UNIVERSITÀ
DEGLI STUDI
FIRENZE

DOTTORATO DI RICERCA IN

SCIENZE CLINICHE

CICLO XXXV

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"Microbiota composition and systemic inflammation in HIV+ patients of different ethnicities"

Settore Scientifico Disciplinare MED/46

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Anni 2019/2022

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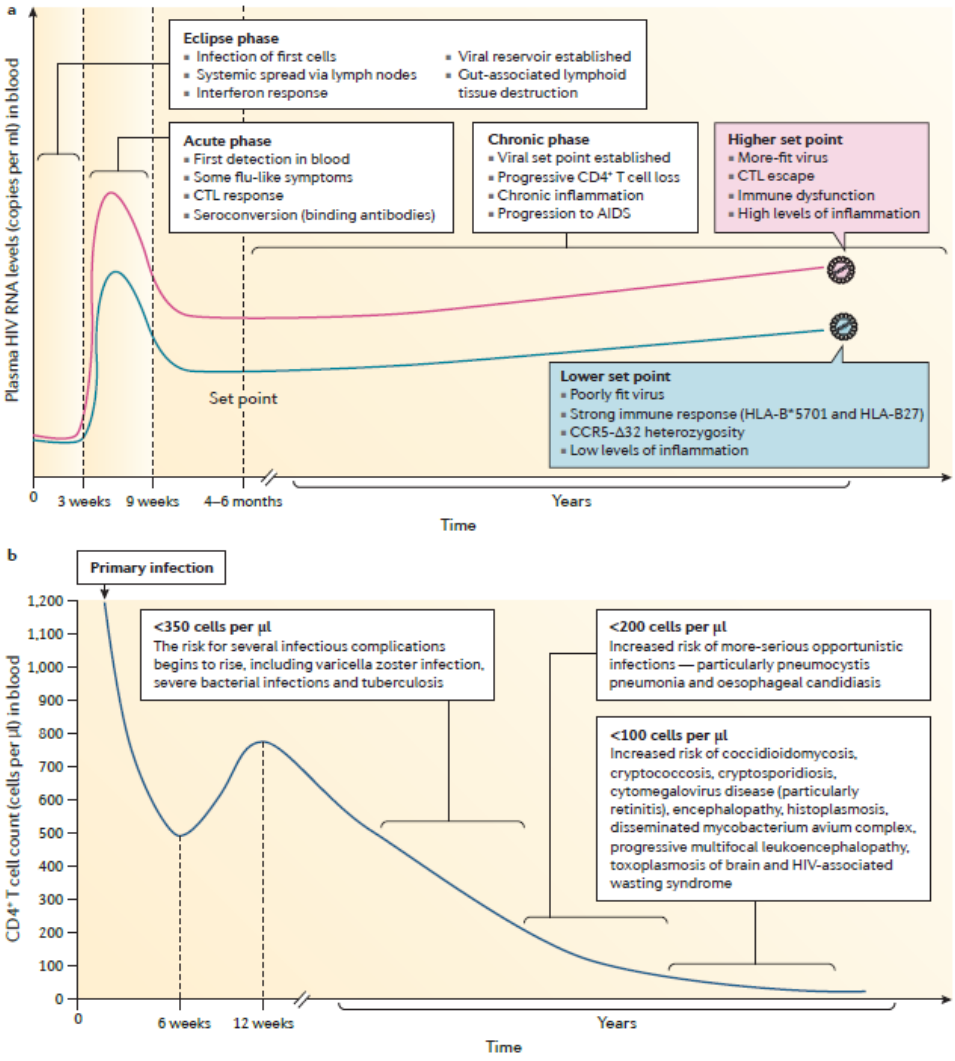
Introduction

1. HIV

The human immunodeficiency virus (HIV) infection probably spread from non-human primates to humans sporadically throughout the 1900s[1,2]. However, it wasn't until the 1980s that the virus became well known. Two years after the discovery of what would later be referred to as acquired immune deficiency syndrome (AIDS), researchers found the causing virus, HIV[3,4]. Currently, the HIV infection is one of the main causes of morbidity and mortality worldwide[5], focused especially in sub-Saharan Africa. HIV primarily infected $CD4^+$ T cells. After a transmission event, HIV spreads the mucosal tissues, and within days reaches the lymphoid organs. At about day, the virus becomes detectable in the blood and then continues to spread exponentially over the next few weeks, when anti-HIV antibodies levels become detectable, approximately in 30 days. HIV results in a gradual decrease of $CD4^+$ T cells and a variety of immunological abnormalities through multifaceted and still poorly understood processes[6,7]. After some years, significant immunodeficiency develops, followed by infectious or oncological manifestations (**Figure 1**). This last phase is called AIDS. Generally, the disease progresses to death in about 10 years, however in some patients it progresses more rapidly while in others it may never progress or very slowly[8]. For almost 20 years, HIV infection has been treated with antiretroviral therapy (ART). When properly administered, ART is very successful at improving immune function, suppressing HIV replication almost completely, and significantly lowering the chance of becoming AIDS[9].



Deeks, S., Overbaugh, J., Phillips, A. et al. HIV infection. Nat Rev Dis Primers 1, 15035 (2015). https://doi.org/10.1038/nrdp.2015.35

Figure 1 | HIV infection and AIDS. a | During prototypic HIV infection, the transmitted virus first infects target cells in mucosal tissues and then spreads through the lymphoid system (eclipse phase). HIV RNA levels first become detectable after several days and then increase exponentially, reaching a peak a few weeks later, at which point the adaptive immune response results in partial control. HIV antibody responses are largely ineffective owing to rapid viral escape. A steady-state level (set point) of viremia, reflecting complex virus–host interactions, is then established. HIV-mediated destruction of CD4+ T cells leads to immunodeficiency and chronic inflammation. **b |** The typical CD4+ T cell count in an adult is typically between 500 and 1,200 cells per µl. As the CD4+ T cell number declines to <350 cells per µl, the risk for several infectious complications begins to rise, leading to more-advanced disease (CD4+ T cell count <100 cells per µl). Indeed, HIV-associated immunodeficiency increases the risk of Kaposi sarcoma, certain lymphomas and invasive cervical cancer. The US Centers for Disease Control and Prevention defines AIDS on the basis of the presence of HIV infection and either a CD4+ T cell count of <200 cells per µl or an AIDS-defining complication249. CCR5, CC-chemokine receptor 5; CTL, cytotoxic T lymphocyte; HLA, human leukocyte antigen.

1.1 Epidemiology

The highest incidence of HIV prevalence is found in some people groups who have common risk factors in almost all world parts. These key affected populations include homosexual men, intravenous drug users and other closed settings, sex workers and transgender people. Some of these groups, such as drug users or sex workers, has complicated legal and social problems connected to their lifestyle that make them more susceptible to contracting HIV, and prevent their access to resources for prevention and treatment. As previously reported, the prevalence of HIV infection is widespread throughout the adult population in various Sub-Saharan Africa, where the illness load is disproportionately heavy women[10]. More transmission incidents occurred from heterosexual transfer from a partner whose HIV status was unknown or unreported[11]. The countries with highest burden are Swaziland, Lesotho, Botswana and South Africa that is the country with the highest number of HIV-positive people. Less than half of the HIV-infected population is receiving ART despite significant public health investment in almost world countries. Several barriers at various steps of the care pathway prevent full ART implementation, including limited availability and uptake of HIV testing, as no more than half of people with HIV infection have been diagnosed[10].

In Italy, in 2020, the HIV incidence was equal to 2.2 new diagnoses per 100,000 residents. Tuscany, according to the latest data published by the Istituto Superiore di Sanità, continues to have a higher incidence rate than the national one and ranks fifth among the regions. Among the cases diagnosed in Tuscany in the period 2018-20, 187 (34.1% of the total) concern the immigrant population: the most frequent foreign nationalities are Brazil, Peru and Nigeria[12] (**Figure 2**). Notably, little is known about the access of migrants to health services, in particular the HIV test, access to treatment and safer sexual practices. Suboptimal adherence to the ART potentially related to socio-economic factors might explain the slightly lower viral suppression among foreigners. Previously it had been hypothesized that most of the HIV diagnosed among migrants in Europe was acquired in the native country. However, it is unclear whether infections are acquired before or after migration[13]. Limited data are available on migrant populations

to inform policies and practices for these communities, and also to date there are not many studies focused on the microbiota of HIV+ migrants.

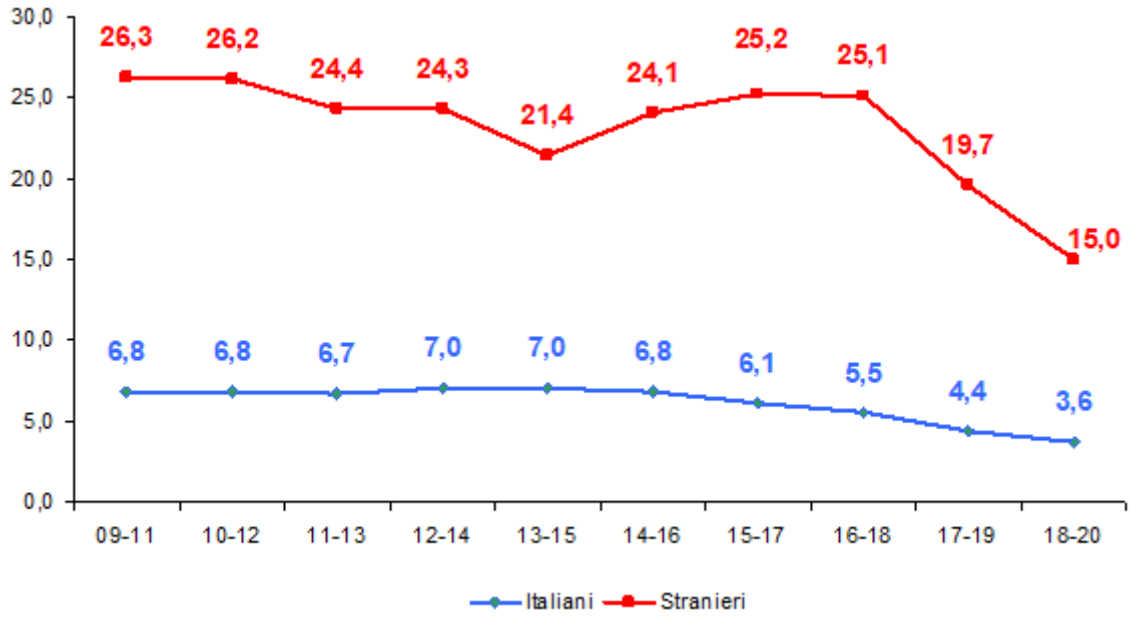


Figure 2. HIV notification rate (per 100,000 residents) by citizenship and year of diagnosis. 2009-2020 years

1.2 Pathophysiology

1.2.1 Virus life cycle

Being a retrovirus, HIV is able to integrate its DNA into the host genome, making it extremely challenging to treat and to eradicate with current medicines. After entering the cell, single-strand RNA is reverse transcripted into HIV DNA, which is then integrated into the host DNA. With the employment of host enzymes, HIV is transcripted, proteins are produced and cleaved, and mature virions are released. The receptor for HIV-1 is the CD4, which is expressed on the surface of monocytes, macrophages, dendritic cells and especially T lymphocytes. In order to be able to enter

cell, HIV also needs a co-receptor, typically the chemokine receptors CCR5 and CXCR4. CCR5 is expressed at high levels in memory T lymphocytes but not on naive T cells, while CXCR4 is expressed on both; in addition, CCR5 is expressed on macrophages and dendritic cells (DC). HIV infects preferentially activated T $CD4^+$ cells which are more permissive to infection than resting cells; on the contrary DC are difficult to infect, therefore they are able to capture and present the virus to neighbouring T lymphocytes[14]. In addition, HIV causes lymphoid tissue fibrosis through several mechanisms, including upregulation of T regulatory cells (Tregs) and release of transforming growth factor-β (TNF- β). Tissue fibrosis persists during long-term effective ART[15]. Much of the harm associated with the virus in both untreated and treated disease probably occurs in these lymphoid structures[16].

1.2.2 Infection and transmission

Much of our knowledge about the starting events of infection comes from studies of the related simian immunodeficiency virus (SIV) infection in macaques. These studies show that productive infection of $CD4^+$ T cells can be detected within the first 2 days of viral challenge. The virus are able to create local foci at the site of infection and then spreads to lymph nodes and other tissues[17,18,19]. During the course of HIV infection, the transmitted viruses evolve, presumably in response to changes in target cell population and immune response to the virus. In some patients, the virus population evolves from CCR5-tropic to CXCR4-tropic[20].

1.2.3 Acute infection

Acute HIV infection is the earliest stage of HIV infection, and it generally develops within 2 to 4 weeks after infection with HIV. The detection of virus in the blood (typically measured as viral RNA levels) is often associated with a short symptomatic phase marked by fever, generalized lymphadenopathy, a nonspecific rash, myalgias and/or malaise. In this stage of acute infection, plasma levels of HIV RNA are often at their highest (approximately 10^6-10^7 copies per ml). The levels of virus decrease when

the immune response develops to a steady-state level often defined viral set point. This level can range from very few copies per ml of blood to approximately 10^6 copies per ml. Notably, the level of the set point is connected with the clinical outcome: people with high viral load set points generally develop AIDS and die more quickly.

1.2.4 HIV reservoir

HIV develops quiescent (or latent) infection within memory $CD4^+$ T cells via mechanisms that are still poorly understood[21]. HIV can also infect long-lived cells such as naive $CD4^+$ T cells, monocytes, macrophages, and possibly other long-lived cells. As long as the cell survives after HIV DNA has been incorporated into the host chromatin, the virus can restart reproduction[22,23]. ART can prevent new cells from becoming infected, but these drugs cannot eradicate infection once the viral DNA is successfully integrated into its target cell. Replication is completely inhibited by ART, therefore replication seems to occur at low levels in at least a patients subset [24,25]. Antiretroviral drug penetration is more difficult in the lymph nodes, which represent a perfect habitat for HIV[26]. This reservoir is also the source of virus recrudescence among effectively treated individuals who interrupt or stop ART.

