UNIVERSIDAD DE LA RIOJA

TESIS DOCTORAL

Título
Effects of different antiretroviral treatments on gut microbiota of HIV-infected patients
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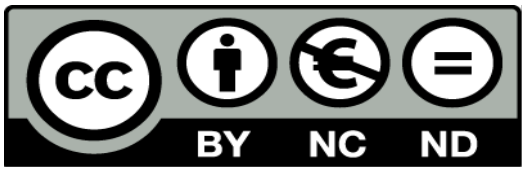
Curso Académico



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Effects of different antiretroviral treatments on gut microbiota of HIV-infected patients

Doctoral thesis

María Jesús Villanueva Millán

2018

Effects of different antiretroviral treatments on gut microbiota of HIV-infected patients

Dissertation for the Degree of Doctor of Philosophy (PhD) with the mention of International Doctor

By

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Logroño, 2018

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Por la presente declaran que:

La memoria titulada **"Efectos de los diferentes tratamientos antirretrovirales sobre la microbiota intestinal de pacientes infectados por el VIH" ("*Effects of different antiretroviral treatments on gut microbiota of HIV-infected patients*")**, que presenta Dña. María Jesús Villanueva Millán, licenciada en Ciencias Biológicas, ha sido realizada en el Centro de Investigación Biomédica de La Rioja (CIBIR) bajo su dirección y reúne las condiciones específicas para optar al grado de Doctor con la mención de "Doctor Internacional".

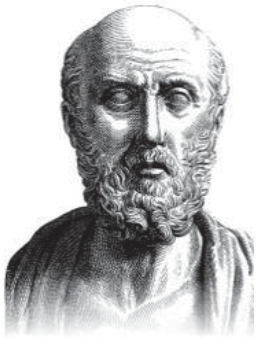
Para que conste donde proceda en Logroño, a 22 de mayo de 2018.

Fdo.: Dr. José A. Oteo Revuelta Fdo.: Dra. Patricia Pérez Matute

A miña nai

La realización de esta tesis doctoral ha sido posible gracias a la beca otorgada por la Consejería de Industria, Innovación y Empleo del Gobierno de La Rioja, beca que he disfrutado durante cuatro años y me ha permitido desarrollar los trabajos que aquí se exponen en el Centro de Investigación Biomédica de La Rioja (CIBIR). Además, los estudios llevados a cabo en humanos incluidos en la presente Tesis Doctoral han sido financiados por Gilead Sciences y el grupo SEINORTE.

La estancia predoctoral se ha podido realizar gracias a la beca (EMBO Short-Term Fellowship) concedida por The European Molecular Biology Organization (EMBO).



Hippocrates

"All disease begins in the gut"

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Abbreviations

AIDS	Acquired immune deficiency syndrome
ALT	Alanine aminotransferase
ART	Antiretroviral treatment
ARV	Antiretroviral
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
BBH	Best blast hit
BT	Bacterial translocation
CCL5	Chemokine (C-C motif) ligand 5
CCR5	C-C chemokine receptor type 5
CDC	Centers for disease control and prevention
CFU	Colony forming units
CSS	Cumulative sum scaling
CVD	Cardiovascular disease
CXCR4	C-X-C chemokine receptor type 4
DBP	Diastolic blood pressure
DMSO	Dimethyl sulfoxide
EACS	European AIDS Clinical Society
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assays

F0	No fibrosis
F1	Portal fibrosis without septa
F2	Portal fibrosis and few septa
F3	Numerous septa without cirrhosis
F4	Cirrhosis or advanced fibrosis
FDA	Food and Drug Administration
FDR	False discovery rate
GALT	Gut-associated lymphoid tissue
GESIDA	AIDS Study Group of the Spanish Society of Infectious Diseases and Clinical Microbiology
GM	Gut microbiota
Gp41	Glycoprotein 41
Gp120	Glycoprotein 120
HALS	HIV-associated lipodystrophy syndrome
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDL	High-density lipoprotein
HDV	Hepatitis D virus
HEV	Hepatitis E virus
HFD	High fat diet
HFD+MVC	High fat diet with maraviroc in drinking water

HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HIV+(cART)	HIV-infected patients on combined antiretroviral therapy
HIV+(naive)	Untreated HIV-infected patients
HIV+MS-	HIV-infected patients without metabolic syndrome
HIV+MS+	HIV-infected patients with metabolic syndrome
HMP	Human microbiome project
HOMA-IR	Homeostasis model assessment insulin resistance
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HS	Heterosexual
HSV	Herpes simplex virus
I-FABP	Intestinal fatty acid binding protein
ICAM	Intercellular adhesion molecule
IBD	Inflammatory bowel diseases
IBS	Irritable bowel syndrome
IFN	Interferon
IgA	Immunoglobulin A
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-17	Interleukin 17

IL-22	Interleukin 22
IVDU	Intravenous drug user
IVDU/HS	Intravenous drug user and multiple heterosexual contacts
LBP	Lipopolysaccharide binding protein
LCA	Lowest common ancestor
LDL	Low-density lipoprotein
LHMP	Lung HIV Microbiome project
LPS	Lipopolysaccharide
M-C	Mother to child
M cells	Membranous or microfold cells
MCP-1	Monocyte chemoattractant protein-1
MetaHit	Metagenomes project of the human intestinal tract
mRNA	Mitochondrial RNA
MEGAN	MEtaGenome Analyzer
MIC	Minimal inhibitory concentration
MS	Metabolic syndrome
MSM	Men who have sex with men
MSR	Macrophage scavenger receptor
MyD88	Myeloid differentiation primary response gene 88
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis

NCEP-ATP III	National Cholesterol Education Program Adult Treatment Program III
Nef	Negative regulating factor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next generation sequencing
OTUs	Operational taxonomic units
PAI-1	Plasminogen activator inhibitor-1
PCA	Principal component analysis
RDP	Ribosomal Database Project
Rev	RNA splicing-regulator
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SARM	Sterile α and HEAT-Armadillo motifs-containing protein
SBP	Systolic blood pressure
sCD14	Soluble CD14 molecule
SCFAs	Short-chain fatty acids
SIV	Simian immunodeficiency virus
SIVcpz	Simian immunodeficiency virus of chimpanzees (*Pan troglodytes*)
SIVgor	Simian immunodeficiency virus of gorillas (*Gorilla gorilla*)
Streptavidin-PE	Streptavidin-phycoerythrin

Tat	Transactivator protein
Th17	T-helper 17 cells
TIR	Toll-interleukin-1 receptor
TIRAP	Toll-interleukin-1 receptor domain-containing adaptor protein, MyD88-adapter-like
TNFα	Tumor necrosis factor alpha
TLRs	Toll-like receptors
TRAM	Toll-interleukin-1 receptor domain-containing adaptor inducing interferon beta-related adaptor molecule
Tregs	Regulatory T cells
TRIF	Toll-interleukin-1 receptor domain-containing adaptor inducing interferon beta
VCAM	Vascular cell adhesion molecule
Vif	Viral infectivity factor
Vpr	Virus protein r
Vpu	Virus protein unique
Vpx	Virus protein x
WHO	World health organization

ABBREVIATIONS REGARDING ANTIRETROVIRAL TREATMENTS

3TC	Lamivudine
ABC	Abacavir
ATV	Atazanavir
AZT	Zidovudine
BIC	Bictegravir
cART	Combined antiretroviral therapy
COBI	Cobicistat
d4T	Stavudine
ddI	Didanosine
DTG	Dolutegravir
DRV	Darunavir
EFV	Efavirenz
ENF	Enfuvirtide
ETR	Etravirine
EVG	Elvitegravir
FPV	Fosamprenavir
FTC	Emtricitabine
IDV	Indinavir
INSTIs	Integrase strand transfer inhibitors
MVC	Maraviroc
NRTIs	Nucleoside reverse transcriptase inhibitors

NRTIs+INSTIs	Nucleoside reverse transcriptase inhibitors and integrase strand transfer inhibitors combination
NRTIs+NNRTIs	Nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors combination
NRTIs+PIs	Nucleoside reverse transcriptase inhibitors and protease inhibitors combination
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NVP	Nevirapine
LPV	Lopinavir
PIs	Protease inhibitors
RAL	Raltegravir
RPV	Rilpivirine
RTV	Ritonavir
SQV	Saquinavir
TAF	Tenofovir alafenamide
TDF	Tenofovir disoprovil fumarate
TPV	Tipranavir
ZVD	Zidovudine

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ABSTRACT

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ABSTRACT

MARÍA JESÚS VILLANUEVA MILLÁN

Effects of different antiretroviral treatments on gut microbiota of HIV-infected patients

The human gut microbiota has a symbiotic relationship with the host and plays a crucial role in the maintenance of health. HIV infection has been associated with a disturbance in gut microbiota (dysbiosis). Increased bacterial translocation and alterations to gut microbiota composition have been observed in HIV-infected patients and contribute to immune activation and inflammation. This Doctoral Thesis demonstrates, in clinical practice, that not only HIV infection has effects on gut physiology and microbial profile, but also different combined antiretroviral therapies modify gut microbiota composition. From all the combinations tested in this study, INSTI-based antiretroviral therapy was associated with levels of systemic inflammation and bacterial translocation similar to uninfected controls, suggesting a healthier gut and potentially lesser HIV-related complications. *In vitro*, Maraviroc did not exert any bacteriostatic effect in the tested strains, and no significant effects were either found in gut microbiota composition when administered to mice fed a standard diet, while several and interesting actions were observed when administered to high-fat diet fed-mice. Although Maraviroc is not actually prescribed as monotherapy, if its immunological actions could be potentiated if administered along with a high fat diet deserves further investigation. Finally, we have also demonstrated that other factors that increase the morbidity and mortality of these patients, such as the coinfection with hepatotropic viruses and the metabolic syndrome, also affects the gut flora, although to a lesser extent than HIV infection itself. However, these effects are not mild and highlight the need for

monitoring these patients even after immunological control with combined antiretroviral therapies.

RESUMEN

MARÍA JESÚS VILLANUEVA MILLÁN

Efectos de los diferentes tratamientos antirretrovirales sobre la microbiota intestinal de pacientes infectados por el VIH

La microbiota intestinal tiene una relación simbiótica con el hospedador y juega un papel muy importante en el mantenimiento de la salud. La infección por el VIH induce una disbiosis intestinal. Varios estudios han demostrado que los pacientes infectados por el VIH presentan alteraciones en la integridad y funcionalidad del tejido gastrointestinal, un incremento en la translocación bacteriana y alteraciones en la composición de la microbiota intestinal, todo lo cual contribuye a una inmunoactivación y, con ello, a un estado inflamatorio crónico. Esta Tesis Doctoral demuestra que no solamente la infección por el VIH tiene efectos sobre la fisiología intestinal y el perfil de la microbiota, sino que también las diferentes terapias antirretrovirales empleadas en la práctica clínica pueden alterar la composición de la microbiota intestinal. De todas las terapias analizadas en este estudio, la basada en los inhibidores de la integrasa se asoció con niveles de inflamación sistémica y translocación bacteriana similar a la de los controles no infectados por el VIH, lo que sugiere la presencia en estos pacientes de un intestino más sano y una menor probabilidad de desarrollo de complicaciones relacionadas con el VIH. *In vitro*, el fármaco antirretroviral Maraviroc no ejerció ningún efecto bacteriostático en las cepas testadas, y tampoco se encontraron efectos significativos en la composición de la microbiota intestinal cuando se administró en ratones alimentados con una dieta estándar. Sin embargo, este fármaco unido a la ingesta de una dieta alta en grasa se asoció con varias alteraciones del perfil microbiano

gastrointestinal. En este sentido, aunque Maraviroc no se prescribe actualmente como monoterapia, sus acciones inmunológicas podrían ser potenciadas al administrarse junto con una dieta alta en grasa. Esta observación debería ser investigada en el futuro. Finalmente, se ha demostrado que otros factores que incrementan la morbi-mortalidad de estos pacientes, como la coinfección por virus hepatotropos y el síndrome metabólico, también afectan a la composición de la flora intestinal, aunque en menor medida que la propia infección por el VIH. Sin embargo, estos efectos no son leves y parecen resaltar la necesidad de monitorizar estos pacientes incluso después del control inmunológico con las terapias antirretrovirales.

1. Introduction

1.1. Microbiota

1.2. HIV infection

1.1. Microbiota

The human body is a perfect culture medium providing nutrients and an environment that maintain the growth of a wide variety of microorganisms. These microbial species comprise what is known as "*human microbiota*" (Jorth *et al.,* 2014;Lynch and Pedersen 2016). Thus, microbiota is defined as the community of microorganisms inhabiting a specific environment, including bacteria, archaea, viruses, and some unicellular eukaryotes. The term microbiome refers to the genomic content of the microbiota (reviewed by Morgan *et al*., (2013)) (Morgan *et al.,* 2013). The human gut microbiome is considered as the second genome of human body, since includes approximately 3.3 million of genes, 150 times more genes than our own genome which contains about 20,000-25,000 genes and whose functions are not yet fully known (Zerbino *et al.,* 2018;Zhu *et al.,* 2010). For this reason, humans are also referred to as "**superorganisms**" (Gill *et al.,* 2006).

The human microbiota is composed mainly of four bacterial phyla: Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria (Ley *et al.,* 2008). The relative abundance of these and other bacterial phyla varies depending on several factors such as different parts of the body (skin, respiratory and urogenital systems, gut, etc.), age, diet, antibiotic intake, geographic location and stress (Figure 1) (Bailey *et al.,* 2011;Morgan *et al.,* 2013;Ursell *et al.,* 2012). Thus, there is a high intra-individual variability, which makes it difficult to establish the exact composition of what is considered as a "normal" (healthy) microbiota (Monda *et al.,* 2017). In this context, international multicenter studies developed by different countries and consortiums attempt to describe this "normal" microbiota. Some of the most relevant projects include the Metagenomes project of the Human

Intestinal Tract (MetaHit) (*www.metahit.eu*) carried out in Europe and Asia and the Human Microbiome Project (HMP) (*https://hmpdacc.org*) conducted in the United States of America (USA). Thanks to the MetaHIT consortium, the human intestinal ecosystem has been classified into three large groups, called enterotypes, according to the relative abundance of three genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3) (Arumugam *et al.,* 2011). These enterotypes have been linked to long-term diets. Specifically, the enterotype 1 is associated with diets rich in protein and animal fat, while subjects with diets based on carbohydrates and simple sugars belong to enterotype 2 (Wu *et al.,* 2011).

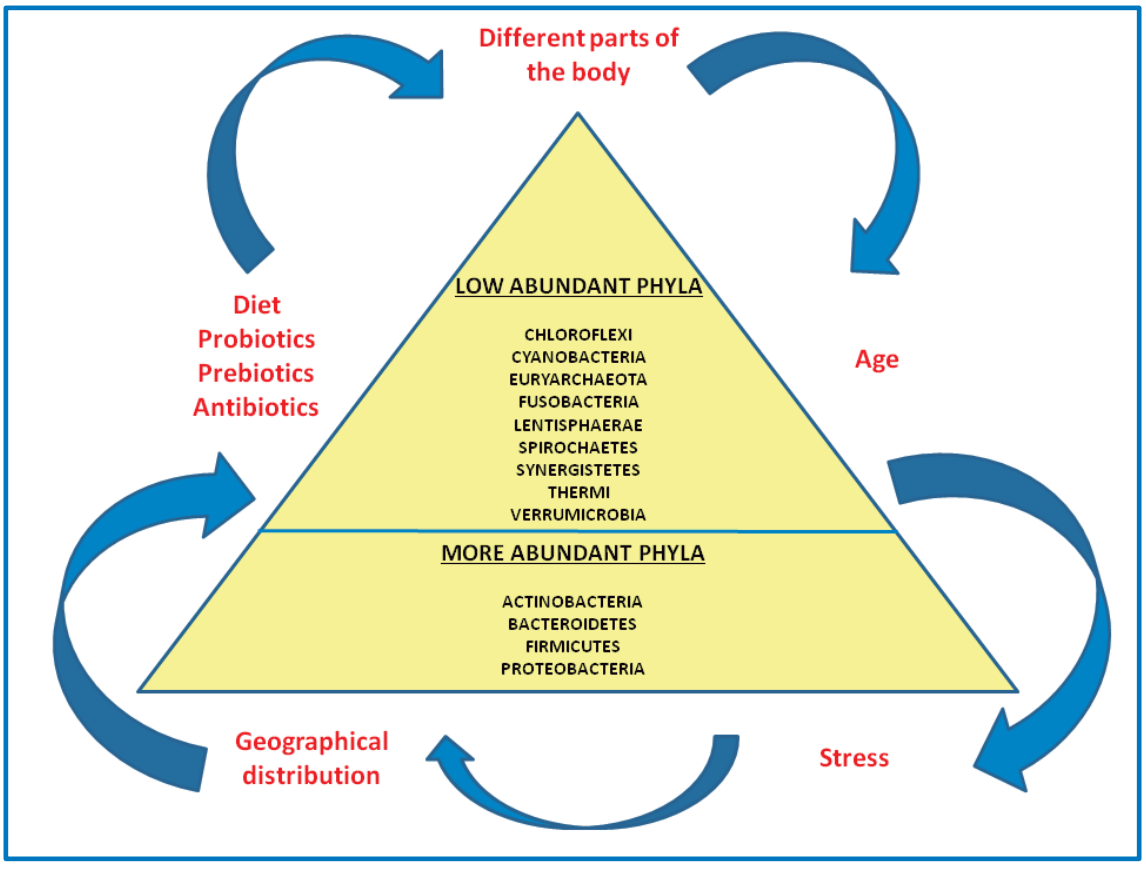


Figure 1. Abundance of the main bacterial phyla in humans and factors that modulate the abundance of these phyla.

Over the years, different techniques have been used to study the microbiota, such as staining techniques, electron microscopy, bacterial culture or PCR techniques. However, conventional microbiology techniques are very limited and only a few species can be studied, due to the fact that the vast majority of bacterial species (60-80%) are not cultivable. Fortunately, nowadays, thanks to the development of next generation sequencing (NGS), also known as high-throughput sequencing, more than 400-500 bacterial species have been identified, which has made possible to overcome the limitations of culture-dependent methods. Therefore, NGS provides a more accurate picture of the bacterial community, being the metagenomic sequencing analysis the gold standard approach. This technique allows the identification of the taxonomic profile of a microbial community. Today, two different approaches are available to carry out the metagenomic analysis: the 16S ribosomal RNA (rRNA) gene sequencing and the whole genome shotgun sequencing (WGS), although the 16S rRNA sequencing is the most popular molecular tool used for this aim. Bacterial 16S rRNA genes comprise nine "hypervariable regions" (V1–V9) that exhibit considerable sequence diversity among different bacteria (Chakravorty *et al.,* 2007;Ranjan *et al.,* 2016). A different percentage of taxa recovery is obtained depending on the 16S rRNA region sequenced. In general, the sequencing of the V3-V4 regions is the most widely used today. However, more accurate estimations of bacterial classifications can be obtained when longer segments or the whole genome are considered (Yarza *et al.,* 2014). There are other concepts that are also important when the microbiota is studied. Metatranscriptomics and metabolomics techniques indicate which genes are expressed in the community and which metabolites are released by these microorganisms (Aguiar-Pulido *et al.,* 2016). These "omics" approaches can offer an integrated study of microbial community to establish the role of each

participant, how they change under different physiological and pathologic conditions and how the members of a microbial community interact with each other and with their environment (Turnbaugh and Gordon 2008). Therefore, understanding the association of a disorder with the microbiome, metatranscriptome and metabolome may guide to improve disease management and for the development of new therapies. Besides the study of bacteria, these omics technologies make possible to study the virus (virome) and fungal (mycobiome) which also play important roles in human health and disease (Conceicao-Neto *et al.,* 2015;Seed 2014).

