Systemic inflammation following long-term successful antiretroviral therapy in people living with HIV (PLHIV)

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Abstract:

Background: Long-term HIV infection, even with successful combination antiretroviral therapy (cART), is associated with an enhanced and accentuated onset of premature-aging or age-related diseases in people living with HIV (PLHIV). No data are available from low- and middle-income countries (LMICs) like India on inflamm-aging. In this study, we attempt to understand the relationshipbetween several 'biomarkers' of inflamm-aging in a well-defined Indian cohort of PLHIV.

Methods: Blood samples were obtained from therapy naïve PLHIV (Pre-ART, n=43), patients on cART (ART, n=53) and age and gender-matched healthy controls (HC, n=41) after screening 714 individuals.Wemeasured telomere length, 92 markers of inflammation, immune activation markers, and HIV-1 reservoir coupled with clinical phenotypes and neurocognitive function assessments using the International HIV Dementia Scale (IHDS).

Findings: Despite a median duration of eight years of cART, sCD14 ($p<0.001$) and sCD163 ($p=0.0377$) was not normalized to the level of HC. Significant differences were observed in 11 inflammatory markers between HC and ART ($p<0.05$). Linear regression analysis showed a significant negative association of HIV-1 positive status on telomere length (-2.687, $p<0.0001$). There was a significant association between HIV status and higher odds of having IHDS≤10 (OR:39.74, $p<0.0001$). A significant negative association of CCL20 (-0.5236, $p=0.0219$) and CCL11 (-1.1608, $p=0.0338$) with HIV-1 reservoir was also observed.

Interpretation: Our study suggests that PLHIV on successful cART in a standardized public-health setting, may be at higher risk of inflamm-aging and age-related inflammatory diseases which may need special intervention and identifies several biomarkers for further mechanistic investigation.

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Keywords: Inflamm-aging, HIV-1 reservoir, cART, LMIC

1. Introduction

The most remarkable achievements in the battle against human immunodeficiency viruses (HIV) is the discovery of efficient, well-tolerated combinational antiretroviral therapy (cART) that has transformed a deadly viral infection into a chronic, manageable disease. In the absence of cure or vaccine, long-term HIV infection, even with successful treatment, is associated with an enhanced and accentuated onset of non-AIDS-related severe pathologies. For some undefined reasons, long-term treated people living with HIV (PLHIV) not only succumb to death at an earlier age than the HIV-uninfected counterparts but also suffer from some maladies that are typically associated with human-aging [1]. Premature onset of immunosenescence and HIV-associated inflammation in patients on cART might be the primary reasons for early aging which has not been reported in uninfected individuals.

Human aging that is characterized by a chronic, low-grade systemic inflammation has been termed as "inflamm-aging" which is a highly significant risk factor for both morbidity and mortality in elderly people [2]. Such inflammatory environment might trigger the development of age-related inflammatory diseases [3], such as atherosclerosis [4, 5], cardiovascular diseases [6], type 2 diabetes mellitus [7], Alzheimer disease [8] etc. During cART, HIV persists in a rare population of long-living, latently infected cells which can contribute to an inflammatory-like state [1].

Unlike in high-income countries (HIC), the cART program in low- and middle-income countries (LMIC) like India, has a public health approach with the standardized regimen for all PLHIV. Following the launch of National AIDS Control Programme (NACP) in India in 2004, a massive scale-up of the access to the cART has occurred. As of December 2016, nearly one million PLHIV were receiving free ART which is 49% of the PLHIV residing in the country [9]. The Indian national first-line ART program recommends the use of one non-nucleoside reverse transcriptase inhibitor (NNRTI), either nevirapine (NVP) or efavirenz (EFV), in the backbone of two nucleoside reverse transcriptase inhibitors (NRTI); zidovudine (AZT) or tenofovir (TDF), and lamivudine (3TC) [10]. Although the perfect adherence remains a challenge, reasonably good response to the

first line therapy is indicating the overall success of the Indian ART program [11]. A recent study from TREAT Asia HIV Observational Database (TAHOD) including India estimated that older people with age of 50 years or older would account for 32% of the PLHIV by 2025 [12]. Therefore, by the expansion of effective cART, together with aging PLHIV, the burden of age-related but the non-AIDS related disease is likely to increase. As the environment can have an enormous impact on age and age-related diseases, and the genetic determinants of variation of healthy aging may vary across populations, studies conducted in HIS might not apply to the LMICs [13].

To understand the HIV-associated inflammation and immune activation with respect to the successful long-termcART, our study aimed to assessthe relationship betweenseveral 'biomarkers' of aging and inflammation, including telomere length as an indicator of "biological aging", host plasma proteome targeting 92 inflammatory markers and two well-characterized immune activation markers (sCD14 and sCD163) coupled to clinical phenotypes and neurocognitive function assessment using the International HIV Dementia Scale (IHDS).Additionally, we also assessed the association between HIV-1 reservoir, and inflammatory and immune activation markers in these long-term treated individuals. This study is the first comprehensive study on inflamm-aging in long-term successfully treated PLHIV which could provide insights into the premature-aging in a standardized public health setting for monitoring PLHIV on treatment.

2. Materials and methods

2.1. Study design and participants

The cohort consists of three groups of individuals: i) PLHIV with successful long-term ART for more than five years (ART herein), ii) treatment-naïve PLHIV with viremia who were initiating therapy (Pre-ART herein) and iii) age and gender-matched healthy individuals without any chronic illness (HC herein). The HIV-positive cohort was recruited from a tertiary care ART Centre, Government Hospital for Thoracic Medicine (GHTM), Chennai, India, attending routine standard-of-care.

