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ROLE OF CART ON GUT MICROBIAL DYSBIOSIS, STUDYING THE GUT/BLOOD MICROBIOTA DURING THE FIRST TWO YEARS OF SUPPRESSIVE CART

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Part I

PART I

HIV INFECTION EPIDEMIOLOGY

In 1981 Center for Disease Control (CDC) di Atlanta (USA) detected the presence of a rare and severe variant pneumonia due to Pneumocystis carinii (today recalled Pneumocystis jiroveci), a commensal protozoa, in young homosexual individuals.[1] Simultaneously, there were many clinical reports of a rare vascular cancer in young individuals, previously diagnosed only in elderly subjects, called Kaposi's sarcoma. In 1983, researchers from Pasteur Institute identified the virological agent as a retrovirus related to HTLV-1 (Human T-Lymphotropic virus type 1), and in 1985 it was possible to detect the presence of specific antibodies to permit to identify its presence on blood samples by blood donors. At the beginner, the virus was recalled LAV and HTLVIII, and finally Human Immunodeficiency Virus (HIV) in 1986 according to WHO. HIV was identified as the cause of AIDS, acquired immunodeficiency syndrome: a complex of different diseases characterized by a deep immune-depression resulting in a vast range of clinical settings[2]. It was also proved that the HIV progenitor is a primates retrovirus: probably multiple episodes of infection between monkeys and humans, in distant african areas during the first decades of 1900, determined the development of the infection from rural to urban zones through the mucosal/skin exposition from contaminated animal biological materials.[3] Indeed, both serotypes, HIV-1 and HIV-

2, respectively, are correlates to SIVcpz (as guest Pantroglodytes troglodytes, a kind of Chimpanzee) and to SIVsm (as guest Cercocebus torquato atys, other Chimpanzee variant located to west Africa area as Guinea Bissau, Sierra Leone, Nigeria and Senegal, with a total prevalence of 1% with low transmission efficacy and long incubation period). UNAIDS reports 2012 evaluated about 35,3 millions of people living with HIV with an incidence of 2,3 millions of new infections per year and 1,6 millions of deaths due to AIDS, whose 75% in sub-saharian Africa regions. Analysing the last years, we can assume that the global prevalence is stable although the number of HIV living people increased due to the improvement of life expectancy in western countries; moreover, the reduction of deaths is due to both the therapies and the reduction of annual incidence. It is important to note that there are two different clinical backgrounds of HIV according the modality of transmission: epidemic model (model I), located in sub-saharian areas, that is essentially heterosexual and paediatric transmission; a partial/selected modality in western countries (model II) that includes intravenous drug users, “sex workers” and their sexual partners, homosexuals and heterosexuals; and a further model (model III) most typical in areas with low HIV transmission, with a mix of the first two models. Some envelope glicoproteins show 4 distinct groups: M (mayor), O (outlier), N (Non M-Non O) e P. The M group is the most diffused on the world, with 9 types (A-B-C-D-F-G-H-J-K) and 6 sub-types (A1, A2, A3, A4, F1, F2);

often the different types are located in different areas; for example B-type in USA and western Europe, E in Thailand; C in India, Ethiopia and southern Africa[4]. Risk groups are intravenous drug users, homosexuals and “sex workers” with their partners.[5] Transmission infection acquisition occurs through sexual transmission (both homosexual and heterosexual), parenteral transmission (blood transfusion o related, syringes use…) and vertical transmission.[6,7]. Mediterranean countries, as Italy, showed a model II transmission modified with high level of infection through intravenous drug users and vertical transmission, with an increase of the infection until to 1995, and subsequently reduction until 2000. In 2012 there were 64.898 cumulative AIDS cases with a stable incidence per year of 3853 new infections, 6.5 per 100.000 residents, with a majority prevalence of heterosexual modality, located especially in northern regions of the country as Liguria, Veneto, Lombardia, Toscana and Emilia Romagna regions[2]. During the last years there were an increase of homosexual transmission, especially in very young subjects, determining the homosexual group as the majority epidemiological group today, as observed in other western countries[8]; although the incidence of these new people very young infected, the median age increased during the last 10 years according analyses from ICONA (Italian COhort of antiretroviral Naïve patients).

HIV INFECTION ETIOLOGY

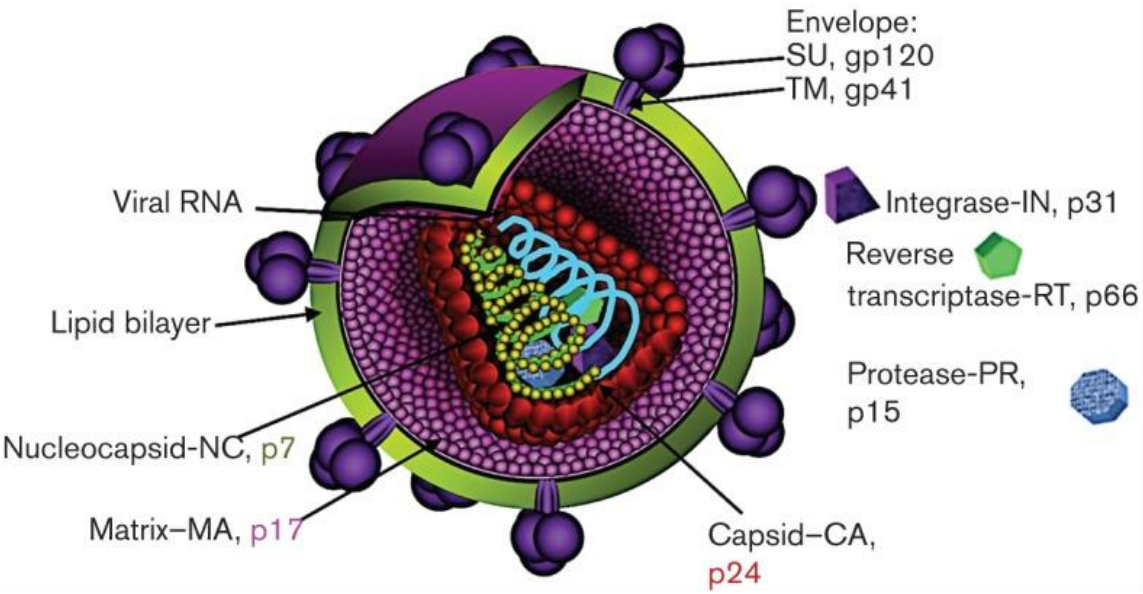
Family Retroviridae: 80-100 nm with envelope, RNA genome with 2 copies of the single-stranded RNA and 3 enzymes: reverse transcriptase, integrase and protease. The reverse transcriptase permits the retrotrascription from RNA to complementary DNA (cDNA); the integrase enzyme is essential for the integration of the viral cDNA to the cell nucleus; the protease works during the final steps of the viral replication cycle.

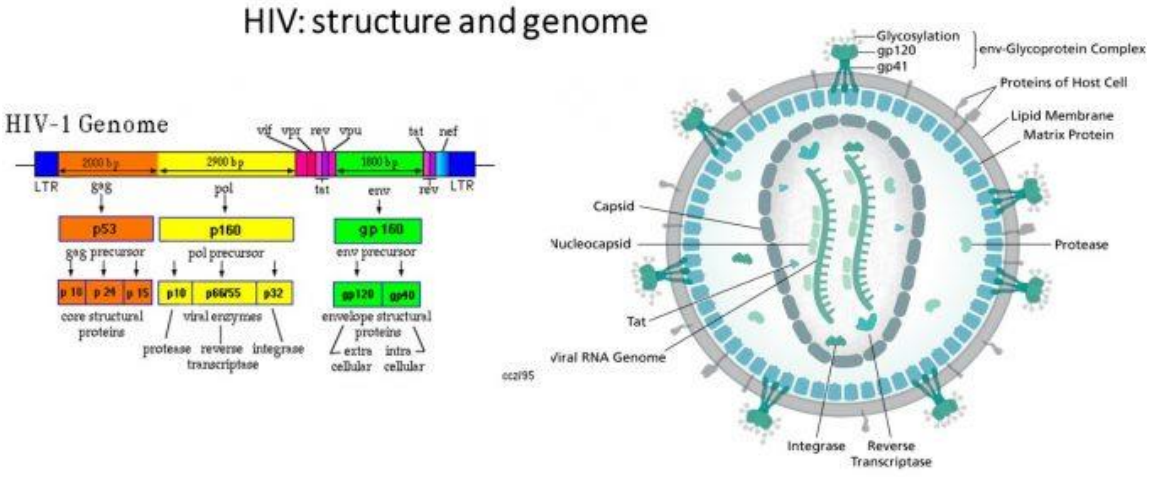
Retroviridae family includes two sub-families:

- Spuma-retrovirinae[9]
- Ortho-retrovirinae

The latter includes 6 genera: A-retrovirus, B-retrovirus, Γ-retrovirus, Delta-retrovirus, Epsilon-retrovirus e Lentivirus.

HIV-1, HIV-2 and SIV are all Lentivirus genus virus. The HIV-1 core includes *p24*, *p7/p9*, the two copies of RNAand the 3 enzymes. The superficial part of the core includes *p17* (a matrix protein) and other 2 glicoproteins: *gp120* and *gp41*. The HIV-genome is 9,7 kb and it is made by 3 genes: *gag, pol* and *env*. Furthermore, there are other 6 optional genes*: tat, rev, vif, nef, vpr, vpu* with different functions[10]. It is important to note that HIV presents a high level of genetic heterogeneity among different geographic areas and in the same individual too[11]; see Fig.1A and 1B.





HIV structure above (Fig 1A); HIV structure and genome below (Fig 1B)

HIV INFECTION CLINICAL SETTING

NATURAL HISTORY

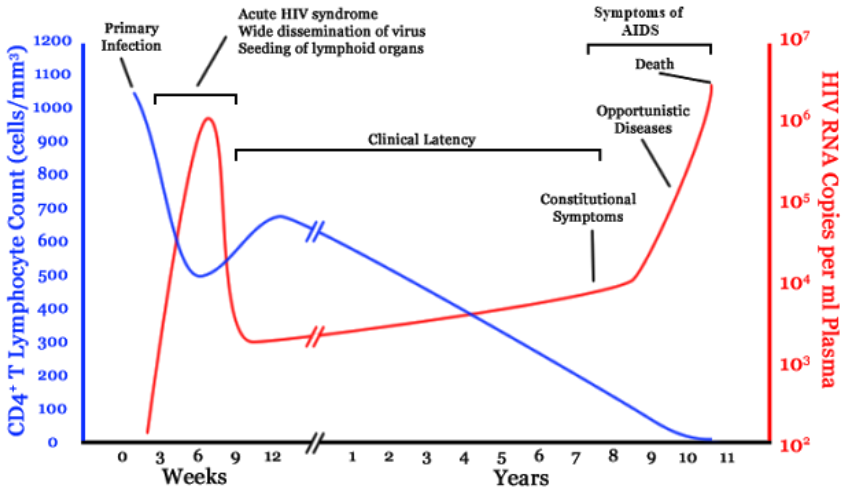
After the entry of the virus there are 3 phases:

- primary infection
- latency period
- advanced symptomatic stage

Primary infection is constituted by both acute clinical syndrome and increase of peripheral blood markers. During acute phase it is possible to observe the presence of fever, sore throat, asthenia, linphadenomegaly, arthromyalgia, nausea, vomit, diarrhoea, hepatosplenomegaly, skin rash. During this first phase, HIV replicates until 10^7 copies (cp)/ml with a significant and improvise reduction of CD4 T cell count; later some weeks, the immune system develops an antiviral response which determines a viral load decay, clinical setting resolution and an almost complete immunologic recovery[12]. During this first symptomatic period it is possible to detect a subsequent and slow significant reduction of CD4+ T cells (50-70 cells/year as CD4 T cells count at blood level) and an increase in CD8+ T cells lymphocytes[13]. Due to the presence of specific marker and after 10 days the viral introduction, it is possible to distinguish the acute infection among 6 acute stages according to Fiebig classification: Fiebig-I with only HIV-RNA detection (viral load, VL), Fiebig-II with VL + p24 antigen detection (possible with 4th generation

test), Fiebig-III with VL + P24 + Elisa IgM antibodies reactive (all these 3 stages with Western Blot (WB) negative), Fiebig-IV with VL+p24+Elisa+ WB indeterminate, Fiebig-V with VL+p24+Elisa+ WB positive without p31 band and finally Fiebig-VI with VL+p24+Elisa+ WB positive in association to p31 band too (within 6 months it is possible to conclude the acute phase of HIV)[14]. After the introduction of the steady-state, there is a balance between the creation and the destruction of new cells, with a long duration of 8-12 years, characterized by this distinct and slow decay of the CD4 T cells number and by a function alteration of immune system, with an important role of CD8 T cells to control the viral replication, without viral eradication, due to the presence of a vast viral reservoir[6,15]. Also, it is possible to detect the viral replication at lymphoid level as HIV-RNA: here, the virus determines a complete rearrangement of the lymphoid tissues[16]. When the immune system shows a deep deterioration, patients start to develop a symptomatic clinical stage of AIDS: at beginner with constitutional not specific symptoms, later with the onset of opportunistic infections (OI), especially with CD4 T cells number below 200/µl, that determine the majority cause of death due to HIV (see Fig. 2). During AIDS stage, it is possible to detect an increase in virological parameters of viral replication both lymph node and peripheral blood[6] (Fig. 3A and 3B).

Fig 2: natural history of HIV infection



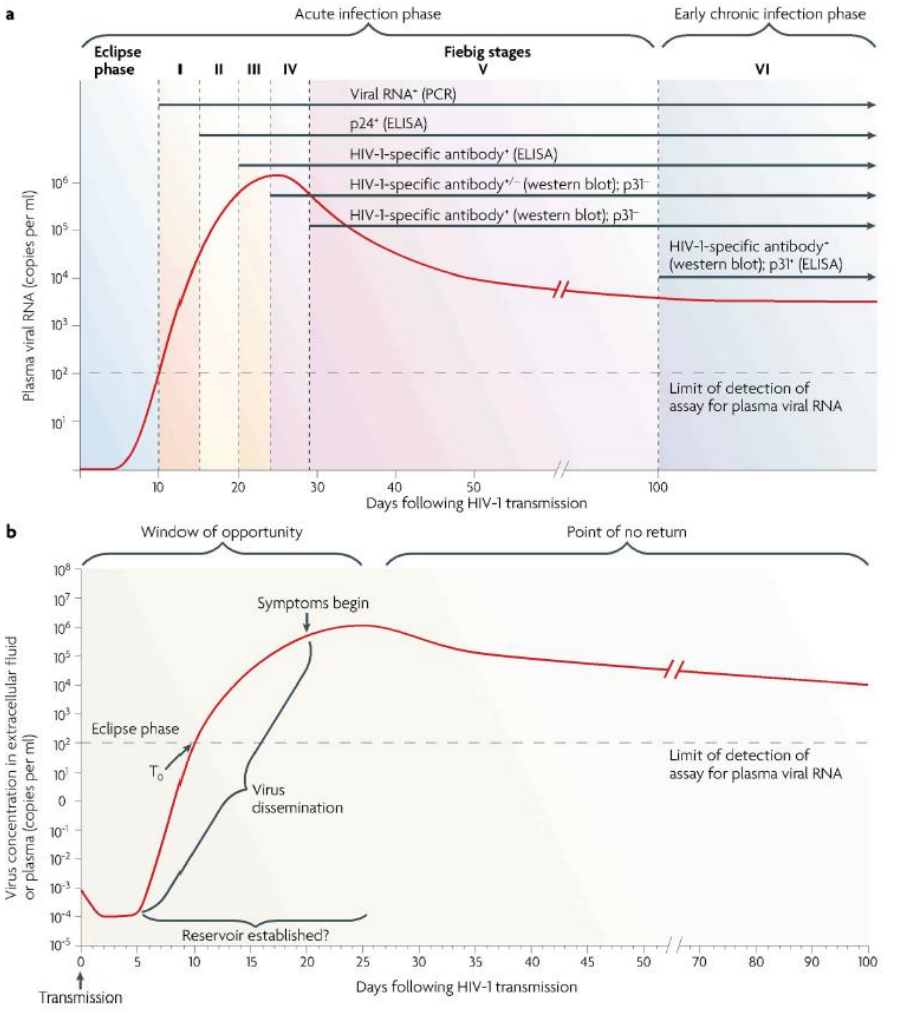


Fig 3A and 3B: Fiebig classification

OPPORTUNISTIC INFECTIONS

Simultaneously the reduction of CD4 T cells count, opportunistic infections become available through the develop of infections and neoplastic disorders. Different etiologic agents can determine different clinical settings. In 1992, CDC of Atlanta modified the previous classification (1987) according two parameters: clinical categories (number 3: A,B and C) and CD4 T cell count (number 3 ranges: 1,2 and 3). This last CDC classification system is different from WHO classification; indeed the CDC classification emphasizes the importance of CD4 lymphocyte testing in clinical management of HIV infected individuals, creating a matrix of 9 exclusive categories. The number indicates different ranges of CD4 T cell count: “1” with CD4 > 500 cell/mmc, “2” with CD4 T cells count between 200 and 500, “3” with CD4 T cells count < 200. The category “A” includes: either the asymptomatic HIV infection, either persistent generalized lymphoadenopathy as well as the acute primary illness; the category “B” includes all symptomatic conditions not included in category A and C; the category “C” includes 20 AIDS events. It is possible to consider an AIDS stage all people with HIV category C (C1, C2 and C3) and all people with CD4 T cells number below 200 (A3, B3 and the previous mentioned category C3). It is always AIDS diagnosis for some opportunistic disorders: Candidiasis (esophageal, trachea and bronchi), Cryptococcosis extrapulmonary, Cryptosporidiosis chronic intestinal (with symptoms >1 month), CMV retinitis or in other sites

(lung, brain), Herpes simplex virus (with mucocutaneous ulcer > 1 month, bronchitis, pneumonia), Kaposi's sarcoma (in subjects <60 years), Micobaterium avium or M. kansasii extrapulmonary, Pneumocystis jiroveci pneumonia, Progressive multifocal leukonecephalopathy, cerebral Toxoplasmosis. For the other AIDS conditions it is mandatory a laboratory confirmation: Coccidioidomycosis extrapulmonary, HIV encephalopathy, Histoplasmosis disseminated, Isosporiasis (chronic > 1 month), Kaposi's sarcoma (with > 60 years), other lymphoma non Hodgkin, cervical cancer invasive, Mycobacterium tuberculosis pulmonary and extrapulmonary, pneumonia recurrent (≥ 2 episodes in 1 year) wasting syndrome and Salmonella bacteremia recurrent[2].

