






## Research Article

# Effects of Tobacco Usage and Antiretroviral Therapy on Biomarkers of Systemic Immune Activation in HIV-Infected Participants

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Like HIV infection, smoking, which is common among HIV-infected persons, is associated with chronic, systemic inflammation. However, the possible augmentative effects of HIV infection and smoking and other types of tobacco usage on indices of systemic inflammation and the impact of combination antiretroviral therapy (cART) thereon remain largely unexplored and represent the focus of the current study. Of the total number of HIV-infected persons recruited to the study ( $n = 199$ ), 100 were categorised as pre-cART and 99 as virally suppressed (HIV viral load  $< 40$  copies/mL). According to serum cotinine levels, 144 and 55 participants were categorised as nonusers and users of tobacco, respectively. In addition to cytokines (IL-6, IL-8, and TNF- $\alpha$ ) and chemokines (IP-10, MIG, IL-8, MCP-1, and RANTES), other biomarkers of systemic inflammation included C-reactive protein (CRP),  $\beta$ 2-microglobulin, and those of neutrophil activation [ICAM-1, L-selectin, matrix metalloproteinase-9 (MMP-9)], microbial translocation (soluble CD14, LPS-binding protein), and oxidative stress (cyclophilin A, surfactant D). These were measured using multiplex bead array, ELISA, and immunonephelometric procedures. Viral suppression was associated with significant decreases in the levels of most of the biomarkers tested ( $P < 0.0037$ - $0.0008$ ), with the exceptions of CRP, cyclophilin A, and MMP-9. With respect to tobacco usage, irrespective of cART status, circulating levels of  $\beta$ 2-microglobulin, cyclophilin A, and RANTES were significantly elevated ( $P < 0.042$ - $0.012$ ) in users vs nonusers. Additional analysis of the groups of tobacco users and nonusers according to cART status revealed high levels of RANTES in pre-cART/tobacco users relative to the three other subgroups ( $P < 0.004$ - $0.0001$ ), while more modest increases in cyclophilin A and MMP-9 ( $P < 0.019$ - $0.027$ ) were observed in comparison with the cART/tobacco user subgroup. Notwithstanding the efficacy of cART in attenuating HIV-associated, chronic systemic inflammation, the current study has identified RANTES as being significantly and seemingly selectively increased in those with active HIV infection who use tobacco, a mechanism which may underpin augmentative proinflammatory activity.

## 1. Introduction

According to UNAIDS, there were approximately 36.7 million people worldwide living with human immunodeficiency virus (HIV) in 2016. Of these, 19.5 million people were accessing combination antiretroviral therapy (cART) globally [1]. With the introduction of cART, the prevalence of AIDS-related mortality has declined significantly, with cART successfully suppressing viral replication to undetectable levels in plasma and increasing circulating CD4<sup>+</sup> T cell numbers; however, it is unable to normalize immune activation [2]. Persistent immune activation and inflammation associated with HIV infection accelerate the process of immunosenescence [3], thereby potentially placing HIV-infected individuals at higher risk of developing infections and non-AIDS-defining diseases such as cancer (especially lung, head and neck, hepatocellular, Hodgkin's lymphoma, cervix, and anus) [4], cardiovascular diseases [5], renal [6] and liver [7] diseases, and neurocognitive impairment [8]. Most of these diseases have also been associated with concomitant tobacco use [9].

The prevalence of cigarette smoking among European and North American adults with HIV infection is higher (40-70%) [9, 10], compared to the general population (15-20%) [11], and these individuals are also less likely to stop smoking [12, 13]. Helleberg et al. reported that HIV-related and non-HIV-related disorders, as well as mortality, are significantly increased in HIV-infected individuals who also smoke cigarettes and that the increased mortality is associated with smoking rather than HIV-related factors [13]. In addition, smoking has been well documented to be a risk factor for respiratory complications associated with HIV infection such as tuberculosis and chronic obstructive pulmonary disease (COPD), as well as cryptococcosis, *Pneumocystis jirovecii*, and bacterial pneumonia [14–16]. HIV-1-infected smokers have also been shown to have decreased immune responses, poorer responses to cART, and a greater risk of biological rebound, compared to HIV-1-infected nonsmokers [17]. In this context, an increased level of hepatic CYP2B6 in smokers could lead to an altered metabolism of its substrates, thereby increasing the possibility of drug-drug interactions between nicotine and antiretroviral drugs (particularly protease inhibitors and nonnucleoside reverse transcriptase inhibitors) all of which are metabolised by cytochrome P450 enzymes [18, 19]. Subtherapeutic plasma levels of antiretrovirals, as well as overproduction of reactive oxygen species (ROS), resulting from these interactions may lead to enhanced viral replication [18, 20].

