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THE ROLE OF MICROBIAL TRANSLOCATION AND GUT MICROBIOTA IN HIV-1 INFECTION

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The role of microbial translocation and gut microbiota in HIV-1 infection

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ABSTRACT

HIV-1 infection is characterized by persistent systemic inflammation and immune activation, even in patients receiving effective antiretroviral therapy (ART). Translocation of microbial compounds from a leaky gut to systemic circulation, so called microbial translocation (MT), is a major driver of the immune activation. Additionally, gut microbiota dysbiosis in HIV-1 infected patients further facilitate and fuel MT.

The objectives of this thesis were to study:

- how different ART regimens and usage of antibiotics affect markers of MT (I, II)
- the alternations of gut microbiota during HIV-1 infection and the effect of ART (III,IV)

In a clinical randomized trial, HIV-1 infected subjects started ART based on efavirenz (n=37) or ritonavir-boosted lopinavir (n=34). Levels of MT markers and of enterocyte death were elevated at baseline (BL), and MT markers declined until follow up after 72 weeks, but the reduction of anti-flagellin IgG antibodies was significant only in lopinavir treated patients. Levels of Intestinal Fatty Acid Binding Protein (I-FABP) remained unchanged at 72 weeks, but were temporarily increased after one month in efavirenz treated patients. 29 subjects with concomitant use of antibiotics had superior reduction of soluble CD14 (sCD14) levels. These data show that choice of ART and antibiotics usage could affect the kinetics of some MT markers.

To further explore the impact of antibiotics usage on MT, we performed a longitudinal study on HIV-1 patients initiating ART without (n=13) or with (n=13) co-trimoxazole (TMP-SMX) as prophylaxis against *Pneumocystis jirovecii*. Following ART, levels of LPS-binding protein (LBP) were reduced only in the TMP-SMX group, whilst levels of sCD14 declined in both groups after one year. The LBP decrease remained significant in a multivariate analysis model adjusting for co-variates including BL CD4+ T-cell count. This study confirmed that concomitant use of antibiotics and ART in severely immune deteriorated individuals may beneficially influence the kinetics of MT markers.

In the third study, the composition of gut microbiota was determined by 16S rRNA sequencing in 28 HIV-1 progressors, 3 Elite controllers (EC) and 9 uninfected controls at BL, and additionally after ten months of ART in 16 subjects. Gut microbiome α-diversity was reduced in HIV-1 infected individuals as compared to controls, and further declined after introduction of ART. At BL, α-diversity was positively correlated with CD4+ T-cell counts, but in contrary several markers of MT/immune activation were inversely correlated. Microbiome of EC had the lowest interindividual variation (ß-diversity), clustering together in PCoA analysis. The bacterial composition at genus level was altered in HIV-1 progressors with higher abundance of *Lactobacillus*, and depletion of *Lachnobacterium*, *Faecalibacterium* and *Hemophilus*. Thus, this study showed that the alternations of gut

microbiota during HIV-1 infection are associated with the level of immune dysfunction, and that almost one year of ART does not restore the shifts in the gut microbiome.

In the last work, we studied the gut microbiome of 16 EC in relation to 32 matched ART naive HIV-1 positive individuals and 16 uninfected controls. The number of observed genera and richness indices Chao-1 and ACE were significantly higher in EC as compared to naive patients. The gut microbiota in EC was enriched in genera *Succinivibrio*, *Sutterella*, *Rhizobium*, *Delftia*, *Anaerofilum* and *Oscillospira*, whilst *Blautia* and *Anaerostipes* were reduced. Determination of inferred bacterial functionality by PICRUSt analysis revealed that carbohydrate metabolism related genes were depleted in EC. In contrary, pathways related to fatty acid metabolism, PPAR-signaling and lipid biosynthesis proteins were more abundant in EC vs naive. The kynurenine pathway of tryptophan metabolism was altered only during progressive HIV-1 infection, and kynurenine tryptophan (K/T) ratio was inversely associated with gut microbiota richness. This study shows that EC have richer gut microbiota than untreated HIV-1 patients with progressive infection, with a unique bacterial composition and a distinct metabolic profile which may be involved in the control of HIV-1.

In summary, data from the studies in my thesis reveal that MT in HIV-1 infection is reduced by ART but also that the choice of ART influences this decline. Additionally, the antibiotics usage may affect the levels of MT. The complexity, composition and functionality of gut microbiota are disturbed in HIV-1 infected individuals with progressive disease, whilst EC have a unique gut microbiota profile that eventually contributes to their control of HIV-1.

LIST OF SCIENTIFIC PAPERS

I. **Vesterbacka Jan**, Nowak Piotr, Barqasho Babilonia, Abdurahman Samir, Nyström Jessica, Nilsson Staffan, Funaoka Hiroyuki, Kanda Tatsuo, Andersson Lars-Magnus, Gisslèn Magnus, Sönnerborg Anders. Kinetics of microbial translocation markers in patients on efavirenz or lopinavir/r based antiretroviral therapy. PLoS One. 2013;8(1):e55038. doi: 10.1371/journal.pone.0055038. Epub 2013 Jan 28.

II. **Vesterbacka Jan**, Barqasho Babilonia, Häggblom Amanda, Nowak Piotr. Effects of Co-Trimoxazole on Microbial Translocation in HIV-1-Infected Patients Initiating Antiretroviral Therapy. AIDS Res Hum Retroviruses. 2015 Aug;31(8):830-6. doi: 10.1089/AID.2014.0366. Epub 2015 Jun 15.

III. Nowak Piotr, Troseid Marius, Avershina Ekatarina, Barqasho Babilonia, Neogi Ujjwal, Holm Kristian, Hov Johannes R, Noyan Kajsa, **Vesterbacka Jan**, Svärd Jenny, Rudi Knut, Sönnerborg Anders. Gut microbiota diversity predicts immune status in HIV-1 infection. AIDS. 2015 Nov 28;29(18):2409-18. doi: 10.1097/QAD.0000000000000869.

IV. **Vesterbacka Jan**, Rivera Javier, Noyan Kajsa, Parera Mariona, Neogi Ujjwal, Calle Malu, Paredes Roger, Sönnerborg Anders, Noguera-Julian Marc, Nowak Piotr. Richer gut microbiota with distinct metabolic profile in HIV infected Elite Controllers. Sci Rep. 2017 Jul 24;7(1):6269. doi: 10.1038/s41598-017-06675-1.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
ART	Antiretroviral therapy
EC	Elite controllers
EFV	Efavirenz
ELISA	Enzyme-linked immunosorbent assay
GALT	Gut Associated Lymphoid Tissue
GI	Gastrointestinal
HIV-1	Human immunodeficiency virus type 1
hS-CRP	High sensitive C-reactive protein
I-FABP	Intestinal Fatty Acid Binding Protein
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
LBP	Lipopolysaccharide Binding Protein
LPV	Boosted lopinavir
MD-2	Lymphocyte antigen 96
MT	Microbial translocation
NGS	Next generation sequencing
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
sCD14	Soluble Cluster of Differentiation 14
sCD163	Soluble Cluster of Differentiation 163
TLR	Toll-like receptor
TMP-SMX	Co-trimoxazole
TNF	Tumor necrosis factor
UNAIDS	Joint United Nations Program on HIV/AIDS

1 INTRODUCTION

Since the appearance of the human immunodeficiency virus (HIV) epidemic, tremendous progress has been achieved in the treatment and care of HIV infected patients. The breakthrough with highly active antiretroviral therapy (HAART) at 1995-96 dramatically decreased AIDS related mortality and reduced prevalence of HIV related conditions. A systemic immune activation was early identified as a hallmark of HIV-1 infection in untreated patients and this feature persists even in patients treated with combined antiretroviral treatment (ART), regardless of undetectable plasma viral load. The chronic immune activation and inflammation contribute to a higher risk of both AIDS related clinical events and to non-AIDS related conditions[1,2]. Following initiation of ART, the levels of both cellular and serum biomarkers of immune activation are thus reduced, but not to the levels present in healthy controls[3,4].
There are several potential contributors fueling the low-grade inflammation, in addition to HIV itself. Co-infections with other viruses inducing an inflammatory response like cytomegalovirus, Epstein-Barr virus and hepatitis B/C may further maintain the inflammatory state[5-7]. Other microbial triggers, like protozoan parasites may contribute to additional inflammation. Thus, in endemic areas, co-infections with malaria and visceral leishmaniasis in HIV patients represent independent causes of intensive immune activation[8,9]. Moreover, translocation of bacteria or bacterial products across a damaged gut-blood barrier, so called microbial translocation (MT), has been proposed to be one of the most important mechanisms behind the chronic immune activation in HIV infection[10].

2 BACKGROUND

2.1 HIV CHARACTERISTICS

HIV is a lentivirus within the *Retroviridae* family, which infects host cells expressing the CD4 receptor, e.g. lymphocytes, monocytes, dendritic and microglia cells. In addition, an interaction with the co-receptors CCR5 or CXCR4 is crucial for viral cell entry[11]. By using the viral enzymes reverse transcriptase (RT) for generation of double-stranded DNA from the viral RNA templates, followed by integration of this proviral DNA into the human chromosomes by HIV integrase, a viral reservoir is established in the human genome. This persistent reservoir is formed very early during the acute infection in the resting memory CD4+ cells[12]. Also other cellular reservoirs exist, such as the pool of macrophages and follicular dendritic cells[13,14]. The viral replication rate is intensive with $10^{10\text{-}11}$ virions produced every day[15], and together with the inborn high error rate of the RT during the DNA synthesis, an enormous viral diversity develops.

Two different types, HIV-1 and HIV-2, infect humans and cause HIV related disease followed by AIDS, if untreated, when the function of the immune system has become severely deteriorated. HIV-1 is phylogenetically classified into several groups: (I) the worldwide spread M (major) group, representing >90% of all HIV infections, which can be further subdivided into 9 subtypes (A-D, F-H, J, K) and circulating recombinant forms (CRFs); (II) N; (III) O and (IV) P. Also unique recombinant forms exist[16]. HIV-2 is categorized into groups A-H[17,18].

The clinical course varies between the two HIV types. Typically, AIDS defining conditions and complications related to HIV-1 infection present 8-10 years after transmission, but a smaller proportion of the patients have an accelerated disease progression already after seroconversion over a period of 2-3 years[19]. The disease progression is slower in HIV-2 infected, and development of low CD4+ T-cell counts complicated by opportunistic infections may be deferred for 20 (-30) years[20,21]. The plasma HIV-2 load is usually very low or undetectable during the asymptomatic course in these patients[22], similar to the HIV-1 infected Elite controllers who are able to spontaneously maintain viral plasma suppression without ART for decades[23].

2.2 EPIDEMIOLOGY

In the 2016 UNAIDS Global AIDS update, an estimated 36.7 million individuals are living with HIV, of whom ~17 million are on ART at the end of 2015[24]. HIV-1 is responsible for most of the global HIV burden. The overall prevalence of HIV-2 infections is low world-wide, and a total of 1-2 million people are expected to be HIV-2 positive. Anyhow, the estimated HIV-2 seroprevalence is 1-5 % in some countries in West Africa, with significant proportions of patients being dually infected with HIV-1[25,26].

In Sweden, the number of diagnosed people living with HIV was 20th of June 2017:7338,

according to the Swedish InfCare HIV quality assurance registry, with 430 newly diagnosed cases in 2016. This corresponds to a prevalence of ~0.07%, which is one of the lowest in Europe[27]. During the last five years, 15-25% of the newly diagnosed patients contracted HIV in Sweden according to reports from treating physicians. However recent data suggest that at least 20% of migrants are infected after arrival to our country[28].

2.3 HIV-1, IMMUNE ACTIVATION AND INFLAMMATION

HIV-1 infection is associated with activation of both the innate and adaptive parts of the immune system. An extensive systemic immune activation starts immediately at acute infection[29], but gradually decreases during the chronic phase, though still present at abnormally high levels[30,31]. Even in well treated patients with undetectable plasma viral load, a chronic immune activation and low-grade inflammation are found[32].

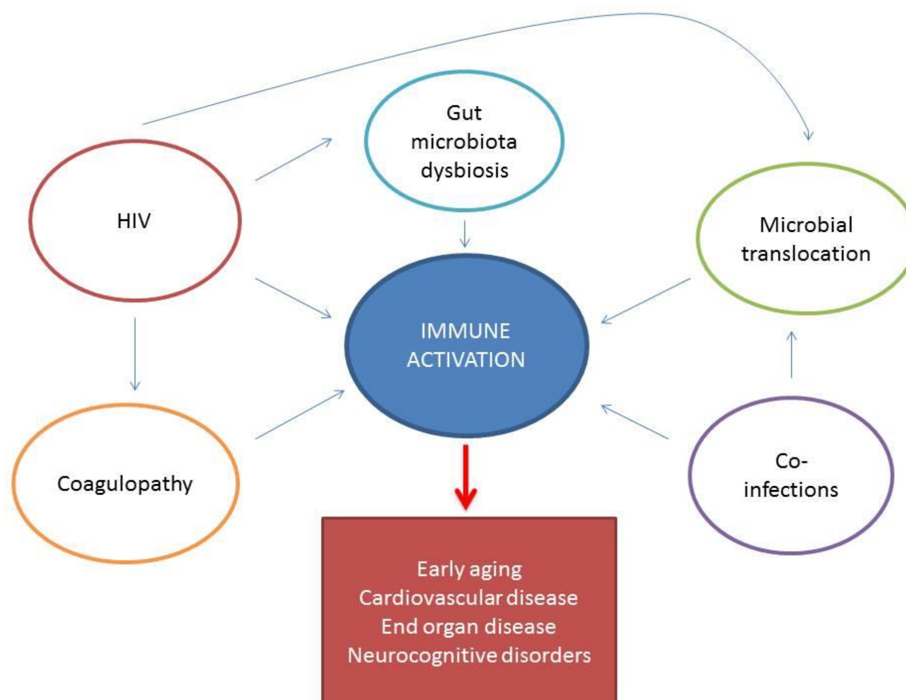

Figure 1. Factors associated with immune activation during HIV-1 infection.

