HIV-1 transcription but not intact provirus levels are associated with systemic inflammation

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ABSTRACT

HIV-1 infected individuals have increased inflammation, which has been associated with age-associated diseases. Plasma markers, cell-associated (ca) virus levels, and ability to stimulate RNA transcription in latently infected cell lines was examined in younger and older HIV-1 infected individuals with suppressed virus. ca-RNA, but not intact provirus level, had positive correlation with plasma D-Dimer levels. The older as compared to the younger group had higher D-dimer levels and a trend toward more ca-RNA, but similar levels of intact proviruses. Even though all measured inflammatory markers were relatively higher in the older as compared to younger individuals, this greater inflammation did not induce more HIV-1 transcription in latently infected cell lines. Inflammation and HIV-1 RNA expression increase with age despite similar levels of intact infectious HIV DNA. While plasma inflammation correlates with HIV-1 RNA expression in peripheral blood mononuclear cells, it does not induce HIV-1 transcription in latently infected cell lines.

Key words: HIV-1 latency; accelerated aging; cell-associated HIV; inflammation; viral transcription

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Summary: Plasma inflammation increases with aging in treated people with HIV. This inflammation associates with intracellular HIV-1 RNA levels, but not the number of intact infectious proviruses. This aging associated inflammation does not stimulate greater HIV-1 RNA transcription in cell lines.

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INTRODUCTION

Although combination antiretroviral therapy (cART) routinely reduces HIV-1 replication below levels detectable by conventional clinical assays, treatment does not eliminate infection. Infection persists in latently infected cells, and virus can re-emerge if cART is interrupted [1]. Further, despite the effectiveness of cART in preventing viremia, low level expression of cell-associated (ca) HIV-1 RNA remains present [2]. People with HIV (PWH) who are not on cART have high levels of systemic immune activation [3]. This inflammation, estimated via plasma cytokines / chemokine levels and cellular activation markers, decreases with cART-induced virus suppression, but it does not normalize to pre-infection levels [4]. Thus, PWH who have suppressed plasma viremia experience chronic systemic inflammation as compared to age-matched HIV-1 uninfected individuals.

Among HIV-1 negative individuals, there is a large body of literature supporting the notion that chronic systemic inflammation increases the risk for atherothrombosis, cancer, and other diseases associated with aging [5, 6]. In the current cART era, HIV-associated non-AIDS diseases, such as atherothrombosis, neurocognitive decline, and cancer, account for most of the morbidity and mortality in PWH who have suppressed virus levels [3]. While this may be expected because PWH are surviving longer and aging, some but not all studies suggest that optimally treated HIV-1 infected individuals have 5 – 10 years loss in life expectancy and increased medical burden compared to risk-adjusted uninfected counterparts [7, 8]. Similar to HIV-1 uninfected individuals, these non-communicable HIV-associated non-AIDS diseases have been associated with elevated biomarkers, such as D-Dimer, C-reactive protein (CRP), soluble (s)-CD14, and interleukin (IL-6) [9-12].

There are potentially multiple etiologies for the persistent chronic inflammation observed in virus suppressed PWH, including the latently HIV-1 infected cells, other pre-existing co-infections, such as cytomegalovirus (CMV), and the irreversible gastrointestinal (GI) tract damage that occurs prior to cART initiation [3]. Previous studies have yielded

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conflicting conclusions about the impact of the persistently HIV-1 infected cells on systemic inflammation. Some but not all studies have shown a correlation between the markers of immune activation and the level of residual DNA and ca-RNA [13-18]. To date, however, no study has examined the association between markers of inflammation and the number of intact, presumably infectious, HIV-1 proviruses. In PWH with sustained virus suppression, the majority of infected cells contain defective proviruses with deletions or hyper mutations [19, 20]. The intact and defective proviruses can yield virus RNA and proteins [21, 22], which potentially allows the host to recognize the provirus harboring cell. This immune response may account for a possible association between chronic inflammation and intact infectious provirus levels. Until recently, PCR-based assays that quantified the level of residual HIV-1 DNA measured both intact and mutated sequences. In this work, we evaluated the association between age, chronic inflammation, and levels of intact proviral DNA and total ca-RNA. We also examined if plasma with varying levels of inflammation can differentially induce HIV-1 RNA expression in latently infected cells.