Moreover, cigarette smoking affects both innate and adaptive (cell-mediated and humoral) immune responses (reviewed by [21–26]) and has been associated with both release and inhibition of proinflammatory and anti-inflammatory mediators. Cells of the adaptive immune system affected by smoking include T helper cells (Th1/Th2/Th17), CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells, CD8<sup>+</sup> T cells, B cells, and memory T/B lymphocytes, while cells of the innate immune system affected by smoking include dendritic cells, macrophages, and natural killer cells [24].

With respect to the proinflammatory activity, the nuclear factor kappa-light-chain-enhancer of activated B cell (NF- $\kappa$ B) signalling pathway is well recognized as one of the main mechanisms underpinning smoking-induced activation of inflammatory responses triggered through activation of the inhibitor of NF- $\kappa$ B kinase. Translocation of active NF- $\kappa$ B to the nucleus then occurs, followed by the induction of a number of genes involved in immune regulation and inflammation [27–30]. Proinflammatory cytokines shown to be upregulated after exposure to cigarette smoke condensate include tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin- (IL-) 1, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and monocyte chemoattractant protein-1 (MCP-1) [28, 30].

The association between HIV, smoking, and increased upregulation and release of potent cytokines and chemokines has been investigated in a number of studies [21, 27, 31], and, notably, it has been suggested that cytokine dysregulation may eventually contribute to HIV disease progression [31]. In addition, cytokines have been associated with the development of viral latency and the maintenance of latently infected CD4<sup>+</sup> T cells during cART, with potentially important implications for viral persistence posing a barrier to HIV cure [32].

Importantly, however, the effects of tobacco usage on the systemic inflammatory profiles of HIV-infected persons are largely unexplored, as is the influence of cART. The primary focus of the current study is on the cumulative effects of active HIV infection and tobacco usage, as well as the impact of cART, on a profile of inflammatory biomarkers.

## 2. Methods

**2.1. Study Population.** Adult ( $\geq 18$  years) HIV-infected participants attending the Antiretroviral Clinic at the Charlotte Maxeke Johannesburg Academic Hospital in Johannesburg, South Africa, were recruited by means of convenience sampling. Ethics approval for the study was obtained from the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, Pretoria, South Africa (Ethics reference number 94/2013), and the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg, South Africa (Clearance Certificate Number M130383). All participants gave informed consent, and a questionnaire was completed containing patient demographic data, and information related to the use of tobacco products. Treatment information was retrieved from clinical records. CD4<sup>+</sup> lymphocyte counts and HIV-RNA levels were measured by standard flow cytometric and PCR-based procedures, respectively, according to the South African National Health Laboratory Services guidelines. In addition, whole blood samples were collected in EDTA vacutainers and processed within 4 hours to separate the plasma component by centrifugation. The resultant plasma was stored at minus 80°C for up to 24 months.

The patients were classified in two groups, *viz.*, those who were cART-naïve ( $n = 100$ ) and those who were virologically suppressed on their cART regimen [HIV-1 plasma viral load (VL) < 40 copies/mL] ( $n = 99$ ).

TABLE 1: Demographic, clinical, and biomarker results of pre-cART and virally suppressed participants.

