Differential signature of the microbiome and neutrophils in the oral cavity of HIV-infected individuals

by

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Abstract

HIV infection is associated with a wide range of changes in microbial communities and immune cell components of the oral cavity. The purpose of this study was to evaluate the oral microbiome in relationship to oral neutrophils in HIV-infected compared to healthy individuals. We evaluated oral washes and saliva samples from HIV-infected individuals (n=61) and healthy controls (n=43). Using 16S-rRNA gene sequencing, we found differential β-diversity using Principal Coordinate Analysis (PCoA) with Bray-Curtis distances. The α-diversity analysis by Faith's, Shannon, and observed OTUs indexes indicated that the saliva samples from HIV-infected individuals harbored significantly richer bacterial communities compared to the saliva samples from healthy individuals. Notably, we observed five species of *Spirochaeta* including *Spirochaetaceae*, *Spirochaeta*, *Treponema*, *Treponema amylovorum*, and *Treponema azotonutricum* were significantly abundant. In contrast, *Helicobacter* species were significantly reduced in the saliva of HIV-infected individuals. Moreover, we found a significant reduction in the frequency of oral neutrophils in the oral cavity of HIV-infected individuals, which was positively related to their CD4 T cell count. In particular, we noted a significant decline in CD44 expressing neutrophils and the intensity of CD44 expression on oral neutrophils of HIV-infected individuals. This observation was supported by the elevation of soluble CD44 in the saliva of HIV-infected individuals.

Overall, the core oral microbiome was distinguishable between HIV-infected individuals on antiretroviral therapy compared to the HIV-negative group. The observed reduction in oral neutrophils might likely be related to the low surface expression of CD44, resulting in a higher bacterial diversity and richness in HIV-infected individuals.

Preface

Research Ethics Approval

This thesis is an original work of Eliana Rosalia Perez Rosero. The research project, of which this thesis is a result, received research ethics approval from the University of Alberta Research Ethics Board, Project entitled “Human Galectin-9 as a novel weapon to reactivate HIV-1 infection” and “Investigating immune correlates of protection in HIV infection”, #Pro00070528 and Pro000064046, respectively.

Contributions

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Chapter 1. Introduction

1.1. Human Immunodeficiency Virus (HIV) Infection

The infection is caused by a viral agent called human immunodeficiency virus (HIV), which causes the disease “the acquired immunodeficiency syndrome (AIDS)” at the late stage of the disease in the absence of treatment (1). The World Health Organization (WHO) defines HIV infection as a major global public health issue (2) constituting one of the main causes of morbidity and mortality worldwide (3). HIV is a retrovirus from the lentivirus genus, as such its genetic material is present as a double stranded RNA (ssRNA), (Fig. 1). There are two subtypes of HIV defined as HIV-1 and HIV-2. HIV-1 is more prevalent and accounts for the most reported infection cases. HIV-2 subtype is endemic in certain parts of west Africa with a milder disease. Both subtypes emerged from the cross-species transmission of the simian immunodeficiency virus (SIV) to humans, which is thought occurred for the first time by blood contact of infected animals with African meat hunters (3,4).

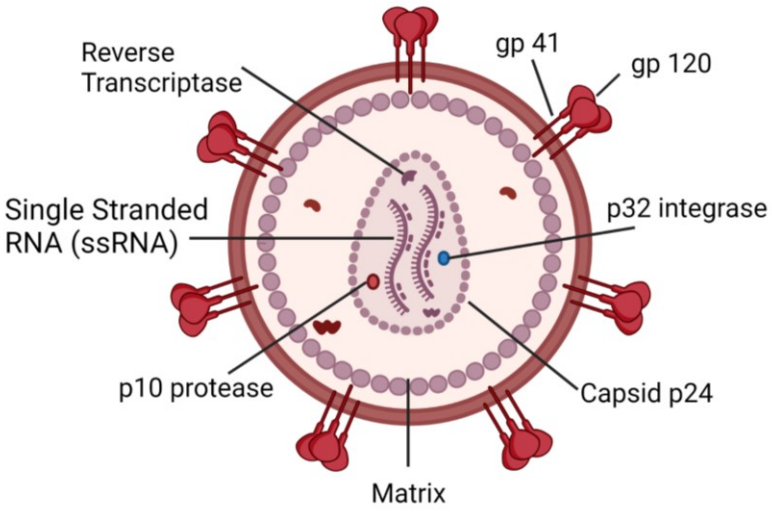


Figure 1 HIV structure, author, created in Biorender software, adapted from (1)

1.1.1. Epidemiology

On a worldwide scale, HIV affected 37.7 million people until the end of 2020 (2). Most of the new cases (incidence) concentrated in the Sub-Saharan African Region (25 million), followed by South, Southeast, and East Asia (4.8 million), Eastern Europe and Central Asia (1.3 million), Latin America (1.5 million), North America (1.3 million), Eastern Europe and Central Asia (1.3 million), Western and Central Europe (860 000), North Africa and Middle East (260 000), the Caribbean region (250 000) and Oceania (51 000) (4). While antiretroviral therapy (ART) has reduced the incidence of HIV especially by heterosexual and mother-to-child transmission from 3.3 million global cases in 2002 to 2.3 million in 2012, the prevalence of HIV has increased over the last several years because HIV infected individuals under ART now can live longer. (4)

In Canada, 62,050 people were living with HIV and 8,300 people had HIV but didn't know or were undiagnosed at the end of 2018. Also, 2,242 newly infected cases were registered until 2018, with a slight increase of 1,960 new cases in 2018 compared to 2016 (5).

1.1.2. Transmission

After contact with body fluids from an HIV-infected person by semen, rectal and vaginal fluids, blood, and breastmilk. Therefore, common routes for transmission are (1,6):

- Sexual transmission: by vaginal or anal intercourse, accounting for most cases.
- The passage from HIV infected mothers to infants during pregnancy, labor, delivery, or breastfeeding.

- Blood or blood products contamination: in blood transfusions, when sharing contaminated needles in drug users, or after accidental needle punctures in health care workers.

1.1.3. Pathophysiology

a) Initial exposure.

After transmission, HIV enters the mucosal barriers as a free virus through inter epithelial cell spaces and/or tissue abrasions, and then carried by infected immune cells, or as a virion attached to tissue-resident dendritic cells (DCs) or Langerhans cells (7). HIV preferentially infects CD4+ T cells, binding its viral protein gp120 with the CD4 receptor, and gp41 to the coreceptors CXCR4 or CCR5 on the surface of T cells, monocytes/macrophages, and dendritic cells (1,3). Inside the cell, the virus releases its genetic material (ssRNA), for posterior reverse-transcription to complementary DNA (cDNA) by the viral enzyme reverse transcriptase (RT). The newly formed cDNA is incorporated into the host genome, and this *provirus* is transcribed and translated to generate viral proteins that together with copies of the viral ssRNA genome will form virions that bud from the surface of the infected cells leading to cell lysis (Fig. 2) (1). From the site of infection, HIV disseminates to draining lymph nodes and later to distal lymph nodes in approximately 2 weeks. However, the virus can remain latent inside the cells, because it is incorporated into the host genome. In this way, HIV builds reservoirs where antiretroviral drugs and the host's antiviral immune mechanisms cannot reach efficiently to eliminate the virus (3,4).

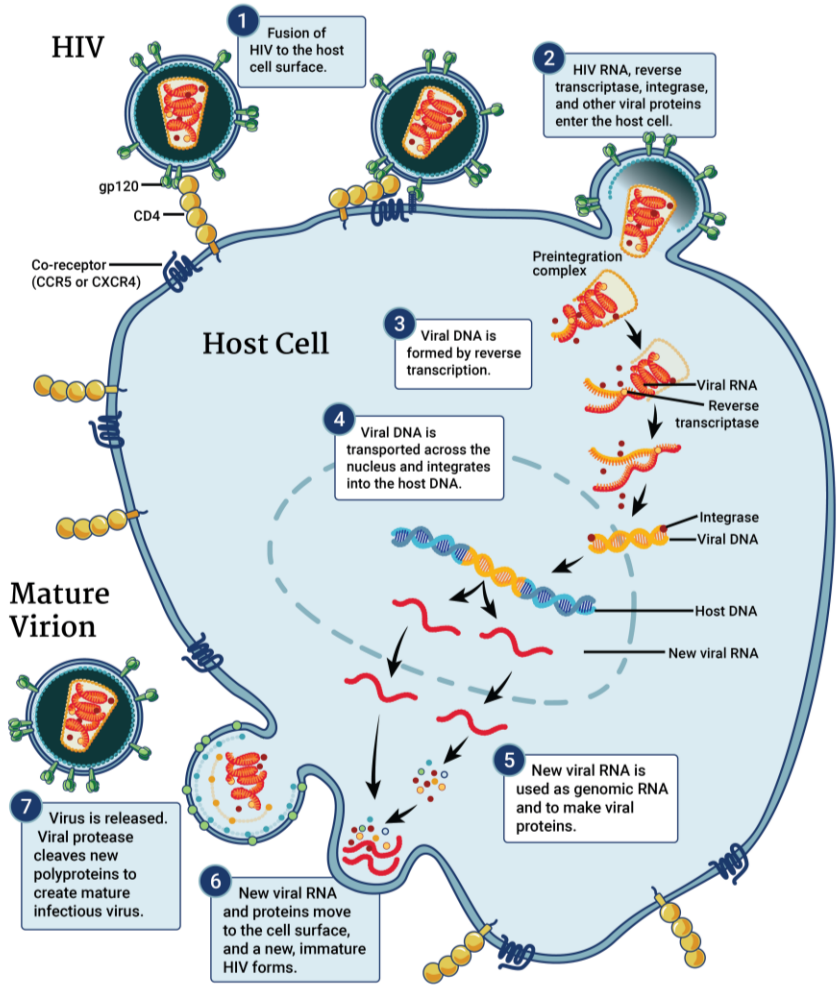


Figure 2. HIV Replication Cycle (8)

b) Primary or Acute Phase.

- ***Signs and symptoms*.** Nonspecific mild flu-like symptoms, that occur after 2-4 weeks of the initial exposure to HIV, are commonly fever, generalized lymphadenopathy, pharyngitis, non-specific rash, myalgia/malaise, and arthralgia (3,9). Less common manifestations include oral ulcers, abdominal pain, diarrhea, weight loss, and photophobia (9).
- ***Viremia.*** The severity of symptoms is related to the amount of viral load present in the blood which in some cases can reach a peak of 10^6-10^7

copies/ml (3). After this transitional viremia, the immune response including natural killer (NK) cells, CD8+ cytotoxic T lymphocytes (CTLs), and B cells suppress viral replication up to 100 times lower than the acute phase leading to a steady-state known as the viral set point (3,7).

- ***CD4 T cell depletion.*** Severe depletion of CD4 T cells and their subtype Th17 cells occurs because of the viral replication, especially in the gut-associated lymphoid tissue (GALT). Gut's depletion of CD4 T cells is thought to permeate the intestinal barrier integrity, promoting bacterial products translocation that will in turn result in inflammation, hyperimmune activation, and microbial dysbiosis over time. (3,9–11).

c) Chronic Phase or Clinical Latency.

The chronic phase in the result of the body's inability to control HIV over time (12). After the acute phase, CD4 numbers are partially and temporarily restored and HIV viremia is controlled by the response of CD8+ CTLs, B cells, and NK cells (1,7). However, HIV will establish body reservoirs within CD4 T cells of the GALT, and lymphoid tissues from where it will proliferate over the years, resulting in a gradual and irreversible depletion of CD4 T cells (1,3,4,7). This process greatly differs between individuals having three types of progressors (12)**.**

- ***Typical Progressors (70-80%).*** Experience a period from 6-8 years of clinical latency, where HIV continues its replication, but no symptoms are observed if CD4 T cell counts are maintained >500 per ul. Over time their CD4 T cells numbers will drop to < 200 per ul (12).

- ***Rapid Progressors (RPs) (10-15%).*** RPs are individuals who progress to AIDS in a short period of 2-3 years after infection. Their immune system does not efficiently control the first viremia observed in the primary phase, thus the latency period is more like an extension of the acute phase, therefore their levels of viremia are maintained high throughout the disease (12).
- ***Long-Term Nonprogressors (LTNP) (<5%).*** LTNPs are individuals that maintain normal CD4 T cell counts, and low viral levels. Their immune system maintains its architecture and function over time. They do not develop symptoms of immunodeficiency and do not progress to AIDS even in the absence of treatment (12,13). LTNPs have healthier CD8+ T cells with greater proliferative, cytolytic, and cytokine secretion capacities, which can be explained by their enrichment with a protective form of the human leukocyte antigen (HLA) allele groups, mainly HLA-B*27 and HLA-B*57 (10,14).

d) Acquired syndrome of immune deficiency (AIDS).

This is the last and more advanced stage of HIV infection (2). It is characterized by significant drop in CD4 T cells counts (< 200 cells/ul) (1), and the development of *opportunistic infections (OIs)* because of their compromised immune system not being capable of providing a robust immune response against infectious agents. Common OIs in AIDS are listed below (15).

- Candidiasis
- Invasive cervical cancer
- Coccidioidomycosis
- Cryptococcosis
- Cryptosporidiosis (Crypto)
- Cystoisosporiasis

- Cytomegalovirus (CMV)
- HIV related Encephalopathy
- Herpes simplex virus (HSV)
- Histoplasmosis
- Kaposi's sarcoma (KS)
- Lymphoma
- Tuberculosis (TB)
- Mycobacterium avium complex (MAC)
- Pneumocystis pneumonia (PCP)
- Pneumonia (Streptococcus pneumoniae)
- Toxoplasmosis
- Wasting HIV syndrome

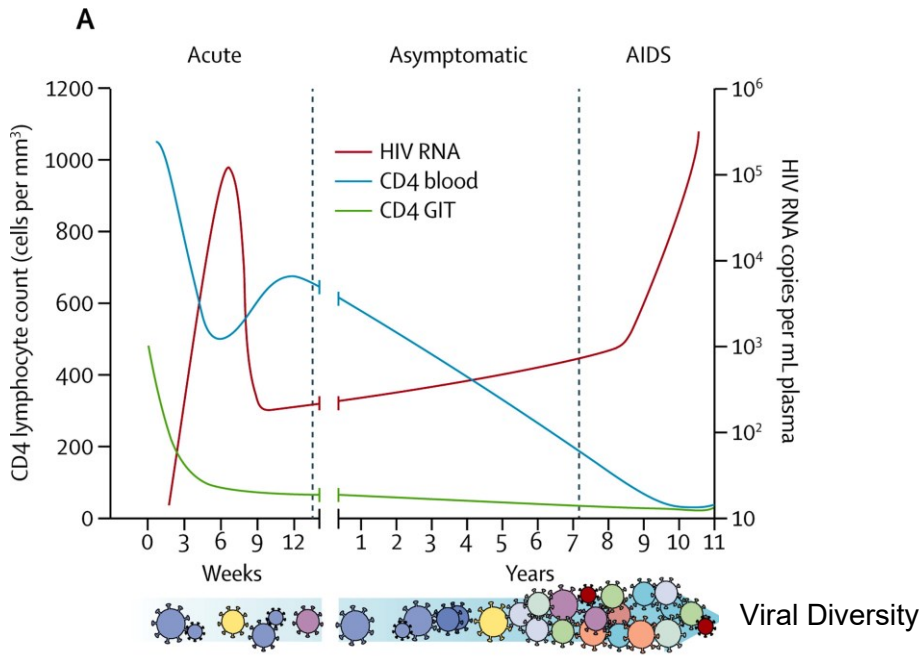


Figure 3. Pathophysiology of HIV infection (4). *Copyright license: 5178400364594*

1.1.4. Immune Response to HIV

a) **Adaptive Immune Response.**

- ***CD4 T cells.*** They are the main target of HIV. During the primary infection, peripheral CD4 T cells experiment a transient and robust depletion, but later upon the magnitude of the immune response, they are partially restored (3). Over time HIV creates a continuous cycle of activation, proliferation, and

destruction of CD4 T cells especially in the GALT (7). Initially, CD4 T cell destruction by HIV is counteracted by an increase in CD4 T cell turnover (up to 3-fold in untreated individuals), a process that will occur until the virus spreads and infects CD4 naïve progenitors destroying them (10) (Fig. 3).