1.2.5 $CD4^+$ T cells decline

During the primary infection, a transient reduction in peripheral $CD4^+$ T lymphocytes' count can often be detected, but these cell counts usually return to near-normal levels after the prime infection resolves and then slowly decline over many years. Gut-associated lymphoid tissue (GALT) is the preferential seat of HIV replication and $CD4^+$ cells death. The severe depletion of T cells due to high levels of HIV replication seems to remodelling the intestinal mucosa make it much permeable, producing a systemic bacterial products' translocation which leads to increased immune activation[27]. The frequency of activated and proliferating $CD4^+$ and $CD8^+$ T cells, many of which are doomed to death even in the absence of infection, increases dramatically and persistently with HIV infection. Different mechanisms are involved in depletion of $CD4^+$ cells

caused by HIV. HIV replication can be directly cytopathogenic, although this is unlikely to account for all cell death, particularly in chronic infection. The HIV infection of T lymphocytes can generate incomplete reverse transcripts (HIV DNA), which stimulates an intense inflammatory response and death of local uninfected cells[28]

1.3 Diagnosis and screening

HIV is transmitted through contact of infected body fluids with mucosal tissue, blood or broken skin. Higher levels of virus in plasma[29] or in genital secretions[30] are factors that augment the infectiousness of a HIV infected individuals. Some virus characteristics, such as higher envelope content, increased cell-free infectivity, increased interaction with dendritic cells and resistance to IFN-α have been associated with increased infectivity. The HIV susceptibility is also influenced by social and structural factors, which may potentially contribute to some of the variations in HIV prevalence and incidence among different groups.

HIV testing algorithms generally have changed over time, as test accuracy has increased. Current US Centers for Disease Control and Prevention and European guidelines for HIV testing[31] recommend that screening be performed with an antigen–antibody assay (these assays are considered to be fourth generation). Positive results must be confirmed with an antibody assay that differentiate between HIV-1 and HIV-2. In order to prevent HIV transmission, it is necessary to detect acute infection, which seems to contribute in a remarkable way to new infections[32,33]. The timely administration will decrease early HIV infection symptoms, possibly minimize the seeding of viral reservoirs, and protect the health of the newly infected patients while lowering the risk of transmission to uninfected partners. Rapid HIV testing, using blood from a finger-stick or collection of oral fluid, can provide HIV-1 infection test result within 30 minutes, however most currently licensed rapid tests have limited sensitivity for detecting acute HIV infection. More-sensitive fourth-generation antigen–antibody tests and/or nucleic acid tests should be included when risk factors are suggestive of acute infection.

1.4 Treatment

The development of combination ART is one of the best achievements of modern medicine. The current combination regimens reduce the level of viremia by several orders of magnitude in a matter of weeks when administered to motivated people. ART is not always completely successful. Drug resistance is still a serious issue that has to be addressed. Even if durable viral suppression is achieved, many individuals fail to restore optimal immune function, even after several ART years.

Currently, a combination of antiretroviral therapy (cART) has proven to be efficient in governing viral replication (**Figure 3**), significantly dropping the risk of transmission and prolonging life expectancy in infected patients, thus decreasing morbidity and mortality.

- **Non-nucleoside RT inhibitors (NNRTIs):** can bind and block the reverse transcriptase subunit of HIV, inhibiting its replication and spreading.

- **Nucleotide RT inhibitors (NRTIs):** act on the HIV RT competing with the host's nucleosides, influencing the nucleotide addition by RT during the DNA polymerization and therefore blocking the formation of new cDNA molecules.

- **Protease inhibitors (PIs):** are able to block HIV-1 protease enzyme, essential for the correct maturation of gag polyprotein, resulting in the production of defective and un-infectious viral particles.

- **Fusion Inhibitors (FIs):** can contrast the HIV-1 fusion end viral entry to the cells

- **CCR5 antagonists:** are able to compete with HIV-1 entrance through the binding of one of its co-receptors.

- **Integrase inhibitors (INIs):** act directly on a viral protein, thus reducing the potential side effects. The drug is able to inhibit the integrase enzyme and consequently the viral integration in the host DNA

- **Post-Attachment Inhibitors:** are able to block the interaction of gp120 and the host co-receptor.

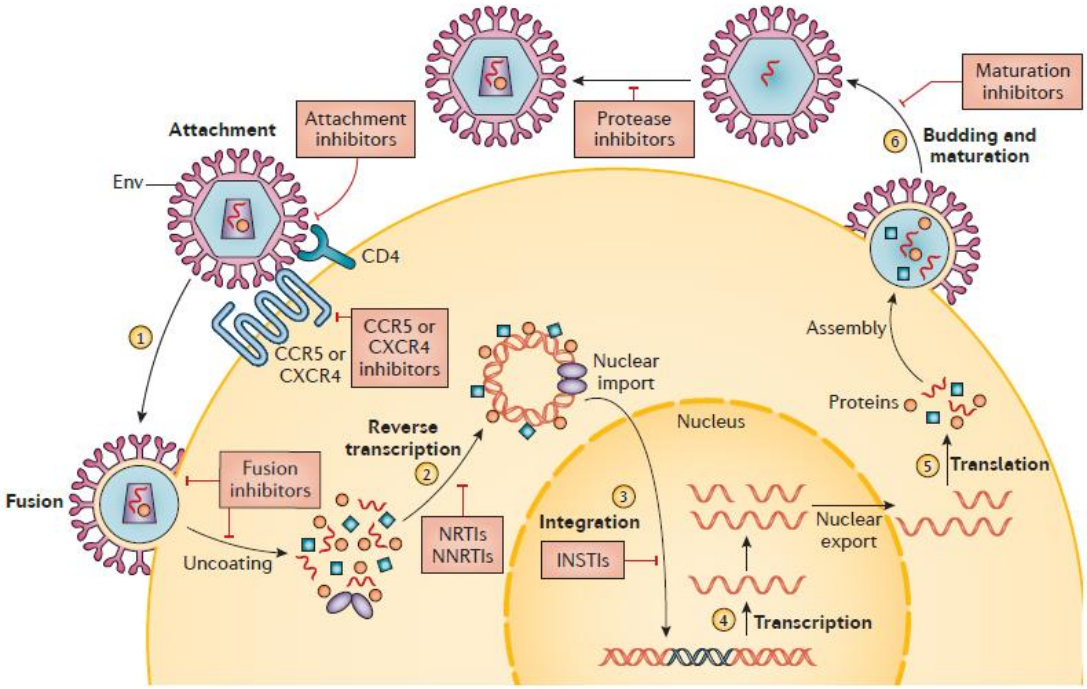


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Figure 3 | The HIV life cycle. HIV enters its target cells via CD4 and either CC-chemokine receptor 5 (CCR5) or CXC-chemokine receptor 4 (CXCR4) through interaction with envelope (Env) glycoprotein (step 1). After fusion and uncoating, the viral RNA is then reverse transcribed into DNA (step 2). The ensuing pre-integration complex is imported into the nucleus, and the viral DNA is then integrated into the host genome (step 3). Mediated by host enzymes, HIV DNA is transcribed to viral mRNAs (step 4). These mRNAs are then exported to the cytoplasm where translation occurs (step 5) to make viral proteins and eventually mature virions (step 6). Each step — HIV entry, reverse transcription, integration and protein maturation — in the HIV life cycle is a potential target for antiretroviral drugs251. INSTI, integrase strand transfer inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor

2. Role of microbiota in immunomodulation

At least two orders of magnitude more genes than the human genome are present in the collective genomes of microbiome members. While the human genome is thought to contain 42,611 genes, only about half of them are estimated to be protein-coding[34], the size of the microbial genome on the examination of gut microbiome metagenomes identified 22,254,436 non-redundant consensus genes[35]. Additionally, the collective microbiota genome adjusts in response to outside influences (such as a change in food) and evolves under the impact of both positive[36] and negative[37] selects, whereas the genome of the host remains constant throughout its lifetime. Beyond just metabolic connections, co-evolutionary processes have created complex interdependencies between the host physiology and microbiota. To maintain homeostasis and decrease the inflammation, the host immune system must engage with the microorganism in a regulated manner[38]. The host-microbiome mutualism is fundamentally based on cooperative behaviour between these two ecosystems, in which one's activities depend on another's, with homeostasis as the ultimate aim. Because of the close connection between the immunity and the microbiota, in particular the gut microbiota, any dysbiosis causes changes in the mucosal immunological response, which can result in the starting of different disorders[39]. The interaction with the microbiome mediates robustness and tenability in the immune network. The idea of the microbiome as an additional organ of the human body effectively captures this self-reinforcing mutualistic host-microbiome coevolution[40]. The immune system is made up of a highly organized collection of innate and adaptive cells, both of which have a high ability for responding to various stimuli and develop memory to combat reinfections. Additionally, due to the use of antibiotics, dietary changes, and a decrease in intestinal parasite illnesses (such as intestinal worms) caused by mutualistic helminths, our microbial allies have evolved into potential foes[41]. Essentially, there are two types of immune responses: innate, which is inherited by an organism, and adaptive, which is learned by exposure to various infections or diseases. The establishment of a complex microbiome coincides with the defined development of the immune system, particularly its adaptive compounds,

reflecting a developing mechanism to maintain a symbiotic interaction with these microorganisms. Microbes therefore influence and regulate the immune system in all aspects[42].

2.1 Microbiota and innate immune system

The intestinal lining is protected by the mucosal immune system, which is made up of lymph nodes, lamina propria, and intestinal epithelial cells (IECs). This mucosal immune system also keeps track of the microbes in the gut[43]. IECs have a significant role in the mucosal innate immune system both in health and illness. The border that divides the host from the microbiota and the environment is formed by a single cell layer of IECs. As a result, IECs must function as both a physical barrier separating self from others and a conduit for the bidirectional communication between the host immune system and the microbiome. Intestinal epithelium is functionally distributed into distinct niches that extend from the top of villi to the bottom of the crypts, despite the single-cell layer arrangement. The bottom of the crypts serves as a niche for LGR5+ intestinal stem cells (ISCs), early progenies of ISCs, as well as Paneth cells that secrete antimicrobial proteins (AMPs) into the lumen, preventing most bacteria from reaching the ISCs niche[44]. Innate immune receptors and the identification of metabolites linked to the microbiome allow IECs to detect (directly and indirectly) the microbiota composition. In particular, the depletion of Toll Like Receptor (TLR) signalling elements such as MyD88[45], TRAF6[46] or whole-body knock-out of NOD2[47], lead to activation of small intestinal adaptive immune cells[45] or enhanced susceptibility in models of intestinal injury[46,47]. In this context, considering that an inadequate innate immune signalling brings to a lower amount of Paneth cells and consequently lower luminal concentration of AMPs and decreased mucin production by goblet cells, more bacteria are able to come into direct contact with the epithelium, promoting mucosal inflammation[48]. IECs perform the function of non-classical antigen presentation cells for microbial components in addition to strengthening the mucosal barrier by the release of AMPs and mucin. IECs in the small intestine capture luminal antigens[49] that ,after

digestion [50], present to the T lymphocytes via MHC class II (MHC-II)[51,52]. Also, LGR5+ ISCs in the crypt express MHC-II presenting $CD4^+$ T cells luminal antigen, which in turn controls the rate of ISCs' differentiation. IFN-γ, IL-17A, and IL-13, pro-inflammatory cytokines, secreted from $CD4^+$ T cell stimulate active differentiation of ISCs, whereas regulating IL-10 attenuates differentiation and encourages self-renewal. Antigen sampling by IECs helps in the selection of inflammatory bacteria to be targeted by humoral and cellular immune responses, which actively control the microbiome composition in addition to altering the regenerative or barrier properties of the epithelium. Indeed, enterocytes sample antigens of mucosa-attaching bacteria such as segmented filamentous bacteria (SFB) by employing clathrin-independent, dynamin-dependent cell division control protein 42 (CDC42) mediated endocytosis[53]. Such microbial adhesion-triggered endocytosis (MATE) results in the induction of a SFB antigen-specific mucosal Th17 response, suggesting a mucosa-cleansing role of MATE[53]. The M cells, a specialized type of absorptive IECs, sample luminal antigens and simultaneously help to mediate the maturation of naïve B cells into IgA-producing plasma cells[54] targeting inflammatory disease-causing bacteria[55] as well as the induction of luminal pathogen-specific Th1, Th17, and Th22 responses[56]. Similar to M cell-mediated induction of cellular responses, goblet cells mediate goblet cell-associated antigen passage (GAP), which supplies luminal antigens to tolerogenic $CD103^+$ DCs in the lamina propria, thereby inducing differentiation of regulatory T (Treg) cells in the steady state. Successful clearance of pathogens requires a swift transition from a tolerant state of the immune system to an inflammatory one. In agreement with this paradigm, tolerogenic GAPs are inhibited during a Salmonella infection, thus allowing an inflammatory response to develop[57].

IECs are also able to recognized and use the microbiota metabolites. In the colon, anaerobic commensal bacteria digest dietary fiber and produce short-chain fatty acids (SCFAs) as by-products. SCFA protect the epithelial barrier by modulating the epithelial cell survival, the mucus layer and the expression of tight junction proteins[58]. Moreover, colonocytes utilized SCFAs as an energy source via the pathway of mitochondrial fatty acid β-oxidation. Due to antibiotic intake, that depleted SCFA producers, colonocytes' metabolism shifts toward glycolysis, which is characterized by low oxygen consumption

and the activation of nitric oxide synthase[59]. The presence of oxygen and nitrate made a perfect environment for facultative anaerobic pathogens such as Escherichia coli[60]. At various stages of cellular development and in different organs, the microbiota affects the growth and function of myeloid cells. The reduction in myeloid-cell growth in the bone marrow that occurs when the microbiota is missing causes the clearance of systemic bacterial infection to be delayed[61]. The SCFAs generated from microbiota may also stimulate myelopoiesis in the bone marrow[61,62]. Antibiotic treatment during pregnancy reduces the number of blood neutrophils and their precursors in the bone marrow in the offspring of mice, while gestational colonization with microbes increases the number of intestinal mononuclear cells in new-born mice[63]. After haematopoiesis, the microbiome also affects the development of myeloid cells. The constant presence of TLR ligands, produced from the microbiome, hastens the neutrophil aging[64]. The microbiome not only affects myeloid cells in circulation, but also the tissue-resident macrophage biology. In germ-free mice, microglia, the central nervous system's macrophages, have a different shape; this trait is partially brought on by deficiency of SCFAs[65]. Microbial SCFAs act as a cue to modify the local macrophages' gene-expression profile in the intestine[66,67]. The movement of myeloid cells throughout the gut is also controlled by the microbiota. The constant replenishment of macrophages in the intestinal mucosa by monocytes that express C-C chemokine receptor type 2 (CCR2) is caused by intestinal microbial colonization. The host's myeloid landscape is substantially altered by commensal microbial colonization, both locally and systemically in mucosal tissues. Myeloid-cell differentiation and function appear to be regulated by local concentrations of microbiota metabolites as well as systemic amounts of microbial products through PRR (Pattern Recognition Receptors) signaling.