HIV INFECTION THERAPY

CLASS DRUGS REGIMEN AND HOW TO START WITH HAART

The introduction of highly active antiretroviral therapy, HAART (or combined antiretroviral therapy cART), has modified the natural history of HIV determining the reduction of AIDS-deaths since 1996[17], although the presence of mutation HIV-related and the onset of side effects drug-related, that compromise both adherence and efficacy[18]. There are more than 20 different drugs, belong to different classes, with a triple combination, essentially, to permit a virologic control[19]. Here the drug classes: reverse transcriptase inhibitor that includes both nucleosidic/nucleotidic reverse transcriptase (NRTIs) and non nucleosidic/nucleotidic reverse transcriptase (NNRTIs), integrase inhibitors (INIs), protease inhibitors (PIs), entry inhibitors (EIs) both fusion inhibitors and CCR5 antagonists. NRTIs are nucleosidic/nucleotidic like structures modified: thymidine related structures: *zidovudine* (AZT) and *stavudine* (d4T); guanosine related structures: *abacavir* (ABC); cytosine related structures: *zalcitabine* (ddC), *lamivudine* (3TC) and *emtricitabine (FTC);* finally adenosine related structures: *didanosine (ddI)* and *tenofovir (*both *TDF* and *TAF).* Normally, 2 NRTIs of different nucleosidic/nucleotidic structures work together as synergic effect, establishing the “backbone” of the therapy plus a third drug (from another drug class). NNRTIs

inhibitors that inhibit the reverse transcriptase with a directly connection to the enzyme (no competitive bind). They are: *delavirdine (DLV), nevirapine (NVP), efavirenz (EFV), etravirine (ETR) and rilpivirine (RPV).* Protease inhibitors are *saquinavir (SQV), ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), amprenavir (APV), fosamprenavir (FPV), lopinavir (LPV), tipranavir (TPV), atazanavir (ATV)* and *darunavir (DRV);* they determine the production of not infected virions*;* important historical class that modified the life expectancy of HIV living people[20]. Entry inhibitors are a small heterogenic class that includes *enfuvirtide* (T-20) and *maraviroc* (MVC): two drugs that cannot be used in the first line regimens as third drug class regimen. Finally the most recent and important class of integrase inhibitors that includes: *raltegravir*, (RAL), elvitegravir/cobicistat (EVG/cobi) and dolutegravir (DTG) stopping the integration of cDNA at the level of nucleus cell.

The aims of HAART are:

- reduction of morbidities and increase of life expectancy
- improvement of the quality of life
- recovery and/or maintenance of immune system functions
- suppression of viral load
- prevention of the transmission of HIV

After the results from START study, all HIV patients have to start the therapy as soon as possible according the regional guidelines.[21] An efficacy response to HAART includes a 16 weeks reduction, a CD8 T

cells reduction after 2 weeks, a slow increase of CD4 T cells number, a reduction of activated T cells phenotypes. Although the recent antiretroviral regimens, a portion of the patients around 20-30% doesn't respond adequately to HAART; probably different factors can contribute to restrict the CD4 T cells recovery: nadir pre-HAART, an advanced age, a persistence detected viral load (often due to bad therapeutic compliance), the presence of microbial translocation and an immunologic unique background specific-individual. After the introduction of HAART, it is not possible to interrupt it due to a risk of a virological rebound and a CD4 T cell count decline[23]. According recent guidelines (DHHS, EACS, SIMIT), the INIs represent one of the most important antiretroviral class due a mix of positive factors: one of which the low level of toxicity; an interesting speed of viral load decay too (highest with INIs); finally formulation often with STR (single tablet regimen) for DTG and EVG/cobi, that is crucial for an efficient durability, to improve the quality of life (reducing pill burden) and to avoid virological failures (HIV-RNA >400 cp/ml after 24 weeks or >50 cp/ml after 48 weeks). According EACS guidelines 2017 in the first regimen we can enumerate many INIs + backbone combination and only one NNRTI based regimen and one PI based regimen:

- DTG/ABC/3TC (with caution for people with cardiovascular risk) or DTG + TAF (or TDF)/FTC
- EVG/cobi/TAF (or TDF)/FTC
- RAL + TAF (or TDF)/FTC

- RPV/TAF(or TDF)/FTC (not for VL > 100.000 copies/ml)
- DRV/cobi/FTC/TAF (or TDF/FTC)

Unfortunately, the 10% of the patients have to change the first line regimen due to toxicity and/or virological failure and/or low therapeutic adherence[23]. No evidence of beneficial effect of a short term four drugs regimen for patients with highly viral load (mega-HAART) with exception of virus with several resistance mutations at baseline; on the contrary, it is possible to switch to dual therapy in presence of toxicity confirmed during a triple regimen, although, until today, we don't know if it is more beneficial the use of a dual therapy (especially the new dual therapy nuc-sparing as DTG/RPV) vs the classical triple therapy (especially containing TAF); probably future studies could solve this question. Other dual therapy include the association between a PI and 3TC (especially DRV/cobi (or rtv) + 3TC or ATV/cobi (or rtv) + 3TC); as well as the association RAL/DRVcobi(rtv) or RAL/ETR or ETR/DRV/cobi (or rtv); not recommended both ATV unbosted and RAL or RAL + 3TC regimens. PI-monotherapy regimen (only DRV based, drug with high genetic barrier) it is not recommended due to the best profile of DRV/cobi (or rtv) + 3TC. Alternative regimens include the use of the other backbone, ABC/3TC, in association to the other third molecules (with exception of DTG/ABC/3TC, first line regimen): indeed this backbone is not recommended for VL > 100.000 cp/ml; the use of other third drug as PIs (ATV/cobi) or NNRTIs (EFV); the use of the dual therapy

RAL/DRV (cobi or rtv); other combinations are optional or not recommended. The choice of the HAART has to consider many factors: the power of the molecules (EVG, DTG, RAL, EFV, DRV) and the power of the combinations; the virological decay speed (INIs>NNRTIs>PIs); the presence of mutations at baseline and the genetic barrier with or without a not perfect therapeutic compliance prevision (low genetic barrier: 3TC, FTC, NVP, EFV, DLV, RPV, T-20, PIs without RTV/cobi; intermediate genetic barrier: INIs as RAL and EVG/cobi, ETR, ddI, TDF, ABC; high genetic barrier: DTG, PIs with cobi/RTV, AZT, d4T; possible better immunologic outcome (MVC, not proved for INIs); allergy (cross reaction between sulfamidic molecules and DRV); drug-drug interactions (regimens with cobi or RTV), life-style of the patient and its role for a good adherence (better STRs with/without no food assumption); CPE score in case of HIV encephalopathy (high CPE score for DTG, DRV, AZT, IDV, NVP). New drugs are *bictegravir*, a new INI DTG-like, as STR in combination with TAF/FTC, and a new NNRTI, *doravirine*, in a STR combination with TDF/3TC. Some studies are on-going to discover new formulation long-acting and new drugs for entry-inhibitors and the new class of maturation-inhibitors.

DRUG RESISTANCE

HIV produces about 10^9-10^{10} virions/day; with vast genetic variability due to new mutations by reverse transcriptase (one mutation every 10.000 nucleotides that means one mutation every single cycle). In this scenario other factors can contribute to develop drug resistances: the use of drugs with low power, the therapeutic adherence and the pharmacocinetic interactions. Several studies showed the low number of mutations by virological failures during a PI-first regimen compared to NNRTI first regimen. In any case, NNRTI based regimens show a better profile because well tolerated: in this case, 3 molecules, with low genetic barrier, can maintain a durability (with a good therapeutic compliance) due to the difficult for HIV to create 3 mutations for the same regimen. INI based regimen seem to be promising molecules, especially DTG based regimens which was superior to DRV in Flamingo study[22]. It is mandatory to perform a resistance test before to start an HAART regimen, often showing a wild type, and especially in case of virological failure; indeed, it is important to note that in 10% of patients that starting HAART, there are mutations not detectable from plasma due to the presence of a wild type which has a high speed replication fitness. The first resistance test could detect NRTIs mutations, recalled TAMS, with two different patterns: TAM1 (M41L, L210W, T215Y) and TAM2 (D67N, K70R, T215F, K219Q/E) with a possible cross resistance to

ABC, ddI e TDF especially with TAM1 profile and in presence of other mutations: G333E/D, N348I, A360T, A371V e Q509L. In particular, the N348I is often associated both to AZT resistance and to NNRTI drugs as NVP and EFV. Other type of mutations for this class are: M184V, Q151M complex, K65R and L74V/I. With exception of ETR (a second generation NNRTI), all other NNRTI molecules are low genetic barrier molecules both first generation (DLV, EFV, NVP) and second generation (RPV). NNRTI mutations include: K103N/S, V106A/M, Y181C/I/V, Y188L/C/H e G190A/S/E. For ETR not all mutations are important: Y181I/V presents a high score in association to L100I, K101P, Y181C and M230L. The 103N doesn't confer resistance to ETR, so it is possible to use ETR after EFV failure; unfortunately RPV has got a low resistance genetic barrier: K101P, Y181I/V, and especially E138K that invalidate the use of EFV and ETR too. Into PI class regimens, major mutations for third generation drugs (DRV and TPV) are: V11I, V32I, L33F, I74V, I50V, I54L/M, T74P, G73S, L76V, I84, L89V (V11I and L89V are DRV related). For INIs, RAL related-mutations show Q148H/K/R, N155H or Y143R/H/C. Association among Q148H and G140S determines resistance both RAL and EVG. For EVG there are: T66I, E92Q, S147G, Q148R/H/K and N155H. After this scenario of INIs resistances, it could be possible to remedy with the introduction of DTG-high dosage: 60% of success for multi-experienced patients, until the presence of mutation 148 (crucial for DTG)[19].

DRUG TOXICITY HAART RELATED

The management of HIV infection includes the monitoring of side effects drug-related. Lipodistrophy represents one of the most important side effect due to the use of old antiretroviral agents, as tymidine-related molecules and PI regimens[23,24,34]. Glucidic and lipidic alterations are common with PI, some NNRTI as EFV and backbone[40]; for example, insuline-resistance or diabetes with respectively 10% and 5% of incidence occur during PI based regimens treatment through the block of GLUT1 and GLUT4[25]. All these conditions can determine the onset of metabolic syndrome and an increase in cardiovascular events, especially with ABC and PIs. Other important endocrinologic targets for antiretroviral agents are the bone metabolism with the onset of osteoporosis[26,27] especially due to PIs[28] and some NRTIs (TDF), and thyroid function alterations that is correlated to d4T use[29,30]. At gut level it is important to enumerate different gastrointestinal disorders as dyspeptic syndrome, loss of appetite, diarrhoea, nausea, vomit, pyrosis, abdominal pain, meteorism and constipation[31]; often PI-related. A specific target is the liver, whose hepatotoxocity is related to NRTIs and PIs, with final result of liver steatosis[32,36] or sever hepatitis with some NNRTI as NVP; finally, unique and particular is the increase of indirect bilirubin related to ATV administration[33] (Gilbert syndrome-like). Central nervous system is altered during EFV administration[34] with dizziness, insomnia, nightmares and depression; the latter recently

associated to DTG and RPV too. Peculiar is the mitochondrial toxicity NRTIs related that can determine lactic acidosis, hepatic steatotis, pancreatitis and peripheral polyneuropathy with myopathy[35]. Allergic reactions can occur with all drugs, especially NNRTIs class regimen with hypersensitivity reactions within 12 weeks[36], from classical Steven-Johnson to hypersensitivity reaction ABC-related to HLA-B 5701 aplotype. The kidney is another target often due to the action of IDV and TDF[37,38,39], with nephrolithiasis or kidney failure and/or Fanconi syndrome due to tubular damage[42]. Rhabdomyolysis is peculiar for RAL whose appearance determines the suspension of the treatment.

IMMUNOPAHOTEGENESIS AND ROLE OF IMMUNE-ACTIVATION ON THE HIV INFECTIONS PATHOGENESIS

HIV INFECTION IMMUNOPATHOGENESIS, T-CELLS ELEVATED TURNOVER AND IMMUNE-ACTIVATION MARKERS

For every clinical phase previous described, HIV uses the immunologic cells to spread out through the different compartments. After the entry through mucosal sites, HIV infects the T cell lymphocytes, macrophages and dendritic cells stabilizing in its new reservoir, which includes spleen, lymph nodes and lymphoid tissues. The first contact involves the gp120-gp41 with CD4 receptor which is present in many different sub-types of immunologic cells as T cells, macrophages/monocytes and dendritic cells. The first contact permits the consecutive second contact between sp120 and one of the two most important chemokines co-receptors: CCR5 and CXCR4: this second contact is mandatory for HIV to infect the cells. There are HIV virus species specific for CCR5, recalled R5 or M-tropic (M from macrophage), on the contrary, other HIV species virus specific for CXCR4, recalled X4, are also T-tropic (from T cells tropic); finally there are HIV-species with tropism for both co-receptors: dual-tropic. M-tropic viruses express tropism for macrophages, monocytes and lymphocytes; on the contrary T-tropic viruses express tropism exclusively for T-cells. It is important to specify that dendritic cells

are infected from M-tropic, lymphocytes have both co-receptors with CD4+memory that are preferably infected from M5: its involvement determines a worse scenario due to both its activation for immunologic response versus the virus, and the chemotaxis effect by these cells to other lymphocytes, emphasizing the transmission[4]. CD4+ memory cells, strong target of HIV, are different from naive and effector cells: memory cells are CD45RO+ CD25+ CD62L+; on the contrary Naive cells are CD45RA+ CD62L+ CCR7+ and CD127+; finally effector memory are CD127+ CD44+ and variable expression of CD62L and CD45RO[45,46,40]. Feature of CD4+ memory is their dependence from naive CD4 cells, due to the higher levels of CD4+ apoptosis after activation compared to CD8+ turnover. Other distinctive aspect is the impossibility to convert an effector CD4+ cell to memory phenotype due to the lower expression of CCR7, that impedes the movement to lympho-nodal tissues[41,45,46,47]. After the entry of the virus, M-tropic virus infects immunologic cells through gut lamina propria, which is the most vast immunologic site of the body. The decay of immunity system at gut level occurs rapidly, without modification of plasma lymphocytes count, especially during the first 3 weeks[51]; successively, during years, there is a switch from M-tropic to T-tropic that determines the final stage of AIDS. Every day there is the production of $100*10^9$ new virions in association to the death of $1\text{-}2*10^9$ CD4 T-cells, with a loss of 50-100 cells/µl /year/plasma[51]. In this scenario it is important to note:

- Modifications of lymphonode architecture
- Death of cells due to activation from other pathogenic triggers (other infections) or HIV its-self
- Destruction of the precursors both direct infection either absence of cytokines
- Infected cells fusion creating giant cells due to the action of X4-tropic viruses through the contact between gp 120 by infected cells to CD4+ of not infected cells.
- CD8+ T-cells activation targeted versus the complexes made between the bond of soluble gp120 and CD4[4].

During the chronic phase the 30% of lympho-nodal CD4+ T cells count have viral RNA; on the contrary in the blood compartment the viral RNA tends to be zero during virologically suppressive HAART; moreover, other important different is the final viral target: memory cells at lympho-node level, naïve for blood compartment. In these conditions, the best reservoir is probably represented by lymphonode and, especially, innate immunity cells[42]. Douek et all described the presence of an altered and increase turnover T cells with a reduced life-time of all lymphocytes[49] (see fig.4):

- increase in production, but not in survival time, of CD4+ T cells count during HAART

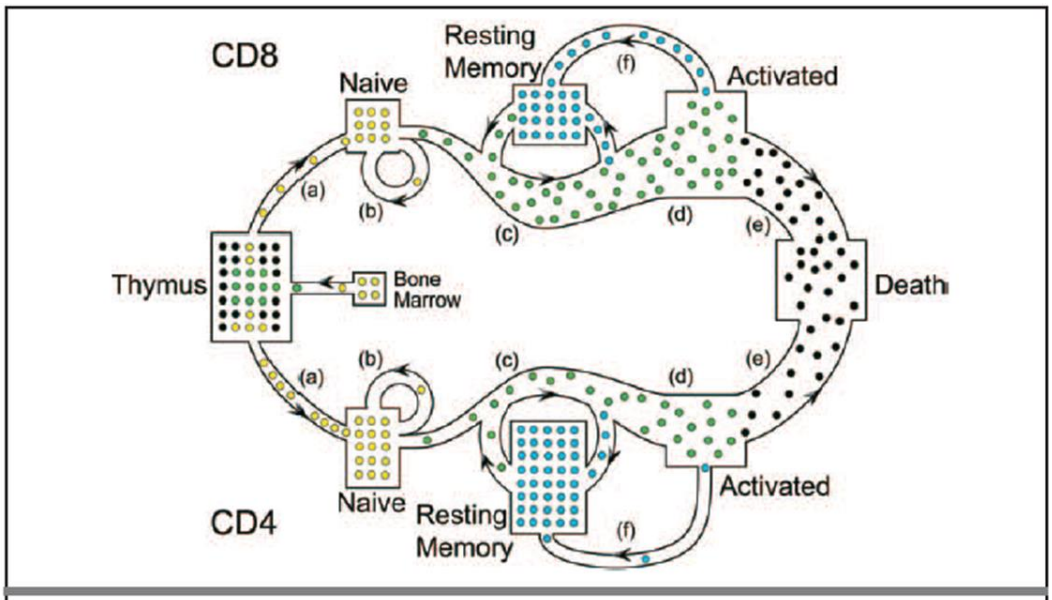
- reduction in CD4+ T cell, memory and naive, due to chronic activation

- reduction of lympho-nodal dendritic cells and plasmacytoid cells

- high number of CD8+ T cells with an inversion CD4/CD8 ratio.

Features of the HIV infections are either the CD4+ T cell count depletion or the elevated turnover T cells. The first feature creates the conditions to develop AIDS events, as quantitative damage; in any case, the second feature is peculiar for chronic infections and represents a qualitative damage. Indeed, the latter could be consequence of reactivation of other viruses (for instance EBV or CMV) or direct consequence by HIV replication that results in T-cells anergy and apoptosis. Moreover, HIV+ presents higher expression of CD38+ and HLA-DR on CD4+ T cells (with a concomitant increase in Fas) and especially on CD8+ T cells compared to controls. Peculiar is the reduction of CD45RA+ CD62L+, Naive cells, both CD4+ and CD8+ T cells with a relative increase in memory activated CD45R0+ CD4+. CD38 is a further activation marker that correlates with short survival in absence of HAART[43]. Steven G Deeks et al[44] have shown an inverse correlation between plasma-HIV-RNA and CD4+ cell count in naive patients; a positive correlation between CD8+ cells and viral load; finally a negative correlation between viral load and activation markers. Probably different components can explain the develop and the persistence of peripheral immune-activation during HIV infection (see fig.5) and its partial response to HAART.

Fig 4: from Douek et al. upper without HIV, below with HIV



. A scheme of T-cell dynamics in a healthy individual. Resting cells along the bone marrow to naive T-cell axis are shown in yellow. Activated T-cells are shown in green. Resting memory T-cells are shown in blue. Dying cells are shown in black. The arrows depict movement of cells between the pools. The sizes of the boxes depicting the pools and the number of cells represent the relative magnitude of those cellular pools, but are not to scale.