- ***T regulatory cells (T regs).*** As a subset of CD4 cells, identified by CD4+, CD25+, FoxP3+ expressing T cells, exhibit immunosuppressive capacities to limit overreacting T cells responses as a mechanism of maintaining homeostasis (10,16). In HIV infection, there are some reports that Tregs expand decreasing the proliferation of other CD4 T cells subsets (10,17). In contrast, there are other reports that HIV infects and depletes Tregs (18). HIV also impacts Tregs effector functions and may impair or enhance their suppressive properties (18). Moreover, T regs can suppress CD8+ CTLs by cell-cell interactions and secretion of soluble factors (e.g. IL-10, IL-35, TGF-β, and adenosine) (19). For example, it is reported that Tregs by constitutively expressing surface Galectin-9 via interaction with T cell immunoglobulin domain and mucin domain (TIM3) on the surface of CD8+ CTLs can suppress their proliferative capacity (14) (Fig. 3).
- ***CD8+ cytotoxic T cells (CTLs).*** They are the most important cells responsible for killing HIV-infected cells, like CD4 T cells and antigen-presenting cells (APCs) (7,10). Upon encounter and recognition of viral antigens presented by APCs, CD8+ CTLs become activated. Following activation, they upregulate surface activation markers and secrete antiviral cytokines (IFN-γ and TNF-α). Also, they will proliferate into antigen-specific CTLs with higher perforin and granzyme B content, to better accomplish their cytotoxic properties by

degranulation (20). The effectiveness of CD8+ CTLs response determines HIV disease progression, while LTNPs show an effective CTL response, progressors are enriched by CD8+ CTLs that over time lose their degranulation capacity (20). Also, CTLs from LTNPs with protective HLA alleles (B*27, B*57) evade suppressive mechanisms of T regs by secreting granzyme B which eliminates T regs they encounter (14).

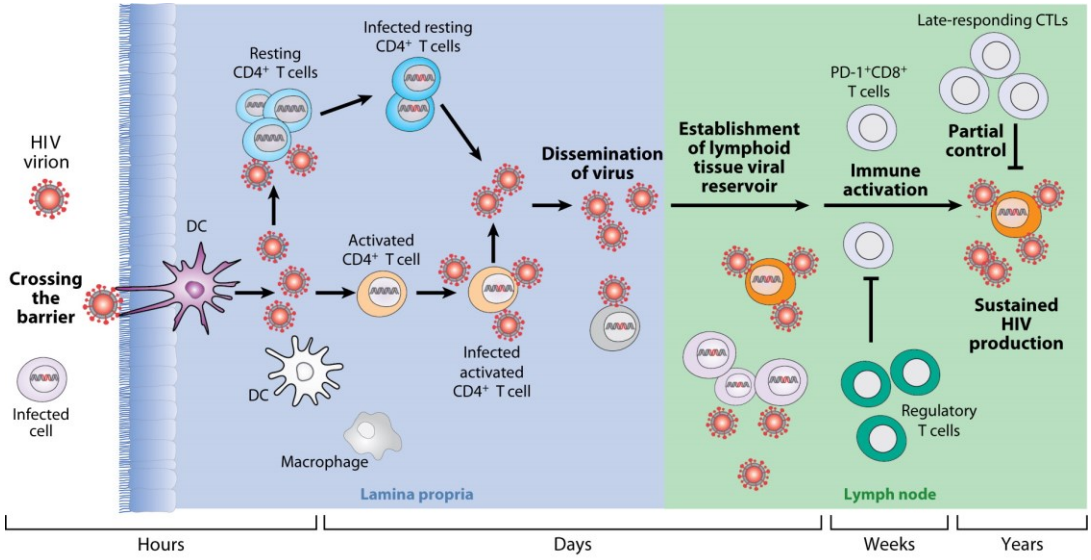


Figure 4. Adaptive Immune Response to HIV- on different stages of disease (7). *As HIV progresses both CD4 and CD8 T cells, will become exhausted upregulating co-inhibitory receptors such as VISTA, PD1, and Galectin-9, losing their capacity to respond to HIV* (21)

b) Humoral Response.

B cells are immune cells responsible for antibody production, that are formed in the bone marrow and continue to develop and differentiate in the peripheral blood and lymph nodes (22). After B cells encounter antigens, they will differentiate into plasma cells and secrete specific antibodies with or without the help of CD4 T cells (22). In HIV infection, 5-7 days postexposure there is an increase in activated memory B cells and short-lived plasmablasts (plasma cells) in the

peripheral blood, possibly due to the increase of cytokine levels during the acute infection (22). However, the antibodies produced at this point of the infection, are directed to glycoproteins of the virus envelope (gp41, gp120) (Fig.1) and do not provide efficient protection, because they can not neutralize the virus (non-neutralizing antibodies) (23). After 3-4 weeks or even months in some cases, neutralizing antibodies appear (24). In response to such antibodies, HIV will mutate to escape antibody neutralization (23). Antibodies directed to HIV also coordinate the phagocytosis of the opsonized virus by innate immune cells, that recognize them through Fc receptors present in almost all innate immune cells (macrophages, dendritic cells, granulocytes, NK cells) (23).

c) **Innate Immune Response.**

- ***Monocytes/Macrophages.*** They can be HIV targets, due to their expression of CD4 receptor, CCR5, and CXCR4 coreceptors, but since they express lower CD4 compared to T cells they become less attractive for HIV infection (25). Monocytes are the precursors of the majority of tissue macrophages, in HIV infection they are more activated, differentiated with increased tissue migration capacity (26). Also, monocytes/macrophages can be indirectly infected by HIV when they engulf HIV-infected T cells (26). Infected macrophages in the peripheral tissues become long-lasting viral reservoirs, especially in the brain (microglia) and lungs, where HIV persists even after combined antiretroviral therapy (cART) (25,27,28). Interestingly, a study showed that the bone marrow macrophages (osteoclasts) can also be infected by HIV-1 *in vitro* which

enhances their differentiation having a possible contribution to osteolytic disease in HIV-infected patients (29).

- ***Dendritic Cells (DCs).*** DCs are antigen-presenting cells (APCs) that can be found in the peripheral blood (myeloid and plasmacytoid DCs types) and tissues (Langerhans cells). DCs can be potential targets of HIV because they express CD4, CXCR4, CCR5, and they are also one of the first cells to interact with HIV during sexual transmissions, like Langerhans cells that have an intraepithelial location in the vaginal mucosa. Additionally, DCs express C-type lectins receptors that serve as attachment factors for HIV, for example, intercellular adhesion molecule 3 (ICAM3), grabbing nonintegrin (DC-SIGN or CD209), and langerin (or CD207 only in Langerhans DCs) (30). Through these C-type receptors, DCs will capture HIV for later trans-infection to CD4 T cells during antigen presentation in the lymph nodes, without becoming infected (30). DCs can also serve as reservoirs of HIV or they can react to the virus displaying antiviral responses by intracellular sensing of viral antigens through toll-like receptors 7 and 9 (TLRs) resulting in the secretion of interferon antiviral cytokine (IFN-γ) (10).
- ***NK cells.*** They are antiviral effector cells of the innate immune system (31). They lyse virally infected cells by secreting perforin and granzymes, after the loss of interaction between their inhibitory receptors (iNKRs) with the major histocompatibility complex type I (MHC I) present on healthy cells. HIV is known to prevent downregulation of MHC I on infected cells, mimicking a healthy cell status, thus avoiding NK cell lysis (32). Also, another antiviral

mechanism of NKs is the lysis of antibody-coated infected cells through their Fc receptors (like CD16) a mechanism called antibody-dependent cell-mediated cytotoxicity (ADCC) (32). Alternatively, NK cells fight HIV by secreting chemokines (CCL3,4 and 5) that bind to CCR5 receptors and inhibit HIV cell entrance through receptor competition. Besides, NKs secrete other cytokines like IFN-γ, TNF-α, and granulocyte/macrophage colony-stimulating factor (GM-CSF) to recruit more cytolytic effector cells (32). Additionally, HIV infection causes the upregulation of surface Gal-9 on NK cells which is associated with enhanced secretion of IFN-γ, but impaired Granzyme B, perforin, and granulysin expression (31).

- ***Neutrophils.*** The most abundant immune cells and front-line defenders in the peripheral blood and tissues. Their protective mechanisms include phagocytosis of pathogens, or antibody/complement opsonized pathogens, production of neutrophil extracellular traps (NETs), and secretion of antimicrobial peptides (33–35). It has been shown that HIV can bind to neutrophils and that neutrophil-bounded HIV is more efficient in infecting T cells compared to cell-free viruses (36). Moreover, activated neutrophils enhance their binding to HIV (37). Neutrophils can also bear HIV after the phagocytosis of antibody opsonized infected cells by recognition through their Fc receptors CD16, CD32, CD64, CD89 (38). HIV-infected neutrophils later recognize intracellular HIV by their TLRs 7 and 8, producing NETs in response (39). Neutrophils can also release the surface-bound Gal-9 to CD44 following activation, which subsequently can activate T cells (40). However HIV-infected

individuals with low CD4 counts have neutrophils with reduced phagocytic capacities, and HIV progression is reported to be related to a decrease in neutrophils (neutropenia), a process that occurs because of the destruction of CD34+ hematopoietic bone marrow progenitors, CD4 T cells, and mucosal Th17 cells by HIV (33).

1.1.5. Diagnosis of HIV

Diagnostic tests are performed on blood, which evaluates the presence of viral RNA, viral capsid antigen p24, and antibody-antigen immunocomplexes. Thus, different methods have been developed according to the progression of HIV (Fig. 5) (41,42).

a) Enzyme Immunoassays (EIA).

This method recognizes antibodies directed to HIV or viral p24, according to their sensibility and time of application, they are classified into 4 Generations, summarized in Table 1 (42). Generally, EIAs offer high sensitivity and specificity in detection, but on some occasions, they can report false positives. Thus after positive EIAs results, confirmation by a different type of EIA or a different assay such as Western Blot must be performed (43).

Table 1. HIV Diagnostic EIA (42).

EIA	Principle	Limitations
First Generation	Detect HIV-1 specific IgGs antibodies from the serum of infected patients after 6-8 weeks of infection	Does not detect HIV-specific IgM antibodies Does not detect HIV antigens
Second generation	Detect HIV-1 specific IgGs antibodies from the serum of infected patients that are after 5-8 weeks of infection	Does not detect HIV-specific IgM antibodies Does not detect HIV antigens

Third Generation	Detect both HIV-1/2 IgM and IgG antibodies 3 weeks after infection.	Does not detect HIV antigens
Fourth Generation	Detects both HIV-1/2 IgM and IgG antibodies 2 weeks after infection. Detects HIV p24 HIV antigen 5-7 days after the appearance of nucleic acid.	Miss early infection before antigenemia

b) Western Blot.

This approach tests IgG antibodies that bind to fixed HIV proteins, which after exposure to a substrate creates a pattern that is read as positive, negative, or indeterminate. Traditionally this has been used after EIA to corroborate results (42).

c) Qualitative polymerase chain reaction (PCR).

This is a method that detects viral nucleic acid in human secretions after its amplification. It is a very sensitive test, especially during the early phase of the infection (42). It can detect small amounts of virus, useful to evaluate the infection in babies that carry maternal antibodies to HIV up to 15 months of age, and immunocompromised infected individuals that cannot produce significant levels of antibody response against the virus (43).

d) Rapid Tests.

This is an antibody-based test that detect IgG and IgM anti-HIV antibodies, suitable for oral fluids, whole blood, plasma, or serum. Their results can be read in around 20-30 min, making them a good option for remote places where lab testing is limited. They can also be used for self-testing, although their effectiveness will be compromised especially in the early stages of the disease (42).

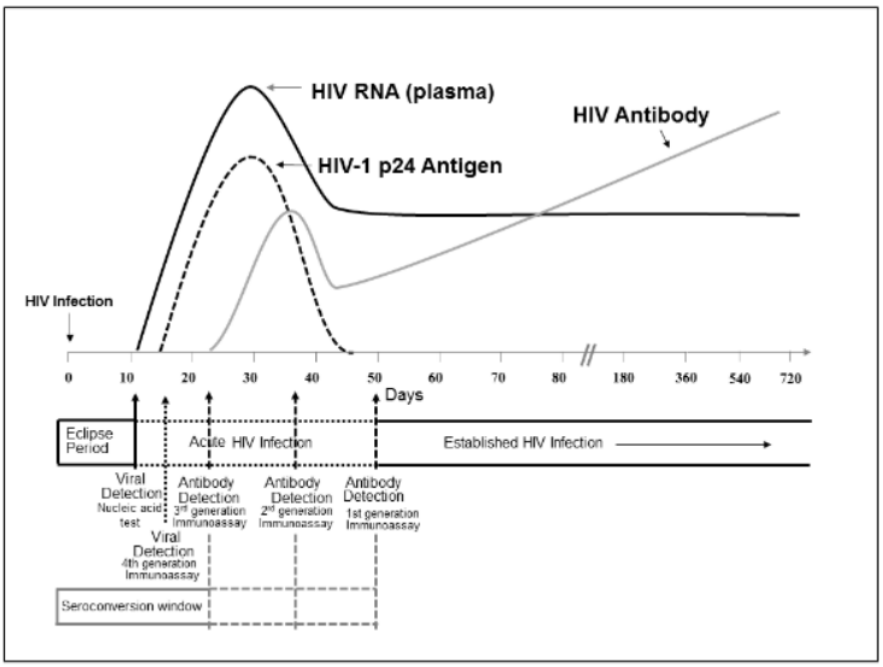


***Figure 5. Diagnostic markers of HIV by sequence of appearance** (40)*

1.1.6. Treatment of HIV

There is no cure for HIV infection, but antiretroviral therapy (ART) suppresses viral replication converting HIV into a chronic disease rather than a death sentence (44). Antiviral drugs target different stages of the HIV replication cycle (Fig. 6, Table 2). Standard ART regimen will combine 2 nucleoside reverse transcriptase inhibitors with a non-nucleoside reverse transcriptase inhibitor, protease, or integrase inhibitor (4). The combination of tenofovir, emtricitabine, efavirenz in a single pill, that is very common for daily intake (44). After starting ART plasmatic viral load will decrease to undetectable concentrations after 3 months, however, CD4 Tcells may rebound to close to normal levels is some patient but not in all. It is different among individuals depending on the early start of ART and the viral set point at the beginning of the infection (4). Clinical trials have shown the benefits of starting early ART, and also the

benefits of immediate ART as a preventive method after a possible exposure (e.g. a sexual encounter, pregnancy, and breastfeeding) to reduce the risk of HIV transmission.

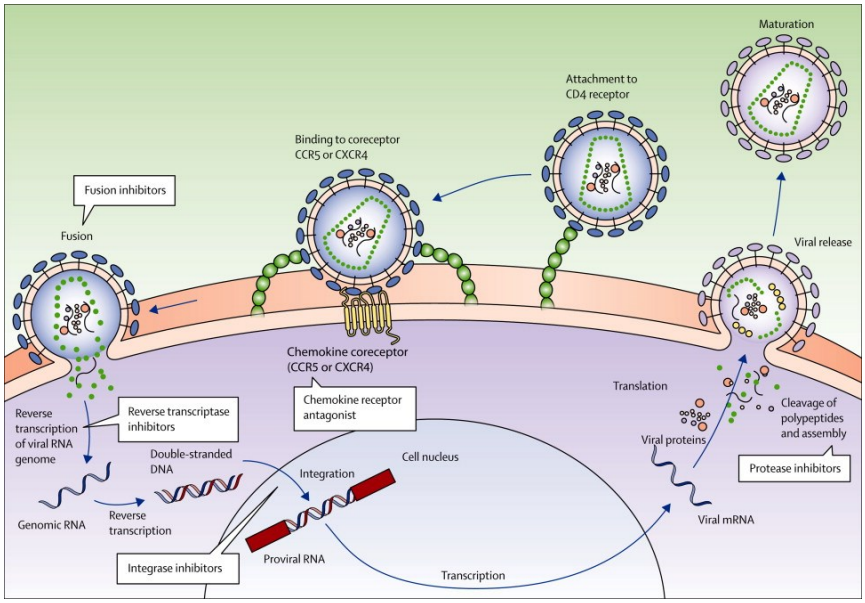


Figure 6. HIV drugs and their targets along HIV's life cycle *(4). Copyright license: 5178400364594*

Table 2. *Antiretrovirals for HIV Treatment (3,4,44)*

Class	Drug
Nucleoside Reverse Transcriptase Inhibitor (NRTIs)	- Tenofovir - Abacavir - Zidovudine - Stavudine - Lamivudine/ Emtricitabine
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	- Efavirenz - Nevirapine - Etravirine
Integrase Inhibitors	- Raltegravir - Dolutegravir - Elvitegravir
Protease Inhibitors	- Fosamprenavir - Atazanavir - Darunavir - Lopinavir - Saquinavir (Ritonavir)
Binding or Entry Inhibitors (CCR5 or CXCR4)	Maraviroc
Fusion inhibitors	Enfuvirtide

1.2. The oral cavity and HIV infection

The oral cavity is a unique environment, where immune protective mechanisms, mainly saliva and its components, immune cells, and mucosal epithelium maintain tolerance against a diverse repertoire of commensal microbes and food antigens, restricting the growth of pathogens. (45) (46).