	Pre-cART <i>n</i> = 100	Virally suppressed <i>n</i> = 99	<i>P</i> value
Age (years)	33 (29–37)	34 (31–41)	0.102
Gender	Female = 64 (64%)	Female = 54 (54.5%)	0.175
CD4 (cells/ $\mu$ L)	209 (115–305)	348 (240–464)	<b>0.0001</b>
Current smoker	32 (32%)	23 (23.2%)	0.167
Cotinine (ng/mL)	0 (0–87.3)	0 (0–9.5)	0.139
MIG (pg/mL)	2610.61 (1353.1–4350.02)	949.26 (694.21–1371.34)	<b>0.0001</b>
IL-6 (pg/mL)	4.51 (2.69–8.14)	2.26 (1.41–3.12)	<b>0.0001</b>
IL-8 (pg/mL)	22.0 (10.76–45.36)	8.2 (5.64–12.47)	<b>0.0001</b>
IP-10 (pg/mL)	1896.98 (963.88–2990.94)	723.13 (567.82–977.66)	<b>0.0001</b>
MCP-1 (pg/mL)	23.21 (13.39–41.81)	5.13 (0–21.66)	<b>0.0001</b>
TNF- $\alpha$ (pg/mL)	5.04 (0–14.14)	0 (0–2.44)	<b>0.0001</b>
RANTES (pg/mL)	1203.68 (326.58–2444.85)	563.22 (313.21–935.84)	<b>0.0037</b>
MMP-9 (ng/mL)	32.08 (18.77–56.51)	39.17 (28.54–53.7)	<b>0.038</b>
L-selectin (ng/mL)	1189.85 (1029.95–1418.19)	924.86 (781.39–1108.35)	<b>0.0001</b>
ICAM-1 (ng/mL)	351.51 (187.97–530.18)	185.42 (107.04–240.38)	<b>0.0001</b>
sCD14 (ng/mL)	11750.64 (8039.79–17467.5)	9029.48 (6836.25–12,373)	<b>0.0003</b>
Surfactant-D (ng/mL)	288.17 (169.5–448.52)	210.95 (123.73–340.87)	<b>0.003</b>
LBP (ng/mL)	2734.865 (1740.25–4286.065)	1151.46 (956.65–1441.26)	<b>0.0001</b>
$\beta$ 2-microglobulin ( $\mu$ g/mL)	2.98 (2.12–3.88)	1.7 (1.5–2.09)	<b>0.0001</b>
hsCRP ( $\mu$ g/mL)	3.84 (1.08–8.65)	3.03 (1.02–6.28)	0.194
Cyclophilin A (ng/mL)	33.79 (15.56–58.85)	33.01 (19.5–51.05)	0.794

**2.2. Measurement of Circulating Biomarkers of Immune Activation.** The selection of biomarkers used in this study was based on previous findings with the inclusion of several additional markers [33, 34].

**2.2.1. Cytokines.** IL-6, IL-8 (CXCL8), IL-17, monokine induced by gamma interferon (MIG, CXCL9), IFN- $\gamma$ , monocyte chemoattractant protein-1 (MCP-1, CCL2), interferon gamma-induced protein-10 (IP-10, CXCL10), TNF- $\alpha$ , and regulated on activation, normal T cell expressed and secreted (RANTES) were quantified using the Bio-Plex suspension array platform together with Bio-Plex Pro™ assay kits (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer’s instructions.

**2.2.2. Neutrophil Activation Markers.** Biomarkers of neutrophil activation included L-selectin (CD62L), intercellular adhesion molecule-1 (ICAM-1, CD54), and matrix metalloproteinase-9 (MMP-9, gelatinase B) (R&D Systems, Minneapolis, MN, USA), as well as IL-8 (as mentioned above), the levels of which were determined by conventional ELISA kits.

**2.2.3. Oxidative Stress Marker [35].** Cyclophilin A (Elabscience, Wuhan, China) levels were measured by standard ELISA.

**2.2.4. Microbial Translocation and Monocyte Activation.** Soluble CD14 (sCD14) (biomarker of monocyte activation) (Abcam, Cambridge MN, USA) and surfactant protein D

(biomarkers of the integrity of the lung epithelium) (Hycult Biotech, Plymouth Meeting, PA, USA) and lipopolysaccharide-binding protein (LBP) (R&D Systems, Minneapolis, MN, USA) levels were determined using standard ELISA methods.

**2.2.5. Infection and Inflammation.** C-reactive protein (hsCRP) and  $\beta$ 2-microglobulin levels were assayed by using high-sensitivity nephelometry (Siemens Healthcare Diagnostics, South Africa).