1.1.1. Gut microbiota

For many years it was estimated that the human body has approximately 10^{14} bacteria, being the number of bacteria within the gastrointestinal tract 10 times higher than the cells of the human body. In fact, the gastrointestinal tract houses up to 1,000 different bacterial species which includes about two million genes (Azad *et al.,* 2013;Fujimura *et al.,* 2010;Harris *et al.,* 2012;Quigley 2013). However, the exact number of human and bacterial cells in the organism has been reviewed and recalculated in a recent study carried out by Sender *et al*., (2016). In this article, authors have revised the estimations taken into account the most up-to-date information about the number of human and bacteria cells in the body published by previous works. After their recalculation, they concluded that the number of bacteria in the body is actually of the same order as the number of human cells (Sender *et al.,* 2016). Anyway, the topic is under discussion.

From oral cavity to anus, bacteria density and diversity increases in the jejunum/ileum and in the large intestine in comparison with the stomach and duodenum. Therefore, the highest cell density is found in the colon. Concretely, there are approximately 10^{12} CFU/mL, with anaerobic bacteria

outnumbering aerobic bacteria by a factor of 100 to 1,000:1. In fact, the bacteria present in the colon are mainly anaerobes due to the low concentrations of oxygen present there; the microbiota has simply adapted to survive in this hostile environment (Figure 2). At any given level of the gut, the microbiota composition also varies along its diameter, with certain bacteria tending to be adherent to the mucosal surface, while others predominate in the lumen. Bacteria residing at the mucosal surface or within the mucus layer are those most likely to participate in interactions with the host immune system, while those present in the lumen may be more relevant to metabolic interactions with food or other products of digestion (reviewed by Quigley 2013) (Quigley 2013).

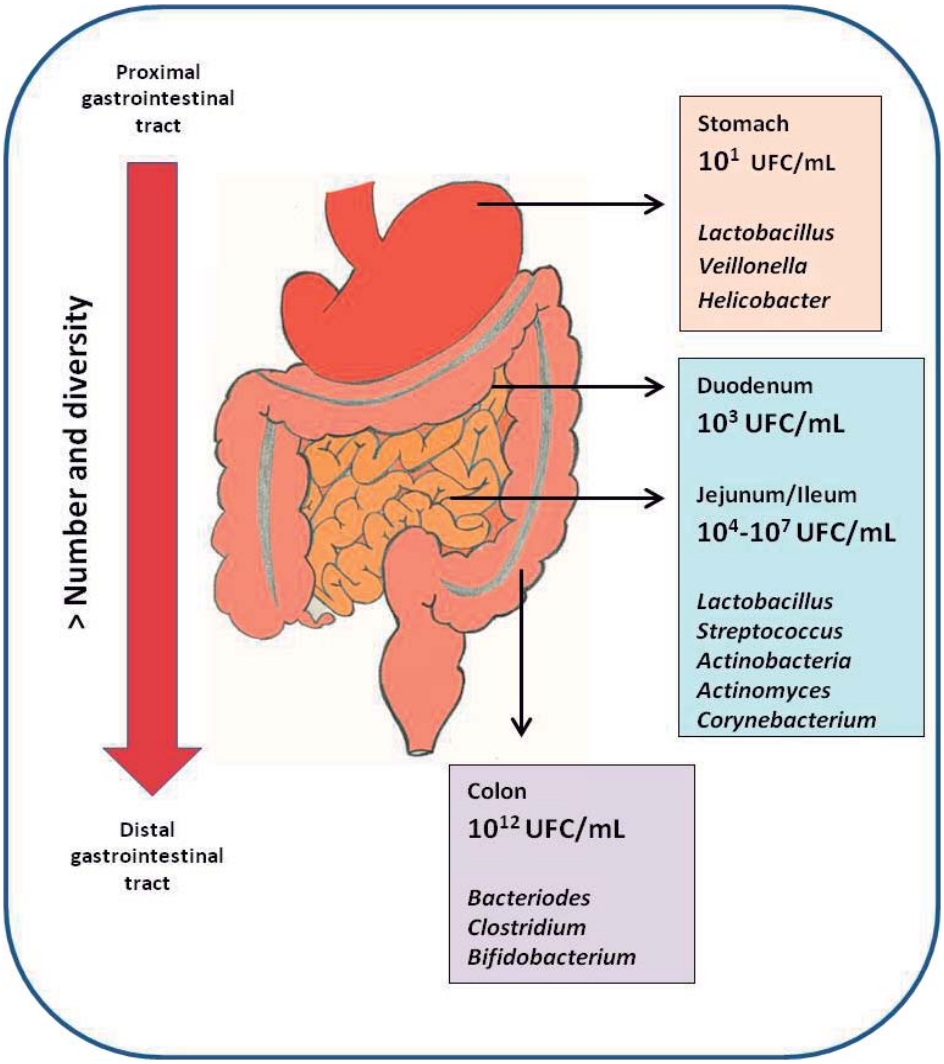


Figure 2. Bacterial distribution along the gastrointestinal tract.

The human gut commensal microbiota maintains a symbiotic relationship with the host, allowing the balanced induction of protective responses to antigens and pathogens (Malys *et al.,* 2015). Although there are several aspects to be elucidated, it is known that microbiota play a crucial role in the maintenance and development of the immune system, in the metabolism and in the body homeostasis in general (Thursby and Juge 2017). More specifically, among other functions, intestinal microbiota participates

in nutrient digestion producing short-chain fatty acids (SCFA), synthesis of vitamin K and folic acid, metabolism of bile salts, drugs and xenobiotics, gut motility, prevention of the invasion of pathogenic microorganisms, proliferation and differentiation of the intestinal epithelium and also participates in the development and modulation of the immune system (Quigley 2013). Because of the pivotal importance of these functions, the microbiota is considered the **last organ of the body** (Baquero and Nombela 2012).

1.1.1.1. Functions of the gut microbiota

1.1.1.1.a. Metabolism

Gut microbiota (GM) is an important factor that affects energy harvest from the diet and energy storage in the host. Indeed, GM exerts a strong influence on host lipid and cholesterol metabolism. In order to carry out these metabolic functions, the enzymes from intestinal microbiota such as glycoside hydrolases and polysaccharide lyases have the ability to extract energy from inaccessible nutrients, as these enzymes are able to cleave glycoside linkages present in some plant polysaccharides and dietary fibers (Backhed *et al.,* 2004;Backhed *et al.,* 2005). The indigestible polysaccharides, as oligofructose (inulin), are metabolized by colonic microbiota to oligosaccharides and monosaccharides and then fermented to SCFA, particularly to acetate, propionate, and butyrate. Butyrate provides energy for cellular metabolism, while acetate and propionate can act as substrates for gluconeogenesis and lipogenesis (Shen *et al.,* 2013). Microbiota also influences host cholesterol metabolism and several mechanisms have been proposed for these actions. Thus, bacterial conversions of bile acids (such as the formation of secondary bile acids) are likely to play a role, as they affect

enterohepatic circulation, *de novo* synthesis of bile acids, emulsification, and cholesterol absorption (Martinez *et al.,* 2009).

GM also contributes to synthesize essential amino acids and vitamins, such as folate (Brenchley and Douek 2012;Gill *et al.,* 2006).

Finally, GM is also able to perform a range of biotransformations on xenobiotics, such as drugs and their metabolites, in ways that can affect their absorption and bioavailability (Wilson and Nicholson 2009).

1.1.1.1.b. Protective role

A pivotal role of GM is to protect the intestine against colonization by exogenous pathogens and potentially harmful indigenous microorganisms. Some of the mechanisms involved in these protective actions include direct competition for limited nutrients and the modulation of host immune responses (Kamada *et al.,* 2013). The intestinal mucus layer is able to keep mutualism by keeping bacteria at bay and restricting overt immune stimulation (Purchiaroni *et al.,* 2013). Bacteria can inhibit the growth of their competitors by producing antimicrobial peptides or proteins known as bacteriocins (Guarner and Malagelada 2003). Concerning the influence of microbiota in the host immune system, accumulating evidence indicates that microbiota regulates the development and/or function of different types of immune cells in the intestine. For instance, some members of the microbiota, such as specific species of Clostridia-related bacteria and *Bacteroides fragilis*, can facilitate beneficial immune responses through the development of steady-state T helper 17 lymphocytes and the induction of regulatory T cells (Tregs), respectively (Kamada and Nunez 2013). Moreover, commensal microbiota induces maintaining host-intestinal microbial T cell mutualism (Geuking *et al.,* 2011).

1.1.1.1.c. Trophic role

GM modulates the proliferation and differentiation of colonic epithelial cells through the SCFA produced. In this context, butyrate is considered as the SCFA with the strongest effect. Butyrate can modify the microstructure of the small and large intestine and it is able to accelerate intestinal mucosa maturation during the development or even to induce its repair after injury. In addition, butyrate reduces apoptosis of normal enterocytes in the small intestine through its influence on gene expression and protein synthesis (Guilloteau *et al.,* 2010).

1.1.1.1.d. Other functions

Gut is a highly innervated organ that possesses its own nervous system known as the enteric nervous system that is in constant communication with the central nervous system. GM produces neuroactive substances such as catecholamines, histamine, and other biologically active substances, including neuromodulators and neurotransmitters, that can stimulate host neurophysiology either through direct interaction with receptors within the gastrointestinal tract or following absorption/passive diffusion through the gut wall and entering into the portal circulation (Lyte 2013;Wang *et al.,* 2010). Recently, it has been shown that cerebral metabolites are influenced by normal intestinal microbiota through the microbiota-gut brain axis and indicates that normal intestinal microbiota is closely connected with brain health and disease, development, attenuation, learning, memory, and behavior (Matsumoto *et al.,* 2013).

Apart from the microbiota-gut-brain axis, it is also important to mention the gut-liver axis, which refers to the close anatomical and functional relationship between the gastrointestinal tract and the liver. Such

association includes transfer of molecules associated with the gut microbiome to the liver and on the other way round. Non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease, and its incidence is increasing worldwide. Growing body of evidence has begun to indicate that gut-liver axis malfunction and specifically small intestinal bacterial overgrowth, intestinal dysbiosis, and increased intestinal permeability is a leading factor in the development and progression of NAFLD and obesity (Paolella *et al.,* 2014).

1.1.1.2. Establishment of gut microbiota

For many years it was believed that the gastrointestinal tract was essentially sterile at birth. However, some authors have shown that meconium houses a complex microbial community. Thus, the fetal intestine may be in contact with microbes present in the swallowed amniotic fluid (Ardissone *et al.,* 2014;Gosalbes *et al.,* 2013;Hu *et al.,* 2013). The intestinal microbiota of neonates is subsequently colonized by microbes acquired from the mother and the surrounding environment and by the end of the first year infants have an individually distinct microbial profile (Mackie *et al.,* 1999;Rodriguez *et al.,* 2015). The composition of the neonatal microbiota in early life is influenced by several factors such as the type of birth (vaginal birth *vs*. caesarean), diet (breast milk *vs*. formula), sanitary conditions, antibiotics, and supplementation with prebiotics and/or probiotics (Mueller *et al.,* 2015;Rautava *et al.,* 2012). It is important to highlight that this initial microbiota establishment could have an impact in the risk of developing several childhood diseases that may persist to adulthood such as asthma, allergic disorders, chronic immune-mediated inflammatory diseases, type 1 diabetes, obesity, and eczema (Hoskin-Parr *et al.,* 2013;Munyaka *et al.,* 2014;Rodriguez *et al.,* 2015). Once microbiota has been established in these

early years, the microbial composition increases in both diversity and richness. It reaches the highest complexity in adults, with several hundred species-level phylotypes. Each human individual reaches a homeostatic climax composition, which likely remains relatively stable during most of a healthy adult's life. However, GM can be punctually modified by different factors, such as the use of antibiotics, probiotics, or prebiotics, stress, etc. At the late stages of life, the microbiota show greater inter-individual variation, being less diverse and more dynamic and characterized by a higher Bacteroides to Firmicutes ratio, increases in Proteobacteria and decreases in *Bifidobacterium* (Gur *et al.,* 2015;Ottman *et al.,* 2012;Rodriguez *et al.,* 2015) (Figure 3).

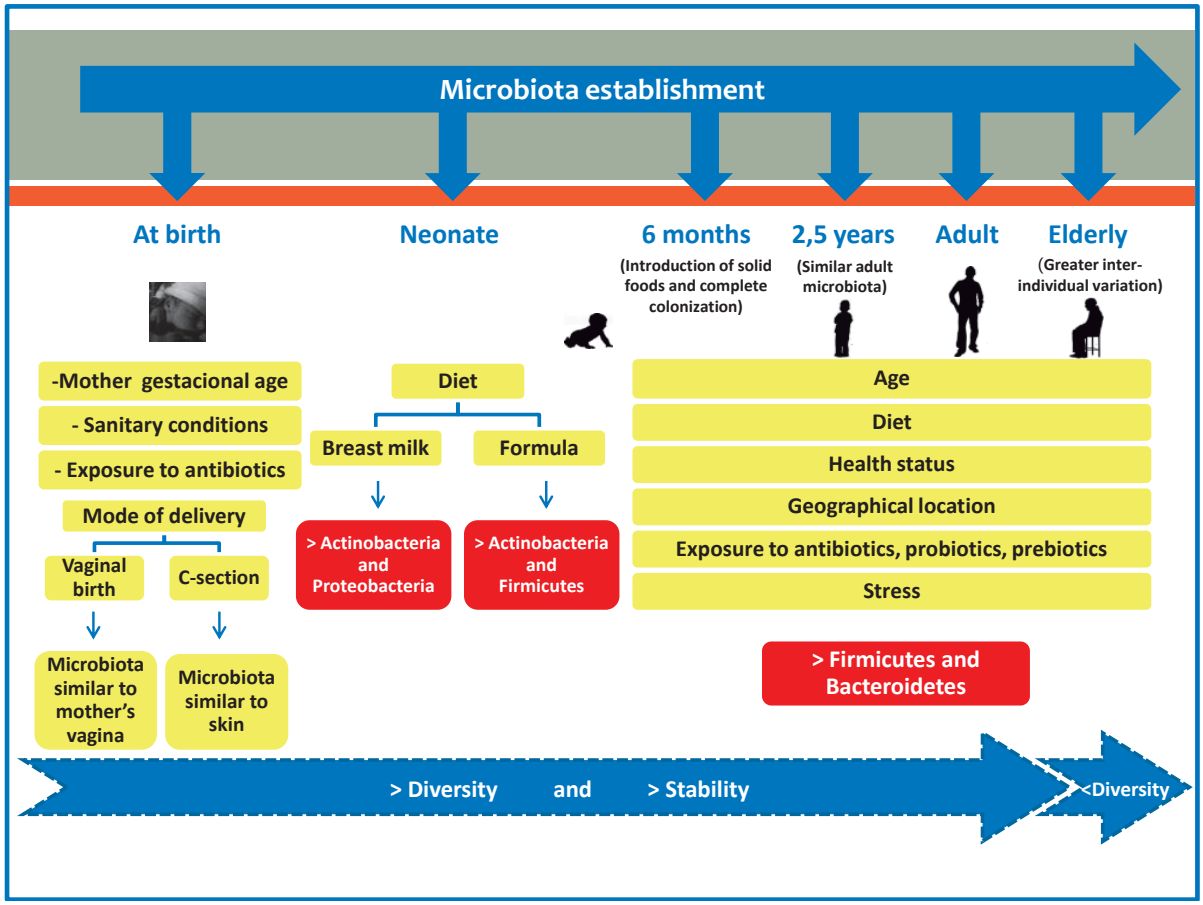


Figure 3. Factors involved in gut microbiota establishment from newborn to adult.

1.1.1.3. Homeostasis and dysbiosis

Under normal conditions, there is a homeostatic equilibrium within microbial communities, but also between microorganisms and the host. An alteration in this balance can occur in response to different perturbations. There is a threshold of stress/perturbation that can be tolerated before the system changes towards a different state of equilibrium. This is known as **resilience**. An alteration of the homeostatic equilibrium is known as **dysbiosis**. In general, dysbiosis can occur due to a loss of microorganisms with beneficial properties and/or excessive growth of harmful bacteria and/or loss of microbial diversity (DeGruttola *et al.,* 2016). In recent years, alterations in GM composition have been associated with different diseases, such as obesity, diabetes, inflammatory bowel diseases (IBD), neurological disorders, allergic asthma, atopic dermatitis or rheumatoid arthritis (Arrieta and Finlay 2014;Cani *et al.,* 2008;Castillo-Alvarez and Marzo-Sola 2017;Lane *et al.,* 2017;Maeda and Takeda 2017;Mangiola *et al.,* 2016;Patterson *et al.,* 2016;Penders *et al.,* 2013). However, to date, it is unknown whether the changes of GM are the causes or the consequences of these diseases, with the exception of the human immunodeficiency virus (HIV) infection. In fact, and as it will be described afterwards, it has been demonstrated that HIV-infection induces a significant damage to gut-associated lymphoid tissue (GALT) that can cause changes in GM composition and, consequently, affects health (Dillon *et al.,* 2014;Dinh *et al.,* 2015;Lozupone *et al.,* 2013;McHardy *et al.,* 2013). For this reason, modulate GM has become a promising therapeutic strategy in diseases associated with microbiota dysbiosis. These "dysbiosis therapy" include the use of antibiotics, probiotics, prebiotics, postbiotics, symbiotics and fecal microbiota transplantation (Cammarota *et al.,* 2014;Vemuri *et al.,* 2017;Vieira *et al.,* 2016;Wischmeyer *et al.,* 2016).

1.1.1.4. Bacterial translocation in gastrointestinal tract

Gut is the primary route by which we are exposed to antigens, and for this reason, it houses the largest immune organ in the body, the GALT. Although most immune cells present in the gut are activated, under normal conditions there is an inmmune tolerance state to prevent excessive inflammatory reactions to innocuous food antigens. This protects the organism against the development of several inflammatory and autoimmune diseases (reviewed by Pabst and Mowat (2012)) (Pabst and Mowat 2012).

The intestinal barrier is mainly composed of the mucus layer, the epithelial layer, and the underlying lamina *propria*. The epithelial layer is covered by a mucous coat rich in mucins and antimicrobial peptides. Epithelial cells are joined by tight junctions thanks to proteins such as occludin, claudin, and zonulin-1 that prevent the passage of molecules between cells. This layer also hosts intraepithelial lymphocytes, membranous or microfold cells (M cells), mucus-producing goblet cells and bacteriocin-producing paneth cells. The lamina *propria* houses a large quantity of immune cells. Thus, it contains cells of the innate (such as monocyte, macrophages, dendritic cells, basophil, and mast cells) and adaptive immune systems (including lymphocytes T cells, lymphocytes B cells, and immunoglobulin A (IgA) secreting plasma cells) (Belkaid and Hand 2014;Konig *et al.,* 2016).