1.2.1. Salivary Immune Factors

Saliva is an aqueous, and hypotonic body fluid of the oral cavity, a mixture of exudates from salivary glands, and crevicular fluid (47). It is made of 98.5% water, 1% organic, and 0.5% inorganic components. Organic components are mainly proteins (amylase, mucin, peroxidase, lysozyme, cortisol, immunoglobulins), glucose, cholesterol, fatty acids, triglycerides, urea, uric acid, and steroid hormones (cortisol). Inorganic components are electrolytes (Na^+, $Cl^–$, Ca^{2+}, K^+, $HCO_3^–$, $H_2PO_4^–$, $F^–$, $I^–$ and Mg^{2+}) (48) (47). Also, saliva contains a large number of oral microorganisms, and some epithelial and immune cells (49). Saliva has several important functions, including mucosal protection, pH maintenance, microbial control (antibacterial, antifungal, antiviral), remineralization of teeth, bolus formation, conversion of starch into maltose or glucose (by amylase), and taste mediator (50). Moreover, saliva can reflect pathological changes, acting as a mirror of systemic diseases, thus many of its components can be used as biomarkers for infectious diseases, neoplasias, hormonal disorders, autoimmune and cardiovascular diseases with diagnosis and prognosis application(51). Despite the presence of HIV in the saliva, its oral transmission is a rare event (52). Saliva is a hypotonic medium and disrupts the membrane of HIV-infected lymphocytes (53). Additionally, some salivary components can bind to HIV and neutralize it (46). However, following HIV progression local protective mechanisms are unable to protect

the host. Hence, leading to the appearance of oral opportunistic infections (e.g. Kaposi's sarcoma, oropharyngeal candidiasis, hairy leukoplakia, necrotizing gingivitis, and periodontitis) that are associated with AIDS (54).

Many salivary components have antimicrobial functions (summarized in Table 3). But only some of them have antiviral and/or anti-HIV functions as described below (Fig. 8),

Table 3. Antimicrobial factors in the saliva (55)

Innate Immunity	Cationic antimicrobial peptides	• α,β defensins • Cathelicidin (LL-37) • Histatins 1, 3 • Adrenomedullin • Azurocidin
	Adhesive proteins mediating bacterial agglutination	• Mucins • Salivary agglutinin • Surfactant protein A • B2 microglobulin • Proline rich proteins (PRPs) • Fibronectin
	Metal ion chelators	Calprotectin, Lactoferrin
	Protease inhibitors	Cystatins, Secretory leukocyte protease inhibitor (SLPI)
	Enzymes against bacterial cell galls	Lysozyme, Peptidoglycan recognition proteins 3 and 4
	Peroxidases	Salivary peroxidase, Myeloperoxidase (neutrophils)
Adaptive Immunity	Immunoglobulins	IgA, IgG

a) Immunoglobulins (Igs).

Their main function is to bind/agglutinate pathogens, inactivating them. There are 2 main Igs in the saliva IgG (1-10%), and secretory IgA (SIgA, 90-98%). IgG has serum origin and reaches the oral cavity through the crevicular fluid. SIgA is produced by plasma cells originated from committed B cells, residing in the MALT (Mucous associated lymphoid tissue) predominantly of the salivary glands (56). B cells class switch and secrete IgA, after receiving stimulation from T helper CD4 Tcells or APCs (57).

- ***Secretory Immunoglobulin A (SIgA).*** Constitutes the major antibody in the saliva, with antiviral and antibacterial opsonizing properties (46). In HIV infection, salivary IgA neutralizes free HIV binding to its gp120, thus inhibiting its attachment to target cells, enzyme activity, and movement across the oral epithelium (58) (46). Also, a decrease in the levels of salivary and serum IgA occurs in HIV-infected individuals, possibly as an indirect effect of CD4 T cells depletion (59) (60). In support, we have observed a decrease in the concentration of salivary IgA, although not statistically significant according to Mann-Whitney statistical test for non- parametrical data (Fig. 7).

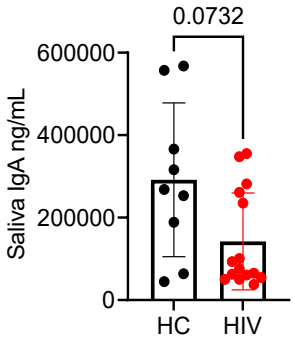


Figure 7. Saliva IgA concentrations in HIV-infected patients versus HCs, measured by ELISA *(Abcam, Cat.# ab196263)*

b) Cationic peptides.

Cationic peptides are positively charged (15-20 aminoacids) with antiviral activity against HIV and other enveloped viruses, Gram+ and Gram – bacteria, fungi, and parasites. They include cathelicidins and defensins (55).

- ***Cathelicidins.*** They are also found in the lungs, intestine, and skin. They are produced by neutrophils, mast cells, lymphocytes, keratinocytes, and epithelial cells. The LL-37 cathelicidin has been identified as an antiviral against HIV and influenza viruses, basically by binding to their membranes and creating pores for membrane rupture (46,56,61).
- ***Defensins.*** There are α-defensins (HNP1, 2, 3, 4) from neutrophils, and β-defensins (hBD1,2,3,4) from mucosal cells (56). They inhibit HIV replication by binding gp120 of HIV and decreasing the expression of CXCR4 on the surface of cells (46). Also, α and β defensins bind to the CD4 receptor, and they can inhibit HIV-1 replication at a step before reverser transcription (62). Moreover, HIV increases the expression of Hbd-2,3 mRNA in oral epithelial cells (63).

c) Metal ion chelators / Lactoferrin.

Secreted by salivary glands and neutrophils, it is an iron-binding glycoprotein with strong anti-HIV activity, because it competes with HIV for the binding to CXCR4, one of its receptors on T cells, as well (64). Lactoferrin also binds directly to gp120 of HIV, inhibiting HIV replication. (62).

d) Enzymes / Lysozyme.

It is produced by the salivary glands, especially the sublingual salivary gland, as well as by neutrophils and macrophages. It is also present in tears, egg whites,

and human milk (65). Lysozyme's anti-HIV properties are attributed to its binding to the CD4 receptor on host cells. Also, lysozyme degrades viral polysaccharides and RNA transcripts (46,62).

e) Protease Inhibitors.

- ***Secretory Leukocyte Protein Inhibitor (SLPI).*** It is a serine protease inhibitor, that protects the mucosa from excessive proteolytic activity (66). It is produced by macrophages, neutrophils, epithelial cells, and salivary glands (46). Its anti-HIV activity occurs by binding to annexin II on macrophages, avoiding HIV to bind to it through phosphatidylserine, which is acquired from the host after budding. (62).
- **Cystatins.** Are proteins that inhibit bacterial cysteine proteases denying nutrient uptake from bacteria suppressing their growth (55). In the case of HIV, they inhibit viral cysteine processes, as well (46).

f) Adhesive proteins.

- ***Thrombosphodin, acidic proline-rich proteins (PRPs).*** Thrombospondin is a glycoprotein that suppresses the infectivity of bacteria and protozoa. PRPs are proteins that constitute nearly 70% of the total protein fraction of human saliva. These 2 factors, interfere with HIV cell entrance by binding to gp 120 (46).
- ***Mucins.*** Produced by acinar cells of salivary glands (submandibular, sublingual). Mucins agglutinate viral particles, isolated salivary Muc5b, and 7 have inhibitory activity against HIV (55) (67).

- ***Salivary agglutinins:*** They are large glycoproteins, from parotid secretion and able to agglutinate large numbers of bacteria by their scavenger receptor (55). They bind and detach gp120 from the virus, damaging it (46).

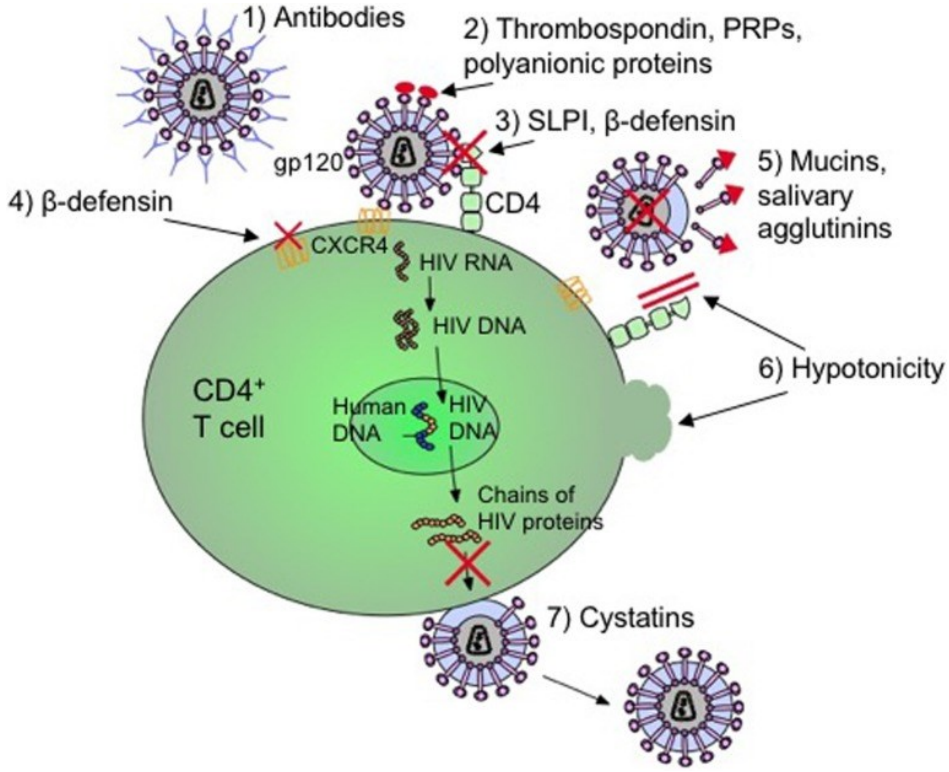


Figure 8. Salivary factors that inhibit HIV-1 infection (46).

1.2.2. Epithelial disruption, microbial dysbiosis, and translocation

In the genital, intestinal, and oropharyngeal mucosa, epithelial cells express CXRC4, CCR5, galactosylceramide (GalCer) receptors, which are targets for HIV binding and entrance into the epithelium. Then, by transcytosis HIV reaches deeper epithelial layers to end up in the lamina propria (68) (69). HIV also disrupts the epithelial tight junctions' proteins, for instance, after 24 hours epithelial cells incubated with HIV showed impairment in their tight junction proteins (claudin 1,2,3, occludin, and ZO-1), resulting in increased tissue permeability. HIV-infected epithelial cells secrete inflammatory

cytokines TNF-a, IL-6, IL-8, increasing epithelial disruption (70) (68). Moreover, the loss of tight junctions results in bigger paracellular spaces that favor HIV passage across the epithelium (68). Additionally, epithelial disruption causes bacterial translocation from the outside (lumen) to the interior of the gut's tissue, causing dysbiosis or disturbing the homeostasis between the host and the commensal microbiota (71). Consistently it has been shown that HIV-infected individuals have decreased bacterial diversity in their gut, increasing pathogenic species from Proteobacteria phylum and decreasing commensal Bacteroidetes phylum (71). Dysbiosis does not improve even after the start of ART, and as a result, a vicious circle of inflammation and continuous activation of CD4 T cells takes place in the gut, contributing to the viral replication and consequently CD4 T cells depletion, especially of the subsets Th1, Th17, Th22 (72). Similarly, bacterial dysbiosis in the oral cavity of HIV-infected individuals has been reported in several studies, analyzing the bacterial microbiome from the saliva and finding changes associated with periodontitis (73), gingivitis (74), the start of antiretroviral therapy (75) (76), decreased pulmonary function (77) and pneumonia risk (78), age and CD4 T cell counts (79), etc. This will be further discussed in chapter 2.

1.3. Galectin-9 (Gal-9)

Gal-9 is a β-galactoside-binding protein with different immunomodulatory functions, including cell aggregation, adhesion, proliferation, death, and inflammation (40) (80). Plasma levels of Gal-9 increase in pathologic conditions, like viral infections including HIV (81). Also, Gal-9 impacts neutrophil functions, increasing or decreasing chemotaxis, enhancing phagocytosis, and production of reactive oxygen species (ROS) (82). It has been reported that Gal-9 is highly expressed on the surface of neutrophils from the peripheral blood in healthy individuals. However, HIV-1 infection results in downregulation of surface Gal-9 as CD4 T cell counts decrease. (40). This suggests neutrophil activation results in Gal-9 shedding from neutrophils. This was supported by the downregulation of Gal-9 after *in vitro* stimulation with bacterial lipopolysaccharide (LPS) (Fig. 9). Therefore, neutrophils can be an important source of plasma Gal-9, given their abundance in the peripheral blood. Furthermore, secreted Gal-9 from neutrophil can interact with other cells resulting in their activation (e.g. CD4 and CD8 T cells) (40).

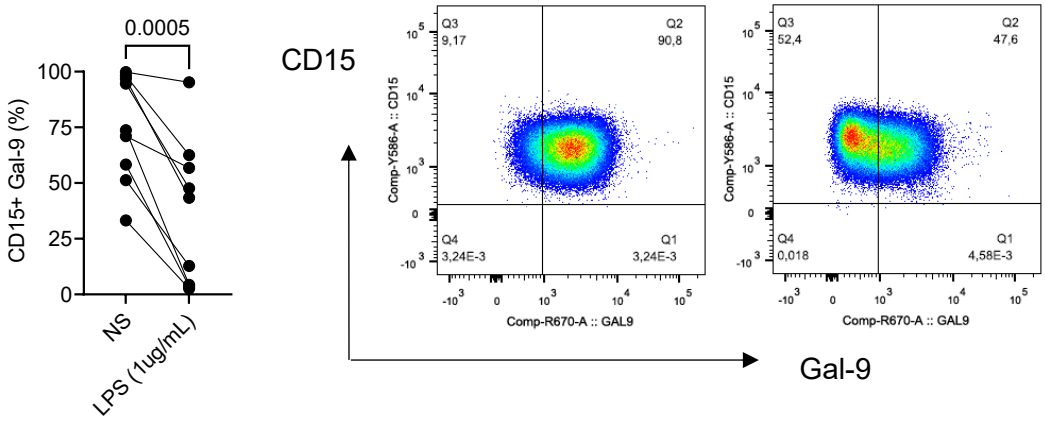


Figure 9. Downregulation of surface Gal-9 on blood neutrophils. (A) Cumulative data of neutrophils from the peripheral blood of HCs (n=8) after 3 hours in-vitro stimulation with LPS (100ng/mL) ***(B)*** Flow cytometry plot of 1 HC.

1.4. CD44- Hyaluronan Receptor

In T cells, one of the receptors for Gal-9 is CD44 (81). CD44 is a transmembrane glycoprotein expressed by hematopoietic and non-hematopoietic cells such as endothelial, epithelial cells, fibroblasts, keratinocytes, (83). CD44 is a common adhesion molecule, that participates in the attachment of immune cells to hyaluronan or hyaluronic acid (HA) of the extracellular matrix (ECM) (84). Adhesion of CD44 on immune cells and its ligand HA promotes cell activation and extravasation to the extracellular space. Particularly in neutrophils, CD44-HA acid interaction enhances their phagocytosis and IL-8 cytokine production (84). CD44 also binds to E-selectins (CD62E) on endothelial cells, therefore CD44 plays an important role in neutrophils' tethering, rolling- arrest, and adhesion (85). For instance, in the liver, endothelial cells capture neutrophils via CD44-HA (86). Also, CD44-/- knockout mice, showed less neutrophil emigration and adhesion after injection with neutrophil-activating chemokine (MIP-2) (87).