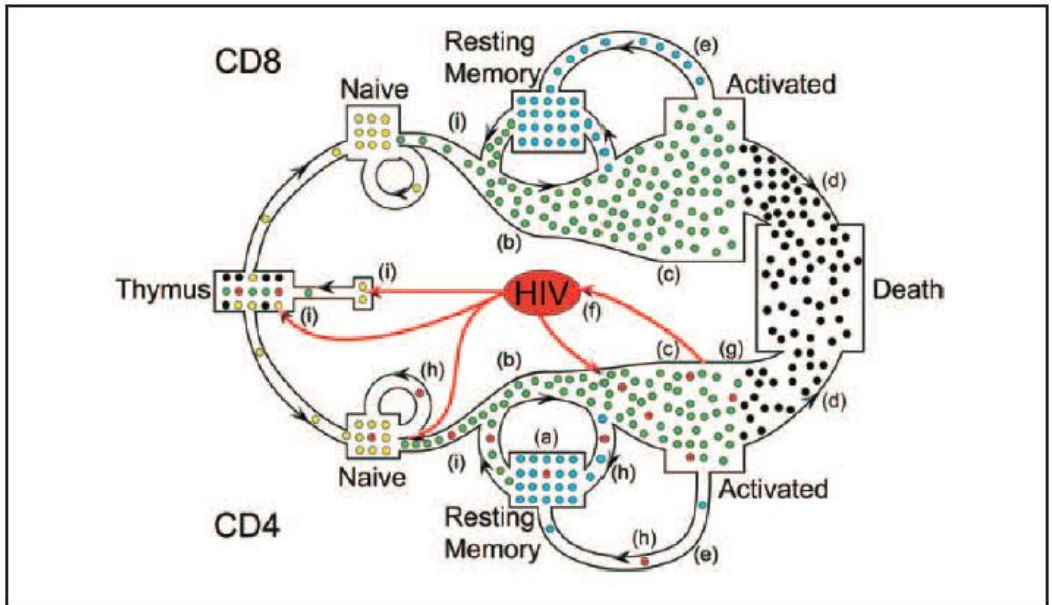
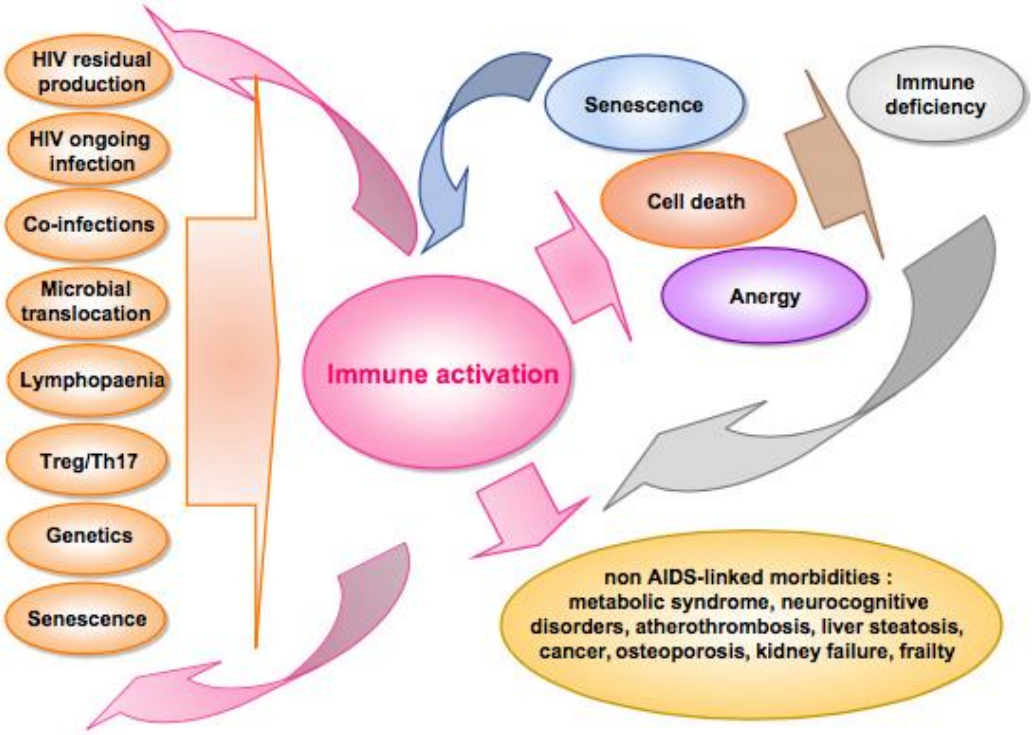


Figure 2. *A scheme of T-cell dynamics in an individual in the chronic phase of HIV-1 infection. The colors of the cells are as in figure 1. HIV-1-infected T-cells are shown in red. The red arrows depict both direct and indirect negative effects of HIV-1 on T-cell production and/or survival, including destruction of lymphoid organ architecture.*

Fig 5: possible causes of immune-activation



MICROBIAL TRANSLOCATION AND GUT INFLAMMATION DURING HIV INFECTION AND IMMUNERECOVERY HAART RELATED

One of the most captivating hypotheses of immune activation, is the theory of microbial translocation, defined as the passage of bacterias or bacterial components, from the gut to extra-intestinal sites, such as plasma, linfonondes, spleen, liver. One kind of evidence that corroborates this hypothesis is the presence of positive correlation between the microbial translocation of ecological bacterial niches, especially some components, such as LPS, and immune-activation. Indeed today we can consider it as a marker of microbial translocation. LPS is a common component of gram negative bacterias, that increases in course of sepsis. It constitutes the 40% of external cell wall and it circulates bind to LBP (protein binding LPS) and/or soluble CD14 (sCD14), a monocyte protein. There is a complex among LPS/LBP/sCD14 that binds the TLR4-MD2 receptor: a classical PRR (pathogen recognition receptor) that recognises PAMPs (pathogen associated molecular pattern), in this specific case the LPS. The subsequent signaling determines proliferation and immunologic activation with increase in cytokines[45]. Brenchley et al. have shown higher plasma LPS concentration in patients HIV+ with CD4+ T cell count below 200 and with AIDS diagnosis[46]. Merlini et al

have shown the presence of polymicrobic flora translocating into the peripheral blood of HIV+ patients with inadequate immune recovery during effective cART[47]. Recently, Tincati et all.[48] have shown anatomical abnormalities that accompany gut injury during HIV infection that include the impairment of the intestinal junctional complex: reduced expression of cadherins, claudins, occludins especially in patients with no adequate immunerecovery; moreover, it was found an alteration of permeability measurers among between HIV+ and HIV- individuals as well as inflammatory markers with an increase in faecal calprotectin at gut level. Faecal calprotectin is produced by gut neutrophils and gut activated monocytes, as occurs in other gut inflammatory diseases, with the batteriostatic and micostatic function, above the normal cut off (> 50 μg/g of feces). Regard to calprotectin there are two conditions: soluble and cell-membrane-bound; the first is produced by neutrophils and monocytes after immunologic activation by LPS, IL-1β, C5a, IL-6 and TNF-α; the second works as adhesion component between cells and extracellular matrix. All these evidences permit to emphasize the relevance of the mucosal gastrointestinal tract as key factor to control comorbidities and improve the clinical outcome of HIV patients. Another complementary aspect is the presence of antibodies, IgM, IgG and IgA class, specific for the endotoxin core of LPS, recalled EndoCAb, that there are in all subject as natural immunologic response to bacterial fragments passage, and that are reduced in HIV

subjects compared to HIV negative controls[49]: the reduction of these antibodies, during elevated microbial translocation, occur because the ELISA test for EndoCAb measures the free part of the antibodies not bound to LPS, so, with a concomitant increase of LPS, there are more immunologic complexes and a subsequent reduction of free EndoCAbs. Hazenberg e al.[50] have shown that the most important recovery occurs during the first 4 weeks after introduction of antiretroviral therapy, although without obtain the normal CD4+ and CD8+ cell count, even after years of HAART. Also, the introduction of HAART determines a reduction of immune activation with an increase in EndoCAb and a reduction in all other microbial translocation markers and immune-activation parameters, without a complete normalization[51].

FAECAL MICROBIOTA

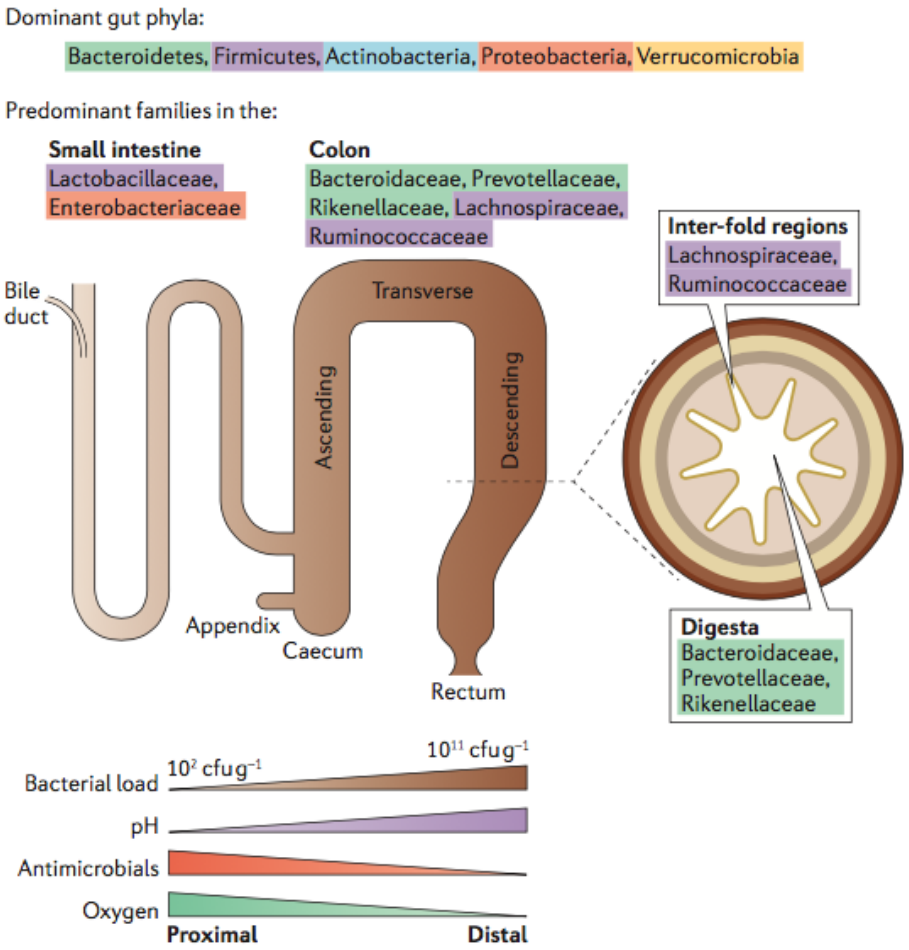
MICROBIOTA IN HEATLHY INDIVIDUALS

The human microbial ecosystems comprises bacteria, archaea, eukaryotes and viruses which colonize different body habitats including the gut, skin, vagina, and respiratory tract. Several studies showed the central role of commensal bacterias at gut level for immunologic, metabolic and integrity functions[52,53]; indeed germ free animals have dramatic level of destruction of gut mucosal integrity and impairment of its immune system. At gut level the total number of prokaryotes located as ecological niches includes $14*10^{13}/10^{14}$ bacterias recalled microbiota, with an amount of genetic material of $3*10^{6}$, that is 100 times higher the value of the human genome ($3*10^{4}$), through 1000 bacterial species, more than 150 genera[54], recalled microbiome; although the two terms are different, it is important to note that microbiota and microbiome are used interchangeably by scientific community. The vast majority of bacteria in the colon are strict anaerobes which are able to perform saccharolytic fermentation of non-digestible substrates from dietary fibres, making energy and nutrients for host and symbiont communities. Few divisions have been identified in the human gut: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Verrucomicrobia and Fusobacteria. The human gastrointestinal microbiota is dominated by the first four main bacterial phyla whose

99% of species identified are mainly to the two first phyla, Firmicutes and Bacteroidetes, representing together 70% of the total microbiota[55], with different distribution according the gastrointestinal tract (see fig. 6A) and with Bacteroides, Faecalibacterium and Bifidobacterium as the most abundant genera although in association to higher variable faecal relative abundance among individuals[56] (see Fig. 6B). The human microbiota is individual specific and relatively stable during adult period but with strong differences according to the age from childhood (with higher Clostridium spp and Bifidobacteria spp.) to elderly age (increase in Firmicutes/Bacteroidetes ratio); moreover other important factors could influence the diversity of microbiota: both gender and sexual behaviours, the BMI (with different microbiota profile in obese individuals), the kind of diet, the geographic area and, of course, the presence of antibacterial agents or pre/probiotics. Other condition that could also modify the distribution of microbiota are the different gastrointestinal disorders as intestinal inflammatory diseases, metabolic diseases as diabetes, autoimmune disorders as celiac disease or other condition not present in healthy population[57] (see Fig.7). The microbiome is essential for gut health through its metabolic function using the diet carbohydrates which are fermented to short chain fatty acids (SCFA) in the colon: acetate, propionate and butyrate. SCFAs are the major metabolic products of anaerobic fermentation, with total concentrations of 50–200 mM in the colon,

that can bind specific immunologic receptors and, subsequently, to be used as sources of energy, as regulators of gene expression for epithelia integrity and for immunologic interactions. If acetate is a fermentation product for most gut bacterias, the other two SCFA, propionate and butyrate, are considered healthy SCFAs, with a different subset of bacteria producers. Priopionate is metabolized through two pathways: the succinate and the propanediol pathways. The succinate pathway is found mainly in Bacteroidetes and in the Negativicutes class of Firmicutes (but also in some members of Ruminococcaceae); the propanediol pathways is used by Lachnospiraceae (Roseburia inulinivorans and Blautia species) and other few bacterial species. Butyrate is the final metabolic step from butyryl-CoA, which proceeds either via butyryl-CoA:acetate CoA-transferase or via phosphotransbutyrylase and butyrate kinase. Butyrate-producing species are distributed among different taxa, with propionate-producing species, especially in the two predominant families of human colonic Firmicutes, Ruminococcaceae (essentially Faecalibacterium prausnitzii) and Lachnospiraceae (especially Eubacterium, Roseburia, Anaerostipes and Corpococcus genera) and in other Firmicutes families as Erysipelotrichaceae and Clostridiaceae[58].

Fig.6A and 6B


Dominant gut phyla:
Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, Verrucomicrobia
Predominant families in the:
Small intestine
Lactobacillaceae, Enterobacteriaceae
Colon
Bacteroidaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae, Ruminococcaceae
Inter-fold regions
Lachnospiraceae, Ruminococcaceae
Bile duct
Transverse
Ascending
Descending
Appendix
Caecum
Rectum
Digesta
Bacteroidaceae, Prevotellaceae, Rikenellaceae
Bacterial load
10^2 cfu g^{-1}
10^{11} cfu g^{-1}
pH
Antimicrobials
Oxygen
Proximal
Distal

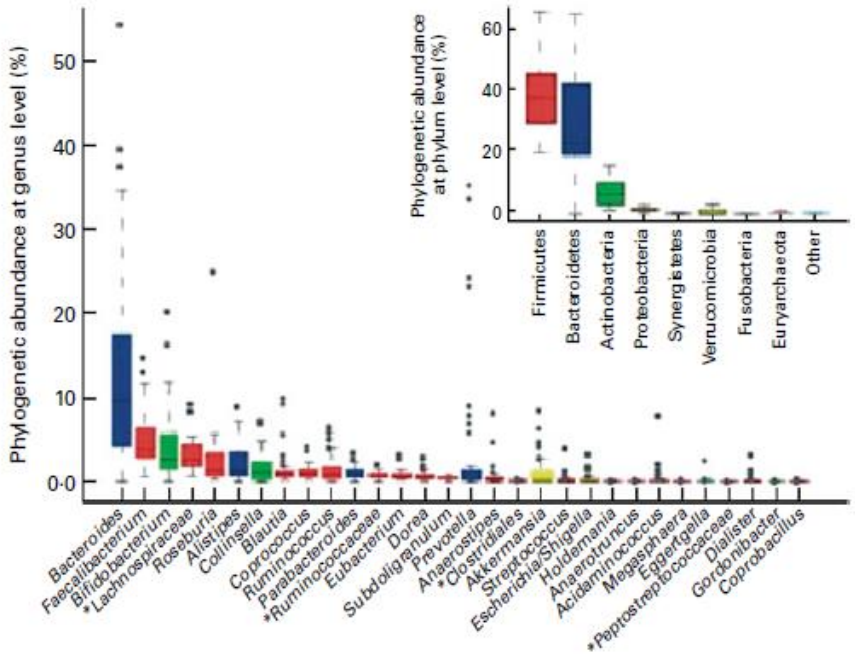
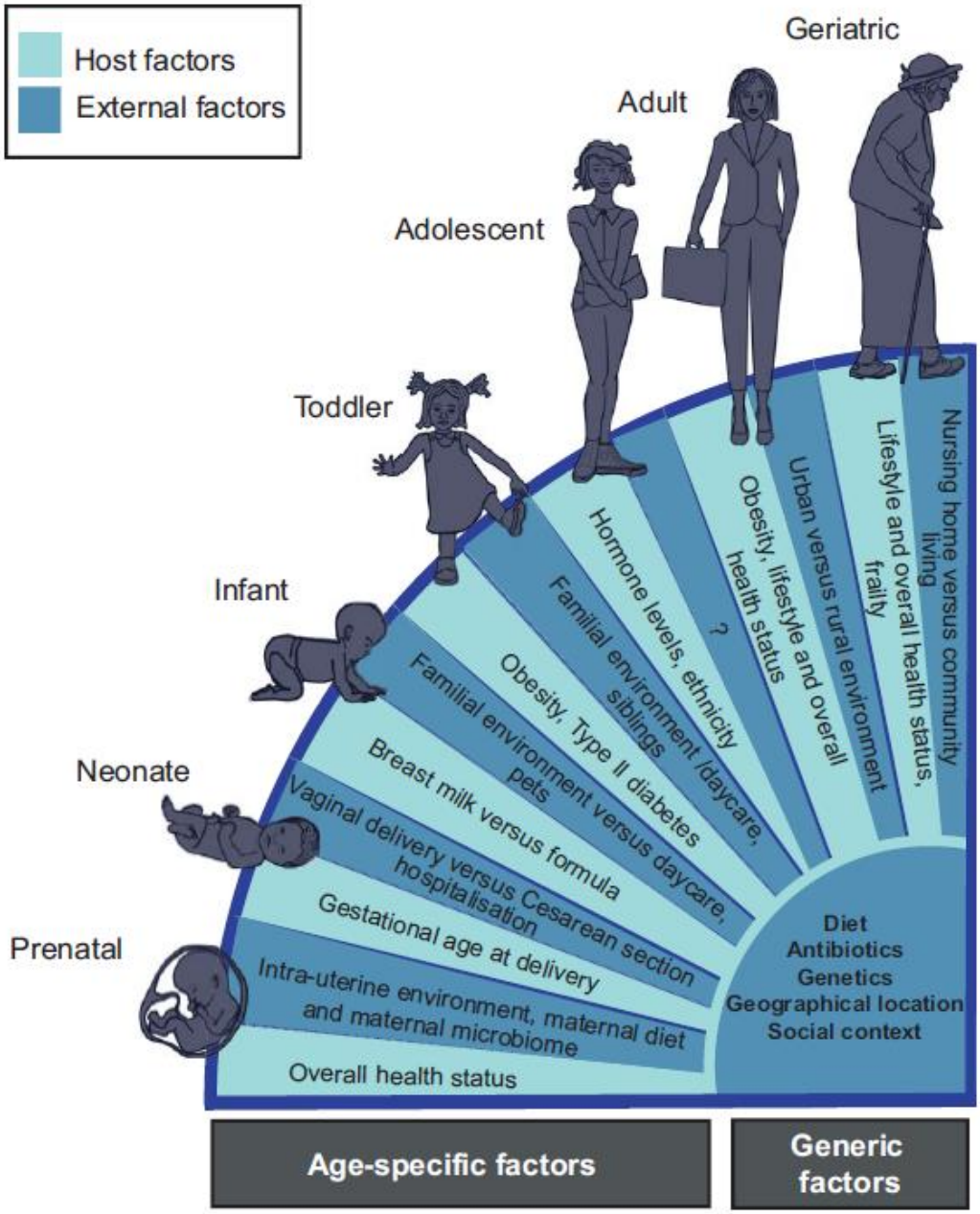

Phylogenetic abundance at genus level (%)
50
40
30
20
10
0·0
Bacteroides
Faecalibacterium
Bifidobacterium
*Lachnospiraceae
Roseburia
Alistipes
Collinsella
Blautia
Coprococcus
Ruminococcus
Parabacteroides
*Ruminococcaceae
Eubacterium
Dorea
Subdoligranulum
Prevotella
Anaerostipes
*Clostridiales
Akkermansia
Streptococcus
Escherichia/Shigella
Holdemania
Anaerotruncus
Acidaminococcus
Megasphaera
Eggerthella
*Peptostreptococcaceae
Dialister
Gordonibacter
Coprobacillus
Phylogenetic abundance at phylum level (%)
60
40
20
0
Firmicutes
Bacteroidetes
Actinobacteria
Proteobacteria
Synergistetes
Verrucomicrobia
Fusobacteria
Euryarchaeota
Other