Several components of the innate immune system are activated in HIV-1 infection. The myeloid dentritic cells (mDCs), presenting processed antigens to T-cells in lymph nodes with subsequent T-cell activation, as well as interferon-γ producing plasmacytoid dendritic cells (pDCs) display transcriptional profiles associated with immune activation *in vivo*[33]. Enteric mDCs become activated by mucosal bacteria like Prevotella, which may lead to both local and systemic immune activation[34]. Activation of monocytes/macrophages is represented by

increased systemic levels of sCD14, sCD163[35-37] and inflammatory cytokines (TNF-α, interleukin (IL)-1 and IL-6)[38,39]. Also the neutrophils are activated with high surface expression of program death ligand 1 (PD-L1), and with upregulation of TNF-α and IL-6 production[40,41]. Furthermore, overexpression of activation markers on natural killer (NK) cells with reduced cytolytic capacity has been reported in HIV-1 infection[42].

The cellular immune activation during HIV-1 infection has been extensively explored, and T-cell activation markers may predict disease progression in untreated patients[43]. Persistent activation of CD4+ and particularly CD8+ T-lymphocytes is evident in the vast majority of naive patients[4,30]. Common cellular markers of immune activation include surface expression of HLA-DR and CD38. The CD4/8 T-cell ratio is also a useful tool for indirect prediction of immune activation[44].

The magnitude of CD8+ T-cell immune activation early during HIV-1 infection may predict the CD4+ cell decline independent of the viral load[45]. It has been assumed, that most CD4+ T-cells will undergo apoptosis secondary to exhaustion and senescence caused by the activation[46]. ART partially reverses this process, but does not fully restore the CD4+ T-cell dysregulation and function[47]. Recently, another mechanism behind loss of CD4+ T-cells has been elucidated. Depletion of these cells by pyroptosis, a process where the pool of resting CD4+ cells (~95% of the CD4+ T-lymphocytes) die bycaspase-1 programmed cell death seems to be the major cause behind the loss of CD+ T-cells in HIV-1 infection[48]. This way of cell death is associated with release of pro-inflammatory cytokines like IL-1ß, resulting in an intensive inflammatory reaction. The pyroptosis takes place mainly in lymphoid tissue, and CD4+ T-cells in blood seems to be highly resistant to this type of cell death[49]. Development of liver fibrosis has been associated with this pathway of cell death[50], and the fibrosis in lymphatic tissues observed in HIV-1 infection may be related to this type of inflammatory cell death.

Defects in the B-lymphocyte line may further fuel the inflammation in HIV-1 infection. Memory B-cells have a lower quality of response to HIV in infected subjects[51], and also decreased IgA and IgG responses have been demonstrated[52]. HIV-specific antibody responses are improved by ART, but the overall frequencies of HIV-specific B cells remain abnormally low[51].

Increased activity in the kynurenine pathway of tryptophan catabolism, mediated by the enzyme indoleamine 2,3-dioxygenase 1 (IDO1), has been considered to be another important factor behind the chronic inflammation in HIV infection. IDO1 overactivity in HIV-infection may influence the ratio between gut resident regulatory T-cells and Th17+ cells[53]. This leads to a progressive injury of the epithelial mucosal barrier, followed by increased MT and immune activation[54].

The chronic immune activation causes a low grade inflammation that persists after initiation of ART, and several soluble systemic markers of inflammation are elevated. Typically, increase of CRP, IL-1, IL-6 and TNF-α is observed. Additionally, the coagulation system is

disturbed with elevated levels of D-dimer, tissue factor and von Willebrand factor. The alternations in the inflammation and coagulation cascades contribute to the HIV related complications from the cardiovascular system, musculoskeletal system, end-organ disease in e.g. liver and kidney, malignancies, neurocognitive impairment and accelerated aging[2].

2.4 MICROBIAL TRANSLOCATION

The human gut compartment represents the largest lymphoid organ in the body, and the intestinal mucosa is constantly exposed to external microorganisms. It plays an important role for maintaining the immunological homeostasis, mediated through innate and acquired immunological responses[55]. Gut associated lymphoid tissue (GALT) consists of mesenteric lymph nodes, Peyer´s patches in the small intestine, and follicular aggregates in the large intestine[56]. The mucus layer in the gastrointestinal (GI) tract, consisting of proteins, phospholipids, electrolytes, water, secretory IgA and antimicrobial peptides, forms a physiological barrier against pathogenic microorganisms. Intraluminal granulocytes maintain control from commensal bacterial overgrowth, and submucosal macrophages and lymphocytes eliminate bacteria or bacterial products crossing the structural parts of the intestinal wall. This is based upon lined enterocytes, closely connected with tight junctions (intercellular structures that join GI-epithelial cells firmly together). All these parts form a complex construction, termed the gut-blood barrier. It strictly regulates both passive and active transfer of fluid, nutrients and electrolytes. In HIV-1 infection, most of the different anatomical and physiological parts of the gut-blood barrier are abnormal, as reviewed by Sandler and Douek[57].

The term microbial translocation (MT) refers to translocation of gut resident intraluminal commensal microbial products into systemic circulation without manifest bacteremia. Translocating microbial compounds from bacteria include peptidoglycans from the cell wall[58], lipopolysaccharide (LPS) from the outer cell membrane of gram-negative bacteria[10], flagellin[59,60] and bacterial DNA[61]. Also viral RNA/DNA[62] and ß-glucans from bacterial or fungal organisms may cross the blood-gut barrier[63]. MT has been linked to a spectrum of diseases besides HIV. It has been described e.g. in patients with inflammatory bowel disease (IBD)[64], coeliac disease[65], infectious and alcoholic cirrhosis[66-68], hepatitis B/C virus infection[66,69], and during Dengue infection[70]. Diseases that are characterized by systemic MT are often associated with shifts in the gut microbiota composition.

2.5 MARKERS OF MICROBIAL TRANSLOCATION

There are numerous established markers of MT. Anyhow, to find appropriate tools and markers to estimate the magnitude of MT has been associated with several difficulties. The traditionally most widely used marker is LPS, a major component of the monolayer of outer cell membrane in most gram-negative bacteria. Thus, it is a direct marker of bacterial products translocating to the systemic circulation. The structure of LPS varies between different bacteria, and the innate immune response may differ 100-fold depending on type of bacterial trigger. LPS forms a complex with LPS-binding protein (LBP), membrane

associated or soluble CD14, MD-2 and toll-like receptor 4 (TLR4) on the surface of innate immune cells, preferentially monocytes and macrophages. This starts a cascade of intracellular processes, mediated via several pathways, whereof activation of the transcription factors nuclear factor kappaB (NFκB) and activator protein-1 (AP-1) are the most important[71]. The subsequent gene expression results in production of inflammatory cytokines like interleukins, TNF-α and interferons[72,73] (Figure 2).

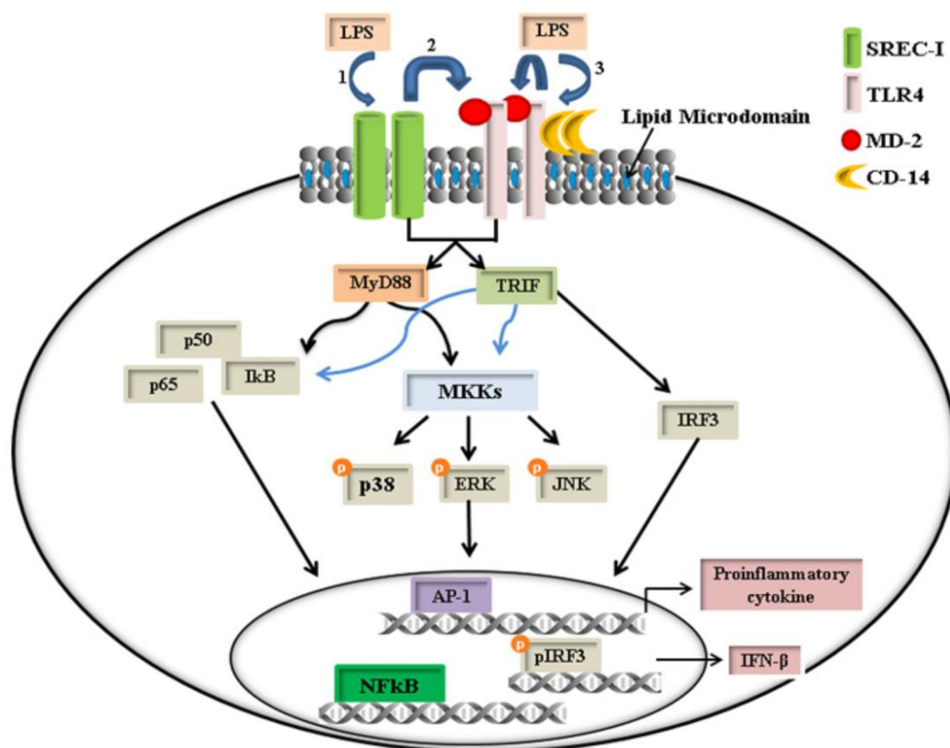

Figure 2. LPS/TLR4 complex related intracellular signaling pathways.

Adapted from: Scavenger receptor SREC-I mediated entry of TLR4 into lipid microdomains and triggered inflammatory cytokine release in RAW 264.7 cells upon LPS activation. Murshid A, Gong J, Prince T, Borges TJ, Calderwood SK. PLoS One. 2015 Apr 2;10(4):e0122529. doi: 10.1371/journal.pone.0122529. eCollection 2015.

Although LPS has been considered to be the most established marker of MT, the analysis process of this marker is associated with technical issues. There is variability between different limulus lysate assay test kits (standard for LPS detection), but also inter-run variability lowers the validity of results. Additionally, the non-fasting condition at sampling can affect LPS levels, and endogenous circulating proteins may inhibit or degrade systemic LPS.

Another direct marker of MT is systemic bacterial DNA. Traditionally, plasma PCR of the conserved 16S ribosomal DNA (16SrDNA) region with Sanger sequencing has been used. Anyhow, the sensitivity and specificity of the method are uncertain with some studies

showing elevated levels of 16SrDNA in HIV positive compared to negative individuals[61,74], in opposite to others[75,76]. Utilizing next generation sequencing (NGS) for characterization of systemic bacterial DNA may enhance the diagnostic accuracy, as discussed by Svärd et al[76].

Several indirect markers of MT are used. LPS-binding protein (LBP) is an acute phase protein, mainly produced in liver and to a smaller extent in pulmonary, gastrointestinal and kidney epithelial cells[77-79]. It is mostly released upon LPS stimuli, but induction may also be triggered by other microbiological structures. Peptidoglycans from gram-positive bacteria and β-glucans from fungal organisms are molecules also recognized as LBP inducers[80].

Membrane bound CD14 is a pattern recognition receptor found mainly on macrophages, recognizing LPS from gram-negative bacteria, peptidoglycans and lipoteichoic acid from gram-positive bacteria, and lipoproteins from spirochetes[81]. Shedding of the membrane bound CD14 gives the soluble form sCD14, which acts as a marker of monocyte activation upon mainly LPS stimuli, thus appropriate as an indirect marker of MT. Anyhow, HIV itself and a broad spectrum of other microbial agents like Dengue, RSV, and Mycobacteria are known triggers of sCD14 production, which has to be considered when interpreting sCD14 results[70,82,83].

Intestinal fatty acid binding protein (I-FABP) is released by enterocytes undergoing cell death, and elevated levels reflect damage to the small intestinal cells. As the enterocytes have a high turnover rate, high plasma I-FABP levels indicate an elevated loss of enterocytes followed by abnormally high intestinal permeability[84]. Elevated levels of I-FABP have been linked to inflammatory bowel diseases, septicemia and also following abdominal surgery[85,86].

Detection of antibody responses against the bacterial antigen flagellin has been studied in patients with IBD (anti-CBir1)[87] and in patients with HIV/AIDS (anti-CBir1, anti-flagellin antibodies)[88,89]. Both antibodies are of IgG type, and may provide insights of the degree of MT over a prolonged period of time due to their long half-lifetime.

2.6 MICROBIAL TRANSLOCATION IN HIV-1 INFECTION

A rapid depletion of mucosal CD4+ T-cells, preferentially affecting the subpopulation of Th17+ T-cells in GALT, starts very early after acquisition of HIV[90]. The IL-17 and IL-22 producing Th17+ cells have essential immunological properties, and are important for the homeostasis of epithelial cells. They produce antimicrobial peptides like defensins, support recruitment of neutrophils on response to intraluminal pathogenic bacteria and fungi, and enhance proliferation of enterocytes[91-94].

Furthermore, the structural integrity of the barrier in GI-tract is impaired due to the HIV infection, leading to a disruption of the epithelial barrier. This is caused by an increased turnover rate of enterocytes and destruction of the important tight junctions starting within the first weeks post-infection[95]. Following oral challenge with lactulose/L-rhamnose, an

increased urine excretion was observed in 20% of asymptomatic HIV patients, and in vast majority of AIDS patients, reflecting the enhanced intestinal permeability[96]. In the early HIV-era, villous atrophy was observed in patients with wasting syndrome[97], with subsequent malabsorption of carbohydrates, proteins, fat and nutrients such as zink and iron.

Abnormally low intraluminal IgA-concentrations due to B-cell dysfunction with decreased proportion of mucosal plasma cells has been associated to HIV infection[98]. Secretory IgA prohibits intraluminal microbes from attachment to and translocation across the epithelial barrier. The local IgA-deficiency may further contribute to less capacity of neutralizing microbial products (Figure 3).

