JCI insight

Irreversible depletion of intestinal CD4+ T-cells is associated with T-cell activation during chronic HIV infection

Osaretin E. Asowata, … , Alasdair Leslie, Henrik N. Kløverpris

JCI Insight. 2021. https://doi.org/10.1172/jci.insight.146162.

Research In-Press Preview AIDS/HIV Gastroenterology

HIV infection in the human gastrointestinal (GI) tract is thought to be central to HIV progression, but knowledge of this interaction is primarily limited to cohorts within westernized countries. Here, we present a large cohort recruited from high HIV endemic areas in South Africa and found that people living with HIV (PLWH) presented at a younger age for investigation in the GI clinic. We identified severe CD4 T-cell depletion in the GI tract, which was greater in the small intestine than in the large intestine and not correlated with years on ART or plasma viremia. HIV-p24 staining showed persistent viral expression, particularly in the colon, despite full suppression of plasma viremia. Quantification of mucosal ARV drugs revealed no differences in drug peneration between the duodemum and colon. Plasma markers of gut barrier breakdown and immune activation were elevated irrespective of HIV, but peripheral T-cell activation was inversely correlated with loss of gut CD4 T-cells in PLWH alone. T-cell activation is a strong predictor of HIV progression and independent of plasma viral load, implying that the irreversible loss of GI CD4 T-cells is a key event in the HIV pathogensis of PLWH in South Africa, yet the underlying mechanisms remain unknown.

Find the latest version:

https://jci.me/146162/pdf

Irreversible depletion of intestinal CD4$^+$ T-cells is associated with T-cell activation during chronic HIV infection

Osaretin E. Asowata[1,2,*], Alveera Singh[1,*], Abigail Ngoepe[1], Nicholas Herbert[1], Rabiah Fardoos[1,3], Kavidha Reddy[1], Yenzekile Zungu[1], Faith Nene[1], Ntombifuthi Mthabela[1], Dirhona Ramjit[1], Farina Karim[1], Katya Govender[1], Thumbi Ndung'u[1,2,5,6], J. Zachary Porterfield[1,7], John Adamson[1], Fusi G. Madela[4], Vukani T. Manzini[4], Frank Anderson[4], Alasdair Leslie[1,2,5] and Henrik N. Kløverpris[1,2,3,5#]

[1]Africa Health Research Institute (AHRI), Durban, South Africa

[2]School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa

[3]Department of Immunology and Microbiology, University of Copenhagen, Denmark

[4]Division Upper Gastrointestinal Tract and Colorectal Surgery, Inkosi Albert Luthuli Central Hospital, University of KwaZulu-Natal, Durban, South Africa

[5]University College London, Division of Infection and Immunity, London, UK

[6]Max Planck Institute for Infection Biology, Berlin, Germany

[7]Department of Microbiology, Immunology, and Molecular Genetics, University of Kentucky, Lexignton, Kentucky, United States

The authors have declared that no conflict of interest exists

*Equal contribution

[#]Corresponding author

Henrik N. Kløverpris,

Africa Health Research Institute (AHRI)
Nelson R. Mandela School of Medicine
5th Floor, K-RITH Tower Building
719 Umbilo Road
Durban, 4083
Department of Immunology & Microbiology
University of Copenhagen
Blegdamsvej 3B
MAERSK Tower, 07.13
DK 2200 Copenhagen N
Denmark
Phone: +27 +27 (0)74 546 6625, +27 (0)31 260 4991, +45 29720910
henrik.kloverpris@ahri.org

Running title: CD4 T-cell depletion in the HIV infected GI tract

Abstract

HIV infection in the human gastrointestinal (GI) tract is thought to be central to HIV progression, but knowledge of this interaction is primarily limited to cohorts within westernized countries. Here, we present a large cohort recruited from high HIV endemic areas in South Africa and found that people living with HIV (PLWH) presented at a younger age for investigation in the GI clinic. We identified severe CD4 T-cell depletion in the GI tract, which was greater in the small intestine than in the large intestine and not correlated with years on ART or plasma viremia. HIV-p24 staining showed persistent viral expression, particularly in the colon, despite full suppression of plasma viremia. Quantification of mucosal ARV drugs revealed no differences in drug peneration between the duodemum and colon. Plasma markers of gut barrier breakdown and immune activation were elevated irrespective of HIV, but peripheral T-cell activation was inversely correlated with loss of gut CD4 T-cells in PLWH alone. T-cell activation is a strong predictor of HIV progression and independent of plasma viral load, implying that the irreversible loss of GI CD4 T-cells is a key event in the HIV pathogensis of PLWH in South Africa, yet the underlying mechanisms remain unknown.

Introduction

HIV-1 associated alterations within the gastrointestinal (GI) tract were first described in 1984 by Kotler and colleagues, who reported histologic abnormalities in the GI mucosa of infected patients (1, 2). HIV-1 infection and replication is far more prevalent in the CD4 T-cells of the GI mucosa (3–6) than in blood CD4 T-cells (5, 7–9), leading to rapid and severe loss of GI $CD4^+$ T-cells following infection. The resulting intestinal damage and microbial translocation (10) is thought to precipitate systemic inflammation and chronic immune activation, which ultimately drives disease progression (10). Crucially, these events are not fully reconstituted by antiretroviral treatment (ART) (11–15), and may have long-term health consequences for the growing number of PLWH on ART. This includes an elevated risk of non-infectious comorbidities (16, 17), such as cardiovascular disease, renal failure, liver disease, neurocognitive disease, osteoporosis, diabetes and gut-associated cancers (17–23). However, the direct link between HIV associated intestinal epithelial barrier break down, CD4 loss in the GI tract and HIV pathology has yet to be established, representing a major hindrance in the development of treatment strategies to restore gut homeostasis.

The intestine is the largest outward facing organ in the body and it contains more activated CD4 T-cells and tissue resident HIV reservoirs than any other human tissue site (24–26). Persistent viral replication within the gut HIV reservoir and high levels of HIV DNA in the gut $CD4^+$ memory T-cells is a major obstacle for HIV eradication (6, 27). ART initiation during early acute HIV-1 infection reduces immune activation and limits reservoir size, but does not appear to prevent its formation or lead to its eradication after prolonged

78 treatment (26, 28–30). The reasons for these are not clear, but may include sub-optimal
79 ARV drug penetration into the gut mucosa (14, 27, 31, 32). Indeed, recent data suggests
80 that some regions of the gut may have little or no exposure to antiretroviral drugs during
81 ART (33).
82
83 The majority of studies investigating HIV related gut immunopathology have been
84 conducted on Caucasian populations and/or carried out in westernized countries.
85 Intestinal health and immunity, however, is influenced by genetics, diet, microbiome and
86 comorbidities, and thus varies greatly between different populations. Therefore, the
87 immunopathology of HIV in the GI tract of these study populations may be distinct from
88 Sub-Saharan African populations, where 2/3rd of the global HIV population live. In
89 addition, for practical reasons, previous work has focused primarily on the large intestine.
90 As a result, the impact of HIV infection on the small intestine, where the immune
91 composition, physiology and microbiome are distinct, remains less understood (34–37).
92
93 Here, we present data collected from a large cohort of more than 500 study subjects
94 undergoing clinically indicated endoscopy or colonoscopy that were recruited from
95 extremely high HIV-1 endemic areas within KwaZulu-Natal, South Africa. The HIV
96 infected participants presented to the GI clinic at a younger age and gut pinch biopsies
97 obtained from their colon, rectum and duodenum revealed severe and irreversible HIV
98 associated CD4 T-cell depletion, in particular within the small intestine. This was
99 associated with persistent HIV expression, as shown by histological staining of HIV-p24
100 in gut CD4 T-cells. This was greatest in the colon, in which CD4 depletion was less

severe, suggesting target cell availability may affect reservoir size in the GI tract, in addition to the poor pentration of ART drugs observed. The degree of GI CD4 T-cell depletion is directly correlated with immune activation of blood CD4 T-cells only in PLWH, which strongly predicts HIV disease progression, independant of viral load (38). Plasma markers of systemic immune activiation, however, were elevated in all study participants and did not correlate with GI CD4 T-cell levels, suggesting these biomarkers are not directly linked to HIV pathology per se. This cohort represent a large and unique resource to further study intestinal health and HIV reservoir dynamics in PLWH from areas of rural Sub-Saharan Africa, which sits at the center of the global HIV epidemic.

Results

Diverse GI diagnoses and increased risk of biliary pathology in PLWH

To study the impact of HIV on the intestinal mucosa, we recruited 529 adult participants from KwaZulu-Natal, South Africa, presenting in the GI clinic for either endocscopy, including ERCP (small intestine) or colonoscopy (large intestine) procedures between the years 2015 and 2019. Participants were from area of high HIV endemicity, and 170 (32%) where infected with HIV (Table 1). Eighty four percent of HIV infected individuals had undetectable plasma HIV viral load due to antiretroviral therapy (ART); with HIV diagnosed for a median of 6.3 years of which 5.0 years were reported on ART and median CD4 counts of 647 cells/mm3. The 29 HIV infected individuals with detectable viremia (VL>20 RNA copies/ml plasma) were diagnosed for a median of 0.3 years with CD4 T-cell counts of 297 cells/mm3 and median viral load of 29,350 RNA copies/ml plasma (Table 1). Less than 3% presented with active Tuberculosis (TB) infection, but previous TB infection were enriched in HIV infected individuals (4% vs 24%, P<0.0001) (Supplemental Table 1) consistent with increased risk of TB infection in HIV infected individuals.

In this cohort, clinical diagnoses were dominated by non-infectious morbidities such as biliary pathology, gastrointestinal (GI) bleed, esophageal disorders and pain within the chest and abdomen (Supplemental Figure 1). The most common indications for colonoscopy were cancer, polyps, constipation/diarrhoea and ano-rectal disorders, while for upper endoscopies these were dominated by biliary, esophageal and abdominal complications. In 45 participants (9%) no obvious GI related diagnosis following

investigation were identified (Supplemental Figure 1). Stratifying participants based on HIV status, showed that females and black ethnicity were enriched in the HIV positive group, consistent with the demographic of the HIV epidemic in this region (Figure 1A). Overall, the GI complication of biliary pathology were enriched in the HIV positive group (OR 1.2, 25.3%, 39/154, P=0.02) by multivariate analysis (Figure 1A). Stratifying clinical diagnoses by ART status, revealed that biliary pathology was enriched in ART treated (OR 3.0, 95% CI 1.8–5.0, P<0.0001), but not in ART untreated individuals (OR 2.0, 95% CI 0.7–5.7, P=0.27) (Figure 1B), while both HIV plasma viral suppressed and viremic participants showed consistent enrichment for biliary pathology (both OR 2.5, 95% CI 1.4 to 4.3, P=0.0050) (Figure 1C). Taken together, these clinical diagnosis showed a diverse spectra of GI complications of which frequencies of inflammatory disorders, including inflammatory bowel disease, diverticulitis, prostatitis, ulcerative colitis and inflammation not otherwise specified, where overall less frequent to that observed in Caucasian cohorts (39) and highlights distinct GI related complications in this Sub-Saharan African cohort. However, with the exception of biliary pathology, clincal presentation of HIV infected and unifected subjects was similar and, therefore, unlikely to significantly skew the comparison of these groups.

Early presentation of GI related complications in HIV infected individuals

The HIV epidemic in South Africa is known to be discordant for ethnicity, age and gender (41–43). We therefore stratified our cohort by ethnicity and found a skewed distribution of non-black ethnicity compared to the background provincial data, which is likely to be at least in part explained by differential access to tertiary healthcare units, in addition to

potential differences in genetic and environmental factors (Figure 2A, Supplemental Table 1). Overall, HIV prevalence was higher in women, reaching >60% infection in the 25-44 year age group (Figure 2B), which is consistent with recent population level studies from the same region (40, 41). Strikingly, while females and males presented to the clinic for GI complications at a similar age, HIV infected participants presented a median of 12 years earlier than HIV-1 uninfected participants (41 years, IQR, 34-52 vs. 53 years, IQR, 40-63 years; $P<0.0001$) (Figure 2C) irrespective of ART treatment status, with females and males presenting 13 and 6.5 years younger, respectively. We found no age differences within the three indicated procedures (colonoscopy, upper endoscopy and ERCP), but in each procedure, HIV infection was associated with an overall younger population (Figure 2D).

In a subset of HIV infected participants full blood parameters where available, including white and red blood cell analysis. Comparsion with HIV uninfected controls, this revealed reduced CD4 T-cell (median 858 vs 641 vs 297 cells/ul) and elevated CD8 T-cells (median 610 vs 670 vs 1040 cells/ul) counts in both viral suppressed and viremic groups compared to HIV uninfected participants (Suppl Figure 2). In addition, HIV infection was associated with elevated neutrophils and basophils, and with increased neutrophil to lymphocyte ratio (NLR), suggesting overall increased inflammation irrespective of viral suppression (Supplemental Figure 2A). Analysis of platelets and red cell parameters showed significant changes including reduced red cell counts with skewed width distribution and volume, and reduced mass and hemoglobulin levels (Supplemental Figure 2B). These data are consistent with HIV-1 infection leading to GI pathology in Sub-Saharan African

cohorts (40, 41) and suggests that GI complications may arrive earlier in HIV infected individuals with reduced CD4 T-cell counts and overall increased systemic inflammation.