Fig.7


Host factors
External factors
Geriatric
Adult
Adolescent
Toddler
Infant
Neonate
Prenatal
Nursing home versus community living
Lifestyle and overall health status, frailty
Urban versus rural environment
Obesity, lifestyle and overall health status
?
Hormone levels, ethnicity
Familial environment /daycare, siblings
Obesity, Type II diabetes
Familial environment versus daycare, pets
Breast milk versus formula
Vaginal delivery versus Cesarean section
Gestational age at delivery
Intra-uterine environment, maternal diet and maternal microbiome
Overall health status
Diet
Antibiotics
Genetics
Geographical location
Social context
Age-specific factors
Generic factors

ECOLOGICAL MEASUREMENTS: A AND B DIVERSITY

To study the differences from human gut microbiome, it's possible to use a NGS (next generation sequencing) technology where it is mandatory the DNA extraction followed by amplification and sequencing of ribosomial 16S DNA, a relatively short sequence highly conserved within a species, with high-throughput DNA sequencing; subsequently the identification through taxonomic alignment compared to reference sequences in database, with a similarity at least of 97% (today it is possible until 99%) for single taxa. After all this pipeline, often using a package of software (for example Qiime2), it will be possible to obtain all the OTUs (operational taxonomic unit) from phylum to species (phylum, class, order, family, genus, species), and in some cases the differentiation among ASVs (amplicon sequence variants) that allow to discriminate between two bacterias belong to the same species. An important aspect is linked to the portion of the r16SDNA selected to amplify: some authors amplified the hyper-variable V4 regions, instead of V3-V4 or other regions; it is important to specify that the choice of the r16SDNA region could determine a different ability to better distinguish species that belong to specific taxa instead of others. In any case, we can assume that the different methods are comparable although this bias[59]; on the contrary it is not possible to compare the features found in other biological materials as anal swab and/or gastrointestinal biopsies. The analyses of comparison of relative abundance for each taxonomic

level is a simple and initial step to analyse the differences among groups; anyway, given the great amount of bacterias and the difficulty in comparing these results, many authors prefer to evaluate the microbiota distribution using ecological diversity parameters, such as α and β diversity, that broaden the features found with relative abundance. The concept of "α and β diversity" was introduced in ecology by R.H. Whittaker: the former is the number of the species in a specific geographic community; the latter is the number of communities in a specific geographic area; finally, γ diversity is the product of α and β diversity. In humans, α diversity measures the level of diversity within individual samples, while β diversity measures the level of diversity (or dissimilarity) between samples[60]. Two important concepts are related to α diversity: richness and evenness. Richness is the number of different species represented in an ecological community, landscape or region. It is simply a count of species, and it does not take into account the abundances of the species or their relative abundance distributions. On the contrary, evenness refers to how close in numbers each species in an environment is. There are different parameters to compare α diversity: according to observed parameter it is possible to evaluate how many species there are, without capture the relatedness among species (a qualitative measure of community richness); PD (phylogenetic diversity or Faith's PD) determines how close the related species count are each other; so the latter is a measure of the level of

evolutionary diversity (measure of tree of life) and it indicates a qualitative measure of community richness that incorporates phylogenetic relationships between the features. Unfortunately both, observed and PD, don't consider the distribution. Another richness

$$S_{est} = S_{obs} + \left(\frac{[(f]_1)^2}{2f_2}\right)$$

method is Chao1:

It emphasizes the importance of rare OTUs found in a sample: number of observed species add the ratio of the square of singletons (only saw one sample) divided by two times the number of doubletons[61]. Community diversity indices combine species richness and abundance into a single value of evenness. Gotelli et al. specified that communities that are numerically dominated by one or a few species exhibit low evenness while communities where abundance is distributed equally amongst species exhibit high evenness. Two of the most widely used indices are the Shannon (or Shannon-Wiener) index (Shannon, 1948) and Simpson's index (Simpson, 1949). Shannon index quantifies the entropy in strings of text:

$$H_{SH} = -\sum_{i=1}^{S} p_i \log_2 p_i$$

if practically all abundance is concentrated to one type, and the other types are very rare (even if there are many of them), Shannon entropy approaches zero; when there is only one type in the dataset, Shannon

entropy exactly equals zero. Because Shannon includes richness and abundance, it is often considered a diversity index instead of a richness or evenness index; according to other authors it is possible to simplify it as a quantitative measure of community richness; to solve this problem, some authors used the Shannon evenness index: this latter index presumes that the most equitable community could have equal number of OTUs (Shannon index divided Ln(OTUs) or Pielou's evenness): becoming a measure of community evenness. Simpson index measures the degree of concentration when individuals are classified into types. The measure equals the probability that two entities taken at random from the dataset of interest represent different types, or the same type if we consider

$$H_{SI} = 1 - \sum_{i=1}^{S} p_i^2$$

inverse Simpson: :

Because every single index expresses different α diversity informations, it is possible to use all or some of these indexes[62,63,64]. B diversity measures use the principle coordinates analysis, PCoA (any distance matric, not only Euclidean distance) through a non matrix multidimension scale (NMDS) where there are the two principle component analysis (PCA). The most used β diversity metrics are: Jaccard distance: a qualitative measure of community dissimilarity;

$$J(A,B) = \frac{|A \cap B|}{|A \cup B|} = \frac{|A \cap B|}{|A| + |B| - |A \cap B|}.$$

Jaccard index measures the similarity between finite sample sets, and it is defined as the size of the intersection divided by the size of the union of the sample sets; on the contrary 1-Jaccard index is the Jaccard distance that is complementary to the Jaccard coefficient, so a dissimilarity measure. Bray-Curtis

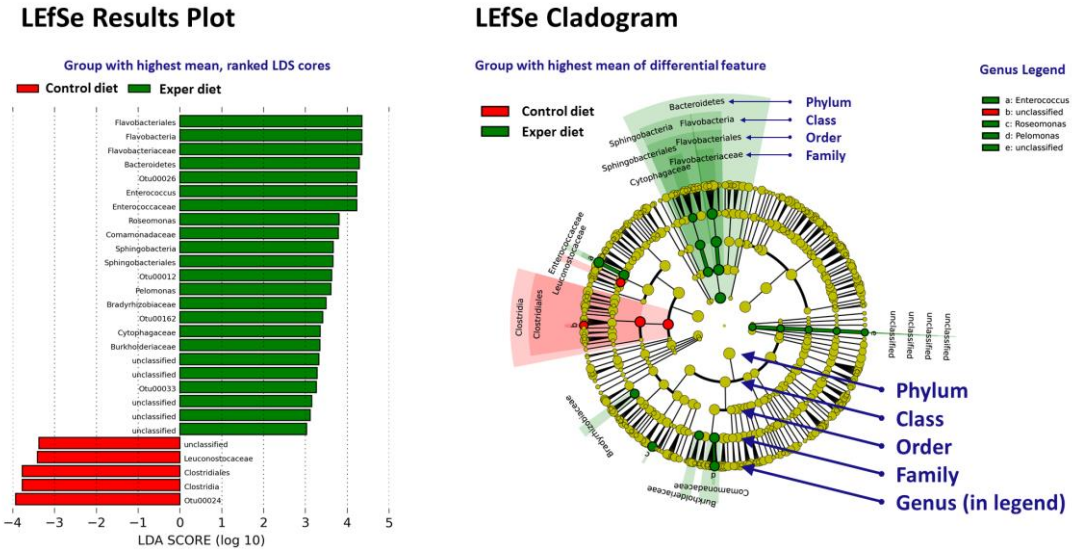
$$BC_{ij} = 1 - \frac{2C_{ij}}{S_i + S_j}$$

distance

is a quantitative measure of community dissimilarity; where 2C{ij} is the sum of the lesser values for only those species in common between both sites. S{i}and S{j} are the total number of specimens counted at both sites: how much does one sample «undershoot» the other. Between 0 and 1, where 0 means the two sites have the same composition (that is they share all the species), and 1 means the two sites do not share any species. Both (Jaccard and Bray-Curtis) don't consider the relatedness among species; so, after the inclusion of this information, it is possible to use the UniFrac distances where d= «0» that means identical community, 1 unrelated community, 0,5 related community. It measures "the percentage of observed branch lenght unique to either sample"; Unweighted unifrac only uses presence/absence, so emphasizes the minor bacterias, as a

qualitative measure of community dissimilarity that incorporates phylogenetic relationships between the features; Weithed unifrac considers count of relative abundances (it weights the branch lengths by the abundances of bacterias, so emphasizes the dominant bacterias), as a quantitative measure of community dissimilarity that incorporates phylogenetic relationships between the features. B diversity could be represented by either PCoA and Ward's method criterion to generate the hierarchical clusters with the UniFrac taxonomic distance measures[65]. Finally, another way to measure the β diversity is the use of LEfSe analyses: LDA Effect Size (LEfSe)[66] is an algorithm for high-dimensional biomarker discovery and explanation that can identify taxonomic groups characterizing the differences between two or more biological conditions. LEfSe identifies features that are statistically different among biological classes; subsequently, performs additional tests to assess whether these differences are consistent with respect to expected biological behaviour. The linear discriminant analysis effect size often uses a threshold of 2.0 for the logarithmic LDA score for discriminative features in addition to the strategy for multi-class analysis set to 'all-against-all'. LEfSe cladograms, compared to plots, represents the differences between two groups including the taxonomic informations (see Fig 8).

Fig 8


LEfSe Results Plot
Group with highest mean, ranked LDS cores
Control diet
Exper diet
Flavobacteriales
Flavobacteria
Flavobacteriaceae
Bacteroidetes
Otu00026
Enterococcus
Enterococcaceae
Roseomonas
Comamonadaceae
Sphingobacteria
Sphingobacteriales
Otu00012
Pelomonas
Bradyrhizobiaceae
Otu00162
Cytophagaceae
Burkholderiaceae
unclassified
unclassified
Otu00033
unclassified
unclassified
unclassified
unclassified
Leuconostocaceae
Clostridiales
Clostridia
Otu00024
−4 −3 −2 −1 0 1 2 3 4 5
LDA SCORE (log 10)
LEfSe Cladogram
Group with highest mean of differential feature
Control diet
Exper diet
Bacteroidetes
Flavobacteria
Sphingobacteria
Flavobacteriales
Sphingobacteriales
Flavobacteriaceae
Cytophagaceae
Phylum
Class
Order
Family
Genus Legend
a: Enterococcus
b: unclassified
c: Roseomonas
d: Pelomonas
e: unclassified
Enterococcaceae
Leuconostocaceae
Clostridia
Clostridiales
unclassified
unclassified
unclassified
unclassified
Bradyrhizobiaceae
Burkholderiaceae
Comamonadaceae
Phylum
Class
Order
Family
Genus (in legend)

MICROBIAL DYSBIOSIS IN HIV INFECTION

In the course of chronic HIV infection the GALT, the vastest immunologic compartment located at gut level, shows a dramatic deterioration, that comprises substantial loss of mucosal T-cells, disruption of intercellular tight junctions, pro-inflammatory infiltrate. An important alteration described in HIV-related GALT damage is dysbiosis with disruption of the residents ecological niches. Dysbiosis represents a change in the numbers of microbes and/or a change in the diversity of the microbiota due to specific diseases. A clear definition of dysbiosis is difficult because there is not agreement about the term of eubiosis due to intra-individual differences among distinct body sites and inter-individual variation among healthy individuals[67,68]. Some authors suggested an unbalance of microbiota with an increase in pathogenic bacteria (Pseudomonas aeruginosa e Candida albicans) and a decrease in other healthy genera (Bifidobacteriaceae e Lactobacilli) compared to controls[69,70]; it is important to note that before the introduction of NGS, all the previous works used the qPCR to detect specific taxa. After introduction of NGS, it was possible to detect alterations in composition and degree diversity, whose grade is difficult to evaluate[71], with alterations of links between resident bacterias and immunity system, promoting gut and systemic inflammation with a possible association to tryptophan metabolism[72]. Lozupone at al. in 2013 described a cluster separation with PCoA according Unifrac,

from faecal samples, between chronic naïve HIV subjects and controls with higher α diversity indexes (Shannon and PD)[73]. As relative abundance abundances of Prevotellaceae, Erysipelotrichaceae, Veillonellaceae families and Clostridium cluster XIII and Desulfovibrio genera compared controls; on the contrary HIV negative subjects showed increase in Bacteroidaceae, Rikenellaceae and Porphyromonadaceae. After this crucial study, new topics were described: the unbalance in Bacteroidetes phylum (Prevotella-rich/Bacteroides-poor); an increase in α diversity instead of decrease, as occurs in other disease model; the effect of HAART; the impact of the diet or other factors that could justify these results. Indeed the Prevotella-rich microbiota resembles what described in healthy individuals in agrarian cultures of Malawi and Venezuela and associated to carbohydrate-rich, protein- and fat-poor diets. An evidence that an altered profile of Bacteroidetes phylum could play a pathogenetic role was suggested by other authors[74,75] confirming this unbalance at mucosal level (with an interesting decrease in Firmicutes and increase in Proteobacteria phylum, the latter not confirmed from faecal samples), that was associated with increased activated colonic T cells and myeloid DCs, in untreated[79] patients. Similar findings were reported in treated patients[80] with a reduced α diversity in both studies. Moreover, PCoA showed a microbiota associated with increased mucosal cellular immune activation, microbial translocation and blood T cell activation[79]. In a first review,

it was postulated a possible pathogenic role of Prevotella-richness community due to its association to an increased prevalence of cardiovascular disease and/or atherosclerosis, probably through the TMAO pathway and other species as Desulfovibrio, which is increased in HIV infection[74]. An important contribute was added by Noguera-Julian et al.[76] that emphasized the important to control for sexual behaviour: MSM (men sex men) had an higher α diversity compared to other HIV+ epidemiological groups; moreover, within the MSM group, HIV was associated with reduced α diversity. This discovery can clarify why many authors have found contrasting data from different cohorts[77].

Few studies have thus far have investigated the impact of different antiretroviral agents[78,79] or the impact of the introduction of HAART with a longitudinal design[80].

PART II

BACKGROUND

In the recent years, HIV-infected individuals have been reported to harbor a distinct gut microbiome (Zilberman-Schapira G et al, BMC Med 2016; 14:83; Lozupone CA et al, Cell Host Microbe 2013; 14:329-339; McHardy IH et al, Microbiome 2013; 1:26; Mutlu EA et al, PLoS Pathog 2014; 10:e1003829), that have been associated to HIV infection itself, antiretroviral treatment, sexual orientation (Noguera-Julian M et al, EBioMedicine 2016; 5:135-146; Lozupone CA et al, Gut Microbes 2014; 5:562-570; Yu G et al, AIDS 2014; 28:753-760 18, 61, 62+klatt science). Gut microbiome involvement in HIV transmission and pathogenesis is increasingly being recognized (Dillon SM et al, AIDS 2016; 30:2737-2751; Gootenberg DB et al, Curr Opin Inf Dis 2017; 30:31-43; Williams B et al, AIDS Res Hum Retroviruses 2016; 32:935-941; Vujkovic-Cvijiin I et al, Sci Transl Med 2013; 5:193ra91; Nowak P et al, AIDS 2015; 29:2409-2418), yet the findings and interpretation of the results diverge significantly among studies (Li SX et al, Clin Phramacol Ther 2016; 99:600-611), due to differences in cohort size, sampling, lack of adjustment for confounding factors, such as sexual practice and diet (Noguera-Julian M et al, EBioMedicine 2016; 5:135-146; Shanahan F et al, Gut 2017; 66:1709-1717).

Among the most consistent alterations described in untreated HIV, dramatic subversion of the *Bacteroidetes* phylum with an unbalanced *Prevotella*/*Bacteroides* pendulum, and the *Proteobacteria* phylum with enrichment in *Enterobacteriaceae*. Indeed, several sets of evidence have been provided to link IA and microbioma. In untreated HIV, several authors have described important alterations in enteric microbiota, particularly in phyla such as *Proteobacteria*, *Bacteroidetes* and *Firmicutes* with a shift toward a *Bacteroides*-poor/*Prevotella*-rich profile and an enrichment in *Enterobacteriaceae*, a Gram-negative family of the phylum *Proteobacteria*, known to be involved in microbial translocation (Klase Z et al, Mucosal Immunol 2015; 8:1009-1020), in turn contributing to residual immune activation (Klase Z et al, Mucosal Immunol 2015; 8:1009-1020; Dillon SM et al, Mucosal Immunol 2014; 7:983-994; Tincati C et al, AIDS Res Ther 2016; 13:19; Zevin AS et al, Curr Opin HIV AIDS 2016; 11:182-190).

Both cross-sectional and longitudinal studies have shown a limited effect of suppressive cART in restoring gut microbiome (Pinto-Cardoso S et al, Curr Opin HIV AIDS 2018; 13:53-60; Deusch S et al, AIDS 2018; 32:1229-1237; Ji Y et al, Emerging Microbes & Infections 2018; 7:113; McHardy IH et al, Microbiome 2013; 1:26)[80,68,81,78,79,72,82]. Indeed, despite cART-treated HIV+ patients showed a gut microbiota composition different from the viraemic individuals, they also display a community structure distinct from

HIV- healthy subjects (McHardy IH et al, Microbiome 2013; 1:26; Lozupone CA et al, Cell Host Microbe 2013; 14(3):329-39; Nowak P et al, AIDS 2015; 29:2409-18. Taken together, data to link IA and gut microbiome and persistent alteration in cART, would suggest that gut microbiota alterations could contribute to the pathogenesis of residual disease that persist during suppressive antiretroviral therapy. Antiretroviral compounds could themselves further promote dysbiosis (Shilaih et al, Antiv Ther 2018; 23(1):93-94; Li SX et al, Clin Pharmacol Ther 2016; 99:600–611), and different cART regimens differentially impact the gut microbiome, markers of microbial translocation, inflammation/immune activation and the gut epithelial barrier damage, with cART-experienced individuals on protease inhibitors being worse off (Nowak P et al, AIDS 2015; 29:2409-2418; Pinto-Cardoso S et al, Sci Rep 2017; 7:43741; Villanueva-Millan MJ et al, J Int AIDS Soc 2017; 20:1-13).

An exciting challenge that has been somehow neglected so far is the understanding of the contribution of the "circulating microbiota" to HIV-associated co-morbidities and its association with microbial translocation during effective cART. We have previously shown the presence of polymicrobic flora translocating into the peripheral blood of HIV+ patients with inadequate immune recovery during effective cART[47], leading to wonder whether a diverse microbial composition into the blood might simply reflect the changes into the gut or might take actively part to the pathogenesis of HIV infection. Interestingly,

recent studies have demonstrated a role of blood microbiota in the onset of diabetes and athero-thrombotic disease in the general population[83,84], possibly confirming a role of blood dysbiosis in non-AIDS related co-morbidities. Despite the importance of the gut microbiome in HIV infection has been widely recognized, little is known on how HIV-related dysbiosis is associated with microbial translocation (MT) and gut damage and how long-term cART fits within this complex scenario. Thus, we decided to explore (i) the long-term effects of cART on markers of gut damage/MT and gut/blood microbiota composition, with a focus on the role exerted by different drug classes and (ii) the possible associations between gut dysbiosis and peripheral inflammation/immune activation, microbial translocation and intestinal permeability.