As previously mentioned, GM is located in the gut lumen, at the mucosal surface or within the mucus layer, where participates in important functions. Under homeostasis, antimicrobial peptides and the secreted IgA present in the mucus layer prevent the passage of bacteria to the deeper layers of the intestine, maintaining the integrity of the mucosal barrier (Konig *et al.,* 2016;Quigley 2013). However, a passage of viable bacteria,

microbial products or their fragments, such as lipopolysaccharide (LPS), peptidoglycan, lipopeptides, and bacterial DNA through the intestinal epithelial barrier into lymph nodes or the systemic circulation can occur. This is known as bacterial translocation (BT) (Berg and Garlington 1979;Fukui and Wiest 2016;Vaishnavi 2013). Some authors have observed that BT can take place as a spontaneous event in a rate of approximately 5-10% in humans, while in animals this rate is higher, of approximately 10-20% (Berg and Garlington 1979;Sedman *et al.,* 1994). However, several factors are associated with intestinal epithelial barrier dysfunction and increased permeability, which triggers BT and leads to breakdown of immune tolerance. Among these triggering factors are host immune deficiencies and immunosuppression, disturbances in GM composition, high fat diet (HFD) intake, excessive alcohol ingestion, IBD, and other causes that could alter the mucosal barrier permeability (Brenchley and Douek 2012;Cani *et al.,* 2012;Engen *et al.,* 2015;Vaishnavi 2013).

Innate immune sensors such as Toll-like receptors (TLRs) are considered the interface among intestinal epithelial barrier, microbiota and immune system (Gribar *et al.,* 2008;Peterson and Artis 2014). TLRs are expressed in numerous cell types including macrophages, dendritic cells, T lymphocytes, and intestinal epithelial cells. In general terms, they are transmembrane receptors that play an important role in the mechanism of innate immunity of the intestinal epithelium, recognizing bacterial, viral, parasites or self-derived ligands, initiating several signaling cascades and inducing the synthesis and release of factors related to inflammation, such as tumor necrosis factor alpha (TNFα), and/or interleukins 1 (IL-1) and 6 (IL-6). Enterocyte TLRs in a “healthy” state may contribute to intestinal homeostasis, playing a pivotal role in the regulation of intestinal inflammation and immune tolerance but also in the response to invading

pathogens. Thus, the constant exposure of the intestinal mucosal surface to commensal bacteria induces a basal state of activation that ensures mucosal homeostasis through limited inflammatory responses and accelerated restitution and healing in the healthy intestine. Otherwise, after exposure to physiological stressors, such as remote infection, changes on GM composition or high concentrations of LPS, an increased TLR activity occurs. Excessive activation of TLR signaling within the enterocyte may result in a profound damage of the epithelial layer, including the release of proinflammatory citokines, the induction of epithelial damage through apoptosis, and the inhibition of intestinal repair. These effects may be initiated but also propagated by the TLR-activated leukocytes. Taken together, these factors promote BT, resulting in activation of the subepithelial immune system and the development of intestinal inflammation (reviewed by Gribar *et al*., (2008)) (Gribar *et al.,* 2008).

There are different types of TLRs: TLR1 to TLR10 in humans, and TLR1 to TLR9, TLR11, TLR12 and TLR13 in mice. The different TLRs detect a specific pathogen-associated molecular pattern, including endotoxin/LPS (TLR4), bacterial lipoprotein and peptidoglycan (TLR2), flagellin (TLR5), unmethylated CpG DNA (TLR9), double-stranded RNA (TLR3), and single-stranded RNA (TLR7-8). In short, TLRs 1, 2, 4, 5 and 6 are specialized in the recognition of bacterial products, while TLRs 3, 7 and 8 seem to be associated to viral detection. TLR 9 is related with both of them, whereas the role of TLR10 was unknown, therefore, it was considered an orphan receptor without a known agonist or function. However, it was recently demonstrated that this receptor is involved in the induction of innate immune responses to influenza virus infection, suggesting that could play a role in a range of other viral and, perhaps, other microbial diseases (Fukui and Wiest 2016;Lee *et al.,* 2014;Mesonero *et al.,* 2012;Pott and Hornef 2012).

One of the most studied TLRs is the TLR4, which is an innate immune receptor expressed in both immune cells and enterocytes (Frosali *et al.,* 2015). Fukata *et al*., (2004) demonstrated that this receptor is important in intestinal response to injury and in limiting BT (Fukata *et al.,* 2005). TLR4 signaling pathway starts when TLR4 binds to LPS which is in the outer membrane of gram negative bacteria. Concretely, the inflammatory process begins when the lipopolysaccharide binding protein (LBP), which is produced by the liver, binds longitudinally to the surface of LPS micelles. Then, CD14, an LPS coreceptor expressed by peripheral blood monocytes and tissue macrophages, interacts with LBP to obtain a single LPS molecule and rapidly dissociates from the LBP/LPS complex. After the rapid dissociation of CD14/LPS, LBP retains its binding to LPS micelles and recruits another CD14 again. Meanwhile, CD14 delivers a single LPS molecule to myeloid differentiation protein-2 (MD-2) (also known as lymphocyte antigen 96) in a TLR4-dependent manner. Two LPS-bound TLR4/MD2 complexes form a M-shaped dimer, followed by activation of the signaling pathway for the innate immune response. It is important to mention that in those cell types without CD14 receptors (endothelial cells, dendritic cells, fibroblasts, smooth muscle cells, etc.), the signaling pathway is initiated by binding the LPS-LBP complex to the soluble CD14 (sCD14) secreted by monocytes and macrophages, which are circulating in the plasma (Kim and Kim 2017;Mesonero *et al.,* 2012;Shu *et al.,* 2013).

Following LPS recognition, TLR4 undergoes oligomerization and recruits its downstream adaptors through interactions with the TIR (Toll-interleukin-1 receptor) domains. There are five TIR domain-containing adaptor proteins: MyD88 (myeloid differentiation primary response gene 88), TIRAP (TIR domain-containing adaptor protein, MyD88-adapter-like), TRIF (TIR domain-containing adaptor inducing interferon beta (IFN-b)),

TRAM (TRIF-related adaptor molecule), and SARM (sterile α and HEAT-Armadillo motifs-containing protein). Different TLRs utilize different combinations of adaptor proteins to establish downstream signaling. Surprisingly, TLR4 is the only known TLR which uses all these adaptor proteins. TLR4 signaling can be divided into MyD88-dependent and MyD88-independent pathways, which mediate the activation of proinflammatory cytokines (such as TNFα, IL-1 or IL-6) and type I interferon genes, respectively (Figure 4) (reviewed by Lu *et al*., (2008)) (Lu *et al.,* 2008). There are other cell wall receptors that also recognize LPS and induce an inflammatory response such as the MSR (macrophage scavenger receptor), K^+ channels, and CD11/CD18 receivers. In all cases, several transcriptional factors are activated, such as nuclear factor kappa B (NF-κB), activator protein-1 (AP-1), and interferon (IFN) regulatory factors, resulting in the increased expression of genes involved in the inflammatory response (Kim and Kim 2017;Ortiz and Garnacho 2005).

Bacteria and microbial products can be drained from the intestine by the portal vein into the liver, where are recognized by TLRs expressed on Kupffer cells, dendritic cells, hepatic stellate cells, and hepatocytes. Microbial antigens can also reach the systemic circulation. Once there, bacterial products come into contact with a further host-mediated response regulated by cell-surface receptors and circulating factors that bind and clear these products (reviewed by Nakamoto and Kanai (2014) and Brenchley and Douek (2012)) (Brenchley and Douek 2012;Nakamoto and Kanai 2014).

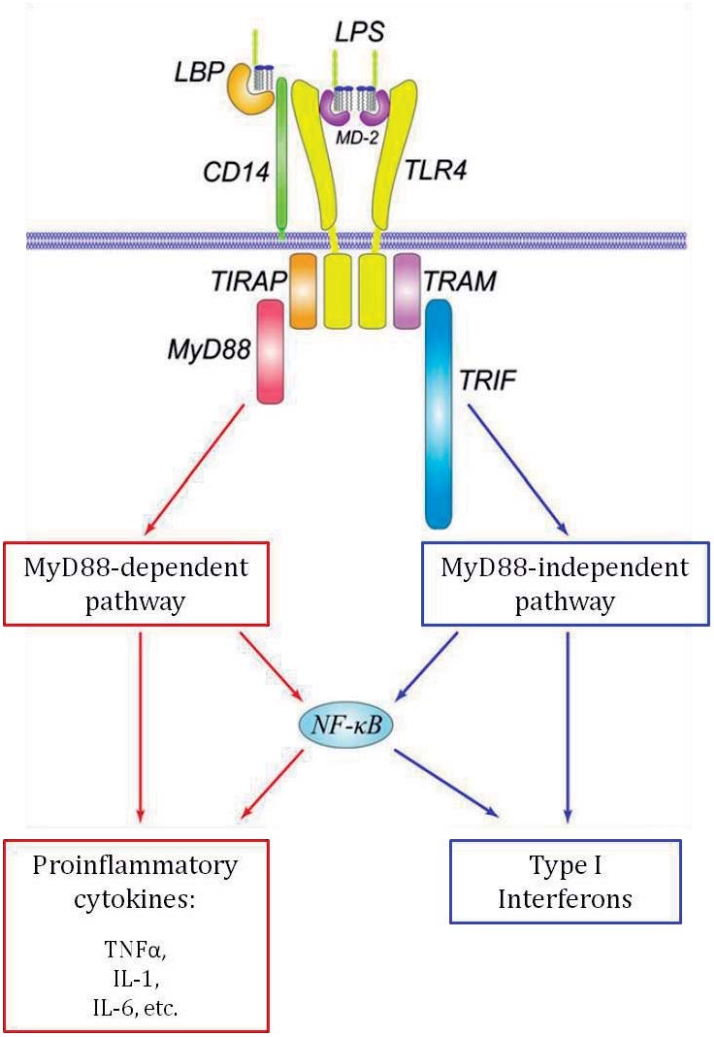


Figure 4. LPS/TLR4 signaling pathway (image adapted from Lu *et al.*, (2008) (Lu *et al.,* 2008)).

CD14, cluster of differentiation 14; IL-1, interleukin-1; IL-6, interleukin-6; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; MD-2, myeloid differentiation protein-2; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa B; TIRAP, toll-interleukin 1 receptor (TIR) domain-containing adaptor protein; TLR4, toll-like receptor 4; TNFα, tumor necrosis factor alpha; TRAM, toll-interleukin 1 receptor (TIR) domain-containing adaptor inducing interferon beta (IFN-b)-related adaptor molecule; TRIF, toll-interleukin 1 receptor (TIR) domain-containing adaptor inducing interferon beta (IFN-b).

1.2. HIV infection

One of the major public health problems reported in our history occurred in 1981 when a high number of homosexual men suffered unusual opportunistic infections and rare malignancies. This "new disease" was recognized in 1982 by the CDCs (Centers for Disease Control and Prevention from USA) as an Acquired Immune Deficiency Syndrome (AIDS), as the immune system of these patients were weakened (MMWR Morb Mortal Wkly Rep 1982a; MMWR Morb Mortal Wkly Rep 1982b;Gottlieb *et al.,* 1981). In record time, 1983, the team of Luc Montaigner at the Pasteur Institute (Paris) and the American team of Robert Gallo reported the discovery of the causal agent of such disease (Barre-Sinoussi *et al.,* 1983;Gallo *et al.,* 1983) that was later named as Human Immunodeficiency Virus (HIV) (Coffin *et al.,* 1986). There was great controversy about who was the first to isolate the virus. However, in 1994, the French team was recognized as the discoverers, since they were the first to isolate the virus. Thus, for this important discovery, Montagnier and his colleague Françoise Barré-Sinnoussi were awarded by the Nobel Prize in Medicine (2008) (Special Commentary of AIDS Journal 2009;Weiss 2008). In 1986, a second type of virus was discovered in AIDS patients in West Africa: HIV-2 (Clavel *et al., 1*986). Thus, AIDS can be caused by HIV types 1 and 2 (HIV-1 and HIV-2). HIV-1 has spread worldwide, while HIV-2 is mainly limited to West Africa and European communities with socioeconomic links to West Africa, such as Portugal. Both types of viruses are very similar, such as in modes of transmission or intracellular replication pathways. However, patients with HIV-2 have lower plasma viral load than patients infected with HIV-1, thus, HIV-2 has a lower transmissibility, and reduced probability of progression to AIDS with a longer asymptomatic period, and, finally, being less pathogenic. The underlying mechanisms for this lower pathogenicity are still poorly understood, however, some authors

have showed important variations among HIV types (German Advisory Committee Blood 2016;Nyamweya *et al.,* 2013;Popper *et al.,* 1999;Vicenzi and Poli 2013). Thus, because the higher pathogenicity, global distribution, and the type of patients of our enviroment, this Doctoral Thesis is focused on the HIV-1-infected patients.

The origin of HIV-1 was the result of the zoonotic transmission of viruses that infect non-human primates in Africa, known as simian immunodeficiency viruses (SIVs). Concretely, SIVs have "jumped" successfully into humans on four independent occasions, crossing species barrier and generating HIV-1. Thus, HIV-1 is divided into four phylogenetically distinct groups: M, N, O and P (Hahn *et al.,* 2000;Sharp and Hahn 2011). The subspecies M, N and O are closely related to the SIV that infects chimpanzees (SIVcpz) (Gao *et al.,* 1999;Hahn *et al.,* 2000;Huet *et al.,* 1990), while the P group, which only exists in people from Cameroon, appears to come from SIV that infects Cameroon's gorillas (SIVgor) (Huet *et al.,* 1990). The M group was the cause of the global pandemic with a majority global distribution (Merson *et al.,* 2008). A recent study placed the origin of the HIV-1 group M around the early 1920s in Kinshasa (Democratic Republic of the Congo). Considering that the hunting of these apes is a common habit in Africa, virus could have been transferred from ape to human through a bite, scratch or the blood of a dead ape getting into an open wound. Then, the virus was spread from Kinshasa to the rest of Africa and to other parts of the world mainly by sex practice and trade (Faria *et al.,* 2014).

Since the beginning of the HIV/AIDS epidemic, more than 70 million people have been infected with the HIV virus and approximately 35 million people have died because of this infection. The latest figures published by the World Health Organization (WHO) indicated that 36.7 million (30.8–42.9

million) people were living with HIV at the end of 2016, being Sub-Saharan Africa the most affected area, with about 4.2% of people living with HIV (*www.who.int/hiv/en*, date accessed: January 2018). With regard to Spain, 86,663 people have been infected with the virus between 1981 and the first half of 2017. Moreover, between the years 1981 and 2015, it was estimated that there were 58,162 deaths attributed to AIDS-related causes. Most of them were men (47,085 male and 11,077 female). In the year 2016, 3,535 new cases of HIV were diagnosed in Spain (*Instituto de Salud Carlos III* (Spain): *http://www.isciii.es/isciii/es/contenidos/fd-servicios-cientifico-tecnicos/fd-vigilancias-alertas/fd-enfermedades/sida.shtml*, date accessed: February 2018). Concerning the Autonomous Community of La Rioja (Spain), the number of new HIV diagnosed has been stabilized in the last years (data from *Dirección General de Salud Pública y Consumo* – Government of La Rioja (Spain)) (Figure 5).

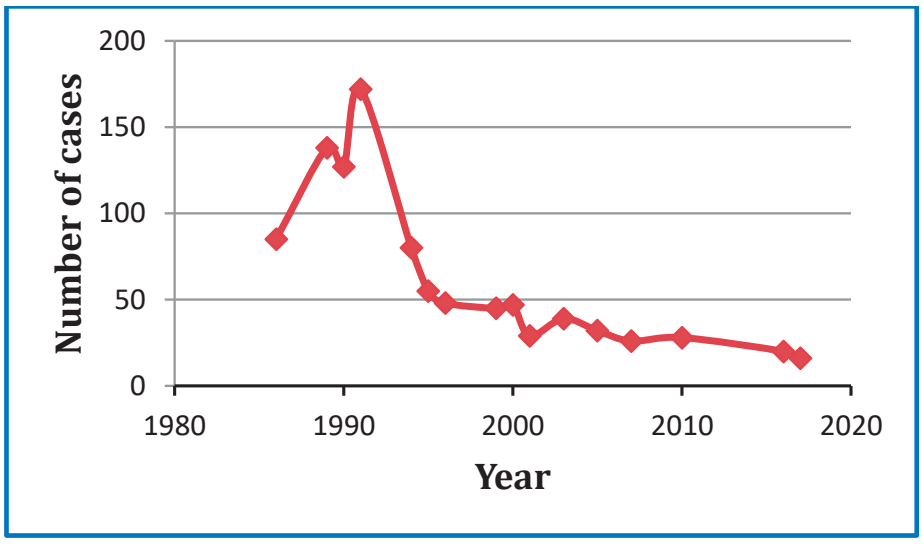


Figure 5. HIV cases reported in the Autonomous Community of La Rioja (data from *Dirección General de Salud Pública y Consumo* – Government of La Rioja (Spain)).

Regarding the mode of transmission, HIV is transmitted from an infected person to another through blood, semen, vaginal secretions and maternal transmission. Thus, HIV has been classically spread through sexual behaviors, needle or syringe use and from mother to child during pregnancy, childbirth and breastfeeding (CDC: *https://www.cdc.gov/hiv/basics/transmission.html,* date accessed: February 2018). Although HIV is mainly a global sexual transmitted infection, the mode of transmission has changed over the years. In our enviroment at the beginning of the HIV/AIDS epidemic the needle/syringe sharing among persons who inject drugs was the leading cause of HIV transmission ahead of sexual contact. However, over recent years this trend has changed, and the sexual contact both men who have sex with men (MSM) and heterosexual has become the first cause of HIV transmission (Figure 6) (*Instituto de Salud Carlos III* (Spain): *http://www.isciii.es/isciii/es/contenidos/fd-servicios-cientifico-tecnicos/fd-vigilancias-alertas/fd-enfermedades/sida.shtml*, date accessed: February 2018). Fortunately, the usage of condoms to prevent the infection by sexual contact, along with the development of effective antiretroviral treatments led to a partial "control" of this infection. However, in recent years, there is a certain relaxation in the population in relation to the sexual practice, which leads to an increase in other sexually transmitted diseases, such as syphilis, gonorrhea or chlamydia infection, as well as HIV.

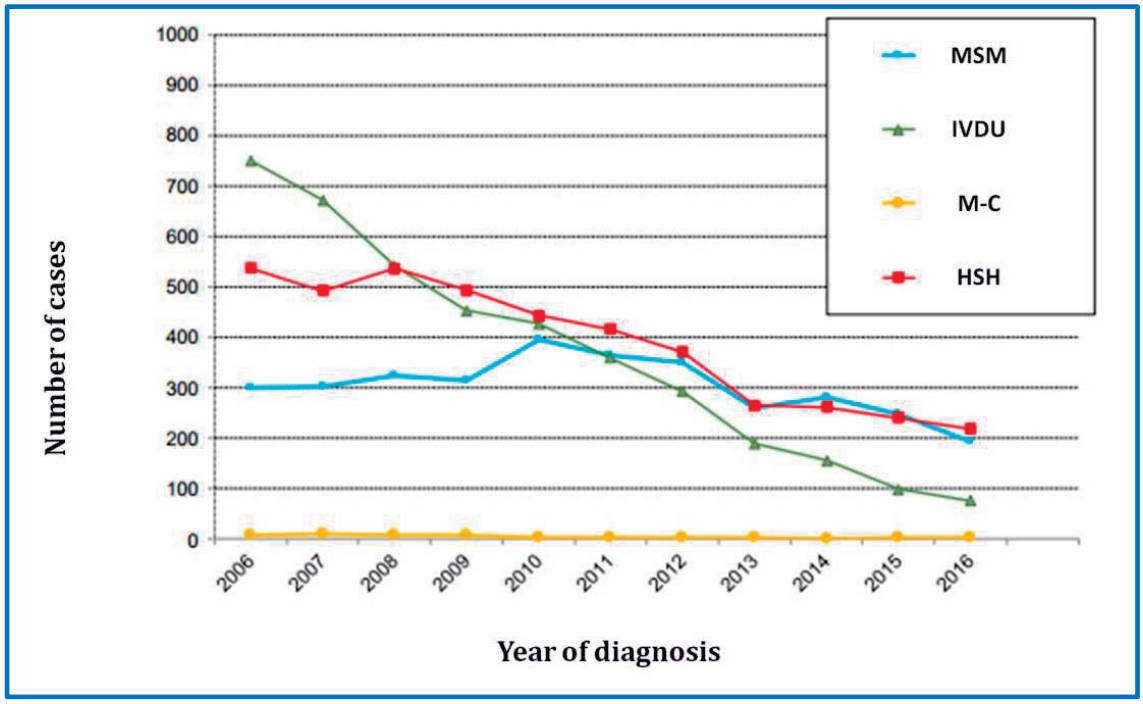


Figure 6. HIV cases reported in Spain divided by mode of transmission (2006-2016) (image adapted from Instituto de Salud Carlos III (Spain) (Instituto de Salud Carlos III: *http://www.isciii.es/isciii/es/contenidos/fd-servicios-cientifico-tecnicos/fd-vigilancias-alertas/fd-enfermedades/sida.shtml,* date accessed: February 2018).
HS, heterosexual; IVDU, intravenous drug user; M-C, mother to child; MSM, men who have sex with men.