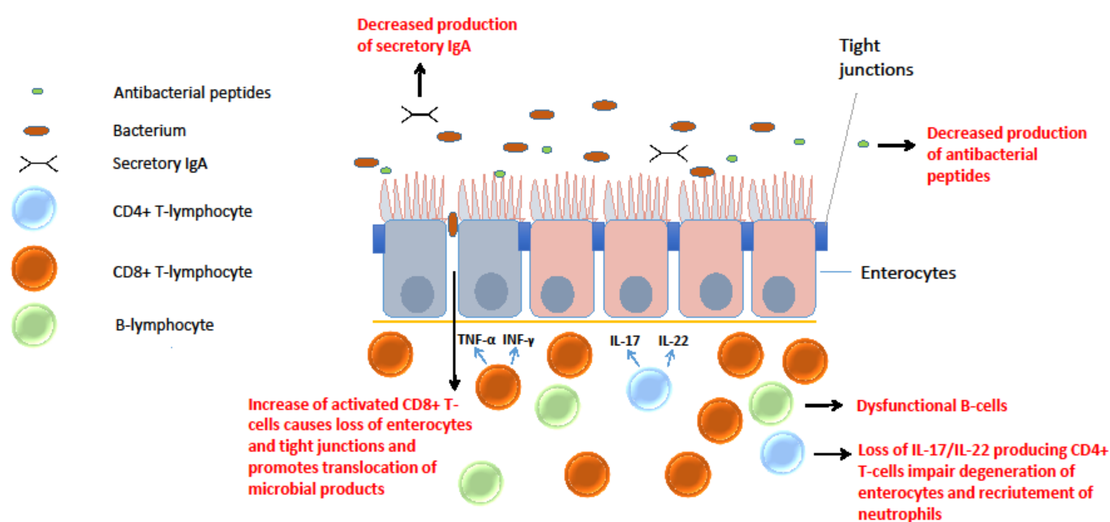

Figure 3. The pathogenesis of HIV-1 induced gut damage. During HIV-1 infection, CD4+ T-cells are lost, and CD8+ T-cells are activated in the submucosal parts of the gut. This is associated with an increased turnover of enterocytes, loss of tight junctions (connecting the epithelial cells strictly together), impaired recruitment of neutrophils and less production of antimicrobial peptides. Additionally, the B-lymphocytes are dysfunctional, and intraluminal IgA concentration is lowered. As a consequence, microbial parts may translocate from gut lumen into systemic circulation, triggering the immune system and contributing to chronic immune activation.

The clearance of MT products from systemic circulation takes place mainly in the liver, delivered via the portal vein. Hepatocytes and specialized liver resident macrophages, (Kupffer cells), which are activated by an innate immune response via TLR4-CD14 complex upon LPS-stimuli, clear most of enteric derived LPS[99]. Kupffer cells are depleted in HIV-hepatitis C co-infection[100], and serum markers of MT have been elevated compared to hepatitis C mono- infected[101]. These data suggest that MT may play an important role in the more rapidly developed fibrosis progress and cirrhosis observed in co-infected patients. Phagocytosis by mucosal macrophages is another crucial mechanism to clear translocating microbial products. In a study of simian immunodeficiency virus (SIV) infected rhesus macaques, Estes et al[95] showed that intestinal macrophages have impaired phagocytic capability, and instead a dysfunctional response on translocating bacterial products is observed, with secretion of inflammatory cytokines. Similar findings have been reported in HIV infected patients, where density of mucosal macrophages was higher, but their

phagocytic skills were impaired[102]. Altogether, these defective compounds involved in human defense against microbial products invading from the intestinal lumen contribute to the leaky-gut syndrome featuring HIV infection.

2.7 MICROBIAL TRANSLOCATION AND ANTIRETROVIRAL THERAPY

ART reduces markers of systemic T-cell activation, but there are conflicting data about the effect on MT. The choice of surrogate MT marker will most likely impact the interpretation of the magnitude of MT. Also co-morbidities, nadir CD4+ cell count, timing and duration of ART may influence level of MT after initiation of ART. Moreover, concomitant use of antibiotics probably may influence some of MT markers, e.g. LPS, LBP and sCD14. Most studies report declining LPS levels after ART initiation[10,103,104], while no reduction was observed in other studies[35,105-107]. This may partly be explained by the different techniques and kits used for determination of the LPS levels, with associated difficulties of measuring LPS. Anyhow, when the more stable marker sCD14 is used, results are still diverging, with some presenting declining[35,105,108] or increasing levels[104,106] after introduction of ART. The trend is the same for most of the other surrogate markers, with the exception of LBP, that seems to decline in most of the studied HIV cohorts after starting ART[109,110]. Two studies on early initiation of ART during acute HIV infection did not demonstrate any positive effect on levels of MT markers[111,112], although this might be due to that ART was started before any significant MT had developed. Such an interpretation is supported by a recent article, where the introduction of ART during acute HIV infection was associated with less immune activation and inflammation in colonic lamina propria at 24 weeks. Though, a significant reconstitution of CD4+ T-cells was observed in only blood and not in the sigmoid biopsies even after 96 weeks[113]. These data illustrate the difficulties of interpreting how organ-specific and systemic immune destruction and recovery are connected with inflammation and immune activation related to MT.

The impact of the individual components in a HIV drug regimen is less explored. Some minor differences in the levels of monocyte activation markers have been observed, with higher sCD14 in patients on NNRTI + boosted protease inhibitors (PI), but without affecting the overall magnitude of MT[114] . Interestingly, monotherapy with PI has also been linked to higher levels of monocyte activation markers (sCD14, sCD163), but still levels of LBP did not diverge from patients with standard ART, thus not indicating increased MT, so other mechanisms than MT may trigger the innate immune activation during PI monotherapy [115]. In another cross-sectional study from Mexico, it was reported that the levels of sCD14 and I-FABP were elevated in patients on long-term ART based on boosted atazanavir or lopinavir, but not in efavirenz treated subjects[116]. All together, these findings raise concerns about the insufficient effects of monotherapy with protease inhibitors. In contrast, viral reservoirs were found to be smaller in patients on NNRTI based ART[117], and the size of the gut reservoir has been linked to MT[118]. To summarize, the effect of specific ART regimens and the individual drugs on MT during chronic HIV infection is uncertain, and has to be further investigated in larger scaled, preferable randomized studies.

2.8 THE HUMAN GUT MICROBIOTA

The total bacterial number in the adult human gut has traditionally been estimated to be ~10^{14}, composed by 500-1000 different species, together creating an estimated biomass of 1.5 kg[119,120]. The vast majority of the commensal bacteria in the gut resides in the colon, and only small fractions are located in the stomach and the small intestine. Though, in a recent paper the number of enteric bacteria was calculated to be ~$4x10^{13}$, suggesting that the commonly accepted bacteria to human cell ratio 10:1 is much closer 1:1[121]. *Bacteroides*, *Clostridium*, *Lactobacillus*, *Fusobacterium*, *Bifidobacterium*, *Eubacterium*, *Peptococcus*, *Peptostreptococcus*, *Escherichia* and *Veillonella* are the most abundant genera forming the commensal gut flora[122]. The human gut microbiome has many crucial biological functions. This bacterial compound facilitates absorption and digestion of nutrients, and also biosynthesizes vitamins like biotin, thiamine and folate. Short-chain fatty acids (SCFA), such as butyrate, propionate and acetate, are of major importance for maintaining the gut homeostasis as they serve as both energy sources for endothelial cells and as signaling molecules[123]. They are produced by commensal Clostridia and Lactobacillales species, as products of fermentation of dietary fibers and end products from carbohydrates[124]. Additionally, the microbiome also confers resistance against invasion of pathogenic bacteria, and interferes with the host's production of antimicrobial peptides. Importantly, it is also deeply involved in development and modulation of the immune system[125]. Early in life, gut colonization with lactobacilli during first two months after birth has been associated with a favorable cytokine pattern at the age of two years, suggesting that specific bacterial species may affect subsets of T helper cells[126].

2.9 GUT MICROBIOTA IN HIV-1 INFECTION

The influence of HIV infection on gut microbial flora was first elucidated in 2008 when the composition of gut microbiome was assessed by fluorescence *in situ* hybridization and quantitative real-time PCR for *Pseudomonas* on fecal samples from 57 asymptomatic and ART naïve HIV patients. The presence of *Pseudomonas aeruginosa* was 92% in HIV infected vs 20% in healthy subjects, corresponding to a 10-fold increase of the *Pseudomonas* proportion of total microbiota. Further, *Candida albicans* was detected in all samples from the HIV infected, compared to 40% from the healthy population[127], advocating a pathological shift in the composition of the microbial gut flora during HIV infection. Determination of gut microbiome with quantitative molecular techniques was first presented in a pilot study by Ellis et al, demonstrating that total bacterial load analyzed by 16SrDNA PCR was lower and the proportion of order *Enterobacteriales* was higher in stools of treatment naïve HIV infected compared to uninfected individuals. Additionally, the CD4+ cell count in duodenal tissue correlated negatively with the fraction of *Enterobacteriales*, and the total bacterial load was also negatively correlated to activation of CD4/8+ cells in duodenal tissue[128], highlighting the importance of the gut microbiome composition as a functional trigger of chronic systemic immune activation. Since then, several studies using NGS have shown a

higher abundance of Proteobacteria and depletion of Firmicutes at phylum level in HIV+ populations[129-131]. More frequently, enrichment of genus *Prevotella* and depletion of *Bacteroides* has been reported in fecal samples[132,133] or gut mucosal tissue[130,134] of untreated chronically HIV infected subjects. Multiple bacterial species involved in production of short-chain fatty acids, e.g. butyrate, are less represented in the fecal flora of HIV+ subjects. Proportions of *Faecalibacterium* spp., *Eubacterium* spp. and *Coprucoccus* spp. have been reduced in several cohorts[130,134,135]. This may influence the turnover of epithelial cells, and affect the tuning of immunological mucosal cells. In the complex interplay between intraluminal microorganisms and GALT, beneficial commensals like Lactobacillales and Bifidobacteria have been linked to mucosal anti-inflammatory properties[136]. The relative amount of both taxa is lowered in fecal flora from an HIV infected population[127,134], and dietary supplementation with prebiotics has been shown to partially restore the shortage[137]. In a randomized trial comparing ART plus maraviroc versus placebo, the systemic and gut CD4+ cell counts were positively correlated to the rectal proportions of Lactobacillales. Additionally, higher proportions of Lactobacillales were associated with less MT in these treatment naïve patients, further indicating the protective qualities of these bacteria. In this longitudinal study, subjects were followed after initiation of ART, and the percentage of gut CD4+ cells correlated to the proportions of Lactobacillales after 48 weeks[138], indicating that recovery of the immune system may be due to the interaction between ART and the fraction of beneficial commensals. According to a recent exploratory work by Dinh et al, where the fecal microbiota from 21 HIV patients on suppressive ART for in median 13 years was profiled, the composition differed significantly from healthy controls. Enrichment of Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Erysipelotrichi, Erysipelotrichales, Erysipelotrichaceae and *Barnesiella* was found in HIV+ individuals[139], and the abundance of pathogenic organisms correlated to markers of MT and systemic inflammation. Thus, dysbiosis of gut microbiota with related MT and immune activation seems to persist for more than a decade even with fully suppressive ART, but supplementation with pre/probiotics may be possible therapeutic strategies. The choice of ART regimen may also be of importance, as only a combination of 2 NRTIs + integrase inhibitor was able to restore the diversity loss and systemic inflammation in a cross-sectional study on HIV-1 patients. But still, in this work no major shifts in the gut microbiome were observed at phylum level before or after initiation of ART[114].

2.10 ELITE CONTROLLERS

Elite controllers (EC) constitute a unique subset of HIV-1 infected individuals with the ability to spontaneously control the HIV-1 replication over time without ART, representing less than 1% of the total HIV-1 population[23]. Until today, there has been sparse data about MT in this group of HIV-1 patients. In the pioneering work by Brenchley et al, EC demonstrated higher levels of LPS compared to uninfected controls, but levels tended to be lower than in the HIV-1 progressors[10]. This finding was confirmed by Hunt et al, who investigated 30 EC, and found that the proportion of activated (CD38+ HLA-DR+) CD4+ and CD8+ cells were higher compared to both HIV-1 negative individuals and patients virologically suppressed by

ART. The plasma LPS levels were also significantly elevated in EC compared to HIV negative controls, consistent with gut MT[140]. Later on, the number and proportion of CD17+ T-cells in gut biopsies from EC have been shown to be similar to HIV-1 negative individuals[141]. In a recent study from Kim et al[142], four EC with a median duration of HIV-1 infection for 18.5 years did have similar plasma levels of LPS and sCD14 at baseline as HIV negative controls, thus without evidence of increased MT in these EC. The blood CD8+ T-cell activation was also similar in both groups, but the CD4/CD8 ratio was lower in EC compared to HIV negative controls, and higher levels of D-dimer and IL-6 were observed in EC. All EC received ART for 6 months, but did not normalize their levels; nevertheless CD4/CD8 ratio was positively affected. Interestingly, a reduction of the mucosal Th17+ cell polyfunctionality was observed after ART discontinuation, but no long-term follow up of MT was performed in order to search for a progressive MT. In a Brazilian cohort, 7 EC and HIV negative controls were found to have comparable sCD14 levels, but in EC with occasional viremic episodes (<30% of frequency of transient viremia between 81 and 400 copies per mL), sCD14 levels were significantly higher compared to healthy controls[143]. Taken these divergent data together, further studies of MT in EC are warranted.

3 AIMS

The general aim was to assess the MT and the composition of gut microbiota in HIV-1 infection. Additionally, we wanted to explore the influence of antiretroviral therapy and antibiotics on MT.

Study I

Vesterbacka Jan, Nowak Piotr, Barqasho Babilonia, Abdurahman Samir, Nyström Jessica, Nilsson Staffan, Funaoka Hiroyuki, Kanda Tatsuo, Andersson Lars-Magnus, Gisslèn Magnus, Sönnerborg Anders. ***Kinetics of microbial translocation markers in patients on efavirenz or lopinavir/r based antiretroviral therapy****. PLoS One. 2013;8(1):e55038*

The aim was to examine how levels of MT markers were longitudinally affected up to 72 weeks after initiation of two different ART regimens in HIV-1 infected individuals, and to investigate the influence of antibiotics use on MT.

Study II

Vesterbacka Jan, Barqasho Babilonia, Häggblom Amanda, Nowak Piotr. ***Effects of Co-Trimoxazole on Microbial Translocation in HIV-1-Infected Patients Initiating Antiretroviral Therapy****. AIDS Res Hum Retroviruses. 2015 Aug;31(8):830-6*

The aim was to determine the levels of MT markers in HIV-1 patients initiating ART with or without co-trimoxazole (TMP-SMX) prophylaxis.

Study III

Nowak Piotr, Troseid Marius, Avershina Ekatarina, Barqasho Babilonia, Neogi Ujjwal, Holm Kristian, Hov Johannes R, Noyan Kajsa, Vesterbacka Jan, Svärd Jenny, Rudi Knut, Sönnerborg Anders. ***Gut microbiota diversity predicts immune status in HIV-1 infection****. AIDS. 2015 Nov 28;29(18):2409-18*

The aim was to investigate the diversity and composition of gut microbiota in treatment naive HIV-1 patients, and to examine the relation between gut microbiota and immune status. Additionally, we studied the effect of ART after one year.

