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Primary HIV Infection Features Colonic Damage and Neutrophil Inflammation yet Containment of Microbial Translocation

Camilla Tincati¹, Valeria Bono¹, Elvira Stefania Cannizzo¹, Delfina Tosi², Federica Savi², Camilla Falcinella¹, Anna Casabianca³, Chiara Orlandi³, Carmelo Luigiano⁴, Matteo Augello¹, Stefano Rusconi⁵, Antonio Muscatello⁶, Alessandra Bandera⁶, Andrea Calcagno⁷, Andrea Gori⁸, Silvia Nozza⁹, Giulia Marchetti¹§ on behalf of the Italian Network of ACuTe HIV InfectiON (INACTION)*

¹Clinic of Infectious Diseases, Department of Health Sciences, ASST Santi Paolo e Carlo, University of Milan, Italy; ²Pathology Unit, Department of Health Sciences, ASST Santi Paolo e Carlo, University of Milan, Italy; ³ Department of Biomolecular Sciences, University of Urbino Carlo Bo, 61029 Fano, Italy; ⁴Digestive Endoscopy Unit, ASST Santi Paolo e Carlo, Milano, Italy; ⁵UOC Malattie Infettive, Ospedale Civile di Legnano, Department of Biomedical and Clinical Biosciences, University of Milan, Italy; ⁶Infectious Diseases Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; ⁷Unit of Infectious Diseases Unit, Department of Medical Sciences, University of Turin, Italy; ⁸Clinic of Infectious Diseases, Department of Pathophysiology and Transplantation, ASST Fatebenefratelli Sacco University of Milan, Italy; ⁹ Infectious Diseases Unit, IRCCS Ospedale San Raffaele, Milan, Italy

Current affiliations:

ESC: ViiV Healthcare, Italy;

CL: Digestive Endoscopy Unit, Grande Ospedale Metropolitano, Bianchi Melacrino Morelli, Reggio Calabria, Italy;

§ Corresponding author: Giulia Marchetti, M.D., Ph.D.

Address: Clinic of Infectious Diseases, Department of Health Sciences, University of Milan, San Paolo Hospital, Milan, Italy, Via di Rudini' 8, 20142, Milan, Italy

Phone: +390281843046; Fax: +390281843054; email: giuila.marchetti@unimi.it

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*Author contributors are listed in the Acknowledgment section.

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Abstract

Introduction: Impairment of the gastrointestinal (GI) barrier leads to microbial translocation and peripheral immune activation which are linked to disease progression. Data in the setting of primary HIV/SIV infection suggest that gut barrier damage is one of the first events of the pathogenic cascade, preceding mucosal immune dysfunction and microbial translocation. We assessed gut structure and immunity as well as microbial translocation in acutely- and chronically-infected, combination cART-naïve individuals.

Methods: Fifteen people with Primary HIV infection (P-HIV) and 13 with Chronic HIV infection (C-HIV) c-ART naïve participants were cross-sectionally studied. Gut biopsies were analyzed in terms of gut reservoirs (total, integrated and unintegrated HIV DNA); tight junction proteins (E-cadherin, Zonula Occludens-1), CD4 expression, neutrophil myeloperoxidase (histochemical staining); collagen deposition (Masson staining). Flow cytometry was used to assess $\gamma\delta$ T-cell frequency (CD3+pan $\gamma\delta$ +V δ 1+/V δ 2+). In plasma we measured microbial translocation (LPS, sCD14, EndoCAB) and gut barrier function (I-FABP) markers (ELISA).

Results: P-HIV displayed significantly higher tissue HIV DNA, yet neutrophil infiltration and collagen deposition in the gut were similar in the two groups. In contrast, microbial translocation markers were significantly lower in P-HIV compared to C-HIV. A trend to higher mucosal E-cadherin, and gut $\gamma\delta$ T-cells was also observed in P-HIV.

Conclusions:

Early HIV infection features higher HIV DNA in the gut, yet comparable mucosal alterations to those observed in chronic infection. In contrast, microbial translocation is contained in primary HIV infection, likely due to a partial preservation of E-cadherin and mucosal immune subsets, namely $\gamma\delta$ T-cells.

Keywords: Primary HIV Infection (PHI), mucosal immunity, gut barrier, microbial translocation

Introduction

The damage of the gastrointestinal (GI) barrier is a key event in the pathogenesis of HIV/SIV infection and arises by different mechanisms.

HIV directly impairs the epithelial barrier^[1] and depletes, aside from CD4+ T-cells, a wide range of IL-17- and IL-22-producing mucosal cell subsets^[2] which contribute to intestinal integrity and restrict commensal bacteria from the systemic circulation. Taken together, these events lead to mucosal impairment^[3, 4], microbial translocation^[5] and peripheral inflammation^[4], which are strictly linked to

disease progression^[6-8] as well as poor CD4+ T-cell recovery on combination antiretroviral therapy (cART)^[9-14].

Given the critical role of the GI tract in HIV infection, understanding the precise timing of the phenomena taking place therein is crucial for the elaboration and timely administration of adjuvant therapeutic interventions which can counteract the key events in the HIV-driven pathogenic cascade.