MATERIALS AND METHODS

STUDY POPULATION

HIV-positive patients were consecutively enrolled at the Clinic of Infectious Diseases, Dept of Health Sciences, University of Milan - ASST Santi Paolo e Carlo, Milan, after providing written, informed consent in accordance with the Declaration of Helsinki. The Institutional Review Board at the ASST Santi Paolo e Carlo specifically approved the study. Inclusion criteria were: age >18 and naïve to cART with any CD4+ count and plasma HIV-RNA. The patients who were enrolled were from the same geographic area of Milan (North of Italy); individuals with signs/symptoms of gastrointestinal diseases were excluded. HIV+ patients underwent blood and faecal sampling prior to cART (T0) and after 12 (T12) or 24 (T24) months of therapy. We also enrolled HIV-negative healthy subjects as controls.

T-CELLS IMMUNEPHENOTYPES

Lymphocyte surface phenotypes were evaluated by flow cytometry on fresh peripheral blood (FACSCanto II; BD Italy). We evaluated activation (CD45R0/CD38), naive (CD45RA) and memory (CD45R0) subsets and IL-7 receptor (CD127) on CD4 and CD8 T-cells. Samples were stained with the following fluorochrome-labeled

antibodies: L/D V500, CD4 PE-Cy7, CD8 PerCPCy5.5, CD38-FITC, CD45R0-PE, CD45RA-FITC, CD127-PE (BD Bioscience).

The following combinations were used: LD/CD8/CD38/CD45R0, LD/CD8/CD4/CD127 and LD/CD4/CD8/CD45RA/CD45R0. FACSDiva 6.1.3 software was used to analyze data.

MICROBIAL TRANSLOCATION MARKERS

Plasma sCD14 and Endotoxin Core Antibodies (EndoCab) were measured by ELISA (R&D systems), in accordance with the manufacturer's instructions. Samples were diluted 1000X and 200X respectively. The total amount of 16S rDNA present in the samples was measured by qPCR in triplicate and normalized using a plasmid-based standard scale (Vaiomer SAS, Labége, France).

URINARY LACTULOSE-MANNITOL FRACTIONAL EXCRETION RATIO (LAC/MAN) AND INTESTINAL FATTY ACID BINDING PROTEIN (I-FABP)

Participants were asked to fast the night before and to collect morning urine before drinking a sugar probe solution containing 5g lactulose and 2g mannitol. Urine was collected for 5 hours following administration of the double sugar solution and participants did not eat or drink (with the exception of water) until the end of the 5-hour collection. The total volume of urine was recorded and a 30mL aliquot

of chlorhexidine-preserved (0.236 mg/mL of urine; Sigma Chemical, St Louis, MO, USA) was frozen and stored for High Performance Liquid Chromatography analysis of lactulose and mannitol (Dionex MA-1 ion exchange column with pulsed amperometric detection on a Dionex Ion Chromatograph 3000, Thermo Scientific, Sunnyvale, CA). The ratio of lactulose and mannitol excretion (LAC/MAN) was assessed. Normal values were considered <0.05[85,86].

Intestinal Fatty Acid Binding Protein (I-FABP) was assessed by ELISA (Hycult Biotech), according to manufacturer's instruction.

FAECAL CALPROTECTIN LEVEL

Calprotectin concentrations were measured by use of a commercial ELISA kit (Immundiagnostik, Bensheim, Germany), according to the manufacturer's instruction. Briefly, two 100-mg samples of feces from a single stool sample from each participant were assayed, and the mean of the two measurements was recorded.

GUT PERSISTENCE SCORE

A gut persistence score was calculated for all drug regimens. Briefly, given the known % of bioavailability for every single cART molecule, the % of RA-dose was arrived at for unchanged drug in feces, converted to a score between 1 (low persistence score) and 4 (high persistence score). This way, a gut persistence score cART

regimen was calculated as the total of the values determined for the single component of the triple regimens.

METAGENOMIC SEQUENCING OF BLOOD AND FAECAL SAMPLES

Fresh stool samples and plasma were collected from each subject, frozen immediately and stored until processing at −80 °C. Total DNA was extracted as previously described[87]. The V3-V4 hypervariable regions of the 16S rDNA were amplified and quantified by qPCR, sequenced with MiSeq technology, and clustered into operational taxonomic units (OTUs) before taxonomic assignment as described[87,88,89]. The targeted metagenomic sequences from faecal and plasma microbiota were analyzed using the bioinformatics pipeline established by Vaiomer SAS from the FROGS guidelines. For bioinformatics pipelines FROGS v1.3.0 and PhyloSeq v1.14.0 were used. Briefly, after demultiplexing of the barcoded Illumina paired reads, single read sequences were cleaned and paired for each sample independently into longer fragments. After quality-filtering and alignment against a 16S reference database, a clustering into OTU (Operational Taxonomic Unit) with a 97% identity threshold and a taxonomic assignment were performed in order to determine community profiles. The list of possible species for each unique sequence was further investigated using the BLASTN program from NCBI Blast against the NCBI 16S Microbial database. Only the

BLASTN hits covering the full query sequence length with an overall sequence identity of 97% or more were considered as possible species. Specifically, through FROGS v1.3.0 amplicons with these parameters were removed: with a length < 350 nt or a length > 480 nt; without the two PCR primers (10% of mismatches were authorized); with at least one ambiguous nucleotides ('N'). OTU identified as chimera (with vsearch v1.9.5) in all samples in which they were present were removed, as well as OTU with an abundance lower than 0.005% of the whole dataset abundance or with a strong similarity (coverage and identity >= 80%) with the phiX (library used as a control for Illumina sequencing runs). With regard to the clustering process: it was produced in two passes of the swarm algorithm v2.1.6. The first pass was a clustering with an aggregation distance equal to 1. The second pass was a clustering with an aggregation distance equal to 3. With regard to the taxonomic assignment: it was produced by Blast+ v2.2.30+ with the databank RDP v11.4 and filtered to ignore "unknown" taxa from the RDP database Finally, through the package PhyloSeq v1.14.0 it was possible to use a set of classes and tools to facilitate the import, storage, analysis, and graphical display of microbiome census data, after the exclusion all samples with less than 5000 sequences from FROGS processing for statistical analyses. The DNA extracted from plasma for sequencing was evaluated by 16S qPCR. The total 16S rDNA found in the samples was measured by qPCR in triplicate and

normalized using a plasmid-based standard scale. The construction of a standard curves allows for a proper quantification of 16S rDNA gene copy in the sample, but also enables the determination of the efficiency, linear dynamic range, and reproducibility of the qPCR assay. In these experiments, the efficiency calculated from the standard curve was required to be between 80-120%, and the R2 of the standard curve greater than 0.980. Melting curve analysis was used for the assessment of heat induced dissociation-characteristics for double-stranded DNA to determine the specificity of the target PCR amplicons, including identification of the presence of non-specific products and primer-dimers. This property was valuable because the presence of secondary non-specific products and primer-dimers could affect the accuracy of the qPCR assay. The specificity of all qPCR products was assessed by the systematic analysis of the post-PCR dissociation curve performed between 60°C to 95°C.

BIOINFORMATIC ANALYSES

Reads obtained from the MiSeq sequencing system have been processed using Vaiomer SAS bioinformatics pipeline. Based on the results, graphical representations were made of the relative proportion taxa for each taxonomic level (phylum, class, order, family, genus, and species) present in individual study samples. Taxa are identified by name in the plot for abundance >1%. Taxa are merged into the “Other” category only if it exists in any sample with

abundance greater than 0.01%. Taxa are merged into the "Multi-affiliation" category when they can correspond to two or more different taxa. A diversity (α-diversity) represents the mean of species diversity per sample in each group/class. Diversity analysis is presented for 1) observed, 2) Chao1, 3) Shannon, 4) Simpson, and 5) inverse Simpson. Principal Coordinate Analysis (PCoA) was performed for comparison of sample groups/class based on four methodologies for β-diversity: 1) Bray-Curtis, 2) Jaccard, 3) Unifrac, and 4) Weighted Unifrac. Finally, the output matrix containing the relative abundance of OTUs per sample was processed with the linear discriminant analysis effect size (LEfSe) algorithm[66] using an α cut-off of 0.05 and an effect size cut-off of 2.0. The functional metagenome has been predicted using PICRUSt v1.1.1[90] as follow: the OTU representative sequences were used to pick OTUs against the GreenGenes reference tree (May 18, 2012 database) at 97% identity in order to convert our initial OTU abundance table into PICRUSt-ready OTU abundance table. The metagenome was predicted for each sample and the related pathways were retrieved from the Kyoto encyclopedia of genes and genomes (KEGG) pathways database and formatted for STAMP analysis.

STATISTICAL ANALYSIS

Continuous variables were expressed as median and interquartile range (IQR), whereas categorical variables were

expressed as absolute numbers and percentages. The different groups of patients and the different time points were compared using Fisher's exact test, Mann-Whitney, Kruskal-Wallis or Friedman paired test as appropriate and the correlations among variables were tested by Spearman Rank correlation. P values <0.05 were considered statistically significant. Data were analyzed with GraphPad 5 Prism (GraphPad-Software).

RESULTS

STUDY POPULATION

We consecutively enrolled 138 HIV-infected patients fulfilling the inclusion criteria. Table 1 shows the main characteristics of the study population. At baseline, 66/138 (48%) started a NNRTI-based regimen, 55/138 (40%) a PI-based regimen, 8/138 (6%) an INSTI-based regimen and the remaining 9/138 (6%) started with mixed regimens. Following 12 and 24 months of suppressive cART, we observed viro-immunological improvements (Tab.1, coupled with decreased T-cell activation, redistribution of memory and naïve T-cell subsets (Fig.2 and Fig.2bis), increased I-FABP ($p<0.0001$) and EndoCAb ($p<0.0001$), yet stable sCD14 levels (Fig.3). The cohort diagram in Fig.1 illustrates the assignment of peripheral blood, urine, and stool samples to each analytical method.

VIRO-IMMUNOLOGIC PARAMETERS, MICROBIAL TRANSLOCATION (MT), INTESTINAL DAMAGE AND INFLAMMATION MARKERS IN A SUB-GROUP OF 41 HIV+ PATIENTS STARTING A FIRST CART REGIMEN

In an unselected sub-group of 41-infected patients who accepted and completed the longitudinal faecal collection (Table 2), we investigated blood/gut microbiota composition following the first

two years of cART and we compared these data to 15 HIV-uninfected healthy controls. Compared to HIV-negative healthy controls, HIV+ patients were younger (p=0.0017), with a higher proportion of MSM (p=0.039). In line with what observed in the entire cohort, two years of cART resulted in viro-immunological recovery, significant decrease of activated CD8 T-cells and a redistribution of memory and naïve T-cells (Table 3). We next assessed circulating markers of gut damage and permeability, and microbial translocation (Figure 4). Upon cART introduction, we observed stable16S rDNA (Figure 4a), and sCD14 levels (Figure 4b), along with a rise in EndoCAb (p=0.0001; Figure 4c) and I-FABP plasma levels (p=0.039; Figure 4d). Interestingly, while faecal calprotectin was stable over time (Figure 4e), it significantly decreased in those HIV+ patients with baseline calprotectin values above the range of normality (i.e. >50μg/g) (p=0.018; Figure 4f). Likewise, despite stable LAC/MAN ratio in the whole cohort (Figure 4g), HIV+ patients starting cART with LAC/MAN ratio greater than 0.05 displayed a significant reduction at T12 (p=0.031; Figure 4h).

FAECAL BACTERIAL COMPOSITION AMONG HIV-INFECTED PATIENTS AND HIV-INDIVIDUALS

We first assessed the faecal α and β diversity, as well as the bacteria relative abundance between HIV+ and HIV- healthy controls. The α diversity richness indexes, but not the evenness indexes, were

higher in HIV+ patients, compared to the control group (observed: p=0.029; Chao1: p=0.011; Shannon p=0.184; Simpson p=0.303; Figure 5a). Following the linear discriminant analysis (LDA) effect size (LEfSe) with LDA score>2 as the cut-off, we found that compared to controls, HIV+ displayed higher *Actinomycetaceae* (p=0.01), *Prevotellaceae* (p=0.003), *Lactobacillaceae* (p=0.003), *Peptococcaceae* (p<0.0001), *Succinivibrionaceae* (p<0.0001) and *Fusobacteriaceae* (p=0.01) and lower *Bacteroidaceae* (p<0.0001), *Ruminococcaceae* (p=0.066) and *Rikenellaceae* (p<0.0001) (Figure 5b). These differences were confirmed by the subsequent analysis of the diverse taxonomic levels (phylum, class, order, family, genus and species). In particular, while we did not find major differences at the phylum level, we observed significant modification within the lower taxonomic levels, such as families and genera (Figure 7). Given that some authors have reported sexual behaviour, particularly men who have sex with men (MSM), as a driving factor of large microbiome differences[76], we performed a sensitivity analysis in 28 MSM vs 13 heterosexuals in HIV+ at each time point. Interestingly, with regard to alpha diversity comparison, in HIV+ group we were not able to detect the same profound alteration of gut microbiota composition when we compared HIV-infected individuals MSM to HIV-infected individuals heterosexuals (that include MSW, female sex and 1 IDU patient) (fig.6). On the contrary, with regard to faecal relative abundance sensitivity analysis performed at taxa family for the 11 families

altered: we confirmed a strong sexual behaviour influence between MSM HIV+ individuals compared to heterosexuals HIV+ individuals (fig. 8a) with the typical Prevotellaceae-rich/Bacteroidaceae-poor unbalance; although, interestingly, we could detect many differences between HIV+ heterosexuals and HIV- heterosexual subjects (fig. 8b). These unique microbial changes in HIV+ subjects lead us to hypothesize diverse metagenomic functions. To answer this question, we used the bioinformatics tool PICRUSt (http://picrust.github.io/picrust), that revealed similar predicted functional metagenomic pathways between the two groups (data not shown).

EFFECT OF 24 MONTHS OF DIVERSE CART REGIMENS ON FAECAL BATERIA COMPOSITION

Having shown a marked dysbiosis in HIV-infected patients, we next asked whether diverse cART regimens might result in modifications of gut microbiota composition. To address this question, we first analysed the impact of cART regimens overall, then we stratified our cohort according to the 3rd drug, i.e. NNRTI, PI or INSTI. The initiation of antiretroviral therapy did not lead to substantial modifications of richness and evenness parameters, with HIV+ patients maintaining significantly higher α diversity compared to healthy controls at T24 (observed: p=0.015 Chao1: p=0.002, Figure 9a). Similarly, the LEfSe analysis showed a modest variation in gut

bacterial composition following 24 months of cART, with HIV+ patients still displaying higher *Prevotellaceae*, *Lactobacillaceae*, *Peptococcaceae*, *Succivibrionaceae* and *Veillonellaceae* but lower *Bifidobacteriaceae*, *Bacteroidaceae*, *Rikenellaceae* and *Ruminococcaceae*, as compared to HIV negative individuals (Figure 9b). In line with the above-mentioned results, the principal coordinate analyses based on β-diversity results (Jaccard and Unweighted Unifrac indexes) revealed a tendency of HIV+ gut microbiome to cluster separately from HIV- healthy controls, irrespective of cART initiation (Figure 9c). Following cART, the relative abundance analysis revealed a significant increase in *Veillonellaceae* (p=0.004; Figure 10a) and a trend towards higher *Desulfovibrionaceae* (p=0.092; Figure 10b), coupled with a parallel decrease in *Lactobacillaceae* (p=0.020; Figure 10c), *Coriobacteriaceae* (p=0.004; Figure 10d) and *Peptococcaceae* (p=0.027; Figure 10e). Furthermore, at genus level we observed an increase in *Allisonella* (p=0.004; Figure 10f), and *Desulfovibrio* (p=0.037; Figure 10g), with a significant decrease in *Lactobacillus* (p=0.020; Figure 10h), *Eggerthella* (p=0.049; Figure 10i) and *Peptococcus* (p=0.027; Figure 10j). Interestingly, this persistent dysbiosis was associated with the continuous mucosal damage, despite cART introduction. Indeed, the levels of the direct marker of intestinal damage (I-FABP) were positively correlated with *Veillonellaceae* both at T12 (r2=0.197; p=0.030;) and T24 (r2=0.156; p=0.017). Interestingly enough, when we stratified patients according

to cART regimens, we found that only NNRTI-based therapy significantly reduced richness (observed: p=0.038; Chao1: p=0.006; Figure 11a-b), but not evenness indexes (Figure 11c-d) over time. Furthermore, the relative abundance analyses showed a different profile at both family and genus levels, with NNRTI-based regimens significantly reducing the families of *Coriobacteriaceae*, *Peptococcaceae* and increasing the *Veillonellaceae* family (Figure 12a-c). On the opposite, INSTI-based regimens resulted in decreased *Peptococcaceae* and increased *Veillonellaceae* families, as well as in higher *Allisonella* genus (Figure 12d-f). No major effects following PI-based regimens were detected. The changes in gut dysbiosis according to diverse cART regimens, was accompanied by stable predicted metagenomics predictions (data not shown). With regard to our "gut persistence score" for each antiretroviral drug, based on the drug bioavailability and the proportion of unchanged drug in feces (courtesy of Dr. Calcagno A., University of Turin, Italy), we wanted to test the hypothesis of an indirect effect of cART on faecal microbial composition. We found that the gut persistence score was similar between PI-, NNRTI- and INSTI-based regimens over time (Figure 13a-b). Furthermore, the gut persistence score did not associate with the faecal bacteria composition (data not shown).

BACTERIA A AND B DIVERSITY AND RELATIVE ABUNDANCE ANALYSES IN PLASMA

Given the presence of microbial bioproducts within the plasma compartment of HIV-infected individuals despite cART introduction[47,70,91], we also explored the composition of the translocating microbiota. The composition of blood microbiota was very different from the one observed within the faecal samples, with significantly higher relative abundance of bacteria belonging to the *Actinobacteria* and *Proteobacteria* phyla (Figure 14a-b), and lower bacteria belonging to the *Firmicutes* and *Bacteroidetes* phyla (Figure 14c-d). Focusing on blood composition alone, while richness parameters (observed and Chao1) did not highlight differences in bacteria composition (Figure 15a-b), evenness parameters (Shannon and Simpson) showed reduced α diversity in HIV+ vs healthy controls ($p=0.02$, $p=0.009$, respectively; Figure 15c-d). B diversity analysis revealed no differences in plasma bacteria composition following the principal coordinate analysis (Figure 15e-f). We only found a higher *Sphingobacteriales* ($p<0.0001$), yet lower *Chitinophagaceae* ($p=0.001$), *Cytophagales* ($p<0.0001$) *Sphingomonadales* ($p=0.01$) and *Enterobacteriales* ($p=0.029$) in HIV-infected individuals compared to healthy controls (Figure 15g). In line with what observed in the feces, cART introduction modified the bacteria composition only partially, with slight variations of *Sphingomonadaceae* ($p=0.04$) *Staphylococcaceae* ($p=0.01$) and *Pseudomonadaceae* ($p=0.031$)

families (Figure 15h). With regard to a possible associations between these plasma taxa and microbial translocation markers, at baseline a weak positive correlation between EndoCab and Cytophagales (r=0.034,p=0.03) and a weak negative trend between Sphingomonadales and 16SDNA (r=-0.29, p=0.06) were found; on the contrary, no correlations between microbial translocation markers and the plasma taxa altered after cART introduction were found (data not shown). A similar trend was observed according to the diverse drug classes. Finally, in order to better investigate the role of cART in modifying bacteria composition, we also explored the faeces to plasma ratio of the different bacteria taxa, failing to find any modification of this ratio after 12 and 24 months of virologically suppressive cART (data not shown).