Study IV

Vesterbacka Jan, Rivera Javier, Noyan Kajsa, Parera Mariona, Neogi Ujjwal, Calle Malu, Paredes Roger, Sönnerborg Anders, Noguera-Julian Marc, Nowak Piotr. ***Richer gut microbiota with distinct metabolic profile in HIV infected Elite Controllers****. Sci Rep. 2017 Jul 24;7(1):6269. doi: 10.1038/s41598-017-06675-1*

The aim was to explore the composition and functionality of gut microbiota in EC as compared to HIV-1 infected patients with progressive disease.

4 METHODS

4.1 SUBJECTS

Paper I

In a Scandinavian randomized clinical phase IV efficacy trial (RCT) trial (NORTHIV), 239 ART naïve HIV-1 infected subjects received allocated intervention. In our substudy, the patients were randomized to ART with either the non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz (EFV) + 2 nucleoside reverse transcriptase inhibitors (NRTIs) once daily (n= 37), or ritonavir-boosted lopinavir (LPV) + 2 NRTIs twice daily (n= 34). Plasma was collected at baseline (BL) and after 72 weeks (w72). Data on antibiotic therapy was available in 63/71 patients, of whom 29 received antibiotics at BL (n= 27) and/or w72 (n= 10), while 34 had not been given antibiotics at any of the two time points.

Paper II

Patients with HIV-1 infection (n= 26) followed at the Department of Infectious Diseases, Karolinska University Hospital, Stockholm, Sweden, who initiated first line ART, were selected from a larger cohort based on sample availability. They were classified into two groups dependent on whether they started ART and concomitantly TMP-SMX prophylaxis against *Pneumocystis jirovecii* (160mg/800mg three times a week) (n= 13) or not (n= 13). Plasma was collected at first visit at the clinic (BL), at one month (FU1) and after one year (FU2). TMP-SMX was started in median 7 days (IQR 1.5-21) and ART 12 days (7.5-15) after BL. In the non-TMP-SMX group ART was initiated 21 days (8.5-52) after BL. ART consisted of two NRTIs: tenofovir (n=15), abacavir (n=6) or zidovudine (n=4) in combination with lamivudine/emtricitabine, and the NNRTI efavirenz (n=7) or one of ritonavir boosted protease inhibitors (PI/r) LPV (n=8), darunavir (n=4) or atazanavir (n=6), with exception of one patient in the group of ART only who was treated with the integrase inhibitor raltegravir + darunavir/r.

Paper III

An observational cohort of 31 HIV-1-infected individuals was recruited from the HIV Outpatient Clinic at Karolinska University Hospital, Stockholm, Sweden. Additionally, a sex and age-matched control group of nine healthy HIV-1-seronegative individuals was included. Stool and peripheral blood samples were collected from all study participants at baseline and for 19 patients at follow-up (median 10 months; interquartile range 4–15) after ART introduction. ART consisted of two NRTIs in combination with a NNRTI (n=8) or a PI/r (n=11). Neither patients nor controls had been prescribed antibiotics or consumed probiotics during the preceding 2 months, or had infectious diarrhea.

Paper IV

Totally, 48 HIV positive subjects and 16 HIV negative controls were recruited from the out-patient HIV clinic at Karolinska University Hospital, Stockholm, Sweden. All viremic progressors were ART naive (naive). Exclusion criteria were inflammatory bowel disease or infectious gastroenteritis within the last four weeks. EC were defined by: (I) HIV positive for ≥ 1 year and with ≥ 3 consecutive viral loads (VLs) <75 c/ml over one year with all previous VLs <1000 c/m, or (II) HIV positive for ≥ 10 years, with ≥ 2 VLs and ≥ 90% of all VLs <400 c/ml. The study subjects were categorized into three groups (EC: n=16; naive: n=32; negative: n=16) and matched by Body Mass Index (BMI), age, gender and sexual practice. Plasma and stool samples were collected from the participants. Four female EC had been on short time ART due to pregnancy (three for 3.5 months, one for 14 days), all more than four years before study entry.

4.2 FLOW CYTOMETRY AND VIRAL LOAD

Determination of CD4/8+ T-cell counts and plasma HIV-1 RNA load was performed as part of the clinical routine with flow cytometry and CobasAmplicor (Roche Molecular Systems Inc., Branchburg, New Jersey, USA), respectively.

4.3 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS AND IMMUNOPHENOTYPING

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-treated blood using Hypaque-Ficoll (GE Healthcare) density gradient centrifugation, counted with Nucleocounter® and finally cryopreserved at -150ºC in fetal bovine serum (Sigma-Aldrich) containing 10% DMSO (Sigma-Aldrich), at a concentration of 10^6 cells/ml of cryopreservation media. At the analysis day, samples were thawed and PBMCs stained for HLA-DR and CD38 as markers of immune activation of CD4+ and CD8+ T- cells, and for FoxP3 and CD25 as markers of CD4+ T-regulatory cells[44].

4.4 MARKERS OF MICROBIAL TRANSLOCATION

At day of sampling, plasma specimens from EDTA-treated blood were frozen at -80ºC to be thawed later. The samples were analysed in a blind fashion in relation to patient identity, clinical data and treatment response. Quantification of LPS was determined by limulus amebocyte assay (LAL, Lonza, Maryland, USA), according to manufacturer´s directives, but with modifications as described by Troseid et al[103]. LBP, sCD14 and I-FABP levels were assessed by enzyme-linked immunosorbent assays (ELISA) (Hycult biotech, R&D Systems, USA and DS Pharma Biomedical Co, Japan; respectively), according to the instructions from respective manufacturer. For the studies with longitudinal design, all samples from the same patient were assayed on the same plate. Antibody titers to flagellin levels were determined by an in-house anti-flagellin specific IgG ELISA[144] using purified flagellin monomers from S. typhimurium (InvivoGen, USA), as it has been shown that human sera have a similar

recognition pattern of flagellin monomers whether isolated from flagellated E. coli or S.typhimurium[145]. Summarily, microwell plates (MWP) were coated overnight with purified flagellin from S. typhimurium (25 ng/well). Plasma samples from the study subjects were diluted 1:1000 and applied to the MWP the day after. After incubation and washing, the MWPs were incubated with HRP-conjugated antihuman IgG. Determination of total IgG levels was performed by commercial ELISA, following the manufacturer's procedure (MABTECH, Nacka, Sweden).

4.5 SOLUBLE MARKERS OF INFLAMMATION AND TRYPTOPHAN CATABOLISM

To assess the level of inflammation in plasma, quantification of IL-6 (R&D, Minnesota, USA), hs-CRP (Abcam, UK), D-Dimer (Technoclone, Austria) and sCD163 (R&D) was done by ELISA. Plasma levels of metabolites from tryptophan catabolism were measured by high-performance liquid chromatography (HPLC) (http://bevital.no). All analyses were performed according to the manufacturers´ instructions.

4.6 FECAL SAMPLE COLLECTION

In paper III, the fecal samples were frozen after donation, and later stored in -70 °C. The stool specimens were weighed, and S.T.A.R. (Stool Transport and Recovery; Roche, Basel, Switzerland) buffer solution was added to each sample at a ratio of ~1 (stool) to 3 (S.T.A.R. buffer) within 1 month. In order to achieve homogenous suspension, the samples were vortexed and then stored at -80 °C before DNA extraction, as previously described[146]. In paper IV, a sterile tube without preservation media for fecal sampling was used when study participants were able to donate feces adjacent to their study visit at the out-patient clinic. The samples were frozen and instantly stored at -80º C. Participants who submitted feces at home were instead using the PSP® Spin Stool DNA sampling tube (Stratec Biomedical). These stool samples were delivered to the clinic by the participant, or instantly sent by post and stored at -70°C according to the manufacturer´s instructions[147].

4.7 EXTRACTION OF DNA FROM STOOL SAMPLES

In paper III, the stool specimens were weighed, and S.T.A.R. (Stool Transport and Recovery; Roche,Basel, Switzerland) buffer solution was added to each sample at a ratio of ~1 (stool) to 3 (S.T.A.R. buffer). Samples were vortexed to achieve homogenous suspension and then stored at -80 °C. The frozen stool samples were thawed on ice. Microcentrifuge tubes(2 mL) containing 250-mg glass beads (<106 μm) were filled with a suspension volume of 0.5 mL of the stool sample. To achieve bacterial cell lysis, homogenization was performed using a MagNaLyser (Roche) twice at 2000 rpm for 40 s, with 40 s cooling between runs. The samples were kept cold during the rest phase to avoid DNA degradation due to overheating. This step was followed by centrifugation at 12 300 g for 5 min. The supernatant lysate solution was then transferred to a new microcentrifuge tube in two replicates (designated parallel A and B) for each of the samples. Fifty microlitres supernatant from the tubes were transferred to a KingFisher 96-well plate as

previously described[146], and DNA extraction from supernatant lysate solution was performed using the Mag ™ mini kit (LGC, Middlesex, UK). In paper IV, PowerSoil DNA Extraction Kit (MO BIO Laboratories, Carlsbad, CA, US) was used, following the manufacturers´ recommendations, respectively.

4.8 SEQUENCING OF GUT MICROBIOTA

In summary, the extracted DNA was amplified by PCR, with primers targeting the variable V3-V4 region from the bacterial 16S rRNA gene according to protocols. The amplified DNA products were washed to remove non-DNA material, followed by attachment of sequencing adapters and dual indices. The sequencing was performed on an Illumina™ platform, generating paired-end reads of 300 bases in each direction. After a second round of cleanup, PCR-amplicons were quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, MA, USA).

4.9 SEQUENCE ANALYSIS

In paper III, QIIME ver.1.8.0 (Quantitative Insights into Microbial Ecology) software was used for analyses of sequences after processing of the reads. From the full dataset, 10 000 sequences per sample were randomly selected in order to guarantee equivalent information. Taxonomy classification was built on Operational taxonomic units (OTUs), requiring 97% cluster identity. α-diversity was calculated using number of observed species, Shannon, and reciprocal Simpson’s diversity indexes. To estimate the ß-diversity, principal coordinate analysis (PCoA) was performed, and assessments were also made with the weighted Unifrac and Bray–Curtis indices[148], using MATLAB R2013a software (MathWorks, USA).

In paper IV, sequencing data was processed using Mothur[149] phylotype approach. Preprocessed sequences were classified using RDP algorithm[150] in combination with 16s rRNA Silva database[151]. To assess α-diversity, richness and Shannon and Simpson indices were computed using R/vegan library[152,153] randomly selecting subsamples of ten thousand counts for each subject.

Bacterial genera count table were normalized to relative abundance measures. These were used to compute Bray – Curtis dissimilarity between each pair of individuals. This index was served as input ordination analysis using non-metric multidimensional scaling (NMDS). Correlation between NMDS plot axis coordinates and inflammation parameters were tested by applying Spearman test. Additionally, a PERMANOVA (adonis) test was performed on this distance matrix to partition different sources of variation using R/vegan package.

The function of bacterial microbiome was inferred using PICRUSt[154] on GreenGenesDB[155] classified phylotypes. Counts were normalized by considering 16S rRNA gene copy number. To infer the gene content, the normalized phylotype abundances were multiplied by the respective set of gene abundances, represented by Kyoto Encyclopedia of Genes and Genomes (KEGG) identifiers estimated for each taxon.

4.10 STATISTICAL ANALYSES

4.10.1 Paper I + II

Non-parametric statistics were applied. Two-tailed Mann-Whitney U-test was used for comparisons between independent groups, and Wilcoxon signed rank test for analyses of longitudinal paired data. Spearman´s rank test was used for determination of correlations between two variables. Differences in levels of MT markers between patients with or without antibiotics adjusted for co-variables in paper I was determined with ANCOVA. In paper II, differences in levels of MT markers at the different sampling points were analyzed with a generalized linear mixed-effects model adjusting for significant co-variables, and this model was based on parametric analyses. Data were analyzed by GraphPad Prism v. 5.02-04, R 2.13.1 and STATA 12 SE/12.The significance level was set at 0.05.

4.10.2 Paper III

Non-parametric statistics were applied. Analyses of data between two independent groups were performed by two-tailed Mann-Whitney U-test, and by Wilcoxon signed rank test for comparisons of longitudinal samples from baseline and follow-up. α-diversity indexes were compared in QIIME software using a t test based on Monte Carlo permutations, whereas Kruskal–Wallis test was used for comparisons of ß-diversity. Correlations were analyzed with Spearman's rank tests. Multivariate linear regression models included only age and sex as covariates because of the small sample size, and assumptions for use of the model were fulfilled. A two-tailed significance level of 0.05 was used. P values were corrected for multiple testing using false discovery rate (FDR). The statistical analyses were performed with SPSS software, version 19.0.

4.10.3 Paper IV

Multiple group differences in diversity indices, bacterial abundances, inflammation and activation markers were analyzed via Kruskal–Wallis rank-based test, and Benjamini–Hochberg correction was applied to correct for multiple testing[156]. Two-tailed Mann-Whitney U-test was used for comparisons of inflammation markers between two groups.

Associations between bacterial genera, functional pathways and inflammatory markers were performed with Spearman´s rank test. Associations with a Benjamini–Hochberg adjusted p-value lower than 0.01 were considered as relevant, and when plotting the heatmap, inflammatory parameters associated with less than two bacteria were discarded. Bacterial genus and functions were ordered in the heatmap using Ward hierarchical clustering.

To evaluate the power of the classification of individuals according to the composition profile of their microbiome, a LASSO penalized logistic regression model was computed for each pair of profiles as previously described[157]. LiblineaR and pROC libraries were used to obtain the regression models, represent ROC curves and estimate accuracy of the model using AUC.

4.11 ETHICAL PERMITS

The studies were approved by The Regional Ethics committee in Gothenburg (Gothenburg Ö 739-03) and Stockholm (2009/1485-31, 2013/1944-31/4, 2014/920-32).

5 RESULTS

5.1 PAPER I+II

The detailed characteristics of HIV-1 infected subjects at baseline are presented in table 2 in paper I and table 1 in paper II. In summary, 71 treatment naive patients starting first line ART with LPV or EFV combined with two NRTIs were included in paper I. Data on use of antibiotics was available for 63 patients. In paper II, naive patients were starting ART with (n=13) or without (n=13) concomitant TMP-SMX prophylaxis. Historical data from healthy controls were used for comparison of levels of MT markers.