Based on our observations of blood neutrophils, they not only co-express abundant surface CD44 and Gal-9 but also Gal-9 is colocalized with CD44. Gal-9 shedding from neutrophil surface takes place following neutrophil activation that makes CD44 interact with the actin cytoskeleton of the cell, promoting cell polarization and movement (40).

1.5. Hypothesis and study aims

As discussed in chapter 1, HIV-1 infection in different ways impacts the oral immunity (46). Some previous studies have described bacterial dysbiosis in the oral cavity of HIV-1 infected patients (75,88). However, this has been the subject of debate. Moreover, there is no information about the frequency and phenotype of neutrophils as the most abundant cells in the oral cavity of HIV-1 infected individuals compared to HIV-negative individuals. Thus, the purpose of this study was to address oral bacterial dysbiosis with a particular interest in neutrophils, given their important role in bacterial containment and tissue homeostasis in the oral cavity (89). I hypothesize that HIV-1 infected individuals harbor a pro-inflammatory environment shaped by neutrophil's phenotype, similar as the gut, where dysbiotic bacteria impact neutrophil's functions (90).

I propose the following *specific aims* to better understand the role of neutrophils in the oral cavity of HIV-infected patients versus HIV-negative individuals.

1. To phenotype neutrophils from oral washes of HIV patients versus healthy controls by flowcytometry analysis, evaluating their expression of surface Gal-9 and CD44. I suggest that neutrophils' that lose Gal-9 become more activated. Additionally, neutrophils' CD44 expression will also be important, considering its role in Gal-9 shedding (40), neutrophil migration, phagocytosis and cytokine production (84), as described previously.
2. To measure soluble biomarkers, mainly inflammatory cytokines, and other biomarkers (soluble Gal-9, and CD44) in the saliva of HIV-1 infected versus healthy controls by Elisa and Multiplex Elisa.
3. To explore changes in the bacterial oral microbiome of HIV-1 infected versus age/sex-matched healthy controls by 16srRNASeq.

Chapter 2. "Differential signature of the microbiome and neutrophils in the oral cavity of HIV-1-infected individuals"

*This study has already been published in Frontiers of Immunology Journal,*09 November 2021 | https://doi.org/10.3389/fimmu.2021.780910.

2.1. Introduction

The oral cavity is a unique environment that comprises immune cells, soluble immune mediators, microbial communities, food antigens/foreign materials, soft and hard tissues (45) (91). Although most of the research has been focused on bacterial communities, the oral cavity harbors a large collection of viruses, fungi, and bacteriophages (92). Saliva flow, soluble salivary components such as antimicrobial peptides (93), immune cells, and mucosal epithelial cells in cross-talk with oral microbiome work together to sustain an immune homeostatic state under normal physiological conditions (52) (57). For example, SLPI (salivary secretory leukocyte protease inhibitor), defensins, sIgA, lactoferrin, and lysosome in the saliva play a protective role against viral infections (e.g. HIV) (46). However, upon the acquisition of HIV infection and disease progression due to the elimination/reduction of HIV-target cells (CD4 and Th17 cells) (46) (11)(94), patients become prone to opportunistic infections such as candidiasis and other oral manifestations (e.g. Kaposi's sarcoma) (54) (95). Notably, elimination of Th17 cells and reduction in IL-17 result in decreased recruitment of innate immune cells especially neutrophils into the oral cavity (96). Besides, HIV-infected individuals with lower CD4 T cell count even when on antiretroviral therapy (ART) exhibit decreased neutrophil frequency in their blood circulation (97).

Neutrophils are the most abundant leukocytes in the blood circulation and also in the oral cavity (98). The interaction of neutrophils with symbiotic microbial communities plays a crucial role in immune homeostasis at the mucosal surfaces such as the oral cavity (99). However, dysbiotic microbiota at the mucosal surfaces (e.g. gingival crevice) activate neutrophils which result in an exacerbated inflammatory response (100). The oral cavity is an important peripheral microenvironment for neutrophils given its richness

with bacterial communities (89). Additionally, bacterial communities can directly or indirectly (e.g. activation of macrophage, dendritic cells, and lymphocytes) influence neutrophil recruitment and activation in oral tissues (99) (101). For example, germ-free mice have decreased neutrophils and their progenitors in the oral cavity compared to wild-type mice (102). It has also been reported that bacterial communities enhance neutrophils aging (103). As such, aged neutrophils exhibit an inflammatory phenotype by increased production of reactive oxygen species (ROS) (104). Moreover, chronic conditions can influence microbial communities at the mucosal surfaces including the oral cavity. For example, diabetes can modify the oral microbiome to exacerbate periodontal disease (105).On the other hand, the dysbiotic oral microbiome has been reported to be associated with colorectal and pancreatic cancers (106) (107) (108). Nevertheless, the impact of HIV infection on the oral microbiome has been controversial. Some studies support microbiome modifications and some do not. These discrepancies could be related to various factors such as the study design, detection methods, and sampling (e.g., saliva, oral wash, or subgingival). For example, it was reported that HIV infection modulates the fungi population, and smoking habit in HIV-infected individuals was associated with greater microbial diversity (109). It appears that HIV infection shifts oral microbial communities towards a dysbiotic state (79). In particular, higher levels of cultivable microbes were isolated from the saliva of HIV-infected individuals compared to the HIV-uninfected group (88) (74). Other clinical variables are associated with alterations in the composition of oral microbial communities in HIV-infected individuals on ART (74). Although the potential influence of ART on the oral microbiome is debatable, it is reported that ART partially reverses HIV-induced oral microbiota alteration (74). ART treatment appeared to be associated with significantly greater

bacterial richness and diversity (75). These studies demonstrate that HIV infection and/or ART adds another layer of complexity to the tight interplay between the immune system and microbial communities in the oral cavity. However, the impact of HIV infection and/or microbial communities on oral neutrophils and vice versa have remained unexplored.

Neutrophils are crucial players in immune homeostasis players in the oral cavity (110). However, in inflammatory conditions such as periodontitis, the accumulation of activated neutrophils can result in tissue damage and bone loss (111). The activation of circulatory neutrophils results in cellular polarization, which facilitates tissue extravasation (112). CD44, a type I transmembrane glycoprotein, is one of the extracellular adhesion molecules that impacts neutrophil rolling and tissue migration (83). As such, lack of CD44 was associated with decreased neutrophil migration to inflamed tissue in CD44 KO mice (87). Besides, the interaction of CD44 with hyaluronate enhances neutrophil phagocytosis *in vitro* (84). Recently, we reported that CD44 interacts with Galectin-9 (Gal-9) on blood neutrophils (40). Gal-9 as a beta-galactosidase binding protein has a wide range of immunomodulatory properties depending on interaction with its corresponding receptors (81). We found that CD44 depalmitoylate during neutrophil activation and facilitates the movement of CD44 out of the lipid raft, and subsequently Gal-9 shedding from neutrophils in HIV-1-infected individuals (40). This process results in increased soluble plasma Gal-9 in HIV-1 infected individuals which subsequently enhances T cell activation via interaction with CD44 on T cells (40). Therefore, Gal-9 shedding from neutrophils might explain a potential source for the elevated plasma Gal-9 in HIV-1 infected individuals (113).

In the present study, we show that HIV-1 infected individuals have a different and richer bacterial composition in their saliva than healthy controls. Besides, we observed a significant reduction in the proportion of oral neutrophils in HIV-1 infected individuals, in particular, in those with lower CD4 T cell count. Additionally, we observed downregulation of CD44 surface expression on oral neutrophils in HIV-infected individuals, which potentially explains their decreased frequency in the oral cavity of HIV 1 infected-individuals.

2.2. Materials and Methods

2.2.1. Study population

For this study, we recruited sixty-one HIV-1 infected individuals including: a) on ART with low CD4 T cell count (< 200 cell/mm^3, n=11); b) on ART with high CD4 count (>200 cell/mm^3, n=40); c) Long-term non-progressors (21) (LNTPs, n=9) and d) ART-naive, n=2) through the Northern Alberta HIV-1 Program in Edmonton, Canada (Supplementary Table 1). Also, a total of 43 healthy controls (HCs) defined as HIV-1, Hepatitis B virus, and Hepatitis C virus seronegative individuals without active oral disease were recruited for comparison. The institutional ethics review boards at the University of Alberta approved the study with the protocols (Pro00070528 and Pro000064046). All study participants gave written informed consent to participate in the study.

2.2.2. Sample collection

Participants avoided eating or drinking for at least 30 minutes before the sample collection. Saliva samples were obtained followed by oral washes from the study participants. Saliva samples were aliquoted and stored at -80°C until use.

Oral wash was performed 5 times using 20 ml of phosphate-buffered saline solution (PBS) for 30 seconds with 3 minutes' intermission between rinses. Samples were centrifuged at 2000 rpm for 10 min, supernatants were discarded, and cell pellets were resuspended in culture media (RPMI-1640) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 1% penicillin-streptomycin (Sigma). Cell suspensions were filtered through 100, 70, and 50 μm sterile strainers (Fischer Scientific), centrifuged and resuspended in culture media for further analysis. Blood samples of 16 HCs and 15 HIV-1 infected individuals on ART were subjected to gradient separation using Ficoll-Paque Premium (GE). The peripheral blood mononuclear cell (PBMC) fraction was removed, and the remaining red blood cell pellet was lysed using red blood cell lysis buffer for 10 minutes (0.155M NH_4Cl, 10mM $KHCO_3$, and 0.1mM EDTA) to isolate polymorphonuclear cells according to our previous methods (40) (114).

2.2.3. Flow cytometry analysis

Fluorophore antibodies with specificity to antigens of human cells were purchased from BD Biosciences, Thermo Fisher Scientific, and/or R&D. We used anti-CD15 (W6D3), anti-Gal-9 (9M1-3), anti-CD44 (515), and anti-CD32 (FLI8.26). Cell viability was evaluated by LIVE/DEAD Kit (Life Technologies) (Cat. L34966). Apoptosis assay was performed using the PE Annexin V Apoptosis Detection Kit I (BD Biosciences) (Cat. 559763) according to the manufacturer's protocol. Stained cells were fixed in 4% paraformaldehyde before acquiring on an LSRFortessa-SORP or LSRFortessa X-20 flow cytometers (BD Biosciences), and data were analyzed using the FlowJo (version 10).

2.2.4. Cytokine, CD44, and Gal-9 measurement

The liquid fraction of the saliva was 2-fold diluted for cytokine quantification. We specifically measured TNF-α, IL-8, IL-6, IL-10, IL-13, IL-1B, IFN-γ using V-plex Plus pro-inflammatory kit from Meso Scale Discovery (MSD) (K15054D-1) according to the manufacturer's instruction and our previous reports (114) (115). Similarly, CD44 (R&D Systems; DY7045-05) and Gal-9 (R&D; DY 2045) concentrations were quantified by ELISA.

2.2.5. Bacterial DNA Isolation

Saliva aliquots from sex and age paired participants were centrifuged and pellets were used for DNA isolation using the QIAamp DNA Mini Kit (cat. 51304). Pellets were mixed with 20 μl of Proteinase K and 200 μl of in-house lysis buffer (100 mL of 0.5 M sodium chloride, 0.005 M tris aminomethane-pH8, 0.05M ethylenediaminetetraacetic acid with pH 8, and 4% sodium dodecyl sulfate), briefly vortexed, and incubated at 56°C water bath for 60 min, followed by 15 min incubation at 70°C. Then 200 μl of buffer AL (from the kit) was added for a final 10-minute incubation at 70°C, accompanied by DNA column extraction according to the manufacturer's instructions.

2.2.6. 16SrRNA Illumina MiSeq Sequencing

V3-V4 variable regions of 16S rRNA were amplified from genomic DNA samples. Amplicons were generated using the following primers: Forward Primer = 5' CGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and Reverse Primer = 5' TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTAC HVGGGTATCTAATCC (IDT). Thus, generated PCR products were indexed using Illumina's Nextera XT kit. Sequencing was performed on Illumina's MiSeq platform using

a 250-bp paired-end sequencing kit at The Applied Genomic Core (TAGC), University of Alberta. Sequencing data were demultiplexed and binned into individual samples according to their barcodes and further bioinformatic analysis was performed using the QIIME2 pipeline (2021.4). The first step of this analysis was to join the paired-end reads (1 and 2) with a minimum of 100 bp overlap and 0 mismatches. Reads were then quality filtered by removing sequences having more than 10 sites with a Phred quality score less than 20. Next, reads were denoised into amplicon sequence variants (ASVs) using the DADA2 method. Taxonomy classification at the phylum, family, and genus levels was done by comparing ASVs to the Green genes bacterial reference database (v. 13.5). Diversity indices (Evenness, Observed OTUs, Shannon's diversity, and Faith's phylogenetic index) and distances between samples (Bray-Curtis, weighted- and unweighted-Unifrac) were all calculated in QIIME2 to profile oral microbiota. Before taxonomy classification and generating alpha and beta diversity metrics, data were rarefied across samples for normalization such that all samples have the same number of total reads.

2.2.7. Statistical Analysis

This was performed in GraphPad Prism 9 (GraphPad Software, Inc.). D'Agostino & Pearson test was used for normality check, being samples non-parametrical Mann-Whitney U tests for unpaired data or Wilcoxon signed-rank tests for paired data were used. When comparing more than two groups, the Kruskal Wallis test was used. Means and standard deviations (mean ± SD) are used to present the data. Correlation analysis was performed by nonparametric Spearman correlation.

2.3. Results

2.3.1. Differential bacterial communities in the oral cavity of HIV-1 infected individuals compared to HCs.

We first compared the two groups in terms of β-diversity using Principal Coordinate Analysis (PCoA) with Bray-Curtis distances. The results indicated a differential clustering of bacterial communities in the saliva of HIV-1 infected compared to healthy individuals (Figure 10 A). Distances within groups versus distances across groups were determined by ANOSIM, which was significant (P=0.001). ADONIS or differences between the group's centroid was also significant (P=0.003). The Alpha diversity analysis by Faith's, Shannon, and observed OTUs indexes showed that the saliva samples from HIV-1 infected individuals harbored significantly richer bacterial communities compared to the saliva samples from HCs (Figure 10 B)

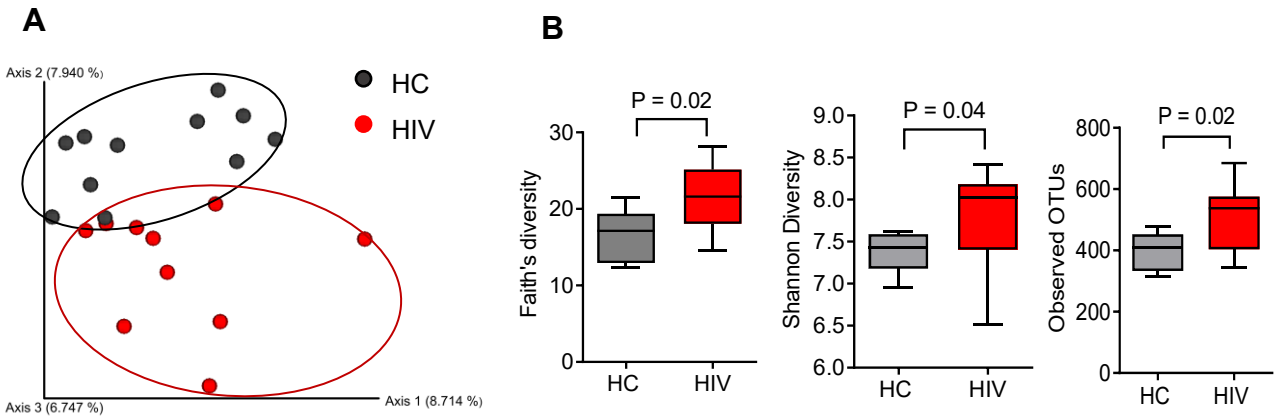


***Figure 10. Bacterial Diversity in the saliva of HIV-1 patients vs HCs.*(A).** Beta diversity in bacterial communities is shown by Principal coordinates analysis (PCOA) of Bray-Curtis Distances, in gray HCs (n=11), in red HIV-1 infected individuals (n=10), and each symbol represents an individual. **(B)** Alpha diversity in bacterial communities determined by Faith's, Shannon's, and observed OTUs indexes.