Older work in acute HIV/SIV infection demonstrated rapid CD4+ T-cell loss at mucosal sites during the very first phases of infection^[15, 16], while little/no microbial translocation was shown in primary HIV infection^[5, 17]. In keeping with these findings, a study in the animal model demonstrated that proteins involved in epithelial integrity are altered 3 day post-SIV infection and precede Th17 depletion which occurs approximately 2-3 weeks later^[18], confirming previous data on impairment of mucosal Th17 frequencies and function no earlier than Fiebig stage III^[19]. Recent work in acutely-infected rhesus macaques showed that proteome changes linked to the transition of epithelial to mesenchymal cells in the gut occur before the mucosal antiviral response^[20].

In untreated Primary (P-HIV) and Chronic (C-HIV) people living with HIV we assessed the gut mucosa in terms of viral reservoir, mucosal fibrosis/inflammation and immunity as well as barrier structure. We also measured microbial translocation and gut damage markers in the peripheral blood.

Methods

Study design and population

This is a cross-sectional study enrolling people living with HIV in care at the Clinic of Infectious Diseases, University of Milan, ASST Santi Paolo e Carlo, Milan, Italy. Patients with Primary HIV (P-HIV; n=15) infection were classified based on a positive p24 antigenemia or detectable HIV RNA with a negative or indeterminate Western Blot confirmation assay, according to Fiebig stage^[21]. Individuals with chronic HIV infection (C-HIV, n=13) were also included. Both P-HIV and C-HIV were naïve to cART. Individuals with a known gastrointestinal disease or clinical symptoms were excluded from the study. Blood samples and intestinal biopsies during colonoscopy were obtained following the provision of informed consent which was approved by the Institutional Review Board at the ASST Santi Paolo e Carlo, Milan, Italy. Experiments were carried out by the Laboratory of the Clinic of Infectious Diseases and by the Pathology Unit, Ospedale San Paolo, University of Milan, Italy.

Colonoscopy

Patients underwent routine screening colonoscopy [bowel preparation: Moviprep (Norgine, Marburg, Germany); pre-treatment: midazolam (2mg) and pethidine (50mg)]. Four pinch biopsies were collected in the colon: two biopsies were immediately transported to the Pathology Unit for cryopreservation with Optimal Cutting temperature Compound (OCT) and the other two were processed for flow cytometric

analysis. Following this procedure, all colonic tracts, rectum and distal ileum were biopsied (one pinch biopsy per site) were formalin-fixed (10%) and processed for routine histopathological examination; one colonic biopsy was selected for immunohistochemical staining (see below).

Immunohistochemical staining

One paraffin-embedded biopsy from transverse colon was selected, stained with hematoxylin-eosin (HE), Masson trichrome and antibodies against the following markers: (i) major structural proteins of Tight Junctions (TJs) [Zonula Occludens 1 (ZO-1, 1:200, Zymed) and Cadherin 1 (Cdh1, 1:15000, ABNOVA)]; (ii) CD4 (clone 1F6, 1:50; Leica Microsystems); (iii) CD8 (clone C8/144B Mouse, Dako) (iv) neutrophil infiltration: myeloperoxidase (MPO) (rabbit anti-human MPO, Dako). The Autostainer Dako Omnis was used for the staining.

Expression of TJs proteins in colonic epithelium of study subjects was evaluated as compared to controls, i.e. n=5 surgical resection margins of colonic tissue removed for neoplastic pathology and expressed as follows: i) ZO1: focal, partial or total reduction with respect to the normal localization on the apical part of the cell membrane in 3 high-power fields (HPF); ii) CDh1: reduction considered as expression only in some parts of the cell membrane (baso-lateral, lateral, basal) compared to normal localization on the entire perimeter of the membrane in 3 HPF. CD4+ T-lymphocytes were determined by number of CD4+ cells in 3 HPF of gut lamina propria.

Masson staining

Collagen deposition in the gut was measured through Masson staining on formalin-fixed and paraffin-embedded sections (Bio-Optica Milano Spa). A semiquantitative score was used (0 = no staining, 1 = thin collagen fiber staining, 2 = thick collagen fiber staining, 3 = intense blue bundles staining).

Flow cytometry

Lamina Propria (LP) mononuclear cells were extracted from intestinal biopsies using previously described techniques [22]. Tissue was rinsed with Hanks' Balanced Salt Solution (HBSS, Cellgro, Manassas, VA), then digested with 1–2 mg/ml of collagenase D (Roche, Nutley, NJ) in RPMI containing 1% penicillin, 1% streptomycin, and 1% glutamine (complete RPMI) supplemented with 0.1% BSA for one to two 60 minute treatments. Released LP mononuclear cells (LPMC) from each treatment were passed through a cell strainer. All released cells ultimately pooled were utilized to perform flow cytometry. We evaluated frequencies of $\gamma\delta$ T-cells (CD3+pan $\gamma\delta$ +V δ 1+/V δ 2+) on intestinal biopsies. The following antibodies were used: anti-CD3-APC-H7, anti-TCR $\gamma\delta$ -FITC, anti-V δ 1-APC, anti-V δ 2-PE, (BD Biosciences, La Jolla, California, USA). LIVE/DEAD Viability Dye (Thermo Fisher) was used to exclude dead cells. Samples were acquired with the Flow Cytometer FACSVerse (Becton Dickinson Italia Spa, Milan, Italy). FCS files were then analyzed with FlowJo software.

$\Gamma\delta$ T-cells subsets were also measured in colon samples from representative HIV-uninfected controls.

The gating strategy for $\gamma\delta$ T-cells is shown in Supplemental Figure 1, <http://links.lww.com/QAD/D58>.