1.2.1. Structure of HIV-1 virion

HIV is grouped to the genus Lentivirus (lentis=slow), characterized by long incubation periods, and within the family of Retroviridae, since it has the enzyme reverse transcriptase that allows it to copy RNA into DNA. Figure 7 showed the squematic structure of the HIV virus. The mature, infectious HIV-1 particles have a spherical shape with a diameter of 100-130 nm. The viral envelope is comprised of a lipid bilayer, derived from the host cell. This envelope contains 72 projections which are composed by trimers of proteins, and specifically by the trimers of glycoprotein 120 (gp120) and the

glycoprotein 41 (gp41). Gp41 is associated with the matrix protein p17 that is immediately below of the lipid layer. This membrane covers a conical capsid that is assembled for the capsid protein, termed as p24. The capsid contains two single strands of viral RNA, which are associated with the nucleocapsid proteins (p7), as well as the viral enzymes reverse transcriptase and integrase. The capsid also contains some copies of the viral protease and the accessory proteins Vif (viral infectivity factor), Vpr (virus protein r), and Nef (negative regulating factor), which are not required for viral replication, but help efficiency of this process (Figure 7) (German Advisory Committee Blood 2016;Heger *et al.,* 2015;Kirchhoff 2013). As a retrovirus, the HIV genome contains the retroviral genes *gag*, *pol* and *env* flanked by long terminal repeats, which contain the viral promoter. *Gag* codes for the structural proteins capsid, matrix, and nucleocapsid; *pol* encodes the enzymes reverse transcriptase, protease, and integrase; and *env* encodes the glycoproteins gp120 and gp41. Moreover, HIV genome codes six regulatory genes (*tat* (transactivator protein), *rev* (RNA splicing-regulator), *nef*, *vif*, *vpr*, and *vpu* (virus protein unique)). *Tat* enhances proviral transcription and *rev* is critical for the transport of incompletely or unspliced viral mRNAs into the cytoplasm. The regulatory gen *vpu* is considered an accessory gen, along with *nef*, *vif*, *vpr* genes (Kirchhoff 2013).

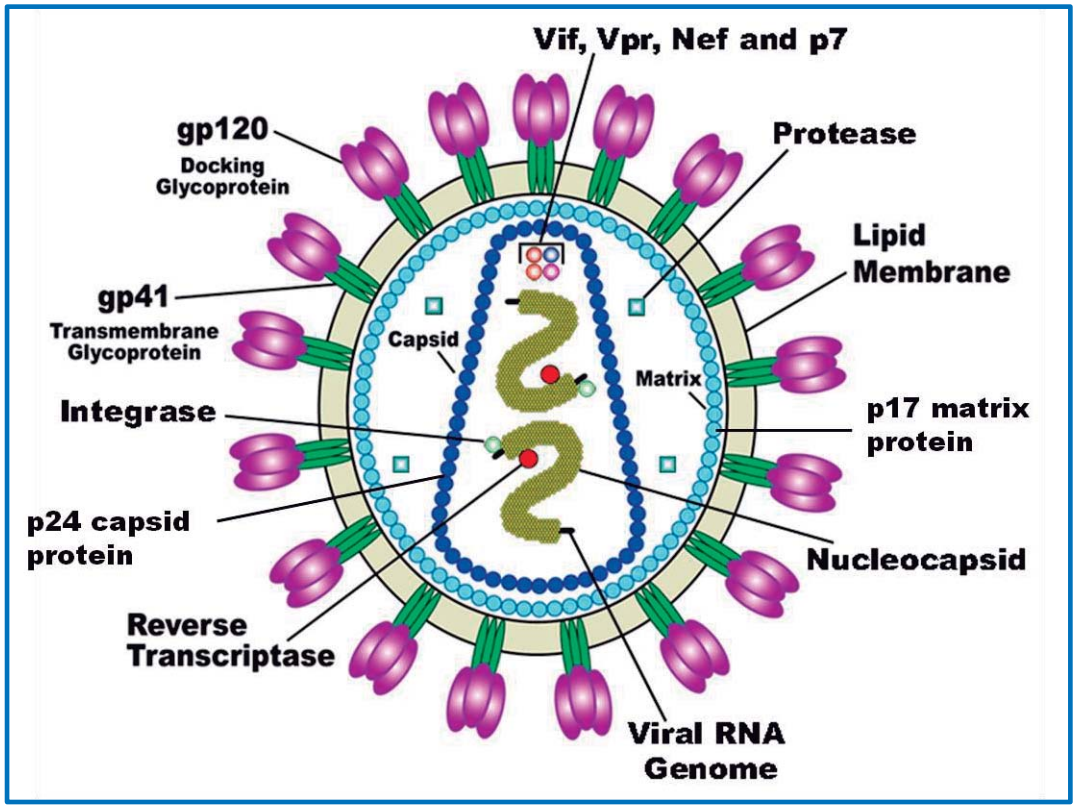


Figure 7. Structure of HIV virion.
Gp120, glycoprotein 120; gp41, glycoprotein 41; nef, negative regulating factor; p17, protein 17; p24, protein 24; vif, viral infectivity factor; vpr, virus protein r.

1.2.2. Replication Cycle

HIV use as cellular receptor the molecule $CD4^+$, being $CD4^+$ T helper cells the primary target for HIV-1 since they express high levels of the HIV-1 receptor CD4 (necessary for binding to and enter cells) and are highly permissive for HIV-1 production. Nevertheless, other immune cells, such as monocytes/macrophages and dendritic cells also express CD4 and HIV-1 co-receptors at the cell surface. The entry of the virus is initiated with the fusion between the $CD4^+$ T cell and the envelope glycoprotein, gp120 from the virus. This binding induces a conformational change in gp120 that increases its affinity for a secondary receptor (co-receptor). The most relevant co-

receptors for the HIV-1 are CCR5 and CXCR4. Thus, besides binding a CD4 receptor, HIV must also bind either a CCR5 or CXCR4 co-receptor protein to get into a cell. Then, gp120 is attached to the co-receptor and further conformational changes in both gp120 and gp41 trigger membrane fusion reaction that leads to translocation of the viral capsid into the cytoplasm. The capsid is taken up by an endosome, and a change in pH in the phagosome induces the viral core release into the cytoplasm of the infected cell. Then, the viral RNA is reverse-transcribed into a double-stranded DNA by the viral enzyme reverse transcriptase. The synthesized viral DNA is transported across the nucleus, where the integrase enzyme integrates the viral DNA into the host cell chromosomal DNA. Once integrated, viral DNA is called provirus. The provirus sometimes can remain in an inactive state for several years, producing few or no copies of HIV. The integrated viral DNA can be transcribed by the host enzyme called RNA polymerase into several copies of new viral RNAs, and some of these new RNAs are transported into the cytoplasm where are translated into viral polyproteins. The transcribed proteins must be cleaved into smaller component proteins through the action of the viral enzyme protease. The new viral RNA and HIV proteins move to the surface of the cell, where are assembled in new immature HIV particles. Finally, viral particles are released from the infected cell and mature infectious virus are formed, and then a new cycle of infection can be initiated (Checkley *et al.,* 2011;Freed and Martin 2006;Stein *et al.,* 1987).

1.2.3. Natural history of HIV-infection

The first period, after HIV infection, 3-4 weeks post-exposition and infection with the virus, is defined as **acute HIV infection.** This phase lasts 6-12 weeks and involves the first detection of HIV RNA in plasma and the formation of HIV-specific antibodies. When the HIV is parenterally

transmitted, the virus enters directly into the bloodstream, however, if the transmission occurs through unprotected sexual intercourse, the virus must deal with the mucous membranes where dendritic cells are present. The virus then traffics within these cells before being transferred to $CD4^+$-T cells (Mogensen *et al.,* 2010;Nasi *et al.,* 2014;Wu and KewalRamani 2006). As previously mentioned, HIV-1 infects primarily $CD4^+$ T cells and cells of the monocyte/macrophage lineage. Following infection, virus or virus-infected cells achieve the draining lymph nodes where activated $CD4^+CCR5^+$ T cells are found and that represent targets for further infection. This facilitates the replication and dissemination of the virus to secondary lymphoid tissue throughout the organism, with a particular predilection for GALT, which is the largest immune organ in the body and where activated $CD4^+CCR5^+$ effector memory T cells are housed at high levels (Mogensen *et al.,* 2010). These lymphocytes, in contrast to what happens in peripheral blood mononuclear cells, are memory T lymphocytes in a continuous state of activation, which makes them very susceptible to HIV infection (Lapenta *et al.,* 1999). In fact, HIV-infection should be considered as a gut disease, as it significantly depletes CD4 T cells from mucosal sites, particularly from GALT (Brenchley *et al.,* 2004). Thus, regardless the route of transmission of HIV infection, the gastrointestinal tract is the primary and major site of viral replication, and also where more $CD4^+$ T-cell are depleted. For this reason, it is important to know the structure of the intestinal immune system. As it is showed in Figure 8, the gastrointestinal immune system is organized into distinct anatomic and functional subcompartments, the inductive and effector sites. Inductive sites include the GALT (such as Peyer's patches and isolated lymphoid follicles) and the gut-draining mesenteric lymph nodes. The lamina *propria* and epithelium constitute the main effector sites, harboring large populations of activated T cells and antibody-secreting

plasma cells (Pabst and Mowat 2012). Peyer's patches have clearly defined T- and B-cell–dependent areas, which are overlaid by epithelium containing M cells. These cells are able to transport antigens into immune inductive sites resulting in initiation of T-cell education and maturation. Once antigen presentation occurs, T and B cells are transported through efferent lymphatics to the draining lymph nodes, and eventually to the peripheral circulation. Then, these cells can reach the mucosal effector sites, where differentiated T cells are able to neutralize antigens and protect the host against invading pathogens. Effector site lymphocytes can be subclassified into lamina *propria* lymphocytes and intraepithelial lymphocytes. The lamina *propria* contains mainly $CD4^+$ T cells, whereas intraepithelial lymphocytes are mainly $CD8^+$ T cells. Lamina *propria* also contains macrophages and plasma cells. Thus, HIV can reach the intestinal tract through blood dissemination and also may pass through the epithelium layer in different ways, as shown in Figure 8: by transcytosis across intact epithelial cells (1) or M cells (2); by adhering to dendrites of mucosal dendritic cells (3); or by direct passage through epithelial breaches (4) compromising epithelium integrity. The lamina *propria* loses most of its $CD4^+$ T cells, while there is an influx of $CD8^+$ T cells. Hence, collagen deposition occurs making difficult the reconstitution of $CD4^+$ T cells. Inductive sites lose their architecture, harboring many apoptotic T and B cells with few $CD4^+$ T cells (excellently reviewed by Mehandru (2007) and Shacklett and Anton (2010)) (Mehandru 2007;Shacklett and Anton 2010).

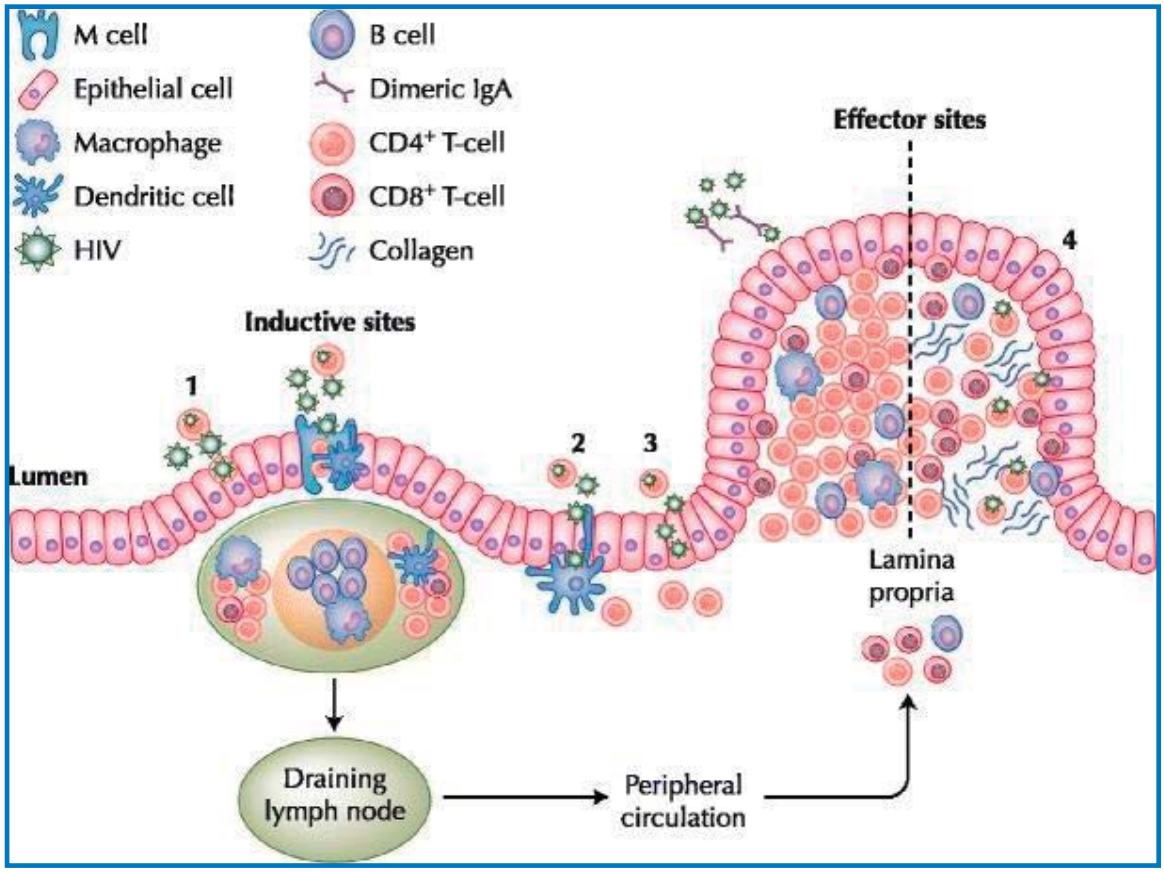


Figure 8. Structure of the intestinal immune system (image taken from Shacklett and Anton (2010) (Shacklett and Anton 2010)).
The gastrointestinal immune system is organized into distinct anatomic and functional subcompartments, the inductive and effector sites. HIV can reach the gastrointestinal tract through blood dissemination and also may pass through the epithelium layer in different ways: by transcytosis across intact epithelial cells (1) or M cells (2); by adhering to dendrites of mucosal dendritic cells (3); or by direct passage through epithelial breaches (4).

Moreover, during the earliest stage of HIV infection, especially in GALT, some HIV-infected cells go into a resting (or latent) state. These cells can remain at this state for years without producing new HIV, but at any time they can become active and start making more HIV mature particules. These reservoirs are comprised by replication-competent linear provirus

integrated into host cellular DNA. During latency, there is highly restricted expression of viral genes (Churchill *et al.,* 2016;Murray *et al.,* 2016). Latent HIV reservoirs are established in cells with slow rate of depletion (Mogensen *et al.,* 2010). HIV-infection also depletes mucosal Th17 cells that play a key role in the antimicrobial defense (Brenchley *et al.,* 2008;Elhed and Unutmaz 2010). The infection of macrophages present in the intestinal mucosa also facilitates the depletion of $CD4^+$ cells, by the transmission of the virus "cell by cell" and also by secreting cytokines that attract and recruit activated T lymphocytes, which, subsequently, are infected, enhancing intestinal infection (Nasi *et al.,* 2014). Therefore, at this time, there is a wide dissemination of virus seeding of lymphoid organs. After 10-14 days post infection HIV can be detected in the whole body, including the nervous system. A peak of plasma viraemia is reached after 3-4 weeks of infection, along with a decline in peripheral $CD4^+$ T cells (German Advisory Committee Blood 2016;An and Winkler 2010;Mogensen *et al.,* 2010) (Figure 9). At the time of peak viraemia, patients could develop symptoms of the acute retroviral syndrome, including fever, fatigue, lymphadenopathy, sore throat, and exanthema (Kahn and Walker 1998). Afterwards, there is a marked reduction of viral replication probably due to virus-specific immune responses that limit viral replication. Then, viral load reaches at steady state with low but constant replication that marks the start of the **chronic phase**. The symptoms that come at this phase usually are resolved as the viral load in the plasma decreases. Thus, immune response increases the circulating T cell numbers at “normal” levels (over 500 cells/mm^3), however, the immune response is not capable to restore the depleted $CD4^+$ T cells in GALT. The chronic phase is also called asymptomatic HIV infection or clinical latency, since is characterized by the absence of symptomatology and lasts an

average of 7–10 years (German Advisory Committee Blood 2016;Fauci *et al.,* 1996;Mogensen *et al.,* 2010).

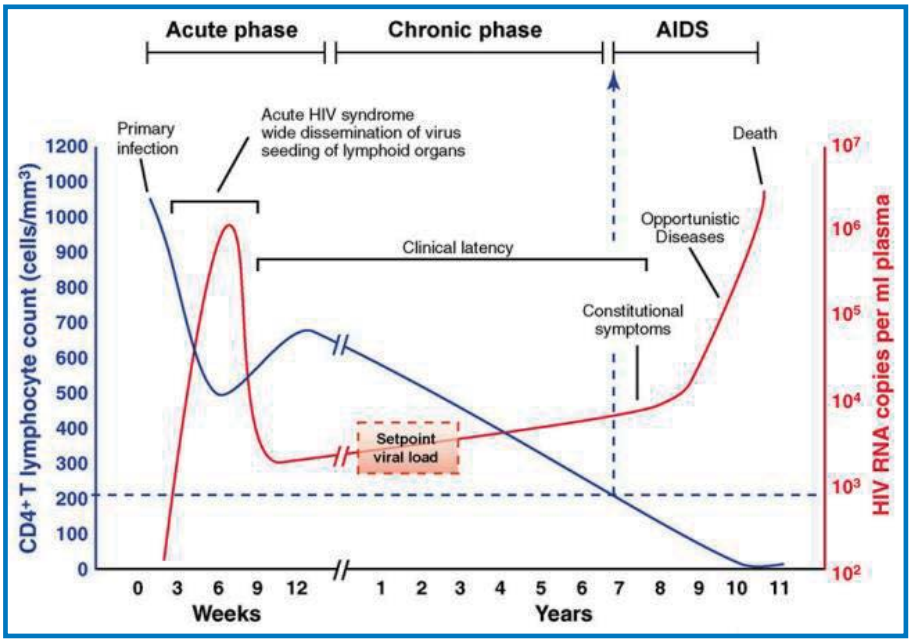


Figure 9. Stages of HIV infection (image taken from An and Winkler (2010), which was adapted from Pantaleo *et al*., (1993) (An and Winkler 2010;Pantaleo *et al.,* 1993)).