To further determine which bacterial communities were different between the groups, we compared them at different levels. At the phylum level, we found Spirochaetes' phylum was significantly enriched in the saliva samples of HIV-1 infected individuals compared to HCs (Figure 11A and 11B).

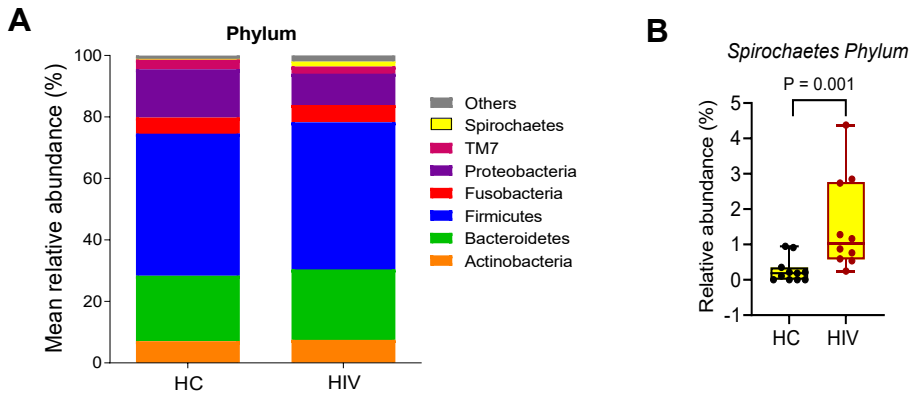


Figure 11. Salivary Bacteria at the phylum level in HCs vs. HIV-1 infected **(A)** Comparison of the saliva microbiota at phylum level in HCs versus HIV-1 infected group. **(B)** Comparison of *Spirochaete's* phylum in the saliva of HCs versus HIV-1 infected individuals

When bacterial communities were compared at the species level (Figure 12A), we observed that five species of Spirochaeta including *Spirochaetaceae*, *Spirochaeta*, *Treponema*, *Treponema amylovorum*, and *Treponema azotonutricum* were significantly abundant in the saliva of HIV-1 infected individuals compared to HCs (Figure 12B). The volcano plot shows further differences in bacterial species among the groups. Species that were enriched in the saliva of HIV-1 infected individuals are shown in the right upper quadrant, and bacterial species that were less prevalent in the saliva of HIV-1 infected individuals are shown in the upper left quadrant of the volcano plot (Figure 12B). Bacterial species with mean counts less than 1 were not accounted for. We found that bacterial communities belonging to *Spirochaetes*, *Bacteroidetes*, *Firmicutes*, and *TM7* species were significantly enriched in the saliva samples from HIV-1 infected individuals, respectively (Figure 12C-G). In contrast, we noted a significantly lower abundance of

Proteobacteria phylum (in particular *Helicobacter*) in the saliva of HIV-1 infected individuals compared to HCs (Figure 12G). Overall, our results show a significant difference in bacterial communities in the oral cavity of HIV-1 infected compared to HCs

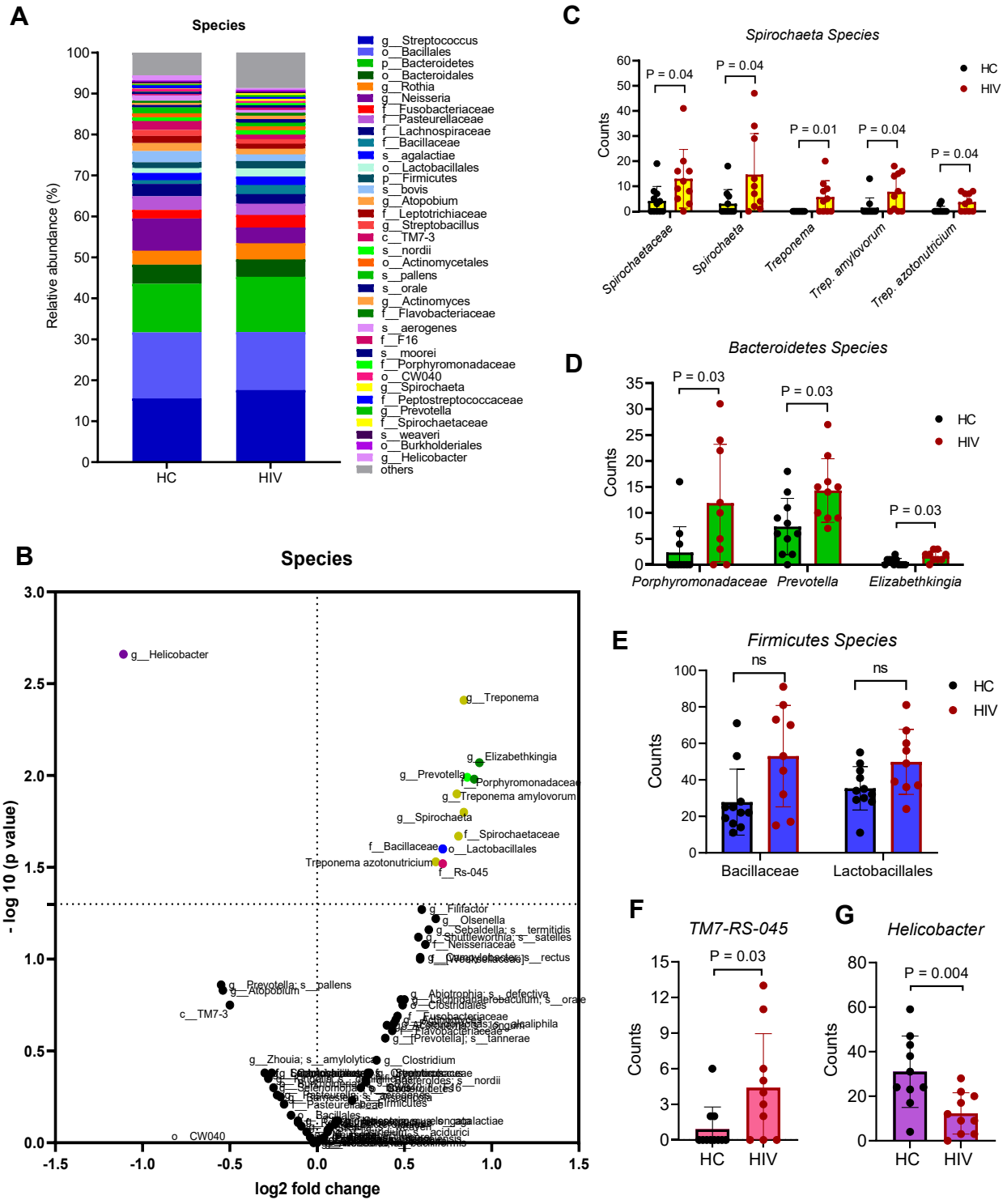


Figure 12. Salivary bacteria at the species level in HCs vs. HIV-1 infected individuals (A) Relative abundance of bacterial species. **(B)** Volcano plot of bacterial species, those who exceeded the dotted line marked at 1.3 were considered significant. **(C)** Comparison of unclassified *Spirochaetaceae, Spirochaeta, Treponema, Trep. amylovorum*, and *Trep. Azotonutricum* species **(D)** Comparison of *Bacteroidetes* species *Prophyromonadaceae, Prevotella, and Elizabethkingia*. **(E)** Comparison of *Firmicutes* species *Bacillaceae and Lactobacillales* **(F)** Comparison of (TM7-RS-045) species **(G)** Comparison of *Helicobacter* species.

2.3.2. A lower proportion of neutrophils in the oral cavity is related to the clinical status of HIV-1 infected individuals

To better understand the possible role of immune components of the oral cavity in HIV-1 infected individuals on the bacterial composition, we focused on neutrophils as the most abundant immune cells in the oral cavity (116). In agreement with previous reports, we found that neutrophils were the most abundant cells in the oral cavity. However, HIV-1 infected individuals had significantly lower percentages of neutrophils in their oral cavity compared to HCs (Figure 13A and 13B). Notably, we observed significantly lower percentages of neutrophils in the oral cavity of ART-naive and patients on ART compared to HCs (Figure 13C and 13D). In contrast, we found a similar proportion of oral neutrophils in the oral cavity of HIV-1 infected LTNPs compared to HCs (Figure 13C and 13D). These observations suggest that the disease status may impact the frequency of neutrophils at the mucosal surfaces.

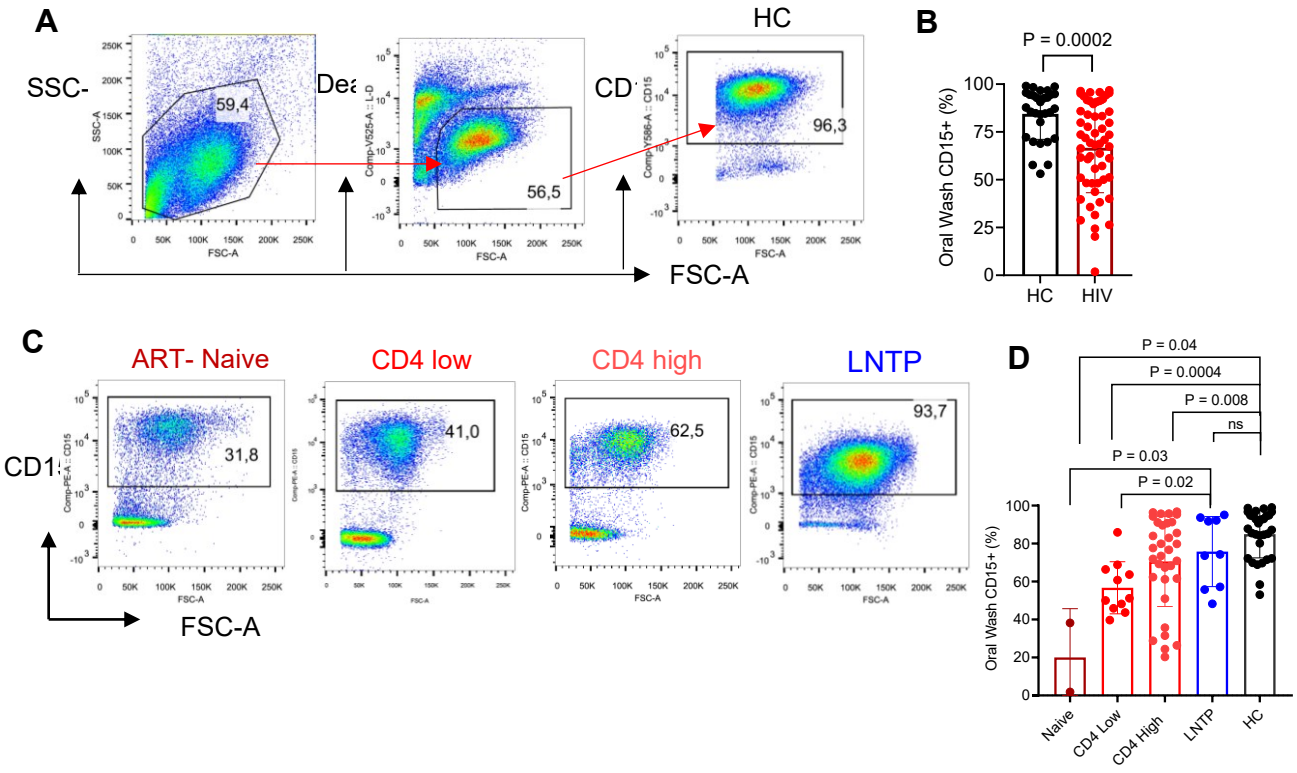


Figure 13. Neutrophils in the oral cavity are decreased in HIV-1 infected individuals **(A)** Representative flow cytometry plots for CD15⁺ neutrophil identification in the oral wash. **(B)** Cumulative data of percentages of CD15⁺ cells in the oral washes of HCs compared to HIV-1 infected individuals. **(C)** Representative flow cytometry plots, and **(D)** cumulative data of neutrophils in oral washes of different HIV-1 infected individuals (ART-naïve, on ART with low CD4 T cell count (< 200 cells/ul) or high CD4 T cell count (> 200 cells/ul), and long-term non-progressor (LTNP) compared to HCs.

2.3.3. Gal-9 is downregulated on the surface of blood but not oral neutrophils in HIV-1 infected individuals

Recently, we showed that Gal-9 is downregulated from the surface of blood neutrophils in HIV-1 infected individuals compared to HCs (40). Therefore, we decided to investigate whether this was the case for oral neutrophils. Our observations reconfirmed that the frequency of Gal-9 expressing blood neutrophils was significantly lower in HIV-1 infected individuals compared to HCs (Figure 14A and 14B). However, we found a smaller portion of Gal-9 expressing oral neutrophils compared to their siblings in the blood,

without any significant difference between the HIV-1 infected individuals on ART and HCs (Figure 14A and 14B). Moreover, we measured the intensity of Gal-9 and found that the Mean fluorescence intensity (MFI) of Gal-9 was significantly lower on the surface of blood neutrophils from HIV-1 infected individuals (Figure 14C and 14D). However, this was not the case for the oral neutrophils (Figure 14C and 14D).

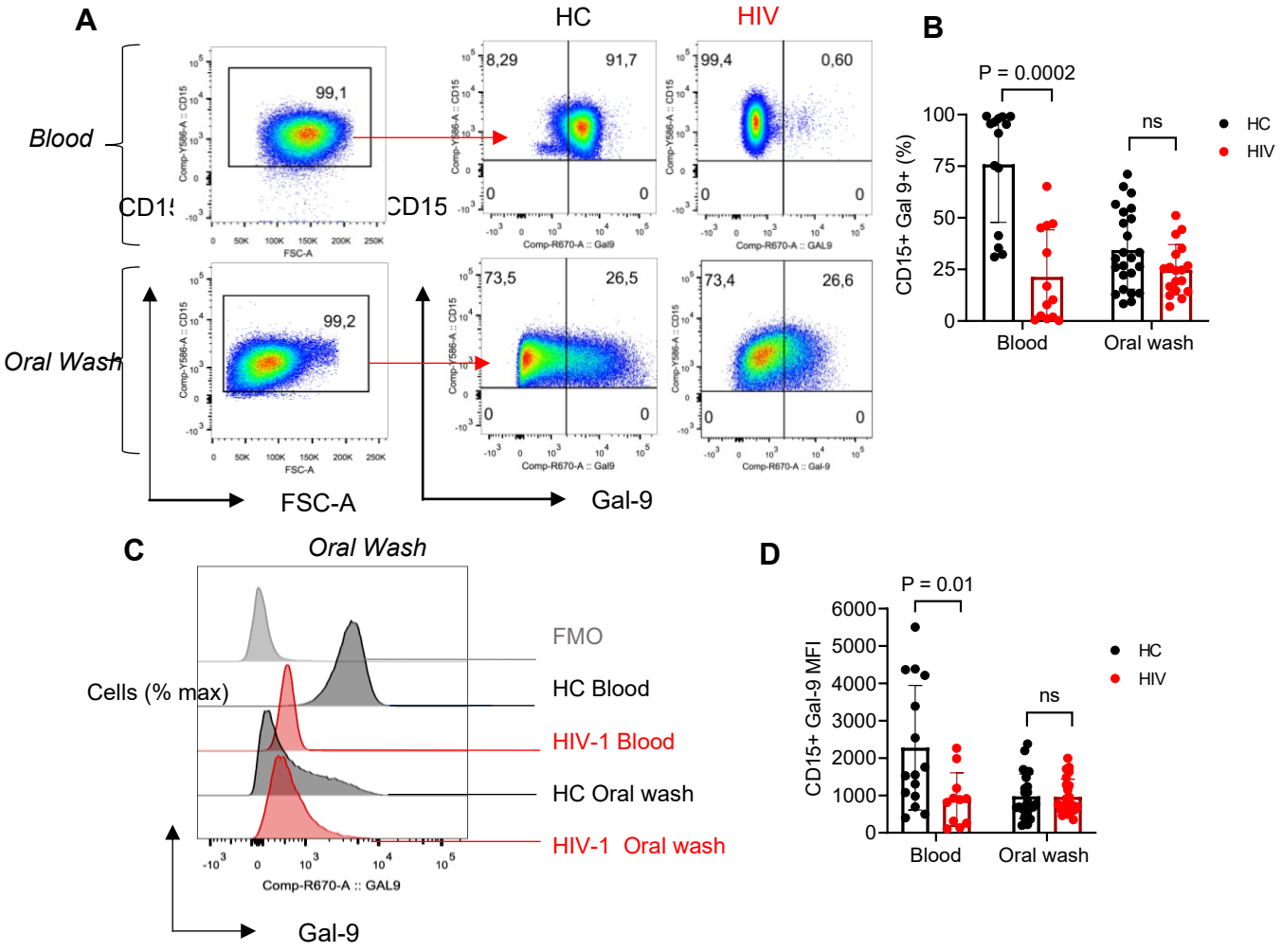


Figure 14. Low expression of surface Gal-9 in oral neutrophils of HIV-1 infected individuals vs HCs

(a) Representative flow cytometry plots, and (b) cumulative data of Gal-9 surface expression in neutrophils from the blood and oral washes of HIV-1 and healthy individuals. (c) Representative histogram plots, and (d) cumulative data of Gal-9 expression measured by the Mean Fluorescence Intensity (MFI) on blood and oral neutrophils of HIV-1 -infected and healthy individuals.