For the ART group, we screened 258 patients who were already on first-line treatment as per national guidelines for more than five years with two NRTIs and one NNRTI and stable CD4 counts. We used the following inclusion criteria: age between 35 years and 60 years, without any current co-infections like active tuberculosis or hepatitis C virus (HCV) infection, no comorbidities like diabetes mellitus, no evidence of cardiovascular diseases or any chronic illness, and adherence >90% by self-reported adherence and pill count. Finally, 55 patients matched our inclusion criteria and consented to the study. Samples were also collected from treatment-naïve HIV-infected individuals with viremia using the following inclusion criteria were used: no active tuberculosis or diabetes and no illicit drug users (people who inject drugs). After screening 166 patients, 41 gender-matched individuals were included in the study. Plasma viral load were measured by either *Abbott* RealTime HIV-1 assay (Abbott, US) or COBAS TaqMan 48 version 2.0 (Roche, US). In the ART group, two patients showed a viral load >150 copies/mL (4000 and 1800 copies/mL respectively) and were excluded from the study.

We screened 295 healthy individuals in and around Chennai, India and finally included 43 HC using the following inclusion criteria: without any chronic illness, active tuberculosis or HCV infection, comorbidities like diabetes mellitus, evidence of cardiovascular diseases and no anti-inflammatory medications for past one month.

The overall study design is presented in Figure 1.After first-time counseling and obtaining informed consent to participate in the study, 15 mL of venous blood was collected from the study subjects.

2.2. Assessment of neurocognitive function using International HIV dementia scale (IHDS)

The neurocognitive function test was performed using IHDS [14]in the ART and HC group of individuals. Study participants were first asked to remember four words (dog, hat, bean, and red) in the Tamil language (one second per word) which should be recalled at the end of the test. After a brief introduction of the method, the participants were asked to perform three subtests of the IHDS, i.e., i) motor speed assessment or a nondominant finger-tapping test, ii) Psychomotor speed assessment or a nondominant Luria hand sequence test, and iii) memory recall test to recall the four

words. Sum of the scores of each subtestwas taken as the total score of IHDS for each. Cutoffs of ≤10 composite IHDS score were indicative of potential risk of cognitive impairment.

2.3. Proteomic profiling of the plasma soluble factor

Plasma samples from Pre-ART, ART and HC groups were used for the analysis of the soluble proteome using Proximity extension assay (PEA) technology (Olink Bioscience AB, Uppsala, Sweden).[15] We selected the Olink® Inflammation Panel that includes 92 inflammation-related protein biomarkers. These biomarkers were also part of several disease areas including cancer (n=65), cardiovascular diseases (n=47), neurological impairments (n=41), and renal dysfunction (n=23). We also measured two extensively used biomarkers of immune activation sCD14 (Human CD14 Quantikine ELISA Kit R&D Systems, UK) and sCD163 (Thermo Scientific™ Pierce™ Human CD163 Kit, Thermo Scientific, USA).

2.4. Peripheral blood mononuclear cells' Telomere length

Genomic DNA was extracted from PBMCs using the QIAampDNA Mini Kit (Qiagen, Germany).The average telomere length was measured using the Absolute Human Telomere Length Quantification qPCRAssay Kit(AHTLQ; ScienCell Research Laboratories, US) as per manufacturer's instruction.

2.5. Total HIV-1 DNA quantification using IC-qPCR as a marker for HIV-1 reservoir

To quantify total HIV-1 DNA from PBMCs internally controlled qPCR (IC-qPCR) was performed as described [16]. IC-qPCR was performed in duplicates of 500ng DNA using Takara Premix Ex Taq™ (Probe qPCR) (Takara, Japan). Primers were used targeting HIV-1 LTR and Beta-globin. Total HIV-1 DNA copy numbers were calculated based on the linear equations of the 10-fold Beta-globin standard curve derived from Jurkat cells and the 10-fold pNL4-3 plasmid standard curve, diluted in 50 ng/μL of Jurkat DNA to mimic clinical samples and normalized to obtain HIV-1 DNA copies per million PBMCs.

2.6. Statistical analysis and data visualization

The Mann Whitney U test, Chi-square test, and one-way analysis of variance (ANOVA) were performed to identify differences in means of protein expression values (NPx) of different groups in the cohort under study. Pair-wise comparison between means of each group was also carried out. A post hoc test using Tukey Honest Significant Differences (TukeyHSD) method was executed to obtain the pair-wise ANOVA results. Linear and logistic univariate and multivariate regression were used with the outcomes of telomere length and IHDS≤10, respectively, to investigate the association of HIV-status and HIV-treatment duration and these markers of cellular aging. This analysis was performedin R. We did not correct for multiple comparisons. A heatmap was generated to visualize the clustering of samples based on protein expression using gplotsv3.0.1 packages in R. The similarities between each sample in the cohort concerning protein expression was also visualized in a multi-dimensional scaling plot (MDS) using the R package edgeR.

2.7. Ethical Clearances

The study was approved by the Institutional Ethics Committee of the National Institute for Research in Tuberculosis (NIRT IEC No: 2015023 and TRC IEC No: 2011001) and Institutional Review Board Committee of Government Hospital for Thoracic Medicine (GHTM-27102015). All the study participants gave written informed consent. Patient identities were anonymized and delinked before analysis.

3. Results

3.1. Patients' clinical characteristics

The cohort characteristics are presented in **Table 1**. All the three cohorts are gender matched. At sampling, there was no difference in median age between ART and HC groups (45 vs. 46 years) but relatively lower age in the Pre-ART group. In the ARTgroup, the median (IQR) duration of treatment was 8 years (6-10 years). Among the ART patients 57% (30/53) were on zidovudine, lamivudine, and nevirapine (ZDV/3TC/NVP) and remained 43% (23/53) were on tenofovir, lamivudine, and efavirenz (TDF/3TC/EFV). All the ART group patients-initiated treatment in the chronic phase of infection with median (IQR) CD4 count of 186 (100-280) cells/µL.