Irreversible depletion of duodenal CD4$^+$ T-cells despite long-term ART

Next, to investigate alterations in CD4$^+$ and CD8$^+$ T-cell homeostasis within the small and large intestine during HIV-1 infection, we quantified T-cells from fresh duodenum, colon and rectum biopsies in 228 study participants (Figure 3A,B). The CD4:CD8 ratios in the GI tract of HIV uninfected individuals were overall lower than those found in matched uninfected blood samples, and showed compartment specific differences between the small (duodenum) and large (colon/rectum) intestine ($P<0.01$), but similar ratios between colon and rectum (Figure 3C) (42). HIV-1 infection reduced CD4:CD8 T-cell ratios within the duodenum, colon and blood that was not restored by full ART mediated viral suppression in plasma (Figure 3D). Consistent with altered CD4:CD8 ratios, the frequency of CD4$^+$ T-cells, as % of CD45$^+$ cells, was significantly reduced in the duodenum, the colon/rectum and blood of viremic participants compared to HIV uninfected participants ($P<0.001$) (Figure 3E). The CD4 depletion remained highly significant in the duodenum of virally suppressed participants ($P<0.001$), but was less pronounced in the colon and no longer reached statistical significance ($P=0.1$). The blood compartment overall showed consistent CD4 depletion (see Supplemental Figure 2A), although the median change was lower than that observed in the colon for viral suppressed individuals (Figure 3F). Duodenal CD4 T-cell depletion, irrespective of plasma viral suppression, remained significant when we controlled for age, gender and ethnicity with overall less impact in the large intestine (Figure 3G). Direct comparison of

CD4 T-cell levels in blood and duodenum/colon combined, showed significant positive correlation in HIV infected ($ART^{+/-}$) participants (r=0.39; P=0.0015), but not among HIV negative participants (r=0.10; P=0.4) (Figure 4A). In HIV viremic individuals, we found a strong negative correlation between plasma VL and blood CD4 levels (r=0.57; P=0.02), but not between plasma viral loads and gut CD4 levels (r=0.002; P=0.99) (Figure 4B). To explore disconnect between plasma viremia and GI CD4 T-cells further, we used available treatment initiation data from the GI clinic and found no overall correlation between gut CD4 T-cell levels and ART duration (r=0.05; P=0.7) (Figure 4C). Breaking this down by compartment, we observed a failure to reconstitute CD4 T-cells in the duodenum even after 10 years of ART (2.5 fold reduction vs HIV unifected, P=0.04; Figure 4C). The same trend was not observed in the colon, which might indicate improved reconstitution in this compartment, although fewer samples were available for this analysis. Blood CD4 T-cell levels showed a trend towards CD4 restoration (r=0.24; P=0.13) with only a 1.3 fold reduction (P=0.2) in participants receiving ART more than 10 years (Figure 4D), which is consistent with a 1.1 reduction in absolute CD4 T-cell counts (HIV^- vs HIV^+ VL<20 ART>10years, median 858 vs 795 cells/mm^3, P=0.99). Taken together, CD4 T-cell frequencies within the small and large intestine from >200 GI patients show that long-term ART fails to restore CD4 T-cell levels in the small intestine despite long term and fully suppressive ART.

Compartment specific HIV-1-p24 detection and CD4 depletion in the gut mucosa of ART treated individuals

Studies of non-SubSaharan African cohorts show that CD4 T-cells within the large intestinal mucosa are a major site for viral infection and replication even during plasma viral suppression (4, 43). We first quantified CD4 expressing cells in colon, after exclusion of GALT-like structures (Figure 5A), and duodenum tissue sections (Figure 5B). We found severe depletion of duodenal CD4 cells in both virally suppressed ($P<0.05$) and viremic ($P=0.0001$) participants, corresponding to 4- and 10-fold reduction, compared to HIV uninfected GI participant controls, respectively (Figure 5C), but no significant CD4 depletion in the colon. This is consistent with our findings from flow cytometric analysis of these compartments with severe CD4 T-cell depletion in particular observed within the duodenum (see Figure 3 above).

Next, we tested HIV-1 persistence in the gut by co-staining tissue sections for HIV-p24 and CD4 (Figure 5D,E) and (Supplemental Figure 3A). We found HIV-p24 protein in both the duodenum and colon tissues of both viral suppressed and viremic individuals that co-localised with CD4 expression and most often within the lamina propria (Figure 5D,E). We detected more HIV-p24 protein in the colon of both plasma viral suppressed and viremic individuals compared to that of the duodenum compartment, suggesting that compartment specifc differences exist in the gut for HIV detection (Figure 5D). We confirmed HIV detection by digital droplet PCR and found a trend towards more HIV DNA in the duodenum and colon compared to blood, despite low overall CD4 frequencies in the gut compared to the blood compartment (Supplemental Figure 3B). To examine the relative contribution of CD4 expressing macrophages to the overall HIV-p24 expressing cells, we included CD68 staining and found relatively high frequencies of CD4 and CD68

co-expressing cells and with more HIV-p24^{+}CD68^{+} cells co-expressing CD4 in the colon compartment compared to the duodenum, suggesting that macrophages also may contribute to overall increased level of HIV-p24 in the colon, at least in the plasma viral suppressed participants (Supplemental Figure 3C-E). Thus, we found higher HIV-p24 expression in the colon and lower target cell availability in the duodenum in both suppressed and viremic participants, suggesting that compartment specific effects may contribute to the dynamics of the HIV reservoir in the gut.

Poor ARV mucosal drug penetration levels in the GI tract does not explain compartment specific differences in PLWH.

Next we tested if the mucosal HIV-p24 protein detection and CD4 T-cell depletion in the presence of plasma viral suppression could be linked to poor ARV penetration into the GI tissue. For this, we established a liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) approach to accurately quantify the concentration of the first line ART regimen drugs used at the time of study (44); Tenofovir (TDF), Emtricitabine (FTC), Efavirenz (EFC), in fresh mucosal tissue and matched plasma (Figure 6A). Using this methodology, we observed significantly lower concentrations of FTC ($P<0.0001$) and EFV ($P<0.0001$), but not TDF, in intestinal tissue compared to paired plasma, when normalizing by tissue mass (see methods) (Figure 6B). In participants with detectable plasma ARV, FTC was only detected in 45% (5/11) tissue samples, compared to 86% (12/14) and 93% (14/15) for TDF and EFV, respectively (Figure 6B). Importantly, however, levels of all three drugs were not different between the duodenun and colon compartments (Figure 6C). Moreover, in a subset of participants for whom % CD4^{+} T-cell

frequencies and drug levels were available, we consistently found CD4 T-cell depletion in both blood and tissue (Figure 6D) despite detection of all three ARVs in both compartments (Figure 6E).

Although, it is not clear from these data whether the reduced levels of FTC and EFV directly contribute to persistent viral replication or CD4 depletion in the GI tract, they do indicate that the differences in CD4 T-cell depletion and HIV-p24 detection between the duodenum and colon are not a consequence of differential ARV drug penetration into these two distinct gut compartments. These data suggest that penetration of some ARV drugs into the gut mucosa is reduced, but that this alone does not explain the lack of CD4 T-cell reconstitution observed within the small intestine.

Persistent systemic T-cell activation correlates with gut CD4 T-cell depletion only in PLWH

Several studies report that both persistent T-cell activation and systemic inflammation, despite ART viral suppression, is linked to increased risk of morbidity and mortality in PLWH (43, 45–49). Therefore, to test the association between GI CD4 T-cell depletion and activation and systemic T-cell activation in this GI cohort, we measured HLA-DR expression as a surrogate marker for T-cell activation by flow cytometry (Figure 7A). As all GI participants are undergoing medical examination (see Figure 1), we compared HLA-DR expression in the blood of HIV^- participants from the GI cohort to that of healthy non-GI HIV^- donors from an unrelated control cohort (42) and found no significant differences (Figure 7A). By contrast, HLA-DR expression was upregulated on blood CD4 and CD8 T-cell subsets in both HIV viral suppressed and viremic participants, confirming HIV driven

T-cell activation in these individuals (Figure 7B). Next we measured HLA-DR expression on GI CD4 T-cells (Suppl Figure 4) and found overall higher steady state expression compared to the blood compartment (P<0.001) (Figure 7C), which is expected, given the persistent exposure of GI T-cells to the gut microbiome. Surprisingly, we did not see significantly increased HLA-DR expression on duodenum or colon CD4 T-cells in the viral suppressed individuals, and activation was actually lower in the viremic group, reaching significance in the colon (P=0.01) (Figure 7D).

Next we tested the relationship between blood T-cell activation and CD4 T-cell frequency, and found a direct inverse correlation in the blood of HIV infected individuals with CD4 T-cell activation (r=-0.61; P=0.001), that was not observed in the HIV uninfected group (r=-0.01; P=0.9), or for CD8 T-cell activation in either group (Figure 7E). Next, we correlated HLA-DR expression on blood CD4 and CD8 T-cells to mucosal CD4 T-cell frequency and consistently found a strong inverse correlation in HIV infected individuals with activation of both CD4 T-cells (r=-0.51; P=0.03) and CD8 T-cells in the blood (r=-0.61; P=0.06)(Figure 6F). Again, this trend was not observed in HIV uninfected participants (r=-0.05; P=0.9 and r=0.2; P=0.5). Therefore, these data suggest a potential mechanistic link between HIV mediated CD4 T-cell depletion in the GI tract and systemic CD4 and CD8 T-cell activavtion, which is a strong predictor of HIV disease progression independent of viral load (51).

Elevated intestinal and inflammatory markers are not specific to HIV GI pathology and do not correlate with HIV associated depletion of gut CD4 T-cells

315 Several previous studies have investigated the association between HIV infection, loss of
316 gut barrier integrity and immune activation using plasma markers of innate immune
317 activation, soluble CD14 (sCD14), and of elevated gut epithelial turnover, intestinal fatty
318 acid-binding protein (I-FABP) (49–54). We measured these markers in our GI study
319 participants and additional samples from an unrelated HIV uninfected healthy participants
320 not undergoing GI investigation (Figure 8). Compared to these non-GI controls, we found
321 elevated sCD14 ($P<0.0001$) and I-FABP ($P<0.0001$) levels in all GI participants, and
322 detected no significant differences between HIV infected and uninfected individuals in the
323 GI cohort (Figure 8A). To confirm this, we measured additional plasma markers of
324 inflammation (IL-6, TNF-a), macrophage activation (sCD163), a further marker of
325 intestinal barrier break down Zonulin, and the anti-inflammatory souble IL-33 receptor
326 sST2, (Supplemental Figure 5); and found no significant differences between HIV infected
327 and uninfected individuals from the GI cohort.
328
329 Taking all individuals togther, we observed a positive correlation between sCD14 and I-
330 FABP, consistent with a mechanistic link between elevated gut epithelial turn over and
331 systemic innate immune activation in this cohort of subjects with GI complications ($r=0.4$;
332 $P=0.002$) (Figure 8B). However, there was no correlation between gut CD4 T-cell
333 frequency and matched plasma I-FABP or sCD14 levels, and no correlation with
334 activation of blood CD4 T-cells (Figure 8C). When individuals were grouped by HIV
335 status, we also found no correlation between these plasma markers and GI CD4 T-cell
336 frequency or blood CD4 T-cell activation in individuals with suppressed viremia (Table 2).
337 There was a significant negative association between I-FABP and GI CD4 T-cell

frequency, and a non-significant positive association with blood T-cell activation in the small number of viremic subjects tested.

Taken together, these data suggest that gut barrier break down and systemic innate immune activation are likely to be general markers of GI distress, are not unique to HIV infected individuals and are unlikely to occur as a direct consequence of GI CD4 T-cell depletion. Importantly, they also imply that systemic T-cell activation does not occur as a direct result of GI barrier breakdown, at least in virally suppressed individuals.