The myeloid arm of the innate immunity is only one aspect of the microbiota's influence[68]. In the microbiota missing mice, the innate immune system's lymphocyte branch known as innate lymphoid cells (ILCs) develops correctly, nonetheless, commensal microbial colonization is necessary for ILCs to operate properly[69,70,71]. The ILC family consists of cytotoxic cells (natural killer cells) and non-cytotoxic subsets (ILC1, ILC2 and ILC3). Most studies regarding the influence of microbiota on ILCs focused on ILC3, whose depletion produce a loss of bacterial containment at intestinal

level. The microbiota also influences ILC3 interactions with other immunological components. The presentation of microbial antigens by ILC3s limits commensal-specific T-cell responses[72] to maintain tolerance to commensal bacteria[73]. Finally, the microbial sensing and the production of IL-1β by intestinal macrophages drive granulocyte–macrophage colony-stimulating factor (GM-CSF) secretion by ILC3s, which is required for macrophage function and the induction of oral tolerance[74].

2.2 Adaptive immune response and microbiota

2.2.1 Humoral immunity and microbiota

Through their effects on microbial location and function, antibodies play a crucial role not only in the development of early-life cross-talk with the microbiota but also in the maintenance of lifelong engagement with microbial partners. While IgAs have long been acknowledged for this critical function, more recent research has shown that other isotypes, including IgGs, are also involved in the microbiota-immunity interplay[75]. Anyway, The IgA antibodies are a crucial component of mucosal barriers' immunity. IgA and IgM are able to dimerize and pentamerize through the J chain polypeptide, which allows for their release into the lumen via polymeric immunoglobulin receptors. Secretory IgA and IgM provide protection against toxins and pathogens but also control host-commensal interactions by controlling microbiota composition, localization, and function[76] and limiting aberrant adhesion to epithelial surfaces[77]. Intestinal IgA^+ plasma cells (PCs), which make up 80% of all PCs in humans, are responsible for the majority of antibodies' production[78]. IgA antibodies coat a portion of the gut microbiota (GM)[79,80], and studies on germ-free mice showed that the intestinal IgA^+ PCs are heavily dependent on microbial colonization[81]. Early seminal studies uncovered a T cell-independent arm of the IgA response to commensal bacteria[82] and revealed that lack of activation-induced cytidine deaminase (AID)-dependent antibodies leads to aberrant expansion of anaerobic bacteria and isolated lymphoid follicle (ILF) hyperplasia[83]. The Mucosal IgA responses to the microbiota are thought to be induced locally by B1 and

B2 precursors, via both T-independent (TI) and T-dependent (TD) pathways. The spectrum of IgA responses to commensal bacteria has been explored thanks to the sequencing of microbiota members bound to IgA. This revealed that IgA predominantly targets bacteria resting in the small intestine[84,85]. T cells appear to be unessential for most commensal IgA binding, while select taxa such as SFB and Mucispirillum spp. require TD responses for IgA coating[85]. According to earlier research, mucosal IgA responses may not have the properties of classical memory and may be capable of adapting to shifts in the microbiota composition. In fact, novel antibacterial responses outcompete established mucosal IgA specificities, enabling the mucosal immune system to react to a continually shifting microbiota[86,87].The evolution of antibodies is tightly linked to their role in maintaining homeostatic relationships with the microbiota, however it's needed to well define how certain subsets or pathways affect the regulation of host-microbiota homeostasis at various developmental stages or in particular settings.

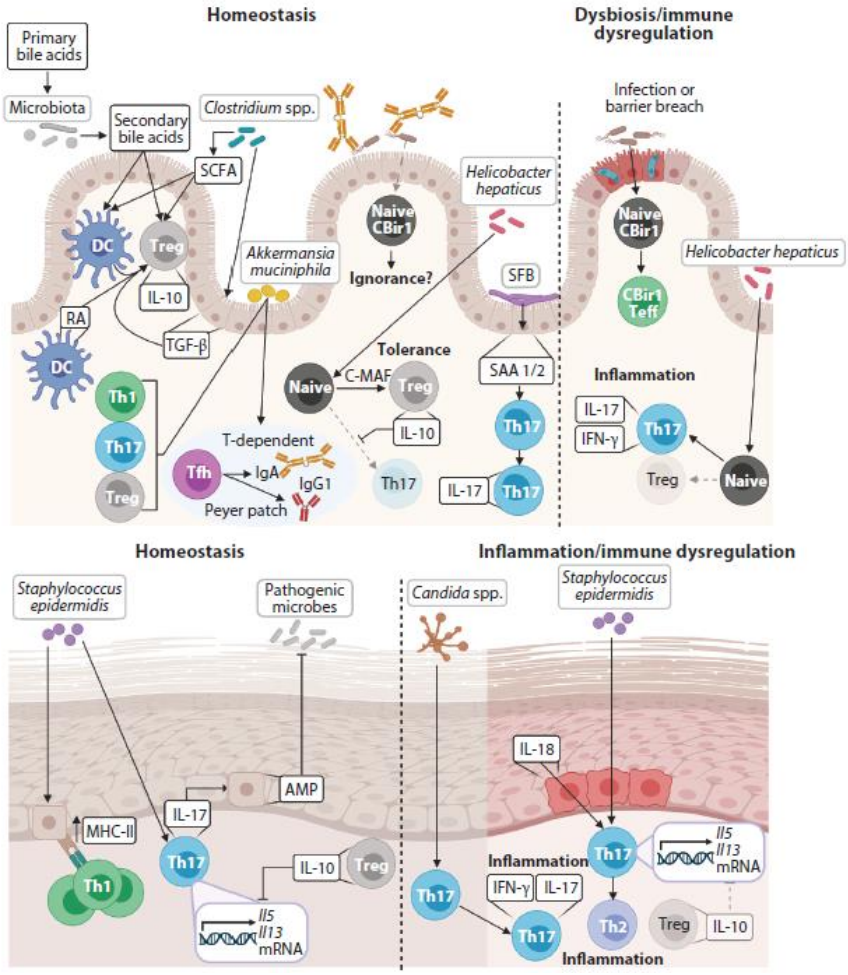
2.2.2 T cells and microbiota

Microbes at all barrier surfaces constitutively engage the immune system promoting the induction of cognate responses, including non-inflammatory responses directed at the microbiota itself[88]. Even though the microbiota as a whole can support all components of host immunity, under steady state conditions specific species or bacteria groupings may have a more significant impact on the immune system than others. SFB are an typical example of such a keystone species in the gastrointestinal tract[89]. As mentioned above, these gram-positive anaerobic spore-forming bacteria infiltrate the terminal ileum of mice and exert a dominant influence on the mucosal immune system by encouraging the differenzation of Th17 cells and inducing the IgA synthesis. Notably, the SFB-specific Th17 cells influence local and systemic inflammatory responses in addition to supporting local antimicrobial defences. Through their strong adherence to Peyer's patches and epithelial cells, SFB have close interactions with their hosts[90,91]. Only a small number of microbes have been shown to be able to induce and boost the proliferation of effector T cells, according to experiment in germ-free mice. For example, *Coprobacillus* induces the IL-10 producing T cells, *Bifidobacterium longum*

induces Th1 cells, and *Bifidobacterium adolescentis* promotes the Th17 cells[92,93]. The majority of commensal bacteria and fungi that colonize the skin can encourage commensal-specific T cell responses. The low density of resident bacteria and their location in highly specialized appendages like hair follicles and sebaceous glands may contribute to the skin's capacity to react quickly to incoming microbes[94]. Commensal *S. epidermidis*-specific T cells build up in the epidermis, where they consistently work on keratinocytes in an IL-17-dependent way to improve the production of antimicrobial peptides and the host defences against subsequent infections[95]. Through barrier tissues, commensal-specific T cells retain their capacity to significantly improve the tissue immunity and induce heterologous immunization[96]. Furthermore, the stimulation of T cell responses that remodel or repair tissue is one of the several processes by which the microbiota affect the tissue repair[97,98]. During tissue damage, release of type 2 cytokines and particularly IL-13 by commensal-specific T cells, contributes to their ability to promote tissue repair[98]. To promote local immunity and tissue adaptability to damage, commensal-specific T cells can take advantage of tissue residency and cell-intrinsic flexibility. In addition to directing the development of responses against the microbiota, tissues also offer particular checkpoints that favour the functional licensing of commensal-specific T lymphocytes. The commensal-specific T lymphocytes accumulate in tissues, whereupon they produce cytokines like IL-17A and IL-22, the production of which is closely regulated by tissue-specific stimuli, to carry out their local function. For instance, local IL-1 synthesis in the skin is necessary to allow cutaneous T cells to release IL-17A[99], while the vast majority of Th17 responses to the GM can develop in missing of this cytokine[90]. By releasing chemokines that are locally involved in T cell tropism, such as CXCL9, CXCL10, and CCL20, epithelial cells can also coordinate responses to the microbiota in the skin and gastrointestinal tract[100]. The maintenance of tissue homeostasis is related with the standard immune program against the microbiota, but the fate of commensal-specific T cells is highly contextual and can be influenced by host activation status, the milieu, and the microbiota composition. In this scenario, the microbe *Akkermansia muciniphila* affects a wide range of biological outcomes in both mice and humans, from host metabolism to response to checkpoint treatment[101]. *A. muciniphila* exclusively promotes the development of the T follicular

(Tfh) cells in a gnotobiotic model with nine-member bacterial community. However, in the context of a complex microbiota, the *A. muciniphila*–specific T cells can adopt highly diverse fates, including differentiation into Th1, Th17, and Treg cells[102] (**Figure 4**).

It is unknown whether commensal-specific T cells are maintained independent of continuous cognate antigen stimulation in a manner similar to that of virus-induced tissue-resident memory T cells or IELs[103,104]. Commensal-specific memory T cells gradually drop over time, and it is still unclear whether the microbiota can support true immunological T cell memory at rest or during an inflammatory response[105].



Control of Immunity by the Microbiota; Ansaldo E, et al, Annual Review of Immunology 2021 39:1, 449-479

Figure 4. Microbiota-specific T cell immunity in homeostasis and disease.

2.2.3 The link between microbiota and regolatory T cells

In order to maintain tissue homeostasis supporting the microbiota's propensity to activate the immune system, both constitutive (such as barrier structures and mucus) and induced immunoregulatory responses are needed. Indeed, the microbiota, in addition to promoting immunity, can support various immunoregulatory pathways; and the tissue activation threshold is defined by the balance of these responses. Numerous inflammatory conditions, such as inflammatory bowel diseases, are examples of how failure to control reactions to the microbiota can have disastrous effects on the host[106].

A non-redundant function of FOXP3+ Tregs is to maintain immunological homeostasis[107]. The induced Treg cells in the gut regulate immune responses to antigens orally taken, including those produced from the microbiota. This phenomenon is promoted by a complex cellular network, including specialized antigen-presenting cells, as well as a milieu enriched in TGF-β, retinoic acid, and microbiota-derived metabolites[108,109,110]. It has been long recognized that oral tolerance, a phenomenon associated with the induction of FOXP3+ Tregs[110], cannot be induced in microbiota free situation[111]. In fact, under germ-free condition or during antibiotic therapy, certain Tregs' subsets are decreased [112,113]. The hypothesis that Treg cells are enriched for TCRs specific for microbiota-derived antigens within the gut, and in particular the colon, is supported by the use of microbe-specific transgenic T cells. While in germ-free settings only few microbes promote the induction of effector responses, a large number of isolates or microbiota-derived consortia are able to promote the Tregs' induction and function[102,92,112]. This supports the ideas that the induction of Treg cells is a dominant feature of microbes associated with health and that these responses are promoted by microbiota-derived, canonical, and redundant factors. The microbiota also specifically induces a subset of Treg cells expressing RORγt with a preferential tropism for the colon lamina propria[114,115]. The accumulation of RORγt-expressing Treg cells occurs between two and three weeks after birth which coincides with weaning associated with a profound immune reprograming and a shift in microbiota composition.

Different microbiota-associated factors can promote the optimal Tregs' development and function. One of the many biological processes supported by the SCFAs is the

activation of Treg cells[116,117,118]. For example, the cell surface polysaccharides of *Bifidobacterium bifidum* promote the induction of IL-10-producing Treg cells in a TLR2-dependent manner, and specific microbe-associated molecules, such as the *B. fragilis*-derived capsular factor polysaccharide A, can influence the dendritic cell function promoting the Treg cell induction[119,120].

3. HIV and microbiota interaction

As previously mentioned, the microbiota has a significant impact on immunological development and function[96,121,122]. In the last few years, murine studies have revealed the critical role of specific gut microbes communities in different systemic inflammatory diseases[123]. In patients with HIV infection, the systemic inflammation is considered the primary contributors to morbidity and mortality despite ART[124]. In view of these consideration, the gut microbiota (GM) role in HIV infection has garnered great interest.

It has been extensively researched what causes both the pathologic inflammation during untreated HIV infection and the chronic inflammation that occurs with antiretroviral therapy. The disturbances that affect the gastrointestinal immune barrier during HIV infection include dysfunction of cell involving in the regulation of microbiota composition[125]. For example, the IL-17 is able to induce the expression of antimicrobial peptides remodelling the GM composition, and its loss in mouse models leads to an altered microbiota intensifying systemic inflammation[126]. Furthermore, an altered microbiota can originate from macrophage function disruption and can cause both local and systemic inflammatory pathology[127,128]. Numerous studies have investigated the GM composition of HIV-infected patients compared to healthy controls[129,130,131132]. Results of these studies share several common patterns among the HIV cohort: an enrichment of *Erysipelotrichaceae*, *Enterobacteriaceae*, *Desulfovibrionaceae*, and *Fusobacteria*, a depletion of *Lachnospiraceae*, *Ruminococceae*, *Bacteroides*, and *Rikenellaceae*.