3.2. Soluble monocyte activation markers in Pre-ART, ART and HC

We tested the plasma monocyte activation markerssCD14 and sCD163 in the three groups. As expected, Pre-ART group has higher sCD14 (**Fig 2a**) and sCD163 (**Fig 2b**) plasma levels compared to HC ($p<0.0001$, Mann Whitney U Test). Interestingly despite a median duration of eight years of cART, there is no statistically significant difference between Pre-ART and ART group in sCD14. The median level of sCD163 was lower in ART compared to Pre-ART group (30014 pg/mL vs. 68192 pg/mL, $p<0.0001$, Mann Whitney U Test) but not normalized to the level of HC ($p=0.0377$, Mann Whitney U Test). Additionally, we did not find any significant correlation between duration of cART and sCD14 (Spearman r: 0.163; $p=0.2432$) and sCD163 (Spearman r: 0.154; $p=0.2720$) levels in plasma.

3.3. Soluble plasma inflammation markers in Pre-ART, ART and HC

We have also tested 92 plasma inflammation markers in the three groups. Among the 92 proteins, 75 were detectable in >50% of the samples and were used for the analysis. Among the samples tested two Pre-ART and one HC sample did not pass the quality control, thus, they were excluded from the analysis. The hierarchical clustering analysis (HCA) with false discovery rate (FDR) <0.001 identified clustering of 79% (31/39) of Pre-ART samples together separately along with two samples from HC and one from ART group **(Fig 3a)**. Another seven Pre-ART samples also clustered together but within the HC and ART group of samples. Some of the ART and HC clustered separately from Pre-ART but intermingled with each other. HCA result is consistent with the MDS plot (**Supplementary Fig 1**) and principal component analysis. Among the 75 proteins, 41 had a different level of achieved statistical significance ($p<0.05$, TurkeyHSD) at least in on the comparisons (ART vs Pre-ART, Pre-ART vs HC and HC vs ART) (**Fig 3b**). As expected there were several inflammatory markers with a statistically significant differential protein level between

Pre-ART and ART (n=38) and Pre-ART and HC (n=29). There were 11 significantly different proteins between HC and ART with uniquegroup-specific4E-BP1 in the comparison. Five proteins differentiate among the three groups (CD8A, TRANCE, CD5, SLAMF1, and CCL23) (**Fig 3b**). The level of soluble CD8A, 4E-BP1, SLAMF1 and CCL23 in ART group of individuals did not normalized to the level of HC (**Fig 3c**). The level of soluble plasma TRAIL, NT-3, CD5 and TRANCE went down significantly in ART group compared to the HC and Pre-ART groups of individuals. While the level of ADA, MMP-1 and CST-5 gone up significantly in ART group compared to the HC and Pre-ART groups of individuals (**Fig 3c**). The complete comparison was given as supplementary Table 1.

3.4. Association of telomere length with HIV- status and inflammation markers

Telomere length analysis was performed only in two groups (HC and ART), as PBMCs were not available for the Pre-ART group. ART group had statically significant shorter telomere length than chronological age-matched HC (Median (IQR): 1.89 (0.95 – 3.78) vs. 5.151(3.207- 6.765), $p<0.001$).Linear regression analysis, after adjusting for chronological age shows a significant negative association of HIV-1 positive status on telomere length (-2.6870, 95%CI -4.0188,-1.7152, $p<0.0001$). In the ART group alone, there was not a statistically significant association between duration of treatment and telomere length after adjusting for age alone or additionally adjusting for markers of disease progression. We investigated biomarkers as mediators of the relationship between HIV status and telomere length as we saw there was a strong association between HIV status and reduced telomere length. After adjustment for HIV-1 status, age, years of treatment and one of each of the inflammatory biomarkers, in HC and ART group, there was not strong evidence to support any of the biomarkers as mediators of the relationship between HIV status and telomere length with or without adjustment for treatment duration. In the ART group alone, after adjusting for age, gender, duration of treatment, HIV-1 reservoir, CD4 count at initiation, CD8/CD4 ratio and sCD14, we observed CXCL1 and TGF-αto have a significant association with increased telomere length (0.2905, 95%CI: 0.0029,0.5780 p=0.0479) and (0.7865, 95%CI:0.1003,1.4727, p=0.0262),

respectively. We also found that IL-10RA was significantly associated with decreased telomere length (-1.7901, 95%CI: -3.5133,-0.0667 p=0.0423).

3.5. Cognitive impairment with HIV status and inflammation markers

There was a significant association of HIV status and higher odds of having a potential cognitive impairment (IHDS≤10)(OR: 39.7404, 95%CI: 9.9156, 272.4432, p<0.0001) when adjusted only for age and additionally adjusting for the duration of treatment further increased the estimated odds of cognitive impairment for HIV-1 status. Among the ART group of individuals, 75% (40/53) had IHDS≤10 while in HC it was only 2% (2/29), indicating compromised cognitive function. However, in the ART group, there was not a significant association between duration of treatment and odds of IHDS≤10 with or without adjustment for markers of disease progression including HIV-1 reservoir, CD4 at treatment initiation, CD4 and CD8 ratio and sCD163 which earlier reported as plasma biomarkers of neurocognitive impairment.

3.6. HIV-1 reservoir and soluble plasma biomarkers

The median (IQR) total pro-viral DNA count in the ART group was 2.870 (2.631-3.156) $\log_{10}$ copies/mL. The HIV-1 reservoir is significantly negatively associated with the duration of treatment by univariate linear regression (-0.1898, 95%CI:-0.3370, -0.0425; p=0.0128). However, after adjusting for CD4 at treatment initiation, CD4 at sampling, CD4:CD8 ratio at sampling as well as treatment regimen, no significant association was observed (-0.155, 95%CI: -0.3396,0.0296 p=0.0968). Upon further investigation the relationship between inflammatory biomarkers and the HIV-1 reservoir, we found a significant negative association of CCL20 (-0.5236, 95%CI:-0.9657, -0.0815, p=0.0219) and CCL11 (-1.1608, 95%CI:-2.2265, -0.0951, p=0.0338) with HIV-1 reservoir after adjusting for age, gender, duration of treatment, CD4 at treatment initiation, CD4 and CD8 ratio and sCD14.