Discussion

This study presents clinical and immunological changes associated with HIV infection in a large cohort of individuals attending a tertiary GI clinic for investigation. We found an overall HIV prevalence of 32%, that reached >60% in younger women; with 83% of all PLWH being on fully suppressive ART. Flow cytometric analysis of gut biopsies from more than 200 GI participants revealed severe depletion of CD4 T-cells associated with HIV, in particular within the small intestine, compared to colon and blood compartments. This was most severe in the small intestine and was not reversed by full plasma viral suppression and long-term ART treatment. Greater CD4 T-cell depletion within the small intestine compared to the colon of PLHW was confirmed *in situ*, by flourescence microscopy. Interestingly, higher CD4 T-cell levels within the large intestine in particpiants under full plasma viral suppression, was accompanied by more abundant HIV infected CD4 T-cell and CD68 macrophages, as shown by HIV-p24 protein detection. This implies that target cell availability may play an important role in governing the HIV viral reservoir in the GI tract. Importantly, although the level of both EFV and FTC appeared to lower in the gut than the plasma, we found no evidence of discordant ARV drug penetrations between the duodenum and colon, suggesting that ARV levels does not explain these differential effects. CD4 T-cell frequency in the gut of PLWH was inversely correlated with activation of circulating T-cells, a biomarker which correlates strongly with HIV disease progression (55). This was not observed in HIV uninfected indivduals, despite elevated levels of plasma markers of innate immune activation and increased gut barrier permeability, such as sCD14 or I-FABP, respectively. Previous studies examining HIV associated gut pathology have observed elevated levels of these and other plasma

368 markers in relation to HIV negative healthy controls (49, 52, 56, 57). Uniquely, as these
369 data are generated from individuals all attending a clinic for intestinal complications, this
370 demonstrates that such markers are elevated in individuals with GI complications in
371 general, and not just those with HIV infection. It has been argued that the observation of
372 increased gut permeability with immune activation in PLWH suggests a mechanistic link
373 between HIV infection, increased gut permeability and the persistent immune activation
374 that drives HIV progression (54, 57–59). Here we show, however, that systemic immune
375 activation of both CD4 and CD8 T-cell, at least, is not linked to gut permeability per se,
376 but rather to depletion of GI CD4 T-cells. However, we cannot rule out that sustained GI
377 tract damage and microbial translocation may also contribute to persistent systemic
378 immune activation. To our knowledge, this is the largest study of GI participants recruited
379 within a high HIV prevalence area in Sub-Saharan Africa and unequivocally identifies the
380 lack of immune reconstitution throughout the intestinal mucosa as a persistent problem
381 in PLWH despite long-term ART.
382
383 Although this study was not powered to determine differences in clinical presentation in
384 PLWH, we observed a significant increase in biliary pathologies in this group. Although
385 further work would be needed to explore this further, it is in line with previous studies (60)
386 and suggest that HIV infection precipitates cholangiopathy (61, 62) despite plasma viral
387 suppression in most individuals. In addition, we detected a low frequency (<3%) of
388 inflammatory disorders, including bowel diseases, compared to non-African cohorts
389 undergoing clinically indicated GI investigation (39). This underline the potential
390 differences between these and Sub-Saharan African GI cohorts, and demonstrates the

391 importance to study HIV gut pathology in relevant populations for better representation of
392 the HIV epidemic.
393
394 Although ART has successfully decreased HIV associated morbidity and mortality (63,
395 64), we found that virally suppressed HIV infected participants in our cohort presented a
396 median of 12 years earlier to the GI clinic than HIV uninfected attendees, predominantly
397 with non-infectious comorbidities. This early GI pathology associated with HIV could imply
398 premature immune ageing in this group, irrespective of plasma viral suppression (15) that
399 precipitates GI complications. However, we cannot rule out that HIV infected participants
400 are better linked to care than uninfected participants, which can result in earlier clinical
401 investigation.
402
403 The severe CD4 T-cell depletion observed throughout the large and small intestine is
404 consistent with early observations in Caucasian cohorts (3, 4) and further underpins the
405 lack of immune reconstitution in the gut (65). We observed a more pronounced CD4 T-
406 cell depletion in the duodenum compared to the colon. This could be due to an increased
407 abundance of of lymph node structures, such as peyers patches, cryptopatches or
408 isolated lymphoid follicles (ILF) in the lamina propria of the large intestine and requires
409 further validation, ideally in colon and duodenum samples from the same participants.
410 Strikingly, we observed absolutely no correlation between the duration of ART and GI
411 CD4 T-cells, suggesting that these cells are either continually being depleted, or that the
412 CD4 niche in this tissue has been irreparably damaged and cannot be repopulated.
413 Recent data show that intestinal lamina propria CD4 T-cells are essential for regulating

intestinal stem cells and epithelial cell differentiation in the crypt base (66, 67). The blood compartment showed a trend towards CD4 reconstitution, which may indicate late ART initiation in the majority of HIV infected GI participants (68) and further emphasize that intestinal CD4 levels, in particular within the small intestine, do not recover despite long-term ART (65) in contrast to treatment initiation during early acute HIV infection (69).

The observation of more pronounced CD4 T-cell depletion in the duodenum compared to the colon was shown by both flow cytometry and histology. This is a potentially important observation, as, for practical reasons, many studies on the impact of HIV on the GI tract have limited their study to the colon. As CD4 loss was less pronounced in this compartment and appears to be somewhat better restored by ART, limiting obseravations to the colon may miss key aspects of persistent GI pathology in PLWH. It is important to note, however, that we were unable to obtain paired duodenum and colon samples from the sample individuals. It is therefore possible that compartment specific difference may in part be explained by differences in the HIV patients presenting to the clinic for either upper or lower GI tract endoscopy.

Although the life expectancy of people living with HIV has been prolonged by the improvement in ART over the last few years (70), the gut has been implicated as a potential reservoir for ongoing HIV-1 replication in the setting of ART suppressed viremia (26). We tested if discordant ARV levels between duodenum and colon mucosal tissue, with plasma matched controls, could explain the difference in CD4 T-cell depletion. We found no evidence of compartment specific ARV levels, but that duodenal CD4 T-cell

depletion occurs in confirmed ARV detected mucosal tissue, although based on a few individuals available. Thus, reduced ARV levels in mucosal tissue compared to plasma levels (14), does not necessarily explain the lack of CD4 T-cell reconstitution in this cohort. One caveat remains that comparing ARV levels in plasma to homogenized gut tissue cells may be suboptimal instead of using tissue matched PBMCs and normalized by cell numbers. However, previous studies have showed a correlation between plasma and PBMC ARV levels (71–76). Whether ongoing viral replication could be linked to reduction or absence of ARVs in gut mucosal tissue (33) remains to be tested in this cohort. Finally, HIV-p24 detection in both the small and large intestine, despite full viral suppression, may also be linked to ARV drug pharmacokinetics (77, 78). In plasma samples, we found evidence of treatment failure in which 3 ARVs were detected in 40% of viremic patients and highlights the vulnerability of this population where ART adherence over long period of time is challenging (79) and also could accelerate GI complications in this region (80).

Our GI cohort underpins the central role of the intestine in HIV-1 pathology and demonstrates poor restoration of gut mucosal immunity, in particular within the small intestine, by otherwise effective ART. This ongoing GI study cohort is a unique opportunity to further reveal the unknown mechanisms of GI impairement that are central to both HIV pathogenesis and its associated comorbidities in the growing population of individuals receiving ART in Sub-Saharan Africa, a distinct and critical population that is under studied. Future elected mucosal tissue sample collection from matched large and small intestinal tissue sites, combined with better characterized participants with available long-

460 term clinical information from this region, will be a key resource for more in depth
461 understanding of the mechanisms underlying HIV pathology in the GI tract.

Methods

Study participants and sample collection

Patients presenting to the gastrointestinal (GI) surgical unit of Inkosi Albert Luthuli Central Hospital (IALCH) were recruited into this study after obtaining written informed consent. IALCH is an 846-bed central and tertiary hospital in the eThekwini district of KwaZulu-Natal (KZN) Province in South Africa. It provides regional and tertiary services to KZN as well as for referrals from the neighbouring Eastern Cape Province. Duodenum, colon and rectum pinch biopsies with participants-matched blood samples were obtained during endoscopy, endoscopic retrograde cholangiopancreatography (ERCP) and colonoscopy procedures. Blood samples were also obtained from a healthy HIV uninfected volunteer study cohort (42). Clinical information, including HIV status and demographic details of these participants, was collected using a structured questionnaire. HIV status was confirmed using the COBAS® TaqMan® HIV-1 Test (Roche). Full blood count and viral load were done using the Sysmex XE-5000™ Automated Hematology Analyzer and NUCLISENS EASYQ® HIV-1 (BioMerieux), respectively.

Mononuclear cells isolated from blood and gut

Blood was collected in BD vacutainers with sodium heparin (Becton Dickinson). Peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll-Histopaque 1077 (Sigma-Aldrich) density gradient centrifugation. Gut biopsies (2 to 4 pinches) were removed by the operating GI surgeon and transported to the laboratory in cold Phosphate-Buffered Saline (PBS) (pH 7.2). The PBS was decanted from the tubes containing the gut biopsies, which are about 5-8 mm in size, and they were incubated in

epithelial strip buffer (PBS; 0.5M EDTA; 1M DTT; FBS and Penicillin/Streptomycin) in a 37ºC water bath for 10 minutes, with occasional agitation. Thereafter, the epithelial strip buffer was removed, and the tissues were digested in a buffer containing Collagenase-D (0.5 mg/ml; Roche) and DNase-I (20 µg/ml; Sigma-Aldrich) for 30 minutes in a 37ºC water bath with occasional agitation. Digested tissue was passed through a 70µM cell strainer to isolate the cells, and these cells were washed with PBS.

Flow cytometry

PBMC and gut mononuclear cells were stained with antibody mixtures for a minimum of 20 minutes at room temperature. Cells were washed twice with PBS and acquired immediately or fixed in 2% paraformaldehyde for later acquisition within 24 hours. The BD FACSAria Fusion flow cytometer was used for acquisition of sample data. A minimum of 100,000 total events was collected for each sample, and data analysis was done using FlowJo software (version 9.7.2 or higher, TreeStar). The antibody mixture consists of Live/Dead Fixable Near-IR Cell Marker (Invitrogen, Thermo Fisher Scientific) and an optimized antibody cocktail for the different phenotypic staining that included the following antibodies anti-CD45 (BD Biosciences 560777) anti-CD3 (BioLegend 317330) anti-CD4 (BD Bioscience 564651), anti-CD8 (BD Bioscience 563795), anti-HLA-DR (Biolegend 307606).

Measurement of drug levels in gut and plasma

The concentration of ARV drugs was simultaneously quantified in plasma and gut tissue samples using liquid chromatography coupled to tandem mass spectrometry (LC-

MS/MS). The tissue samples were homogenized using a TH Omni international homogenizer/soft tissue probe combination (Omni-International, Georgia, USA) with a short pulse (15 sec) in 500 µl of cold acetonitrile: water (1:1, v/v) solution, then processed for analysis. The drug concentration data were normalized to the average mass of the tissue sample set and used a tissue density of 1.06 grams/mL to relate tissue weight to tissue volume as previously described (75). The ARV drug analytes were extracted from calibration standards (STDs), quality control (QCs) samples, and study samples using protein precipitation followed by LC-MS/MS analysis. Human plasma was used as a surrogate biological matrix for the preparation of quantitative STDs and QCs, due to the limited availability of control (healthy donor/drug-free) gut tissue sample. Matrix effect studies to compare drug quantitation between spiked plasma and homogenized gut tissue samples were performed and showed equivalency with less than 15% variability in analyte peak areas.

The extracted analytes were chromatographically separated on an Agilent Zorbax Eclipse Plus C18 (2.1 x 50mm, 3.5 µm) HPLC column, at 40°C, using gradient elution with a combination of mobile phase A, which consisted of water with 0.1% formic acid and B, acetonitrile with 0.1% formic acid. A sample volume of 20 µl was injected, the flow rate was set to 0.2 ml/minute and the total run time was 20 minutes. All ARV drug analytes were analysed in the positive ionization mode, using 6-Aminonicotinic acid (6-ANA) as an internal standard except for efavirenz which was analysed in the negative ionization mode using deuterated efavirenz (EFV-d5) as an internal standard. Data were acquired using selected reaction monitoring (SRM) and processed using AB Sciex Analyst Software, version 1.6.2, on an AB Sciex Triple Quad™ 5500 mass spectrometer.

Histology and fluorescent immunohistochemistry

Multiplex fluorescent immunohistochemistry staining was performed using the Opal™ 4-Color Manual IHC Kit 50 Slides (PerkinElmer, USA) as directed by the manufacturers. Duodenum and colon tissue samples fixed in 4% formalin between 2 days and a month were paraffin-embedded. 4μm sections were cut on glass slides and the slides were baked at 60ºC overnight. Then, the combined process of deparaffinization, rehydration and antigen retrieval of the tissue sections was done using the PT-Link Pre-Treatment instrument (Dako) and 1x Envision Target Retrieval Solution, High PH (Dako). Then, slides were incubated for 1 minute in distilled water and equilibrated in 1x EnVision FLEX Wash Buffer (Dako) for 5 minutes at room temperature. Then, the slides were incubated in Peroxidase blocking solution (PerkinElmer) for 10 minutes and washed in 1x wash buffer (Dako) immediately at room temperature. The slides were then incubated in Bloxall blocking solution (PerkinElmer) for 10 minutes, and then in primary antibody-1 for 30 minutes at room temperature. Slides were then washed for 5 minutes in 1x wash buffer and incubated in Secondary Opal Polymer Horseradish Peroxidase (HRP) Mouse and Rabbit (PerkinElmer) for 30 minutes. The Opal Polymer HRP is recommended for human tissues with a mouse or rabbit primary antibodies. Then, the slides were washed twice in 1x wash buffer, drained and the sections were incubated in Opal Fluorophore (PerkinElmer) diluted 1:100 in amplification diluent (PerkinElmer) working solution for signal amplification at room temperature for 10 minutes. The slides were then washed for 5 minutes in 1x wash buffer at room temperature. Afterwards, antibody stripping via microwave treatment was done by placing the slides in a slide jar with pre-warmed buffer

1x AR6 (PerkinElmer). The jar was loosely covered and placed in a microwave for 2 minutes at 100% power, 10 minutes at 50% power and 5 minutes at 20% power. Slides were allowed to cool in the dark by placing the slide jar on ice for 20 minutes and the slides were rinsed in distilled water, followed by incubation in the 1x wash buffer for 5 minutes to equilibrate. The microwave step strips the primary-secondary-HRP complex and allows the introduction of the next primary antibody. For the detection of the next target (primary antibody-2), the protocol was restarted at the blocking step using Bloxall blocking solution (PerkinElmer) for 10 minutes. After the third target was detected (primary antibody-3), a working solution of DAPI (PerkinElmer) was applied to the sections as the nuclear counterstain for 5 minutes. The slides were washed in 1x wash buffer for 5 minutes, then in distilled water for 5 minutes and drained. Then, the sections were coverslip with Fluorescence Mounting Medium (catalog number S302380-2; Agilent Technologies) and the edges of the coverslip were sealed with nail polish. Slides were stored in a humidity chamber at 2^{o}C - 8^{o}C until images are acquired.