A potential molecular connection between HIV-associated microbiota alterations and enhanced systemic immune activation is the increased abundance of GM bacteria that can directly stimulate host inflammation. These observations appeared relevant considering the increased abundance of various Proteobacteria including *Enterobacteriaceae*, a family comprised of numerous pro-inflammatory, flagellated, motile members (such as *E. coli, Salmonella, Pseudomonas, Yersinia,* and *Klebsiella*) with the capacity to translocate.

In addition, GM may influence the progression of HIV disease also through its metabolic production, independently of direct immunostimulation. Metabolism of tryptophan through the kynurenine pathway has been implicated in gut barrier disruption in HIV infection. Kynurenine compounds are involved in decreased differentiation of gut barrier-promoting Th17 cells and the activity of the kynurenine pathway correlates with Th17 cell loss, inflammation, and disease progression in HIV-infected patients as reviewed by Routy et al.[133] Systemic kynurenine pathway activity was shown to be correlated with the abundance of bacteria that encode kynurenine synthesis enzymes[134,135] and the GM communities of HIV-infected patients exhibit an increased capacity to catabolize tryptophan to kynurenine[136]. On the contrary, the depletion of SCFAs' producing bacteria, such as *Ruminococcaceae* and *Lachnospiraceae* taxa in HIV+ patients may decrease the production of desirable metabolites.

As repeatedly reported in scientific literature, the oral microbiota is a dynamic community that interacts with the host and drives health or disease through homeostasis or dysbiosis[137]. Often oral lesions are the first symptom for identifying HIV infection, and pharangitis is usually the first manifestation of oral inflammation[138]. Despite the absence of oral lesions in some patients, the persistent state of heightened immune activation and inflammation, which results in microbial translocations and oral mucosal alterations with similar pattern of gastrointestinal disruption[139,140,141]. During disease progression the patients have a CD4 count less than 200 positive cells/µl and at this stage they are more susceptible to opportunistic infection including oral infection as candidiasis. The oral manifestation may be the results of oral microbiota imbalance[140,142,143]. A recent study by Gou at al.[144] performed for the first time a stage comparison of salivary microbial differences in HIV-infected patients, founding that the oral microbiome changes in saliva of untreated HIV-positive patients were characterize by greater alpha-diversity.

Aim of the study

Thirty years after the discovery of the HIV virus and the advances in the treatment of HIV infections, AIDS continues to be one of the top priorities for public health. The last decades have seen a rapid expansion in the understanding of how the microbiota influences the immunity development and function. The aim of the present study was to further characterize the role of microbiome-immunity axis (MIA) in patients infected by HIV-1. First of all, we compared oral and fecal microbiota of treatment-naïve patients with healthy controls (HC) to evaluate if HIV infection was associated with a change in the microbiota composition. Thereafter, we investigated the fecal microbial composition, serum and fecal microbial metabolites, and serum cytokine profile of treatment-naïve patients before starting ART and after reaching virological suppression (HIV RNA < 50 copies/mL) after 24 weeks of ART. Finally, in order to define the role of microbiota and their metabolites in immune reconstitution we compared patients that reached a $CD4^+$ reconstitution (CD4/CD8 ratio ≥ 1) and patients that didn't reach $CD4^+$ (CD4/CD8 ratio < 1) reconstitution despite ART.

Methods

1. Patients and clinical data

The study population is composed by 12 treatment-naïve HIV-infected patients receiving ART (mainly based on integrase inhibitors) enrolled between January 2019 and April 2021 at the Department of Infective and Tropical Disease at University Hospital of Careggi, Florence, Italy (**Table 1**). The study was approved (Rif CEAVC 15035) by local ethical committee (Area Toscana Centro) and written informed consent was obtained from each patient before participation. Patients who had used antibiotics, probiotics, or prebiotics or had experienced diarrhoea or digestive symptoms within the previous 1 month were excluded. Plasma HIV-RNA was measured using Test v1.5 Roche COBAS AmpliPrep, Roche TaqMan HIV-1 Test v2.0 (Roche Diagnostics, Branchburg, NJ, United States) and Siemens Versant K PCR (Siemens Healthcare GmbH, Erlangen, Germany), with lower limits of detection of 50, 20, and 37 copies/mL, respectively. The T cell counts were determined using a FACScanto flow cytometer (BD Immunocytometry Systems). Immunophenotyping of peripheral blood lymphocytes was analysed by three-color flow cytometry (Epics XL Flow Cytometry System; Beckman Coulter, United States). In detail, freshly collected EDTA anticoagulated whole blood was incubated and tested with a panel of monoclonal antibodies directed against fluorescein isothiocyanate/phycoerythrin/peridinin chlorophyll protein combinations of CD3/CD4/CD8, CD3/CD16CD56/CD19, HLA-DR/CD8/CD38, and CD4/CD8/CD28 and isotype controls (Immunotech,France). At each time point (0 and 24 weeks after study enrolment), we collected blood, salivary and fecal samples. After collection, stool and saliva samples were immediately frozen and stored at −80 °C until DNA extraction. Fecal and saliva samples were used to assess the microbiota composition, fecal and blood samples were used also to measure FFAs (free fatty acids), and blood samples were used to measure a panel of 27 selected cytokines. Patients underwent medical visits at 0 and 24 weeks after study enrolment. They also underwent a comprehensive physical examination and medical history inquiry, urine toxicology panel testing, clinical laboratory tests including plasma HIV RNA, specimen collection, and detailed behavioural questionnaire survey. Demographic and clinical data were

collected in a specific questionnaire and reported in an appropriate database, including the time point of follow-up in months; the participant's gender, age, weight, and height; $CD4^+$ and $CD8^+$ T cell counts; the CD4/CD8 ratio; HIV-1 RNA levels, ART, and antibiotic use. If patients had to start antibiotics, they provided a last fecal and salivary sample and the study follow-up was immediately stopped.

	Age	Sex	ART regimen	Timepoints (wk)	Viral load (copies/mL)	CD4+ cells/mm3	CD8+ cells/mm3	CD4/CD8 ratio
1	37	Male	3TC/ABC/DTG	T0	597463	110	420	0.3
				T24	< 20	520	832	0.6
2	38	Male	FTC/TDF/EVG/C	T0	4489	630	670	0.9
				T24	TND	831	740	1.1
3	34	Male	FTC/TDF/EVG/C	T0	165516	253	725	0.3
				T24	TND	504	363	1.4
4	39	Male	FTC/TDF/EVG/c	T0	859883	360	974	0.4
				T24	33	781	986	0.8
5	38	Male	3TC/ABC/DTG	T0	4860	1341	928	1.4
				T24	TND	1881	988	1.9
6	41	Male	FTC/TDF/RPV	T0	213	814	690	1.2
				T24	TND	845	519	1.6
7	25	Male	3TC/ABC/DTG	T0	23098	516	1149	0.4
				T24	< 20	942	1019	0.9
8	22	Male	FTC/TAF/EVG/c	T0	12188	654	1055	0.6
				T24	TND	668	733	0.9
9	48	Male	3TC/ABC/DTG	T0	175	833	1520	0.5
				T24	TND	941	1258	0.7
10	53	Male	3TC/ABC/DTG	T0	40545	863	1196	0.7
				T24	TND	612	515	1.2
11	40	Male	3TC/ABC/DTG	T0	859000	399	980	0.4
				T24	39	648	652	1
12	51	Male	FTC/TDF DTG	T0	4410	884	1066	0.8
				T24	< 20	1130	1261	0.9

Table 1. Patients and clinical data ART: Antiretroviral therapy; 3TC: Lamivudine; ABC: Abacavir; DTG: Dolutegravir; FTC: Emtricitabine; TDF: Tenovir; EVG/c: Elvitegravir/cobi; RPV: Rilpivirine.

2. Fecal and salivary microbiota characterization

Genomic DNA was extracted using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) from frozen (-80°C) saliva and stool samples according to the manufacturer's instructions with modifications as reported in our previous study[145]. Total genomic DNA was captured on a silica membrane in a spin column format and subsequently washed and eluted. The quality and quantity of extracted DNA was assessed using the NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, US) and the Qubit Fluorometer (Thermo Fisher Scientific), respectively. Then, genomic DNA was frozen at -80°C. Subsequently, total DNA samples were sent to IGA Technology Services (Udine, Italy) where amplicons of the variable V3–V4 region of the bacterial 16S rRNA gene, delimited through the primers 341F and 805R, were sequenced in paired-end (2 × 300 cycles) on the Illumina MiSeq platform, according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol.

3. Bioinformatic analysis of 16S rRNA

Demultiplexed sequence reads were processed using the bioinformatic tools in QIIME2 2021.4. In particular, the sequencing primers and the reads without primers were removed using Cutadapt tool. DADA2 was used to perform paired-end reads filtering, merging and chimeras removal steps after trimming low quality nucleotides from both forward and reverse reads (--p-trunclen-f 275 and --p-trunclen-r 168). Hence, ASVs (amplicon sequence variants) were generated and the taxonomic assignments have been performed through Scikit-learn Bayesian Classificator retrained on V3-V4 16S region of SILVA 138.

4. Statistical analysis of 16S rRNA

The statistical analyses on bacterial communities were performed in R 4.1 with the help of the packages phyloseq 1.38.0, vegan 2.6.2, DESeq2 1.32.0 and other packages satisfying their dependencies. The packages ggplot2 3.3.6, dendextend 1.15.1 and ggpubr 0.40 were used to plot data and results. A rarefaction analysis on ASV was

performed on every sample using the function rarecurve (step 100 reads), further processed to highlight saturated samples (arbitrarily defined as saturated samples with a final slope in the rarefaction curve with an increment in ASV number per reads < 1e-5). The observed richness, Shannon and Pielou's evenness indices were used to estimate the bacterial alpha-diversity in each sample using the function estimate_richness from phyloseq. The evenness index was calculated using the formula E = S/log(R), where S is the Shannon diversity index and R is the observed ASV richness in the sample. Differences in alpha-diversity indices were tested using the Mann-Whitney test. PCoAs was performed using Bray-Curtis index on proportional count data of each sample adjusted with square root transformation. A PERMANOVA was used to test the statistical significance of the beta-diversity distances. At different taxonomic ranks, the differential analysis of the abundances has been computed through DESeq2 on raw count data.

5. Evaluation of fecal and serum FFAs by gas chromatography-mass spectrometry

The fecal short chain fatty acids (SCFAs), in particular acetic, propionic, butyric, isobutyric, isovaleric, 2-methylbutyric, valeric, and hexanoic acids, were analysed using an Agilent GC-MS system composed with a 5971 single quadrupole mass spectrometer, a 5890 gaschromatograph, and a 7673 auto sampler. Fecal samples were collected in 15-mL Falcon tubes and stored at -80 °C. Just before the analysis, each sample was thawed, weighted (between 0.5-1.0 g), and added to sodium bicarbonate 10 mmol/L solution (1:1 w/v) in a 1.5 mL centrifuge tube. The obtained suspension was briefly stirred in a vortex apparatus, extracted in an ultrasonic bath (for 5 min), and then centrifuged at 5000 rpm (for 10 min). The supernatant was collected and transferred into a 1.5 mL centrifuge tube (sample solution). The SCFAs were finally extracted as follows: an aliquot of 100 µL of sample solution was added to 50 µL of internal standard mixture, 1 mL of tert-butyl methyl ether, and 50 µL of 1.0 mol/L HCl solution in a 1.5 mL centrifuge tube. Afterwards, each tube was shaken in a vortex apparatus for 2 min and centrifuged at 10000 rpm for 5 min, and finally the solvent layer was transferred into an autosampler vial and analyzed by the GC-MS method. Each sample was prepared and processed, by

the method described above, three times. In addition, serum FFAs, classified as SCFAs (acetic, propionic, butyric, isobutyric isovaleric, 2-methylbutyri, and valeric acids), medium chain fatty acids (MCFAs; hexanoic, heptanoic, octanoic, nonanoic, decanoic, and dodecanoic acids), and long chain fatty acids (LCFAs; tetradecanoic, hexadecanoic, and octadecanoic acids) were analyzed with our previous described GC-MS protocol[146].

Just before the analysis, each sample was thawed. The FFAs were extracted as follows: An aliquot of 300 μL of plasma sample was added to 10 μL of internal standard mixture, 100 μL of tert-butyl methyl ether, and 20 μL of 6 M HCl plus 0.5 mol/L NaCl solution in a 0.5 mL centrifuge tube. Afterwards, each tube was stirred in vortex for 2 min and centrifuged at 10000 rpm for 5 min, and finally the solvent layer was transferred into a vial with a microvolume insert and analyzed.