3.7. Treatment regimen and biomarkers

As there were two sub-groups within the ART group: those on TDF/3TC/EFV (n=23) and those on ZDV/3TC/NVP (n=30), we, therefore, investigated the potential impact of the treatment regimen on the level of inflammatory markers between the two groups in comparison with HC (**Fig 4**). There are 25 plasma inflammatory markers that were significantly different between any of the two comparative analysis i.e. HC vs ZDV/3TC/NVP (n=14, $p<0.05$, Mann Whitney U test), HC vs TDF/3TC/EFV (n=13, $p<0.05$, Mann Whitney U test) and ZDV/3TC/NVP vs TDF/3TC/EFV (n=11, $p<0.05$, Mann Whitney U test). In ZDV/3TC/NVP vs. TDF/3TC/EFV comparison,three unique proteins were significantly different (IL-10RB, TWEAK, and CSF-1) (**Fig 4**).

4. Discussion

In this study that examined a cohort of PLHIV on long-term successful cART from India, we found that despite a median duration of eight years of cART, no difference in median levels of the soluble monocyte activation marker sCD14 between the Pre-ART and ART group. However, the median level of sCD163, was significantly lower in the ART group than in the Pre-ART group, although the level was not normalized to that of the healthy control group. Several soluble inflammatory markers were also not normalized to the levels seen in healthy controls, indicating syatemic inflammation in PLHIV and patients receiving a ZDV/3TC/NVP have higher residual inflammation than PLHIV's on TDF/3TC/EFV regimen. This data suggests that patients on successful cART were clearly at higher risk of age-related inflammatory diseases leading to inflamm-aging.

sCD14 is a marker of monocyte activation in response to a microbial product like lipopolysaccharide (LPS), thus also called a marker of microbial translocation. A study in the US population showed that sCD14 is an independent predictor of mortality in HIV infection [17]. Contrasting results have been reported from different studies on the effect of cART on sCD14. Several studies reported that sCD14 did not decline following short-term [18] or long-term cART [19, 20] while others reported decline [21-23]. Our study is in line with former studies that we did not find any difference between PreART group and patients with median eight years of cART. A high burden of diarrhoeal diseases, coupled with compromised water quality, poor sanitation, and

handwashing [24], PLHIV are at greater risk of elevated microbial translocation which could be one of the reasons for increased monocyte activation by a microbial product that was not restored following cART.

sCD163 is thought to be a more precise monocyte/macrophage activation markers [25], which is also shown be associated with all-cause mortality in HIV-infected individuals [26]. In our study, we observed a significant decrease in the level of sCD163 between PreART and ART groups, but the levels were not normalized to that seen in the healthy state even with successful cART. As sCD163 is a cause of vascular inflammation leading to cardiovascular disease [27]and neurocognitive impairment in HIV-1 infected individuals [28], non-normalization of sCD163 at healthy state increase the chance of both age-associated cardiovascular as well as neurocognitive disease in those individuals.

The age-related inflammatory diseases are more common in PLHIV than in the general population [29]. In our study, we observed several plasma inflammatory biomarkers like CD8A, 4E-BP1, SLAMF1 and CCL23 were not normalized to the level of healthy controls which were earlier associated with age-related diseases. Out of the several plasma proteins tested, TRANCE, NT-3, CD5 and TRAIL were significantly lower level in ART group compare to healthy controls, while CCL23, CST5, CD8A, MMP-1, 4E-BP1, ADA and SLAMF1 were significantly higher in ART group. TRANCE/RANKL produced by osteoblast and other cells including T cells. Patients treated with NRTIs and NNRTIs shows decrease in circulating RANKL [30] which is similar to our data. The low level of TRANCE was shown to be an independent predictor of nontraumatic-fracture affecting osteoclastogenesis [31]. Previous study reported elevated level of sTRAIL in treatment naïve PLHIV and decrease following short term cART initiation [32], our study showed that long-term ART in our cohort did not normalized the TRAIL level to healthy status. Therefore prolonged HIV-1 infection and cART has influenced the normalization of sTRAIL because of immunological dysfunction by both persistent HIV-1 infection and treatment effect. In an earlier study from our group in Swedish patients with two decade long successful therapy showed normalization of sCD5

to a healthy state [33], which is not true in the Indian cohort with median eight years of successful treatment. The decrease NT-3 level in cerebrospinal fluid showed strong co-relation with the severity of neurocognitive impairment in PLHIV [34]. In our cohort NT-3 level was significantly decreased in ART compared to the healthy control which is correlated with the lower IHDS score in these population. Cystatin D (CST5) is not well studied in the context of HIV, however study showed that higher level of serum CST5 along with the TRAIL are biomarkers in traumatic brain injury patients which indicates of neuronal damage in ART patients as the level is higher compared to healthy individuals. Serum soluble CD8 is proposed to be a marker of CD8 T-cell activation in HIV-1 infection [35]. Higher plasma level of sCD8A in our ART group compare to healthy controls indicates there is still persistent infection which could be linked to the higher reservoir. Increased plasma CCL23 level was associated with coronary atherosclerosis [36] suggest that patients in long term ART treatment with higher plasma CCL23 level compare to healthy group has higher risk of developing vascular diseases. HIV-1 hyperactivates mTOR complex1 (mTORC1) for its own viral production and latency reactivation [37]. However no study reported any relation of treatment and plasma 4E-BP1. Higher level of 4E-BP1 in plasma in ART patients compare to healthy and its correlation with HIV-1 associated mTOR pathway mechanism in viral production has to be elucidated in long-term ART suppressive condition.