2.3.4. Soluble Gal-9 is positively correlated with IL-6 in the saliva of HIV-1 infected individuals

We also quantified the concentration of soluble Gal-9 in the plasma and saliva samples. Although soluble Gal-9 was significantly elevated in the plasma of HIV-1 infected individuals as we have reported elsewhere (113), we did not find any difference in the soluble Gal-9 levels in the saliva between the groups (Figure 15A).

Since Gal-9 has been reported as a contributing factor to the cytokine storm in COVID-19 patients (114), we reasoned to evaluate the correlation of saliva Gal-9 levels with pro-inflammatory cytokines. We found that Gal-9 levels were positively correlated with IL-6 in the saliva (Figure 15B). This may suggest the potential role of soluble Gal-9 in the activation status of neutrophils in the oral cavity of HIV-1 infected individuals. However, we did not find such correlation for other pro-inflammatory cytokines (e.g., IL-1β, IL-8, TNF-α and IFN-γ) in the saliva (Supp. Fig 3).

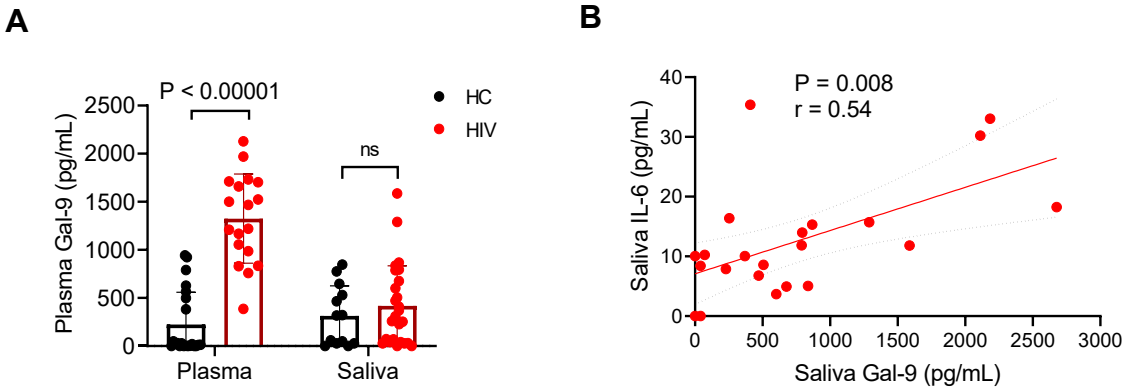


Figure 15. Salivary Gal-9 is correlated with IL-6. **(A)** Cumulative data of soluble Gal-9 in the plasma and saliva of HIV-1 infected and healthy individuals as measured by ELISA. **(B)** Cumulative data of a positive correlation between the saliva Gal-9 with the saliva IL-6 (Spearman correlation, r=0.54, P= 0.008).

2.3.5. Downregulation of surface Gal-9 makes oral neutrophils less apoptotic

IL-6 has been related to increased neutrophil survival (117) and an activated status can prolong the lifespan of neutrophils (118). Thus, we decided to investigate differences in Gal-9+ versus Gal-9- oral neutrophils. Recently, we have reported that stimulation of blood neutrophils with LPS results in the downregulation of Gal-9 at the gene and protein levels (40). Thus, we proposed that activated neutrophils lose their surface Gal-9, which in turn increases their lifespan. Indeed, we found that in HCs and HIV-1 infected individuals, neutrophils that did have surface Gal-9 were less apoptotic compared to their Gal-9 expressing counterparts (Figure 16A and B). These observations suggest that the downregulation of Gal-9 may act as a mechanism of enhanced neutrophil survival.

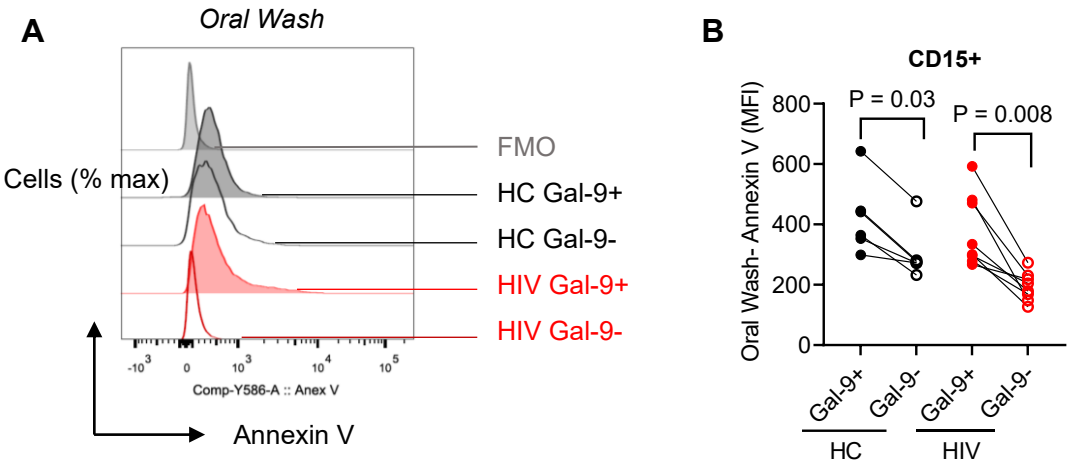


Figure 16. Oral neutrophils with low Gal-9 are more apoptotic. **(A)** Representative histogram plots, and **(B)** cumulative data of apoptosis in oral neutrophils regarding Gal-9 expression measured by Annexin V assay.

2.3.6 CD44 is downregulated from the surface of oral neutrophils in HIV-1 infected individuals

Unstimulated blood neutrophils express high levels of surface Gal-9 which is bound to CD44 (40). Therefore, we decided to determine whether the same pattern exists for oral neutrophils. Similar to our previous finding, we found all blood neutrophils expressed CD44, however, this was about 50% for oral neutrophils (Figure 17A). Interestingly, we observed that the percentage of CD44 expressing neutrophils was significantly lower in the oral cavity of HIV-1 infected individuals (Figure 17A and 17B). Moreover, we found a significant reduction in the intensity of CD44 expression on oral neutrophils from HIV-1 infected individuals versus HCs (Figure 17C and 17D). When the expression of CD44 in the blood and oral neutrophils was analyzed, we found a lower frequency of CD44 expressing neutrophils and even CD44 expression level on oral neutrophils compared to their counterparts in the blood in both HCs and HIV-1 infected individuals (Fig. 17E-F).

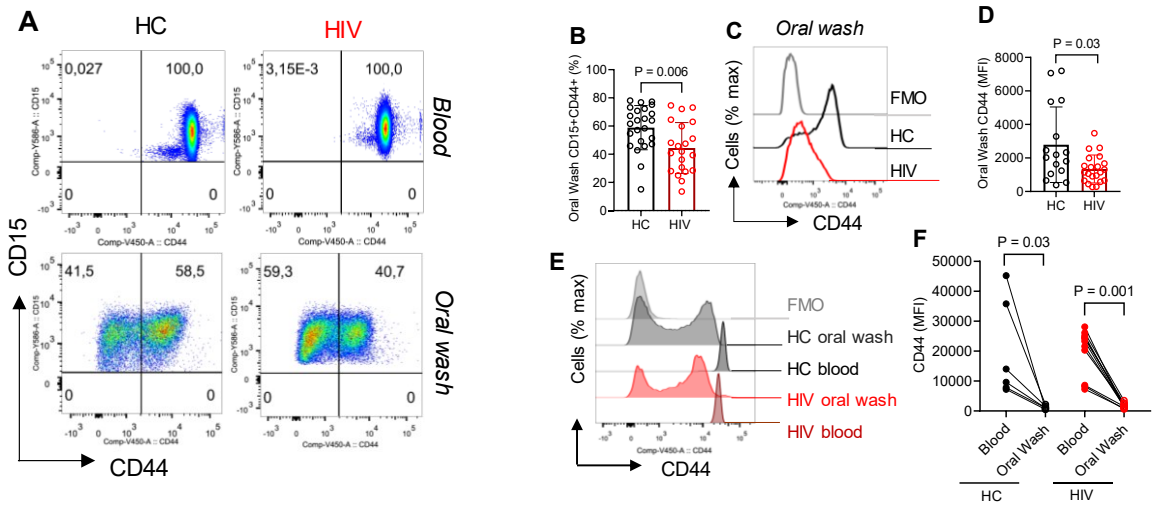


Figure 17. CD44 is downregulated from the surface of oral neutrophils in HIV-infected individuals (**A**) Representative flow cytometry plots of CD44 expression on blood and oral neutrophils. **(B)** Cumulative data of CD44 expression on oral neutrophils of HIV-1 infected vs HCs. **(C)** Representative histogram plots, and **(D)** cumulative data of CD44 expression (MFI) in oral neutrophils of HV-1 infected individuals vs. HCs. **(E)** Representative histogram plots, and **(F)** cumulative data of CD44 expression (MFI) in the blood and oral neutrophils of HIV-infected vs. healthy individuals.

2.3.7. Soluble CD44 is associated with inflammatory cytokines and bacterial diversity

The previous observations led us to measure the soluble CD44 concentration in the saliva, which was higher in HIV-1 infected compared to healthy individuals (Figure 18A). This might explain that oral neutrophils in HIV-1 infected individuals shed CD44 that can be detected in their saliva. Therefore, these findings may suggest that oral neutrophil's surface CD44 is upregulated to facilitate neutrophil migration into peripheral tissues, but once they reach their action site it gets downregulated to keep neutrophils at their destination. Additionally, we found that increased salivary CD44 in HIV-1 infected was positively correlated with salivary proinflammatory cytokines IL-6 and IL-8 in HIV-1 infected individuals (Figure 18C and 18D). Remarkably, we noted a positive correlation between the soluble CD44 with Faith's bacterial diversity index in HIV-1 infected individuals (Figure 18D).

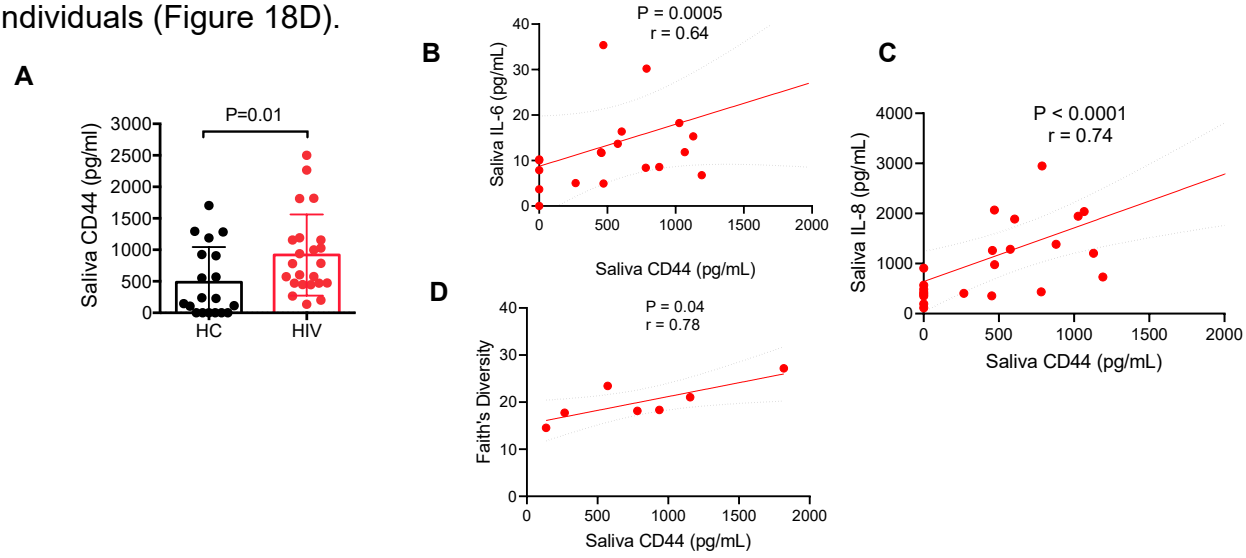


Figure 18. Salivary Soluble CD44 **(A)** Concentrations in HIV-1 infected vs. HCs as measured by ELISA. **(B)** Cumulative results showing a positive correlation (Spearman correlation) between the soluble CD44 with IL-6 in the saliva of HIV-1 infected individuals **(C)** Cumulative results showing a positive correlation (Spearman) between the soluble CD44 with IL-8 in HIV-1 infected. **(D)** Cumulative data showing a positive correlation (Spearman) between the soluble CD44 with bacterial Faith's diversity.

2.3.8. CD32 is highly expressed in oral neutrophils from HIV-1 infected individuals and in CD44⁺ neutrophils

To further determine the activation status of neutrophils, we measured the expression of CD32. We found that CD32 was significantly upregulated in oral neutrophils of HIV-1 infected compared to healthy individuals (Figure 19.A-C). CD32 has been described to participate in the interaction of neutrophils and uptake of IgG-opsonized viral particles (119). Thus, we decided to investigate whether aged oral neutrophils expressing CD44 had different CD32 expressions compared to CD44-negative counterparts. We found that CD44⁺ oral neutrophils had significantly a higher expression of CD32 compared to their CD44⁻ counterparts in both HIV-1 infected and healthy individuals (Figure 20. A). In addition, we noted that although CD44⁺ neutrophils were more activated, both CD44⁺ and CD44⁻ neutrophils in HIV-1 infected individuals had significantly higher expression of CD32 compared to their counterparts in HCs (Figure 20. B-D). These results suggest that neutrophils in the oral cavity of HIV-1 infected individuals have an activated phenotype compared to their counterparts in HCs.

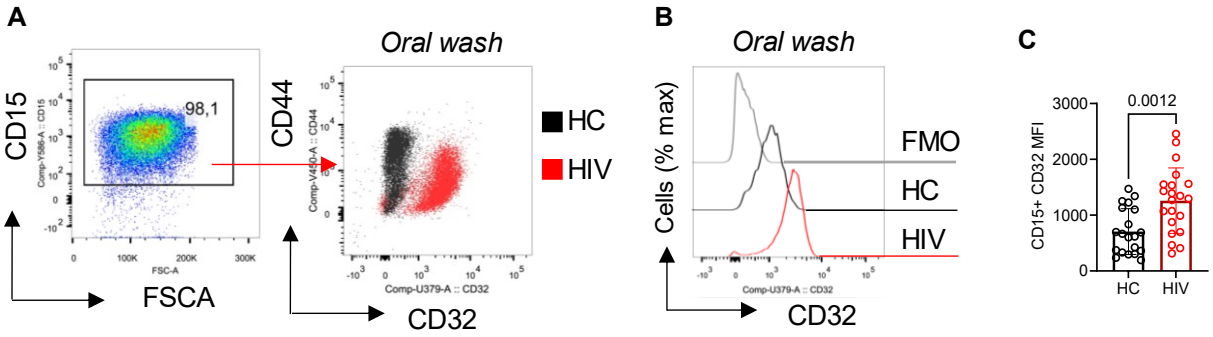


Figure 19. Surface expression of CD32 on oral neutrophils of HIV-1 infected vs. HCs

(A) Representative flow cytometry plots of CD32 expression on neutrophils of an HIV-1 infected vs. a healthy individual. **(B)** Representative histogram plots, and **(C)** cumulative data of CD32 expression (MFI) in oral neutrophils from HIV-1 infected vs. healthy individuals.