6. Serum cytokines' profile

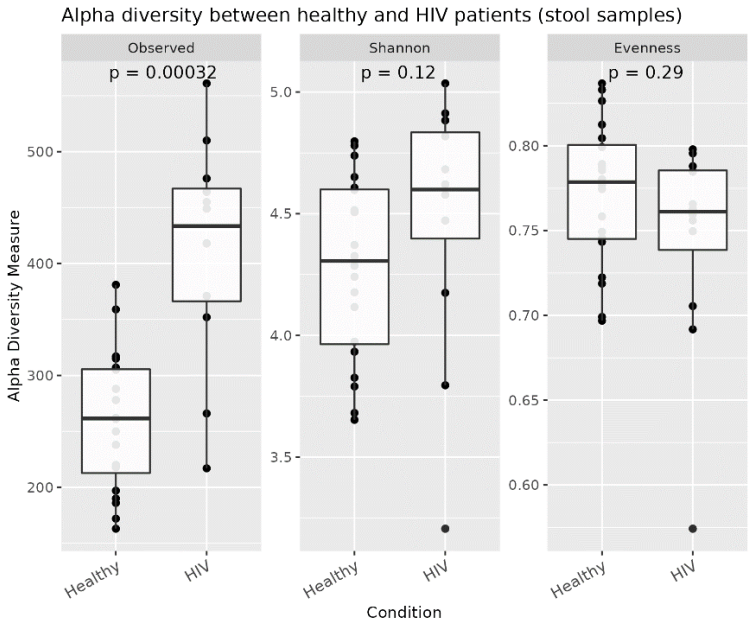
The systemic inflammatory response was evaluated in serum through the testing of 30 cytokines. We used specifically assembled MixMatch Human kits with a Luminex MAGPIX detection system (Affymetrix, Thermo Fisher, Vienna, Austria) and followed the manufacturer's instructions. More specifically, we analysed: granulocyte colony-stimulating factor (G-CSF), interferon (IFN)-γ, IFN-α, interleukin (IL)-1 beta, IL-2, IL-4, IL-6, IL-8, IL-9, IL-10, IL-15, IL-17A, IL-27, IL-5, IL-12p70, IL-13, IL-1a, IL-23, IL-18, IL-21, IL-22, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), tumor necrosis factor-α (TNFα), vascular endothelial growth factor (VEGF)-A, interferon gamma-induced protein 10 (IP-10), granulocyte-macrophage colony stimulating factor (GM-CSF), soluble intercellular adhesion molecule-1 (sICAM1), P-selectin, and E-selectin. The levels of cytokines were estimated using a 5-parameter polynomial curve (XPonent Software, Luminex Corporation, Austin, TX, USA). The Lower and Upper Limits of Quantification (LLOQ and ULOQ) for the cytokines and chemokines are reported in Supplementary Table S1. Out of range concentrations on the lower or upper end of detection were input with values representing 0.5 times the lowest value or 1.5 times the highest value, respectively

Results

1. Fecal and oral microbiota differences between HIV patients and healthy subjects

First of all, to determine if HIV infection was associated with a change in the microbiota composition, we evaluated the differences between HIV patients and healthy controls in fecal and salivary samples. As shown in panel A of the **Figure 5** the analysis of the alpha diversity of fecal samples did not display significant differences for Shannon and Evenness indices, while a significant Richness index (p= 0,00032) revealed a diversity between HC and HIV patients. Instead, the alpha diversity analysis in salivary samples revealed significant difference according to the Richness (p=0,0004) and Shannon (0,0085) indices while did not display significant differences for Eveness index (**Figure 5B**). As expected, the PCoA analysis on normalized ASV counts both for fecal and salivary samples evidenced distinct clusters between HIV patients and HC (**Figure 6**). The stacked bar-plot representation displayed the different relative abundance of both the top five phyla and genera in either fecal (**Figure 7A**) and salivary (**Figure 7B**) samples. In detail, the most abundant phyla in stool samples were Actinobateriota, Bacteroidota, Firmicutes, Proteobacteria and Euryarchaeota while saliva samples showed high abundance of Proteobacteria, Fusobacteriota, Actinobacteriota, Bacteroidota e Firmicutes. Besides, the top five genera in stool samples were *Catenibacterium, Prevotella, Bifidobacterium, Bacteroides* and *Faecalibacterium* while salivary samples showed high abundance of *Proteobacteria, Fusobacteriota, Actinobacteriota, Bacteroidota* and *Firmicutes*.

A



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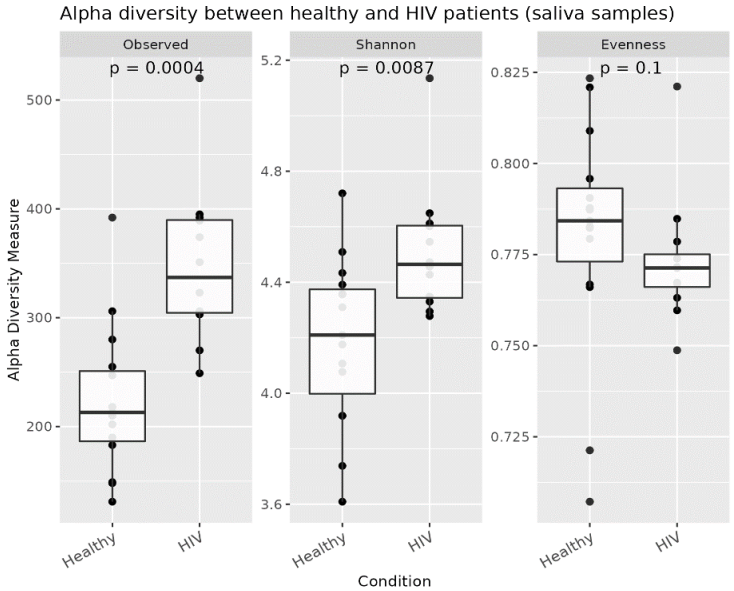


Figure 5: Box-plots showing alpha diversity indices (Observed index, Shannon index, Evenness) in fecal (A) and salivary (B) samples of HIV patients and healthy subjects. Statistical differences were evaluated using Mann-Whitney test for Observed richness, Shannon and Evenness indices. P-values less than 0.05 were considered statistically significant.

A **B**

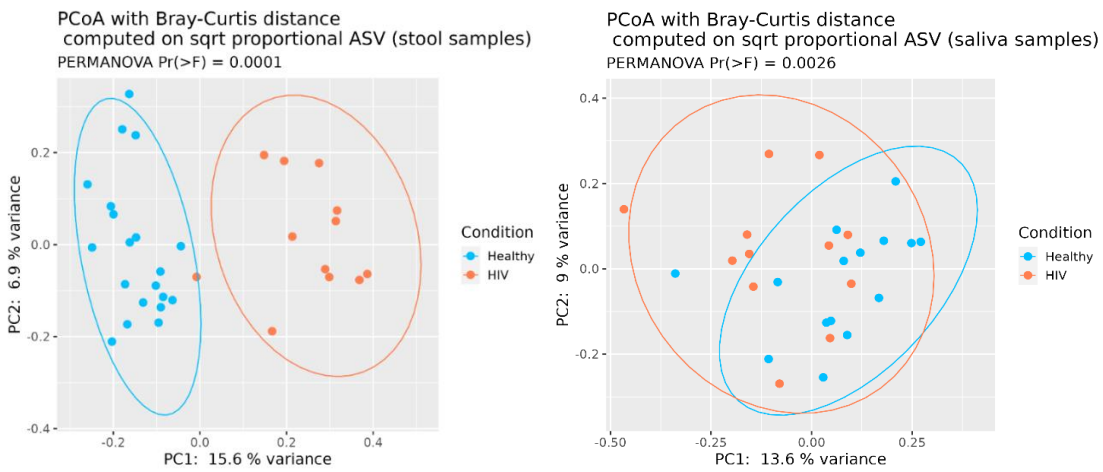
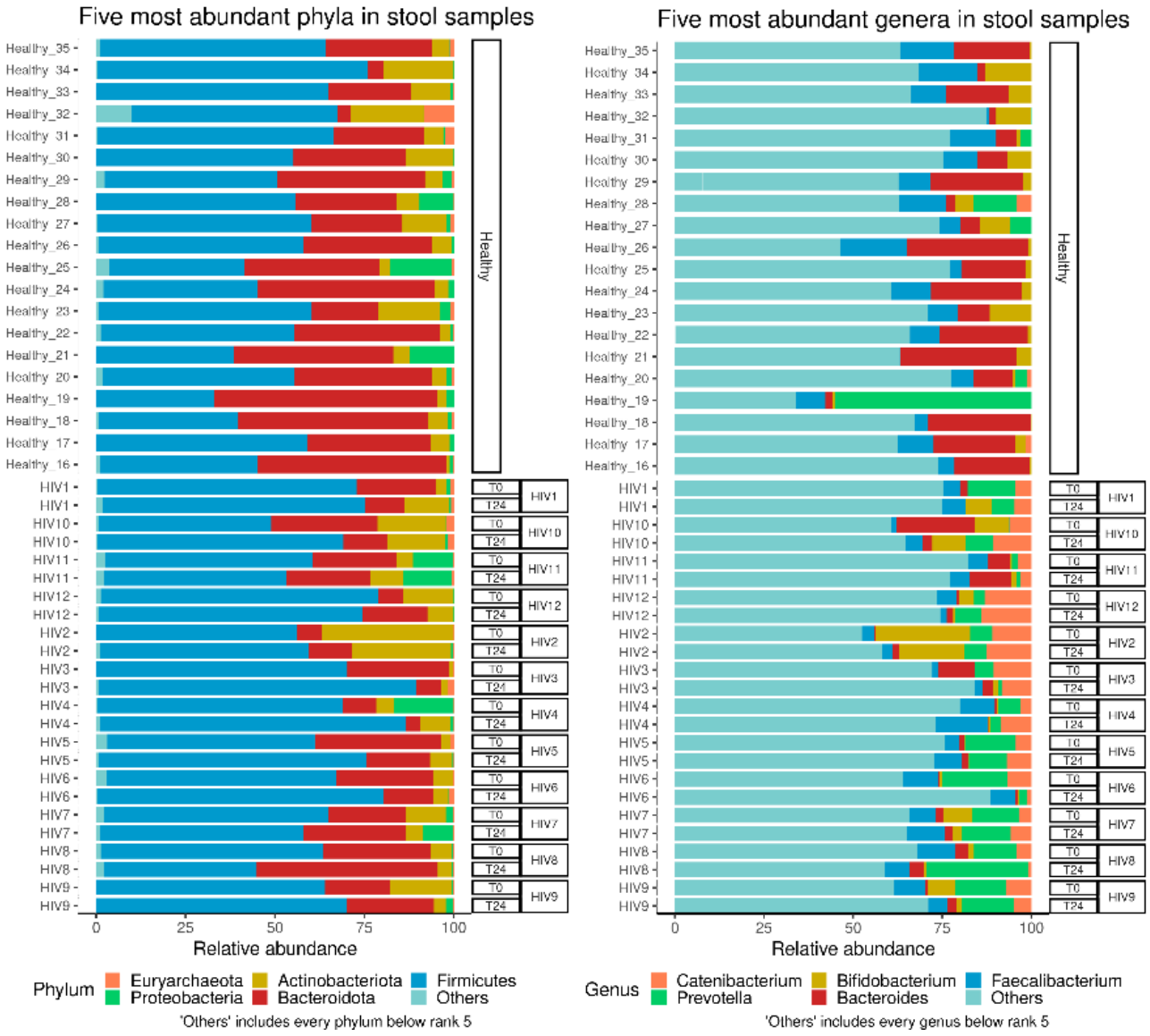


Figure 6: Principal coordinate analysis using Bray–Curtis dissimilarity as a distance metric on square root–transformed percent abundance of identified ASVs in fecal (A) and salivary (B) samples of HIV patients and HC.

A


Five most abundant phyla in stool samples
Five most abundant genera in stool samples
Healthy
HIV1
HIV10
HIV11
HIV12
HIV2
HIV3
HIV4
HIV5
HIV6
HIV7
HIV8
HIV9
T0
T24
0
25
50
75
100
Relative abundance
Phylum
Euryarchaeota
Actinobacteriota
Firmicutes
Proteobacteria
Bacteroidota
Others
'Others' includes every phylum below rank 5
Genus
Catenibacterium
Bifidobacterium
Faecalibacterium
Prevotella
Bacteroides
Others
'Others' includes every genus below rank 5