5.1.1 Levels of LPS

Overall LPS levels at BL were elevated in HIV-infected subjects, and declined in paper I at w 72 in both treatment groups (Figure 4). In paper II, the BL LPS levels correlated with CD4+ T-cell count and were lower in the more immunocompromised (TMP-SMX) group. At one month (FU1), LPS levels increased in the non-TMP-SMX group, but remained unaffected as compared to BL in both groups after one year of ART.

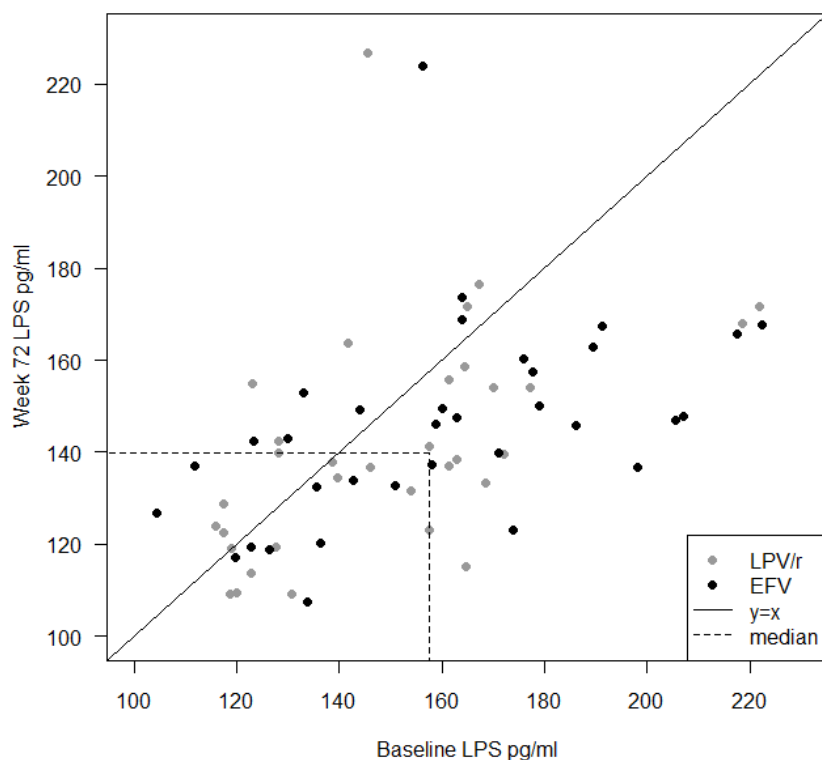

Figure 4. Plasma levels of LPS before and after 72 weeks of ART (paper I).

5.1.2 Levels of sCD14

The BL levels of sCD14 were increased and correlated with VL in both cohorts (ρ=0.42, p= 0.0002; ρ=0.55, p=0.005, respectively), negatively with CD4+ T-cells in paper I (ρ=-0.42, p = 0.0003) and with LBP in paper II (ρ=0.42, p=0.03). Levels were highest in the group with lowest CD4+ T-cell counts (TMP-SMX), and were longitudinally reduced by initiation of ART in all groups of HIV-1 infected patients.

5.1.3 Levels of LBP

LBP levels were analyzed in paper II, and were elevated at BL in TMP-SMX as compared to non TMP-SMX group. A negative correlation (ρ= -0.65) was found at BL between LBP and CD4+ T-cell counts, which had a tendency to be most prominent in TMP-SMX group. LBP levels in TMP-SMX group were longitudinally distinctly reduced at FU2. No fluctuations in levels of LBP were found in the non-TMP-SMX group between BL and FU2, but this group had low LBP levels already at BL, almost at the same levels as the healthy controls used in paper IV. The reduction was still significant after adjustment for BL CD+ T-cell counts and viral load in the generalized linear mixed-effects model.

5.1.4 Levels of anti-flagellin antibodies

The levels of anti-flagellin IgG antibodies were assessed in paper I. All HIV-patients had detectable levels at BL. We found a reduction at w72, which was significant only in LPV/r treated individuals after stratifying the patients to their respective treatment group. Total IgG levels were as expected elevated at BL, and declined until w72. The ratio between of anti-flagellin IgG and total IgG was used for verification of the reduction of specific anti-flagellin IgG. Also the ratio was reduced, confirming decline of anti-flagellin specific IgG and not only total IgG. We detected a positive correlation between LPS and anti-flagellin antibodies at BL and w72, but otherwise no associations with any of the other MT markers were present.

5.1.5 Levels of I-FABP

Also I-FABP levels were abnormally high in both cohorts at BL, with further increase at w72 in EFV treated subjects in paper I. We also observed a temporary increase of I-FABP between BL and FU1 in TMP-SMX treatment arm in paper II, but after one year (FU2) there was no differences in I-FABP levels as compared to BL for any of the groups. After stratifying the subjects in paper II by type of NRTI treatment, we found that only the 15 patients starting tenofovir (Tenofovir Disoproxil Fumarate, TDF) had elevated levels of I-FABP at FU1. Additionally we categorized subjects upon increasing or decreasing I-FABP at FU1, and compared the CD4+ T-cell development in each group to investigate whether the transient I-FABP elevation could reflect systemic immunoreconstitution. We observed that

the CD4+ T cell recovery was 103 cells/µl in the group with increasing and 210 cells/µl in the group with decreasing I-FABP levels.

5.1.6 Antibiotics and microbial translocation markers

In paper I, we observed that use of antibiotics at BL (27 /71) or/and ongoing antibiotic treatment was associated with lower levels of sCD14 at w72 after adjusting for significant co-variates with ANCOVA analysis.

In paper II, levels of LBP were lower after one year in TMP-SMX treated individuals, also after adjusting for co-variates with the generalized linear mixed-effects model. A reduction was not observed in the other treatment arm with patients on ART without TMP-SMX treatment. As mentioned above, differential I-FABP levels were found at FU1 with elevation in TMP-SMX group, but this difference could not be confirmed in the multi-variate analysis. No difference in the kinetics of LPS or sCD14 levels was found between HIV patients on ART with or without concomitant use of antibiotics.

5.2 PAPER III+IV

The detailed description of the cohorts´ characteristics at BL is presented in respective Table 1 in paper III+IV. To summarize, we included 31 HIV-1 infected individuals of whom three were EC, and 9 HIV-negative controls in paper III. Blood and fecal samples were obtained at BL and after one year. In paper IV, 48 HIV-1 infected patients and 16 HIV-seronegative controls were included. This was a cross-sectional study, where samples from blood and feces were collected at a single time point.

5.2.1 Gut microbiota diversity in treatment naive patients

5.2.1.1 α-diversity

The α-diversity of gut microbiota represents the diversity of bacteria within each individual. In our cohorts, we found that the number of observed bacterial taxa was lower in HIV-infected patients without ART as compared to seronegative controls (Figure 5), and lowest in the most immunocompromised patients. Further assessment with both Shannon and Simpson index revealed dissimilarities in the fecal microbiota between HIV patients and negative controls, with decreased indices in the HIV-1 infected population. We also found that richness estimated by indices ACE and Chao-1 was lower in HIV-infected patients with progressive disease (Figure 6).

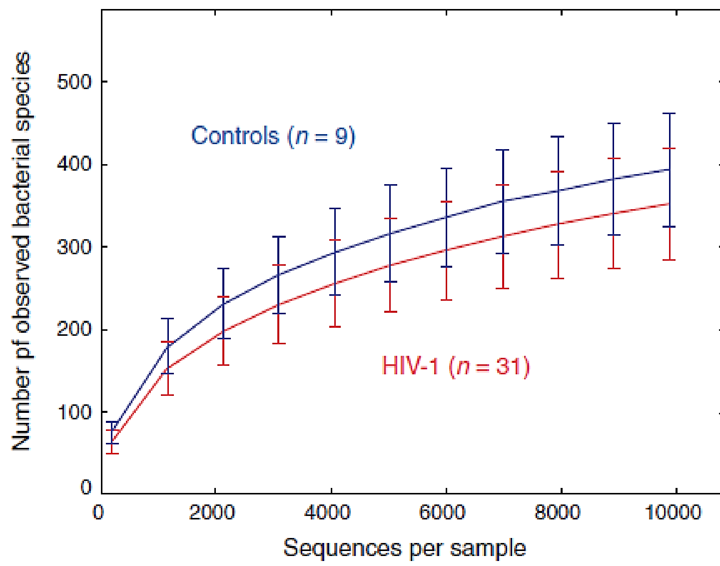

Figure 5. Number of observed bacterial species in relation to number of analyzed sequences (paper III).

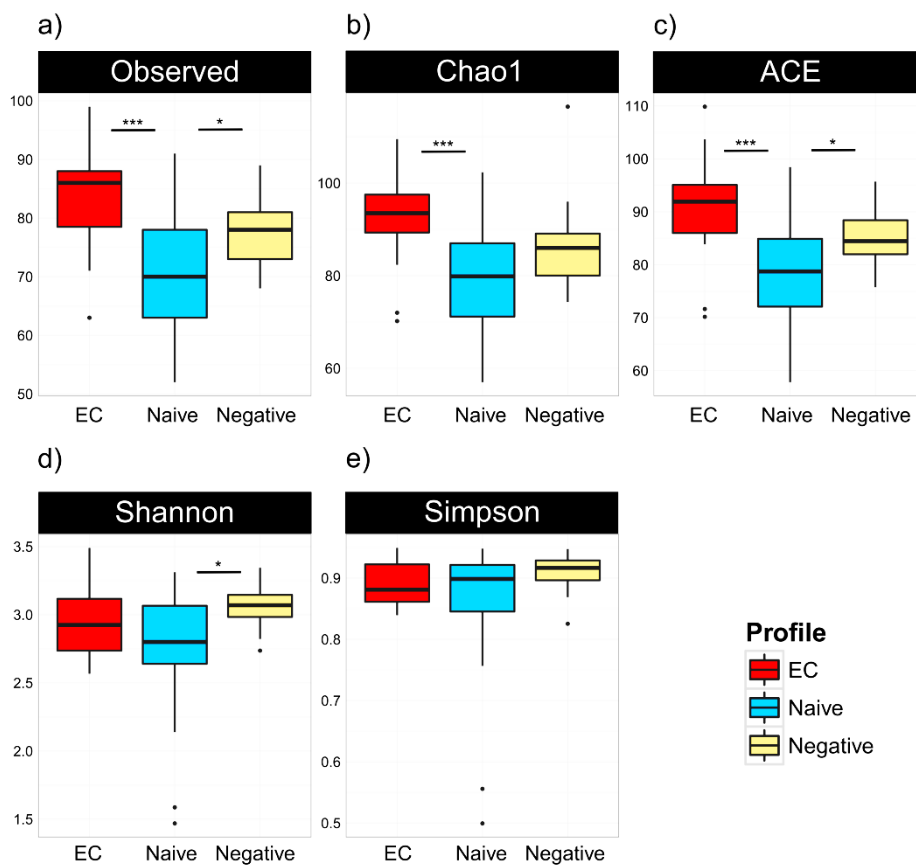

Figure 6. Richness (genus level) and diversity indices in HIV-1 infected and negative controls (paper IV).

5.2.1.2 ß-diversity

ß-diversity represents the inter-individual differences, heterogenecity, of fecal microbiota composition between different populations. Normalized bacterial counts were used for calculation of weighted Unifrac and Bray-Curtis dissimilarity indices, yielding highest ß-diversity in patients with progressive HIV, and lowest in EC as compared to negative controls. Based upon results from calculation of ß-diversity indices, a principal coordinate analysis (PCoA) was performed in paper III, and non-metric multidimensional scaling (NMDS) and LASSO regression analysis in paper IV. The LASSO model analyses revealed that the composition of the gut microbiota was more different in individuals with progressive disease compared to negative and EC. In paper III, PCoA showed that the gut microbiota was overlapping between HIV progressors and negative controls, whilst the three EC were clustering together as presented in Figure 7. This initial observation was confirmed in paper IV, where the EC were clustering together in the NMDS analyses, indicating a unique gut microbiota composition in these individuals (Figure 8).

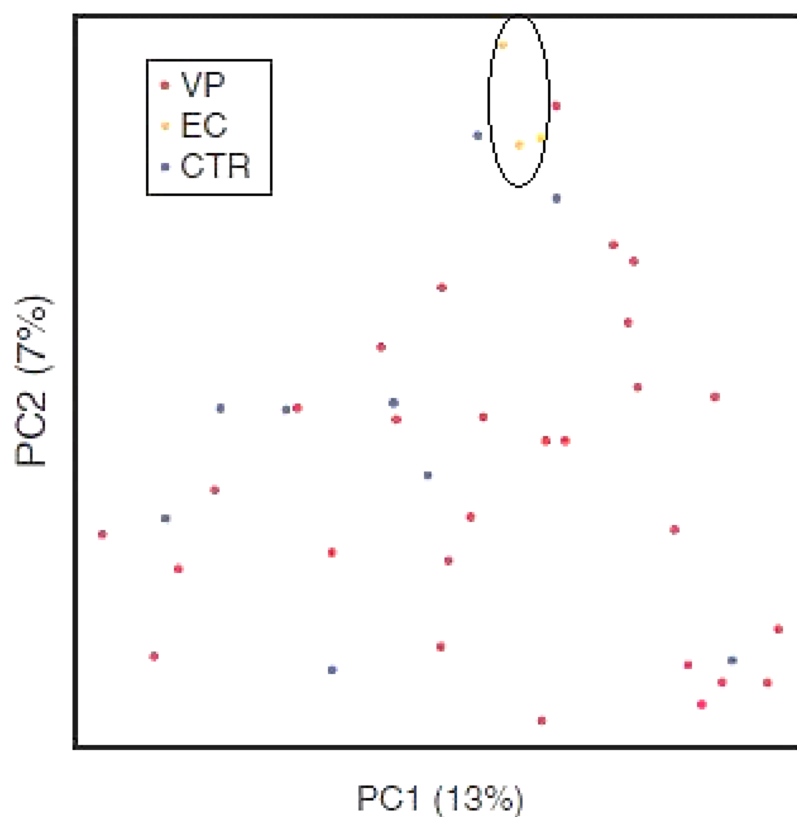

Figure 7. Differences in ß-diversity between viremic patients (VP), Elite controllers (EC), and healthy controls (CTR) in PCoA (paper III).