Markers of microbial translocation

Plasma sCD14, endotoxin core antibodies (EndoCAB) and intestinal FABP (I-FABP) were measured by Sandwich Enzyme Linked Immunosorbent Assay (ELISA) (sCD14: R&D Systems, Minneapolis, Minnesota, USA; Minimum detectable dose (MDD) of human sCD14 <125 pg/mL; EndoCAB and I-FABP: HyCult Biotech, Uden, Netherlands; EndoCAB: MMD: 0.13 GMU/ml; I-FABP: MDD: 47 pg/ml) as per the manufacturers' instructions. Circulating lipopolysaccharide (LPS) was assessed using the limulus amoebocyte lysate (LAL) test (Lonza Group L.T.D., Basel, Switzerland; Labeled Lysate Sensitivity: 0.125 EU/ml), in accordance with the manufacturer's instructions.

Total, Unintegrated, and Integrated HIV DNA Quantification

Cellular DNA was isolated from gut biopsies with the protocol for DNA Purification from Tissues (QIAGEN QIAamp DNA Mini Kit) following manufacturers' instructions. Total and unintegrated HIV-DNA forms were simultaneously analyzed by *TotUFSys* qPCR platform using a single set of specific primers selected in the 5' LTR-Gag highly conserved region of HIV-1 genome, as described by Casabianca et al.^[23]. All PCR reactions were carried out in a 7500 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific Inc., Carlsbad, CA, USA) in a final volume of 100 μ L using the the Hot-Rescue Real-Time PCR Kit Sybr Green (Diatheva s.r.l., Cartoceto [PU], Italy)^[24] assay testing 0.5 μ g (at least in duplicate) and 1.0 μ g of cellular DNA. The HIV-1 DNA copy number was estimated by interpolation of the experimentally determined threshold cycle (Ct) on the standard curve (generated from 10^5 to 10, and 2-copy numbers). Values < 2 copies were arbitrarily considered to be 1 for statistical analyses. The amount of integrated HIV DNA was obtained by subtracting the amount of uDNA from the amount of total HIV DNA (tDNA). Total/unintegrated/integrated HIV DNA copy numbers were normalized (as described in^[23-29]) to 1 μ g of cellular DNA (equivalent to 142857 cells)^[30].

Statistical analysis

Descriptive and statistical analyses were performed with GraphPad Prism 5.1 (GraphPad Inc., La Jolla, California, USA). Categorical variables are presented as number of cases and percentages, continuous variables are presented as median values and interquartile range. Categorical variables were analyzed by two-sided Fisher's exact test, continuous variables were analyzed by non-parametric two-tailed Mann-Whitney test or Kruskal-Wallis test where appropriate. Spearman's correlation test was used to correlate peripheral biomarkers and gastrointestinal variables. A P-value < 0.05 was considered statistically significant.

Results

Study population

Fifteen P-HIV (0/15 in Fiebig stage I; 4/15 in Fiebig stages II/III; 11/15 in Fiebig stages IV/V) and 13 C-HIV were studied. No differences were observed between groups in terms of demographics (Table 1), yet the former presented a significantly higher CD4+ T-cell nadir and a trend to higher CD4+ T-cell count at time of analysis as well as a lower prevalence of AIDS-defining events in their clinical history (Table 1).

P-HIV show higher HIV DNA levels in colon biopsies

Significantly higher gut HIV DNA was found in P-HIV compared to C-HIV, with 6290 copies/10⁶ cells [IQR 838.3-11480] in the former and 455 copies/10⁶ cells [IQR 35-1614] in the latter (p=0.027; Figure 1A). P-HIV also presented higher levels of unintegrated HIV-DNA (189 copies /10⁶ cells, [IQR 70.70-556.5] compared to C-HIV (45 copies/10⁶ cells, [IQR 7-84]) (p=0.014) as well as integrated HIV DNA (6069 copies/10⁶ cells, [IQR 731.5-10862] vs 413 copies/ 10⁶ cells, [IQR 28-1477] p=0.022) (Figure 1A).

Of note, a hierarchal distribution of HIV reservoirs was found in colon tissue according to the duration of HIV infection, with the highest levels of total, unintegrated and integrated HIV DNA in P-HIV in early Fiebig stages (II/III) followed by P-HIV in later Fiebig stages (IV/V) and, finally, C-HIV (Figure 1B). In this respect, the comparison between P-HIV in Fiebig II/III and C-HIV was the only one to result in statistical significance for all HIV DNA measures (total HIV DNA: 12513 copies/ug [IQR 6200-21130] vs 455 copies/ug [IQR 35-1614]; p=0.012); unintegrated HIV DNA: 371 copies/ug [IQR 166.3-591.3] vs 45.50 copies/ug [IQR 7-84]; p=0.025; integrated HIV DNA: 12142 copies/ug [IQR 6034-20538] vs 413 copies/ug [IQR 28-1477]; p=0.009) (Figure 1B).

P-HIV and C-HIV feature comparable fibrosis, CD4+ T-cell depletion and neutrophil infiltration in colon biopsies

Having shown higher HIV DNA in the gut of P-HIV compared to C-HIV, we assessed whether the two groups differed in terms of fibrosis, CD4+ T-cell depletion and neutrophil infiltration at mucosal sites. Collagen deposition in the colon of P-HIV was similar to that detected in C-HIV (2, [IQR 1.75–3] vs 3, [IQR 2–3]; p=0.3; Figure 2A, B) as was the mucosal CD4+ T-cell count (P-HIV: 2, [IQR 1-3]; C-HIV: 2, [IQR 1-3.4]; p= 0.8; Figure 2C, D). We also found CD4+ and collagen colocalization (Figure 2E).