B

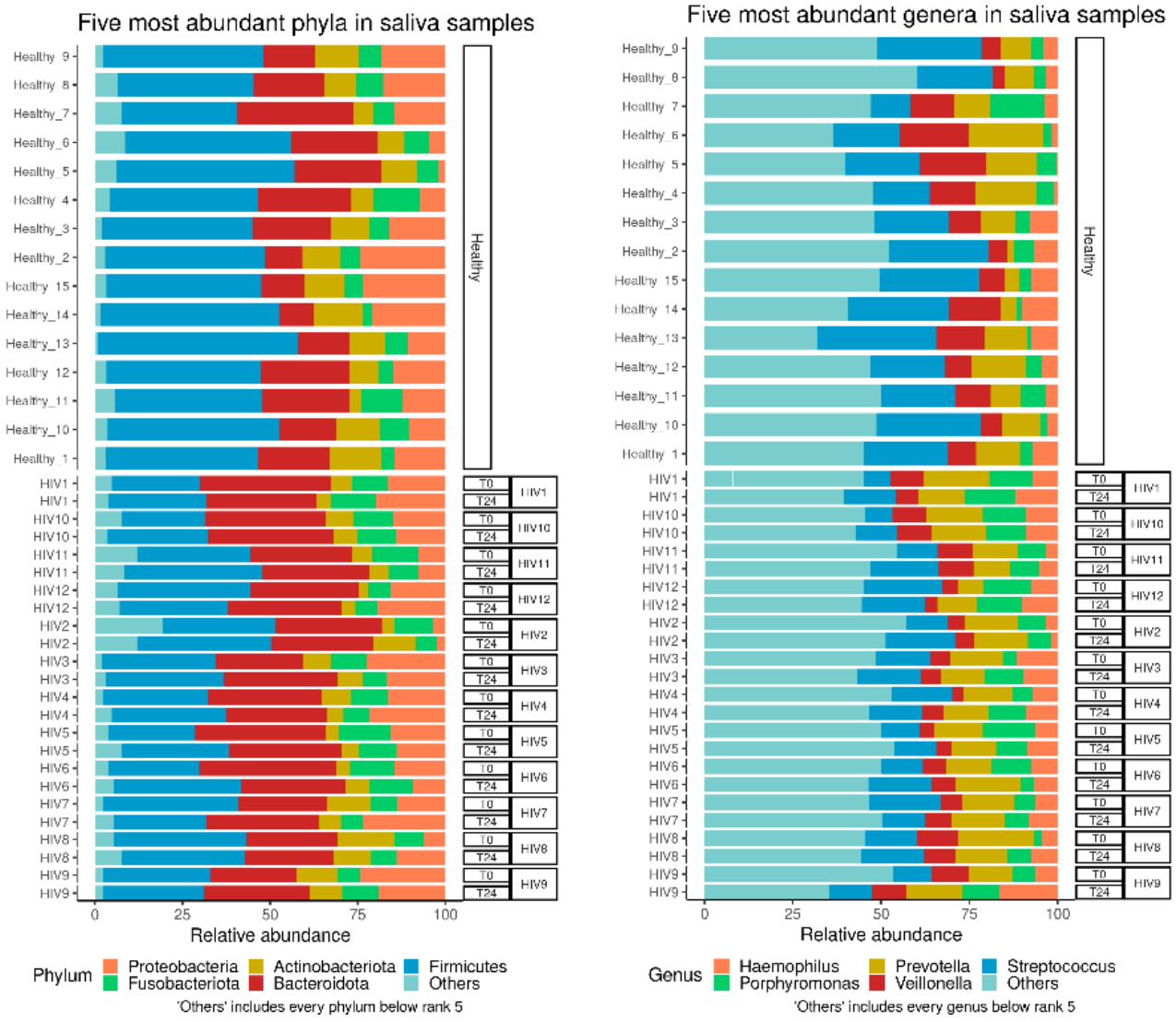
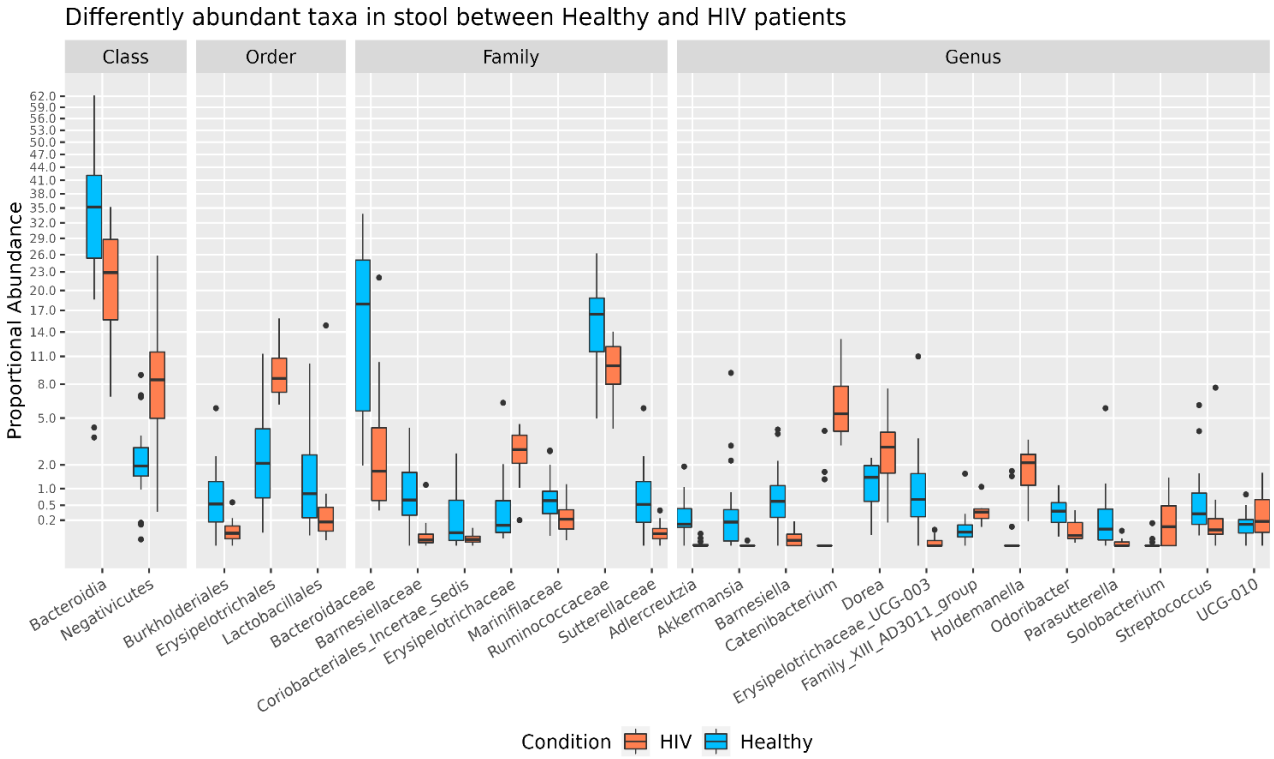


Figure 7: Taxonomic composition of the five most abundant microbial phyla (A) and genera (B) in stool and saliva samples of HIV patients and HC. Bar plot shows the relative abundance of bacterial phyla in each sample.

Significant differences were observed in microbial clades from fecal samples when comparing HIV patients vs HC. In detail, at class level we observed an increased level of Negativicutes and low level of Bacteroidia in HIV patients, while at family level a decrease of Ruminococcaceae and Erysipelotrichaceae. At genus level we observed an increase of *Catenibacterium*, *Dorea* and *Holdemanella* while a decrease of *Barnesiella*

and *Erysipelotrichaceae-UCG-003* (**Figure 8 A**). Regarding the oral microbiota, differential abundance analysis in salivary samples highlighted that only two different families resulted differentially abundant in HIV patients respect to HC. In detail, compared to HC, HIV infected patients had higher salivary abundance of Porphyromonadaceae and Spirochaetaceae (**Figure 8 B**).

A



B

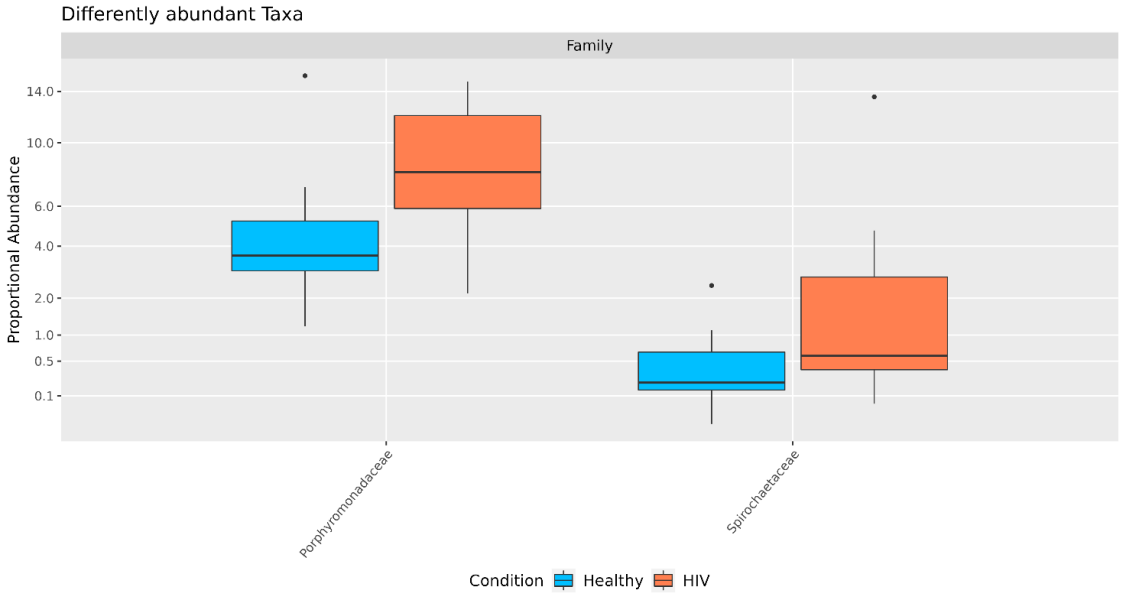


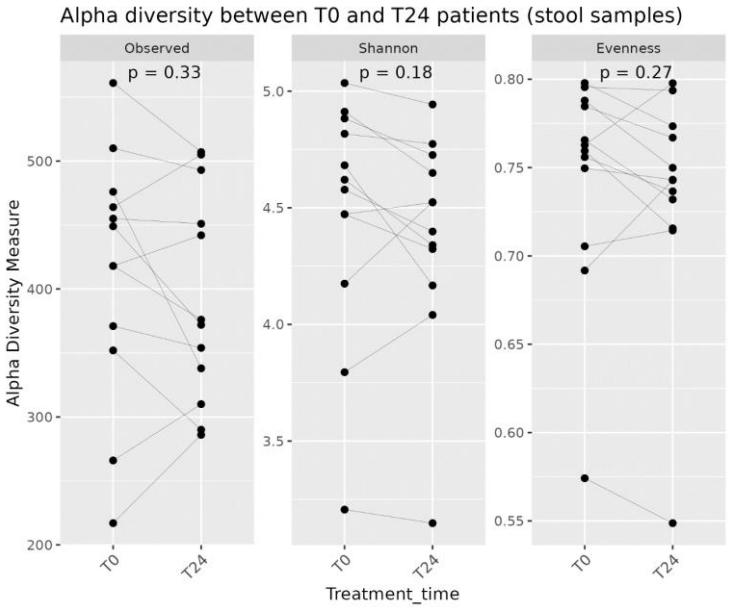
Figure 8. Boxplot showing the results of taxa differential abundance analysis between the fecal (A) and the salivary (B) samples from HIV patients and HC. All results have an adjusted p-value < 0.05.

2. Microbiota, metabolic and inflammatory profiles after ART

2.1 Fecal and salivary microbiota

For the second project aim we compared the metabolic and inflammatory signature, the fecal and oral microbiota before and after ART, in order to document the potential changes occurring in HIV infection during ART. We analysed the longitudinal variation of fecal and salivary microbiota composition in patients at two different time points: T0, corresponding to a high viremia condition (HIV- RNA > 50 copies/mL) and T24 corresponding to a viral suppression condition (HIV-RNA ≤ 50 copies/mL). The Richness, Shannon, and Evenness indices did not find significant differences in the alpha diversity of fecal samples (**Figure 9A**). Instead, the alpha diversity of salivary samples did not display significant differences for Richness, Shannon, and Evenness indices (**Figure 9B**). To probe the dissimilarity of patients' microbiota abundance profiles we carried out a cluster analysis and PCoA on normalized ASV counts. The PCoA showed a substantial proximity of each patient at T0 and T24 both in fecal and saliva specimens, suggesting that, overall, the abundance profile of the single patient was not affected by the 24 weeks' therapy (**Figure 10**).

A



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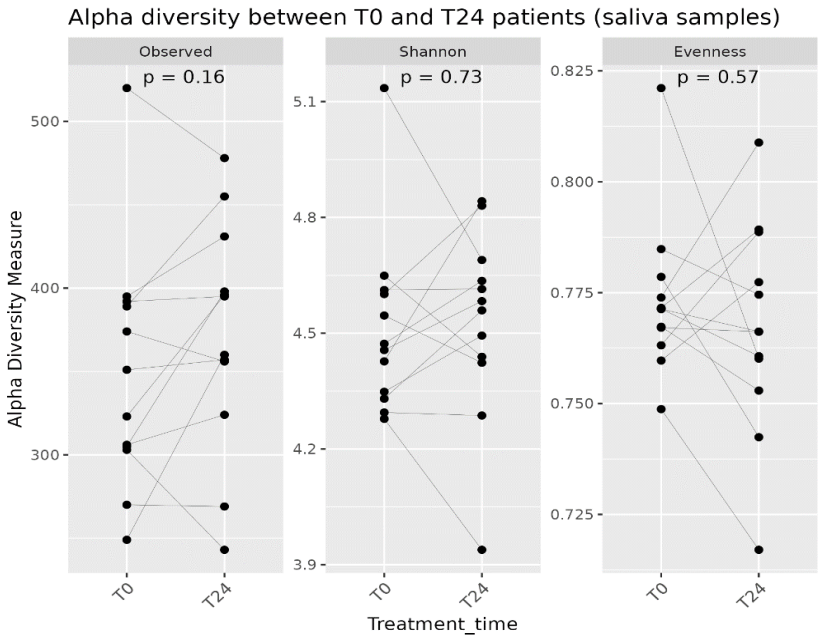


Figure 9. Box-plots showing alpha diversity indices (Observed richness, Shannon and Evenness) in fecal (A) and salivary (B) samples of HIV at high viremia (T0) and viral suppression condition (T24). Statistical differences were evaluated using Wilcoxon paired test for Observed richness, Shannon and Evenness indices. P-values less than 0.05 were considered statistically significant.

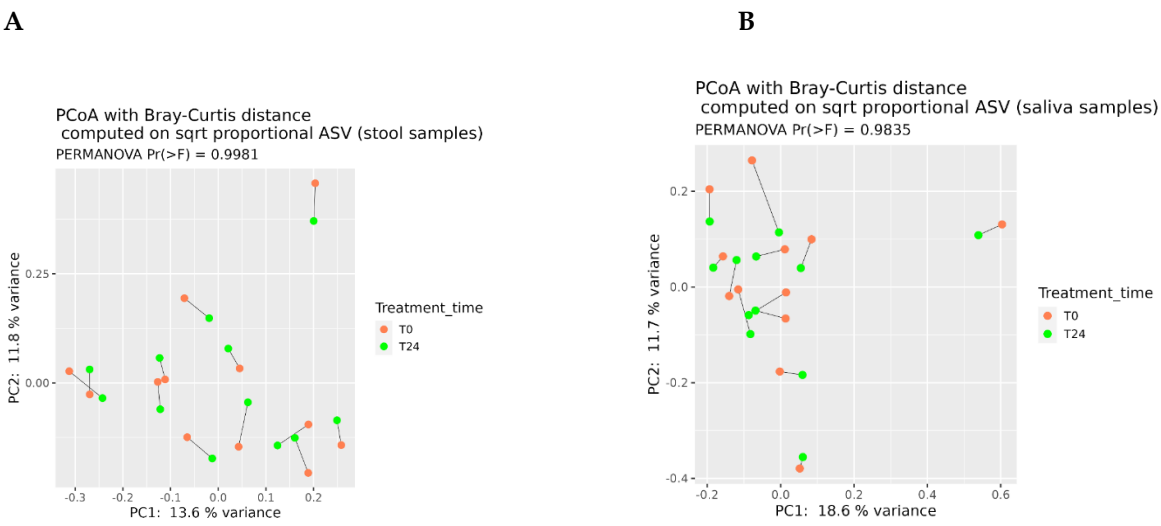
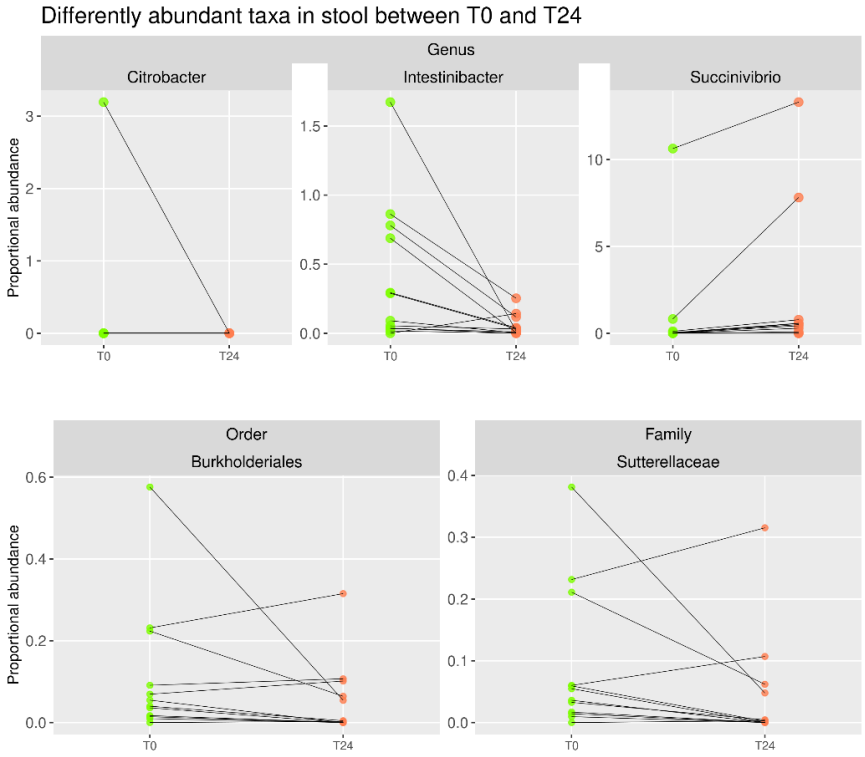


Figure 10. Principal coordinate analysis (PCoA) conducted with Bray-Curtis dissimilarity of the bacterial communities of fecal (A) and saliva (B) samples among high viremia (T0) and viral suppression condition (T24).