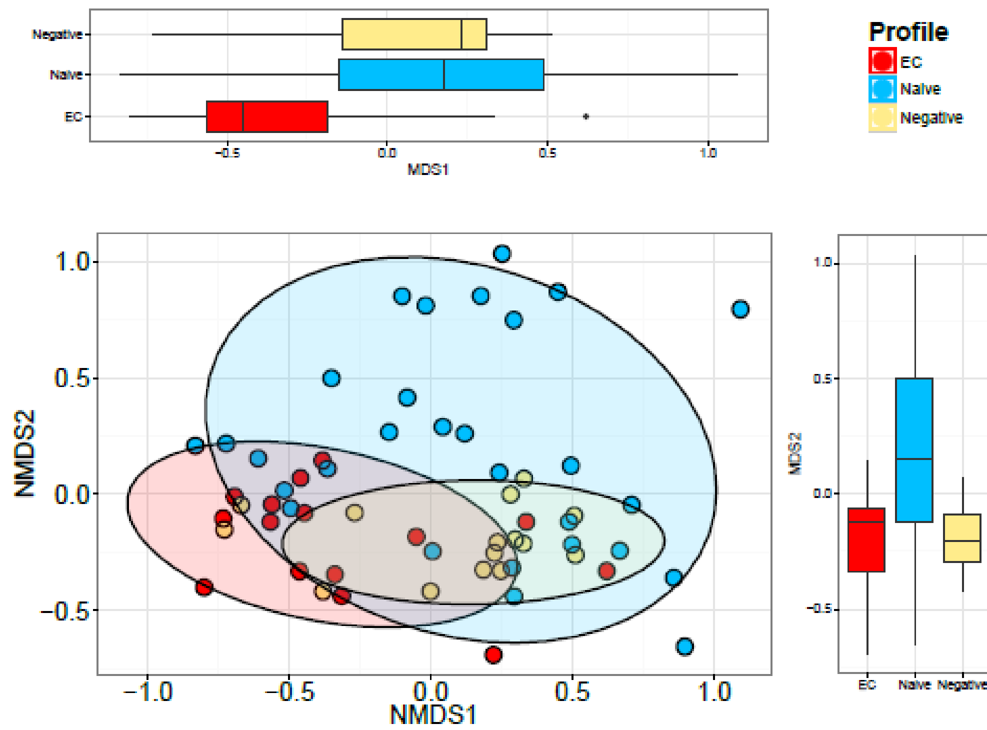

Figure 8. Clustering of Elite controllers (EC) in NMDA analysis (paper IV).

5.2.2 Composition of gut microbiota in treatment naive patients

Description of the bacterial community is built on a hierarchic ranked-based classification system with several taxonomic levels. In bacteriology, the highest level is phylum, followed by class, order, family, genus and species. We analyzed the BL fecal bacterial composition at several levels. In paper III, Firmicutes, Bacteroidetes, and Actinobacteria were the most abundant phyla, and no significant differences between HIV-1 seropositive and negative individuals were detected (Figure 9). Some differences were found between patients with progressive disease and EC, with a higher relative abundance of Actinobacteria and lower proportion of Bacteriodetes in HIV progressors.

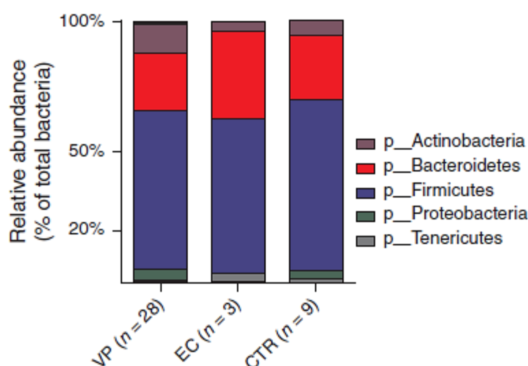

Figure 9. Distribution of five most abundant bacterial phyla in ART naive patients and negative controls (paper III).

At genus level, we found several compositional differences between HIV-infected and negative controls. The relative abundance of *Lachnobacterium*, *Faecalibacterium*, *Hemophilus*, *Anaerofilum*, *Delftia*, *Oscillospira*, *Sutterella* and *Rhizobium* was reduced, and proportions of *Lactobacillus*, *Blautia* and *Anaerostipes* were increased in HIV progressors compared to negative controls (Figure 10). The compositional differences between EC, HIV progressors and negative controls are described in paragraph 5.2.6.

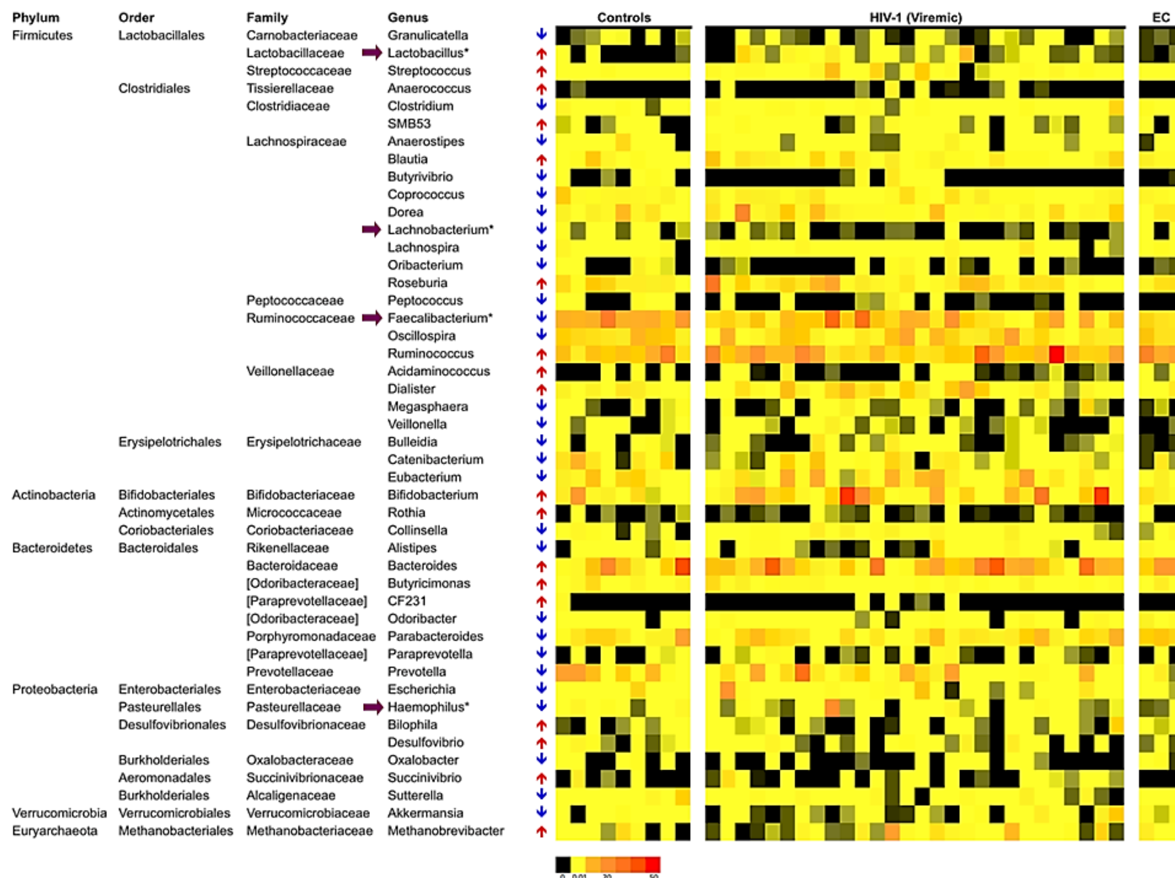

Figure 10. Heat map illustrating differences in bacterial genera between controls, untreated viremic HIV-1 individuals and Elite controllers (EC) (paper III).

5.2.3 α-diversity, immune status and inflammation

In treatment naive patients, the number of observed unique bacterial sequences in paper III and IV correlated to the CD4+ T-cell count in the univariate analyses ($\rho=0.59$, $p=0.009$; $\rho=0.58$, $p<0.0001$, respectively). After adjustment for covariates sex and age in a multivariate regression analysis in paper III, the independent association between the observed bacterial species and the CD4+ T-cell count persisted. For every gain of a bacterial species, the CD4+ T-cell count increased with 0.88 cells/μL. After adding a third followed by all covariates to the model, it was shown that only the number of observed species and LPS were

independently associated with CD4+ T-cell counts. The number of observed species and Shannon α-diversity index were negatively correlated to markers of MT (LPS, LBP), and soluble markers of immune activation (sCD14, sCD163, CD4/8+ T-cell ratio). We also observed strong negative correlations (ρ ~ -0.6) between proportions of activated CD4/8+ T-cells and observed genera/richness indices ACE and Chao-1 in paper IV. No significant correlations between α-diversity and soluble markers of inflammation were found in this cohort.

5.2.4 Tryptophan catabolism and gut microbiota

The serum kynurenine/tryptophan (K/T) ratio is commonly used as a marker of IDO1 activity, and is elevated during HIV infection[158]. Our analyses confirmed that K/T ratio is increased in patients with progressive HIV-1 infection, but we found no evidence of abnormal IDO1 activity in EC compared with negative controls. Additionally, levels of several metabolites from tryptophan degradation were abnormal in HIV progressors; xanthurenic and kynurenic acid levels were lower and anthralinic acid levels higher. Interestingly, K/T ratio correlated negatively to the number of observed genera and richness indices ACE and Chao-1. Furthermore, we observed significant correlations between NDMS axis 2 and tryptophan, xanthurenic acid and K/T ratio in the NMDS analysis, with a separation of HIV progressors from EC and negative controls, indicating a different tryptophan catabolism related to microbiota differences between these groups of individuals. At genus level, we were able to associate only *Rhizobium* with K/T ratio, and these were negatively correlated to each other.

5.2.5 Effects of ART on gut microbiota

ART had significant effect on the gut microbiota after longitudinal follow up. 19 patients were starting ART, and after almost one year the α-diversity was further reduced as compared to BL, with lowered number of observed species and Shannon index. In contrary, ß-diversity increased as a consequence of bacterial taxa changes within the individuals. The composition of microbiota was altered at both phylum and genus level after introduction of ART. Fecal proportions of genus *Prevotella* (phylum Bacteroides) were significantly depleted at follow up, and the abundance equaled that in negative controls. In phylum Firmicutes, abundance of genera *Lachnospira*, *Oribacterium* and *Oscillospira* was reduced. Finally, genus *Sutterella* (phylum Proteobacteria) was less prevalent in fecal microbiota after ART.

5.2.6 Elite controllers

5.2.6.1 Microbial translocation, inflammation and tryptophan catabolism

The levels of MT markers LBP and sCD14 were low in our cohort of EC, and equal to those in seronegative controls. However, levels of inflammatory parameters hs-CRP and IL-6 were elevated in EC compared to negative controls. We found no evidence of increased cellular immune activation in EC as compared to controls when assessing the proportion of activated CD38+ HLA-DR+ CD4/8 T-cells. Plasma levels of tryptophan, the related catabolism

metabolites and K/T ratio were same in EC and controls, thus lower than in HIV progressors. All together, these data suggest that EC don´t have increased MT or immune activation, but persistent low-grade inflammation as compared to healthy controls.

5.2.6.2 Gut microbiota

Analyses of EC revealed a richer gut microbiota with higher number of observed genera and richness indices compared to HIV progressors, but not different from negative controls. As mentioned earlier, EC had less inter-individual variation of the gut microbiota composition than negative controls and HIV progressors, clustering together in the multidimensional scaling analyses PCoA and NMDS who are based on the ecological dissimilarity indices. Sequencing data showed that the abundance of several taxa was differential in EC. At phylum level, Bacteroidetes were enriched and both Proteobacteria and Actinobacteria depleted in EC vs HIV progressors. At genus level, *Succinivibrio* and *Sutterella* were more abundant in EC as compared to both HIV progressors and negative controls. Moreover, genera of *Rhizobium*, *Delftia*, *Anaerofilum* and *Oscillospira* were more enriched in EC vs HIV progressors, but were not significantly different from negative controls (Figure 11).

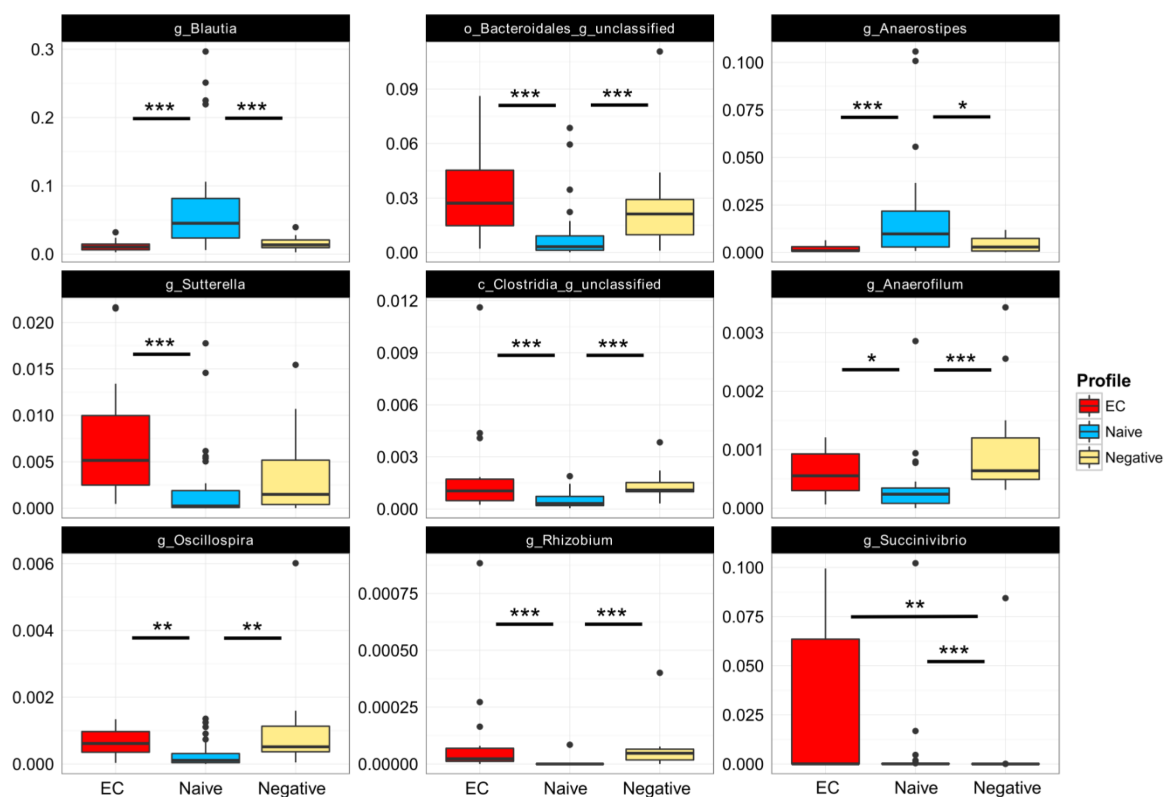

Figure 11. Compositional differences in fecal microbiota between Elite controllers (EC), naive patients and negative controls (paper IV).