Moreover, in our sub-group analysis of the treated HIV group, we found several significant associations all of which revealed higher inflammatory markers levels in patients treated with ZDV/3TC/NVP, than in those treated with TDF/3TC/EFV. The virological efficacy studies indicated that TDF/3TC/EFV is equal or superior to other regiments.[38, 39]Studies have shown that the NRTIs like ZDV and 3TC, can cause accelerated aging by depletion of mitochondrial DNA via inhibition of the mitochondrial specific DNA polymerase-γ [40, 41]. Use of NVP also showed neurocognitive impairment [42]. Therefore we propose to use TDF/3TC/EFV as a first-line regimen that might avoid accelerated the aging process in PLHIV partly.

In our study, we observed a strong association between higher odds of having a potential cognitive impairment with HIV-1 positive status. The IHDS [14]was adapted from the HIV Dementia Scale (HDS)[43]mainly for a non-English speaking population, and it was earlier used for the Indian population [44]. It was observed 35% of the treatment naïve Indian patients have IHDS<10 [44]. Following the use of cART, the severe form of HAND have declined, but the prevalence of a milder form of the HAND is continuously stable or has even increased [45, 46]. Despite the earlier studies showing that HIV-1 subtype C circulating in India (HIV-1C_{IN}) hasa lower magnitude of neurovirulence [47-49], a substantial proportion (75%) of the patients who initiated treatment at the chronic stage of infection with very low CD4 count had cognitive impairment [50]. Based on the findings we postulated that though HIV-1C_{IN} circulated in India has lower neurovirulence than HIV-1B or HIV-1C circulating in Africa, late initiation of the therapy could potentially be the cause of impaired cognitive function which cART fails to restore.

Chronic inflammation and immune activation during cART are proposed to be important parameters contributing to HIV persistence [51, 52]. However, a recent study showed that the markers of immune activation (sCD14 and sCD163) and inflammation (hsCRP and IL-6) were not associated with the level of a persistent HIV-1 reservoir following median seven years of treatment [20]. Our study is in line with this previous study as we found no co-relation of sCD14 and sCD163 plasma levels with HIV-1 reservoir size. After adjusting several parameters including age and CD4 count at initiation of therapy, several inflammatory markers including IL-6 did not correlate with the HIV-1 reservoir either. As our inflammatory markers panel was larger than any other earlier studies reported, we observed that the two chemokines eotaxin-1 (CCL11) and CCL20 (also known as Macrophage Inflammatory Protein-3 [MIP-3]) were negatively associated with the HIV-1 reservoir.

Eotaxin-1and its seven-transmembrane G protein-coupled receptor (GPCR) CCR3 have been shown to modulate the HIV-1 entry of dual-tropic and some macrophage (M)-tropic strains [53] and eotaxin can inhibit the efficiency of this process also in microglia [54-56]. CCR6 and its ligand

CCL20 (CCR6/CCL20 axis) may be involved in HIV pathogenesis and immunity [57]. CCR6 and its isoform CKR-L3 that mainly found on dendritic cells (DCs), memory T cells and selected B cell subtypes also act as a co-receptor for certain HIV-1 isolates [58, 59]. Another study also showed thatHIV-1 latency could be established in resting $CD4^+$ T cells infected with HIV-1 after exposure of CCL20 [60] and CCR6 is a marker for memory $CD4^+$ T cells that harbor the highest levels of HIV-1 proviral DNA in infected individuals [61]. CCL20 has also been shown to be an anti-HIV-1 microbicide in the female genital tract [62]. We observed a negative association of both CCL11 and CCL20 with the total HIV-1 reservoir with longer duration of successful therapy. Several studies suggested that cytokines may play an important role in the seeding of the latent reservoir in the acute HIV-1 infection as well as they can promote long-term viral persistence during suppressive cART (reviewed in [63]). Based on all these findings we, therefore, hypothesize that cytokine receptors that can regulate the HIV-1 entry into the cells (like CCR3/CCL11 axis) and cell surface receptor ligands which have anti-HIV-1 activities, like CCR6/CCL20 axis, are key modulators for the HIV-1 persistence during suppressive cART. Understanding the molecular mechanism of those cytokine signaling pathways responsible for HIV-1entry, anti-HIV-1 activity and latency may provide attracting candidates for therapeutic strategies for remission or reduction of the viral reservoir during suppressive cART.

Our study has some limitations that merit comments. First, the patient population that was selected for this study are among the best pool of successfully treated individuals' trough the Indian National ART program. The findings may not generalize to the general population of treated individuals. Second, due to lack of earlier studies in the settings, the design of our study, and sample size limitations, the conclusions that can be drawn are limited to associations with modest significance. Also, a large number of tests were run, and the most significant results are highlighted, for this reason, the results should be considered hypothesis generating. Third, the patients were not monitored virologically as a standard of care, and we have only virological data at the time of sampling. Any potential viral blips may confound the inflammatory markers. Finally, the reservoir

quantification was done with total HIV-1 DNA, not the replication HIV-1 reservoir. However, our study is the first comprehensive study on inflamm-aging in the long term successfully treated PLHIV from an LMICs which uses a standardized public health approach for monitoring PLHIV on treatment.