**2.3. Smoking Status.** Cotinine, as an objective measure of tobacco use, was measured using an ELISA procedure (Calbiotech, Spring Valley, CA, USA), with levels above 15 ng/mL taken as being positive for active smoking [36]. Because of the prevalence of usage of snuff among older, black South African females in particular, with reported frequencies of around 43%-48% [37, 38], together with the inability of measurement of serum cotinine to distinguish between active smoking and usage of inhaled snuff, we have chosen to use the term “tobacco users” as opposed to “smokers” in the current report.

**2.4. Statistical Analysis.** Data followed a nonnormal distribution, and distribution-free statistics were therefore employed. Descriptive results are given as proportions and frequencies, as well as medians and interquartile ranges (IQR). Tests of association were performed by chi-square or Fisher’s exact

TABLE 2: Demographic, clinical, and biomarker results according to tobacco usage.

	Tobacco nonuser <i>n</i> = 144	Tobacco user <i>n</i> = 55	<i>P</i> value
Age (years)	33 (29.5–39)	34 (30–39)	0.827
Gender	Female = 93 (64.6%)	Female = 25 (45.5%)	<b>0.014</b>
BMI (kg/m <sup>2</sup> ) ( <i>n</i> = 77; <i>n</i> = 23)	26 (23–30)	23 (21–27)	0.084
CD4 (cells/ $\mu$ L)	283 (181–366)	294.5 (184–334)	0.815
VL (copies/mL) ( <i>n</i> = 76; <i>n</i> = 23)	40 (40–40)	40 (40–40)	1.000
Cotinine (ng/mL)	0 (0–0)	100 (84–100)	<b>0.0001</b>
MIG (pg/mL)	1202.27 (705.05–2381.51)	1586.55 (1118.17–3140.22)	0.070
IL-6 (pg/mL)	2.9 (1.73–5.36)	3.12 (2.26–6.11)	0.404
IL-8 (pg/mL)	11.19 (6.07–23.96)	12.47 (9.06–31.27)	0.296
IP-10 (pg/mL)	923.06 (618.67–1821.06)	1177.58 (699.38–2030.72)	0.249
MCP-1 (pg/mL)	13.91 (3.58–27.86)	21.66 (7.19–31.99)	0.114
TNF- $\alpha$ (pg/mL)	0.57 (0–5.69)	1.14 (0–10.24)	0.505
RANTES (pg/mL)	552.31 (279.88–934.91)	1059.49 (436.52–2043.34)	<b>0.012</b>
MMP-9 (ng/mL)	34.74 (22.36–53.7)	39.17 (26.77–67.08)	0.161
L-selectin (ng/mL)	1060.95 (881.17–1283.41)	1076.63 (809.64–1223.38)	0.543
ICAM-1 (ng/mL)	223.31 (133.7–351.51)	205.88 (130.82–427.59)	0.827
sCD14 (ng/mL)	10177.13 (6859.92–14065.46)	10696.59 (7720.14–14664.51)	0.516
Surfactant-D (ng/mL)	239.33 (138.28–365.35)	286.61 (184.47–370.3)	0.143
LBP (ng/mL)	1512.9 (1085.55–2803.38)	1941.39 (1168.96–3235.82)	0.305
$\beta$ 2-Microglobulin ( $\mu$ g/mL)	1.99 (1.52–2.97)	2.33 (1.82–3.06)	<b>0.042</b>
hsCRP ( $\mu$ g/mL)	3.56 (1.14–7.48)	2.71 (0.817–6.85)	0.349
Cyclophilin A (ng/mL)	29.19 (16.3–51.73)	39.44 (23.46–73.61)	<b>0.033</b>

tests as appropriate, or by the Kruskal-Wallis test, used for comparing two or more independent samples.

### 3. Results

A total of 199 participants were recruited for the study of whom 100 were cART-naïve and 99 were virologically suppressed on cART. Participants had a median age of 33 years [interquartile range (IQR) 30–39 years], all but 3 were Black African, and the majority (59.3%) were women. One hundred and thirty-eight participants (69.4%) admitted to have ever smoked, and 55 (27.6%) were current tobacco users as determined by cotinine levels. Fifty-three participants used snuff and 55 smoked cigarettes and had been doing so for a median duration of 5 (IQR 4.5–15) and 19 (IQR 17–27) years, respectively, with 38 using both. Smokers smoked on average 5 (IQR 3–7) cigarettes per day. Males were significantly more likely to use tobacco products than females were (37% versus 21.2%;  $P = 0.014$ ). All cART-treated participants were on standard first-line treatment consisting of tenofovir disoproxil fumarate, emtricitabine, and efavirenz which had been started a median of 14 (IQR 14–19) months earlier,

and all participants had an undetectable VL at the time of the study.