The unlabelled primary antibodies used in this study were anti-HIV p24 (clone: Kal-1, Dako) used as the first antibody in the staining cycle, diluted 1:10 in antibody diluent (PerkinElmer), then anti-CD4 (clone: 4B12, Dako) which was the second antibody in the staining cycle and it was pre-mixed, followed by anti-CD68 (clone: KP1, Dako) as the third antibody in the cycle, diluted 1:200 in antibody diluent (PerkinElmer). The fluorophores were FITC (product number FP1487001KT; PerkinElmer) for anti-HIV p24 (clone: Kal-1, Dako), Texas-Red (product number FP1488001KT; PerkinElmer) for anti-CD4 (clone: 4B12, Dako) and Cy5 (product number FP1497001KT; PerkinElmer) for anti-CD68

(clone: KP1, Dako) signal generation. These fluorophores were diluted 1:100 in amplification diluent. Images of the tissue sections were acquired using the TissueFAXS software (TissueGnostics) connected with a Zeiss Axio Observer Z1 inverted microscope (Olympus). The quantitative analysis of the cells of the different phenotypes within the images was done using the TissueQuest quantitation software (TissueGnostics). Isotype and non-antibody controls were included in the experiment to rule out non-specific staining and autofluorescence.

Measurement of blood and gut HIV-1 DNA using digital droplet PCR (ddPCR)

Total PBMC, and duodenum and colon tissue mononuclear cells from study participant were subjected to DNA extraction using DNeasy Blood & Tissue Kits (QIAGEN). Total HIV-1 DNA and host cell concentrations in the DNA extracts were estimated using BIO-RAD ddPCR, using primers and probes covering HIV-1 5′ LTR-gag HXB2 coordinates 684–810 (81) (forward primer 5′-TCTCGACGCAGGACTCG-3′, reverse primer 5′-TACTGACGCTCTCGCACC-3′ probe/56-FAM/CTCTCTCCT/ZEN/TCTAGCCTC/31ABkFQ/, and human RPP30 gene (82) forward primer 5′-GATTTGGACCTGCGAGCG-3′, reverse primer 5′-GCGGCTGTCTCCACAAGT-3′, probe/56-FAM/CTGACCTGA/ZEN/AGGCTCT/31ABkFQ/). ddPCR was performed using the following thermocycler program: 95 °C for 10 minutes, 45 cycles of 94 °C for 30 seconds and 60 °C for 1 minutes, 72 °C for 1 min. The droplets were subsequently read by the

598 BIO-RAD QX100 droplet reader and data were analyzed using QuantaSoft software
599 (BIO-RAD).

600

601 **Markers of microbial translocation and inflammation**

602 The concentration of some of the plasma markers was measured by ELISA using Human
603 soluble CD14 DuoSet ELISA (R&D Systems, catalogue number: DY383), Human
604 FABP2/I-FABP DuoSet ELISA (R&D Systems, catalogue number: DY3078) and Human
605 Zonulin/Haptoglobin ELISA kit (ARP American Research Products, catalogue number:
606 E01Z0004) per manufacturer's instructions. Also, Milliplex multiplex assays using
607 Luminex was used to determine the concentration of soluble ST2 and CD163 using the
608 Human Cardiovascular Disease Magnetic bead panel 5 (Milliplex, catalogue number:
609 HCVD5MAG-67K) per manufacturer's instructions. The concentration of IL-6 and TNF-α
610 was determined using the Human TH17 magnetic Bead Panel (Milliplex, catalogue
611 number: HTH17MAG-14K) per manufacturer's instructions.

612

613 **Statistics**

614 Descriptive and categorical data were represented as medians with interquartile ranges,
615 minimum and maximum values and differences were analyzed using the Mann-Whitney
616 U-test. The Kruskal-Wallis test was performed for multiple comparisons between HIV
617 uninfected and each of the HIV-1 infected groups. Bivariate correlations were determined
618 by the Spearman rank correlation test. A multivariate logistic regression model was used
619 to estimate the association between HIV-1 and GI comorbidities at a 95% confidence

620 interval (CI). Statistical analyses were performed using GraphPad Prism (version 8) and
621 STATA (version 15) software, and p-values <0.05 were considered statistically significant.

622 **Study Approval**

623 This study was approved by the Biomedical Research Ethics Committee (BREC) of the
624 University of KwaZulu-Natal (REF: BE 021/13) following its ethical standards and with the
625 1964 Helsinki declaration and its later amendments.
626

627 **Author contributions**

628 OA and AS performed experiments. FN and MN recruited study participants. FN and NM
629 consented participants in the GI ward. DR, FK coordinated human specimens. OA, AS,
630 AN, RF, YZ, KG, NH and KR contributed to experimental work. FM, VTM, FA contributed
631 surgical human tissues samples. NM, FN, FK and TN contributed sample collection. HK,
632 OA, AL prepared the manuscript. TN, ZP, JA, AL provided intellectural input. HK
633 conceptualized and provided funding.
634

635 **Acknowledgments**

636 HNK is supported by The Wellcome Trust (202485/Z/16/Z). AL is supported by the
637 Wellcome Trust (210662/Z/18/Z). This work was supported through the Sub-Saharan
638 African Network for TB/HIV Research Excellence (SANTHE), a DELTAS Africa Initiative
639 [grant # DEL-15-006]. The DELTAS Africa Initiative is an independent funding scheme of
640 the African Academy of Sciences (AAS)'s Alliance for Accelerating Excellence in Science
641 in Africa (AESA) and supported by the New Partnership for Africa's Development

642 Planning and Coordinating Agency (NEPAD Agency) with funding from the Wellcome
643 Trust [grant # 107752/Z/15/Z] and the UK government. The views expressed in this
644 publication are those of the author(s) and not necessarily those of AAS, NEPAD Agency,
645 Wellcome Trust or the UK government. HK and AS was supported by SANTHE.

Table 1. HIV diagnosis and treatment duration in GI cohort (n=529)

	HIV^+ all, n=170/529 (32%)	$HIV^+ART^+VL<20$, n=101/120 (84%)	HIV^+ART^-, n=23/160 (14%)
Time HIV diagnosed, years (IQR)	6 (1.5-10.1)	6.3 (1.6-10.1)	0.3 (0.2-1.1)
Time on ART, years (IQR)	5.5 (1.7-8.0)	5.0 (1.6-7.7)	NA
Time ART naïve, years (IQR)	NA	2.1 (0.4-7.7)	NA
CD4 count cells/mm^2 (IQR)	629 (380-810)	647 (465-813)	297 (155-363)
HIV RNA copies/ml plasma (IQR)	NA	NA	29,350 (205-147,500)

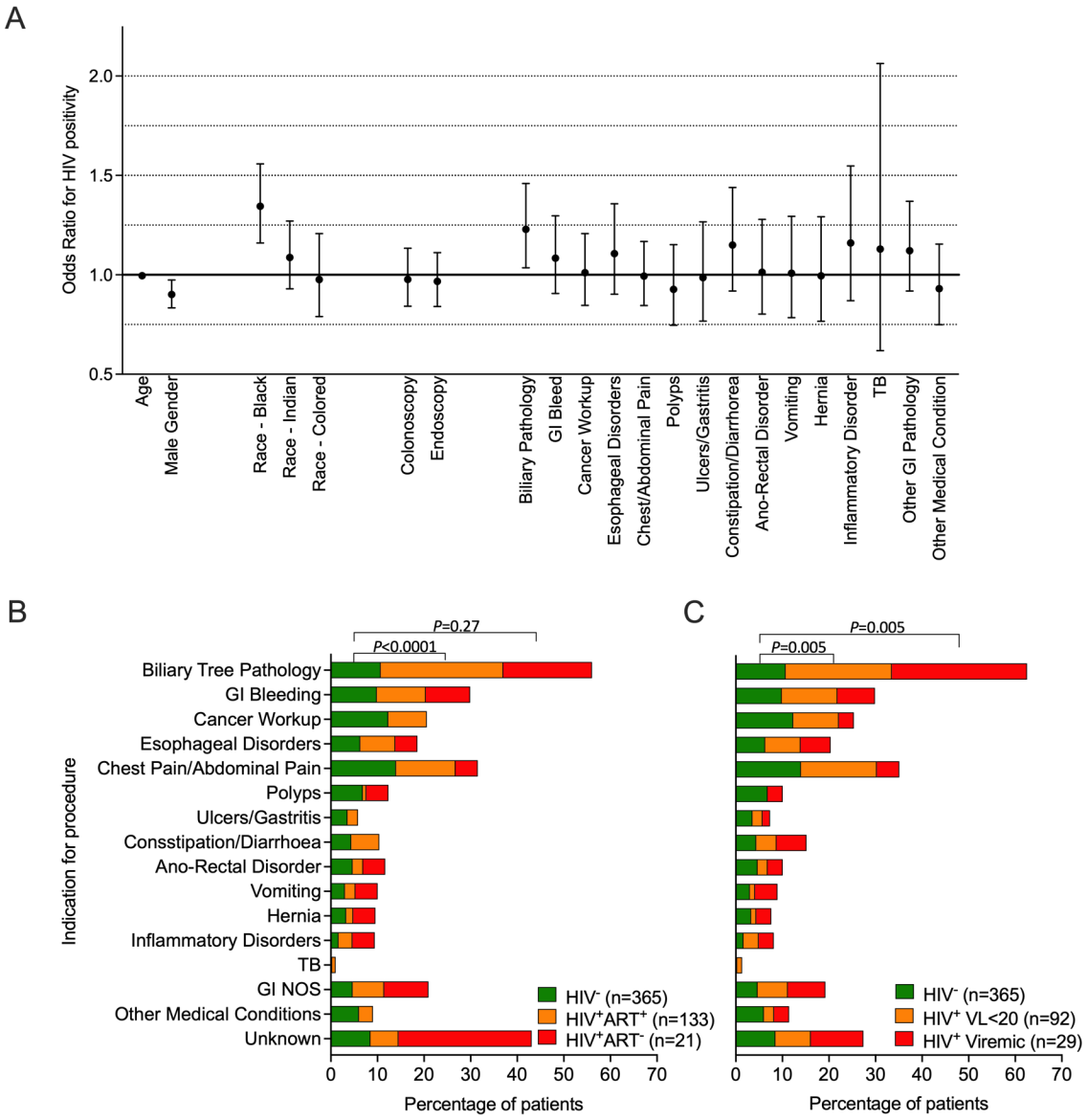
646

647

Table 2. Plasma marker correlations CD4 and HLA-DR

	*All	*HIV$^-$	*HIV$^+$ VL<20	*HIV$^+$ Viremic
sCD14 vs %CD4 in gut	0.9, -0.01 (40)	0.5, 0.16 (18)	0.2, 0.37 (15)	0.9, -0.04 (7)
IFABP vs %CD4 in gut	0.6, -0.08 (42)	0.14, 0.35 (19)	0.7, -0.09 (16)	*0.006, -0.94 (6)*
sCD14 vs %CD4 HLA-DR blood	0.6, 0.08 (51)	0.6, 0.11 (27)	0.6, -0.14 (19)	0.8, -0.14 (5)
I-FABP vs %CD4 HLA-DR blood	0.3, 0.13 (54)	0.9, -0.001 (30)	0.4, 0.19 (19)	0.1, 0.76 (5)

*Pearson correlation P-value, r –Pearson correlation coefficients, n – biological replicates


A
Odds Ratio for HIV positivity
2.0
1.5
1.0
0.5
Age
Male Gender
Race - Black
Race - Indian
Race - Colored
Colonoscopy
Endoscopy
Biliary Pathology
GI Bleed
Cancer Workup
Esophageal Disorders
Chest/Abdominal Pain
Polyps
Ulcers/Gastritis
Constipation/Diarrhorea
Ano-Rectal Disorder
Vomiting
Hernia
Inflammatory Disorder
TB
Other GI Pathology
Other Medical Condition
B
P=0.27
P<0.0001
Biliary Tree Pathology
GI Bleeding
Cancer Workup
Esophageal Disorders
Chest Pain/Abdominal Pain
Polyps
Ulcers/Gastritis
Consstipation/Diarrhoea
Ano-Rectal Disorder
Vomiting
Hernia
Inflammatory Disorders
TB
GI NOS
Other Medical Conditions
Unknown
Indication for procedure
HIV- (n=365)
HIV+ART+ (n=133)
HIV+ART- (n=21)
0 10 20 30 40 50 60 70
Percentage of patients
C
P=0.005
P=0.005
HIV- (n=365)
HIV+ VL<20 (n=92)
HIV+ Viremic (n=29)
0 10 20 30 40 50 60 70
Percentage of patients

653 **Figure legends**

654 **Figure 1. Surgical indications of participants in GI study cohort. A**. Odds ratios for
655 HIV positivity associated with demographic variables and procedural indications.
656 **B**. Surgical indications stratified by HIV and ART status (n=519). **C**. Surgical indications
657 stratified by HIV and viral load status (n=486).