Of note, P-HIV displayed a trend to lower CD8 T-cells (1, IQR [1-2] vs 3, [1.5-4]; p=0.05 and higher CD4/CD8 ratio (1.5, [1-3] vs 0.8 [0.3-1.75]; p=0.15) in colon tissue compared to C-HIV (Figure 2D).

We next measured the degree of neutrophil infiltration in colon and found no differences in MPO staining between P-HIV and C-HIV (Figure 2 F, G).

Higher colon CDh-1 expression in P-HIV than C-HIV

Having shown a similar degree of neutrophil infiltration and collagen deposition in the colonic mucosa of P-HIV and C-HIV, we next investigated gut barrier integrity through an IHC study of junctional complex (JC) proteins (CDh-1 and ZO-1).

Both P-HIV and C-HIV displayed CDh-1 and ZO-1 protein expression preferentially located in lateral and basal zones respect to controls (Figure 3A). However, a trend to higher lateral and baso-lateral CDh-1 expression and greater staining intensity was observed in P-HIV compared to C-HIV ($p=0.12$; Figure 3B).

No significant differences were found between groups in terms of ZO-1 expression (data not shown).

Microbial translocation is contained during acute HIV infection

We then asked whether the partial preservation of JCs proteins in P-HIV translated into the containment of microbial translocation from the gut lumen to the systemic circulation.

Overall, measures of microbial translocation and intestinal barrier impairment were lower in P-HIV compared to C-HIV. Indeed, the former displayed significantly lower LPS (188.8 pg/ml [IQR 145.1-234.2] vs 345.8 pg/ml [IQR 216.4-368.3], $p=0.006$) (Fig. 4A), sCD14 (1.58 $\mu\text{g/ml}$ [IQR 1.05-2.208] vs 5.953 $\mu\text{g/ml}$ [IQR 3.95-11.17], $p=0.0002$) (Figure 4B) and I-FABP (186.9 pg/ml [IQR 127.7-406] vs 1210 pg/ml [IQR 367.4-1644], $p=0.03$) (Figure 4C). No differences between groups were found in EndocAb levels ($p=0.1$; Figure 4D).

No statistical correlation was found between plasma biomarkers (IFABP, EndocAb, sCD14) and gastrointestinal variables (collagen deposition, neutrophil infiltration, E-cadherin, mucosal immune subsets) (Supplemental Figure 2, <http://links.lww.com/QAD/D58>).

Mucosal $\gamma\delta$ T-cells are linked with gut damage and microbial translocation

To further comprehend the possible correlates of preserved gut epithelial integrity and reduced microbial translocation in P-HIV, we lastly investigated a subset of mucosal-associated immune cells, V δ 1/V δ 2 $\gamma\delta$ T-cells.

A trend to higher gut total $\gamma\delta$ T-cells (P-HIV: 7.8 [IQR 6.4-10.0]; C-HIV: 5.8 [IQR 4.8-7.0]; $p=0.11$) (Supplemental Figure 3A, <http://links.lww.com/QAD/D58>) and V δ 1 cells (P-HIV: 3.56 [IQR 2.36-3.76]; C-HIV: 2.95 [IQR 1.98-2.77]; $p=0.28$) was observed in P-HIV (Supplemental Figure 3B, <http://links.lww.com/QAD/D58>), yet P-HIV and C-HIV displayed similar V δ 2 (P-HIV: 1.2 [IQR 1.0-1.6]; C-HIV: 1.2 [IQR 1.0-1.7]; $p=0.96$) (Supplemental Figure 3C, <http://links.lww.com/QAD/D58>). Total $\gamma\delta$ T-cells and the V δ 1 subset inversely correlated with collagen deposition ($r=-0.67$, $p=0.06$) and circulating sCD14 ($r=-0.62$, $p=0.09$) (Supplemental Figure 2, <http://links.lww.com/QAD/D58>).

Discussion

GI barrier damage is a pathogenic feature of HIV infection, leading to a “leaky gut” with translocation of microbial bioproducts from the mucosal lumen to the systemic circulation, causing immune activation which is a driver of disease progression in untreated disease^[31]. Combination antiretroviral therapy (cART) only partially reverts such damage^[10-12], and the ongoing alterations within the GI tract continuously fuel microbial translocation and inflammation, thus contributing to non-infectious comorbidities and premature aging in virally-suppressed individuals^[8]. Further, despite significant decreases in total peripheral^[32] and integrated gut HIV DNA following cART initiation in early Fiebig^[33], HIV reservoirs persist in the gastrointestinal mucosa, making viral eradication impossible through antiviral treatment alone.

Understanding when the above-mentioned events precisely occur in the natural course of HIV infection, is of utmost importance in the best framing of HIV pathogenesis and HIV-associated comorbidities.

In the present work we show higher tissue reservoirs in early HIV infection, yet similar neutrophil infiltration and collagen deposition to those observed in chronic HIV infection. In contrast, plasma microbial translocation markers were significantly lower in the former.

We first evaluated the HIV reservoir in P-HIV and C-HIV through the study of total, unintegrated and integrated HIV DNA forms. Total HIV DNA allows for the simultaneous quantification of all forms of HIV DNA in infected cells which play a different role in HIV pathogenesis^[34]. Indeed, the study of unintegrated and integrated HIV DNA provides insights into the dynamics and composition of the HIV reservoir^[24, 35-38], however, these are not regarded as standard markers of the reservoir.