MATERIALS AND METHODS

Study Population

We used samples from an existing cohort aimed at examining the interaction between HIV and aging [23]. This cohort prospectively recruited PWH in 2 age –stratified groups, a younger 18-35 years of age and an older ≥ 50 years of age group. No participants between 35 to 50 years of age were enrolled. All participants had HIV-1 infection for an undetermined duration, but they had been on cART for a minimum of 6 months with plasma HIV-1 RNA levels less than 50 copies/ml as assessed by commercial assays. Those with active hepatitis B or C, ≥ five viral blips since cART initiation, or recent immunomodulatory therapy (oral or injected corticosteroids, plaquenil, azathioprine, methotrexate, biologic therapies, systemic interferon, local interferon, or chemotherapy and receipt of an HIV vaccine) were excluded.

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Plasma and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque centrifugation techniques, aliquoted, and stored at -80 C until future use.

Virologic Assays Cellular DNA and RNA were obtained from the same PBMC aliquot using All Prep DNA/RNA mini kit (Qiagen). Total HIV-1 DNA and number of total cells was quantified as previously described with minor modifications [24]. PCR amplicons were used as templates for the quantitative standards rather than plasmid DNA. HIV-1 ca-RNA quantification was based on an *in vitro* transcribed RNA standard and using a 1-step reverse-transcription quantitative polymerase chain reaction (RT-qPCR) reaction with 4X Fast Virus Master Mix (Applied Biosystems). Primer and probe sequences are available upon request. All samples were assessed in triplicate, a minimum of two independent times. A recently described intact proviral DNA assay was used to estimate the quantity of intact inducible HIV-1 proviruses [20]. Briefly, HIV-1 regions psi (ψ) and HIV-1 envelope were targeted in a multiplex droplet digital PCR. Double and single positive droplets were deemed as having intact and defective provirus respectively. Negative droplets contained no DNA or cellular DNA lacking HIV-1. Total cell copies and the level of DNA shearing was estimated by amplifying and quantifying the human RPP30 gene at two different positions. The total number of intact proviruses were estimated after correcting for the estimated DNA shearing as detailed previously [20]. Droplet digital PCR was accessed through the Boston University Genome Sciences Institute.

Inflammatory plasma assessments: Stored frozen plasma samples were thawed and assayed for tumor necrosis factor (TNF) – alpha, IL–6, sCD14, and sCD163 using bead-based multiplex (Milliplex) and for D-dimer and CRP using ELISA (RD Systems and Abcam) kits as described previously [25, 26]. Assays were run according to manufacturer's instructions. Quantification was performed on a Magpix (Luminex) instrument equipped with xPNENT 4.2 software and a luminometer. Values were based on a standard curve, and any reading below the standard curve was assigned the lowest quantifiable value. Plasma

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measurements were not available for all participants because of limited sample quantities and degradation.

RNA expression among latently infected cell lines

HIV-1 latently infected cell lines (J-Lat 6.3 and ACH-2) were obtained from the NIH AIDS Research Reagent and Reference Program. J-Lat 6.3 and ACH-2 are T cell lines carrying a single defective and infectious HIV-1 proviral copy respectively [27, 28]. These cells were cultured and passaged in RPMI containing 10% fetal bovine serum (FBS) and supplemented with 100 U/mL penicillin/streptomycin (RPMI complete) [29]. Approximately 10^5 latently infected cells were incubated in 200 µL RPMI complete or RPMI supplemented with 10% plasma from PWH and 100 U/mL penicillin/streptomycin in a 96 well plate. After 24 hours, cells were either stimulated with TNF-alpha (5 ng/mL) or no cytokines. Twenty four hours after incubation, RNA and DNA was isolated from the cultured cells. All incubation conditions were examined in duplicate independent wells and pooled for nucleic acid extraction. Although ACH-2 harbor an infectious provirus, HIV-1 replication could not be measured in our assay because antiretroviral medications were present in all the patient plasma.