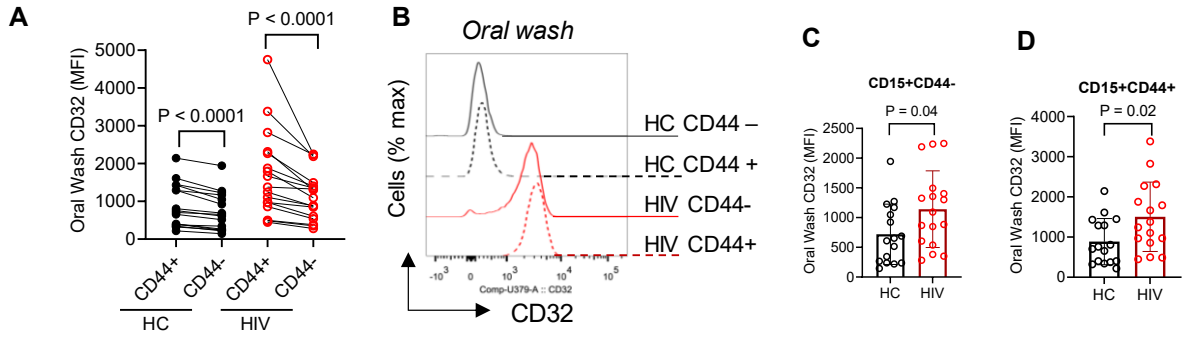
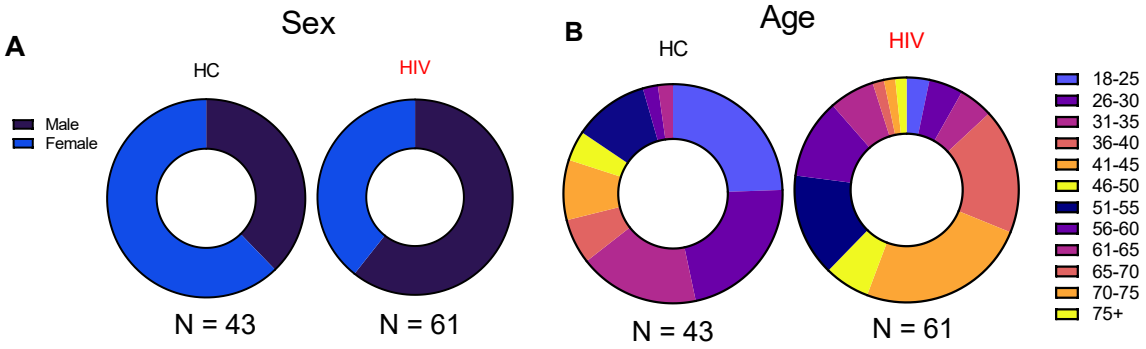


Figure 20. CD32 expression on oral neutrophils related to CD44 in HCs vs. HIV-1 infected individuals **(A)** Cumulative data of flow cytometry stainings of neutrophils from oral washes and their co-expression of CD44 and CD32 in HIV-1 infected individuals shown in red and HCs in black. Note the higher expression of CD32 in neutrophils of HIV -1 infected individuals. **(B)** Representative histogram plots, and cumulative data of CD32 expression (MFI) in **(C)** $CD44^-$, and **(D**) $CD44^+$ populations of oral neutrophils from HCs and HIV-1 infected individuals..

2.3.9. Patients' demographics and oral health associated factors

To better understand whether observed changes were associated with other variables in both groups, we examined their sex, age, medications, oral health (brushing, flossing, using mouthwash), and other habits (smoking, alcohol, and recreational drug use) into our analysis. We found that groups were differently distributed based on their sex (P=0.028) (Figure 21A). While HCs consisted of 16 Males and 27 females, the HIV 1 cohort was composed of 37 males and 24 females. In terms of age, for statistical analysis, age was dissected in groups, 18-30, 31-40, 41-50, 51-75+, and differences were evaluated by Chi-square test (P<0.0001). HCs had more participants between18-50 years compared to HIV-1 infected individuals who were 50 plus years old (Figure 21B). Excluding LTNPs and naïve patients, the rest of HIV-1 infected individuals were receiving ART. Also, some HIV-1 infected individuals (n=33) had other comorbidities (mainly diabetes, high cholesterol, and blood pressure, rheumatoid arthritis, anxiety-

depression, and chronic pain). In contrast, a small portion of HC participants (n=13) had underlying conditions and were receiving related medications at the time of examination. Also, the participants had differences in their habits as was analyzed by Fischer's exact test. The HIV-1 infected group had a significantly higher number of smokers compared to HCs ($P < 0.0001$). The same pattern was true for recreational drug/cannabis in HIV-1 infected individuals compared to HCs (P=0.002) (Figure 21C). However, alcohol consumption was similar between the groups (Figure 21C). Regarding oral health, HIV-1 infected individuals flossed less often compared to HCs (P= 0.0001) (Figure 21C). In terms of brushing, mouthwash use, and the self-reported evaluation for bleeding on brushing (a tool for periodontitis risk prediction), we did not find any difference between the groups (Figure 21C). Finally, we correlated the proportion of neutrophils in oral washes ($CD15^+$ cells) with habit practices in HIV-1 infected versus HCs. We found that HCs tend to have more neutrophils in their oral wash compared to HIV-1 infected individuals regardless of their habits (Figure 21D). Also, we noted that alcohol consumption, daily brushing, mouthwash use, and flossing significantly impacted neutrophil % in HCs vs HIV-1 infected individuals, respectively. Of note, the self-report for bleeding on brushing did not impact neutrophil percentages in the oral wash.



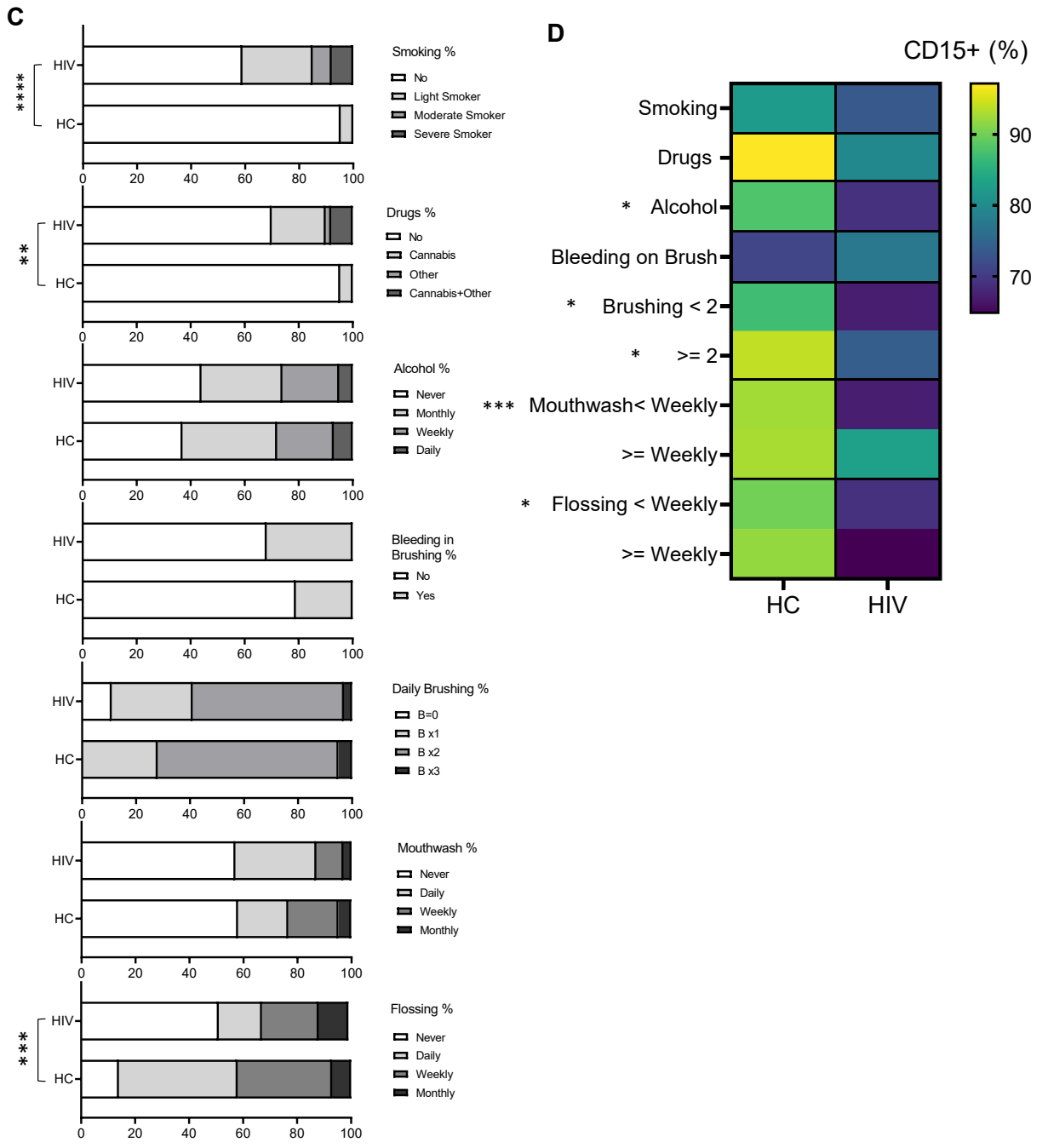


Figure 21. Demographics and habits assessment of participants. **(A)** Sex distribution with 37% and 63% females and males, respectively in HCs, 61% and 39% for males and females in HIV-1 infected group. **(B)** Age distribution among participants. HC group was enriched with younger population according to the Chi-square test (P<0.0001). **(C)** Cumulative data illustrating participant's habits such as smoking, drug use, alcohol consumption, daily oral health. **(D)** Cumulative data showing the correlation of neutrophils' percentages with study participants' habits.

2.4. Discussion

We investigated microbial communities in the saliva of HIV-1 infected and age-sex-matched HCs. We found differential bacterial compositions in the saliva of HIV-1 infected compared to healthy individuals. Our results are in agreement with another report that demonstrated differential bacterial clusters in the oral washes of a larger cohort of HIV-1 infected individuals versus HCs regardless of other contributing factors (e.g. smoking, missing teeth, gingivitis, candida infection, etc.) (74). We observed differences in bacterial α-diversity (e.g. Shannon and Faith's indexes) and bacterial richness, which supports HIV-1 associated salivary dysbiosis. Although previous work investigated dysbiosis of the tongue (120) and periodontal (121) tissues in HIV-1 infected individuals, our results support that saliva expectoration provides a non-invasive, less expensive, and informative approach for oral cavity microbiome-related studies. In addition to microbiome studies, our study bridges the correlation between HIV-1 immune status, oral soluble mediators, and oral neutrophils. In previous work, a major difference in the phyla of *Bacteroidetes*, *Firmicutes*, *Proteobacteria,* and *TM7* in the saliva of HIV-1 infected individuals versus HCs has been documented (122). Our results were somewhat similar in identifying increases in oral *Spirochaetes, Bacteroidetes, Firmicutes,* and *TM7* in HIV-1 infected individuals. Such differential oral microbiome composition may be related to systemic inflammation and impaired pulmonary function in HIV-1 infected individuals as a contributing factor to chronic obstructive pulmonary disease (COPD) (77). Since all of our study subjects were on ART, we were unable to investigate the potential effects of ART on the oral microbiome. However, it was reported that the dysbiotic oral microbiome was not fully restored after effective ART, although some microbiota were restored (123). Furthermore, ART, viral

load, and CD4 T cell count differentially contribute to salivary dysbiosis resulting in reduced or increased different bacterial species (79).

It is difficult to differentiate between various factors without longitudinal studies pre-infection, pre-ART, the type of ART regimens, and well-matched HIV-1 infected untreated controls sampling. In general, oral health, habits such as smoking and drinking may influence the oral microbial communities. Although in our cohort we did not observe any difference between the HIV-1 infected vs. the control group in terms of periodontitis, higher bacterial diversity and richness have been reported in HIV-1 infected individuals with severe periodontitis (73). Therefore, oral health can have direct effects on the microbial communities as increased bacterial diversity and richness have been associated with periodontal disease (124). Prior work also reported gingival bleeding, decayed teeth, periodontal pockets, and smoking habits as important contributing factors to oral dysbiosis (125). In our study, we observed that the HIV-1 infected group was more enriched with smokers, recreational drug users, and individuals who had poor adherence to oral health. These factors may influence the general oral health and subsequently the microbial composition of the oral cavity. For example, smoking has been associated with alteration in the oral microbiome. In particular, it has been reported that smoking enhances bacterial colonization in the upper respiratory tract in HCs, and is even more pronounced in HIV-1 infected individuals (77,126). Although a greater abundance of *Streprococcus* mutants, *lactobacillus,* and *candida* species have been found in the saliva of HIV-1 infected individuals (120), we did not find such differences. Instead, we discovered a significantly higher abundance of *Spirochaetes* (*Spirochaetacea*, *Spirochaeta, and Treponema), Porphyromonadaceae, Prevotella, Elizabethkingia, TM7* in the saliva of HIV-1 infected individuals. These

observations are in agreement with a report showing an increased abundance of *Prevotella* in HIV-1 infected individuals (79). Similarly, an increase in *Porphyromonadaceae* in the saliva of HIV-1 infected individuals was reported at the start of ART when compared to 24 weeks later (75). Interestingly, the *Porphyromonadaceae family* harbors the well-known periodontal pathogens *Phorphyromona gingivalis* and *Tannerella Forsythia* (127,128). It is reported that *Phorphyromona gingivalis* partners with HIV-1 virus to co-infect mucosal epithelial cells, *in vitro* (129). Based on this observation, the abundance of *Phorphyormonas* in the oral cavity may facilitate HIV 1 acquisition at the mucosal sites. However, further studies are required to support this hypothesis. *TM7-RS-045* or *Saccharibacteria* is a recently discovered commensal oral bacteria that abides at the expense of *Actynomices* bacteria, however, its role in the oral cavity is not well defined (130). In our cohort, we observed an increase of *TM7*, although we did not observe any difference in the abundance of *Actinomyces species*. A previous study, in support of our results, found a higher abundance of *TM7, Treponema, and Prevotella* in the oral washes of HIV-1 infected individuals (74). Particularly, *Treponema denticola* associated with *Porphyromonas gingivalis, and Tanerella forsythia* form the “red complex”, which are the main pathogenic bacteria involved in periodontitis (131) (132) (124). Therefore, consistently our data suggest that HIV-1 infected individuals have increased bacterial communities associated with periodontal conditions. Besides, *Prevotella* is reported to be more abundant in the gastrointestinal tract (GI) of men who have sex with men (133). Furthermore, a higher abundance of *Prevotella* is related to a lower abundance of Th17 cells and IFN-I genes expression in the GI of HIV-1 infected individuals (134). Moreover, the presence of *Prevotella* is associated with increased HIV-1 acquisition in the genital tract (135). We found *Elizabethkingia* was another

abundant bacteria in the saliva of HIV-1 infected individuals. *Elizabethkingia* is a multidrug-resistant bacteria associated with life-threatening infections in immunocompromised individuals (136). Thus, the presence of bacterial species like *Porphyromonadacea* and *Elizabethkingia* in the saliva of HIV-1 infected individuals may predispose them to such bacterial infections.