Table 1 shows the demographic, clinical, and biomarker results of participants according to cART status with comparisons between the groups. Participants did not differ according to age or gender, but treatment-naïve participants had significantly lower CD4 counts and higher levels of all biomarkers tested, except for MMP-9, which was lower, and hsCRP and cyclophilin A, which were comparable to values observed in virally suppressed participants. Apart from gender, which is shown as frequency and percentage, all results are given as median and IQR throughout.

Table 2 shows the demographic, clinical, and biomarker results of participants according to tobacco usage, as determined by cotinine level, with comparisons between the groups. Tobacco users were more likely to be male and had comparable age, CD4 counts, and VL than nonusers, but significantly higher levels of RANTES (Figures 1 and 2),  $\beta$ 2-microglobulin, and cyclophilin A.

Figure 1 displays the distribution of the RANTES values, illustrating that the tobacco users have significantly

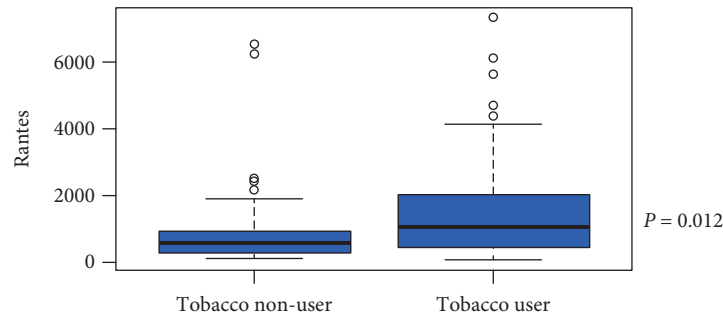


FIGURE 1: Boxplot of RANTES according to tobacco usage.

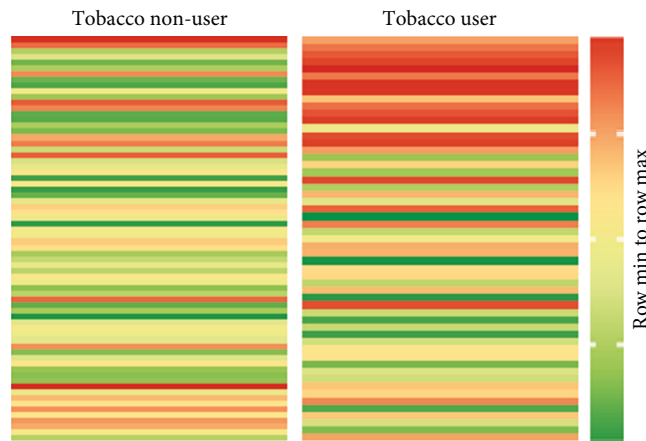


FIGURE 2: Heat map of RANTES according to tobacco usage.

higher levels of RANTES than nonusers. Additionally, Figure 2 provides an immediate visual summary of the information showcasing those high values found in the tobacco users group.

Table 3 shows the demographic, clinical, and biomarker results of participants according to combined cART status and tobacco use. No differences were found in hsCRP between any of the groups, and cyclophilin A only differed between treatment-naïve tobacco users and nonusers, with levels significantly higher in the former group. RANTES was strikingly higher in the treatment-naïve tobacco users when compared with all the other groups. Treatment-naïve and virologically suppressed tobacco users differed significantly in all biomarkers (all higher in the former) apart from MMP-9, sCD14, and surfactant-D. A similar pattern was observed in treatment-naïve and virologically suppressed tobacco nonusers, except that no difference was observed for RANTES.

When treatment-naïve tobacco users were compared with nonusers, differences were only found in RANTES, MMP-9, and cyclophilin A, whereas no differences were found in the virally suppressed group. Finally, treatment-naïve tobacco users had significantly higher levels of all biomarkers, except for MMP-9, than nonusers on cART, while tobacco users on cART and nonusers not on cART had variable patterns with MMP-9 higher in the former group and MIG, IL-6, IL-8, IP-10, MCP-1, TNF- $\alpha$ , L-

selectin, ICAM-1, LBP, and  $\beta$ 2-microglobulin higher in the latter group.