Statistical Analysis

All HIV-1 RNA and DNA levels and plasma cytokine concentrations were $\log_{10}$ transformed to yield normalized distributions. Comparisons among groups were done using Fisher's exact test, Chi-square test, t-test (normal distributions), and Mann Whitney test (non-parametric distributions). Associations were examined using Pearson and Spearman correlation for normally distributed and non-parametric data respectively. In the multivariable linear regression analysis, the dependent variable was either plasma D-dimer, ca-RNA, or intact proviral DNA levels. Co-variates included in the initial model were age, gender, ethnicity/race, pre-therapy plasma virus level, nadir and enrollment absolute CD4 count, years on therapy, and the antiretroviral anchor. Covariates were removed from this multi-variable model in a stepwise backward fashion if the p-value was > 0.10, starting with the weakest predictor. All p-values are based on two-sided tests. Benjamini Hochberg (BH) and

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Bonferroni corrections were used to account for multiple comparisons. All tests were done in GraphPad Prism 8.2.1.

Study Approval

All studies were approved by the Boston University, Brigham and Women's and Massachusetts General Hospital institutional review board. Written informed consent was received from participants prior to inclusion in the study.

RESULTS

Study Population

We examined 57 HIV-1 cART treated and virologically suppressed individuals who were between the ages of 18-35 (n = 23) or ≥50 (n = 34) years (Table 1). Women were part of the older but not the younger group. The younger group contained a significantly higher proportion of non-White, non-Black participants. The younger as compared to older group had higher pre-cART plasma HIV-1 levels and recorded nadir and enrollment absolute CD4 count although the differences were not statistically significant. The younger as compared to the older group had been on cART for a significantly shorter duration of time. The antiretroviral agent anchoring the cART regimen was not different among the groups. As expected, a greater number of the older as compared to younger individuals had pre-existing conditions.

Cell-associated HIV-1 RNA but not intact proviral DNA levels correlate with plasma inflammation.

We measured six different plasma inflammatory markers. Plasma D-dimer levels showed a significant correlation with ca-HIV-1 RNA levels ($\rho = 0.36$, $p = 0.006$, Fig. 1A). This D-dimer association remained statistically significant after adjusting for multiple comparisons (6 independent tests, BH $p = 0.036$ or for the Bonferroni method any unadjusted p-value < 0.008). Although there were no significant associations with the other plasma markers, there was a statistical trend with sCD14 ($\rho = 0.23$, $p = 0.09$) and sCD163 ($\rho = 0.25$, $p = 0.09$). sCD163 data, however, was not available from 9 participants.

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Multivariable linear regression analysis was used to account for the baseline demographic differences among the older and younger group (Table 1). In the final model, ca-RNA level, age, pre-therapy $\log_{10}$ plasma HIV-1 copies, and ethnicity/race were predictive of plasma D-dimer levels (Table 2). Specifically, for every 10 fold increase in ca-RNA, there was a 0.23 $\log_{10}$ increase in plasma D-dimer level.

To our knowledge, no prior study has examined associations with intact proviral DNA levels and markers of inflammation. Intact proviruses may potentially induce greater host immune responses because of low level spreading infection or higher expression of structurally-intact virus proteins. We assayed intact proviral DNA in a median of 96,674 PBMCs (range 22,469 to 3,483,606). There was no significant difference in the number of cells examined among the older (median 87,124, range 22,469 to 412,421 cells) as compared to the younger group (median 107,645, range 29,568 to 3,483,606 cells, p = 0.23). Intact proviral DNA levels in bulk PBMC (median 2.1, range -0.01 – 3.4 $\log_{10}$ per 10^6 PBMC) were higher in our cohort than that previously documented in bulk PBMC from another cohort of virus suppressed PWH [30]. This observed variation could be attributed to differences in the duration of suppressive cART (median 3.3 versus 9 years) among the cohorts because intact proviruses may decay with prolonged cART [20]. There was a statistical trend in the association between the number of intact proviral DNA and sCD163 (ρ= 0.28, p = 0.05, Fig. 1B), but this was not adjusted for multiple comparisons. No associations were observed with the other plasma inflammatory markers.