After this phase, the number of $CD4^+$ T cells continues to fall to very low levels and this marks the start of **AIDS phase** (An and Winkler 2010;Douek 2003;Mogensen *et al.,* 2010) (Figure 9). In fact, this phase occurs when the number of CD4 cell drops below 200 cells/mm^3 or when opportunistic infections and specific neoplasms develop (AIDS-defining condition), thus, the immune response is weakened. The time to develop AIDS can vary without therapy from 2 to 25 years or more since the primary infection (German Advisory Committee Blood 2016;Mogensen *et al.,* 2010) (Figure 9).

Patients under an effective antiretroviral treatment showed a reduction of viral load to undetectable levels, as well as recovery of $CD4^+$ T cell numbers (Okoye and Picker 2013).

1.2.4. Bacterial translocation in HIV infection

Depletion of gastrointestinal $CD4^+$ T-lymphocytes during HIV infection is followed by alterations to lymphoid tissue architecture, which leads to a loss of the integrity and function of the mucosal barrier (Brenchley *et al.,* 2004;Stebbing *et al.,* 2005). Loss of immune protection of the intestinal mucosa allows translocation of microbial products (such as LPS) into the lamina *propria* of the gastrointestinal tract and, eventually, into the systemic circulation (Miedema *et al.,* 2013;Nasi *et al.,* 2014;Sandler and Douek 2012). BT was firstly described in HIV-infected patients in 2006 by Brenchley *et al*., (2006) (Brenchley *et al.,* 2006). As mentioned above, bacterial products, such as LPS, induce a significant increase in proinflammatory cytokine production via TLR, contributing to immune activation and inflammation (Meier and Altfeld 2007). BT has been shown to persist throughout the course of the infection by the virus and contributes to the immune activation that is observed in the chronic phases of the infection (Cassol *et al.,* 2010;Klatt *et al.,* 2013;Marchetti *et al.,* 2013) (Figure 10).

Although mucosal CD4 T-cell depletion takes place during the acute phase of the HIV infection, Brenchley *et al*., (2006) showed that plasma LPS levels are not enhanced in this phase. Conversely, they observed increased levels of LBP and sCD14 instead, suggesting that LPS translocation is rapidly countered by the host immune response. As HIV infection enters its chronic phase, higher plasma levels of LPS were found, which seems to indicate that the consequences of the CD4 T-cell depletion and mucosal damage are not manifest until the chronic phase or that there is transient mobilization of

factors that neutralize circulating LPS (Brenchley *et al.,* 2006;Marchetti *et al.,* 2013).

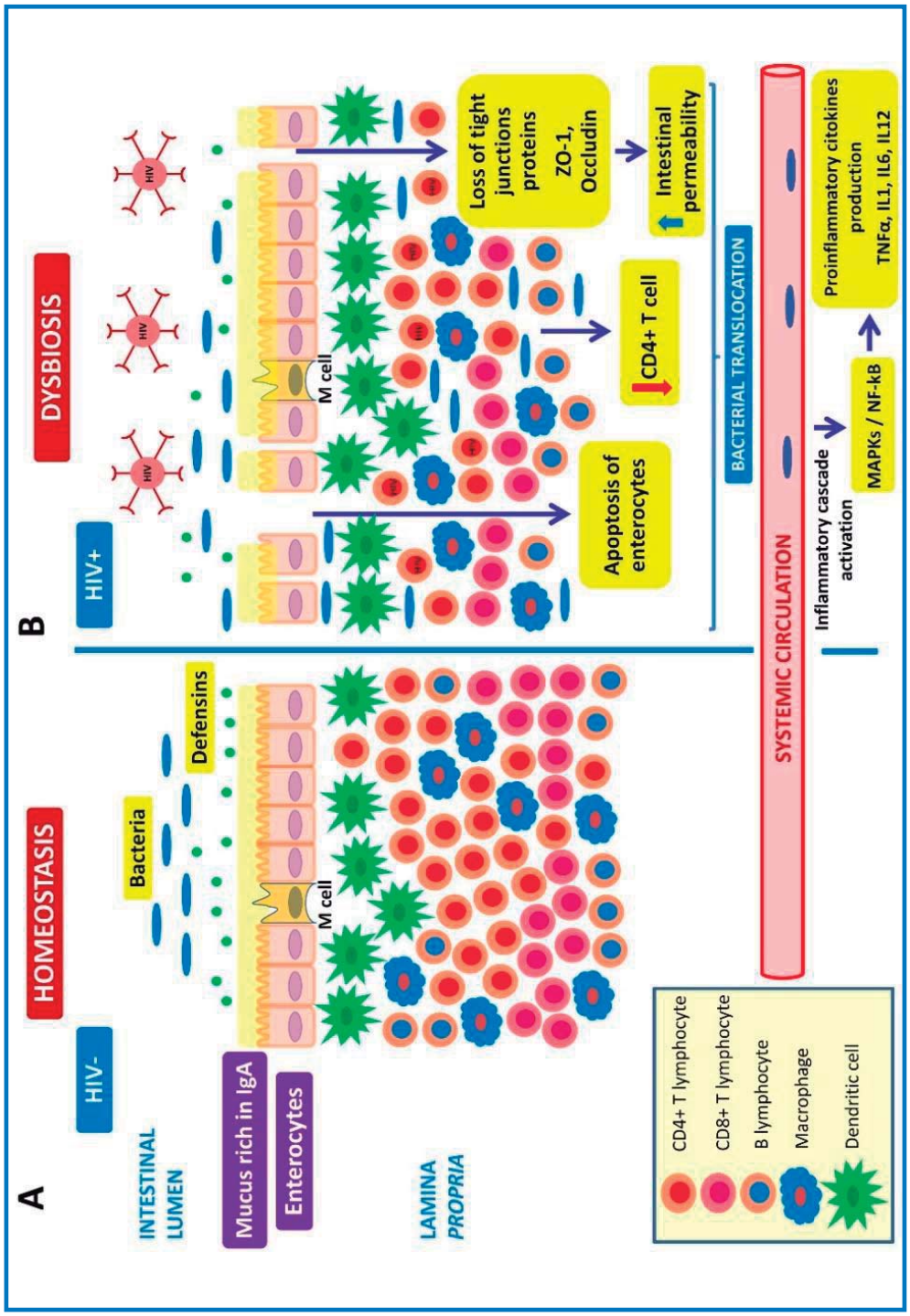


Figure 10. (A) Gut homeostasis; (B) gut dysbiosis after HIV infection.

The degree of BT is linked to the severity of HIV progression independent of viraemia and several studies have demonstrated that increased BT and proinflammatory cytokines are partially responsible for HIV-related comorbidities, which implies a significant increase in morbidity and mortality (Dillon *et al.,* 2014;Dinh *et al.,* 2015;Hsu *et al.,* 2013;Mutlu *et al.,* 2014;Nowak *et al.,* 2015). Anyway, and spite of the relevance of the topic, there are not so many studies regarding this question in HIV infection.

It is important to emphasize that gut microbiota are also altered after HIV infection, resulting in microbiome dysbiosis, which further exacerbates BT, epithelial barrier disruption, inflammation, and mucosal immune functioning (reviewed by Zevin et al., (2016)) (Zevin *et al.,* 2016).

1.2.5. Antiretroviral treatments

Fortunately, the discovery of the HIV replication cycle in human $CD4^+$ T-cells, along with technological advances, enabled the development of potential drug targets to slow or halt different steps within the HIV cycle. Concretely, the currently approved antiretroviral drugs are able to halt viral replication at six different stages, including binding (co-receptor antagonists), fusion (fusion inhibitors), reverse transcription (nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs) and non–nucleoside reverse transcriptase inhibitors (NNRTIs)), integration (integrase strand transfer inhibitors (INSTIs)) and proteolytic cleavage (protease inhibitors (PIs)), as observed in Figure 11 (Arts and Hazuda 2012;Barre-Sinoussi *et al.,* 2013;Pau and George 2014).

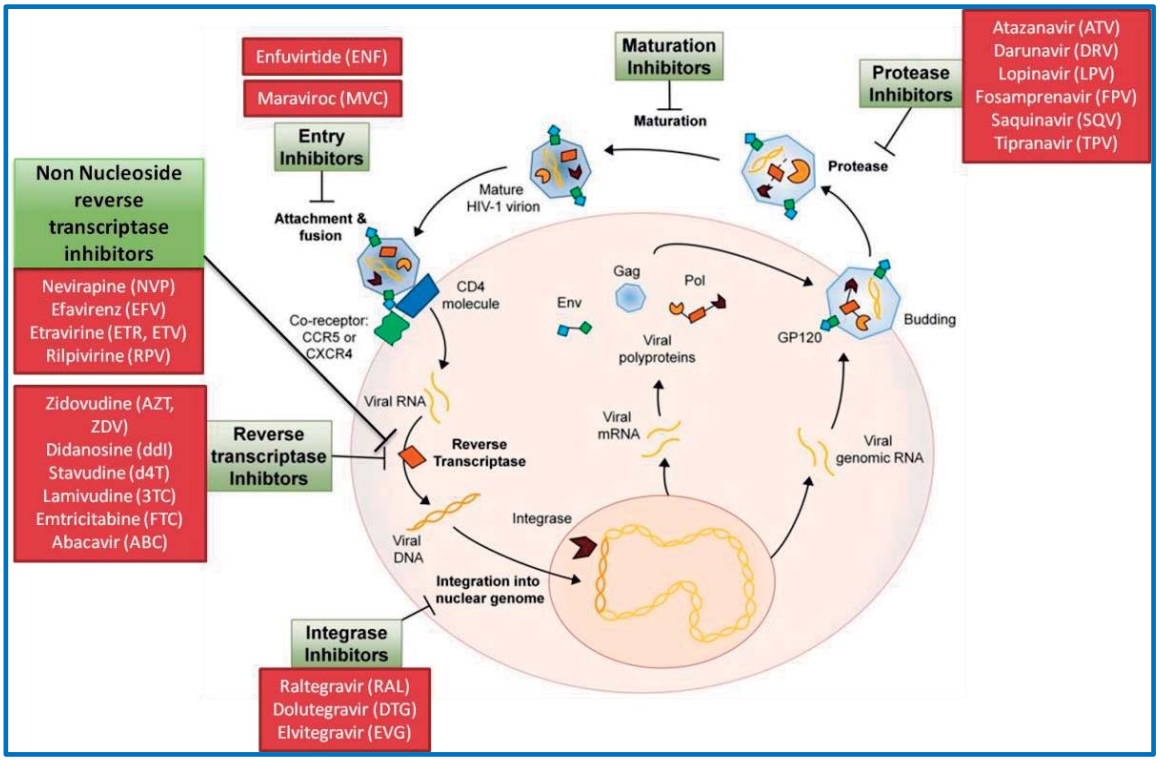


Figure 11. HIV-1 replication cycle with the drugs currently approved for HIV infection by the AIDS Study Group (GESIDA) of the Spanish Society of Infectious Diseases and Clinical Microbiology (image adapted from Smith *et al*., (2012) (Smith *et al.,* 2012)).

In 1987, the hope for a treatment for HIV infection came with Zidovudine (AZT), a NRTI, which had been developed years before for the treatment of cancer (Fischl *et al.,* 1987). In the following years, other drugs were developed for delaying the progression of HIV infection to AIDS. Thus, in the early 1990s, with the discovery of the first antiretroviral drugs, a variety of them administered as monotherapy were introduced to slow the virus progression in HIV-infected patients. In spite of initial successes, these monotherapies became ineffective because the ability of the HIV virus to develop resistances to single drug therapies. In 1995, the development of a new antiretroviral class (PIs), along with the introduction of the combined

antiretroviral therapy (cART) led to a "new era" in the therapy of HIV infection. All this meant a significant step forward in the management of HIV infection, reducing morbidity and mortality in all stages of HIV infection (Arts and Hazuda 2012;Deeks and Volberding 1995;Hoenigl *et al.,* 2016). In fact, the combination of at least two distinct molecular targets/drugs is the actual basis to prevent the evolution of drug resistance and for HIV-treatment. Thus, as shown in Figure 12, different initial combination regimen for ART-naive adult HIV-positive persons are recommended by the AIDS Study Group (GESIDA) of the Spanish Society of Infectious Diseases and Clinical Microbiology. Thus, thanks to this antiretroviral drug combination HIV-infection has became a chronic disease (Arts and Hazuda 2012;Gardner *et al.,* 2008), GESIDA: *http://gesida-seimc.org/category/guias-clinicas/antirretroviral-vigentes/*, date accessed: February 2018). There are also other cART that can be used for rescuing when virological failure or toxicities or other conditions happen. cART can decrease viral loads to undetectable levels and produces significant restoration of $CD4^+$ cells in the peripheral blood, more specifically its main goals are: long-term suppression of viral replication; reduce the morbidity associated with HIV infection and prolong survival; improve the quality of life; restore and preserve immune function; and prevent HIV infection. Although cART is highly effective at inhibiting HIV replication, several mechanisms contribute to HIV persistence during cART, including HIV latency, HIV induced immune dysfunction, and possibly persistent low-level viral replication in compartments and reservoirs, which allow the pathogenic disease processes (Arts and Hazuda 2012;Martinez-Picado and Deeks 2016). At intestinal level, cART only restores partial and slow $CD4^+$ cells and it is not capable of eliminating viral reservoirs, which are also resistant to clearance by the immune system of the host (Mavigner *et al.,* 2012;Mehandru *et al.,* 2004). Besides, it has been

observed that HIV-infected patients are in a state of inmunoactivation despite cART that could be related to the increased BT observed in patients with chronic HIV infection (Dinh *et al.,* 2015). This chronic activation may contribute to the development of different co-morbidities, such as atherothrombosis and other cardiovascular diseases, neurocognitive disorders, liver steatosis and osteoporosis (Younas *et al.,* 2016).

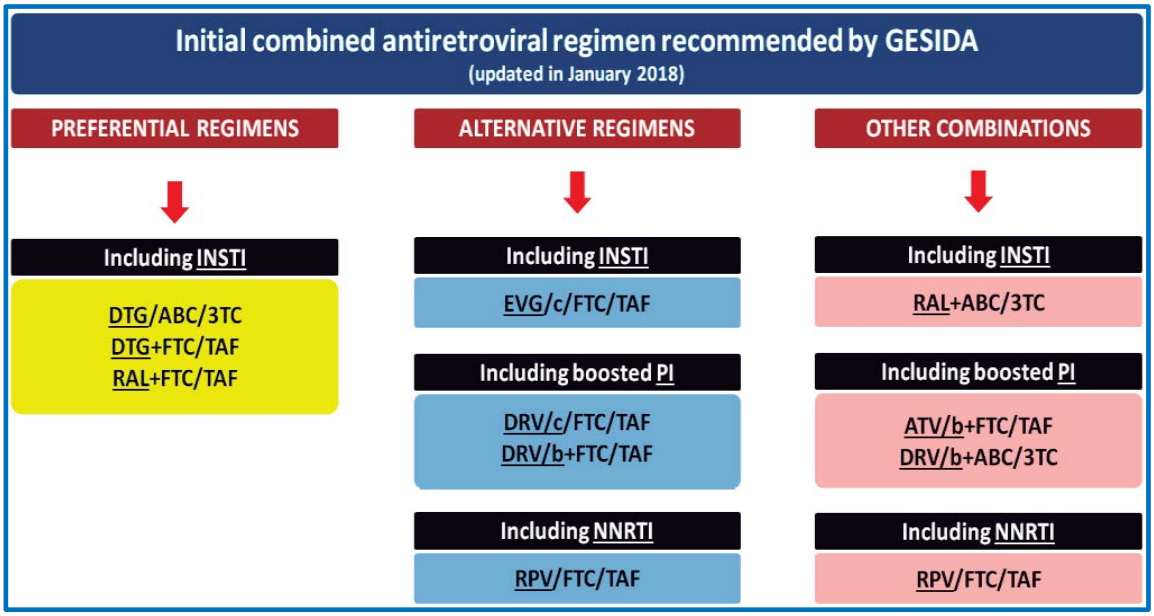


Figure 12. Initial combination regimen for ART-naive adult HIV-positive persons recommended by the AIDS Study Group (GESIDA) of the Spanish Society of Infectious Diseases and Clinical Microbiology (*http://gesida-seimc.org/category/guias-clinicas/antirretroviral-vigentes/, date accessed: February 2018*).

1.2.5.1. Nucleoside and nucleotide analogue reverse transcriptase inhibitors

Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs) were the first class of antiretroviral drugs approved for use by the United States Food and Drug Administration (FDA), and to date they are the "backbone" treatment when combined with a second class of antiretroviral

agents (Young 1988). NRTIs are administered as prodrugs, as these drugs need to be phosphorylated by cellular kinases to exert their antiviral effect. Therefore, NRTIs are phosphorylated within the host cell to their active diphosphate or triphosphate metabolites, which inhibit the enzymatic activity of the viral reverse transcriptase by incorporating into the nucleotide analogue resulting in the termination of the growing viral DNA chain or by competing with the natural substrate of the enzyme, halting the conversion of viral RNA into double stranded DNA (Arts and Hazuda 2012;Pau and George 2014). In 1987, Zidovudine (AZT) was the first approved antiretroviral drug for patients with CD4 count below 200 cells/mm^3 or with AIDS defining conditions (Fischl *et al.,* 1987). Currently, and as showed in Figure 12, GESIDA approves the use of the following NRTIs: zidovudine (AZT, ZDV), didanosine (ddI), stavudine (d4T), lamivudine (3TC), emtricitabine (FTC) and abacavir (ABC). The usage of the nucleotide reverse transcriptase inhibitors tenofovir disoprovil fumarate (TDF) and tenofovir alafenamide (TAF) is also authorized by both Societies (GESIDA: *http://gesida-seimc.org/category/guias-clinicas/antirretroviral-vigentes/*, date accessed: February 2018; EACS: *http://www.eacsociety.org/guidelines/eacs-guidelines/eacs-guidelines.html*, date accessed: February 2018). Regarding drug metabolism, NRTIs are not metabolized via the CYP450, thus, they are less likely to have drug-drug interactions (Vadlapatla *et al.,* 2014). Apart from ABC, the majority of NRTIs need an adjustment of dosage in patients with renal insufficiency (Pau and George 2014).

It is important to mention that serious toxicities have been observed in HIV-infected patients under treatment with older NRTIs, especially AZT and d4T, mostly related to their effects on human cellular mitochondrial DNA. Since these drugs are inhibitors of mitochondrial DNA polymerase gamma, they inhibit the transcription of mitochondrial RNA (mRNA), which

leads to mitochondrial dysfunction. This is associated with an increase in the levels of reactive oxygen species (ROS), promoting oxidative stress. In adipose tissue, an increase in ROS levels may lead to inhibition of cell differentiation and, instead, is associated with death of adipocytes with a loss of subcutaneous fat that could lead to clinical lipoatrophy. For this reason, these drugs are not included within the recommended initial regimens for naive patients. Older NRTIs are also associated with other severe toxicities such as lactic acidosis and hepatic steatosis (De Pauw *et al.,* 2009;Lewis *et al.,* 2003;Villarroya *et al.,* 2009), GESIDA: *http://gesida-seimc.org/category/guias-clinicas/antirretroviral-vigentes/*, date accessed: February 2018). Actually used NRTIs such as ABC, 3TC and FTC are associated with less toxicities, since these drugs are weaker inhibitors of mitochondrial DNA polymerase gamma (Moyle 2005). However, there is a long-lasting controversy about whether the usage of ABC is associated or not with an increased risk of cardiovascular disease in HIV-infected patients (Alvarez *et al.,* 2017). Regarding the nucleotide reverse transcriptase inhibitors, the first approved prodrug of tenofovir (TFV) TDF is associated with significant renal toxic effects and a decline in bone mineral density. However, the development of the novel prodrug of TFV TAF, that is available to patients to date, has improved renal and bone safety compared to TDF-containing regimens (Huhn *et al.,* 2017;Wang *et al.,* 2016).