DISCUSSION

It is now widely accepted that, aside from the direct effect on gastrointestinal mucosal immunity, HIV infection is characterized by gut microbiome compositional and functional changes, not fully reverted by cART[80,68,81,69,92,74,93,77,94,95,96]. However, the causal relationship between altered intestinal microbiota composition, gut damage and cART is still an open question that needs to be answered in order to improve microbiome-targeted therapies. We hereby describe that 24 months of viro-immunological effective cART, while able to substantially reduce immune activation in the periphery, rescuing circulating T-cell immunephenotypes, still fail to fully restore gut health. In particular, our finding of a significant rise in plasma I-FABP levels suggest an enduring enterocyte damage despite 24 months of cART, expanding on previous data by Chevalier et al. that show rising I-FABP in a cohort of acutely treated HIV+ patients (Chevalier et al. Plospath 2013), in all suggesting that even the promptest and long-term cART does not prevent gut mucosal damage. Alongside persistent mucosal injury, our data demonstrate no substantial control over microbial translocation within 24 months of cART, as shown by stable sCD14 and 16s rDNA, and increasing EndoCAb. Unexpectedly, despite the strong signal of inefficient gut repair, specific clinical indexes of gut permeability (i.e. LAC/MAN and calprotectin) were slightly amended selectively in those patients

starting cART with advanced gut damage, to possibly reflect the unproductive attempt of cART to partially fix mucosal barrier. Interestingly, according to our data, the persistence of gut damage on 2-year suppressive cART seems accompanied the limited effects on faecal microbiota composition, featured by modest increase in *Negativicutes*, *Selenomonadales*, *Veillonellaceae*, and decrease in *Lactobacillaceae*, *Peptococcaceae*, *Coriobacteriaceae*. While some of these modifications might be helpful in restoring the balance between microbiome and immune system[97,98,], other changes, such as the appearance of *Allisonella* (*Veillonellaceae* family) or *Desulfovibrio* (*Desulfovibrionaceae* family) genera, might even promote the gut damage, possibly worsening disease progression[99,100,101]. This last hypothesis is further supported by our findings of a direct correlation between *Veillonellaceae* family and the marker of enterocyte damage I-FABP. Indeed, in line with literature[68,80,82,92,102], our cohort of chronically HIV-infected individuals maintained a profound dysbiosis, characterized by higher faecal α-diversity, a distinct cluster separation according to PCoA analysis and a *Prevotellaceae*-rich/*Bacteroidaceae* poor profile, as compared to HIV- individuals. The enhancement or impoverishment of some key species has been associated with markers of disease progression. In particular, the depletion of butyrate-producing bacteria has been associated with increased microbial translocation and immune activation[58,103], supporting the role of specific bacteria population in the HIV

pathogenesis. Additionally, our finding of higher faecal α-diversity in HIV+ patients seems to be in contrast with previous studies[80] and may be attributed in part to differences in sexual practices. Indeed, MSM were found to have greater faecal microbial differences than not-MSM, irrespective of HIV infection[76]. In our cohort, however, the sensitive analysis on HIV+ patients according sexual behaviours for alpha diversity didn't confirm the differences between MSM and heterosexuals at each time point, suggesting that factors other than sexual practice might have influenced the microbiota composition. However, we could observe only a Prevotellaceae-Succinivibrionaceae-rich/Bacteroidaceae-poor unbalance after the sensitivity analysis of faecal relative abundance at family level in HIV + group; moreover, this unbalance seems to partially disappear after the exclusion of MSM individuals by the other subjects; in parallel, other significant differences appeared between HIV+ heterosexuals and HIV negative heterosexuals, with the involvement of other taxa. This way, these data suggest a different setting of microbial dysbiosis according to the epidemiology of our cohort study with the recommendation to stratify in the future any cohorts according to sexual behaviour. The evaluation of faecal bacteria compositional changes according to different ARV classes indicates a pattern similar to that described in the whole cohort. Aside from a small NNRTI-mediated decrease in α-diversity richness indexes, which is an independent indicator of gut microbiome restoration, and has been proposed by Nowak et al.[80] to

reflect immune reconstitution, we did not highlight marked changes according to PI- or INSTI-based combinations, with the exception of the changes already described in the whole cohort. Our observations are in contrast with recent data showing on one hand a worsening of microbial translocation and endothelial damage due to PI-based regimens[78] and on the other hand a restoration of gut microbiota richness following INSTI-containing regimens[79], possibly reflecting the diverse type of sampling, time on cART and methods used for extraction and sequencing of microbial DNA. We should also acknowledge that, given the low CD4 count at cART initiation, 19% of our cohort was on antibiotic prophylaxis, that was promptly interrupted when patients reached a good immune recovery levels (generally within the first year). Thus, we could not exclude that part of the small modifications observed in gut microbiota composition might be mediated by antibiotic suspension. Besides, some cART regimens, particularly protease inhibitor-based combinations, could induce non-infectious diarrhoea[104,105], so it is also possible that the changes in microbiome might reflect these side-effects. We next focused our research on the characterization of blood microbiota composition, given the role of microbial bioproducts within the plasma compartment in disease progression[47,91]. Our analyses revealed a different microbial composition between plasma and faecal samples, in terms of both α and β diversity, suggesting on one hand a selective passage through the gut barrier, possibly mediated by

specific immunological carrier, and on the other hand a specific immune control over potentially pathogenic microorganism. Interestingly, we described higher presence of *Proteobacteria* phylum in contrast to the low intestinal abundance. Given that *Proteobacteria* in blood have been associated with the onset of cardiovascular events[83], our observations suggest a role of microbiome dysbiosis in possibly supporting the development of cardiovascular co-morbidities during chronic HIV infection. Several limitations in our study should be acknowledged: (i) our inability to study bacterial composition, gut damage and immunity at gut mucosal site, that might have shed light on the causal relationship between microbiota and immune system over the course of cART, (ii) the lack of an extensive analysis of microbial function, which would certainly widen the understanding of the possible interaction(s) between gut microbial community and damage, as well as peripheral immune homeostasis, (iii) the lack of a standardized questionnaire on food habits and (iv) the use of a non-validated drug penetration score into the feces, given the well-known distinctive ability of tissue penetrations exerts by the various drugs[106,107]. A further bias in our study might be represented by the younger age in control group. It is well known that aging affects gut microbiota composition[108], however during adulthood (25-50 years) the composition tends to be stable[109,110], thus we could assume that despite the statistical difference in age, the two study groups are homogeneous. In conclusion, despite the viro-immunological benefits,

long-term effective cART, irrespective of drug classes, resulted in persistent gut damage that associates with gut dysbiosis. Besides, the finding of a compositional shift in faecal vs plasma microbiota, with the enrichment of proteobacteria in peripheral blood, opens new perspective on the clinical implication of circulating bacteria and HIV-associated non-communicable co-morbidities. To our knowledge, this is the first study assessing the impact of 24 months of cART on both faecal and plasma microbiota composition. Whether the persistence of dysbiosis fuels intestinal damage and the consequent microbial translocation, or whether the HIV-mediated pro-inflammatory environment linked to GI damage and MT promote the dysbiosis still need to be elucidated. Further studies are needed to dissect the molecular mechanisms behind the interaction of microbiome and antiretroviral drugs, possibly taking into account other key players, such as the virome[111].

FIGURES AND TABLES

Table 1. Epidemiological, Clinical and HIV-related features of HIV-infected cohort.

	T0	T12	T24	p value
Age, years (IQR) *	38 (31-46)	-	-	-
Sex males, n (%) °	122 (88)	-	-	-
Risk factors for HIV infection, n (%)°		-	-	-
Heterosexual	43 (31)			
MSM	89 (65)			
IDU	6 (4)			
Hepatitis coinfection, n (%) °		-	-	-
HCV	8 (5.8)			
HBV	3 (2.1)			
HCV/HBV	2 (1.4)			
Time since 1st HIV diagnosis, months (IQR) *	11.5 (2-40)	-	-	-
AIDS diagnosis before cART, n (%)°	14 (10.1)	-	-	-
CD4 T-cell count, cell/mmc (IQR) *	312 (210-385)	510 (403-634)	608 (461-764)	**<0.0001**
CD4 T-cell, % (IQR) *	19 (15-23)	28 (23-32)	31.5 (25-36)	**<0.0001**
CD4/CD8 ratio, (IQR) *	0.31 (0.23-0.41)	0.59 (0.42-0.71)	0.71 (0.54-0.88)	**<0.0001**
HIV-RNA, Log cp/ml, (IQR) *	5.03 (4.55-5.38)	1.59 (1.59-1.59)	1.59 (1.59-1.59)	**<0.0001**

Note:*Data are median (IQR). °Data are n (%). IQR: Interquartile range. MSM: men-sex men; IDU: intravenous drug users; p value for comparison between T0, T12 and T24. Friedman Test was used.

Table 2. Epidemiological, Clinical and HIV-related features of HIV-infected cohort.

	HIV + (N=41)	HIV negative (N=15)	p value
Age, years (IQR) *	42 (31.5-50.5)	29 (24-33)	**0.0017**
Sex, male (n) (%) °	37 (90)	15 (100)	0.56
Sexual behaviours MSM, n (%) °	28 (68,2)	5 (33.3)	**0.03**
BMI, n (IQR) *	22,66 (22,02-25,18)	22,71 (20,98-24,91)	0.86
Vegetarian/vegan diet, n subjects (%) °	3 (7,3)	0	0.55
Ongoing antibiotic prophylaxis, n (%) °	8 (19,5)	0	0.09
Hepatitis coinfection, n (%) °	2 (4,8)	0	1
First cART regimen, n (%)		n/a	n/a
NNRTI-based	25 (61)		
PI-based	9 (22)		
INSTI-based	7 (17)		

Note: *Data are median (IQR), statistical analysis Mann-Whitney test °Data are n (%), statistical analysis Fisher's Exact Test. IQR: Interquartile. cART: combination of antiretroviral therapy; NNRTI non-nucleoside reverse transcriptase inhibitor; PI protease inhibitor; INI: integrase inhibitor.

Table 3. Peripheral T-cell phenotypes, microbial translocation and gut damage markers in a sub-group of 41 HIV+ patients starting a first cART regimen

	T0	T12	T24	p
Viro-immunological parameters				
CD4 T-cell count, cell/mmc (IQR)	342 (259-431)	539 (442-686)	618 (464-787)	**<0.0001**
CD8 T-cell count, cell/mmc (IQR)	1000 (874-1272)	860 (648-1124)	786 (660-1069)	0.322
CD4/CD8 ratio, (IQR)	0,31 (0,24-0,44)	0,62 (0,48-0,78)	0,73 (0,56-1,06)	**<0.0001**
HIV-RNA, cp/ml, (IQR)	63734 (22751-205583)	<40	<40	**<0.0001**
T-*cell phenotypes*				
CD38+CD8+, %	12 (6-18)	2 (1-3)	2 (1-3)	**<0.0001**
CD38+CD45R0+CD8+, %	5 (3-12,75)	1 (0-1)	1 (0,50-1)	**<0.0001**
CD127+CD4+, %	10 (8-12)	17 (12,75-21)	20 (16-25)	**<0.0001**
CD127+CD8+, %	25 (21-32)	24 (19,75-28)	26 (21-29,5)	0.326
CD45R0+CD8+, %	21 (16,75-31,75)	13 (9-17,25)	13 (9-16,5)	**<0.0001**
CD45RA+CD8+, %	19 (13,25-23)	19 (14,75-22)	21 (16-25)	0.549
CD45RA+CD4+, %	6 (3-9)	7,5 (5,75-14,25)	12 (7-18,5)	**0.0002**

Note: *Data are median (IQR), statistical analysis Friedman Test, with Dunn's multiple comparison. IQR: Interquartile range.

Figure 1. Cohort diagram illustrates the assignment of peripheral blood, urine, and stool samples to each analytical method

We consecutively enrolled 138 HIV-infected patients fulfilling the inclusion criteria. All 138 patients underwent blood sampling for immune phenotyping and plasma quantification of microbial translocation (sCD14, EndoCAb) and gut damage markers (I-FABP). In an unselected sub-group of 41-infected patients, plasma samples were used to assess 16S rDNA levels and microbiota composition. In these 41 patients, we also collected urine for LAC/MAN ratio assessment and stool for microbiota analysis and calprotectin quantification. 15 HIV-uninfected healthy subjects were enrolled as controls: blood sampling and stool were collected for microbiota composition analyses.

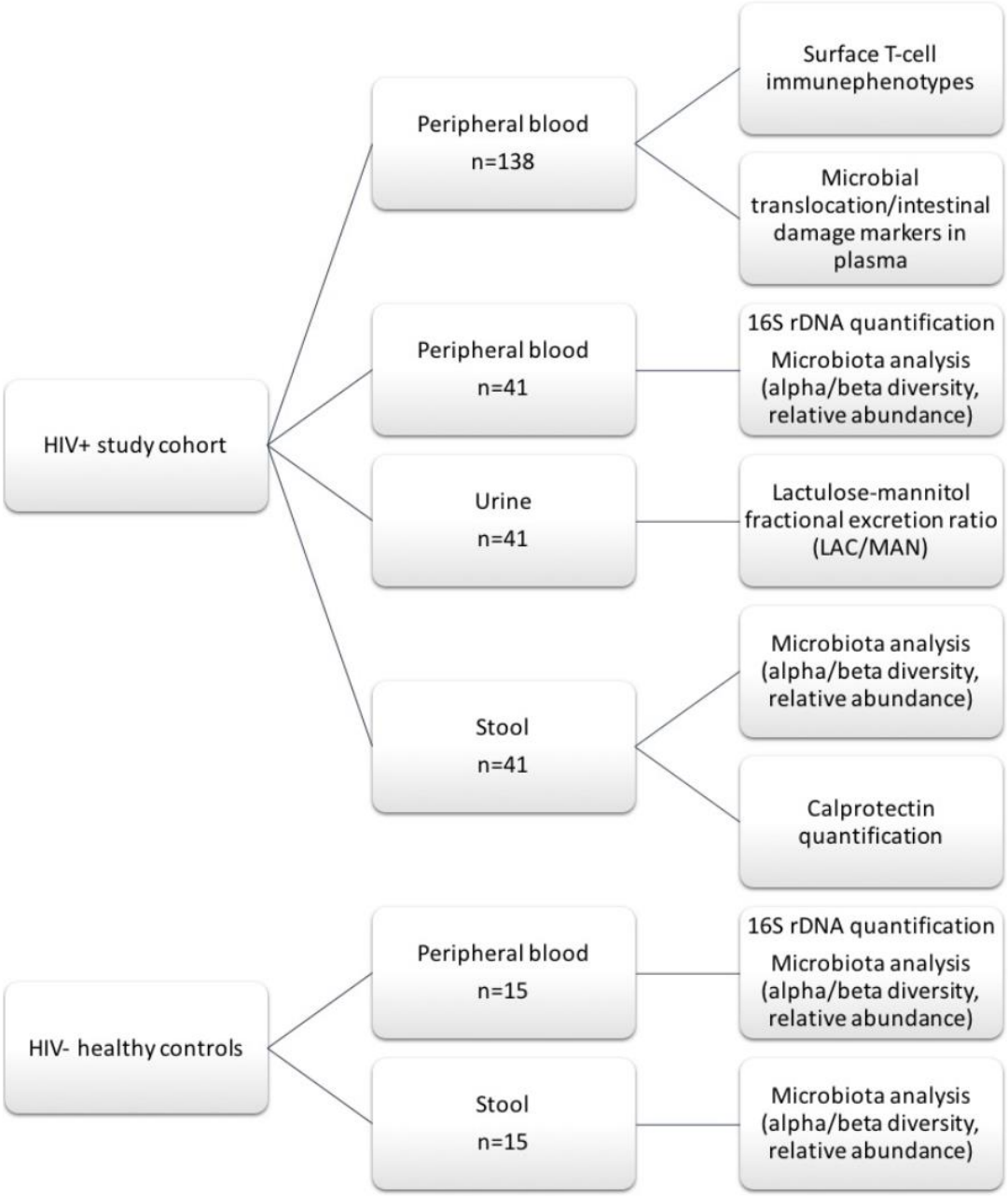


Figure 2. T-cell activation and maturation following 12 and 24 months of suppressive cART

Detection on plasma of T-cell immune phenotypes by flow cytometry on fresh peripheral blood from baseline (naïve, T0) to 12 and 24 months (T12 and T24, respectively) after introduction of cART. **a-b.** significant reduction in activated (CD38+) and memory activated (CD38+CD45R0+) CD8 T-cells (both p<0.0001); **c-d.** significant increase in CD4- and CD8-expressing CD127 (p<0.0001, p=0.0065 respectively); **e.** significant reduction in memory CD45R0+CD8 T-cells (p<0.0001); **f-g**: significant increase in naïve CD45RA+CD8 T-cells (p=0.056) and CD4 T-cells (p<0.0001).

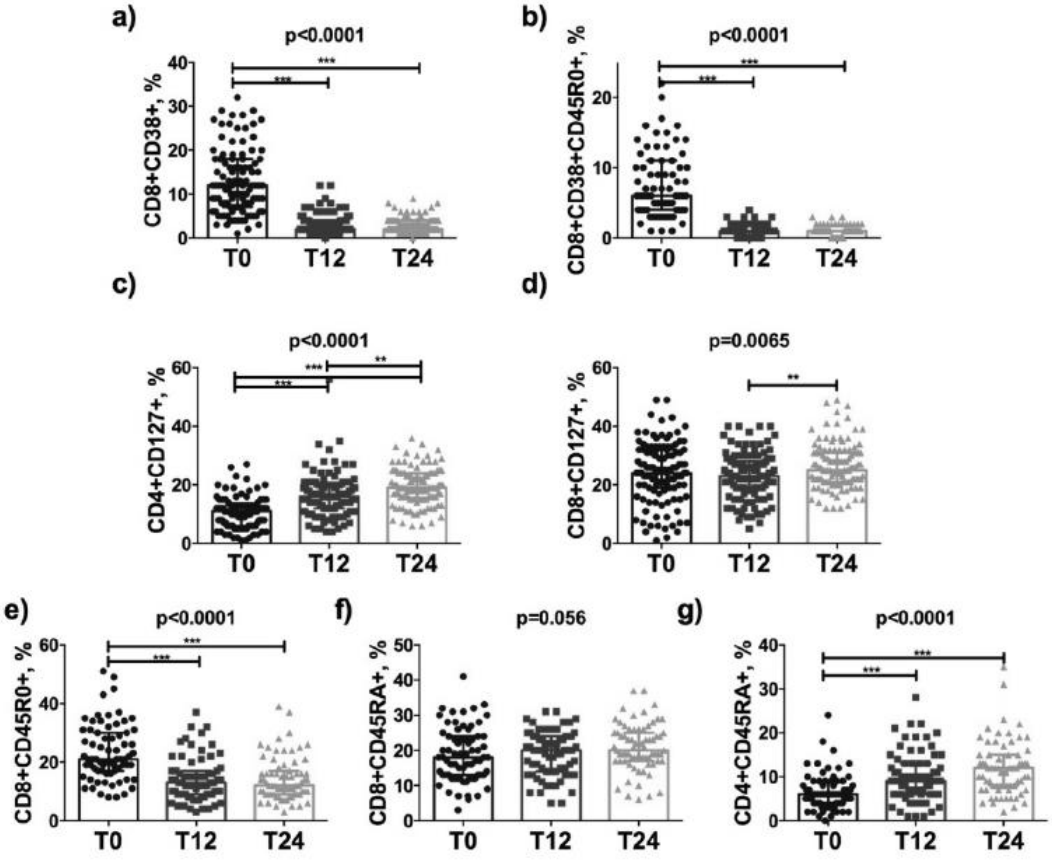


Fig 2 bis. Plot/histograms for gate stragety

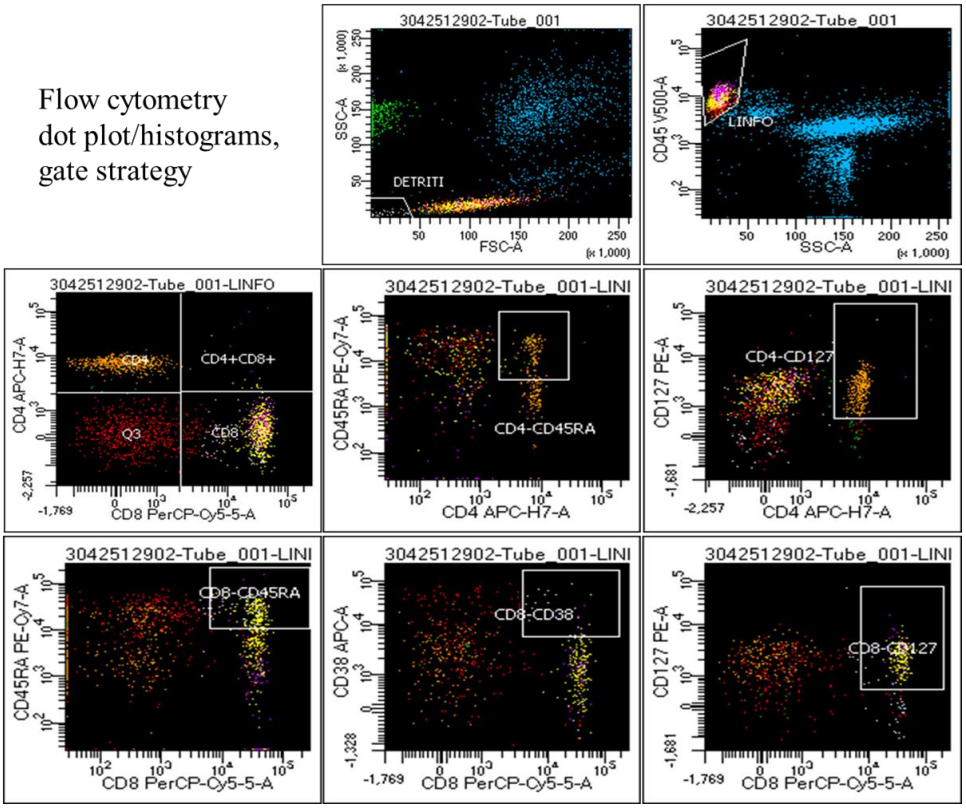


Figure 3. Microbial translocation (MT) and intestinal damage markers following 12 and 24 months of suppressive cART. Quantification of MT and intestinal epithelial damage markers by ELISA assay before and after cART introduction. sCD14s: soluble CD14 soluble); I-FABP: intestinal fatty acid binding protein; EndoCAb: Endotoxin core antibodies. **a.** no significant differences in sCD14 (p=0.548), **b.** significant increase in plasma levels of I-FABP before 24 months of suppressive cART (p<0.0001). **c.** significant increase in circulating EndoCAb levels (p<0.0001).