Aging is associated with inflammation and non-significantly higher ca-HIV RNA levels

Higher plasma inflammatory markers has been observed with increasing age [31, 32]. The multivariable linear regression analysis also suggested that with every year of age, there was 0.02 $\log_{10}$ increase in plasma D-dimer level (Table 2). The median of all six measured plasma inflammatory markers was higher in the older as compared to the younger group (Table 3), although after correction for multiple comparisons, only the D-Dimer (unadjusted p = 0.0006, BH p-value 0.004, Fig. 2A) difference was statistically significant.

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We hypothesized that ca-HIV-1 RNA, but not intact DNA, would be higher in the older as compared to the younger group because ca-RNA but not intact provirus level was associated with plasma inflammation (Fig. 1 and Table 2). Indeed, ca-RNA was higher in the older as compared to younger group although the difference only showed a statistical trend ($p = 0.08$, Fig. 2B). Older as compared to the younger PWH had longer duration of cART therapy (Table 1), and this prolonged drug exposure may impact ca-RNA levels. In multivariable linear regression analysis, one year of older age was associated with 0.007 $\log_{10}$ higher ca-HIV-1 RNA ($p = 0.11$) after adjusting for duration of cART therapy. The other baseline demographic characteristics did not have a significant association with ca-HIV-1 RNA. Thus although not statistically significant, ca-RNA trended higher in the older as compared to younger PWH.

On the other hand, intact proviral DNA level did not demonstrate a statistically trending difference among the two age-stratified groups (Fig. 2C). Age ($\beta = .43$, $p = 0.67$), duration of cART therapy ($\beta = -0.31$, $p = 0.30$) and absolute CD4 count at enrollment ($\beta = -0.0001$, $p = 0.74$) also did not predict the intact proviral DNA levels in univariate linear regression analyses. There were also no significant associations in multivariable linear regression analysis. Although not statistically significant, the negative correlation aligns with previous observation that intact provirus levels decrease with prolonged duration of cART-mediated virus suppression [20].

Plasma inflammation and aging does not enhance HIV-1 RNA production in latently infected cells

The directionality of the association between systemic inflammation and HIV transcription remains uncertain. Although not mutually exclusive, an inflammatory milieu may drive HIV-1 transcription or alternatively intracellular HIV-1 RNA expression may induce inflammation. Our observed association between ca-RNA level and plasma inflammation (Fig. 1 and Table 2) does not distinguish between these possibilities. We examined the impact of the plasma inflammatory milieu on HIV-1 transcription to further explore this relationship. We examined two different latently infected cell lines (J Lat 6.3 and ACH-2) because HIV-1 transcriptional

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capacity in latent cells varies depending on the integration site and other cell characteristics [30]. Thus, we expected varying results from the 2 different cell lines. Cell lines were incubated in media containing 10% plasma from PWH [27, 28]. A 10% plasma fraction was used because J-Lat 6.3 and ACH-2 cell cultures are routinely done with RPMI containing 10% FBS. The level of HIV-1 transcription was also measured in the cell lines in the presence and absence of TNF-alpha because this cytokine has been previously shown to stimulate HIV-1 RNA transcription [27, 28]. HIV-1 RNA was detectable in both cell lines over a range of TNF-alpha concentrations demonstrating that HIV-1 transcription could be induced with minimal cytokine stimulation. (Supplementary Figure 1A and 1B).

Although the older as compared to younger group had higher median levels of all the six measured inflammatory markers (Table 3), HIV-1 RNA relative to DNA levels trended higher among JLat cells incubated in plasma from the younger as compared to older PWH without (p = 0.05) but not with TNF-alpha (p = 0.23, Fig. 3A). RNA relative to DNA levels were also higher in ACH-2 cells incubated in plasma from the younger as compared to older PWH with (p = 0.04) but not without TNF-alpha (p = 0.38, Fig. 3B). As a positive control, higher HIV-1 RNA was observed in the presence as compared to the absence of TNF-alpha in both cell lines conditioned with participant plasma, implying that the patient plasma did not prevent HIV-1 transcription from the latent provirus (Fig. 3A and 3B). There was no correlation in the observed transcription induction in the presence of participant plasma among the two different cell lines (data not shown). This suggests that the higher pre-existing inflammation present in plasma does not induce greater HIV-1 transcription in these two different cell lines.