On the other hand, we found that *Helicobacter* was significantly decreased in the saliva of HIV-1 infected individuals. The presence of *Helicobacter pillory* in the dental plaque of HIV-1 infected individuals with *H. pillory*-induced gastritis has been reported (137) (138). However, its correlation with the stomach infection has been challenged (139) despite reports that the oral cavity should be considered as the secondary site for its colonization (140). However, our results are in agreement with another report that indicated the reduced frequency of *H. pillory*-induced gastric infection in HIV-1 infected individuals (141).

Our further analysis in understanding the immune components of the oral cavity in HIV-1 infected individuals demonstrated a significant decrease in the proportion of oral neutrophils which was associated with disease progression (e.g. CD4 T cell count). Notably, LTNPs exhibited the same frequency of neutrophils in their oral cavity compared to HCs. This observation provides another novel insight into the uniqueness of this rare group of HIV-1 infected individuals as we have reported elsewhere (14,19,142). Therefore, considering the crucial role of oral neutrophils in immune homeostasis in the oral cavity, their lower frequency may predispose HIV-1 infected individuals to opportunistic infections. As such, it is possible to speculate that decreased frequency of oral neutrophils in individuals with lower CD4 T cell count may reflect the depletion of Th17 cells at their mucosal sites (143). Subsequently, a lower Th17 cell

population reduces neutrophils' recruitment to the oral cavity. On the other hand, lower Th17 cells at the mucosal sites of the oral cavity may predispose HIV-1 infected individuals to oral candidiasis, considering the protective role of IL-17 against *Candida albicans* (144). Although we were unable to investigate the cross-talk between neutrophils and the oral microbiome, the salivary increase of *Treponema* species may potentially be related to neutrophil dysfunction in HIV-1 infected individuals (145). Deficiency in neutrophils chemotaxis and polarization in HIV-1 infected individuals (146) might explain another reason for reduced neutrophil frequency in HIV-1 infected individuals. On a supporting note, we observed significantly reduced expression and frequency of $CD44^{+}$ neutrophils in the oral cavity of HIV-1 infected individuals. CD44 is expressed on neutrophils and contributes to neutrophil crawling and extravasation (87). Therefore, lower CD44 expression on neutrophils from HIV-1 infected individuals may provide another underlying mechanism for their impairment. Subsequently, we found elevated levels of soluble CD44 in the saliva of HIV-1 infected individuals compared to HCs. The role of soluble CD44 in the saliva of HIV-1 infected individuals is still unknown and required further investigation. However, the salivary CD44 appears to be a surrogate marker in detection of head and neck squamous cell carcinoma (HNSCC) (147). Additionally, we found that CD44 was positively correlated with IL-6 in the saliva of HIV 1 patients. Interestingly, IL-6 apart from its inflammatory activities, is highly secreted by tumorigenic mammary cells (148) and lung tumorigenic epithelial cells (149), having a possible role in the development of cancers (150). Besides, in vitro stimulation with IL-6 promoted neutrophil degranulation (151). Thus, our results point out that increased levels of CD44 and IL-6 in the saliva of HIV 1 patients possibly activate neutrophils in the oral cavity.

We are aware of many study limitations that may have influenced our results. Although we attempted to have age-sex-matched study subjects, due to the COVID-19 pandemic we had limited options in terms of cohort access. We also noted a higher prevalence of cigarette smoking, people with substance use disorder, and individual with poor oral health in the HIV-1 infected group, which might have influenced our results. Moreover, we were unable to analyze the impact of ART on neutrophils and/or microbiome in our cohort. Also, HIV-1 infected individuals had more underlying conditions compared to the HCs. However, for the microbiome studies, we selected subjects without major underlying health conditions apart from HIV-1 infection. Finally, due to a very low cell yield in the oral washes, we were unable to perform functional studies to better characterize/compare neutrophil functions.

In summary, our data provide a novel insight into the impact of HIV-1 infection on oral neutrophils. In particular, we discovered that oral neutrophils exhibit an activated phenotype but with lower frequency in the oral cavity of HIV-1 infected individuals. More importantly, we found elevated levels of soluble salivary CD44 which was positively correlated with Faith's diversity of the microbiome.

Overall, our results support the differential oral microbiome diversity and richness in HIV-1 infected individuals. Although further studies in larger cohorts are required, our results provide a novel insight into the immune-microbiota relationship in the oral cavity.

2.5. Author Contributions

E. P. performed most of the immunological and microbiome-related experiments, analyzed the data, and wrote the first draft. S. H. performed some of the experiments. J. J. assisted with 16S rRNA data analysis. C. O. and S. T. recruited HIV-1 infected individuals for the study. P.P. provided resources and scientific advice. S.E. conceived

the original idea, designed and supervised all of the research, assisted in data analysis, and re-wrote the manuscript.

2.6. Founding

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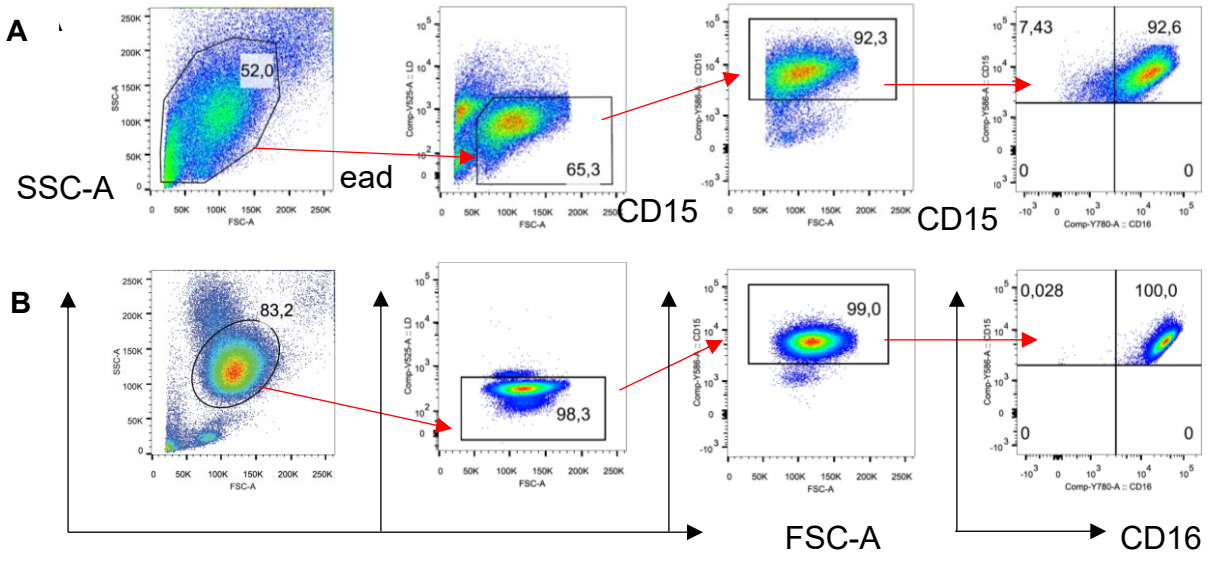
Appendix

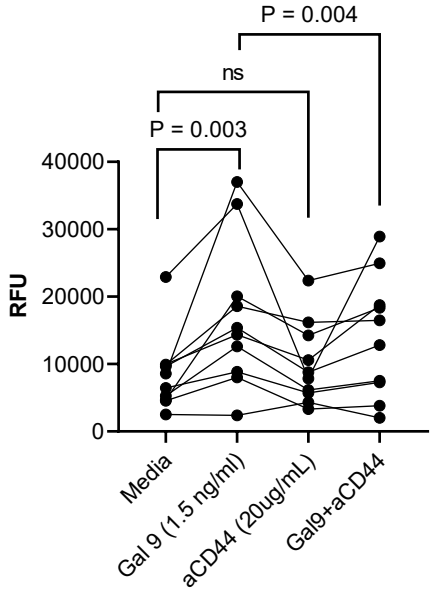
Supplemental Fig 1. Purity plots for oral and blood neutrophils

A. Gating strategy for oral neutrophils showing that all gated CD15+ cells identified as neutrophils are also CD16+ cells. B. Same gating for blood neutrophils.



Supplemental Fig 2. Neutrophil migration in relationship to Gal-9 and CD44

Using the Cell Invasion Assay (Cat. No. ECM 555, Millipore), neutrophils from 6 HCs, were left to migrate towards chemoattractant N-Formyl methionyl-leucyl-phenylalanine (fMLP, 50 uM) in the presence or absence of Galectin 9 (1.5 ng/ml), and recombinant anti-CD44 antibody (20 ug/mL). Gal-9 enhanced neutrophil migration.



Supplemental Fig.3. Inflammatory cytokines in the saliva of HIV-1 infected individuals versus healthy controls (HC).

Cytokines were measured using V-plex Plus pro-inflammatory kit from Meso Scale Discovery (MSD) (K15054D-1).

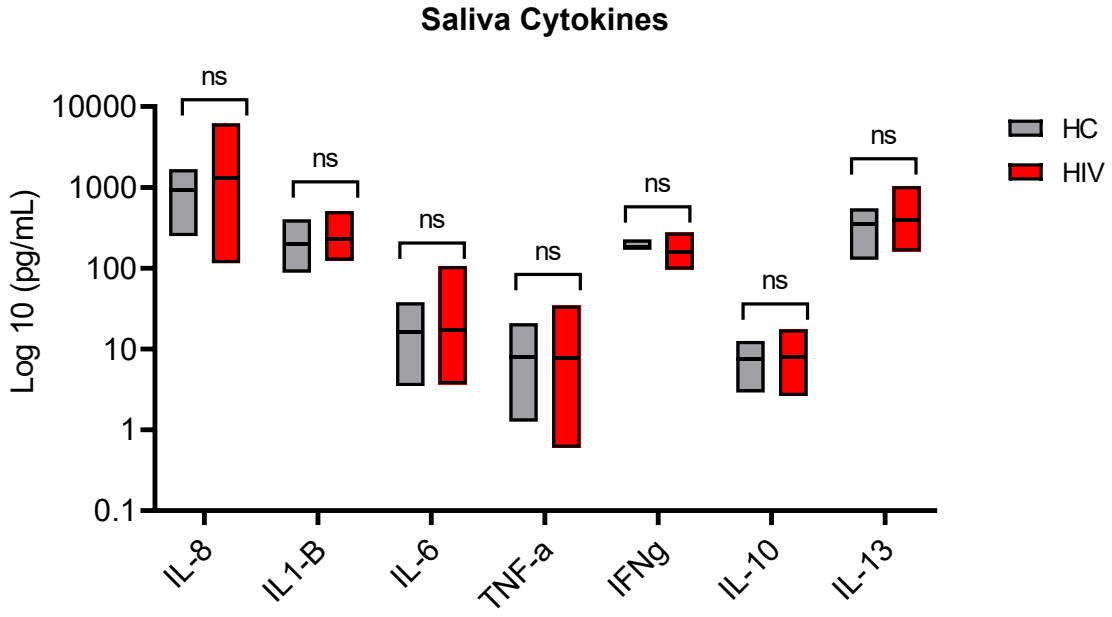


Table 4. HIV-1 infected individuals demographics

TH=ART patients with CD4 High>200 cells/mm3, TL=ART patients with CD4 Low<=200 cells/mm3, L= Long-term non progressor, N= Naïve ART

Red= Samples used for 16SrDNA sequencing

Patient Code	CD4 absolute (cells/mm3)	Viral Load (copies/mL)	Sex	Age
TH01	640	< 40	Female	26-30
TH02	590	< 40	Male	51-55
TH04	880	< 40	Male	51-55
TH05	730	< 40	Male	41-45
TH06	740	< 40	Male	61-65
TH09	560	< 40	Male	56-60
TH10	870	< 40	Male	46-50
TH11	800	< 40	Male	41-45
TH12	650	< 40	Female	41-45
TH13	630	< 40	Male	61-65
TH15	740	< 40	Female	41-45
TH16	550	< 40	Male	46-50
TH17	710	< 40	Female	31-35
TH18	760	< 40	Male	36-40
TH19	560	< 40	Female	36-40
TH20	570	< 40	Female	36-40
TH21	650	< 40	Male	41-45
TH170	720	< 40	Male	36-40
TH173	340	804	Male	31-35
TH175	354	< 40	Female	36-40
TH177	651	< 40	Female	18-25
TH178	590	< 40	Male	41-45
TH180	460	< 40	Male	46-50
TH182	302	54	Female	41-45
TH185	750	< 40	Male	41-45
TH186	590	< 40	Female	46-50
TH187	920	< 40	Male	36-40
TH189	208	314	Male	51-55
TH190	910	< 40	Female	56-60
TH191	780	51	Male	51-55

TH193	570	< 40	Female	51-55
TH194	348	< 40	Male	66-70
TH196	1200	< 40	Male	61-65
TH197	402	< 40	Male	71-75
TH198	1464	< 40	Male	56-60
TH199	539	< 40	Male	18-25
TH201	N/A	< 40	Male	26-30
TH202	539	< 40	Male	36-40
TH204	315	< 40	Male	41-45
TL01	140	< 40	Male	75+
TL03	170	< 40	Male	56-60
TL04	20	< 40	Male	41-45
TL05	140	< 40	Female	41-45
TL07	100	< 40	Female	56-60
TL08	40	< 40	Female	36-40
TL09	160	< 40	Male	56-60
TL171	166	< 40	Female	36-40
TL174	152	< 40	Male	51-55
TL179	161	< 40	Male	51-55
TL184	110	< 40	Male	51-55
L01	720	< 40	Male	61-65
L02	250	< 40	Female	41-45
L04	570	< 40	Female	41-45
L05	1030	< 40	Female	51-55
L06	640	< 40	Female	26-30
L07	920	< 40	Male	31-35
L08	560	< 40	Female	36-40
L09	700	< 40	Female	36-40
L10	970	< 40	Female	41-45
N01	230	N/A	Male	41-45
N02	160	N/A	Male	56-60

Table 5. Healthy Controls Demographics

Red= Samples used for 16SrDNA sequencing

Patient Code	Sex	Age
HC1	Male	26-30
HC2	Male	26-30
HC3	Female	41-45
HC4	Female	41-45
HC5	Male	18-25
HC6	Female	31-35
HC7	Female	56-60
HC8	Female	46-50
HC9	Female	18-25
HC10	Female	26-30
HC11	Female	18-25
HC12	Female	26-30
HC13	Female	26-30
HC14	Female	41-45
HC15	Female	36-40
HC16	Male	31-35
HC17	Female	51-55
HC18	Female	18-25
HC19	Male	18-25
HC20	Male	18-25
HC21	Female	26-30
HC22	Female	26-30
HC23	Female	26-30
HC24	Male	26-30
HC25	Female	51-55
HC26	Female	18-25
HC27	Female	18-25
HC28	Female	31-35
HC29	Male	51-55
HC30	Male	31-35
HC31	Male	31-35
HC32	Male	31-35
HC33	Female	18-25
HC34	Male	18-25
HC35	Female	26-30
HC36	Male	46-50
HC37	Female	36-40
HC38	Male	61-65
HC39	Male	41-45
HC40	Female	41-45
HC41	Male	26-30
HC42	Female	51-55
HC43	Female	51-56

Table 6. Questionnaire Results

Frequencies and percentages are presented. The Chi-square test was applied to calculate the p-value for age. For all other parameters, Fischer's exact test was used.

		HC (n=43)		HIV (n=61)		
		Counts	%	Counts	%	p-value
Age	18-30	21	49	5	8	0.0003
	31-40	9	21	14	23	
	41-50	6	14	19	31	
	51-60	6	14	16	26	
	61-75	1	2	6	10	
	75+	0	0	1	2	
Sex	Male	16	37	37	61	0.028
	Female	27	63	24	39	
Smoking	No	41	95	36	59	<0.0001
	Yes	2	5	25	41	
Alcohol	No	16	37	27	44	0.546
	Yes	27	63	34	56	
Drugs	No	41	95	43	70	0.002
	Yes	2	5	18	30	
Medication	No or only ART	30	70	33	54	0.153
	Yes	13	30	28	46	
Flossing	No	6	14	31	51	0.0001
	Yes	37	86	30	49	
Mouthwash	No	25	58	35	57	0.194
	Yes	10	23	26	43	
Brushing	0 to 1	*14*	33	25	41	0.168
	2 to 3	36	84	36	59	
	(n=24)			(n= 22)		
Bleeding on Brushing	No	19	79	15	68	0.507
	Yes	5	21	7	32	