In conclusion, for the first time in a standardized public health setting for monitoring PLHIV on treatment from an LMIC, we identified several inflammatory soluble markers in long-term successfully treated individuals that are not normalized to the levels seen in healthy individuals. These findings imply that in spite of successful ART, PLHIVs are potentially at higher risk of inflamm-aging and age-related inflammatory diseases. Therefore use of anti-inflammatory drugs may potentially be considered in this population which can reduce the burden of excessive immune activation and inflammation [64]. The effect was greater in those on a ZDV/3TC/NVP treatment regimen than in those receiving TDF/3TC/EFV. HIV-1 persistence did not correlate largely with immune activation or inflammatory markers. Some markers like CCL11 and CCL20, which play a role in regulating HIV-1 entry and have potent anti-HIV activity, could be a key for the design of therapeutic strategies for reduction of the viral reservoir during suppressive cART. However, their precise mechanisms of interactions need to be first investigated.

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Conflict-of-interest disclosure

The authors declare no competing financial interests

Contribution: H.B., S.S.A, N.R.M, M.S., N.C. performed the laboratory experiments; H.B. performed the IHDS assessment; A.T.A. and E.E.G. performed bioinformatics and statistical analysis; U.N. and A.T.A. made the figures. N.P., R.S., V.J., and S.K.T. recruited study subjects and provided the clinical data. P.N. provided the clinical interpretation. U.N. and L.E.H conceived and designed the study; U.N. wrote the first draft of the paper reviewed by H.B., M.S., P.N, L.E.H. All the authors approved the final version of the manuscript.

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691 **Figure Legends:**

692 **Figure 1. Flow diagram of study design and experimental plan:** HIV-1 positive individuals
693 (n=424) and HIV-1 negative healthy controls (n=295) were screened. Following inclusion and
694 exclusion criteria, healthy controls (n=43), and HIV positive ART-experienced subjects (n=53)
695 (grey shed) and ART-naïve HIV-1 positive subjects (n=41) (red shed)were included in the study.
696 Additional inclusion for ART-experienced subjects are marked in orange. The clinical investigation
697 including assessment of neurocognitive function by IHDS and laboratory experiments including
698 evaluation of inflammatory markers, monocyte activation markers, telomere length and HIV-1
699 reservoir quantification were performed.

700 **Figure 2. Plasma immune activation markers:** Soluble CD14 (a) and CD163 (b) in plasma of the
701 three groups of individuals were measured using ELISA.

702 **Figure 3. Plasma inflammation markers:** (d) Hierarchical clustering analysis of ANOVA of
703 significantly differentially expressed proteins with false discovery rate (FDR) <0.001, identified
704 clustering of 79% (31/39) of Pre-ART samples along with two samples from the HC and one from
705 the ART group. The ART and HC samples clustered separately from Pre-ARTsamples but
706 intermingled with each other. (b) Venn diagram of statistically significant protein level in plasma
707 soluble markers. The sum of the numbers in each large circle represents the total number of
708 significantly differentially expressed proteins in plasma in the different groups (HC vs. ART, Pre-
709 ART vs.ART and Pre-ART vs. HC). The overlapping part of the circles represents significantly

different protein among the indicated groups. (c) The eleven soluble proteins that have significantly different levels of expression between HC and ART groups are shown.

Figure 4. The difference in plasma biomarkers between the different treatment and healthy controls. CIRCOS plot was created to visualize the different analysis of 25 plasma inflammatory markers that were significantly different between any of the two comparative groups, i.e. Healthy control vs. ZDV/3TC/NVP or TDF/3TC/EFV and ZDV/3TC/NVP vs. TDF/3TC/EFV. The outer circle showed the genes followed by six circles representing proteins associated with different disease status as indicated by the Olink inflammatory panel. Disease area was selected based on widely used public-access bioinformatic databases, including UniProt, Human Protein Atlas, Gene Ontology (GO) and DisGeNET by a custom-built tool at Olink Ab. Cancer (dark orange), cardiovascular diseases (orange), hepatic disorder (red), inflammatory diseases (maroon), neurological disorder (dark brown) and renal disorder (golden yellow). Followed by this a bar chart of NPX values for different proteins in HC (grey), patients on ZDV/3TC/NVP (light yellow) and those on TDF/3TC/EFV (pink) is presented. The scale was adjusted to +0.2 to -0.2 NPX of higher and lower NPX value of a particular protein in the three groups. Finally, Venn diagram of statistically significant protein levels of plasma soluble markers isshown in the centre. The sum of the numbers in each large circle represents the total number of significantly differentially expressed proteins in plasma among various combinations (HC vs. ZDV/3TC/NVP, HC vs. TDF/3TC/EFV and ZDV/3TC/NVP vs. TDF/3TC/EFV). The overlapping part of the circles represents proteins differently expressed between the groups. The unique proteins are highlighted.

Table 1. Patients' demographic and clinical parameter

Parameter	Treatment naïve (Pre-ART)	Treatment Experience (ART)	Healthy Control (HC)	P values
N	41	53	43	ND
Gender, Female, N (%)	21 (51)	23(43)	22 (51)	0.6734[#]
At sampling				
Age in years, median (IQR)	40 (37-43)	45 (42-49)	46 (40-54)	<0.0001*
CD4 count (cells/µL); median (IQR)	367 (251-578)	667 (476-797)	NA	<0.0001[§]
CD8 count (cells/µL); median (IQR)	1138 (872-1625)	772 (337-1092)	NA	<0.0001[§]
CD4:CD8 ratio, median (IQR)	0.329 (0.1863-0.529)	0.76 (0.575-1.013)	NA	<0.0001[§]
Viral Load, Log_{10} copies/mL, mean (SD)	4.4943 (0.9036)	2.14	NA	<0.0001[§]
Years on treatment, median (IQR)	NA	8 (6-10)	NA	ND
Treatment Regimen, n (%) ZDV+3TC+NVP TDF+3TC+EFV	NA	30 (57%) 23 (43%)	NA	ND
CD4 Count at treatment initiation (cells/µL), median (IQR)	NA	186 (100-280)	NA	ND

NA: Not available, ND: Not Done, *Kruskal-Wallis test, [#]χ^2 test, §Mann-Whitney test