658

659

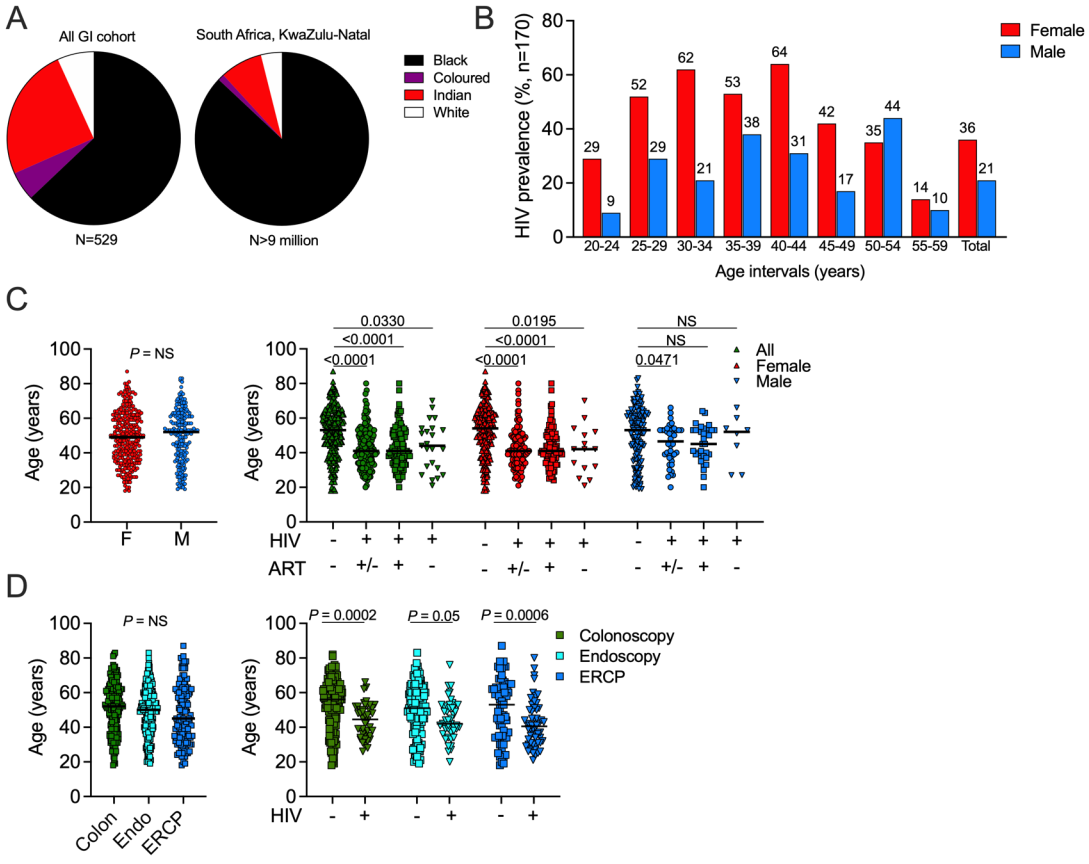

A
All GI cohort
South Africa, KwaZulu-Natal
Black
Coloured
Indian
White
N=529
N>9 million
B
HIV prevalence (%, n=170)
Female
Male
29
9
52
29
62
21
53
38
64
31
42
17
35
44
14
10
36
21
20-24
25-29
30-34
35-39
40-44
45-49
50-54
55-59
Total
Age intervals (years)
C
Age (years)
P = NS
F
M
0.0330
<0.0001
<0.0001
0.0195
<0.0001
<0.0001
NS
NS
0.0471
All
Female
Male
HIV
ART
D
Age (years)
P = NS
Colon
Endo
ERCP
P = 0.0002
P = 0.05
P = 0.0006
Colonoscopy
Endoscopy
ERCP
HIV

Figure 2. Characteristics of study participants. A. Study participants (n=529) stratified by ethnicity compared to the general population of the KwaZulu-Natal province. **B.** HIV prevalence stratified by age group and gender, female (red) and male (blue) (n=170). **C.** Age of presentation to the GI clinic stratified by gender, Female (red) and Male (blue); HIV prevalence and ART status stratified by age and gender. All participants (green), Female (red), Male (blue), p-values by Mann-Whitney U test and Kruskal-Wallis test. **D.** Participants age of presentation for GI surgical procedures irrespective of HIV-1 status; Participants' age of presentation for GI surgical procedure stratified by HIV-1 status. p-values by Kruskal-Wallis test.

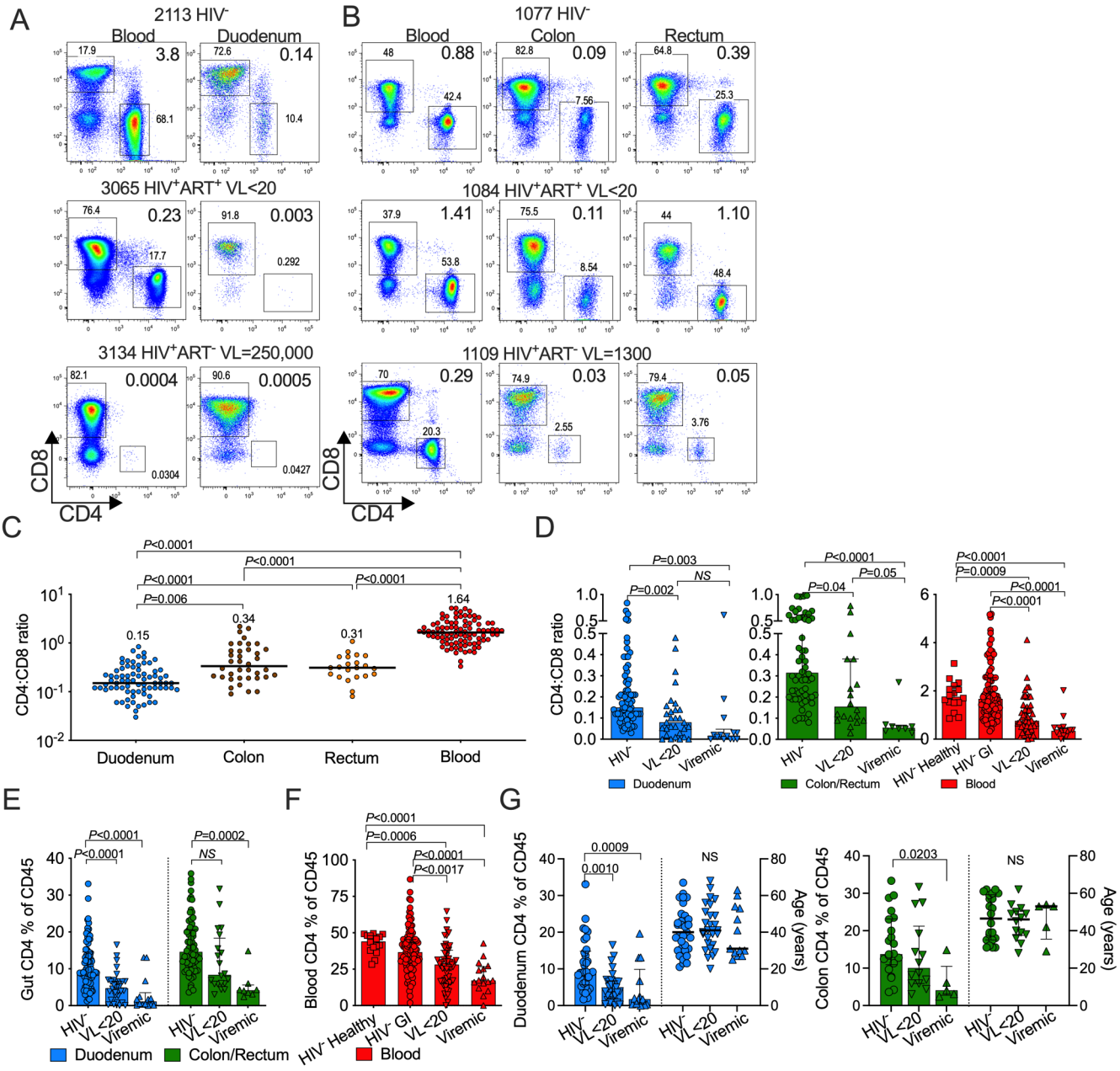

A
2113 HIV-
Blood
Duodenum
17.9
3.8
68.1
72.6
0.14
10.4
3065 HIV+ART+ VL<20
76.4
0.23
17.7
91.8
0.003
0.292
3134 HIV+ART- VL=250,000
82.1
0.0004
0.0304
90.6
0.0005
0.0427
B
1077 HIV-
Blood
Colon
Rectum
48
0.88
42.4
82.8
0.09
7.56
64.8
0.39
25.3
1084 HIV+ART+ VL<20
37.9
1.41
53.8
75.5
0.11
8.54
44
1.10
48.4
1109 HIV+ART- VL=1300
70
0.29
20.3
74.9
0.03
2.55
79.4
0.05
3.76
CD8
CD4
C
CD4:CD8 ratio
P<0.0001
P<0.0001
P<0.0001
P<0.0001
P=0.006
0.15
0.34
0.31
1.64
Duodenum
Colon
Rectum
Blood
D
CD4:CD8 ratio
P=0.003
NS
P=0.002
P<0.0001
P=0.04
P=0.05
P<0.0001
P=0.0009
P<0.0001
HIV-
VL<20
Viremic
HIV- Healthy
HIV- GI
Duodenum
Colon/Rectum
Blood
E
Gut CD4 % of CD45
P<0.0001
P<0.0001
P=0.0002
NS
HIV-
VL<20
Viremic
Duodenum
Colon/Rectum
F
Blood CD4 % of CD45
P<0.0001
P=0.0006
P<0.0001
P<0.0017
HIV- Healthy
HIV- GI
VL<20
Viremic
Blood
G
Duodenum CD4 % of CD45
0.0009
0.0010
NS
Age (years)
Colon CD4 % of CD45
0.0203
NS
Age (years)
HIV-
VL<20
Viremic

Figure 3. Irreversible depletion of duodenal mucosal CD4$^+$ T-cells despite long-term ART. A. Gating strategy to identify CD4$^+$ and CD8$^+$ T-cells in patient-matched blood and duodenum from HIV uninfected and infected participants. **B.** Gating strategy to identify CD4$^+$ and CD8$^+$ T-cells in participants-matched blood, colon and rectum from HIV uninfected and infected patients. **C.** CD4: CD8 ratios of duodenum, colon, rectum and blood from HIV uninfected participants. p-values by Kruskal-Wallis test. **D.** CD4: CD8 ratios of duodenum (blue), colon/rectum (green) and blood (red) from HIV uninfected, HIV-1 infected suppressed and viremic participants. p-values by Kruskal-Wallis test. **E.** CD4% of CD45 cells from the duodenum (blue) and colon/rectum (green) of HIV-1 uninfected, infected virally suppressed and viremic participants. **F.** CD4% of CD45 cells from blood (red) of Healthy HIV uninfected non-GI study participants, HIV uninfected GI study participants, HIV-1 infected suppressed and viremic from our GI cohort. p-values by Kruskal-Wallis test. **G.** Duodenum (blue) and Colon (green) CD4% of CD45 cells stratified by age from HIV uninfected GI study participants, HIV-1 infected suppressed and viremic from our GI cohort. p-values by Kruskal-Wallis test.

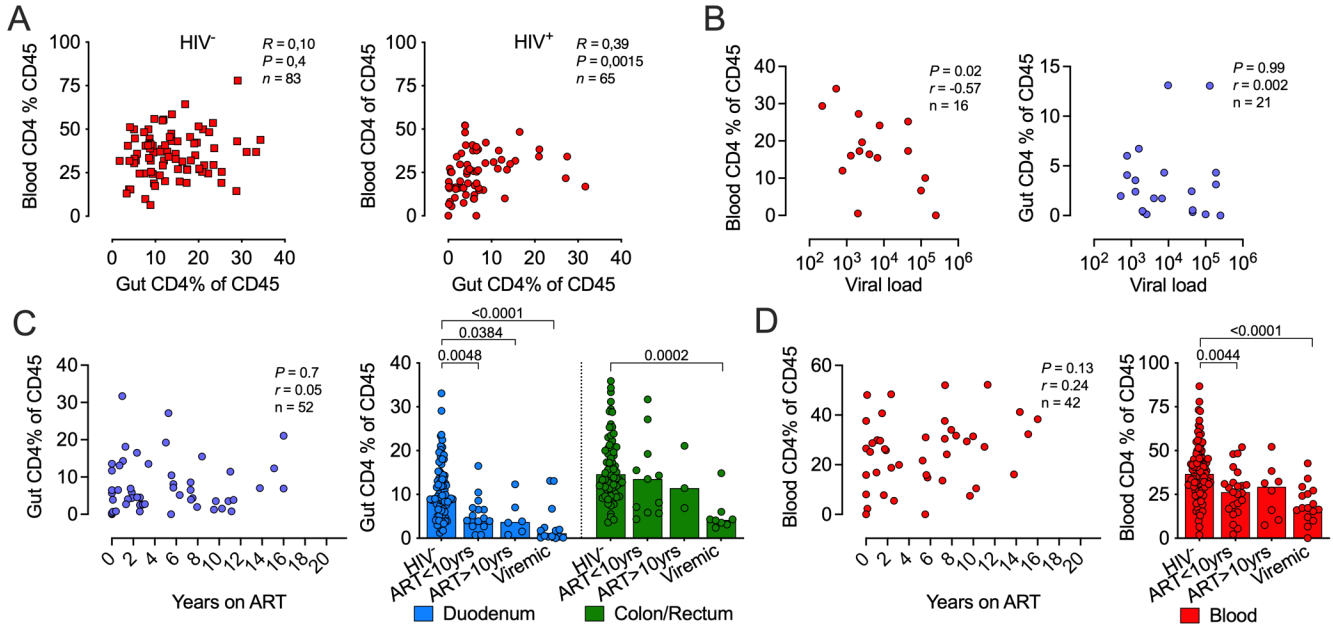

A
HIV-
R = 0,10
P = 0,4
n = 83
Blood CD4 % CD45
Gut CD4% of CD45
HIV+
R = 0,39
P = 0,0015
n = 65
Blood CD4 of CD45
Gut CD4% of CD45
B
P = 0.02
r = -0.57
n = 16
Blood CD4 % of CD45
Viral load
P = 0.99
r = 0.002
n = 21
Gut CD4 % of CD45
Viral load
C
P = 0.7
r = 0.05
n = 52
Gut CD4% of CD45
Years on ART
<0.0001
0.0384
0.0048
0.0002
Gut CD4 % of CD45
HIV-
ART<10yrs
ART>10yrs
Viremic
Duodenum
Colon/Rectum
D
P = 0.13
r = 0.24
n = 42
Blood CD4% of CD45
Years on ART
<0.0001
0.0044
Blood CD4 % of CD45
Blood

Figure 4. Depleted duodenal CD4 T-cells fails to reconstitute even after 10 years of ART. A. Correlation of CD4% of CD45 cells of pooled gut tissues with that of blood among HIV uninfected and infected participants. **B.** Correlation of viral load with CD4% of CD45 cells of blood (red) and gut tissues (blue). **C.** Correlation of participants' duration on ART (years) with CD4% of CD45 cells of gut tissues (blue); CD4% of CD45 cells of duodenum (blue) and colon/rectum (green) tissues stratified by HIV and ART status. D. Correlation of participants' duration on ART (years) with CD4% of CD45 cells of blood (red), correlations by spearman test; CD4% of CD45 cells of blood stratified by HIV and ART status. Correlations by spearman test and P-values by Kruskal Wallis Test for multiple comparisons.