5.2.6.3 Inferred functionality of gut microbiota

To predict the metagenomic inferred functional content of gut microbiota, PICRUSt analysis of gene contents was used based on the KEGG database. At the higher level II, carbohydrate metabolism pathway was decreased in the bacterial metagenome of EC as compared to both HIV progressors and negative patients. Genes encoding cardiovascular diseases and circulatory system pathways were instead enriched in EC as compared to HIV progressors, but not different from negative controls. At the more specific KEGG level III, we found that carbohydrate metabolism related pathways were less abundant in EC vs HIV progressors. These included galactose metabolism, pentose-glucoronate interconversions, pyruvate metabolism and pentose-phosphate pathway (PPP). Additionally, PPP was also less prevalent in EC vs negative controls. The predicted lipid metabolism associated pathways were mostly similar in EC and negative controls, and metagenomics proportions were different from HIV progressors. This was illustrated by lower proportions of genes encoding metabolism of fatty acids and lipid biosynthesis proteins in HIV progressors. EC had significantly higher proportions of genes involved in pathways related to synthesis and degradation of ketone bodies, also compared to negative controls. Anyhow, the secondary bile acid biosynthesis metabolism pathway, that plays an essential role in cholesterol homeostasis, was less represented in EC as compared to the other groups. PPAR (peroxisome proliferator-activated receptors)-signaling pathway, involved in metabolism of lipids, carbohydrates and proteins, was less abundant in HIV progressors compared with EC and controls. Bacterial genes involved in metabolism of tryptophan were less abundant in HIV progressors, but were equally abundant in EC and negative controls (Figure 12).

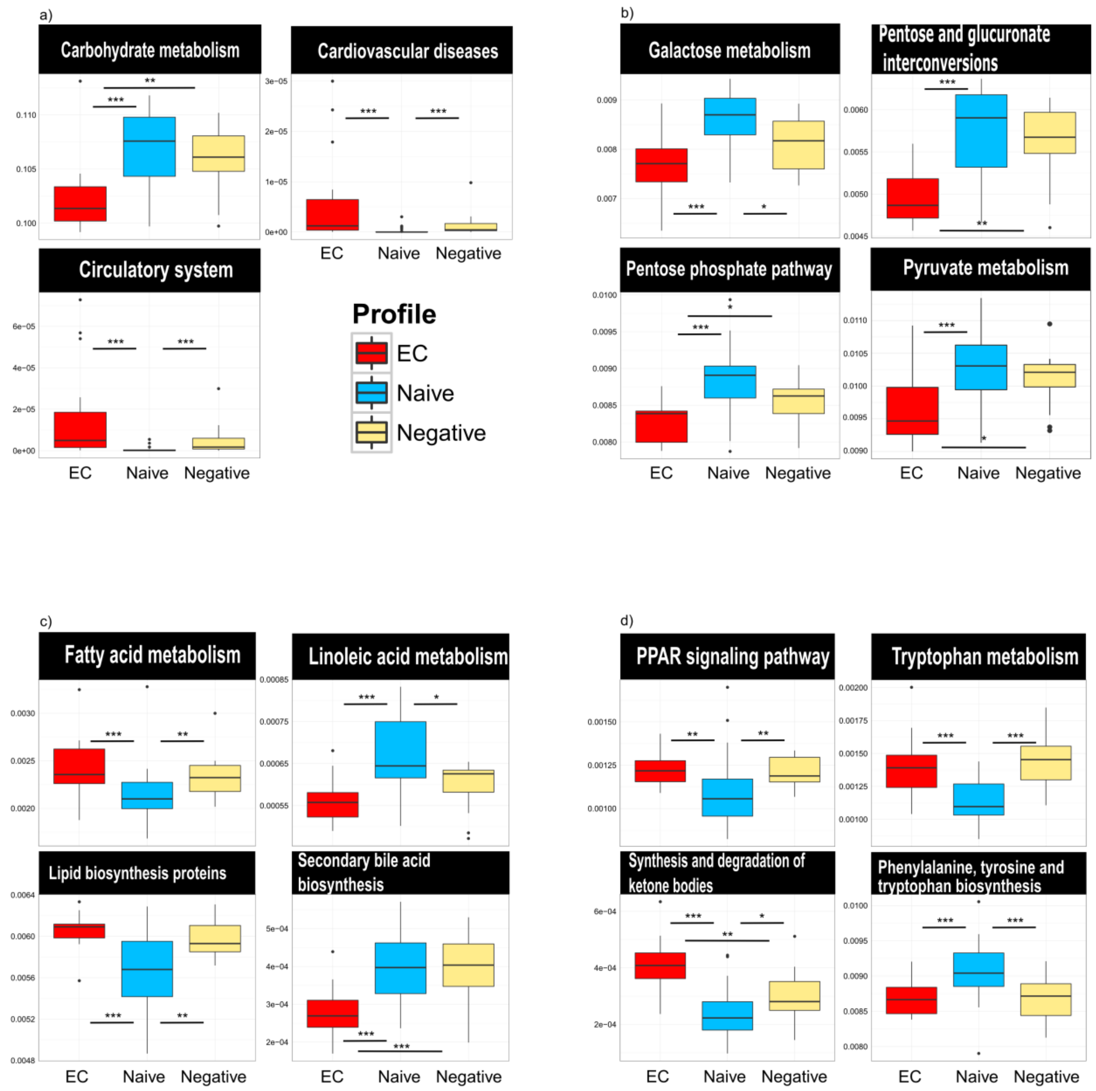

Figure 12. The metagenomic functional content of gut microbiota predicted by inferred PICRUSt analysis (paper IV).

6 DISCUSSION

MT is one of the causes behind the persistent low-grade inflammation in well-treated HIV-1 infected patients[10,159]. Most studies of MT in such patients associate the introduction of ART with declining levels of MT markers, but even in fully virologically suppressed patients, MT is significantly higher than in uninfected individuals[10,35,103,104].

In the first study we addressed the question whether the type of ART influences MT using the samples from an RCT. Our findings of a unified reduction of LPS and sCD14 after 72 weeks of ART in both treatment arms (NNRTI vs PI/r) are similar to previous reports[10,35,103,104,109]. The decreased levels of MT markers may be related to restoration of submucosal Th17+ T-cells, enhanced epithelial cell integrity and/or recruitment of neutrophils in the gut. Nevertheless, LPS and sCD14 levels were still abnormally high after 1.5 years of full viral suppression in our study, and were actually even elevated as compared to patients with active inflammatory bowel disease[64,160].

ART was followed by declining levels of anti-flagellin IgG antibodies, but the reduction was significant only in LPV treated patients. We hypothesize that the choice of ART could directly modulate the composition of bacteria with a reduction of the number of flagellin containing gram-negative bacteria. It has been shown that LPV/r has an antimalarial activity[161], and the older NRTI zidovudine has been found to have a direct antibacteridal effect on several enterobacteria, e.g. *Escherichia coli* and *Klebsiella oxytoca*[162]. Additionally, we have earlier shown that the levels of anti-flagellin antibodies and LPS were lower in HIV patients on TB-treatment in a Vietnamese cohort[89]. Alternatively, the difference may be explained by a more rapid restoration of the blood-gut barrier by LPV as compared to EFV.

In opposite to the other markers, I-FABP levels did not decline in our patient populations during the follow-up. Actually, an increase at w72 was detected in paper I, but it remained significant only for EFV treated patients. In paper II, a transient I-FABP elevation was observed after one month, preferable in individuals with TDF containing regimen. Similarly, in a work by Sereti et al, patients starting very early EFV based ART (within 2-3 weeks of their infection) had increasing I-FABP levels during the first two weeks of treatment, which remained elevated after two years of viral suppression[163]. On the contrary, I-FABP levels were higher in a Mexican cohort of patients treated for more than five years with boosted atazanavir or LPV as compared to EFV treated and seronegative controls, but the cross-sectional study design with relatively few participants limits the conclusions from this work[116]. As I-FABP reflects the death and turnover of enterocytes, and not directly MT, we speculate that the antiretrovirals themselves may have a detrimental effect on enterocytes and suggest that I-FABP has a limited value as estimator of MT.

As antibiotics modulate the diversity and composition of the gut microbiota[164,165], we studied the influence of antibiotics on the level on MT markers in cohorts I and II. Use of TMP-SMX

as *Pneumocystis jirovecii* prophylaxis is recommended to HIV-patients with a CD4+ T-cell count <200/µL, and is frequently started in combination with ART. This antibiotic has a broad antibacterial spectrum, covering both gram-negative and –positive organisms, and is also active against the opportunistic agent *Toxoplasma gondii*. In the paper I, we found that levels of sCD14 were significantly lower at week 72 in patients using antibiotics at BL or in those with ongoing antibiotic treatment, but no effect of antibiotics on any of the other MT markers was observed. Serving as a marker of macrophage/monocyte activation upon a wider range of microbial stimuli and not as a direct marker of MT, levels of sCD14 may better represent the overall innate host response to gut-resident microbial triggers. The importance of sCD14 as an important predictive marker has previously been demonstrated when the mortality risk of HIV infected individuals has been independently correlated to plasma levels of sCD14[36].

In study II, levels of sCD14 tended to be higher in TMP-SMX group at BL, but in this set of patients we could not confirm the antibiotics' effect on sCD14 at FU2 after one year. On the other hand we found that LBP levels were significantly reduced in the group of TMP-SMX treated patients suggesting additional antibiotic effect. Surprisingly BL LPS levels were lower in TMP-SMX group, and no longitudinal alternations in LPS levels were observed at all until end of study. This may reflect that 8/13 patients in this group received antibiotics or antifungals within 6 months from BL, with a compositional shift in the gut microbiome from gram-negative to gram-positive bacteria including Firmicutes[166], altered proportions of fungal organisms[63] or viruses such as *Adenoviridae* and *Anelloviridae*[167] as suggested by others. 61.5 % of TMP-SMX patients had stopped prophylaxis at FU2, and this might have influenced the longitudinal development of LPS levels due to TMP-SMX related alternations in the composition of gut microbiota, which at least partially was inversed when the prophylaxis was completed. LBP induction may origin from bacteria, fungi but also viruses[168], and elevation of LBP levels in the more immunosuppressed group may be caused by LBP release induced by broader microbial triggers. LBP elevation due to IRIS during HIV/TBC co-infection has also been reported[169]. The reduction of LBP levels in the univariate analysis was further confirmed with multivariate analysis. As the results were divergent between LBP and LPS, and LPS detection could be substantially influenced by microbiome shifts and methodological issues[170], we suggest that LBP is a more appropriate marker of MT than LPS.

As our first two studies strongly imposed that MT may be affected by alterations in the gut microbiota, we proceeded with analyses of the fecal bacterial composition in different HIV populations; HIV progressors and EC. We were able to show that the α-diversity in treatment naive HIV progressors is significantly reduced as compared to seronegative controls, similarly to work by others [134,171,172]. The lowest number of bacterial species was detected in the most immunodeficient patients and we could report the novel finding that the α-diversity of gut microbiota is positively correlated number of CD4+ T-cells. The inter-individual diversity was highest in HIV progressors, lower in controls and lowest in EC. This followed observations from e.g. Mutlu et al, who showed a more dispersed distribution of fecal microbiota samples in HIV-infected as compared to negative controls[134].

Others have described dysbiosis in HIV patients , with enrichment of phylum Proteobacteria and depletion of Bacteriodetes, Firmucutes and family Ruminococcaceae and Lachnospiraceae[114,115,134,171,162]. We found significant depletion of *Lachnobacterium* (phylum Lachnospiraceae), *Faecalibacterium* (Ruminococcaceae), *Hemophilus, Delftia*, *Sutterella*, *Rhizobium* (Proteobacteria), *Anaerofilum*, *Oscillospira* (Firmicutes) at genus level in HIV progressors. Several of these genera exhibit immunomodulatory capacities which may disturb the enteric homeostasis. For instance, *Faecalibacterium* is a major producer of the SCFA butyrate, which promotes differentiation of regulatory T-cells in colon[173]. *Sutterella* has been linked to fecal IgA-levels, and may affect differentiation of Th17+ cells[174,175], and members of *Rhizobium* are able to convert tryptophan to indole-3-aceticacid[176], a metabolite that may reduce Th17+ cells and IL-22 production[177,178]. Genus *Lactobacillus*, *Blautia* and *Anaerostipes* were all more abundant in HIV progressors vs negative controls, but *Prevotella* genus was not enriched in our cohort as described by others[132,133,166]. Still, the abundance of *Prevotella* was decreasing in HIV progressors after receiving ART for one year.

Interestingly, we could determine taxa differences between the three EC and HIV progressors. Phylum Bacteroidetes was less and both Proteobacteria and Actinobacteria more abundant in HIV progressors compared with EC.

Due to the low number of included EC in the third study, we aimed to expand our observations of the compositional gut microbiota differences in a larger EC cohort, additionally addressing the metabolic functionality of fecal microbiota. Compared with HIV progressors and negative controls, EC had a higher relative abundance of two genera: *Succinivibrio* and *Sutterella*. We hypothesize that these genera are of special interest and important contributors to immunological control mechanisms in EC, even though their relative abundance is low, only about 0.05-0.5%.Thus, we found a significant positive correlation between proportions of *Sutterella* and CD4+ T-cell counts, and negative association to markers of CD4/8+ T-cell activation. Depletion of *Sutterella* has been observed in gut microbiota during other conditions where the adaptive immune system is involved, e.g. in multiple sclerosis and in lymphoma patients after bone marrow transplantation[179,180]. Genus *Succinivibrio* may be of even more interest, given that we were able to show that initiation of ART was followed by enrichment of *Succinivibrio* in study III. Additionally, others have demonstrated that production of metabolites by members of Succinivibrionaceae was associated with immune recovery after initiation of ART, thus giving a mechanistic explanation behind the relation between abundance of *Succinivibrio* and its immunological effects[181].