DISCUSSION

Even with suppressive cART, some but not all studies suggest PWH have shorter life expectancy as compared to age-matched uninfected individuals [7, 8]. The increased mortality among PWH has been attributed to a higher incidence of age associated morbidities that potentially emerge as a result of chronic inflammation [9-12]. The etiology of the chronic inflammation in individuals with suppressed virus level is likely multifactorial and

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specific drivers remain uncertain. In this study, we show that inflammation as determined by plasma markers associates with intracellular HIV-1 RNA but not intact proviral DNA levels. Furthermore, plasma inflammation increases with age in PWH, similar to what has been observed in HIV-1 uninfected individuals [33]. We also found that *in-vitro* HIV-1 RNA transcription in latent cell line models do not increase in the presence of plasma from older individuals who had greater levels of inflammatory markers. In aggregate, our observations suggest that as PWH age, they have increased systemic inflammation and potentially greater level of HIV-1 transcription, independent of intact proviral numbers. This age-associated inflammation, however, does not induce HIV-1 RNA transcription in latently infected cell lines. Aging is also not associated with higher levels of infectious virus. Our observations suggest that aging may be associated with greater uncontrolled HIV-1 RNA transcription, and this promotes chronic inflammation in the absence of new rounds of virus replication. Larger cohorts need to be examined to confirm our findings.

Previous studies have also examined the link between cellular or plasma markers of inflammation with HIV-1 intracellular DNA and RNA levels. These investigations have yielded conflicting data potentially due to cohort differences, such as the duration of cART and prospective versus cross sectional study design [13-18]. To our knowledge, no previous studies have examined intact proviral DNA, but instead have measured total or integrated DNA, and cell-associated RNA or plasma HIV-1 via ultrasensitive assays. Recently, it has been shown that intact proviral DNA, as opposed to ca-HIV-1 RNA and total HIV-1 DNA levels, correlates better with inducible infectious virus among virus suppressed PWH [20]. Similar intact provirus levels among younger as compared to older PWH on long-term cART implies that there is no difference in ongoing virus replication despite age. Thus, low level infectious virus production does not account for the enhanced plasma inflammation in older PWH.

In contrast to intact proviral DNA, we observed that ca-HIV-1 RNA, a measurement of transcriptional activity, showed a modest link with D-Dimer. The majority of the intracellular HIV-1 RNA as opposed to intact proviral DNA may constitute both infectious and

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non-infectious virus. First, majority of the persistent HIV-1 DNA in virus suppressed PWH is defective and will not yield infectious virus [19]. The integrated defective DNA, however, can still yield HIV-1 RNA, and in some cases this RNA is translated to viral proteins [21, 22]. Second, the small amount of HIV-1 RNA that may lead to infectious virions will likely not yield new productively infected cells because the presence of cART prevents spreading infection. It is important to note that current antiretroviral drugs prevent the generation of infectious virus and subsequent infection, but they do not inhibit HIV-1 transcription and RNA production. Thus, PWH on suppressive cART with no evidence of ongoing virus replication continue to have ongoing HIV-1 RNA production [2].