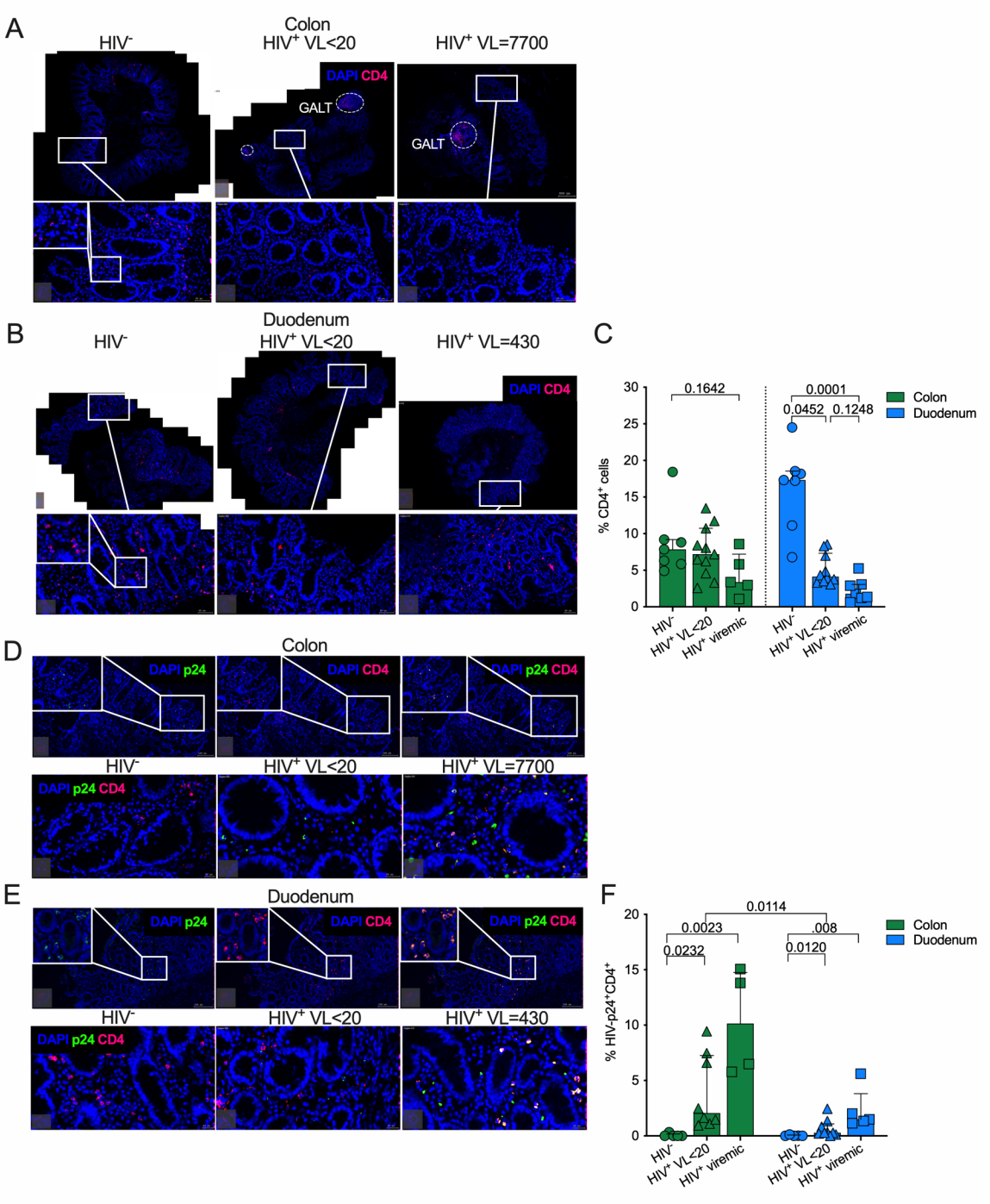

A
Colon
HIV-
HIV+ VL<20
HIV+ VL=7700
DAPI CD4
GALT
GALT
B
Duodenum
HIV-
HIV+ VL<20
HIV+ VL=430
DAPI CD4
C
0.1642
0.0001
0.0452
0.1248
Colon
Duodenum
% CD4+ cells
30
25
20
15
10
5
0
HIV-
HIV+ VL<20
HIV+ viremic
HIV-
HIV+ VL<20
HIV+ viremic
D
Colon
DAPI p24
DAPI CD4
DAPI p24 CD4
HIV-
HIV+ VL<20
HIV+ VL=7700
DAPI p24 CD4
E
Duodenum
DAPI p24
DAPI CD4
DAPI p24 CD4
HIV-
HIV+ VL<20
HIV+ VL=430
DAPI p24 CD4
F
0.0114
0.0023
.008
0.0232
0.0120
Colon
Duodenum
% HIV-p24+CD4+
20
15
10
5
0
HIV-
HIV+ VL<20
HIV+ viremic
HIV-
HIV+ VL<20
HIV+ viremic

Figure 5. Compartment specific HIV-1-p24 detection and CD4 depletion in the gut mucosa of ART treated individuals. A. Representative fluorescent immunohistochemistry image of CD4 T-cells (red) 3proportion of total cells stained with DAPI (blue) in colon tissue sections from HIV uninfected, HIV infected plasma virally suppressed and viremic participants. Bars, 100 μm for full image, 50 μm and 20 μm for large and small inserted images respectively**. B.** Representative fluorescent immunohistochemistry image of CD4 T-cells (red) proportion of total cells stained with DAPI (blue) in duodenum tissue sections from HIV uninfected, HIV infected plasma virally suppressed and viremic participants. Bars, 100 μm for full image, 50 μm and 20 μm for large and small inserted images respectively **C.** Quantification of CD4 T-cells in colon (green) and duodenum (blue). Each dot is representative of a gut tissue section per participants. p-values by Kruskal Wallis test corrected for multiple comparisons. **D.** Representative fluorescent immunohistochemistry image of colon tissues showing HIV-p24 (green), CD4 T-cells (red) and DAPI (blue) from HIV uninfected, virally suppressed and viremic participants. Bars, 100 μm for full image, 50 μm and 20 μm for large and small inserted images respectively. **E.** Representative fluorescent immunohistochemistry image of duodenum tissues showing HIV-p24 (green), CD4 T-cells (red) and DAPI (blue) from HIV uninfected, virally suppressed and viremic participants. Bars, 100 μm for full image, 50 μm and 20 μm for large and small inserted images respectively. **F.** Quantification of HIV-p24 and CD4 T-cells proportion of total cells in colon (green) and duodenum (blue) stained with DAPI from HIV uninfected, virally suppressed and viremic participants. P-values by Mann-Whitney U test.

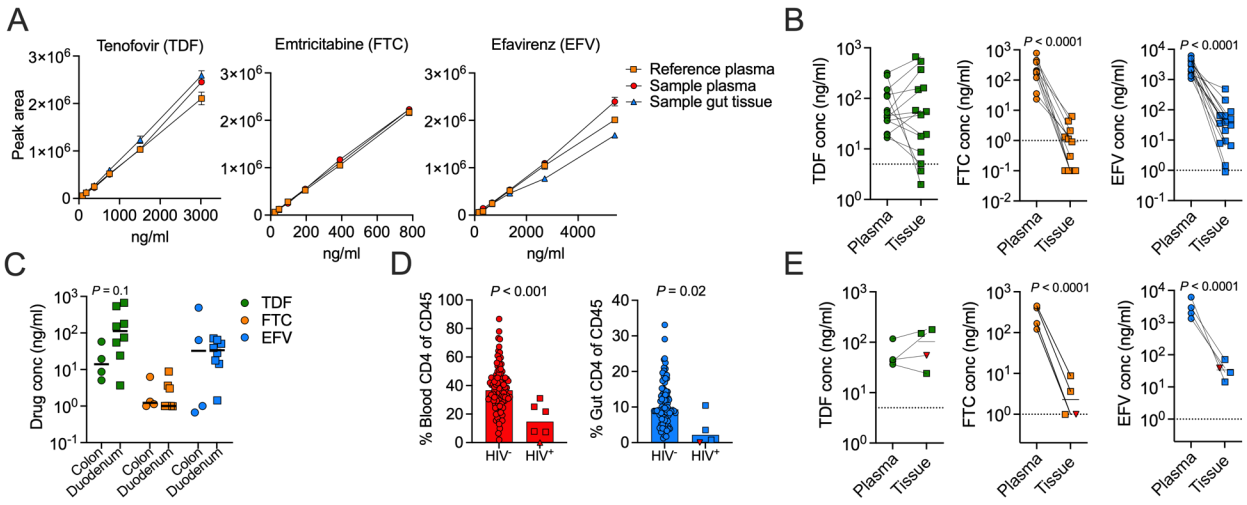

A
Tenofovir (TDF)
Emtricitabine (FTC)
Efavirenz (EFV)
Peak area
ng/ml
Reference plasma
Sample plasma
Sample gut tissue
B
TDF conc (ng/ml)
FTC conc (ng/ml)
EFV conc (ng/ml)
P < 0.0001
Plasma
Tissue
C
Drug conc (ng/ml)
P = 0.1
TDF
FTC
EFV
Colon
Duodenum
D
% Blood CD4 of CD45
% Gut CD4 of CD45
P < 0.001
P = 0.02
HIV-
HIV+
E
TDF conc (ng/ml)
FTC conc (ng/ml)
EFV conc (ng/ml)
P < 0.0001
Plasma
Tissue

Figure 6. ARV drug concentration within plasma and intestinal mucosal tissue sites

A. Quantitation of ARV drugs (TDF, FTC and EFV) in gut tissue and plasma using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), showing biological matrix equivalency between spiked plasma and gut tissue samples. **B**. Comparison of TDF, FTC and EFV concentrations between gut tissue (normalized by wet tissue mass) and patient matched plasma with dotted line showing limit of detection. **C**. ARV quantification stratified by colon and duodenum tissue. **D.** CD4 % of CD45 lymphocytes in blood (red) and tissue (blue) of participants whose ARV concentration was tested, stratified by HIV status. **E.** TDF, FTC and EFV concentrations between gut tissue (normalized by wet tissue mass) and patient matched plasma in subset of participants with available % $CD4^+$ T-cell frequencies of $CD45^+$ cells, with dotted line showing limit of detection. TDF=Tenofovir, FTC=Emtricitabine, EFV=Efavirenz.