A differential abundance of four genera was found between EC/negative controls and HIV progressors; *Rhizobium*, *Delftia*, *Anaerofilum* and *Oscillospira* were all depleted in HIV progressors. Only one genus, *Rhizobium*, correlated with K/T ratio suggesting that these bacteria may be key modulators in microbiota related tryptophan catabolism. As described above, Rhizobia produce the immunomodulatory catabolite indole-3-acetic acid affecting levels of IL-17/22, and should affect the mucosal levels of tryptophan. A lower abundance of

these bacteria may be followed by higher intraluminal concentrations of tryptophan, causing induction of IDO1 from gut resident macrophages, leading to lower serum tryptophan concentrations and higher K/T ratio that we observed in our analyses. This may also be mirrored in the functional analyses, where we found the inferred metagenomic abundance of tryptophan metabolism pathway was reduced in gut microbiota of HIV progressors as compared to EC and negative controls. CD4/8 T-cell ratio may be used as a marker of immune activation, higher ratio reflects less activation. Fecal depletion of genus *Oscillopira* has been associated to the inflammatory Crohn`s disease and obesity[182,183], and it has been speculated that *Oscillopira* probably may produce the SCFA butyrate[184]. In our HIV cohort, abundance of genus *Oscillopira* was strongly positively correlated to CD4/8 T-cell ratio, and negatively to activated (HLA-DR+) CD4/8+ T-cells, and thus lower abundance of this taxa seems to be causing immune activation also in HIV-1 infection. Low abundance of genus *Anaerofilum* has been related to higher intestinal permeability[185], and may in our cohort contribute to the abnormal MT observed in HIV progressors.

Inferred functional analysis of gut metagenome with PICRUSt revealed very intriguing differences between HIV progressors, EC and negative controls. The proportion of bacterial genes encoding pathways involved in metabolism of carbohydrates was significantly lower in EC. More specifically, pentose phosphate pathway, glucoronate interconversions-, and pyruvate metabolism pathways related genes were less represented in EC compared to HIV progressors and negative controls. Also bacterial galactose metabolism was lowest in EC, but this difference remained significant only against HIV progressors. In contrary, we were able to observe that proportion of genes involved in metabolism of lipids and fatty acids was lowest in HIV progressors, with exceptions of secondary bile acid biosynthesis pathway, which was depleted in EC but not different between HIV progressors and controls. Also PPAR (peroxisome proliferator-activated receptors)-signaling pathway, which has a major role in metabolism of fatty acids and lipids but also carbohydrates and proteins[186], was significantly reduced in HIV progressors. In contrary, lineolic acid metabolism pathway was enriched in HIV progressors. This is a long-chain essential fatty acid, precursor of arachidonic acid, which may prevent lymphoproliferation and downregulate adaptive immune cells[187].

Based on our results, we theorize that the bacterial composition and function of gut microbiota upon metabolism of lipids, carbohydrates and other nutrients interfere with and shape the enteric part of the host's immune system. The bacterial genomic pool in EC seems to be unique, favoring metabolism of lipids and with lower bacterial proportions involved in metabolism of carbohydrates and secondary bile acids. This is of extraordinary interest, as intracellular metabolic pathways involved in carbohydrate and lipid metabolism are important regulators of both innate and adaptive immune cells[188]. If the gut microbiota in EC is of importance for maintaining the immunological and sustained viral control in these patients, therapeutic interventions such as transplantation of bacteria with immunomodulatory effects could be one way to proceed to reduce immune activation and inflammation in HIV-1 infection.

Fecal microbiota transplantation (FMT) has also been considered as a therapeutic option in diseases accompanied by gut microbiota dysbiosis and MT. Mb Crohn`s disease and ulcerative colitis are featured by structural damage in the gut wall originating from autoimmune inflammation, and an abnormal shift in the gut microbiome is found during both active and inactive disease. A successful attempt to achieve clinical remission and endoscopic improvement with FMT in an otherwise treatment refractory patient with Mb Crohn was recently reported[189], and remission rates after FMT in patients with ulcerative colitis diagnosed within one year before intervention were superior as compared to more durable disease in a randomized study[190]. These findings warrant more randomized studies in IBD field. FMT as a therapeutic option in metabolic diseases with dysbiotic gut microbiota like diabetes type 2 and obesity has also been proposed as a plausible way to approach alternative treatments for these conditions[191]. For instance, allogenic FMT from lean donors was followed by increased peripheral insulin sensitivity for a period of 6 weeks in patients with hypermetabolic syndrome[192], and several clinical studies on FMT as treatment of obesity are ongoing (www.clincaltrials.gov).

We acknowledge that all results from gut microbiota studies are influenced by external factors. Diet, intake of antibiotics and alcohol all have a major impact[165,193-195], and both sexual practices and exercise modulate the composition and functionality of gut microbiota[196,197]. Travelling has also been associated with rapid shifts in microbiota composition[198]. Additionally, acute conditions like gastroenteritis and many chronic diseases may all contribute to alternations of microbiota. All of these confounding factors are seldom considered in reports from microbiome studies due to the complexity behind these metadata. This may to some extent explain why the results from gut microbiota analyses from different populations and studies are not unified.

7 CONCLUSIONS

HIV-1 infected individuals have elevated levels of microbial translocation (MT) markers, with the exception of Elite controllers. Introduction of ART is followed by declining levels of MT, though not normalized under long term follow up (up to 72 weeks). The choice of ART regimen affects the kinetics of some MT markers. Concomitant use of ART and antibiotics seems to have an additional impact on microbial translocation.

Moreover, we show that gut microbiota dysbiosis features HIV-1 infection, with lower number of represented taxa and reduced intraindividual (α-) diversity. Furthermore, CD4+ T-cell counts and α-diversity are positively correlated in untreated subjects. The inter-individual differences (ß-diversity) are highest in HIV-1 patients with progressive disease, whereas the gut microbiota variability in Elite controllers is much lower, and the gut bacterial composition in these patients is different from other HIV-1 infected patients.

Abundance of several taxa is altered in HIV-1 infected as compared to negative controls, furthermore differences are present also between EC and other HIV-1 infected individuals. Following ART, α-diversity is further reduced and composition modified at genus level.

Functional analyses of the gut bacterial metagenome reveal that Elite controllers have a unique enteric metabolic profile. Bacterial genes encoding carbohydrate metabolism pathways are less abundant compared to both other HIV-subjects and negative controls. Additionally, abundance of pathways related to metabolism of lipids and fatty acids are different in EC from HIV-1 progressors. As metabolites from bacterial digestion of nutrients are deeply involved in the development and control of the human immune system, we suggest that the gut microbiota in Elite controllers may be one of the factors involved in the sustained virological and immunological control observed in this minor set of HIV-1 infected individuals. We also propose that fecal microbiota modulation by supplementation of pre/probiotics or fecal microbiota transplantation may have positive influence on inflammation related HIV pathogenesis in treated HIV-1 patients.

8 FUTURE PLANS AND PERSPECTIVES

The data in this doctoral thesis confirm that microbial translocation features HIV-1 infection even in well treated individuals, and that the bacterial composition and its inferred functionality are profoundly altered as compared to uninfected individuals. Additionally, the gut microbiota in Elite controllers is different from both uninfected and other HIV infected subjects.

As metagenomics analyses suggest that the relation between carbohydrate and lipid metabolism is differential in Elite controllers controlling their infection, further metabolomics studies are warranted in order to estimate the levels of fecal metabolites, e.g. tryptophan catabolites, in the different populations. If such differences are confirmed, it should be explored in what specific mechanisms these metabolites modulate immune cells. One way to proceed could be to investigate the transcriptome of immune cells like CD4/8+ T-cells, B-cells and innate immune cells, and correlate the transcriptome to differential bacterial taxa or metabolites.

Additionally, we found that the richness of the gut microbiota was inversely correlated to K/T ratio, which reflects IDO1 activity that is contributing to hyperinflammation. So far, most interventional studies on probiotics supplementation have focused on suspensions containing single or very few bacterial species. Our observation indicates that alternative therapeutic interventions modulating gut microbiota richness and not only composition are warranted in order to reduce HIV-related inflammation.

9 SAMMANFATTNING PÅ SVENSKA

Trots effektiv behandling med bromsmedicinering kvarstår en kronisk aktivering av immunförsvaret samt låggradig inflammation vid HIV-1 infektion. En viktig mekanism som driver inflammationen är s.k. mikrobiell translokation (MT). Detta innebär att delar från tarmens bakterier, svamp eller parasiter läcker över en av HIV skadad tarm-blod barriär. De mikrobiella komponenterna aktiverar i kroppen både det medfödda och förvärvade immunförsvaret. Detta återföljs av ökad inflammation som anses bidra till den förhöjda risk för sena komplikationer i form av t.ex. hjärt-kärlsjukdom, maligniteter och kognitiva funktionsnedsättningar som observerats hos HIV-patienter.

Syftet med detta doktorandprojekt har varit att studera hur insättande av olika bromsmediciner påverkar graden av MT, samt även hur samtidig användning av antibiotika påverkar MT. Därtill även att undersöka tarmflorans sammansättning vid obehandlad HIV-1 infektion, samt efter insatt bromsmedicinering. Slutligen ville vi även kartlägga tarmflorans sammansättning samt biologiska funktionalitet hos s.k. Elite controllers, sällsynta HIV-1 patienter som utan bromsmedicinering själva kan kontrollera sitt virus och upprätthålla normalt immunförsvar i upp mot 30 år.

I **delarbete I**, en randomiserad klinisk studie, startade obehandlade HIV-1 patienter bromsmedicinering baserad på efavirenz eller lopinavir. Blodmarkörer för MT var förhöjda innan behandling, sjönk efter 72 veckor men var även då förhöjda jämfört med historiska friska kontrollpersoner. Vissa skillnader beträffande nivån på MT markörer observerades mellan de olika typerna av bromsmedicinering. Därutöver påvisades också en signal indikerande att antibiotika gynnsamt påverkade aktiveringen av det medfödda immunförsvaret. Resultaten från denna studie talar för att val av HIV preparat, samt eventuellt också användning av antibiotika kan ha betydelse för inflammationsutvecklingen hos HIV-1 patienter på sikt.

I **delarbete II** påbörjade obehandlade HIV-1 patienter bromsmedicinering med eller utan samtidig trim-sulfa behandling (antibiotika förebyggande mot *Pneumocystis jirovecii* lunginflammation). Även här konstaterades förhöja markörer för MT i blod innan behandling. Patienter som erhöll samtidig behandling med trim-sulfa hade en bättre nedgång av vissa MT markörer, även efter justering för relevanta variabler som grad av immundefekt (antal CD4+ celler) och HIV-1 virusmängd i blod. Resultaten i detta arbete antyder att antibiotika-användning kan påverka markörer för MT, vilket man bör beakta vid tolkning av studier som undersöker MT vid HIV-1 infektion.

I **delarbete III** kartlades den bakteriella tarmfloran hos HIV-1 patienter med 16S rRNA-sekvensering innan, samt efter knappt ett års behandling med bromsmedicinering. Obehandlade patienter uppvisade en lägre bakteriell diversitet (artrikedom) i tarmen, mätt som antal samt proportioner av påvisade arter hos den enskilde individen (alfa-diversitet),

samt därutöver återfanns även en ökad variation av arter på gruppnivå (beta-diversitet). Efter påbörjad bromsmedicinering kunde ingen förbättring av diversiteten iakttas. Alfa-diversiteten var direkt korrelerad till immunstatus mätt som antal (CD4+) immunceller i blod. Flertalet skillnader mellan HIV-1 infekterade och friska kontrollpersoner observerades både på bakteriers släkt- och artnivå, med bl a ökad förekomst av *Lactobaciller* och minskad frekvens av *Lachnobacterium*, *Faecalibacterium* och *Hemophilus* hos HIV-1 infekterade. Detta arbete visar att förändringar av tarmfloran kan kopplas till grad av immundefekt vid HIV-1 infektion, och att förändringarna i tarmfloran består trots nästan ett års behandling med bromsmediciner.

I **delarbete IV** undersöktes den bakteriella tarmflorans sammansättning och funktionalitet baserat på 16S rRNA-sekvensering med PICRUSt analys hos 16 HIV-1 infekterade Elite controllers, hos HIV-1 infekterade med normalt framskridande sjukdom samt HIV negativa kontroller. Till skillnad från övriga HIV-1 infekterade hade Elite controllers tarmflora samma alfa-diversitet som och även störst likhet med floran hos HIV negativa kontrollpersoner. Flera bakteriella släkten var överrepresenterade i tarmfloran hos Elite controllers jämfört med övriga, såsom *Sutterella* samt *Succinivibrio*, och även flertalet skillnader mellan Elite controllers och övriga HIV-1 infekterade kunde identifieras. Vid funktionsanalys av tarmfloran konstaterades att den bakteriella andelen av floran som är inblandad i metabolism av kolhydrater var lägre hos Elite controllers, medan dessa individer istället hade en högre andel bakterier inblandade i metabolism av fetter och olika fettsyror jämfört med övriga HIV-1 infekterade. Denna studie visar således att Elite controllers har en unik tarmflora med en funktionalitet som avviker både från övriga HIV-1 infekterade samt HIV negativa kontrollpersoner. Detta kan möjligen bidra till dessa individers förmåga till att upprätthålla en långvarig egenkontroll av HIV-infektionen.

Sammanfattningsvis visar avhandlingens studier att bromsmedicinering och eventuellt antibiotika minskar graden av MT, samt att val av bromsmedicinering kan vara av betydelse. Dessutom har HIV-1 infekterade individers tarmflora en avvikande sammansättning och funktion, men Elite controllers skiljer sig från övriga HIV-1 infekterade då de besitter en unik tarmflora som möjligen bidrar till deras långvariga kontroll av HIV-1 infektionen.

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