [57]. We observed that higher levels of the growth factor were associated with increased brain volumes. The growth factor has been shown to have neurotrophic effect and enhances survival of neurones in some brain regions [58] and higher levels have been detected in HIV associated CNS diseases [59-61]. Another growth factor related to VEGF, platelet-derived growth factor PDGF-BB was also associated with increased cortical white matter and grey matter volumes. The growth factor has demonstrated promotion of neuronal proliferation and reversal of neurotoxicity mediated by HIV-1 Tat [62]. The growth factor is also involved in astroglial scar formation which confines inflammation to the lesion core and protects the neuronal tissue [63]. Although both VEGF and PDGFBB positive influence on brain volumes, PDGFBB influence was less than that of VEGF. We suggest that the results can guide further clinical studies on the neuroprotective role of both PDGF-BB and VEGF. However, it would be interesting to determine the neuropsychological correlates of plasma VEGF and PDGF-BB to assess how these growth factors impact on the neurobehavioural aspects of HIV. Neuropsychological correlates of VEGF would enable us to confirm that the observed links between higher plasma levels of the growth factors and higher cortical white matter and grey matter volumes were a result of a biological process.

Although TNF- $\alpha$  was associated with all increased brain volumes, cortical white matter, subcortical grey matter and total grey matter, the association was only significant in total grey matter. TNF- $\alpha$  is an inflammatory cytokine whose neurotoxic role in HIV associated neurocognitive disorders is well documented [64-67]. The implications of our findings were contrary to the neurodegenerative role of TNF- $\alpha$  as it was associated with increased brain volumes. Our findings seemed to portray a neuroprotective or neu-



rotrophic role of TNF- $\alpha$  in HIV infection. The neuroprotective role of TNF- $\alpha$  have been demonstrated in experimental models [67] and humans with Alzheimer and vascular dementia [6-69]. Therefore, the neuroprotective or neurodegenerative effects of the cytokine depends on other factors that determine the final effect of the cytokine on the CNS. Detrimental or beneficial effect of cytokine depends on level and time of expression among other factors and this could determine the final effect on CNS. We suggest future studies could perform threshold analysis for direct association studies between the cytokine and brain volumes in order to determine which levels are protective or neurotoxic.

## 4. Materials and Methods

### 4.1. Study participants

A cross-sectional study was conducted on a cohort of 139 HIV positive Xhosa-speaking individuals recruited from primary care HIV clinics in Cape Town, South Africa, that was being investigated for HAND. Inclusion criteria to participate in the study required the following: 1. Age ranging from 18 and 45 years with at least five years of formal education. This age was selected to avoid age-related CNS abnormalities. 2. HIV serostatus, determined by ELISA and then confirmed by Western blot. 3. HIV-1 RNA for plasma viral load measured by the Abbott m2000sp and the Abbott m2000rt analysers (Abbott laboratories, Abbott Park, IL, USA). Exclusion criteria included the following: any major psychiatric condition that could significantly affect cognitive status; confounding neurological disorders including multiple sclerosis and other CNS conditions; head injury with loss of consciousness greater than 30 min; clinical evidence of opportunistic CNS infections and current substance abuse or alcohol abuse as defined by structure interview [70]. This study was approved by the Health Research Ethics Committee (HREC) of Stellenbosch University, Ethics Reference #: S17/02/035.

### 4.2. Plasma cytokine quantification by multiplex assay

Cytokine concentration plasma samples was performed using a 27-plex Biorad Pro Human cytokine assay kit as described earlier (Ruhanya et al., 2021). Quantified cytokines included IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, Eotaxin, basic FG, G-CSF, GM-CSF, IFN- $\gamma$ , IP-10, MCP1, MIP-1 $\alpha$ , MIP-1 $\beta$ , PDGF-BB, RANTES, TNF- $\alpha$  and GM-CSF, VEGF. Briefly, plasma samples were incubated with antibody-coupled beads. Complexes were washed, incubated with biotinylated detection antibody, and subsequently with streptavidin-phycoerythrin, prior to assessing cytokine concentrations. Standard curves were run together with samples using standard cytokines provided in the kit. Plasma cytokine levels were determined using a multiplex array reader from Luminex™ Instrumentation System (Bio-Plex Workstation from Bio-Rad); plasma samples were run in duplicate and cytokine concentrations were calculated as the average of two independent measures using Bioplex Manager Software (California USA).

### 4.3. Structural neuroimaging and volumetric analysis

Acquisition of images and volumetric analysis of specific brain regions of interest (ROI) were performed according to the methods described earlier [72-73]. Briefly high field MRI was used to acquire T1 weighted 3-dimensional magnetization-prepared rapid acquisition gradient echo (MPRAGE) images which provide contrast for segmenting, gray matter, white matter and CSF. The data obtained from MPRAGE was used for volumetric quantification of ROI using Freesurfer software suite (v5.1) (Martinos Center, Harvard University, Boston, MA, USA. <http://surfer.nmr.mgh.harvard.edu>). Briefly, MP-RAGE scans were transformed into a template space with the skull stripped and the brain segmented into white matter, gray matter, and ventricles. The brain was further segmented into subcortical and cortical ROIs. Previous studies identify that these ROIs are impacted by HIV neuropathogenesis [74-77].

#### 4.4. Statistical analysis

Stata statistical package version 12.1 (StataCorp, College Station, Texas, USA, 2011) was used for all statistical analysis. Generalized linear modelling was used to determine the association between brain regions of interest and plasma cytokine levels. Partial least squares (PLS) approach which uses multiple linear regression analysis to find the direction of maximum covariance between plasma cytokine levels and volumes of ROI, was used (Kamat et al., 2012). Statistical significance was determined as  $p$  value  $< 0.05$ .

#### 5. Conclusions

The observation that some proinflammatory cytokines were associated with increased cortical white matter and grey matter volumes suggest that these cytokines such as RANTES, MCP-1 and IL-6 are involved in neurodegenerative pathological processes in HIV infection. The other group of cytokines including IL-9, VEGF and PDGF-BB which were associated with increased cortical white matter and grey matter volumes suggest that these cytokines have neuroprotective or neurotrophic role against inflammation mediated neuronal damage. We suggest that those cytokines which were strongly associated with reduced brain volumes like RANTES are potential biomarkers for HAND whilst those associated with increased brain volumes could be explored in search for immunotherapeutic approaches to HAND. However that data need to be collected in longitudinal studies in order observe to changes in both cytokine levels and imaging data over time. We also suggest that threshold analysis be done in order to determine the cut off values associated with each clinical outcome.

#### Author Contributions:

Study conception and design: VR, SE, RP. Acquisition of data: VR, RP, JJ, SS, GN. Analysis and interpretation of data: VR, GN, SE, RG, GJ. Drafting of manuscript and Critical revision: all authors .

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**Institutional Review Board Statement:** This study was approved by the Health Research Ethics Committee (HREC) of Stellenbosch University, Ethics Reference #: S17/02/035.

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Conflicts of Interest:** Declare conflicts of interest or state "The authors declare no conflict of interest.

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