We report significantly higher HIV DNA levels in P-HIV compared to C-HIV. Given the normalization of HIV DNA copy numbers to 1 µg of cellular DNA and the finding of similar colon CD4+ T-cell counts in P-HIV and C-HIV, our results point to increased reservoirs in the earliest stages of acute HIV infection which appear to be driven by individuals in Fiebig II/III. This is in partial disagreement with previous work demonstrating significantly lower gut reservoirs in the earliest Fiebig stage than later P-HIV stages/chronic infection^[33] and may be explained by the absence of individuals in Fiebig I and the small sample size in our study. Of note, both studies show that the HIV reservoir in Fiebig IV/V resembles that of chronic HIV^[33].

We then studied the expression of myeloperoxidase and Masson staining and show comparable colon neutrophil infiltration and fibrosis in C-HIV and P-HIV. Our IHC finding of collagen and CD4+ co-localization is in line with previous work revealing that the disruption of lymphoid tissue architecture is linked to CD4+ T-cell loss in all stages of HIV infection^[39-41], as it suggests that gut fibrosis may be a contributing mechanism of CD4+ T-cell depletion. Of note, P-HIV displayed a trend to lower CD8 and higher CD4/CD8 ratio in the gut, pointing to better gut mucosal immune competence.

HIV-associated gut dysfunction accounts for chronic low-grade inflammation which is linked to metabolic and chronic diseases^[42]. While not equivocal in all studies^[43], convincing evidence indicates a role for microbial translocation in disease progression in PLWH. Indeed, sCD14 and I-FABP resulted independent predictors of all-cause mortality in treated HIV infection^[44] and were associated with adiposity^[45, 46]; similarly, LPS predicted HIV disease progression in cART-naïve individuals^[47] and was linked to insulin resistance^[48, 49]. EndocAb have also been assessed in the study of HIV pathogenesis^[31] as their levels increase as part of the normal humoral response to antigenic stimulation (i.e. gut-derived microbial products chronically present in the systemic circulation)^[31].

Despite comparable gut lymphoid architecture, microbial translocation markers were significantly lower in P-HIV compared to C-HIV. This is in keeping with reported findings of lower LPS and 16s rDNA in P-HIV^[5, 17] and may reflect the timing of microbial translocation which has been shown to occur in later stages of HIV/SIV infection^[5, 17, 18]. Because the passage of microbial bioproducts from the gut to the systemic circulation is a consequence of increased mucosal permeability, we assessed whether differences in gut barrier integrity could explain our findings, demonstrating that the expression of E-cadherin, a protein of the gut junctional complex^[50], was altered in HIV-infected individuals compared to uninfected controls, with a trend to higher expression in P-HIV than C-HIV. Thus, the reported hierarchical distribution of gut junctional proteins may partially explain why microbial translocation is controlled in primary HIV infection^[17]. In addition, our findings shed light on the sequence of events in the HIV-driven pathogenic cascade showing that gut damage may follow reservoir establishment yet precede microbial translocation. It must be noted that Zonula Occludens 1 expression in the colon showed comparable levels in P-HIV than C-HIV, suggesting that gut barrier dysfunction is, tantamount to the other structural abnormalities described above, an extremely rapid-evolving process in primary HIV infection.

Mucosal immunity also plays a pivotal role in the regulation of gut barrier function and control over microbial translocation^[2]. We hereby show that mucosal V δ 1 cells were lower in P-HIV compared to uninfected controls, yet higher than C-HIV, suggesting their progressive depletion during untreated HIV infection. Together with our results of a negative correlation between gut $\gamma\delta$ T-cells and sCD14/collagen, our findings suggest that these cell subsets may hinder microbial translocation through the maintenance of gut architecture.

Limitations of the present work include the exploratory nature of the study, due to the small sample size and the multiple comparisons performed without adjustment.

In conclusion, in primary HIV infection we show increased tissue reservoirs, impairment of the gut epithelial barrier and mucosal architecture with neutrophil infiltration, yet containment of microbial translocation which may depend on the partial preservation of E-cadherin 1 and mucosal $\gamma\delta$ T-cells in early HIV infection.

Acknowledgements

Author contributions

Camilla Tincati cared for and enrolled study subjects, analysed and interpreted the data, designed the figures and wrote the manuscript. Elvira Stefania Cannizzo performed the experiments, analysed and interpreted the data, designed the figures, and drafted the manuscript. Valeria Bono analysed data and designed the figures. Delfina Tosi and Federica Savi performed the experiments on gut tissue, analysed data and drafted the manuscript. Camilla Falcinella and Matteo Augello cared for and enrolled study subjects. Anna Casabianca and Chiara Orlandi performed HIV DNA on gut tissue. Carmelo Luigiano performed colonoscopies. Stefano Rusconi, Antonio Muscatello, Alessandra Bandera, Andrea Calcagno, Andrea Gori and Silvia Nozza coordinated the INACTION Study group and edited the manuscript. Giulia Marchetti conceived and designed the study, interpreted the results and wrote the manuscript.