The taxonomic composition analysis of fecal samples showed that more than 98% of the sequences were classified into four phyla: Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. On the other hand, the paired comparison of the abundance of single microbial ranks revealed some significant differences between the two samples groups. In detail, the genus *Succinivibrio* was found to be significantly increased in viral suppression condition. On the contrary, the viral suppression was related with a decrease in the *Intestinibacter* genus (median abundance, ~1%) (**Figure 11A**). Moreover, the paired comparison of the abundance of single microbial ranks in saliva samples showed a significant reduced abundance only for the Absconditabacteriales family (**Figure 11B**).

A



B

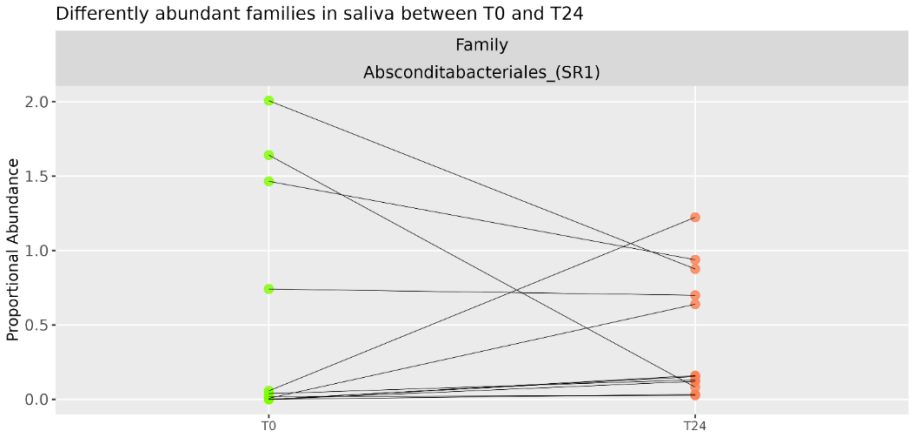


Figure 11. Segment plots depicting taxa with significantly differences between high viremia (T0) and viral suppression (T24) conditions. The lines connect paired samples and highlight the differences in normalized abundances.

2.2 Analysis of fecal SCFAs

As we described minor changes in fecal microbiome profile, we wondered if the GM metabolic activity had been altered as well, and whether this activity might be masked by simply examining the microbiota composition. In order to evaluate alterations in GM metabolic activity, the levels of linear and branched SCFAs were measured in fecal patients'samples. However, the analysis of linear SCFA (acetic, propionic, butyric, and valeric acids), and branched SCFA (isobutyric, isovaleric, and 2-metilbutyric acids) abundance did not reveal any significant change after 24 weeks of therapy for each patient.

2.3 Analysis of serum FFAs

Finally, as we did not report significant alterations in the fecal SCFAs distribution, we wanted to observe if there were any other alterations in metabolic output, by analysing both microbial and host derived FFAs in serum. As known, the impairment of gut integrity due to dysbiosis condition, leads to translocation of microorganisms and its compounds from the intestinal mucosa to the bloodstream, which is considered a major driving force of chronic immune activation even in patients successfully treated with ART and achieving stable virologic suppression. The analysis of serum FFA levels showed a significant change of two SCFAs at T24 compared to the baseline. In particular, propionic and butyric acids were increased in viral suppression condition (**Figure 12**).

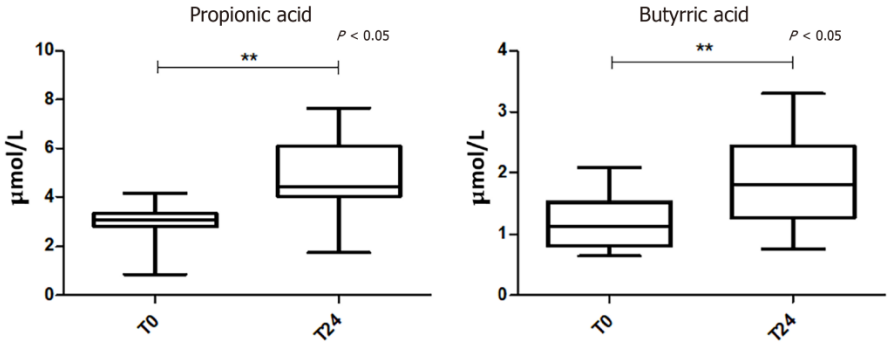


Figure 12. Boxplots showing statistically different levels of serum short-chain fatty acids between high viremia and viral suppressor patients, assessed by the Wilcoxon test. P value < 0.05 was considered statistically significant.

2.4 Molecular inflammatory profile

As known, the GM dysbiosis is linked to aberrant immune responses, in fact these alterations may induce the interruption of gut epithelial barrier integrity with subsequent microbial translocation, increased inflammation, and immune activation, which are often accompanied by abnormal differentiation of immunological cells. Since we detected significant variations of microbial communities between high viremia and viral suppression conditions, we characterized also the serum immunological profile by evaluating a specific panel of cytokines between the two mentioned conditions. Among the 27 cytokines tested, we detected a significant reduction of IP-10 (P = 0.0244) and a significant increment of IL-8 levels (P = 0.0547) in the high viremia setting (**Figure 13**).

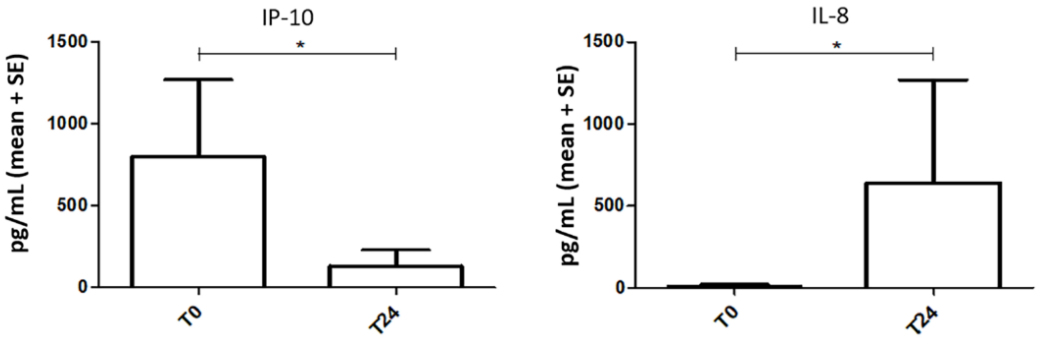


Figure 13: Boxplots showing statistically different levels of serum cytokines between high viremia and viral suppressor patients, assessed by the Wilcoxon test. A P value < 0.05 was considered statistically significant.

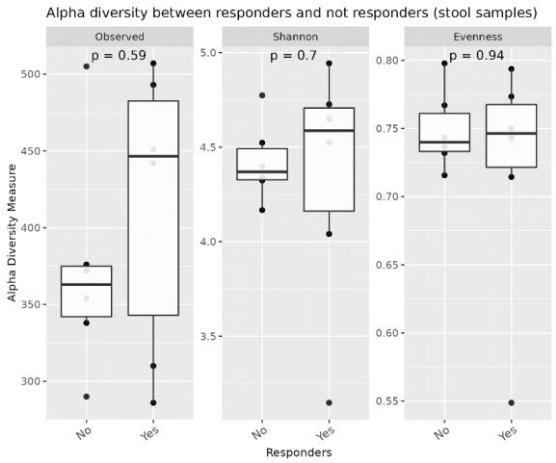
3. Association of microbiota composition, metabolic and inflammatory profiles with $CD4^+$ T-cell counts

3.1 Fecal and oral microbiota composition

In the last study part, we divided the patients into two groups: Immunological responders (IRs) and immunological non-responders (INRs), based on the CD4/CD8 ratio > 1 or < 1, respectively. We did not find significant differences in the alpha diversity on fecal

samples, while for salivary samples we documented a trend for Observed richness index (P=0.041) but no significant differences for Evenness and Shannon indices (**Figure 14 A-B**). In this scenario, the analysis of fecal microbiota revealed that members of the *Alistipes* genus were significantly increased in responders while *Escherichia-Shigella* genus was decreased in responders. Finally, the abundance of single microbial ranks in salivary samples revealed, at phylum level, an increase of Proteobacteria and a decrease of Campilobacterota in IRs (**Figure 15 A-B**).

A



B

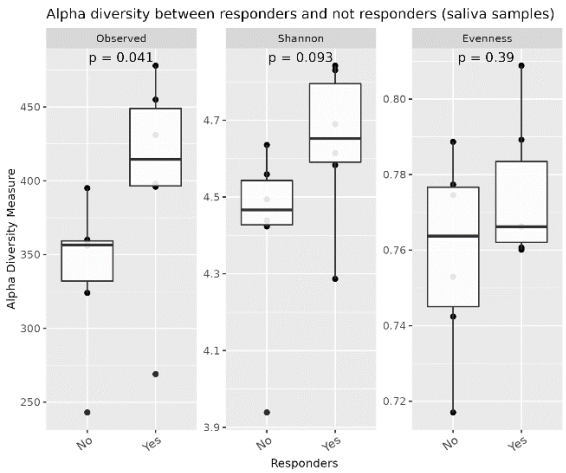
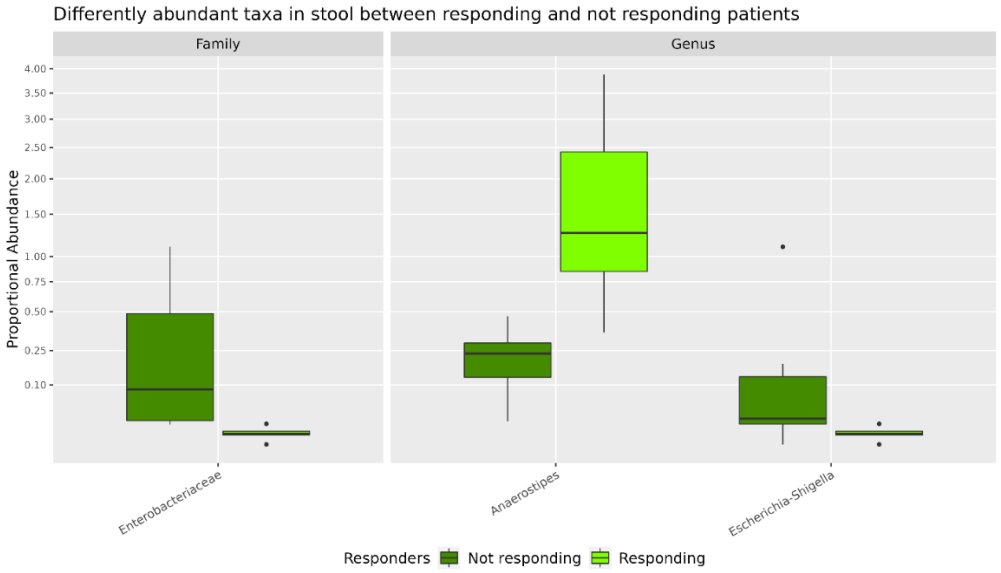


Figure 14. Box-plots showing alpha diversity indices (Observed richness, Shannon and Evenness) in fecal (A) and salivary (B) samples of HIV IRs and INRs. Statistical differences were evaluated using Mann-Whitney test for Observed richness, Shannon and Evenness indices. P-values less than 0.05 were considered statistically significant.

A



B

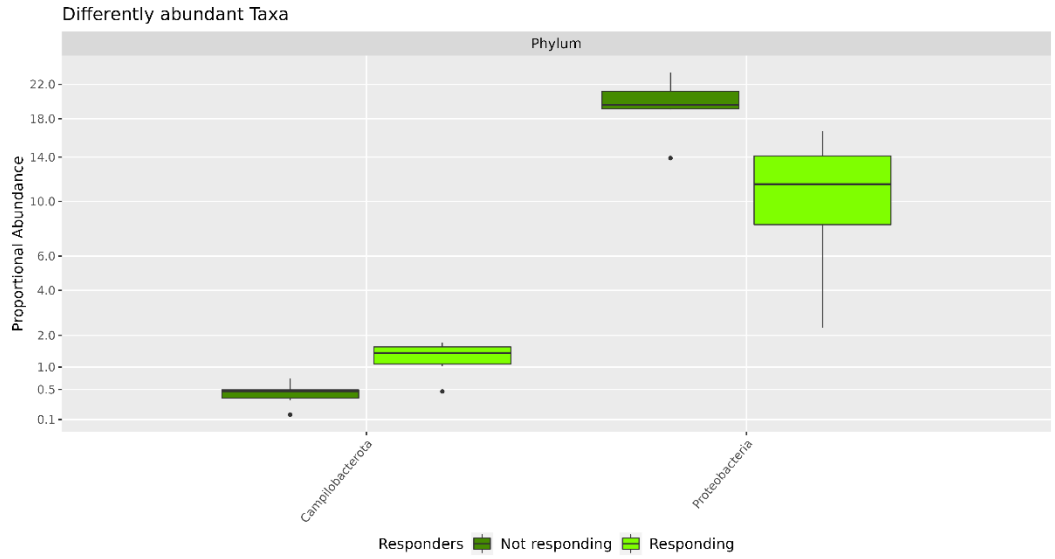


Figure 15. Boxplot showing the results of taxa differential abundance analysis between the fecal (A) and the salivary (B) samples of HIV IRs and INRs. All results have an adjusted p-value < 0.05.