1.2.5.2. Non nucleoside reverse transcriptase inhibitors

Unlike NRTIs, non nucleoside reverse transcriptase inhibitors (NNRTIs) do not need intracellular phosphorylation to exert their pharmacologic action. Members of this class are noncompetitive inhibitors of RT, thus, the binding of NNRTIs results in a change of the spatial conformation of the substrate-binding site, which reduces polymerase

activity (Arts and Hazuda 2012;Pau and George 2014). At this time, both GESIDA and EACS approve the use of four NNRTIs: nevirapine (NVP), efavirenz (EFV), etravirine (ETR, ETV) and rilpivirine (RPV) (GESIDA: *http://gesida-seimc.org/category/guias-clinicas/antirretroviral-vigentes/*, date accessed: February 2018; EACS: *http://www.eacsociety.org/guidelines/eacs-guidelines/eacs-guidelines.html*, date accessed: February 2018). Despite the fact that these drugs produce a potent virologic suppression, NNRTIs are limited because of their drug-drug interactions since they are highly metabolized by CYP450. Thus, special attention should be paid when these drugs are prescribed with other agents that are also highly metabolized by CYP450 (Ma *et al.,* 2005). In general terms, NNRTIs are generally safe and well tolerated, although NVP is associated with rash and hepatotoxicity, whereas the use of EFV can cause central nervous system adverse effecs as well as lipoatrophy (Rojas *et al.,* 2016;Usach *et al.,* 2013).

1.2.5.3. Proteinase inhibitors

Proteinase inhibitors (PIs) perform its pharmacologic action by binding HIV proteases, blocking the proteolytic activities of the enzyme, thus, the new infectious virions cannot be formed (Pau and George 2014). Ritonavir (RTV) was the first approved PI but it is not currently use as anti-HIV agent because of its side effects and high doses required. Nowadays, RTV is used at low doses as a PI booster. GESIDA approves the use of six PIs, including atazanavir (ATV), darunavir (DRV), lopinavir (LPV), fosamprenavir (FPV), saquinavir (SQV), and tipranavir (TPV) (GESIDA: *http://gesida-seimc.org/category/guias-clinicas/antirretroviral-vigentes/*, date accessed: February 2018). In addition of these treatments, the EACS also approves the use of indinavir (IDV). However, this drug is associated with intolerable side

effects, such as lipodystrophy or renal and urologic toxicity (EACS: *http://www.eacsociety.org/guidelines/eacs-guidelines/eacs-guidelines.html*, date accessed: February 2018) (Jao and Wyatt 2010;Lv *et al.,* 2015;Pau and George 2014). Therapies in naive patients with PIs must include a booster, either RTV or cobicistat (COBI). RTV is recommended with most PIs, while COBI has been recommended to only boost ATV and DRV (GESIDA: *http://gesida-seimc.org/category/guias-clinicas/antirretroviral-vigentes/*, date accessed: February 2018). This is due to the fact that RTV is a potent inhibitor of the CYP3A4 isozyme, the primary enzyme involved in the metabolism of most PIs. Thus, this drug reduces the metabolism of concomitantly administered PIs, resulting in an increase in absorption and prolongation of the half-life, enabling the use of the PI in lower doses and dosing frequency (Boffito 2006;Zeldin and Petruschke 2004).

Metabolic abnormalities are the major side effects associated with the use of PIs, including lipodystrophy, insulin resistance, hyperglycemia, metabolic syndrome, and dyslipidemia. Thus, its long-term usage may lead to cardio-metabolic dysfunction (Coffinier *et al.,* 2008;Reyskens *et al.,* 2013). ATV and DRV, are associated with fewer side effects in comparison with the others PIs (Lv *et al.,* 2015).

1.2.5.4. Integrase strand transfer inhibitors

This antiretroviral class is the most recently produced, being raltegravir (RAL) the first integrase strand transfer inhibitor (INSTI) approved by the FDA in 2007 (Temesgen and Siraj 2008). INSTIs catalyze the formation of covalent bonds between the host and viral DNA, which blocks the virus integrase enzyme and avoids the incorporation of viral DNA into the host chromosome. Concretely, INSTIs selectively inhibit the strand transfer reaction, hence its name (Arts and Hazuda 2012;Pau and George

2014). Both GESIDA and EACS approve the use of three INSTIs: RAL, dolutegravir (DTG) and elvitegravir (EVG). A fourth drug, bictegravir (BIC), is currently in the process of being approved (GESIDA: *http://gesida-seimc.org/category/guias-clinicas/antirretroviral-vigentes/*, date accessed: February 2018; EACS: *http://www.eacsociety.org/guidelines/eacs-guidelines/eacs-guidelines.html*, date accessed: February 2018). This antiretroviral class has a potent antiviral activity and it is generally well tolerated. For this reason, INSTIs are included in the recommended treatment naive patients. EVG is administered with COBI, as it needs to be enhanced (Pau and George 2014) (GESIDA: *http://gesida-seimc.org/category/guias-clinicas/antirretroviral-vigentes/*, date accessed: February 2018).

The three currently approved INSTIs are generally well tolerated in both treatment-naive and treatment-experienced patients, being headache and gastrointestinal effects the most frequent adverse events (Lee and Carr 2012).

1.2.5.5. CCR5 antagonists

Maraviroc (MVC) is the only CCR5 antagonist approved for clinical use in patients infected with R5-tropic HIV-1 (GESIDA: *http://gesida-seimc.org/category/guias-clinicas/antirretroviral-vigentes/*, date accessed: February 2018; EACS: *http://www.eacsociety.org/guidelines/eacs-guidelines/eacs-guidelines.html*, date accessed: February 2018). MVC, as CCR5 antagonist, binds to human CCR5 receptor on the cell membrane, then, inhibits the interaction of the HIV gp120 and the CCR5 receptor for CCR5-tropic HIV. Nevertheless, MVC is not able to block viral entry of CXCR4 tropic HIV or HIV that uses both CCR5 and CXCR4 for cell entry (Pau and George 2014). For this reason, MVC must be prescribed exclusively in patients with

infection with HIV-1 strains that are R5-tropics. The HIV-1 tropism is analyzed by genotypic methods, which determine the sequence of the V3 region of the viral envelope (gp120) (GESIDA: *http://gesida-seimc.org/category/guias-clinicas/antirretroviral-vigentes/*, date accessed: February 2018). An important disadvantage of MVC is that co-receptor usage may change along HIV infection, thus, the tropism of HIV-1 should be measured before starting treatment and also when a treatment failure occurs with an antagonist of CCR5 (Pau and George 2014). MVC is extensively metabolized by CYP3A4, thus, MVC needs a dose adjustment when administered with agents that modulate the activity of CYP3A4 (Abel *et al.,* 2009). Because of this drug interaction, the need for testing the HIV tropism and the discomfort of twice per day usage, MVC is not commonly used in clinical practice (Pau and George 2014;Tremblay *et al.,* 2013). In addition, clinical trials have reported adverse effects of MVC, such as upper respiratory tract infections, allergic reactions, fever, hepatotoxicity, and cardiovascular disorders (AIDSinfo website from the USA Department of Health and Human Services: *https://aidsinfo.nih.gov/drugs/408/maraviroc--hiv-treatment/0/patient*, date accessed: February 2018).

1.2.5.6. Fusion inhibitors

Enfuvirtide (ENF) is the only fusion inhibitor approved for use in HIV-infected patients (GESIDA: *http://gesida-seimc.org/category/guias-clinicas/antirretroviral-vigentes/*, date accessed: February 2018; EACS: *http://www.eacsociety.org/guidelines/eacs-guidelines/eacs-guidelines.html*, date accessed: February 2018). The fusion inhibitor ENF binds to gp41 and prevents its conformational change necessary for the fusion of the viral and cellular membrane, therefore, inhibiting viral entry into host cells (Matos *et al.,* 2010). A disadvantage of this agent is that it is only available in an

injectable formulation and needs to be administered twice daily. Thus, most patients have injection site reaction such as pain, erythema, induration, etc., along with the discomfort of this type of administration. Concerning side effects, clinical trials have reported an increase in the incidence of pneumonia in HIV-infected patients under ENF (Pau and George 2014) (EACS: *http://www.eacsociety.org/guidelines/eacs-guidelines/eacs-guidelines.html*, date accessed: February 2018).

1.2.6. Metabolic alterations in HIV-infected patients

1.2.6.1. Effects of HIV infection per se on metabolism

It was demonstrated that HIV itself is associated with a state of persistent inflammation and immune activation, metabolic abnormalities, and vascular dysfunction (Beltran *et al.,* 2015). In general, HIV infection leads to the activation of various inflammatory pathways, causing the release of cytokines. Several of these cytokines cause endothelial activation and alter its functionality. Moreover, HIV causes direct endothelial cell damage, increasing endothelial permeability, promoting apoptosis and increasing the expression of adhesion molecules (E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1)). HIV infection also produces an increase of various activation markers on monocytes/macrophages (sCD163, sCD14, and $CD14^{+}/CD16^{+}$ monocyte expansion) (Beltran *et al.,* 2015;Dhawan *et al.,* 1997;Jia *et al.,* 2001;Oshima *et al.,* 2000;Ren *et al.,* 2002) (reviewed by Beltran *et al*., 2015). Furthermore, HIV blocks the adenosine triphosphate-binding cassette transporter A1 (ABCA-1) pathway, suppressing reverse cholesterol transport from arterial wall macrophages to HDL particles, leading to cholesterol accumulation and promoting conversion of macrophages into foam cells within atherosclerotic plaques. Also, HIV promotes atherogenesis decreasing HDL cholesterol and

its major protein component, knows as apolipoprotein A1, which participates in lipid metabolism. HIV also decreases LDL particle clearance, as well as increases triglycerides and VLDL cholesterol (Beltran *et al.,* 2015;Grunfeld *et al.,* 1992;Norata *et al.,* 2011;Rose *et al.,* 2008) (reviewed by Beltran *et al*., 2015). Moreover, HIV causes oxidative stress, altering the mechanisms of DNA repair and leading to the accumulation of oxidative DNA damage (Aukrust *et al.,* 2005).

As it was previously mentioned, HIV induces BT that causes proinflammatory cytokines secretion and immunoactivation, leading to the development of a chronic inflammatory state with deleterious effects on health (Miedema *et al.,* 2013;Nasi *et al.,* 2014;Sandler and Douek 2012). Thus, it was suggested that BT can be a predictor of disease progression, poor immune restoration, and non-AIDS morbidity (such as atherosclerosis, dyslipidemia, and insulin resistance), regardless of viraemia (Kelesidis *et al.,* 2012;Pedersen *et al.,* 2013).

1.2.6.2. Effects of cART on metabolism

The introduction of cART in HIV-infected patient significantly reduced the HIV-related morbidity and mortality, and consequently, survival rates and quality of life increased in patients with access to this treatment (Krentz *et al.,* 2005;Pacheco *et al.,* 2009). Thus, HIV infection became a chronic disease that requires life-long treatment. However, the prolonged use of the antiretroviral drugs are associated with the development of long-term toxicities, as previously mentioned, such as insulin resistance, osteoporosis, lipodystrophy, dyslipidaemia, hyperglycaemia, hypertension, and cardiovascular disease (CVD) besides of kidney and bone toxicities (Obirikorang *et al.,* 2016;Wu *et al.,* 2012). Moreover, as life-expectancy increased, these patients are exposed to the effects of "aging" itself (Paula *et*

al., 2013). In fact, treated HIV patients show signs of premature and accelerated aging. The acceleration of the aging process of the immune system leads to immunosenescence, which is characterized by continuous activation of the immune system and a low-grade inflammation. Thus, patients are predisposed to co-morbidities and natural aging symptoms more frequently seen in elderly people in the general population. Therefore, HIV patients have higher rates of CVD, non-AIDS cancers, frailty (loss of muscle mass, osteoporosis, and muscle weakness), kidney or liver disease, and neurologic complications (such as dementia) compared to uninfected subjects (Alejos *et al.,* 2014;Choi *et al.,* 2009;Deeks *et al.,* 2012;Desquilbet *et al.,* 2007;Negredo *et al.,* 2017;Smith *et al.,* 2012;Triant *et al.,* 2007).

The chronic inflammatory state due to HIV virus infection along with the prolonged use of the antiretroviral drugs, especially those that affect the mitochondria, could lead to the development of different metabolic alterations such as the well-known HIV-associated lipodystrophy syndrome (HALS). This syndrome is characterized by a loss and/or accumulation of fat and called as lipohypertrophy (fat accumulation on the abdominal area), lipoatrophy (fat reduction in peripherical regions), and/or mixed lipodystrophy (association of lipoatrophy with lipohypertrophy) (Sacilotto *et al.,* 2017). As previously stated, NRTIs are known to inhibit the mitochondrial DNA polymerase and, therefore, contributes to mitochondrial toxicity, apoptosis and loss of adipose mass. Besides NRTIs, PIs can also cause severe mitochondrial damage by increasing oxidative stress and diminishing mitochondrial function, which increase apoptosis, and, therefore, could also lead to the development of HALS (Obirikorang *et al.,* 2016;Perez-Matute *et al.,* 2013;Wu *et al.,* 2012). Although older NRTIs were associated with mitochondrial toxicity and other alterations in adipose tissue, fortunately, this is nowadays less common thanks to the clinical advances achieved in the

development of ARTs (Margolis *et al.,* 2014). However, the persistent immune activation and chronic inflammation, as well as the usage of PIs, have been nowadays associated with the development of metabolic syndrome (MS) in HIV patients (Nasi *et al.,* 2017;Swami 2016). MS is a collection of cardiometabolic risk factors including obesity, hypertension, dyslipemia, and insulin resistance, increasing the risk for CVD and type 2 diabetes mellitus (Roberts *et al.,* 2013). The prevalence of MS in HIV-infected people has become quite similar than that reported for the general population with no HIV-infection (Nguyen *et al.,* 2016). In fact, the prevalence of MS in HIV-infected patients ranges from 7–47 % depending on the MS definition and the study design used, but in all causes, the presence of this syndrome should be taken into account when designing health strategies, since the presence of MS *per se* or its separate components could be responsible for an increased cardiovascular risk in these patients and for a significant reduction of their quality of life (Naidu *et al.,* 2017;Worm and Lundgren 2011).

1.2.6.3. In vitro and in vivo models to study the effect of different antiretroviral drugs on metabolism

RAL and DRV antiretroviral drugs are known to be effective options for both antiretroviral-naive and experienced patients, with few reported side effects. For this reason, our group became interested in the study of the molecular mechanisms that could explain the lack of toxicity of RAL and DRV in metabolism. For this purpose, an *in vitro* model was used, particularly 3T3-L1 adipocytes (derived from mice). The INSTI RAL showed the absence of siginificant actions on adipogenesis and glucose and lipid metabolism in adipocytes, which could explain, at least in part, the neutral metabolic effects of RAL in clinical studies (Perez-Matute *et al.,* 2011). These results were in

line with other studies in which RAL did not show any deleterious effects in both 3T3-L1 adipocytes and human adipocytes (Minami *et al.,* 2011;Moure *et al.,* 2016). On the other hand, the PI DRV showed minimal effects on mature adipocyte metabolism. Concretely, it was observed that DRV decreased in lipolysis, glucose uptake, and lactate production at the highest concentration used (50 μM:). As this drug needs to be boosted, the effect of co-treatment with RTV was also analyzed, and it was observed that co-treatment with RTV did not induce any further effects on lipolysis and glucose metabolism. Hence, the study carried out by our group suggested that the decrease in lipolysis observed after DRV treatment could explain, at least in part, the lower plasma lipids observed in patients under DRV/r treatment in comparison with other drugs. In addition, the lack of effects of RTV co-treatment on glucose and lipid metabolism emphasizes the safety of this treatment (Perez-Matute *et al.,* 2012). Similar results were observed by the group of Capel *et al*., (2012), although they observed a modestly altered differentiation of murine and human adipocytes, but also a disturbed mitochondrial function when associated with RTV (Capel *et al.,* 2012).

Our group also investigated the effect of MVC on a mouse model of diet-induced obesity. The reason for this was because MVC blocks CCR5 that has as natural ligand the proinflammatory molecule CCL5 (also known as RANTES), which is an important chemoattractant for inflammatory cells. RANTES has been implicated in several pathologies, including atherosclerosis, liver fibrosis, obesity and cancer (Berres *et al.,* 2010;Blanco and Ochoa-Callejero 2016;Huber *et al.,* 2008;Veillard *et al.,* 2004). Our group demonstrated that block CCR5 in mice fed with a HFD was associated with beneficial effects at adipose and hepatic level. Specifically, the addition of MVC in the drinking water in a mouse model of diet-induced obesity was able to counteract the increase in body weight, hepatic triglycerides and

ameliorate the development of hepatic steatosis when compared to untreated mice on a HFD (Perez-Martinez *et al.,* 2014;Perez-Martinez *et al.,* 2018). Moreover, *in vivo* studies showed that MVC is able to decrease adipose tissue inflammation in mice on a HFD by decreasing adipose tissue macrophage recruitment (Perez-Matute *et al.,* 2017). Interesting data have been also obtained with MVC from other studies using murine models designed to prevent or treating atherogenesis, or pulmonary arterial hypertension, or hepatocellular carcinoma or breast cancer, among others (Amsellem *et al.,* 2014;Cipriani *et al.,* 2013;Ochoa-Callejero *et al.,* 2013;Velasco-Velazquez *et al.,* 2012).

1.2.7. Coinfection with hepatotropic viruses

The routes of HIV transmission are similar to some of the hepatotropic viruses such as hepatitis C (HCV) and hepatitis B (HBV). The risk of coinfection with these pathogens is related to a faster progression of hepatic injury. There are five unrelated hepatotropic viruses: hepatitis A (HAV), HBV, HCV, hepatitis D (HDV), and hepatitis E (HEV). Nowadays, HEV is the main cause of acute hepatitis in the world, while HBV and HCV are the main causes of chronic hepatitis, cirrhosis and hepatocarcinoma. To date there is an increase in the number of cases of acute HAV infection among MSM (Kamar *et al.,* 2014;Kokki *et al.,* 2016;Rodriguez-Tajes *et al.,* 2018) (CDC: *www.cdc.gov*, date accessed: February 2018; WHO: *http://www.who.int/csr/don/07-june-2017-hepatitis-a/en/*, date accessed: April 2018). Most of studies are focused on HIV coinfected patients with HCV and HCB. The prevalence of active HCV has improved in our environment with the actual effective treatments, which fell from 34% in 2009 to 11.7% in 2016 (Berenguer *et al.,* 2018). The global prevalence of HBV coinfection is 7.4% (WHO: http://www.who.int/mediacentre/factsheets/fs204/en/, date

accessed: April 2018). Although the impact of HCV or HBV on HIV remains unclear, it was demonstrated that HIV accelerates the natural course of HBV and HCV infection, leading to a faster progression of liver disease to cirrhosis and hepatocellular carcinoma. Regarding HCV-infected patients, it was observed that HIV infection increases levels of HCV viraemia by 2- to 8-fold, resulting in a significant decrease in spontaneous recovery of acute hepatitis (Luetkemeyer 2010;Soto *et al.,* 1997). The advent of cART increased the life expectancy of HIV-infected patients, and, consequently, liver disease emerged as the major cause of mortality and morbidity in those HIV patients coinfected with hepatotropic viruses (Sulkowski 2008).