Italian Network of ACuTe HIV InfectiON (INACTION): Nicola Squillace (Monza); Giuseppe Tambussi, Marco Ripa, Raffele Dell'Acqua (H San Raffaele Milano); Andrea Antinori, Carmela Pinnetti (INMI Spallanzani Roma); Gianfranco Orofino, Ilaria De Benedetto, Micol Ferrara (Torino); Cristina Mussini, Vanni Borghi, Federica Carli (Modena); Benedetto Maurizio Celesia (Catania); Lucio Cosco, Carlo Torti (Catanzaro); Gabriella d'Ettorre (Umberto I Roma); Antonio Di Biagio (Genova); Emanuele Focà, Eugenia Quiros-Roland (Brescia); Antonina Franco (Siracusa); Diego Ripamonti, Franco Maggiolo (Bergamo); Roberto Gulminetti, Massimiliano Fabbiani (Pavia); Sandro Piga, Marzia Garau, Marco Campus (Cagliari); Tiziana Formenti, Sonia De Rose, Alessia Lai (H Sacco Milano); Antonella Cingolani (H Gemelli Roma); Giordano Madeddu (Sassari).

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Figure 1. HIV DNA quantification in gut biopsies. HIV DNA copies per 10^6 cells were quantified in gut biopsies from P-HIV (n=10) and C-HIV (n=8) (A) and according to Fiebig stage (B; Fiebig II-III, n=4; Fiebig IV-V, n=6). Red dots indicate P-HIV individuals in Fiebig stages II-III.

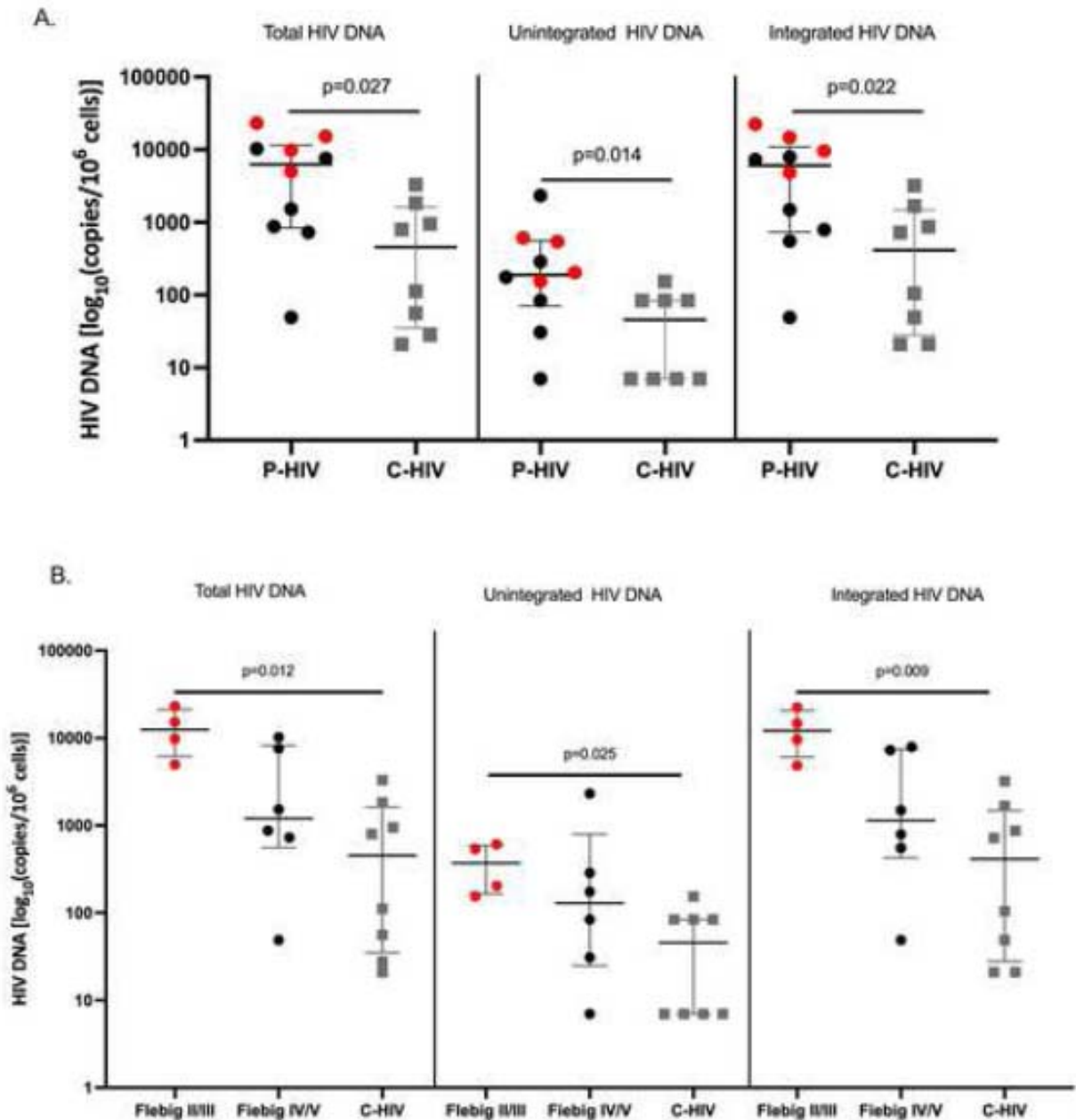


Figure 2. Collagen deposition, T-cell counts and neutrophil infiltration in gut biopsies. Representative sections from lamina propria of Healthy Control (HC, left), P-HIV (middle) and (C-HIV) (right) were stained with trichrome to identify collagen fibers (A). Collagen deposition was higher in the GALT of HIV- infected, compared to HC individuals. Magnitude of fibrosis (blue staining intensity) was determined by using a semiquantitative score and the mean percentage area that stained positive is shown in B (P-HIV, n=10; C-HIV, n=5). The dotted line indicates the median value in representative HIV-uninfected controls (n=5). Sections of colon biopsy from HC (left) and P-HIV (middle) and C-HIV (right) individual were stained with antibodies against CD4 and images were captured for quantitative image analysis (C). CD4 and CD8 T-lymphocytes were determined by number of cells in 3 HPF of gut lamina