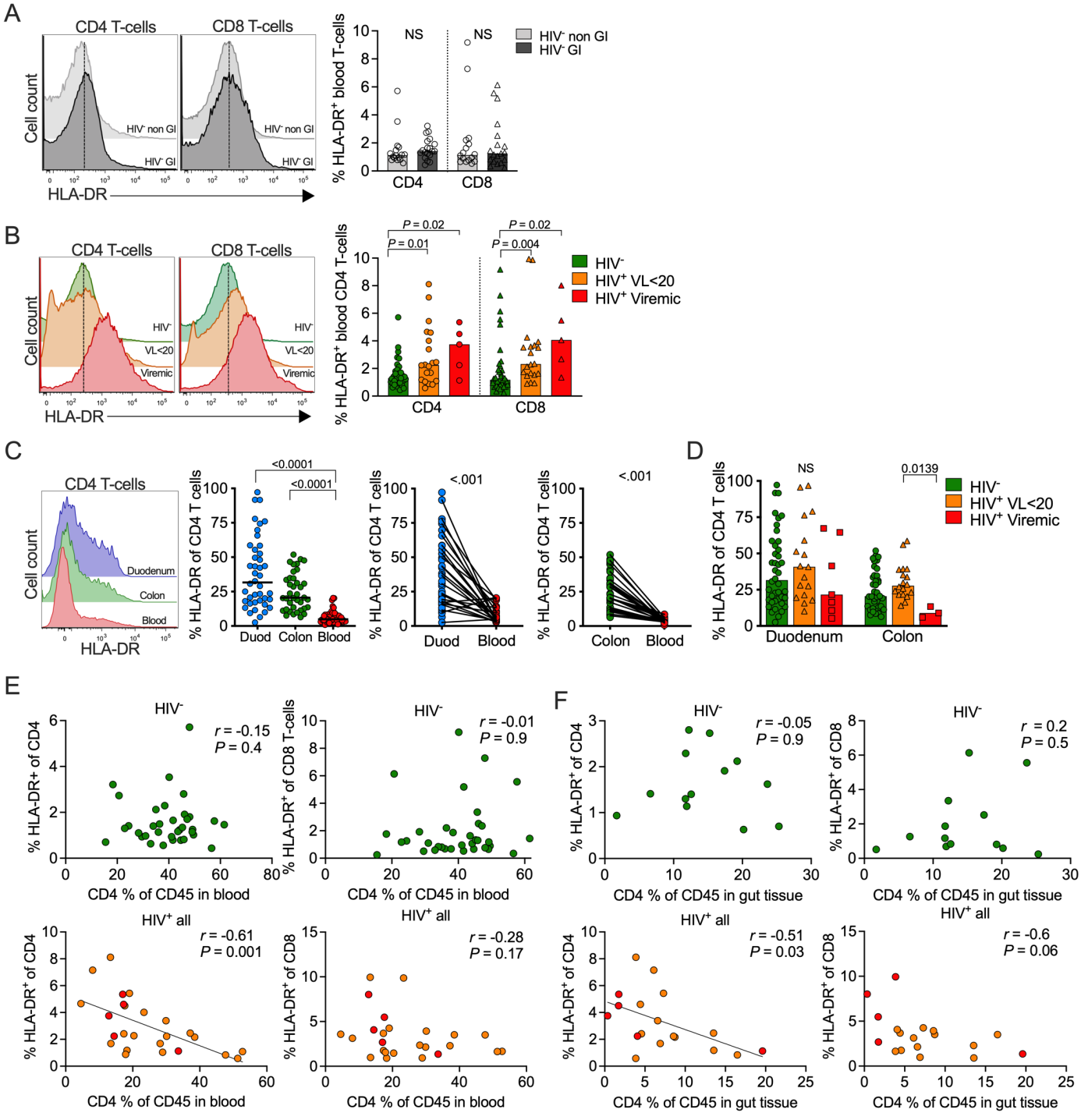

A
CD4 T-cells
CD8 T-cells
Cell count
HIV- non GI
HIV- GI
HLA-DR
% HLA-DR+ blood T-cells
NS
NS
HIV- non GI
HIV- GI
CD4
CD8
B
CD4 T-cells
CD8 T-cells
Cell count
HIV-
VL<20
Viremic
HLA-DR
% HLA-DR+ blood CD4 T-cells
P = 0.02
P = 0.01
P = 0.02
P = 0.004
HIV-
HIV+ VL<20
HIV+ Viremic
CD4
CD8
C
CD4 T-cells
Cell count
Duodenum
Colon
Blood
HLA-DR
% HLA-DR of CD4 T cells
<0.0001
<0.0001
Duod
Colon
Blood
<.001
Duod
Blood
<.001
Colon
Blood
D
% HLA-DR of CD4 T cells
NS
0.0139
HIV-
HIV+ VL<20
HIV+ Viremic
Duodenum
Colon
E
HIV-
r = -0.15
P = 0.4
% HLA-DR+ of CD4
CD4 % of CD45 in blood
HIV-
r = -0.01
P = 0.9
% HLA-DR+ of CD8 T-cells
CD4 % of CD45 in blood
HIV+ all
r = -0.61
P = 0.001
% HLA-DR+ of CD4
CD4 % of CD45 in blood
HIV+ all
r = -0.28
P = 0.17
% HLA-DR+ of CD8
CD4 % of CD45 in blood
F
HIV-
r = -0.05
P = 0.9
% HLA-DR+ of CD4
CD4 % of CD45 in gut tissue
HIV-
r = 0.2
P = 0.5
% HLA-DR+ of CD8
CD4 % of CD45 in gut tissue
HIV+ all
r = -0.51
P = 0.03
% HLA-DR+ of CD4
CD4 % of CD45 in gut tissue
HIV+ all
r = -0.6
P = 0.06
% HLA-DR+ of CD8
CD4 % of CD45 in gut tissue

Figure 7. Persistent systemic T-cell activation correlates with gut CD4 T-cell depletion. A. Gating strategy to identify $CD4^+$ and $CD8^+$ T-cells activation marker, HLA-DR in blood, comparing HIV uninfected non-GI and GI participants. **B.** T-cell activation ($CD4^+HLA\text{-}DR^+$ and $CD8^+HLA\text{-}DR^+$) in blood from GI cohort participants stratified by viral load. P-values by Kruskal-Wallis test. **C.** CD4 T-cell activation in duodenum (blue), colon (green) and blood (red) from HIV uninfected GI participants. P-values by Kruskal-Wallis test. **D.** CD4 T-cell activation in HIV uninfected GI participants (green) compared with HIV-1 infected virally suppressed (orange) and viremic (red) participants from duodenum and colon. P-values by Kruskal-Wallis test. **E.** Correlations of CD4% of CD45 cells in blood with % of $CD4^+$ and $CD8^+$ T-cell activation among HIV uninfected and HIV infected participants. **F.** Correlations of CD4% of CD45 cells in gut tissues with % of $CD4^+$ and $CD8^+$ T-cell activation among HIV uninfected and HIV infected participants. P and r values by pearson correlations.

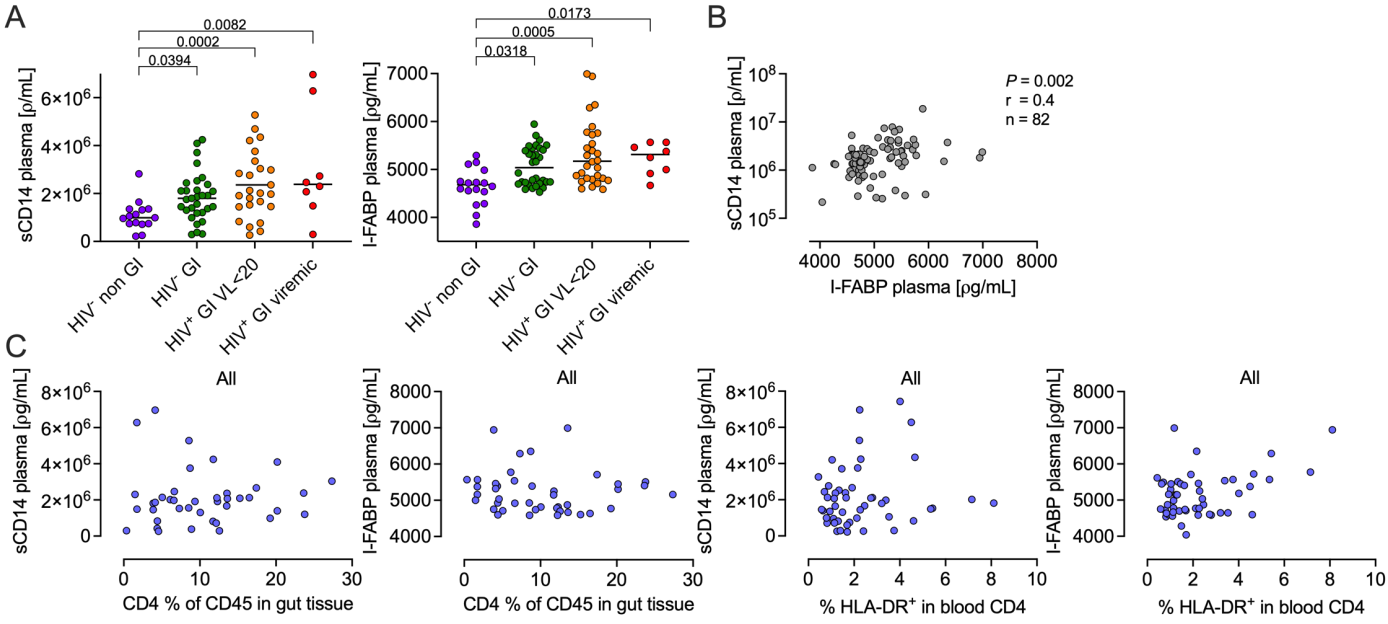

A
0.0082
0.0002
0.0394
sCD14 plasma [μg/mL]
6×10^6
4×10^6
2×10^6
0
HIV⁻ non GI
HIV⁻ GI
HIV⁺ GI VL<20
HIV⁺ GI viremic
0.0173
0.0005
0.0318
I-FABP plasma [pg/mL]
7000
6000
5000
4000
B
sCD14 plasma [μg/mL]
10^8
10^7
10^6
10^5
P = 0.002
r = 0.4
n = 82
4000 5000 6000 7000 8000
I-FABP plasma [pg/mL]
C
All
sCD14 plasma [pg/mL]
8×10^6
6×10^6
4×10^6
2×10^6
0
0 10 20 30
CD4 % of CD45 in gut tissue
I-FABP plasma [pg/mL]
8000
7000
6000
5000
4000
0 10 20 30
CD4 % of CD45 in gut tissue
sCD14 plasma [pg/mL]
0 2 4 6 8 10
% HLA-DR⁺ in blood CD4
I-FABP plasma [pg/mL]
0 2 4 6 8 10
% HLA-DR⁺ in blood CD4

Figure 8. Elevated intestinal and inflammatory markers are associated with GI pathology but do not correlate with HIV associated depletion of gut CD4 T-cells. A. Increased sCD14 and I-FABP among HIV-1 infected virally suppressed (orange) and viremic participants (red) compared with healthy HIV uninfected participants (purple). P-values by Kruskal-Wallis test. **B**. sCD14 correlates positively with I-FABP. P and r values by pearson correlations. **C.** Lack of correlation between CD4% of CD45 cells in gut tissues with plasma sCD14 and I-FABP**;** lack of correlation between % of activated CD4 in the blood with plasma sCD14 and I-FABP.

References

1. Kotler DP, Gaetz HP, Lange M, Klein EB, Holt PR. Enteropathy Associated with the Acquired Immunodeficiency Syndrome. *Annals of Internal Medicine* 1984;101(4):421.
2. Batman PA et al. Jejunal enteropathy associated with human immunodeficiency virus infection: Quantitative histology. *Journal of Clinical Pathology* 1989;42(3):275–281.
3. Mehandru S et al. Primary HIV-1 Infection Is Associated with Preferential Depletion of CD4 $^{+}$ T Lymphocytes from Effector Sites in the Gastrointestinal Tract. *The Journal of Experimental Medicine* 2004;200(6):761–770.
4. Brenchley JM et al. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *Journal of Experimental Medicine* 2004;200(6):749–759.
5. Veazey RS, Lackner AA. HIV swiftly guts the immune system. *Nature Medicine* 2005;11(5):469–470.
6. Chun T et al. Persistence of HIV in Gut-Associated Lymphoid Tissue despite Long-Term Antiretroviral Therapy [Internet]. *The Journal of Infectious Diseases* 2008;197(5):714–720.
7. Nabel G, Baltimore D. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* 1987;326(6114):711–713.
8. Schieferdecker HL, Ullrich R, Hirseland H, Zeitz M. T cell differentiation antigens on lymphocytes in the human intestinal lamina propria.. *Journal of immunology (Baltimore, Md. : 1950)* 1992;149(8):2816–22.
9. Mattapallil JJ, Douek DC, Hill B. Massive infection and loss of memory CD4+ T cells in multiple tissu... - PubMed - NCBI2005;434(April):1093–1097.
10. Mudd JC, Brenchley JM. Gut Mucosal Barrier Dysfunction, Microbial Dysbiosis, and Their Role in HIV-1 Disease Progression [Internet]. *Journal of Infectious Diseases* 2016;214(suppl 2):S58–S66.
11. Estes JD, Haase AT, Schacker TW. The role of collagen deposition in depleting CD4+ T cells and limiting reconstitution in HIV-1 and SIV infections through damage to the secondary lymphoid organ niche [Internet]. *Seminars in Immunology* 2008;20(3):181–186.
12. Schacker TW et al. Lymphatic tissue fibrosis is associated with reduced numbers of naïve CD4+ T cells in human immunodeficiency virus type 1 infection [Internet]. *Clinical and Vaccine Immunology* 2006;13(5):556–560.
13. Sanchez JL et al. Lymphoid fibrosis occurs in long-term nonprogressors and persists with antiretroviral therapy but may be reversible with curative interventions [Internet]. In: *Journal of Infectious Diseases*. Oxford University Press; 2015:1068–1075
14. Fletcher C v. et al. Persistent HIV-1 replication is associated with lower antiretroviral drug concentrations in lymphatic tissues [Internet]. *Proceedings of the National Academy of Sciences* 2014;111(6):2307–2312.
15. Deeks SG, Tracy R, Douek DC. Systemic Effects of Inflammation on Health during Chronic HIV Infection [Internet]. *Immunity* 2013;39(4):633–645.
16. Ake JA et al. Noninfectious Comorbidity in the African Cohort Study. *Clinical Infectious Diseases* 2019;69(4):639–647.
17. Guaraldi G et al. Premature Age-Related Comorbidities Among HIV-Infected Persons Compared With the General Population. *Clinical Infectious Diseases* 2011;53(11):1120–1126.