The last years have been crucial in the fight against HCV. Few years ago, physicians only had therapies based on the combination of weekly pegylated interferon-α and daily doses of ribavirin to treat this infection. The efficacy of these therapies was not higher than 50%, and their mechanism of action was not direct against the virus, but was based on enhancing the immune system. In 2011, the arrival of the first generation direct-acting antiviral agents and more recently the ability of the new ones agents have change the natural history of the infection since we can eliminate HCV in more than 95% of the treated infected people. Unlike the previous therapies, these new regimens cause fewer side effects and they require a shorter duration (Barth 2015;Pawlotsky *et al.,* 2015). Regarding HBV, there is a safe vaccine, which prevents HBV infection by immunization with 90%–95% effectiveness (Chang and Chen 2015). However, there are no effective treatments for eliminating HBV as occurs in HCV infection. Chronic hepatitis B infection can be treated with oral antiviral agents, including entecavir or tenofovir. Both agents provide sustained suppression of HBV replication and clinical benefit in most HBV-infected patients (Soriano *et al.,* 2017) (*www.who.int/en,* date accessed: January 2018).

The pathogenic effects associated with HBV and HCV co-infections are highly variable ranging from acute (including fulminant hepatic failure in HBV infection) to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Liang 2009;Nakamoto and Kaneko 2003). Thus, the normal liver architecture is lost by the excessive accumulation of extracellular matrix proteins including collagen, leading to fibrosis progression and subsequent cirrhosis (Bataller and Brenner 2005). However, the effect of HBV and HCV seems not to be limited to liver and also results in failure in immunological recovery in HIV-infected patients despite cART (Bhaumik 2015). It is important to mention the strong relationship between the liver and the intestine, which is termed as gut-liver axis. This linkage is characterized by bidirectional traffic, for instance, the transport of nutrients and other signals from the intestinal lumen to the liver through the portal circulation, and the release of bile acids secreted by hepatocytes into the small intestine through the biliary tract that have direct effects on bacteria, causing damage to the bacterial DNA (Poeta *et al.,* 2017;Szabo 2015). Under normal conditions, the integrity of the intestinal epithelium and the regular control of the immune system in the intestine reduce the passage of bacterial products to the liver through the portal system. In turn, the liver as the second largest immune organ in the body hosts a large amount of immune cells, maintaining a "state of immune tolerance" in the absence of inflammation. In the presence of an alteration in GM composition and an alteration in the intestinal epithelium integrity, a greater BT occurs. These bacteria and bacterial products can reach the liver through the portal circulation, disrupting the state of immune tolerance and promoting liver inflammation. The passage of bacteria and bacterial products to the liver leads to the activation of immune cells and, as consequence, proinflammatory cytokines are produced, which, in turn, could increase further intestinal permeability, creating a vicious cycle (Arab *et al.,*

2017;Poeta *et al.,* 2017;Szabo 2015). Thus, because of the liver damage caused by these viruses have effects on GM "in both directions", coinfection with hepatotropic viruses is a factor that must be considered when microbiota is described in coinfected HIV patients.

1.2.8. Microbiota & HIV infection

1.2.8.1. Alterations of microbiota in blood, semen and vagina in HIV-infection

For a long time it was assumed that the human plasma of healthy individuals was sterile, however, some groups observed numerous bacteria in blood culture from healthy participants (Damgaard *et al.,* 2015;McLaughlin *et al.,* 2002;Nikkari *et al.,* 2001). This has been confirmed by a recent metagenomic study carried out in different fractions of the blood (buffy coat, plasma and red blood cells) from healthy volunteers, in which they observed a diversified microbiome that differs between blood fractions and donors. Concretely, Paisse *et al*., (2016) found a dominance of Proteobacteria (more than 80%) and Actinobacteria (6.7%-10% depending on the fraction), which differs dramatically from the phyla predominant in the gut (Firmicutes and Bacteroidetes) (Paisse *et al.,* 2016;Turnbaugh *et al.,* 2006). Thus, bacterial DNA can be found in the blood of healthy and immunocompromised patients (Oteo *et al.,* 2017). Regarding HIV-infected patients, Li, *et al*., (2012) found that the amount of bacterial DNA in blood was lower in healthy controls than in HIV-infected patients without receiving cART, being the bacteria belonging to the Pseudomonadales order the predominant component in HIV patients. Moreover, they found that the bacterial elements found in blood were very similar than those living in the gut, suggesting BT (Li *et al.,* 2012). The study by Merlini *et al*., (2011)

observed that the polymicrobic flora circulating in peripheral blood in HIV-infected patients is not markedly modified by a cART (Merlini *et al.,* 2011).

Apart from blood, HIV could also be transmitted through semen and, to our knowledge, only one group has analyzed the semen microbiome and its relationship with local immunology and viral load in HIV infection. Liu *et al*., (2014) have observed that *Streptococcus*, *Corynebacterium*, and *Staphylococcus* are common semen bacteria, regardless of HIV status. *Ureaplasma* was the more abundant genus belonging to Mollicutes class in HIV-uninfected men, while *Mycoplasma* dominated after HIV infection. They also found that HIV infection was associated with decreased semen microbiome diversity and richness, which were restored after six months of cART. In addition, semen bacterial load of HIV-infected patients correlated with several pro-inflammatory semen cytokines (including IL-6, TNFα, and IL-1b) and with semen HIV viral load, which could suggest that semen microbiome has a role in HIV sexual transmission. (Liu *et al.,* 2014).

Regarding the female reproductive tract, the mucosal immune system of this region is one of the first lines of defenses against HIV-infection and other pathogens (Vitali *et al.,* 2017). Lying superficial to the epithelial cells of the vaginal tract is the microbiota, which exists in a symbiotic relationship with the female host. This vaginal microbiota living on the mucus layer helps to inactivate the HIV by secreting H_2O_2 or by decreasing the pH of the environment, among other mechanisms (Haase 2005). In fact, lactic acid and SCFAs produced by vaginal microbiota have reported antimicrobial and immune modulatory activities indicating their potential as biomarkers of disease and/or disease susceptibility (Aldunate *et al.,* 2015).

The current concept of a “healthy” vaginal microbiota includes that dominated by only one or two species, the most common of which are

Lactobacillus iners, *Lactobacillus crispatus*, *Lactobacillus jensenii* and *Lactobacillus gasseri*. These bacteria are known to be the most common dominant species in the vaginal microbiome of Caucasian, Asian, Black and Hispanic women (Huang *et al.,* 2014). However, alterations of diet, inflammation, menstrual cycle, usage of hormonal contraceptives and infection with other viruses (HPV (Human papillomavirus) or HSV (Herpes simplex virus)) can affect the composition and activity of the vaginal microbiota, which may enhance the chance of HIV infection (Murphy *et al.,* 2014). Bacterial vaginosis is a symptomatic clinical condition characterized by a significant reduction of *Lactobacillus* (*L. iners* and *L. crispatus*) populations and overgrowth of anaerobes such as *Prevotella bivia* and Lachnospiraceae (Hummelen *et al.,* 2010). Although bacterial vaginosis has been consistently linked to increased susceptibility to HIV-infection (Atashili *et al.,* 2008;Hummelen *et al.,* 2010;Schellenberg *et al.,* 2012), it is becoming increasingly clear that a lower bacterial diversity, even in the absence of bacterial vaginosis, might also confer greater susceptibility to disease (Anahtar *et al.,* 2015;Gosmann *et al.,* 2017;Jespers *et al.,* 2017). Concerning cART, it was observed that certain microbiota present in female genital tract decreased the vaginal antiretroviral drug concentrations in HIV-infected women. Hence, optimizing antiretroviral concentrations used for biomedical HIV prevention in women could be necessary in some cases (Donahue *et al.,* 2017).

1.2.8.2. Alterations of microbiota in oral cavity and airway in HIV-infection

It was demonstrated that HIV-infection is associated with alterations in the microbiota of the respiratory tract (Lawani and Morris 2016;Twigg *et al.,* 2017). Most of the studies focused on lung microbiota derived from the

Lung HIV Microbiome Project (LHMP) (*https://lhmp.bsc.gwu.edu/*) (Cui *et al.,* 2014). This project was driven by the recognition that pulmonary complications continued to be a major causes of morbidity in HIV-infected individuals even in the era of cART (Grubb *et al.,* 2006). To date, evidence suggests that lung microbiota in HIV-infected individuals with preserved CD4 counts is similar to uninfected individuals. However, in individuals with more advanced disease present an altered alveolar microbiota characterized by a loss of richness and diversity, but an increase in beta diversity differences between individuals. These differences decline with cART, but even after effective therapy the alveolar microbiota in some HIV-infected individuals contain increased amounts of characteristic bacteria, some of which have been previously associated with chronic lung inflammation (Twigg *et al.,* 2017). Despite these findings, other studies have failed to find out such differences in lung microbiota when comparing non-HIV infected individuals and HIV-infected patients (Beck *et al.,* 2015). A very recent study has suggested that microbial communities and their interactions with the host may have functional metabolic impact in the lung (Cribbs *et al.,* 2016). However, more studies are needed in order to have a profound knowledge of the influence of such microbiota on HIV-associated complications.

HIV-infection has also been associated with a variety of oral manifestations and oral microbiota has been suggested to be involved in such HIV-related complications (Li *et al.,* 2014;Moyes *et al.,* 2016). Thus, Dang *et al*., (2012) observed a shift of microbial composition in the lingual region, which was related to the viral load in early-stage HIV patients (Dang *et al.,* 2012). ART has also significant effects on salivary microbial colonization (Li *et al.,* 2014). In addition, a recent study has also observed that the site of collection of oral microbiota could be determinant, as plaque and saliva showed a distinct microbial composition, and, only in saliva, minor but

significant differences were observed when compared non-HIV infected patients with HIV-infected patients (Kistler *et al.,* 2015). Thus, there is still lot of work to do in order to standardize the best sample to analyze and in which conditions to provide clinical significance.

1.2.8.3. Gut microbiota composition in HIV infection

As previously mentioned, HIV infection is associated with disturbances at intestinal level. In this context, several studies have described the main changes that occur in gut in HIV-infected patients, more specifically on GM, as a way to develop new strategies to reduce inflammation and, therefore, to improve HIV-associated immune dysfunction and associated pathologies (excellent reviews such as Ziberman-Schapira *et al*., (2016); Dubourg *et al*., (2017); Dillon *et al*., (2016); Tincati *et al*., (2016); El-Far and Tremblay (2018)) (Dillon *et al.,* 2016;Dubourg *et al.,* 2017;El-Far and Tremblay 2018;Tincati *et al.,* 2016;Zilberman-Schapira *et al.,* 2016).

1.2.8.3.1. Bacterial diversity/richness

HIV infection has been mostly associated with a reduced bacterial diversity in gut (McHardy *et al.,* 2013;Mutlu *et al.,* 2014;Nowak *et al.,* 2015). However, some authors did not find differences in bacterial diversity when comparing healthy controls with untreated HIV-infected patients (HIV+(naive)) and under cART (HIV+(cART) (Dillon *et al.,* 2014;Dinh *et al.,* 2015;Ling *et al.,* 2016;Vujkovic-Cvijin *et al.,* 2013), whereas Lozupone *et al*., (2013) observed a significant increase in HIV+(naive) compared to those under cART (Lozupone *et al.,* 2013). A study carried out in stomach fluid also observed a reduced bacterial diversity in HIV-infected people (von Rosenvinge *et al.,* 2013). Therefore, more studies with a greater number of

patients are needed to better understand what is happening and to associate these findings with the inflammatory-associated alterations observed in HIV-infected patients (Tables 1 and 2).

Table 1. Metagenomic studies in HIV-infection and impact on microbial diversity/richness.

Author	Sample	Control	HIV+(*naive*)	HIV+(cART)	α-diversity/richness	Is ART able to restore gut microbiota diversity?
Gastrointestinal tract						
Lozupone *et al.*, (2013)	Stool	13	3 Recent HIV-infected + 11 chronic HIV-infected untreated	6 (short-term) + 8 (long-term)	Untreated HIV+ patients had significantly higher alpha diversity compared to ART-treated and HIV negative individuals	Partially
McHardy *et al.*, (2013)	Rectal mucosal	20	20	20	HIV infection resulted in a slight reduction of alpha diversity in untreated HIV+ patients subjects that was reversed to near equivalence with negative individuals in ART-treated subjects	Partially
Vujkovic-Cvijin *et al.*, (2013)	Recto sigmoid biopsy	9	6 HIV-infected untreated and 1 HIV-infected long-term non-progressor	18	No significant differences between HIV+ and negative individuals	No

Author	Sample	Control	HIV+(*naive*)	HIV+(cART)	α-diversity/richness	Is ART able to restore gut microbiota diversity?
Dillon *et al.*, (2014)	Rectal swab, rectal aspirate and colon biopsies	14	18	-	No significant difference was found	-
Mutlu *et al.*, (2014)	Stool and colon and terminal ileum biopsies	22	-	21	HIV+ patients had less alpha diversity than negative individuals	-
Dinh, *et al.*, (2015)	Stool	16	-	21	No significant difference was found	-
Nowak *et al.*, (2015)	Stool	9	28 (19 were analyzed after ART introduction) + 3 ellite controllers	19	Decreased alpha diversity in untreated HIV+ patients compared to negative individuals	Partially
Ling *et al.*, (2016)	Stool	16	35	32	No significant difference was found	Partially

Author	Sample	Control	HIV+(*naive*)	HIV+(cART)	α-diversity/richness	Is ART able to restore gut microbiota diversity?
Pinto-Cardoso *et al.*, (2017)	Stool	10	-	33	Reduced	No
Oral cavity and airway						
Li *et al.*, (2014)	Saliva	10	10	10 (same naive patients after 6 months on HAART)	Decreased oral biodiversity in HIV-infected persons relative to uninfected controls	Partially
Kistler *et al.*, (2015)	Saliva Plaque	37	-	37	Reduced bacterial diversity in the saliva of HIV-positive patients compared to HIV-negative individuals	-
Dang *et al.*, (2012)	Oral swab of the dorsal tongue surface	9	6	6	Chronic HIV infection leads to substantial disruptions in the community structure of the lingual microbiota although no significant differences in the number of bacterial species was found between treated or untreated HIV infected groups and healthy controls	-

Author	Sample	Control	HIV+(*naive*)	HIV+(cART)	α-diversity/richness	Is ART able to restore gut microbiota diversity?
Beck *et al.*, (2015)	BAL Oral washes	86 67	18 14	38 30	No significant differences were found among groups	No
Cribbs *et al.*, (2016)	BAL	20	-	39	No significant differences were found between the lung microbiota of HIV-infected and HIV-uninfected groups	-
Semen						
Liu *et al.*, (2014)	Semen	22	27	27 (same naive patients after 6 months on ART)	HIV infection was associated with decreased semen microbiome diversity	Yes

Table 2. Other metagenomic studies focused on microbial diversity.

Author	Sample	Control	HIV+	α-diversity
Gastrointestinal tract				
Yu *et al.*, (2014)	2 rectal swab (1-5 weeks)	32 samples 1 41 samples 2	41	No significant differences were found when examining sample 1 Reduced alpha diversity in sample 2
Noguera-Julian *et al.*, (2013)	Stool	57	296	Decreased alpha diversity in HIV+ patients compared to negative individuals
Von Ronsenvinge *et al.*, 2013	Stomach fluid	21	4	Reduced bacterial diversity in HIV-infected people
Brain				
Branton *et al.*, (2013)	Brain tissue	6	4	No significant differences were found among groups
Genital site				
Gosmann *et al.*, (2017)	Recto- cervical swab	205	31	HIV acquisition is Increased in women with high- diversity, low Lactobacillus abundance

1.2.8.3.2. Microbial Composition

Several studies have observed a characteristic profile in HIV-infected patients (untreated chronic infection) compared to a control/non-infected population. In general terms and as summary, depletion in Clostridia class and *Bacteroides spp*., an overgrowth of Enterobacteriaceae, and an increase in the genus *Prevotella* have been largely observed in HIV-infected patients (reviewed by Dubourg *et al*., (2017)) (Dubourg *et al.,* 2017).

Concerning specific alterations in microbiota in the right colon and terminal ileum, the study of Mutlu *et al*., (2014) showed that HIV-patients under ART presented loss of commensals as well as a gain of some pathogenic bacterial taxa compared to uninfected controls (Mutlu *et al.,* 2014). It is important to mention that HIV-infected men with lower markers of BT, higher $CD4^+$ T lymphocytes and lower viral loads before ART intake showed higher proportions of Lactobacillales in distal gut. During ART, higher proportions of gut Lactobacillales were associated with higher CD4, less BT, less systemic immune activation and less T lymphocyte proliferation, and higher $CD4^+$ T lymphocytes in the gut (Perez-Santiago *et al.,* 2013), corroborating the role of Lactobacillales in HIV-infection in distal gut, similarly to what was observed in the female genital tract. Finally, among species belonging to the *Bacteroides* genus, *Bacteroides fragilis*, which has been found to be significantly reduced in rectosigmoid biopsies (Vujkovic-Cvijin *et al.,* 2013) as well in colon biopsies of HIV-infected patients (Dillon *et al.,* 2014), may also play a key role in the adaptive immune system, since it was demonstrated that this bacteria exerts immune-regulatory effects (Deng *et al.,* 2016;Round and Mazmanian 2010).

Regarding fecal microbiota, Lozupone *et al*., (2013) observed that HIV+(naive) patients had a higher abundance of Prevotellaceae (*Prevotella*), Erysipelotrichaceae (*Catenibacterium* and *Bulleidia*), Veillonellaceae (*Dialister* and *Mitsuokella*), *Clostridium* cluster XIII and the genus *Desulfovibrio* compared with a non-HIV-population. In contrast, the control population had higher amounts of Bacteroidaceae (*Bacteroides*), Rikenellaceae (*Alistipes*), and Porphyromonadaceae (*Parabacteroides*) than HIV-infected patients (Lozupone *et al.,* 2013). Similarly, a pilot study observed, through real-time PCR, an increase in the proportion of Enterobacteriales and Bacteroidales in naive HIV patients with respect to a

control population and demonstrated important associations between this different bacterial composition and systemic immunity parameters in the HIV-infected patients (Ellis *et al.,* 2011).

More recent studies have shown a greater abundance of Proteobacteria phylum (which promote inflammation) and, on the contrary, a lower abundance of Bacteroidia class (known to limit inflammation) in naive HIV patients, which has been associated with increased cell activation T, a lower secretion of lymphocytes IL-17/IL-22 and a greater presence of inflammatory markers (Vujkovic-Cvijin *et al.,* 2013). A very recent study has also observed that a lower abundance of several *Bacteroides* species influence and impair the functions of the invariant natural killer T cells which are known to limit BT and chronic pathologic immune activation in HIV-1 infection (Paquin-Proulx *et al.,* 2017). In addition, among the Bacteroidales, the Rikenellaceae family was reported to be decreased during HIV infection and the genus *Alistipes* seems the most affected although these data have not been interpreted yet (Dubourg *et al.,* 2017).

The study by Dillon *et al*., (2014) also found a greater presence of Proteobacteria and, on the contrary, a lower abundance of Firmicutes in naive HIV patients (Dillon *et al.,* 2014). Among the Firmicutes phyla, particular consideration had been given during the last years to *Faecalibacterium prausnitzii*, an obligate anaerobe belonging to Lachnospiraceae family with known anti-inflammatory properties. Depletion of Lachnospiraceae has been reported during HIV infection (Dillon *et al.,* 2014;McHardy *et al.,* 2013;Mutlu *et al.,* 2014).