propria; the CD4/CD8 ratio was calculated by dividing CD4 by CD8 (D) (P-HIV, n=10; C-HIV, n=5). Representative zoom showing CD4+ (left, brown) and collagen colocalization (right, blue) in a representative C-HIV (E). Representative sections from lamina propria of HC (left), P-HIV (middle) and (C-HIV) (right) were stained to detect neutrophil infiltration by myeloperoxidase (MPO) (F). Magnitude of neutrophil infiltration (brown staining intensity) was determined by using a semiquantitative score and the mean percentage area that stained positive is shown in G (P-HIV, n=10; C-HIV, n=5). The dotted line indicates the median value in representative HIV-uninfected controls (n=5).

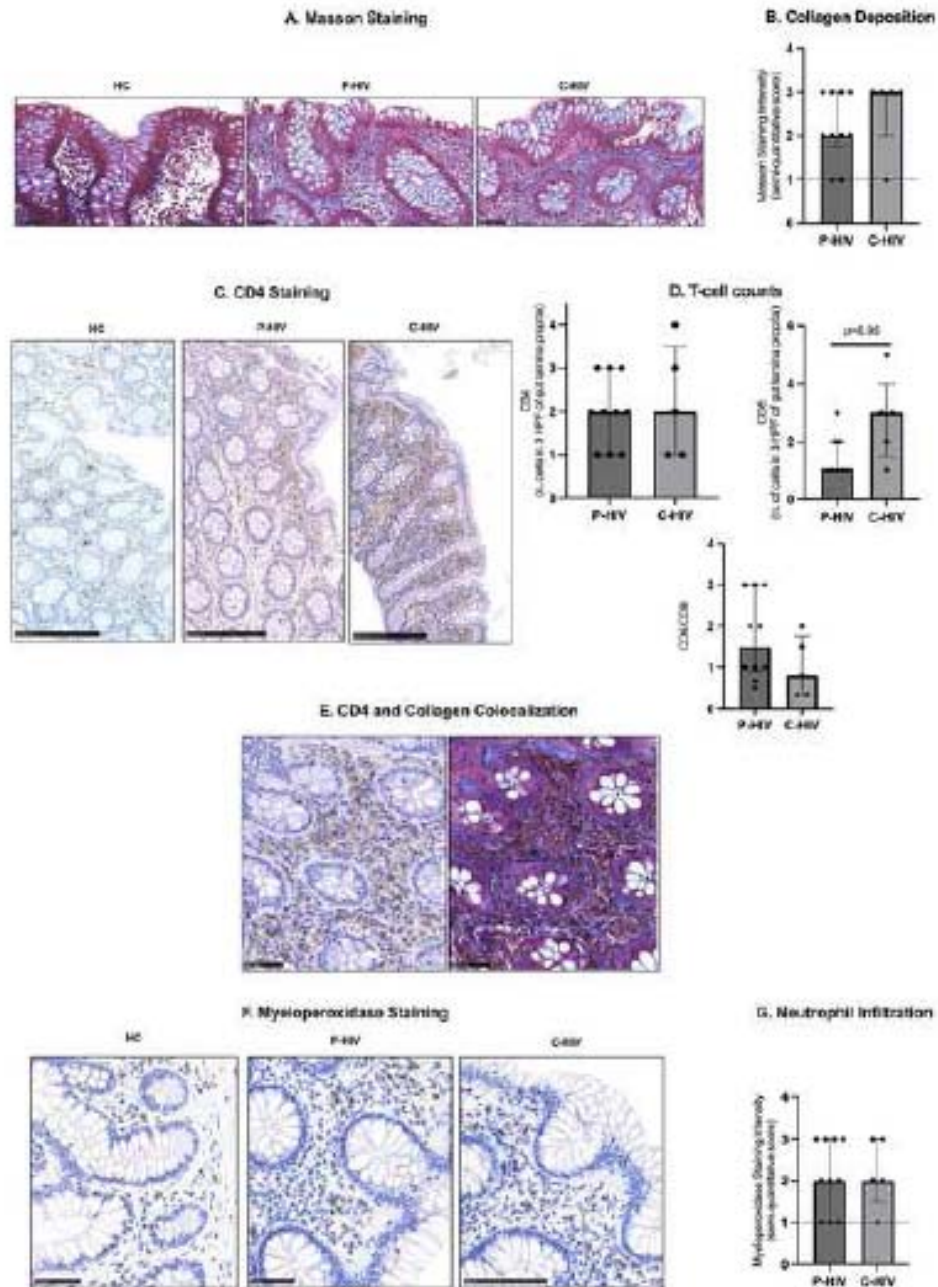


Figure 3. Tight junction protein expression in colon tissue of P-HIV and C-HIV. Representative sections from lamina propria of a Healthy Control (HC, left), P-HIV (middle) and C-HIV (right) were stained to identify E-cadherin (Cdh1, brown) (A). The mean percentage area that stained positive for Cdh1 is shown in B (P-HIV, n=10; C-HIV, n=5). The dotted line indicates the median value in representative HIV-uninfected controls (n=5).