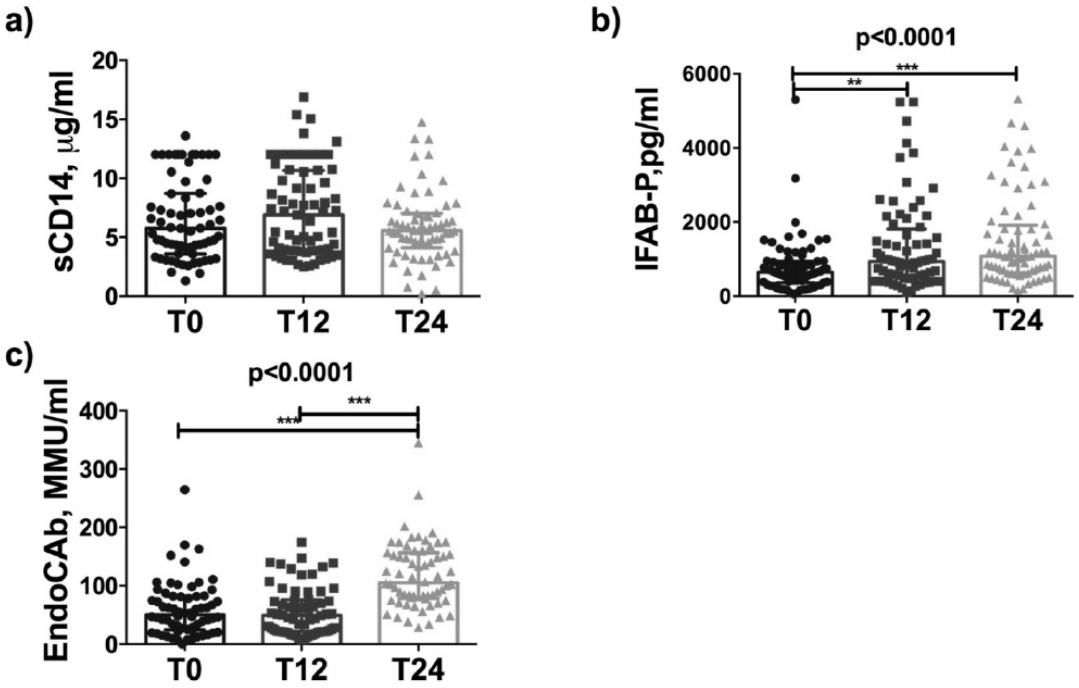


Figure 4. Plasma 16S rDNA, gut inflammation and permeability markers following 12 and 24 months of suppressive cART

a-b. No modifications following cART introduction in 16S rDNA plasma levels (p=0.72) and sCD14 levels (p=0.102). **c-d.** Significant increase in EndoCAb (p=0.0001) and I-FABP plasma levels (p=0.039). **e.** Faecal calprotectin was stable over time in the entire cohort. **f.** Significant decrease of calprotectin in HIV+ patients with baseline values above the range of normality (i.e. >50µg/g, p=0.018). **g-h.** Permeability test, urinary lactulose-mannitol fractional excretion ratio (LAC/MAN) at baseline (T0) and after 12 months the introduction of cART (T12). **g.** Stable levels of LAC/MAN (p=0.657) in the whole cohort. **h.** HIV+ patients starting cART with LAC/MAN ratio greater than the "normality cut-off" 0.05 displayed a significant reduction at T12 (p=0.031).

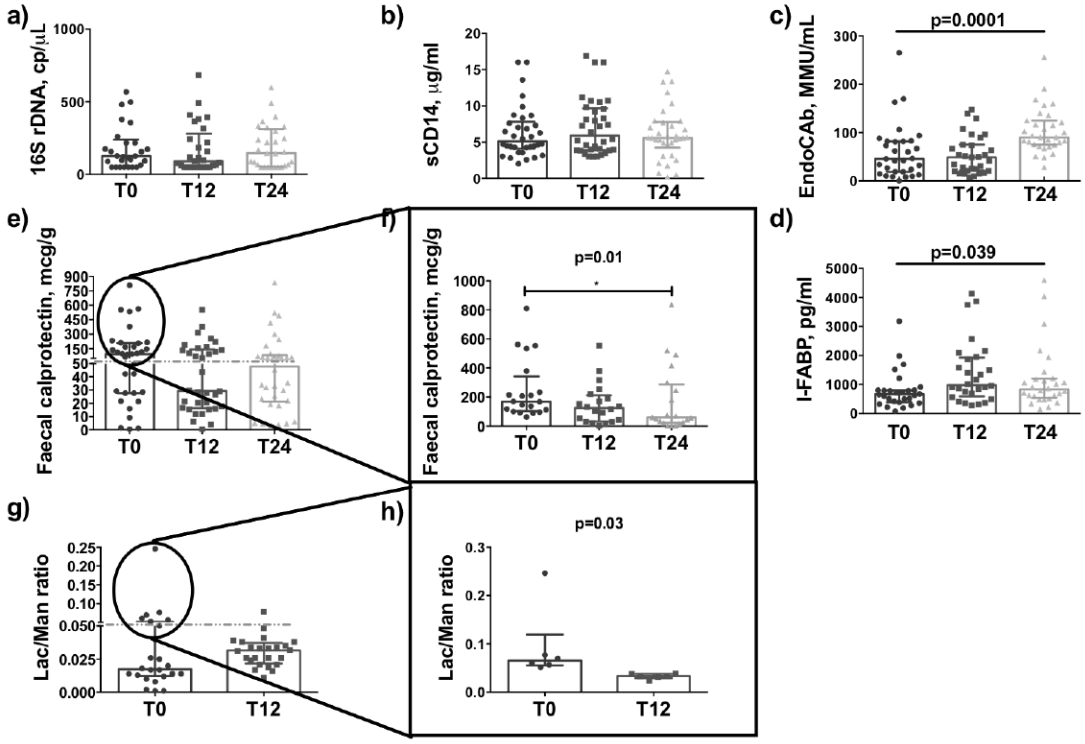


Figure 5. Comparison of faecal microbiota composition between HIV-infected individuals and HIV- controls

a. Faecal diversity (α-diversity) represented as the mean of species diversity per sample in each group/class according to Observed, Chao1, Shannon and Simpson at baseline. We found a significant increase in HIV+ patients compared to controls according to richness parameters (Observed p=0.029; Chao1: p=0.011); no differences according to evenness parameters (Shannon p=0.184; Simpson p=0.3033) were observed. **b.** Linear discriminant analysis (LDA) effect size (LEfSe) with LDA score>2 log as the cut-off at baseline. We showed only significant results at taxonomic level of family with higher abundance in HIV+ patients represented in red, while higher families in healthy controls are represented in green.

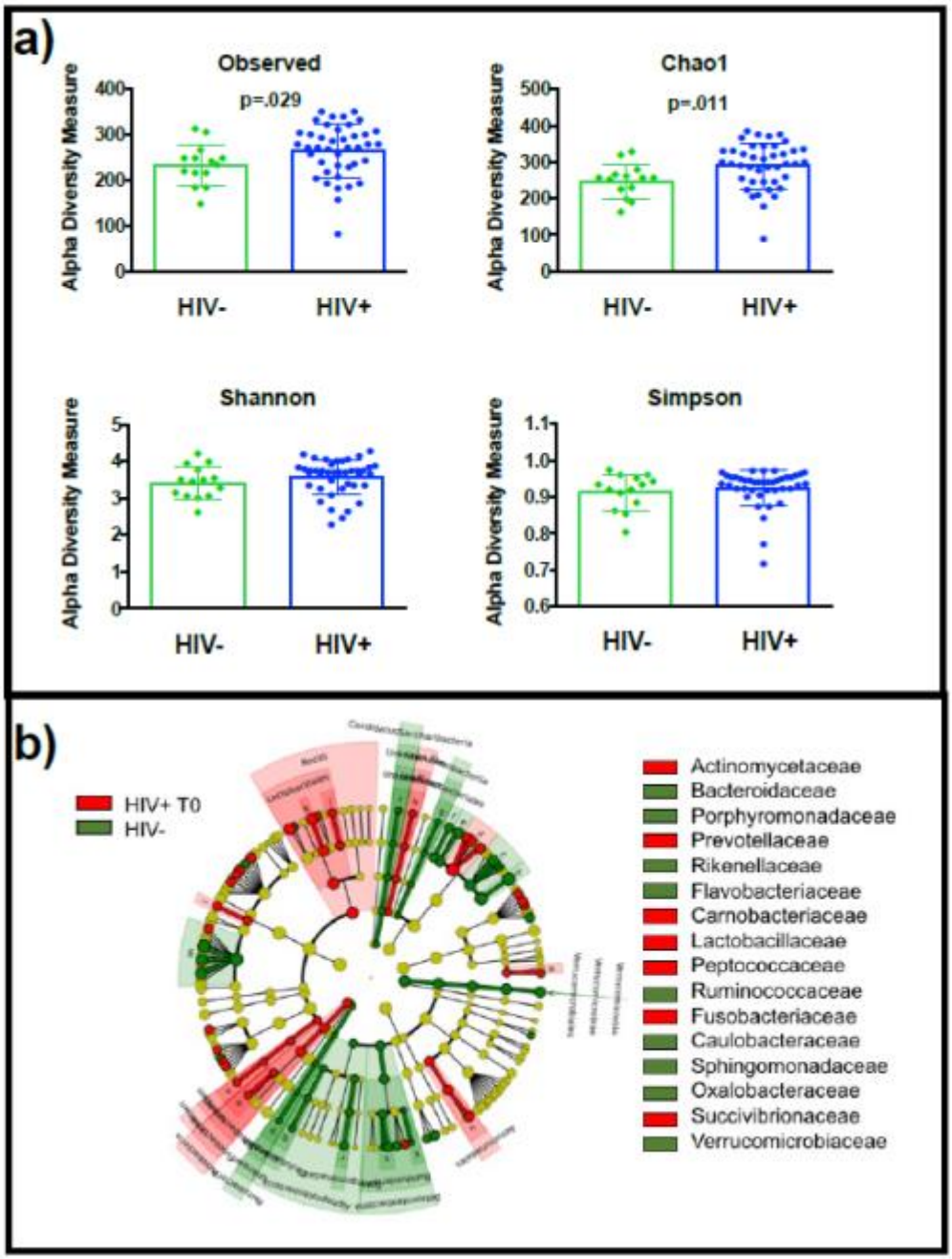


Figure 6. Comparison of faecal microbiota composition between HIV-infected individuals MSM and HIV+ heterosexuals individuals (MSW, female and 1 IDU patient)

Faecal diversity (α-diversity) represented as the mean of species diversity per sample in each group/class according Observed, Chao1, Shannon and Simpson at baseline. No differences according to all indexes were observed.

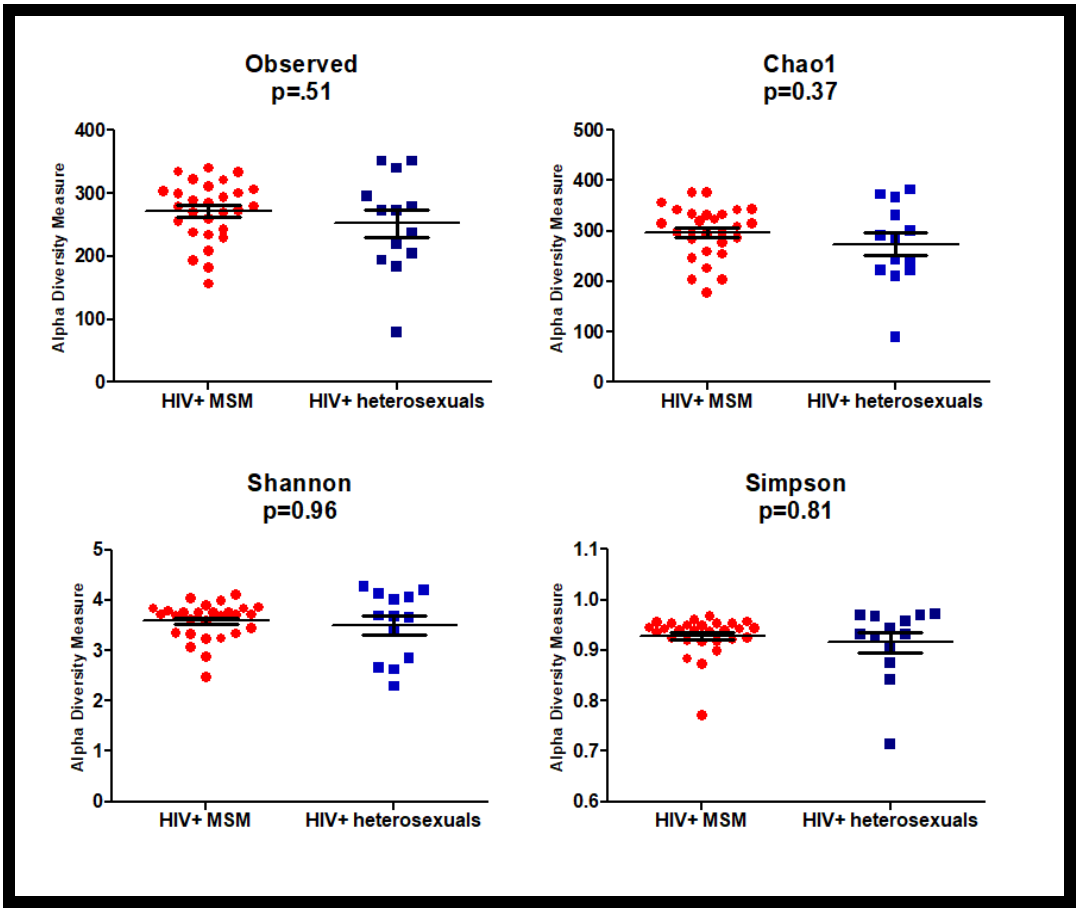


Figure 7. Faecal relative abundance at each taxonomic level (phylum, class, order, family, genus) between HIV-infected individuals and HIV- controls
Faecal relative abundance, the presented data refer to the comparison between HIV+ and HIV- individuals. ↑ indicates increase with significant p values < 0.05, ↓ indicates decrease with significant values < 0.05 with exception for taxa Bifidobacteriales, Carnobacteriaceae, Acidaminococcus, Bifidobacterium, Collinsella and Granulicatella that show trends with p values between 0.05 and 0.07.

Phylum	Class	Order	Family	Genus
Firmicutes=	Bacilli ↑	Lactobacillales ↑	Lactobacillaceae ↑	Lactobacillus ↑
			Streptococcaceae ↑	Streptococcus ↑
			Carnobacteriaceae ↑	Granulicatella ↑
	Clostridia=	Clostridiales =	Lachnospiraceae =	Howardella ↑
				Dorea ↑
				Butyrivibrio ↑
				Ruminococcus2 ↑
			Ruminococcaceae ↓	Ruminococcus ↓
				Faecalibacterium ↓
				Acetivibrio ↑
			Peptococcaceae ↑	Peptococcus ↑
	Negativicutes =	Selenomonadales =	Acidaminococcaceae =	Acidaminococcus ↑
			Veillonellaceae =	Mitsukella ↑
				Megasphera ↑
Bacteroides =	Bacteroidia =	Bacteroidales =	Porphyromonadaceae =	Odoribacter ↓
				Parabacteroides ↓
			Prevotellaceae ↑	Prevotella ↑
				Alloprevotella ↑
			Bacteroidaceae ↓	Bacteroides ↓
			Rikenellaceae ↓	Alistipes ↓
Actinobacteria =	Actinobacteria =	Bifidobacteriales ↓	Bifidobacteriaceae ↓	Bifidobacterium ↓
		Actinomycetales =	Actinomycetaceae ↑	Actinomycetes ↑
		Coriobacteriales =	Coriobacteriaceae =	Collinsella ↑
				Olsenella ↑
				Slackia ↑
				Enterorhabdus ↑
Proteobacteria =	Gammaproteobacteria =	Aeromonadales ↑	Succivibrionaceae ↑	Succinivibrio ↑
Fusobacteria=	Fusobacteria =	Fusobacteriales ↑	Fusobacteriaceae ↑	Fusobacterium ↑

=: similar relative abudance in HIV+ pre-cART vs HIV- healthy controls;
↑: higher relative abundance in HIV+ pre-cART vs HIV- healthy controls
↓: lower relative abundance in HIV+ pre-cART vs HIV- healthy controls

Figure 8. Faecal relative abundance at baseline for 11 selected faxa family. **a.** Faecal relative abundance, the presented data refer to the comparison between HIV+MSM and HIV+ heterosexuals (which include MSW, female individuals and 1 IDU MSW subject). **b.** Faecal relative abundance, the presented data refer to the comparison between HIV+ heterosexuals and HIV- heterosexuals. ↑ indicates increase with significant p values < 0.05, ↓ indicates decrease with significant values < 0.05 with exception for taxa Peptococcaceae and Streptococcaceae that show trends with p values between 0.05 and 0.06.

a	b
Family	**Family**
Lactobacillaceae=	**Lactobacillaceae↑**
Streptococcaceae=	**Streptococcaceae↑**
Carnobacteriaceae=	Carnobacteriaceae=
Ruminococcaceae=	**Ruminococcaceae↓**
Peptococcaceae=	**Peptococcaceae↑**
Prevotellaceae↑	Prevotellaceae=
Bacteroidaceae↓	Bacteroidaceae=
Bifidobacteroidaceae=	Bifidobacteroidaceae=
Actinomycetaceae=	**Actinomycetaceae↑**
Succinivibrionaceae↑	**Succinivibrionaceae↑**
Fusobacteroidaceae=	Fusobacteroidaceae=

Figure 9. Faecal α and β diversity analyses following 24 months of cART
Faecal α diversity (α-diversity) represented as the mean of species diversity per sample in each group/class according to Observed, Chao1, Shannon and Simpson during cART. We failed to find any changes following 24 months of cART, whit HIV- maintaining lower richness indexes (Observed p=0.015, Chao1 p=0.002). **b.** Linear discriminant analysis (LDA) effect size (LEfSe) with LDA score>2 log as the cut-off between baseline (T0) and after 24 months (T24). Upper part: in green HIV+ T24 and in red HIV+ T0. Lower part: in green HIV-, in red HIV+ at T24. **c.** The principal coordinate analyses (PCoA) based on faecal β-diversity results (Jaccard, upper; Unweighted Unifrac below, indexes) between HIV+ patients (T0 blue, T12 red, T24 orange, healthy controls green dots). We observed a cluster separation between healthy control and HIV+ irrespective of cART introduction.