Recent studies suggest that the presence of HIV-1 RNA alone in the absence of protein or infectious virus induces inflammation. These recent investigations argue that nuclear export of HIV-1 intron containing RNA induces an innate immune response in CD4+ T cells, dendritic cells, and macrophages [34, 35]. In addition to these *in-vitro* HIV-1 studies, *ex-vivo* and animal studies also support the notion that endogenous retroviral RNA expression can induce inflammation [36]. This endogenous retroviral expression induced inflammation has been proposed as a contributor to age-associated inflammation, and aging has been associated with de-repression of endogenous retrovirus transcription [36]. Consistent with these data, we observed that HIV-1 RNA levels trended higher in the older as compared to the younger PWH even though the older group had been on suppressive cART for a longer time. This observed difference should not have a binary interpretation just because it was not statistically significant. Indeed, higher intracellular HIV-1 RNA levels have also been observed among suppressed HIV-1 older as compared to younger individuals in another study [14]. Although directionally consistent, a significant association between ca-HIV-1 RNA and D-dimer was only observed in the younger ($\rho = 0.50$, $p = 0.01$) but not the older PWH ($\rho = 0.16$, $p = 0.37$), possibly due to smaller sample sizes. Together these data suggest that aging is correlated with lower HIV-1 transcriptional control, although non-virus processes may also be impacting the associated higher inflammation observed in older PWH.

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Our *in-vitro* studies further show that the greater inflammatory mediators present in plasma samples from older as compared to the younger PWH did not impact the HIV-1 transcriptional landscape in latent cells. Our observations would argue that pre-existing inflammatory milieu present in older PWH does not induce HIV-1 transcription in latently infected cells. We acknowledge that these *in-vitro* studies do not perfectly mimic the *in-vivo* conditions where majority of infected cells are present in tissues rather than peripheral blood and infected cell characteristics, such as integration site, impact transcriptional capacity. Collectively, our observations suggest that enhanced inflammation observed with aging does not promote greater HIV-1 transcription, but rather the presence of HIV-1 RNA in the absence of any new rounds of infection promotes innate immune activation [34, 35]. Our model argues that novel drugs that lower the levels of intracellular HIV-1 RNA, something that current antiretrovirals cannot accomplish, may impact the chronic inflammation observed in PWH, especially as they age.

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Author Contributions

AO, NL, and MS designed the research studies and analyzed the data. AO, CC, JSC, AO, and MS performed experiments. NL provided clinical samples. AO, JSC, NL and MS provided input regarding data interpretation. MS wrote the manuscript with input from the other authors.

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Table 1. Study population and demographics

	Total n = 57	Younger n = 23	Older n = 34	p-value Younger vs. Older
Age years, median	52 (22 – 76)	29 (22 – 35)	57 (50 – 76)	-
Female, n (%)	4 (7.0)	0 (0)	4 (11.8)	0.14[a]
Ethnicity/Race				0.02[b]
White Non-Hispanic, n (%)	34 (59.6)	11 (47.8)	23 (67.6)	
Black Non-Hispanic, n (%)	18 (31.6)	7 (30.4)	11 (32.3)	
Hispanic or other, n (%)	5 (8.8)	5 (21.7)	0 (0)	
Last pre-therapy plasma HIV-1 RNA Log_{10} copies/ml, median (Number missing data)	4.72 (1.92 - 7.82) (n = 6)	5.00 (3.17 – 7.82) (n = 2)	4.64 (1.92 – 7.66) (n = 4)	0.11[c]
Nadir CD4+ T cells cells/mm^3, median (Number missing data)	282 (9 – 1028) (n = 1)	337 (16 – 1028) (n = 0)	262 (9 – 803) (n = 1)	0.20[c]
% CD4+ T cells at enrollment cells/mm^3, median (Number missing data)	308.5 (9 – 1028) (n = 1)	369 (25 – 1028) (n = 0)	301 (9 – 921) (n = 1)	0.27[c]
Years on therapy, median	3.33 (0.69 – 22.57)	2.19 (0.69 – 6.74)	4.72 (0.86 – 22.57)	0.0005[c]
Antiretrovirals				0.92[b]
NNRTI[d]	38 (67)	15 (65)	23 (68)	
Protease inhibitor	8 (14)	3 (13)	5 (15)	
Integrase inhibitor	11 (19)	5 (22)	6 (18)	
Comorbidities				