3.2 SCFA profiles in serum and fecal samples

As we observed significant variations in the composition of the fecal microbiota between IRs and INRs, we assessed if there were any other alterations in the fecal and serum microbial metabolites as linear and branched SCFAs. We documented significant

changes in isobutyric (P = 0.01), isovaleric (P = 0.04), and 2-methylbutyric (P = 0.04) acids, which were increased in IR fecal samples while we did not detect significant differences in serum samples (**Figure 16**)

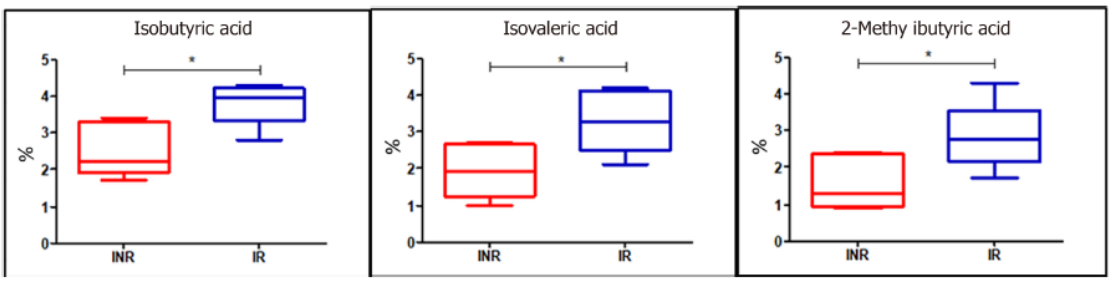


Figure 16: Boxplots showing statistically different fecal short-chain fatty acid abundances between immunological responders and immunological non-responders, assessed by the Mann-Whitney test. P value < 0.05 was considered statistically significant.

3.3 Molecular inflammatory profile

Since we detected significant variations of microbial communities between IRs and INRs, we also evaluated the serum immunological profile. However, cytokine levels did not show significant variations between the IRs and INRs.

Discussion

The microbiome may be influenced by chronic immunological dysfunction in HIV infection, that causes persistent inflammation and an increased risk of mortality. Numerous studies have linked the GM dysbiosis, or altered composition, to HIV[134,130,132], while the oral microbiota still needs to be understood. The fine mechanisms governing the interplay between the host immunity and HIV-1 as well as the modifications to microbiota structure and function are still unknown. To clarify the intricate relationships between the different actors of the "microbiota-immunity" axis, we explored the fecal and oral microbiota composition and function and associated serum inflammatory response and FFA composition in patients undergoing ART in different HIV infection settings. First of all, in order to understand the impact of HIV on the microbiota-immunity axis we compared the oral and fecal microbiota and the systemic inflammation of HIV infected patients and healthy controls.

Our data highlight a decrease of the Bacteroidia and an increase of Negativicutes class class in the stool of HIV+ patients. In detail, the, Negativicutes are Gram-negative bacteria with outer membrane structure rich of LPS. Recently their abundance among patients with HIV infection has been positively correlated with high level of inflammatory cytokine such as IFN-γ and IL-1ß[147], two cytokines that mediate the LPS-induced immune response[148].

On the other side, in the same fecal samples, we observed low level of Bacteroidia class, associated with anti-inflammatory properties and maintenance of gut homeostasis. Furthermore, our fecal data confirmed an increased amount of *Erysipelotrichaceae* family (Catenibacterium), negatively correlated with levels of the anti-inflammatory cytokines IL-19 and IL-35[147] in HIV+ patients. In agreement with you data, the bacterial family of *Erysipelotrichaceae*, which is contained within the separate class Erysipelotrichia, was on the whole found to be increased in association with HIV infection with a greater abundance, as documented in different studies[132,134,149]; only one study reported a decreased abundance of *Erysipelotrichaceae* [150]. Finally, the Erysipelotrichaceae are described as adhesive and potentially pathogenic[151] strain, why they are found to be positive associated with obesity[152,153] and colorectal cancer[154,155].

In addition, our results showed that HIV infected patients had low levels of *Ruminococcaceae* family, that plays both protective and disruptive roles within the GM community, such as the production of anti-inflammatory SCFA[147,156] or the degradation of host mucus and potentially supporting the inflammatory process in IBD[157]. *Ruminococcaceae* species in the gut induces expansion of Treg cells that can down-regulate harmful inflammatory responses[158]. Additionally, the SCFA metabolites produced by commensal bacteria from this and other clades support intestinal barrier integrity by influencing the energy metabolism of epithelial cells and the activation of Treg cells[118]. The aforementioned defensive physiological phenomena can therefore be impacted by abnormalities in these microorganisms' abundances. Paneth cells[159], macrophages[160], epithelial cells[161], and Th17 cells[162] are some of the gut immune barrier elements that are aberrant in HIV infection and are in charge of modulating the GM makeup, raising the possibility of a changed microbiota as a result of HIV infection.

Regarding oral microbiota, our analysis confirmed the data previously reported by other studies, with an increased level of *Porphyromonadaceae* family in saliva of HIV patients respect to HC. The *Porphyromonadaceae* have been associated with the human oral cavity, and are knower as an important periodontal pathogens[163].

In this work, we also carried out a longitudinal analysis to compare the gut and oral microbiota before and after "viral suppression" (T24). Only in few longitudinal studies, where HIV-1-infected participants were followed after starting ART, the data obtained on bacterial flora showed that shifts in the fecal microbiota persisted in some patients[132,164]. Our results, according to the longitudinal study conducted by Dillon et al[130], showed modest changes in the GM composition after ART and we did not assess significant differences in phylum composition. However, the paired comparison of the abundance of single bacterial taxa showed a significant alteration at the genus level between the two sample groups. In detail, after viral suppression, the genus *Succinovibrio* was significantly increased while the genus *Intestinibacter* was significantly decreased. We speculate that the persistent inflammation, the HIV latency throughout the gut, and the direct effects of antiretroviral drugs on the microbial population, may be the cause of the minor difference between the two groups. Additionally, our findings are consistent with other previous longitudinal investigations

in primates[165], which allowed us to account for potential confounders in human study outcomes. In agreement with our results the rare genus *Succinovibrio* was found high in stool samples of Japanese patients under ART[166]. Previous studies suggest that the *Succinivibrio* may be associated with defects in gastrointestinal functions, such as diarrhea and abdominal pain. As far as it concerns the oral microbiota we observed slight change only in the family of Asconditabacteriales.

Considering the microbiota role in influencing the immune system through the bacterial metabolites[116,167], we evaluated the SCFAs' level in peripheral blood and stool samples in order to have a more accurate assessment of microbial metabolism after the ART. Acetic, propionic, and butyric acids are the primary SCFAs, and they are produced by gut bacteria, especially those of the *Firmicutes* phylum, when fibers are fermented[168]. In this study, we observed significant change of two serum SCFAs after the ART, in particular, propionic and butyric acids were increased in "viral suppression" condition. This changed FFA profile would suggest that the SCFAs' production pathway plays a part in controlling the HIV "microbiota-immunity" axis during effective ART. Quite the opposite, we did not observe any significant SCFAs' changes in fecal samples. As previously highlight in literature, in the colon the 95% of produced SCFAs is rapidly absorbed by large intestinal mucosal cells while the other 5% is secreted in the feces[169]. We have already mentioned the fundamental role of SCFAs in the immunity modulation and in detail, the butyrate may reduce the gut inflammation by inducing the Tregs and modulating the activation of antigen-presenting cells[170]. We might suppose that the bacterial flora reacts to inflammation by boosting the synthesis of lipid mediators that are anti-inflammatory and pro-solving and circulate in the bloodstream. In line with these observations, several studies have documented that butyrate-producing bacteria are specifically decreased in stool samples from HIV-infected individuals compared to healthy controls[170,171].

Regarding the inflammation tone, there is consensus that a pro-inflammatory status remains active even after ART starting in most patients[172,124]. In order to evaluate the inflammatory status after ART, we measured a panel of selected multifunctional cytokines in serum. We observed a decrease of IP-10 after the treatment, confirming the downregulation of this chemokine production in HIV patients during ART[173,174]. IP-10

is involved in trafficking immune cells to inflammatory sites, and it is considered an important pro-inflammatory factor in the HIV disease process. It has been observed that its levels can be decreased, but not to normal levels, by ART administration. Interestingly, IP-10 was consistently associated with HIV disease progression (based on CD4+ counts)[175], suggesting its potential use as an indicator of HIV infection and/or a therapeutic target for HIV treatment[176].

On the other hand, in agreement with recent data, we documented a significant increased trend of IL-8 levels with suppressed viral load after 24 weeks of ART. Indeed, increased IL-8 levels were observed in HIV-infected patients on ART[177]. It has been shown that during HIV-1 infection, IL-8 plays a critical role in the recruitment of CD4+ T cells to the lymph nodes, thus generating more targets for viral replication. Our results may suggest that the increased IL-8 levels may represent a hallmark of chronic inflammation in HIV+ patients on ART. In agreement, Wada et al[178] observed significantly higher circulating IL-8 levels in HIV+ patients on ART with suppressed viral load in comparison to HIV-uninfected controls.

Finally, in order to investigate the microbiota role in the immune system reconstitution, we divided our patients in two distinct cohorts: patients with CD4/CD8 ratio < 1 (with insufficient reconstitution of $CD4^+$ T cells' amount), despite achieving virologic suppression after 24 weeks of ART, and those with CD4/CD8 ≥ 1, which reached a robust reconstitution of $CD4^+$ T cells' number. In detail, we found an increase level of Enterobacteriaceae family and *Escherichia-Shigella* genus in non-responding patients. Our data were in line with previous studies, suggesting an anti-correlation with the CD4/CD8 ratio and a positive correlation with the CD8 + CD57+ T-cell which was the hallmark of immunosenescence in human HIV infection[179,90]. Interestingly, the Enterobacteriaceae family was found to be negatively correlated with blood CD4+ T-cell numbers and positively associated with indicators of inflammation, colonic T cell activation, and monocyte activation (sCD14)[130,180].

Regarding the oral microbiota, we obtained an interesting result at Phylum level since we observed a high abundance of Proteobacteria in salivary samples of IR patients respect to INR. Numerous systemic illnesses are known to be linked by noticeable changes in the composition of the salivary microbiota, according to the most recent

investigations. It is already known that high levels of Proteobacteria is associated with host problems to maintain a balanced microbial community in the gut. Consistent findings have commonly revealed that dysbiosis during metabolic disorders often includes an increased prevalence of Proteobacteria[181,182]. In addition, mice lacking Toll-like receptor (TLR)-5 developed transmissible spontaneous colitis and dysbiosis, which was associated with abnormal expansion of Proteobacteria[183]. These findings imply that genetically predisposed mice are exposed to chronic colitis by a transiently unstable gut microbiota dominated by Proteobacteria. Studies in other mouse models with mutations affecting the adaptive immune system lend support to the concept that dysregulated innate immune responses drive the proliferation of Proteobacteria that, in turn, causes intestinal inflammation. For instance, as previously reported the primary immunoregulatory cytokine necessary for immunological tolerance to native microbiota is the IL-10; mice lacking IL-10 developed spontaneous colitis as a result of their intolerance to the intestinal flora[184]. Recently we investigate the alterations of the inflammatory profiles and the ileal bacterial population, analysing the molecular immune response and the GM community of Crohn's disease patients, revealing an increased Proteobacteria abundance [185]. An enriched level of Proteobacteria was revealed also in stool samples of HIV viremic untreated group compared with HIV-uninfected controls by Vujkovic-Cvijin et al. However, no differences in the relative abundance of Proteobacteria or Enterobacteriaceae between HIV-uninfected controls and patients receiving HAART were reported[180]. In conclusion, all this data focused on gut microbiota, while are still poor scientific literature regarding the abundance of Proteobacteria at oral level. Studies on human immune dysregulations such as HIV have also indicated that the oral microbiota is strongly related to the immune responses in immunodeficiency patients. Furthermore, the high frequency of opportunistic oral infections in HIV-infected patients and its correlation with CD4 + T cell levels suggest that the oral microbiota could be linked to various immunological responses to ART. Few studies have addressed the interaction of oral microbiota with different immune responses to ART received by the HIV-infected patients[186]. So, we could speculate that high abundance of pro-inflammatory taxa, such as Proteobacteria are involved in the maintenance of oral dysbiosis and are able to support oral opportunistic infections.

Conclusions

In conclusion, our findings offered a new perspective on the effects of HIV infection and in particular on the intricate relationship between microbiota, immune system and HIV. As expected, we observed that patients infected with HIV-1 displayed a different microbiota both at gut and oral level respect to healthy controls. Moreover, we demonstrated that, despite a successful ART and the achievement of virologic suppression, HIV patients displayed a fecal and oral microbiota with unchanged overall bacterial diversity. In addition, although the ART efficacy of in virologic suppression, not all HIV patients are able to reach the immune reconstitution (ratio CD4/CD8≥1). Finally, comparing immunological and non-immunological responders' patients we obtained a very interesting data at oral level, outlining the possible role of Proteobacteria in inducing and/or maintaining the inflammation tone.

It's undeniable that our study has some limitations, especially the limited number of enrolled patients. Therefore, these pioneering results appear very interesting and a better understanding of the interplay between the oral and gut microbiome may encourage the design of new strategies to modulate dysbiotic microbiota in inflammatory diseases.

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