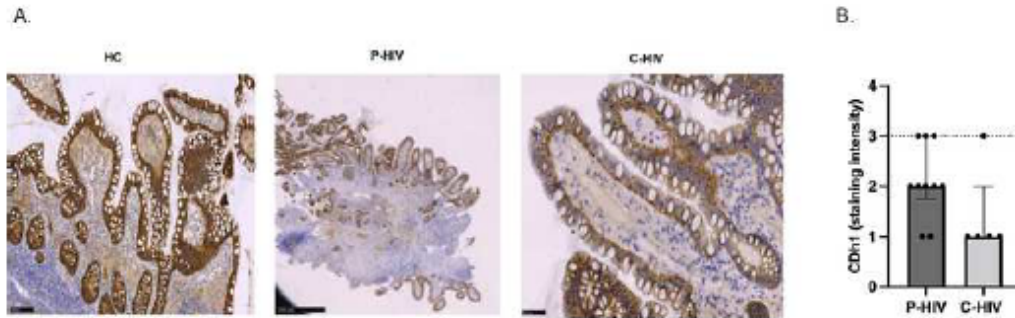


Figure 4. Markers of microbial translocation and intestinal damage in plasma. Markers of microbial translocation (LPS, sCD14, EndoCAb) and intestinal damage (I-FABP) were measured in plasma samples from P-HIV (n=8) and C-HIV (n=8) (A-D). The dotted line indicates median values in representative HIV-uninfected controls (n=5).

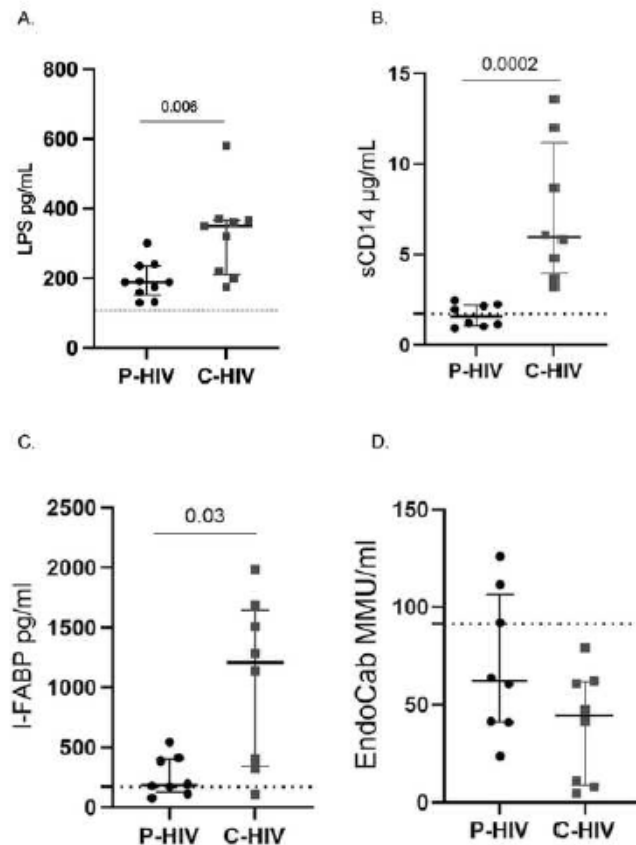


Table 1. Epidemiological, Clinical and Immunological Characteristics of the Study Groups

	P-HIV (N=15)	C-HIV (N=13)	p value
Age [years], median (IQR)	42 (30.5-47.5)	41 (31-60)	0.52
Male sex, n (%)	13 (87)	11 (85)	1
Risk Factors for HIV infection			
Heterosexual, n (%)	5 (33)	4 (31)	0.20
MSM, n (%)	9 (60)	6 (46)	
IDU, n (%)	1 (7)	0	
Other/unknown, n (%)	0	3 (23)	
HBV/HCV co-infection, n (%)	2 (13)	0	0.48
Nadir CD4+ count [cell/mm³], median (IQR)	474 (330-576)	237 (50-422)	0.0074
CD4+ count [cell/mm³], median (IQR)	518 (359-662)	378 (176-508)	0.060
CD4%, median (IQR)	25 (20-32)	17 (16-24)	0.067
CD8+ count [cell/mm³], median (IQR)	1060 (778-1532)	835 (717-1013)	0.17
CD8% at colonoscopy, median (IQR)	46 (42-60)	53 (48-64)	0.47
CD4/CD8 ratio, median (IQR)	0.67 (0.36-0.74)	0.34 (0.24-0.44)	0.12

HIV-RNA [log10 copies/mL], median (IQR)	5.1 (4.4-6.4)	5.4 (2.8-5.4)	0.19
Fiebig stage, n (%)			
I	0	N/A	/
II/III	4 (27)	N/A	
IV-V	11 (73)	N/A	
AIDS-defining conditions or nadir CD4 cell count <200/mm³, n (%)	1 (7)	6 (46)	0.029

Note: data regarding continuous variables are presented as median (interquartile range; IQR), statistical analyses: unpaired t-test, while data regarding discrete variables are presented as absolute numbers (%), statistical analyses: Fisher's exact test or Chi-square test, as appropriate. N/A: not applicable; MSM: men who have sex with men; IDU: intravenous drug use.

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