#### 4. Discussion

Our findings add substantially to the limited literature pertaining to the immunomodulatory effects of cART *per se*, especially in African populations. As described previously by us and others, most biomarkers of systemic inflammation in adults infected with HIV decrease progressively and significantly following the implementation of virally suppressive cART irrespective of viral subtype B or C [33, 39–42]. This scenario is strongly supported by the findings of the current study, specifically with respect to the test cytokines (IL-6, TNF- $\alpha$ ), chemokines (IP-10, MIG, MCP-1, and RANTES),  $\beta$ 2-microglobulin, and two of the three biomarkers of microbial translocation (sCD14, LBP). These findings underscore the apparent utility of measurement of IP-10, in particular, as an objective indicator of responsiveness to cART [33, 39, 42, 43], a contention which is in keeping with the role of this chemokine in the pathogenesis of HIV infection [43–46].

Although previous studies have described elevations in the levels of individual biomarkers associated with neutrophilic inflammation, specifically ICAM-1 [47], L-selectin [48], and IL-8 [49], characterization of the effects of cART on a profile of biomarkers associated with systemic activation

TABLE 3: Demographic, clinical, and biomarker results according to treatment status and tobacco use.

	Tobacco user				Tobacco nonuser				P value			
	1 cART-n = 32	2 cART + n = 23	3 cART-n = 68	4 cART + n = 76	1 vs 2	1 vs 3	1 vs 4	2 vs 3	2 vs 4	3 vs 4		
Age (years)	33.5 (28.5-37)	35 (31-41)	32 (29-37.5)	34 (30.5-40)	0.165	0.420	0.192	0.099	0.365	0.087		
Gender	F = 19 (59.38%)	F = 6 (26.09%)	F = 45 (66.18%)	F = 48 (63.16%)	<b>0.007</b>	0.260	0.358	<b>0.0004</b>	<b>0.0008</b>	0.357		
CD4 (cells/ $\mu$ L)	211 (137.5-305)	344 (315-466)	209 (106-307)	351.5 (237.5-454.5)	<b>0.0001</b>	0.413	<b>0.0001</b>	<b>0.0001</b>	0.267	<b>0.0001</b>		
Cotinine (ng/mL)	100 (100-100)	100 (67.3-100)	0 (0-0)	0 (0-0)	0.363	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	0.361		
MIG (pg/mL)	2696.0 (1786.43-3460.14)	1118.17 (787.12-1383.5)	2501.3 (1110.43-5854.07)	905.33 (677.94-1328.77)	<b>0.0001</b>	0.143	<b>0.0001</b>	<b>0.0001</b>	0.334	<b>0.0001</b>		
IL-6 (pg/mL)	3.55 (2.37-8.99)	2.26 (1.84-4.4)	5.04 (2.8-8.14)	1.84 (1.31-3.12)	<b>0.046</b>	0.132	<b>0.0001</b>	<b>0.002</b>	0.060	<b>0.0001</b>		
IL-8 (pg/mL)	18.46 (9.91-47.13)	9.06 (7.35-17.6)	22.72 (10.76-41.09)	7.35 (4.78-12.47)	<b>0.013</b>	0.247	<b>0.0001</b>	<b>0.0008</b>	0.090	<b>0.0001</b>		
IP-10 (pg/mL)	1947.09 (1330.02-2701.1)	695.91 (545.75-865.47)	1821.06 (833.45-4174.76)	751.84 (570.31-1008.06)	<b>0.0001</b>	0.114	<b>0.0001</b>	<b>0.0001</b>	0.236	<b>0.0001</b>		
MCP-1 (pg/mL)	26.31 (16.49-41.29)	5.13 (0.99-23.72)	22.69 (11.84-41.81)	5.13 (0-20.62)	<b>0.0001</b>	0.193	<b>0.0001</b>	<b>0.0003</b>	0.337	<b>0.0001</b>		