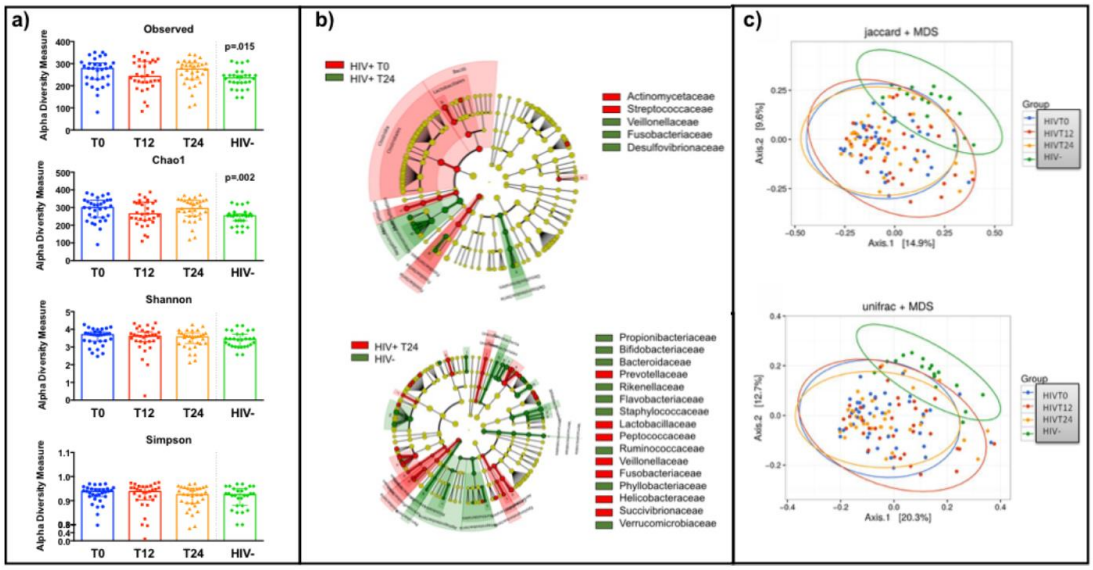


Figure 10. Faecal relative abundance of bacteria Family and Genus taxa following cART introduction

The presented data refer to taxa at family and genus levels in HIV + patients before (T0) and after cART (T12, T24). **a.** significant increase in *Veillonellaceae* family (p=0.004). **b.** increasing trend in *Desulfovibrionaceae* (p=0.092); **c-d-e.** significant decrease, respectively, in *Lactobacillaceae*, *Coriobacteriaceae* and *Peptococcaceae* families (p=0.020, p=0.004, p=0.027 respectively). **f-g.** significant increase in Allisonella and Desulfovibrio genera (p=0.004, p=0.037 respectively). **h-i-j:** significant decrease, respectively, in Lactobacillus, Peptococcus and Eggerthella genera (p=0.020, p=0.049, p=0.027 respectively).

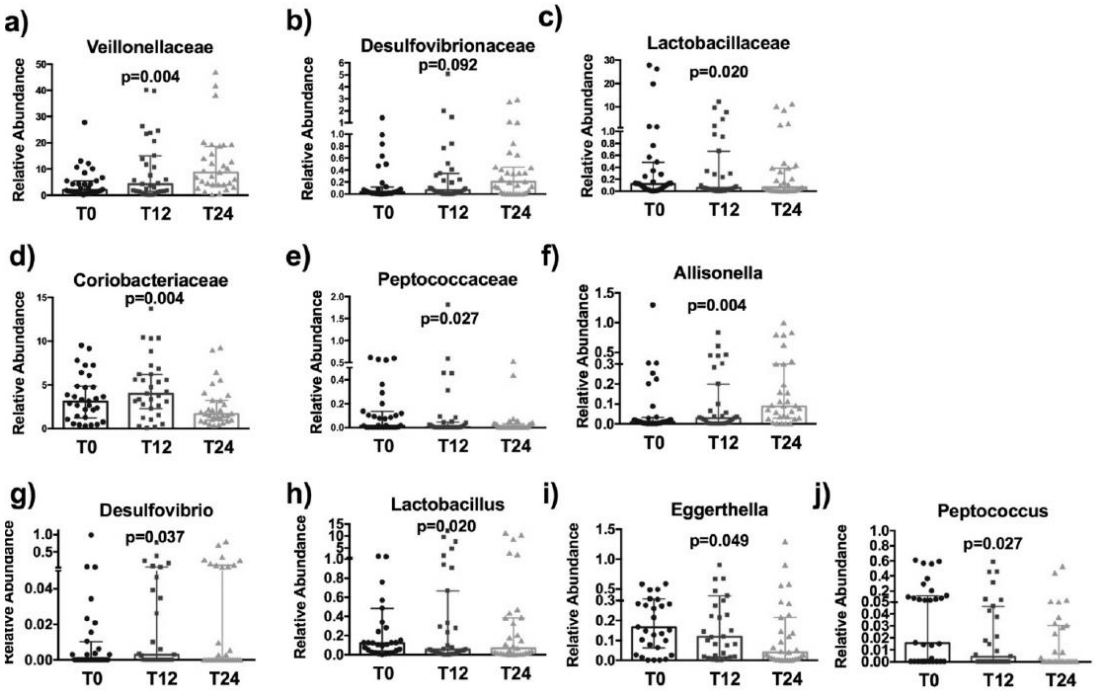


Figure 11. Faecal α diversity Indexes according to different cART regimens
Comparison of α diversity measures in HIV+ patients during cART subdivided according different cART regimens (PI, NNRTI and INSTI based regimens) according to Observed (**a**), Chao1 (**b**), Shannon (**c**), Simpson (**d**): no significant differences in PI- and INSTI-based regimens in all measures; no significant differences in NNRTI-containing regimens according Shannon and Simpson measures, significant decrease only at T12 in NNRTI regimens patients according to Observed and Chao1 respectively p=0.038, p=0.006).

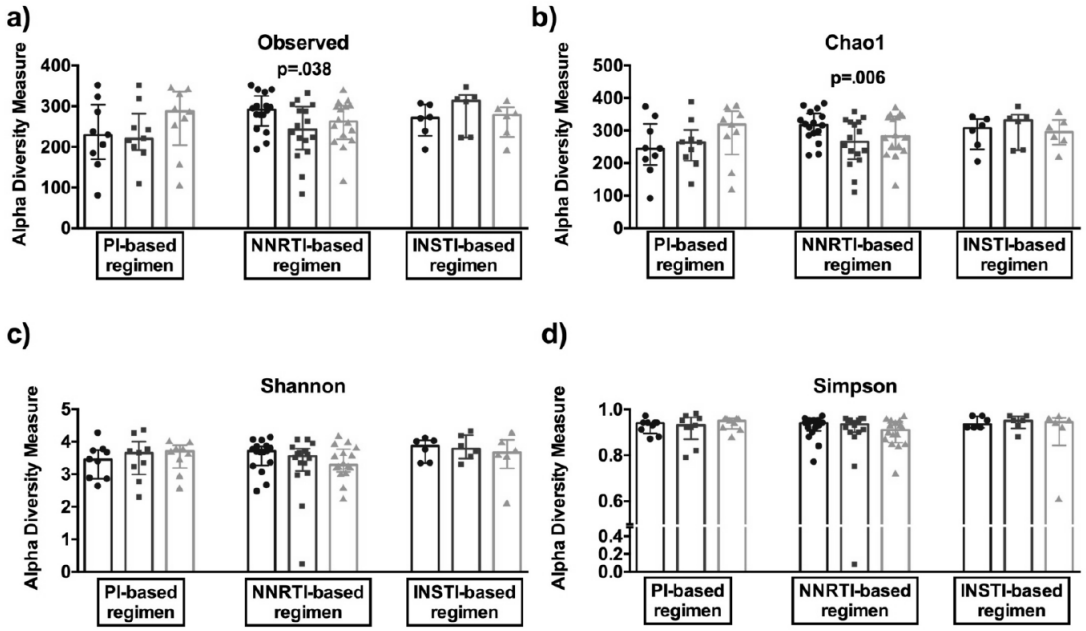


Figure 12. Faecal relative abundance analyses in HIV+ on NNRTI- or INSTI-based regimens. Comparison of faecal relative abundance taxa according to cART regimens; **a.** decrease in *Coriobacteriaceae* following NNRTI-based therapy (p=0.018); **b.** decrease in *Peptococcaceae* following NNRTI-based therapy (0.027); **c.** increase in *Veillonellaceae* following NNRTI-based therapy (p=0.029); **d.** decrease in *Peptococcaceae* following INSTI-based therapy (p=0.003); **e.** increase in *Veillonellaceae* following INSTI-based therapy (p=0.029); **f.** increase in *Allisonella* following NNRTI-based therapy (p=0.006).

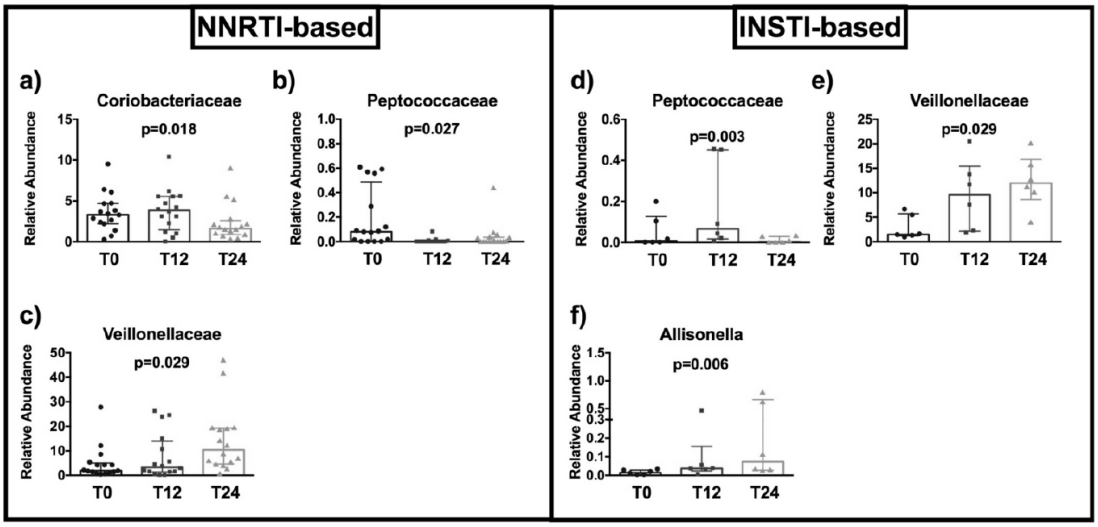


Figure 13. Gut Persistence Score according to different cART regimens.
Comparison between faecal antiretroviral drug Gut Persistence Score according to 3 different cART regimens at T12 (**a**), at T24 (**b**): no significant differences, Kruskal-Wallis test.

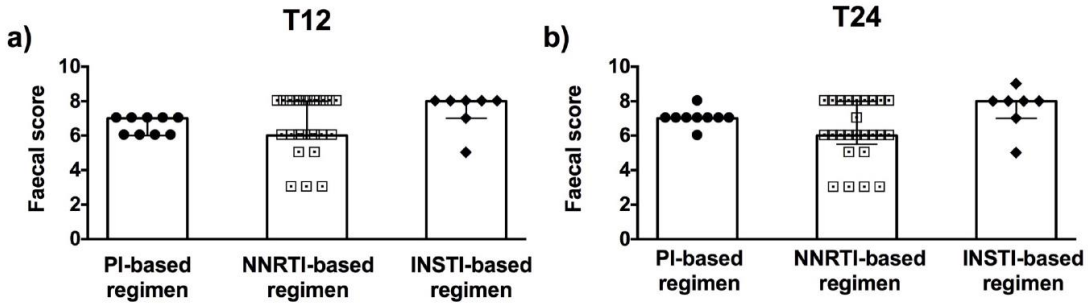


Figure 14. Faecal and Plasma Phylum Distribution
a-b. The relative abundance of Actinobacteria and Proteobacteria phyla was higher in plasma than in feces in all the study groups. **c-d**. The relative abundance of Firmicutes and Bacteroidetes phyla was lower in plasma, as compared to feces, in both healthy controls and HIV-infected patients prior and after cART introduction.

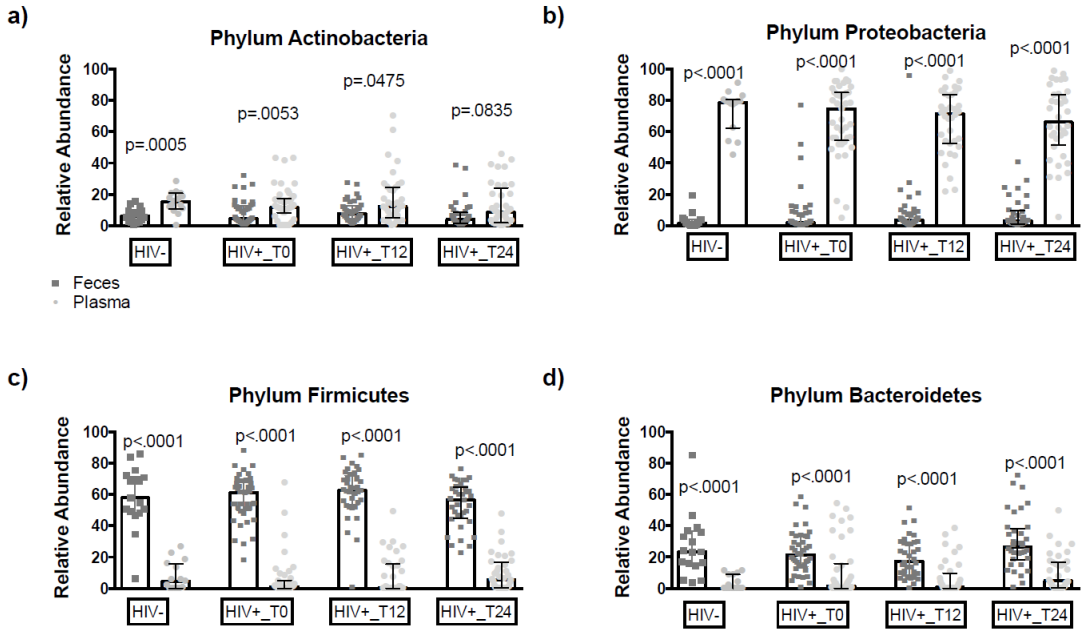
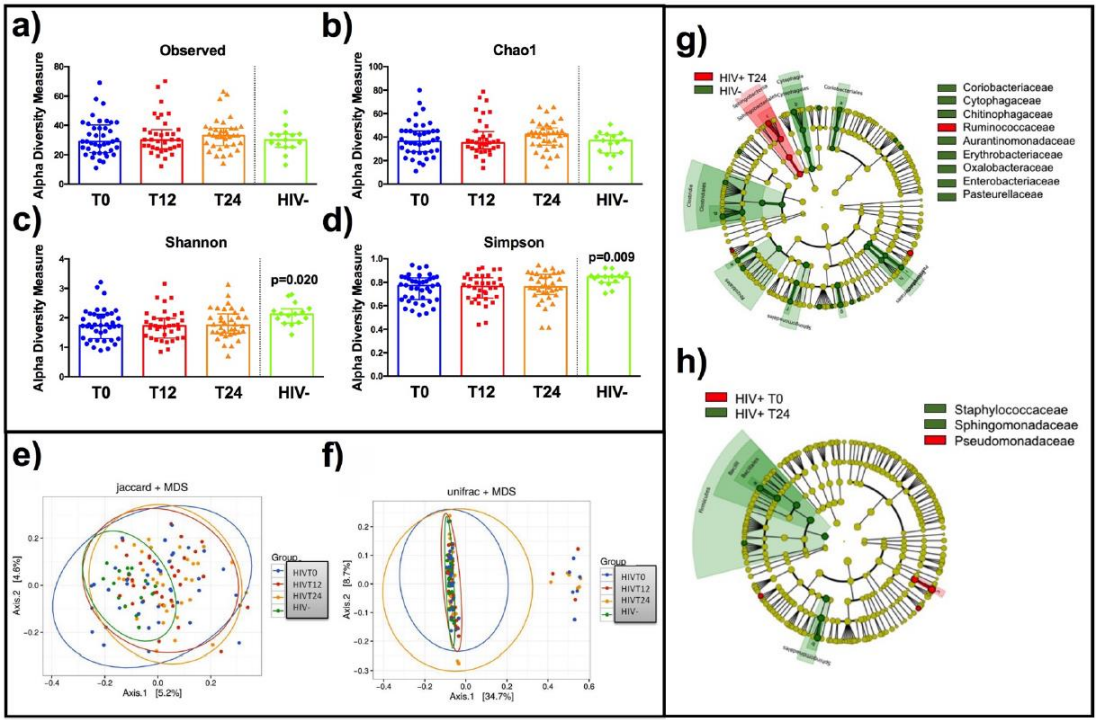


Figure 15. Plasma microbiota composition: α and β diversity analyses

Plasma α diversity (α-diversity) represented as the mean of species diversity per sample in each group/class according to **a.** Observed **b.** Chao1 **c.** Shannon and **d.** Simpson before (T0) and after12 (T12) and 24 (T24) months of cART, vs HIV-negative healthy controls. No significant differences following cART initiation were found. On the opposite, significant differences between HIV+ and HIV- controls at all time points, according to evenness parameters (Shannon p=0.020; Simpson p=0.009) were observed. **e-f**. The principal coordinate analyses (PCoA) based on plasma β-diversity results revealed no significant differences. **g-h.** Linear discriminant analysis (LDA) effect size (LEfSe) with LDA score>2 log as the cut-off. **g.** HIV- controls (green), HIV+ patients T24 (red): higher *Sphingobacteriales* (p<0.0001), yet lower *Chitinophagaceae* (p=0.001), *Cytophagales* (p<0.0001) *Sphingomonadales* (p=0.01) and *Enterobacteriales* (p=0.029) in HIV+ patients **h**. HIV+ T0 (green), HIV+ T24 (red): slight variations of *Staphylococcaceae*, *Sphingomonadaceae* and *Pseudomonadaceae* families following 24 months of suppressive cART.



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