ZDV: zidovudine, 3TC: lamivudine, NVP: nevirapine, TDF: tenofovir and EFV: efavirenz

Cross-Sectional Study

Screening (2015-2018)

Healthy Control (n=295)

Treatment Experienced (n=258)

Treatment Naive (n=166)

Inclusion Criteria

No
Diabetes, Tuberculosis, Chronic illness, Cardiovascular diseases, anti-inflammatory drugs

No
Diabetes, Tuberculosis, ilicit Drug use

- First Line treatment
- Duration of ART >5y
- No other medication
- Stable CD4 count
- Viral load <150 c/mL

Study Cohort

Healthy Control (n=43)

Treatment Experienced (n=53)

Treatment Naive (n=41)

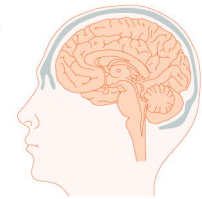
Clinical Experiments

International HIV Dementia Scale

Only for healthy controls and treatment experienced group

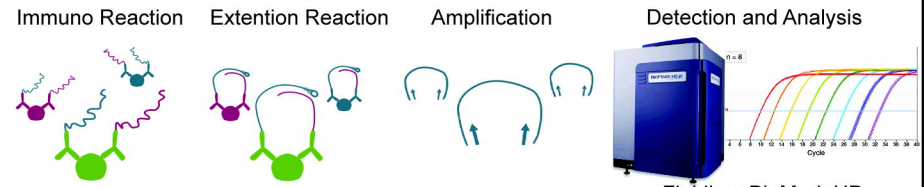
1. Motor Speed
2. Psychomotor Speed
3. Memory Recall

Sacktor *et al.* AIDS 2005,19:1367–1374



Laboratory Experiments

Olink® Inflammation panel



Fluidigm BioMark HD

Monocyte Activation Markers

sCD14 and sCD163

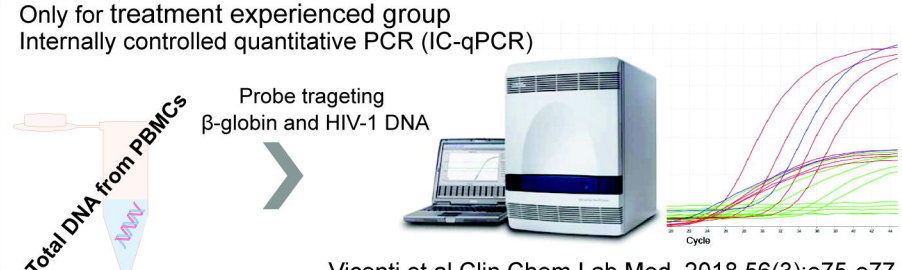
R&D Systems and Invitrogen

Telomere Length

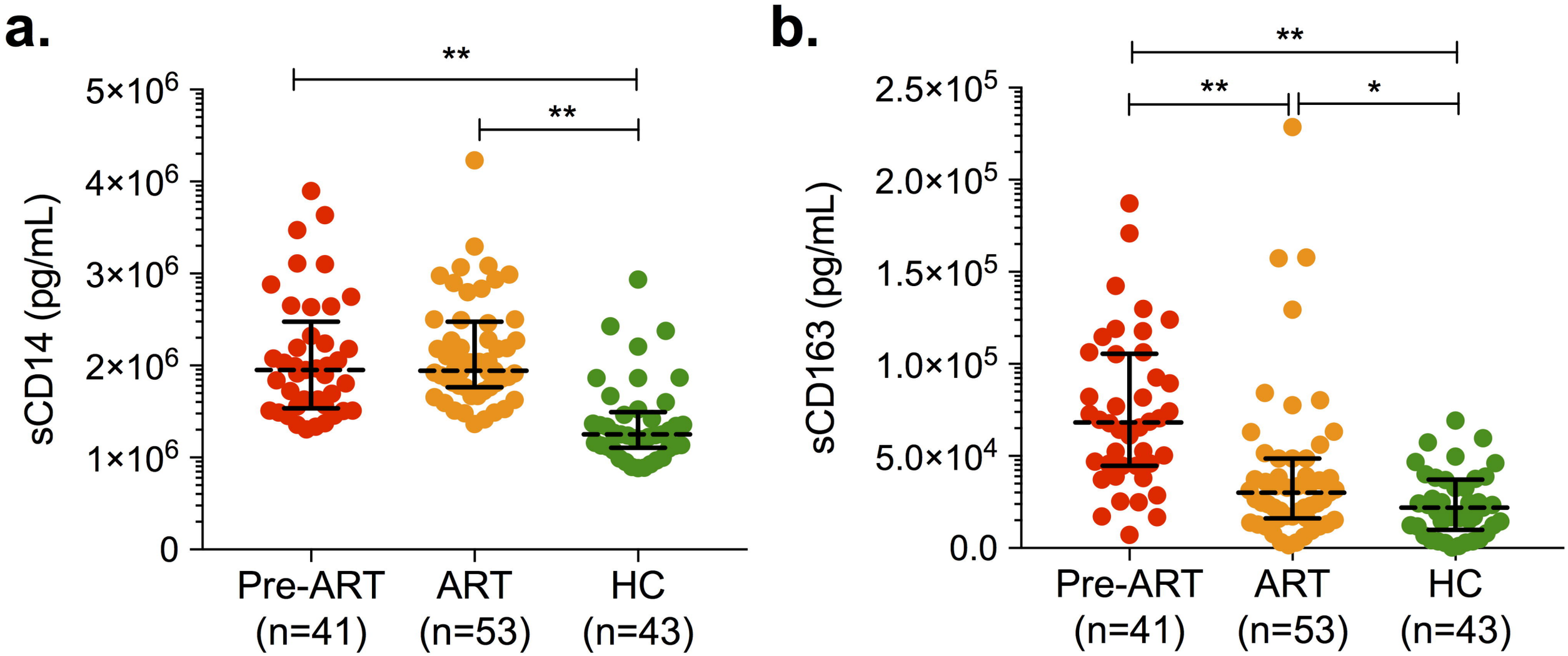
ScienCell™ Research Lab Inc.