TNF- $\alpha$ (pg/mL)	7.64 (0-15.44)	0 (0-1.14)	4.39 (0-11.54)	0 (0-2.44)	<b>0.0001</b>	0.187	<b>0.0001</b>	<b>0.0001</b>	0.217	<b>0.0001</b>		
RANTES (pg/mL)	1698.37 (560.79-3655.84)	488.02 (226.59-1114.67)	478.55 (214.02-1785.06)	564.24 (317.82-861.03)	<b>0.001</b>	<b>0.004</b>	<b>0.0002</b>	0.382	0.491	0.372		
MMP-9 (ng/mL)	38.28 (19.66-73.02)	39.17 (33.85-50)	28.54 (18.77-50)	38.28 (28.54-54.64)	0.284	<b>0.049</b>	0.461	<b>0.017</b>	0.285	<b>0.012</b>		
L-selectin (ng/mL)	1165.21 (932.31-1327.36)	924.86 (614.35-1092.43)	1189.85 (1045.45-1455.75)	932.22 (802.57-1108.35)	<b>0.0008</b>	0.163	<b>0.0005</b>	<b>0.0001</b>	0.233	<b>0.0001</b>		
ICAM-1 (ng/mL)	360.41 (185.34-537.23)	153.38 (113.1-205.88)	351.51 (195.56-507.0)	190.6 (100.89-240.38)	<b>0.0006</b>	0.339	<b>0.0001</b>	<b>0.0001</b>	0.436	<b>0.0001</b>		
sCD14 (ng/mL)	11285.24 (8922.59-15044.95)	9202.32 (7296.24-13,749)	12846.1 (7273.22-18604.36)	8993.65 (6678.74-12346.9)	0.112	0.385	<b>0.007</b>	0.051	0.222	<b>0.0003</b>		
Surfactant-D (ng/mL)	325.17 (178.82-388.71)	273.49 (184.47-354.23)	272.63 (165.24-460.77)	186.31 (114.11-315.62)	0.290	0.433	<b>0.006</b>	0.3161	0.057	<b>0.0016</b>		
LBP (ng/mL)	2844.95 (1943.9-4057.12)	1103.18 (828.71-1441.26)	2640.38 (1627.25-4494.89)	1158.02 (972.33-1443.51)	<b>0.0001</b>	0.311	<b>0.0001</b>	<b>0.0001</b>	0.369	<b>0.0001</b>		
$\beta$ 2-Micro-globulin ( $\mu$ g/mL)	2.97 (2.26-3.5)	1.88 (1.52-2.14)	2.98 (1.84-4.5)	1.69 (1.47-2.08)	<b>0.0001</b>	0.193	<b>0.0001</b>	<b>0.0001</b>	0.174	<b>0.0001</b>		
hsCRP ( $\mu$ g/mL)	2.25 (0.86-6.93)	3.15 (0.77-6.85)	4.34 (1.15-11.75)	2.96 (1.14-6.28)	0.443	0.105	0.482	0.101	0.418	0.061		
Cyclophilin A (ng/mL)	40.01 (26.2-76.32)	36.7 (19.5-64.29)	24.7 (14.6-56.64)	30.96 (19.51-47.82)	0.437	<b>0.027</b>	0.074	0.063	0.136	0.258		

of these cells has not, to our knowledge, been described previously. In this context, our observations that administration of cART is associated with significant decreases in the concentrations of ICAM-1, L-selectin, and IL-8 is consistent with systemic activation of these cells [50, 51] and a beneficial, albeit secondary, anti-inflammatory effect of therapy. This interpretation is supported by the observation that virally suppressive cART was also associated with a significant decrease in serum surfactant D, a biomarker of oxidative stress [52].

According to the findings of two studies, South African HIV-infected participants, particularly males, like their European and North American counterparts, have high rates of cigarette smoking with frequencies of 52–71% recorded in males and 13–28% in females [34, 37, 53], with somewhat lower rates recorded in another study which was undertaken at the same HIV clinic as the current study [54].