18. Achhra A et al. Short-term weight gain after antiretroviral therapy initiation and subsequent risk of cardiovascular disease and diabetes: the D:A:D study. *HIV Medicine* 2016;17(4):255–268.
19. Mocroft A et al. Development and Validation of a Risk Score for Chronic Kidney Disease in HIV Infection Using Prospective Cohort Data from the D:A:D Study. *PLOS Medicine* 2015;12(3):e1001809.
20. Freiberg MS et al. HIV Infection and the Risk of Acute Myocardial Infarction. *JAMA Internal Medicine* 2013;173(8):614.
21. Petoumenos K et al. Predicting the short-term risk of diabetes in HIV-positive patients: the Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) study. *Journal of the International AIDS Society* 2012;15(2):17426.
22. Deeks SG, Phillips AN. HIV infection, antiretroviral treatment, ageing, and non-AIDS related morbidity. *BMJ* 2009;338(jan26 2):a3172–a3172.
23. Goulet JL et al. Do Patterns of Comorbidity Vary by HIV Status, Age, and HIV Severity?. *Clinical Infectious Diseases* 2007;45(12):1593–1601.
24. Kuo HH, Lichterfeld M. Recent progress in understanding HIV reservoirs. *Current Opinion in HIV and AIDS* 2018;13(2):137–142.
25. Ndung'u T, McCune JM, Deeks SG. Why and where an HIV cure is needed and how it might be achieved. *Nature* 2019;576(7787):397–405.
26. Estes JD et al. Defining total-body AIDS-virus burden with implications for curative strategies. *Nature Medicine* 2017;23(11):1271–1276.
27. Yukl SA et al. The Distribution of HIV DNA and RNA in Cell Subsets Differs in Gut and Blood of HIV-Positive Patients on ART: Implications for Viral Persistence. *The Journal of Infectious Diseases* 2013;208(8):1212–1220.
28. Ananworanich J et al. HIV DNA Set Point is Rapidly Established in Acute HIV Infection and Dramatically Reduced by Early ART.. *EBioMedicine* 2016;11:68–72.
29. Ananworanich J et al. Virological and immunological characteristics of HIV-infected individuals at the earliest stage of infection.. *Journal of virus eradication* 2016;2(1):43–48.
30. Yukl SA et al. Challenges in Detecting HIV Persistence during Potentially Curative Interventions: A Study of the Berlin Patient. *PLoS Pathogens* 2013;9(5):e1003347.
31. Belmonte L et al. The intestinal mucosa as a reservoir of HIV-1 infection after successful HAART. *AIDS* 2007;21(15):2106–2108.
32. Lafeuillade A et al. Rectal cell-associated HIV-1 RNA: A new marker ready for the clinic. *HIV Clinical Trials* 2009;10(5):324–327.
33. Thompson CG et al. Heterogeneous antiretroviral drug distribution and HIV/SHIV detection in the gut of three species. *Science Translational Medicine* 2019;11(499):eaap8758.
34. van Marle G et al. Compartmentalization of the gut viral reservoir in HIV-1 infected patients [Internet]. *Retrovirology* 2007;4. doi:10.1186/1742-4690-4-87
35. Veazey RS et al. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* 1998;280(5362):427–431.
36. Guadalupe M et al. Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. [Internet]. *Journal of virology* 2003;77(21):11708–17.
37. Agace WW, McCoy KD. Regionalized Development and Maintenance of the Intestinal Adaptive Immune Landscape [Internet]. *Immunity* 2017;46(4):532–548.

854 38. Deeks SG et al. Immune activation set point during early HIV infection predicts subsequent
855 CD4+ T-cell changes independent of viral load. *Blood* 2004;104(4):942–947.
856 39. Shi HY et al. Ethnicity Influences Phenotype and Outcomes in Inflammatory Bowel Disease:
857 A Systematic Review and Meta-analysis of Population-based Studies. *Clinical Gastroenterology*
858 *and Hepatology* 2018;16(2):190-197.e11.
859 40. Ramjee G et al. A decade of sustained geographic spread of HIV infections among women in
860 Durban, South Africa. *BMC Infectious Diseases* 2019;19(1):1–9.
861 41. Kharsany ABM et al. Community-based HIV prevalence in KwaZulu-Natal, South Africa:
862 results of a cross-sectional household survey. *The Lancet HIV* 2018;5(8):e427–e437.
863 42. Dong KL et al. Detection and treatment of Fiebig stage I HIV-1 infection in young at-risk
864 women in South Africa: a prospective cohort study. *The Lancet HIV* 2018;5(1):e35–e44.
865 43. Gordon SN et al. Disruption of Intestinal CD4+ T Cell Homeostasis Is a Key Marker of
866 Systemic CD4+ T Cell Activation in HIV-Infected Individuals. *The Journal of Immunology*
867 2010;185(9):5169–5179.
868 44. Meintjes G et al. Adult antiretroviral therapy guidelines 2017. *Southern African Journal of*
869 *HIV Medicine* 2017;18(1):1–24.
870 45. Hunt PW, Lee SA, Siedner MJ. Immunologic Biomarkers, Morbidity, and Mortality in
871 Treated HIV Infection. *Journal of Infectious Diseases* 2016;214(suppl 2):S44–S50.
872 46. Brenchley JM et al. Microbial translocation is a cause of systemic immune activation in
873 chronic HIV infection. *Nature Medicine* 2006;12(12):1365–1371.
874 47. Guadalupe M et al. Viral suppression and immune restoration in the gastrointestinal mucosa
875 of human immunodeficiency virus type 1-infected patients initiating therapy during primary or
876 chronic infection.. *Journal of virology* 2006;80(16):8236–47.
877 48. Hattab S et al. Comparative impact of antiretroviral drugs on markers of inflammation and
878 immune activation during the first two years of effective therapy for HIV-1 infection: an
879 observational study. *BMC Infectious Diseases* 2014;14(1):122.
880 49. Hunt PW et al. Gut epithelial barrier dysfunction and innate immune activation predict
881 mortality in treated HIV infection. *Journal of Infectious Diseases* 2014;210(8):1228–1238.
882 50. Kuller LH et al. Inflammatory and coagulation biomarkers and mortality in patients with HIV
883 infection. *PLoS Medicine* 2008;5(10):1496–1508.
884 51. Tien PC et al. Inflammation and mortality in HIV-infected adults: Analysis of the FRAM
885 study cohort. *Journal of Acquired Immune Deficiency Syndromes* 2010;55(3):316–322.
886 52. Tenorio AR et al. Soluble markers of inflammation and coagulation but not T-cell activation
887 predict non-AIDS-defining morbid events during suppressive antiretroviral treatment. *Journal of*
888 *Infectious Diseases* 2014;210(8):1248–1259.
889 53. Justice AC et al. Does an index composed of clinical data reflect effects of inflammation,
890 coagulation, and monocyte activation on mortality among those aging with HIV?. *Clinical*
891 *Infectious Diseases* 2012;54(7):984–994.
892 54. Sandler NG et al. Plasma levels of soluble CD14 independently predict mortality in HIV
893 infection. *Journal of Infectious Diseases* 2011;203(6):780–790.
894 55. Wilson EMP, Sereti I. Immune Restoration After Antiretroviral Therapy: The Pitfalls Of
895 Hasty Or Incomplete Repairs. *Immunological Reviews* 2013;254(1):343–354.
896 56. Sereti I et al. Persistent, Albeit reduced, chronic inflammation in persons starting
897 antiretroviral therapy in acute HIV infection. *Clinical Infectious Diseases* 2017;64(2):124–131.

898 57. Cassol E et al. Persistent Microbial Translocation and Immune Activation in HIV-1–Infected
899 South Africans Receiving Combination Antiretroviral Therapy. *The Journal of Infectious*
900 *Diseases* 2010;202(5):723–733.
901 58. Kamat A et al. A plasma biomarker signature of immune activation in HIV patients on
902 antiretroviral therapy. *PLoS ONE* 2012;7(2). doi:10.1371/journal.pone.0030881
903 59. Marchetti G, Tincati C, Silvestri G. Microbial translocation in the pathogenesis of HIV
904 infection and AIDS. *Clinical Microbiology Reviews* 2013;26(1):2–18.
905 60. LisaM.Chirch J. *HIV and GI Tract Complications*. Clinical Gastroenterology; 2019:
906 61. Imai K et al. Progressive HIV-associated cholangiopathy in an HIV patient treated with
907 combination antiretroviral therapy. *Internal Medicine* 2016;55(19):2881–2884.
908 62. Naseer M, Dailey FE, Al Juboori A, Samiullah S, Tahan V. Epidemiology, determinants, and
909 management of AIDS cholangiopathy: A review. *World Journal of Gastroenterology*
910 2018;24(7):767–774.
911 63. Deeks SG, Lewin SR, Havlir D V. The end of AIDS: HIV infection as a chronic disease. *The*
912 *Lancet* 2013;382(9903):1525–1533.
913 64. Palella FJ et al. Declining morbidity and mortality among patients with advanced human
914 immunodeficiency virus infection. *New England Journal of Medicine* 1998;338(13):853–860.
915 65. Mehandru S et al. Lack of mucosal immune reconstitution during prolonged treatment of
916 acute and early HIV-1 infection. *PLoS Medicine* 2006;3(12):2335–2348.
917 66. Takashima S et al. T cell-derived interferon-γ programs stem cell death in immune-mediated
918 intestinal damage. *Science immunology* 2019;4(42). doi:10.1126/sciimmunol.aay8556
919 67. Biton M et al. T Helper Cell Cytokines Modulate Intestinal Stem Cell Renewal and
920 Differentiation. *Cell* 2018;175(5):1307-1320.e22.
921 68. Lundgren JD et al. Initiation of antiretroviral therapy in early asymptomatic HIV infection.
922 *New England Journal of Medicine* 2015;373(9):795–807.
923 69. Schuetz A et al. Initiation of ART during Early Acute HIV Infection Preserves Mucosal
924 Th17 Function and Reverses HIV-Related Immune Activation. *PLoS Pathogens* 2014;10(12).
925 doi:10.1371/journal.ppat.1004543
926 70. Siddiqi AEA, Hall HI, Hu X, Song R. Population-based estimates of life expectancy after
927 HIV diagnosis: United states 2008-2011. *Journal of Acquired Immune Deficiency Syndromes*
928 2016;72(2):230–236.
929 71. Patterson KB et al. HIV. Penetration of tenofovir and emtricitabine in mucosal tissues:
930 Implications for prevention of HIV-1 transmission [Internet]. *Science Translational Medicine*
931 2011;3(112). doi:10.1126/scitranslmed.3003174
932 72. Thompson CG, Cohen MS, Kashuba ADM. Antiretroviral pharmacology in mucosal tissues
933 [Internet]. *Journal of Acquired Immune Deficiency Syndromes* 2013;63(SUPPL. 2).
934 doi:10.1097/QAI.0b013e3182986ff8
935 73. Patterson KB et al. Differential penetration of raltegravir throughout gastrointestinal tissue:
936 Implications for eradication and cure [Internet]. *AIDS* 2013;27(9):1413–1419.
937 74. Veselinovic M et al. HIV pre-exposure prophylaxis: Mucosal tissue drug distribution of RT
938 inhibitor Tenofovir and entry inhibitor Maraviroc in a humanized mouse model [Internet].
939 *Virology* 2014;464–465(1):253–263.
940 75. Asmuth DM et al. Tissue Pharmacologic and Virologic Determinants of Duodenal and Rectal
941 Gastrointestinal-Associated Lymphoid Tissue Immune Reconstitution in HIV-Infected Patients
942 Initiating Antiretroviral Therapy [Internet]. *The Journal of Infectious Diseases* 2017;216(7):813–
943 818.

944 76. Thurman AR et al. Differences in local and systemic TFV PK among premenopausal versus
945 postmenopausal women exposed to TFV 1% vaginal gel [Internet]. *Journal of Acquired Immune*
946 *Deficiency Syndromes* 2018;78(1):82–92.
947 77. Kis O et al. HIV-1 alters intestinal expression of drug transporters and metabolic enzymes:
948 Implications for antiretroviral drug disposition. *Antimicrobial Agents and Chemotherapy*
949 2016;60(5):2771–2781.
950 78. Fletcher C V. et al. The Lymphoid Tissue Pharmacokinetics of Tenofovir disoproxil fumarate
951 and Tenofovir alafenamide in HIV-infected Persons. *Clinical Pharmacology & Therapeutics*
952 [published online ahead of print: 2020]; doi:10.1002/cpt.1883
953 79. The TenoRes Study Group. Global epidemiology of drug resistance after failure of WHO
954 recommended first-line regimens for adult HIV-1 infection: A multicentre retrospective cohort
955 study. *The Lancet Infectious Diseases* 2016;16(5):565–575.
956 80. Anderson F, Thomson SR. Pancreatitis in a high HIV prevalence environment. *South African*
957 *Medical Journal* 2017;107(8):706–709.
958 81. Malnati MS et al. A universal real-time PCR assay for the quantification of group-M HIV-1
959 proviral load [Internet]. *Nature Protocols* 2008;3(7):1240–1248.
960 82. Hindson BJ et al. High-throughput droplet digital PCR system for absolute quantitation of
961 DNA copy number [Internet]. *Analytical Chemistry* 2011;83(22):8604–8610.
962