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Coronary artery disease	3 (5)	0 (0)	3 (9)	0.27[a]
Hypertension	11 (19)	2 (7)	9 (26)	0.17[a]
Diabetes mellitus	8 (14)	1 (4)	7 (21)	0.13[a]
Hyperlipidemia	14 (25)	1 (4)	13 (38)	0.004[a]
Chronic kidney disease	4 (7)	1 (4)	3 (9)	0.64[a]
Pulmonary disease[e]	8 (14)	3 (13)	5 (15)	0.99[a]
Cancer	6 (11)	1 (4)	5 (15)	0.38[a]
Gastrointestinal[f]	10 (18)	1 (4)	9 (26)	0.04[a]
Neurologic[g]	6 (11)	0 (0)	6 (18)	0.07[a]
Psychiatric[h]	20 (35)	7 (30)	13 (38)	0.58[a]

[a] Fischer's exact test

[b] Chi-square test

[c] Mann-Whitney U test

[d] non-nucleoside reverse transcriptase inhibitor

[e] chronic obstructive pulmonary disease, asthma, history of pneumonia, latent TB.

[f] Barretts esophagus, colonic polyps, diverticulitis, diverticulosis, gastritis, acid reflux, Lynch syndrome, irritable bowel syndrome.

[g] history of meningitis, tremor, neuropathy, seizure disorder, Parkinson's disease, tardive dyskinesia, traumatic brain injury.

[h] depression, anxiety, post-traumatic stress disorder, bipolar disease, episode of psychosis, Asperger's syndrome.

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Table 2. Predictors of plasma D-Dimer levels in multi-variable linear regression analysis[a]

	Estimate (β)	95% confidence interval	p-value
(Intercept)	0.67	-0.24 to 1.58	0.14
ca-RNA	0.23	0.02 to 0.43	0.03
Age	0.02	0.008 to 0.02	<0.0001
pre-cART HIV-1 RNA	0.14	0.03 to 0.24	0.01
Race/ethnicity: Black	0.21	-0.03 to 0.45	0.08
Race/ethnicity: Hispanic/ other	0.41	0.03 to 0.79	0.03

[a] Co-variates included in the initial model were age, gender, ethnicity/race, pre-therapy plasma virus level, nadir and enrollment absolute CD4 count, years on therapy, and the antiretroviral anchor. Only the covariates remaining after removing those with a p-value was > 0.10 are shown.

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Table 3. Plasma inflammatory markers in the younger and the older group

	Younger median (range, number with data)	Older median (range, number with data)	p-value[a]	BH p-value[b]
sCD14 log_{10} (ng/ml)	3.02 (2.83 – 3.43, 23)	3.14 (2.92 – 3.64, 34)	0.01	0.05
sCD163 log_{10} (ng/ml)	2.90 (2.32 – 3.90, 23)	3.08 (2.37 – 3.91, 25)	0.11	
D-Dimer log_{10} (pg/ml)	2.58 (1.97 – 3.44, 23)	3.00 (2.28 – 3.98, 34)	0.0006	0.04
CRP log_{10} (ng/ml)	3.14 (1.97 – 4.41, 23)	3.58 (2.56 – 4.03, 34)	0.06	
TNF-alpha log_{10} (pg/ml)	1.89 (1.75 – 2.03, 23)	1.91 (1.68 – 2.09, 34)	0.12	
IL-6 log_{10} (pg/ml)	1.83 (1.66 – 2.02, 15)	1.94 (1.62 – 2.61, 23)	0.01	0.05

[a] younger versus older t-test p-value without adjusting for multiple comparisons

[b] younger versus older t-test p-value with Benjamini Hochberg (BH) correction for multiple comparisons

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Figure Captions

Figure 1. Correlation between virus measurements and plasma inflammatory markers. Association between intra-cellular HIV-1 RNA and D-Dimer (A) and between intact proviral DNA and s-CD163 (B). Different color symbols show data from the younger (gray circles) and older (black squares) participants. Each figure shows the rho, p-value for a Pearson correlation, and number of data points. The black and dotted lines denote the linear regression and 95% confidence line.