With respect to characterization of the effects of tobacco usage on systemic biomarker profiles, we first categorised the entire cohort of HIV-infected participants into two groups according to low or high serum cotinine levels (nonusers and predominantly male tobacco users accounting for 72.4% and 27.6% of participants in each group, respectively), irrespective of cART. Although tobacco usage was associated with increases in almost all of the test protein biomarkers, consistent with a more systemic inflammatory phenotype, only three of these attained statistical significance, *viz.*, the chemokine, RANTES, a key mediator of T lymphocyte recruitment and activation, and  $\beta$ 2-microglobulin and cyclophilin A. The latter biomarker is released from various cell types, including vascular smooth muscle cells, inflammatory cells, and platelets during systemic oxidative stress, including that caused by smoking [55].

These findings are relevant in the context of a recent study which reported that mild-to-moderate smoking is associated with significant increases in VL, as well as with increases in the systemic concentrations of RANTES and biomarkers of oxidative stress, while no alterations were detected in the plasma levels of several other proinflammatory cytokines/chemokines, specifically IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-8, and MCP-1 [56]. In this context, it is noteworthy that RANTES has been implicated in microbial translocation [57], a process known to drive systemic inflammation, as well as in the pathogenesis of COPD [58, 59] and coronary artery disease (CAD) [60–62], conditions in which smoking is well recognized as being a key risk factor, particularly in HIV-infected individuals who smoke [16, 23, 63]. In addition, elevated plasma levels of cyclophilin A have recently been described in both CAD [55, 64] and COPD [65].

Much less is known about possible relationships between circulating levels of  $\beta$ 2-microglobulin and tobacco usage, particularly in the setting of HIV infection. A few studies have, however, reported on elevated levels of this HLA class I-derived biomarker in the serum and urine of HIV-uninfected smokers. These have been attributed to the cytotoxic activities of smoke-derived toxicants, particularly cadmium, predisposing for possible development of nephropathy [66], malignancy [67], and ischemic stroke

[68]. In those infected with HIV who smoke, exposure to smoke-derived toxicants may also contribute to T cell dysfunction and death [56].

Mostly in keeping with the aforementioned findings, further subdivision of tobacco users/nonusers according to cART status revealed significantly elevated levels of RANTES in particular, as well as cyclophilin A and MMP-9, but not  $\beta$ 2-microglobulin, in tobacco users not on cART (hence with high VL) relative to nonusers not on cART. Interestingly, systemic levels of RANTES were significantly higher in the no cART/user group than in any of the other subgroups (no cART/nonuser, cART/user, and cART/nonuser), as also reported in another study, albeit in a much smaller group of HIV-infected patients of unknown treatment status [69]. Following initiation of cART, levels of RANTES decreased strikingly, attaining levels comparable to those of non-smokers. Aside from direct antiviral activity, these notable effects of cART may also be attributed to attenuation of microbial translocation, a contention which is consistent with the decline in post-therapy levels of LBP. These findings suggest that RANTES may represent a selective biomarker of the harmful, augmentative, proinflammatory interactions of active HIV infection and smoking, while also underscoring the anti-inflammatory benefit of cART. It is however, noteworthy, that conflicting data exist regarding the role of RANTES in HIV infectivity and disease progression [70].

With respect to MMP-9, this proteolytic enzyme, which is mainly produced by activated neutrophils, as well as by monocytes/macrophages, is elevated in the blood and lungs of smokers and is intimately involved in the pathogenesis of COPD [71–74]. The absence of a significant difference in  $\beta$ 2-microglobulin in the tobacco-user versus nonuser groups on cART may reflect the dominant influence of active viral replication on levels of this biomarker.

Limitations of the current study include lack of VL data in the pre-cART group, small numbers of HIV-infected tobacco users when subdivided into cART subgroups, and lack of distinction between active smoking and usage of smokeless tobacco products. Irrespective of these limitations, this study has clearly reinforced the benefit of cART per se in attenuating HIV-associated, harmful, chronic, systemic inflammation, including neutrophilic inflammation, as well as the utility of IP-10 as a biomarker of the efficacy of cART. More importantly, however, the finding, albeit preliminary, that levels of RANTES are considerably and possibly selectively increased in individuals with active HIV infection who use tobacco implies a major role for this chemokine in the pathogenesis of HIV/smoking-related immune dysfunction and organ damage. These findings again highlight the damaging interactions between active HIV infection and tobacco use, underscoring the need to prioritise effective smoking cessation strategies in this population.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

None of the authors have any conflicts of interest to declare.

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