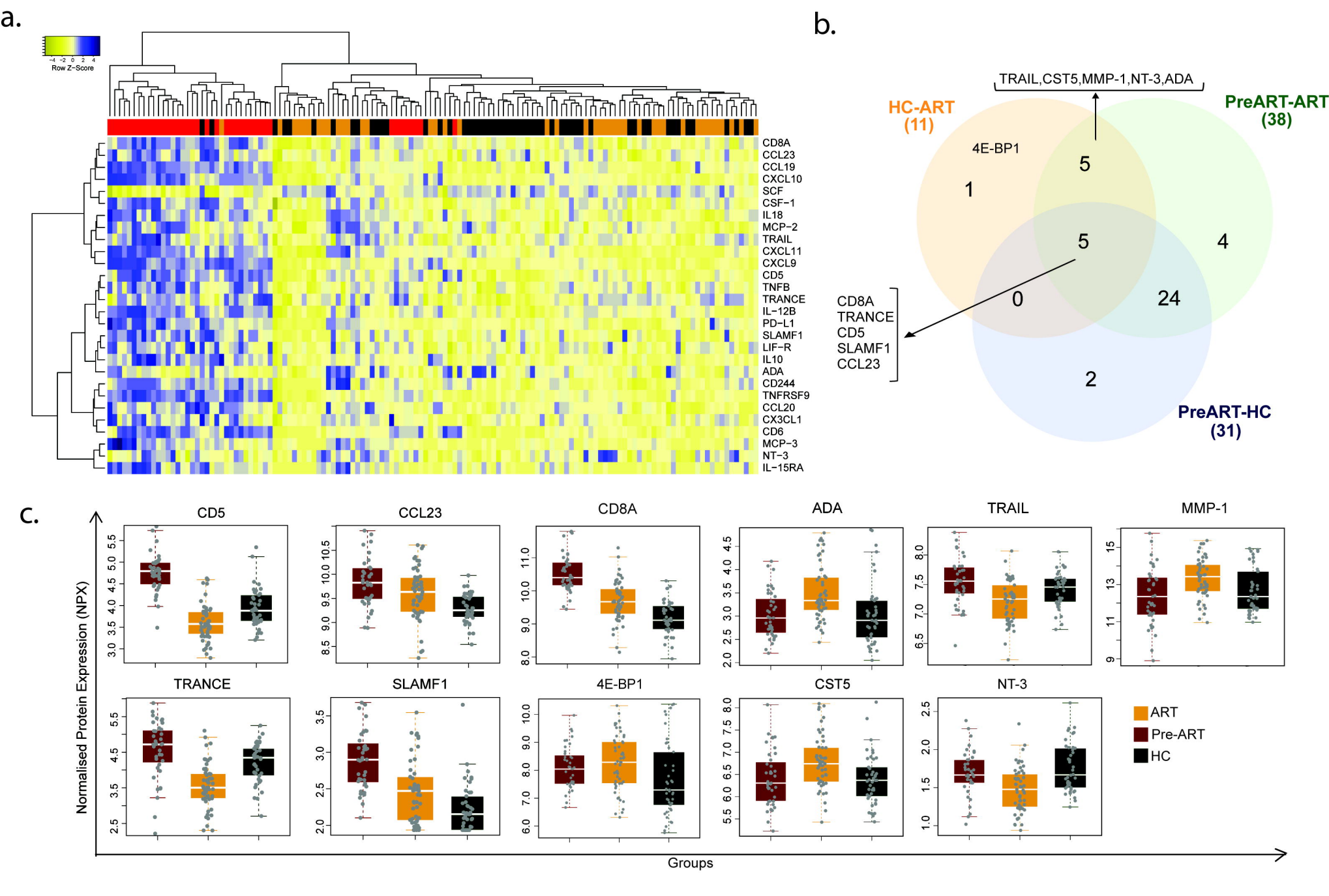
HIV-1 Reservoir Quantification

Only for treatment experienced group
Internally controlled quantitative PCR (IC-qPCR)



Vicenti et al Clin Chem Lab Med. 2018 56(3):e75-e77.


a.
**
**
5×10^6
4×10^6
3×10^6
2×10^6
1×10^6
0
sCD14 (pg/mL)
Pre-ART (n=41)
ART (n=53)
HC (n=43)
b.
**
**
*
2.5×10^5
2.0×10^5
1.5×10^5
1.0×10^5
5.0×10^4
0.0
sCD163 (pg/mL)
Pre-ART (n=41)
ART (n=53)
HC (n=43)


a.
Row Z-Score
CD8A
CCL23
CCL19
CXCL10
SCF
CSF-1
IL18
MCP-2
TRAIL
CXCL11
CXCL9
CD5
TNFB
TRANCE
IL-12B
PD-L1
SLAMF1
LIF-R
IL10
ADA
CD244
TNFRSF9
CCL20
CX3CL1
CD6
MCP-3
NT-3
IL-15RA
b.
TRAIL,CST5,MMP-1,NT-3,ADA
HC-ART
(11)
PreART-ART
(38)
PreART-HC
(31)
4E-BP1
1
5
5
4
0
24
2
CD8A
TRANCE
CD5
SLAMF1
CCL23
c.
CD5
CCL23
CD8A
ADA
TRAIL
MMP-1
TRANCE
SLAMF1
4E-BP1
CST5
NT-3
Normalised Protein Expression (NPX)
Groups
ART
Pre-ART
HC