Figure 2. Older as compared to younger PWH have higher plasma inflammation. Comparison of D-dimer (A), cell-associated RNA (B), and intact proviral DNA (C) among younger (gray circles) and older group (black squares). Black bars show the median and the interquartile range. Asterisk denotes statistically significant difference (Student t-test two-sided p-value < 0.001). The text at the bottom denotes the number of individual data points.

Figure 3. TNF-alpha in the presence of plasma stimulates RNA transcription in latently infected cells. Level of J Lat (A) and ACH-2 (B) HIV-1 RNA per HIV-1 DNA measured in the presence as compared to the absence of 5 ng/ml TNF-alpha and plasma from the younger (gray circles) and older (black squares) PWH plasma respectively. Black lines denote median and interquartile range. The * and *** denotes p-value < 0.05 and < 0.001 respectively. The legend denotes the number of individual data points.

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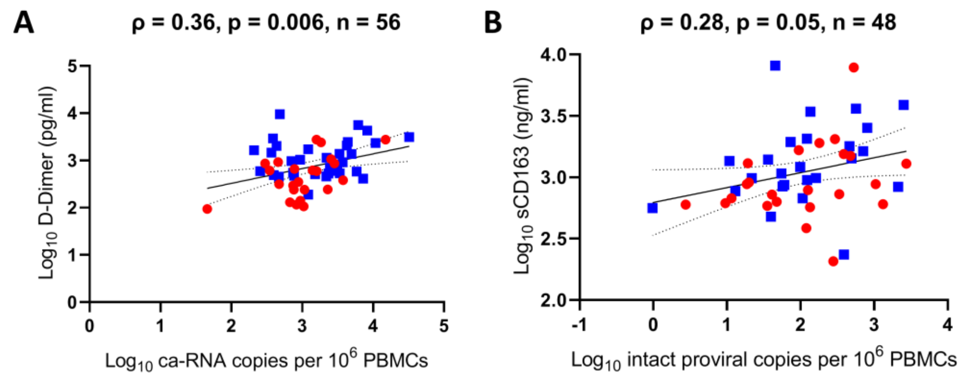

Olson et. al. Figure 1

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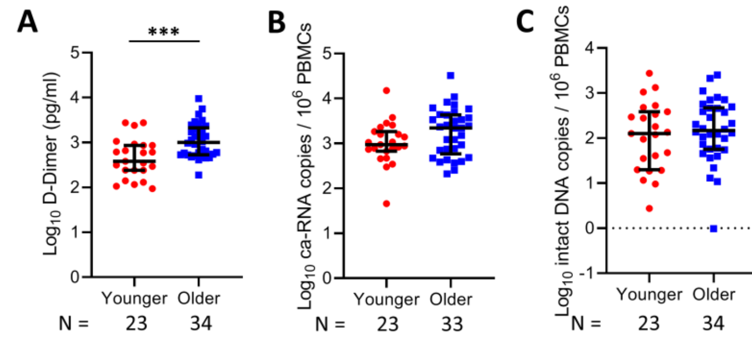

Olson et. al. Figure 2

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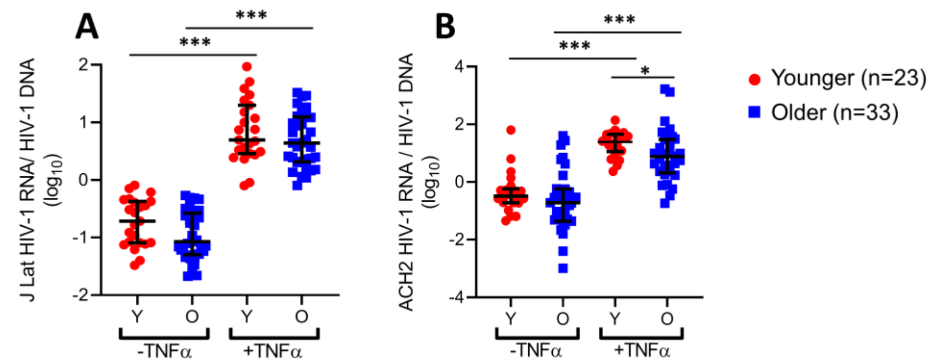

Olson et. al. Figure 3

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