Mutlu *et al*., (2014) showed that GM from HIV-infected patients was enriched with a number of potentially pathogenic bacteria such as *Prevotella* as previously mentioned, and, in contrast, has poor content of the commensal

Bacteroides, similarly to what was observed by McHardy *et al*., (2013) (McHardy *et al.,* 2013;Mutlu *et al.,* 2014). In the same line, Gori *et al*., (2008) reported the predominant opportunistic pathogens in fecal flora of HIV-infected patients. *Candida albicans* and *Pseudomonas aeruginosa* were over-represented in the early stage of the infection. On the other hand, the abundance of protective bacteria, such as Bifidobacteria and Lactobacilli, were decreased when compared with healthy persons (Gori *et al.,* 2008).

A recent study carried out in a Chinese population has observed that the Firmicutes/Bacteroidetes ratio is increased in HIV patients (naive and under ART) with respect to an uninfected population (Ling *et al.,* 2016). Furthermore, HIV+ elite controllers (those that have the capacity to control viraemia in absence of ART) have enriched genera such as *Succinivibrio*, *Sutterella*, *Rhizobium*, *Delftia*, *Anaerolum* and *Oscillospira* but depleted in *Blautia* and *Anaerostipes* (Vesterbacka *et al.,* 2017).

Some contradictory results have arisen, especially those observed with respect to the *Prevotella* genus. Thus, while some studies have observed a significant increase in the abundance of this genus in patients with chronic HIV infection, other studies have not observed it (Nowak *et al.,* 2015;Vazquez-Castellanos *et al.,* 2015;Vujkovic-Cvijin *et al.,* 2013). This may be due, among other factors, to the different methodology used (PCR *vs.* massive sequencing of the 16s and the region chosen from 16s) and to the characteristics of the population (recent infection *vs.* chronic *vs.* naive or low ART).

Recent studies have also highlight the idea that other factors such as sexual orientation or the mode of transmission can also contribute to the different composition of GM in HIV-infected patients and could contribute to the lack of agreement in some of these studies (Noguera-Julian *et al.,*

2016;Volpe *et al.,* 2014;Yu *et al.,* 2014). Thus, the impact of HIV on GM has to be deeply investigated but from a broader context, including different cultures, diets and lifestyles, in order to avoid confounding factors.

Although all results mentioned above contributed to a better understanding of the presence of key microbes at different sites in HIV/AIDS disease progression, the greatest efforts have been carried out in the study of GM in HIV-infection.

1.2.8.3.3. Influence of cART in gut microbiota

Today, very few studies have analyzed the effects of cART on BT and GM composition. Costinuik and Angel (2012) observed that intestinal damage as well as BT are partially, but not completely, restored after cART (Costiniuk and Angel 2012). In fact, it has been observed that both the so-called "partial responders" and those designated as "non-responders" (do not reach 250 $CD4^+$) have similar plasma levels of LPS and sCD14 after 12 months on cART and also without cART (Merlini *et al.,* 2011). Several relative abundances for long-term HIV individuals have been shown to return to levels comparable to HIV uninfected subjects such as for *Peptococcus*, *Catenibacterium*, or *Desulfovibrio* spp, whereas those of *Bacteroides* or *Prevotella* were more similar to those of untreated patients (Lozupone *et al.,* 2013), although, the results regarding *Prevotella* are not conclusive (Lozupone *et al.,* 2013;Nowak *et al.,* 2015).

Despite these studies, little attention has been paid to the impact of distinct classes of antiretroviral drugs on microbiota in order to investigate which combinations are the best to restore the HIV-associated dysbiosis. Our group carried out a pilot study in an animal model of obesity where the effects of MVC were analyzed. This antiretroviral was able to induce several

changes in the abundance of the main bacterial orders present in the gastrointestinal tract (Perez-Matute *et al.,* 2015). Focused on HIV-infected individuals, only two research groups have evaluated the effects of distinct antiretrovirals combinations rather than ART as a whole (Pinto-Cardoso *et al.,* 2017). The study of Nowak *et al*., (2015) addressed the role of NNRTIs *vs*. PIs on GM composition, although no differences were observed (Nowak *et al.,* 2015). However, the study of Pinto-Cardoso *et al*., (2017) observed that patients under PIs showed significantly higher levels of the BT marker sCD14 compared to non-infected subjects. At the taxonomic level, they observed slight differences between treated HIV individuals on either EFV or PI-based antiretroviral regimens and HIV-uninfected participants (Pinto-Cardoso *et al.,* 2017). Surprisingly and to our knowledge, no studies have analyzed the effect of INSTI-based regimens, which are recommended as initial therapy in the current guidelines (GESIDA: *http://gesida-seimc.org/category/guias-clinicas/antirretroviral-vigentes/*, date accessed: February 2018; EACS: *http://www.eacsociety.org/guidelines/eacs-guidelines/eacs-guidelines.html*, date accessed: February 2018).

1.2.9. Gut microbiota & coinfection with hepatotropic viruses

Like HIV infection, several studies have demonstrated that HBV and HCV infections are associated with higher BT in comparison with non-infected subjects. Several studies have showed a disturbed GM composition and BT in patients infected with HBV and HCV compared to non-infected individuals (Aly *et al.,* 2016;Bajaj *et al.,* 2016;Heidrich *et al.,* 2018;Lu *et al.,* 2011;Wei *et al.,* 2013). In fact, Sandler *et al.* (2011) observed higher plasma levels of the bacterial translocation markers LPS, intestinal fatty acid binding protein (I-FABP) (indicating enterocyte death), and sCD14, as well as of the inflammatory marker IL-6 in HCV- and HBV-infected individuals in

comparison with uninfected subjects (Sandler *et al.,* 2011). Several works have analyzed the BT in HIV coinfected patients, although most of them were carried out in HIV/HCV-coinfected patients (French *et al.,* 2013;Marchetti *et al.,* 2014;Merchante *et al.,* 2018;Nystrom *et al.,* 2015;Sacchi *et al.,* 2015;Tudesq *et al.,* 2017). In general terms, they have observed a higher bacterial translocation in these patients. However, to our knowledge there are no studies that analyze the GM composition of HIV co-infected patients in comparison with healthy subjects.

1.2.10. Gut microbiota & metabolic syndrome

Gut dysbiosis has been linked with chronic inflammation associated with several pathologies such as MS. In this context, either MS *per se* or its separated components have been associated with higher levels of BT (Gonzalez-Quintela *et al.,* 2013;Sun *et al.,* 2010). Some specific changes in the relative abundance of gut microorganisms have also been observed in non-infected patients with MS (Lim *et al.,* 2017) or in those subjects suffering from any of the components of this syndrome (reviewed by Festi *et al*., (2014), de Groot *et al*., (2017), Xiao and Zhao (2014)) (de Groot *et al.,* 2017;Festi *et al.,* 2014;Xiao and Zhao 2014). However, some incongruent results have arisen from these studies, due to, among other factors, to the differences in the studied populations. Anyway, what is clear is that MS is accompanied by changes in GM composition in non-HIV patients. However, the association among HIV-infected patients with MS and GM composition has not been addressed yet.

2. Hypothesis

- Several studies have confirmed that HIV infection has direct effects on gut microbiota composition.

- An altered gut microbiota favours bacterial translocation and immune activation which leads to a chronic inflammatory state. HIV-related chronic inflammation is accompanied by an increased risk of developing several pathologies such as cardiovascular diseases, obesity and hyperlipidemia.

- The resilience mechanisms are not able to restore gut microbiota composition and the inflammatory state and persist despite combined antiretroviral therapy (cART).

- Current treatment for HIV infection (cART) is based on suppression of viral replication and restoration of the impaired immune system, which involves a significant reduction in the morbidity associated with HIV infection and a substantital prolongation of survival in these patients.

- The actions of the current antiretroviral regimens on gut microbiota composition, bacterial translocation and the consequent inflammation have not been investigated in depth.

- There are also other factors that might affect gut microbiota composition, such as the coinfection with hepatotropic viruses and the presence of metabolic syndrome.

3. Objectives

The main objective of this thesis was to analyze **the effects of different antiretroviral treatments on gut microbiota composition of HIV-infected patients with and without hepatitis C virus (HCV) coinfection and in presence/absence of Metabolic Syndrome.**

More specifically, the objectives were as follows:

1. To analyze the long-term effects of different combinations of cART on bacterial translocation and gut microbiota composition in HIV-infected patients.

2. To assess the bacterial translocation and gut microbiota composition of HIV-infected patients coinfected with hepatotropic viruses (hepatitis C virus) in comparison with those mono-infected.

3. To analyze the bacterial translocation, inflammation and gut microbiota composition of HIV-infected patients with metabolic syndrome in comparison with HIV patients without metabolic syndrome.

4. To test the effects of Maraviroc on gut microbiota composition in a mouse model of diet-induced obesity/fatty liver.

4. Material and Methods

4.1. Human studies

4.2. Animal study

4.1. Human study

4.1.1. Patients recruitment

Caucasian HIV-infected patients (HIV-1) from the Infectious Diseases Department at San Pedro´s Hospital (Logrono, Spain) as well as healthy (non HIV-infected) volunteers were recruited from September 2013 to April 2014. The group of HIV-infected patients included both untreated patients (naive) and those on cART for at least one year and with viral load <20 cop/mL for at least 6 months. All subjects on cART were immune responders. For both HIV-infected patients and controls, the following exclusion criteria were applied: <18 years old; pregnant women; patients treated with antibiotics, anti-inflammatory drugs, corticosteroids, immunosuppressive drugs or probiotics in the last 3 months; individuals with kidney, coeliac, or inflammatory disease, thyroid disorders, neoplasms, history of intestinal surgery (except appendectomy or cholecystectomy), inflammatory bowel diseases (IBD) (even if inactive), chronic pancreatitis, or any syndrome related to intestinal malabsorption. Patients receiving statins were also excluded because it was demonstrated that statin therapy could cause gut dysbiosis (Caparros-Martin *et al.,* 2017;Nolan *et al.,* 2017).

As a clinical procedure at San Pedro´s Hospital, $CD4^+$ and $CD8^+$ T-cell counts and HIV viral load were measured using flow cytometry and COBAS TaqMan 48 Analyzer, respectively (Roche Molecular Systems Inc., Branchburg, New Jersey, USA). Similarly, viral load of HBV and HCV were quantified for possible coinfection, and, in case of coinfection, degree of liver fibrosis was measured by the non-invasive diagnostic method FibroScan (Echosens, Paris, France). The liver stiffness test was carried out by the physicians of the Infectious Diseases Department. The Fibroscan device was placed in an intercostal space near the right lobe of the liver, and a 50-MHz

wave passed through the liver from a small transducer. Then, the device measured the velocity of the shear wave as this wave passed into the liver (in meters per second). The more rapid the waves passed through the liver, more liver damage/stiffness showed the patient. Results of the measurements were converted to kilopascals (kPa) (Afdhal 2012). Patients were classified depending on liver fibrosis degree according to the METAVIR scoring system: F0: no fibrosis; F1: portal fibrosis without septa; F2: portal fibrosis and few septa; F3: numerous septa without cirrhosis; F4: cirrhosis or advanced fibrosis (Castera 2015).

HIV-infected recruited people were classified according to the cART, the presence of metabolic syndrome or coinfection with hepatotropic viruses in order to answer the different objectives proposed in this thesis. Thus, different cross-sectional studies were performed using the same cohort of patients (i: classification based on type of antiretroviral treatment; ii: classification based on coinfection with hepatotropic viruses; iii: classification based on the presence/absence of metabolic syndrome) (Figure 13).

This study was performed following the Helsinki Declaration and was approved by the Committee for Ethics in Drug Research in La Rioja (CEImLAR) (23 April 2013, reference number 121). All participants provided their written informed consent.

4.1.1.a. Classification based on type of antiretroviral treatment

All HIV-treated patients were on cART (HIV+(cART)) for at least one year and with viral load <20 cop/mL for at least 6 months (n=45). cART-treated patients were classified depending on family treatment: NRTIs and PIs (NRTIs+PIs) (n=15), NRTIs and NNRTIs (NRTIs+NNRTIs) (n=22), and

NRTIs with INSTIs (NRTIs+INSTIs) (n=8). Untreated HIV-infected patients (HIV+(naive)) (n=5) with an average viral load of 54,010 cop/mL (3,550–71,800 cop/mL) and non-infected volunteers (controls) (n=21), as reference group, were also included. The control population was matched for age, gender and body mass index with the HIV-infected group as these factors are known to influence GM composition (Lozupone *et al.,* 2012;Mueller *et al.,* 2015).

4.1.1.b. Classification based on coinfection with hepatotropic viruses

Forty-five HIV-infected patients on antiretroviral treatment (ART) for at least one year and with viral load <20 copies/mL for at least 6 months were ranked in accordance with the presence of coinfection with HBV and/or HCV. Thus, 21 were non-coinfected HIV patients whereas 24 were coinfected with hepatotropic viruses, concretely 23 coinfected with HCV and one with HBV. 21 healthy volunteers were also included in this study. As above mentioned, all HIV patients coinfected with hepatotropic viruses were classified according to the liver stiffness (F0-F1: 37.5%, F2: 16.67%, F3: 33.33%, F4: 12.5%).

4.1.1.c. Classification based on the presence/absence of metabolic syndrome

Fifty-one HIV-infected patients (HIV+) on antiretroviral treatment (ART) for at least one year and with viral load <20 copies/mL for at least 6 months were classified according to the presence of metabolic syndrome (MS) based on the criteria established by the National Cholesterol Education Program Adult Treatment Program III (NCEP-ATP III). Thus, patients with any three of the following five criteria were diagnosed with MS: elevated waist circumference (≥102 cm in men or ≥88 cm in women), elevated

triglycerides (≥150 mg/dL), reduced high-density lipoprotein (HDL) cholesterol (<40 mg/dL in men or <50 mg/dL in women), elevated blood pressure (≥130 mm Hg systolic blood pressure or ≥85 mm Hg diastolic blood pressure) and/or elevated fasting glucose (≥100 mg/dL) (Grundy *et al.,* 2005). Thus, 40 HIV-infected patients did not present MS (HIV+MS-), whereas 11 patients were classified as MS (HIV+MS+).

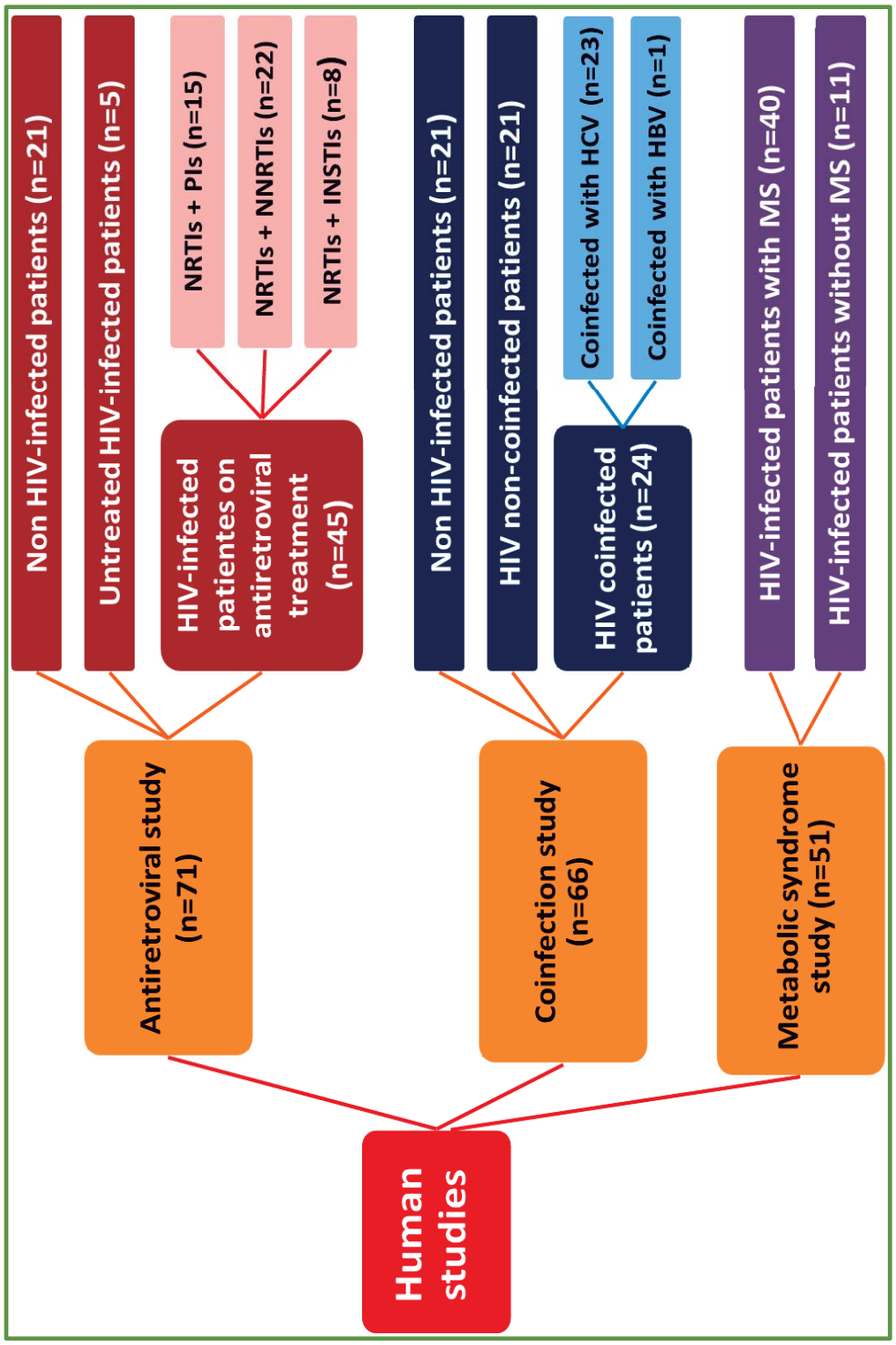


Figure 13. Scheme of the different human studies performed in this Doctoral Thesis

4.1.2. Biochemical parameters and immunological techniques

4.1.2.1. Plasma and serum preparation

Blood samples were collected at San Pedro´s Hospital after 12 h fasting with two different commercial tubes, in order to obtain serum and plasma from blood.

Serum samples were collected in Vacutainer (BD Vacutainer® Plus Plastic Serum Tubes) tubes which contain a gel to help to separate the clot. After collection, the tubes were leaved at room temperature for 30 minutes to allow the blood to clot. Then, the clot was removed by centrifuging at 3,000 xg for 10 min at room temperature.

Vacutainer tubes treated with ethylenediamine tetraacetic acid (EDTA) (BD Vacutainer® spray-coated K2EDTA Tubes) were chosen for collecting **plasma** samples. Cells were removed from plasma by centrifugation at room temperature for 10 minutes at 3,000 xg. The resulting supernatant was designated as plasma.

Following centrifugation, both serum and plasma samples were transferred into clean tubes using micropippetes. The samples were stored at –20°C for subsequent analysis.

4.1.2.2. Biochemical parameters

Plasma levels of glucose, triglycerides, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured at the San Pedro´s Hospital (Logrono) using an AutoAnalyzer (Cobas C711, Roche, Madrid, Spain).

4.1.2.3. Immunological techniques: enzyme-linked immunosorbent assays (ELISA) and Luminex Screening Assay

Enzyme-linked immunosorbent assays (ELISAs) and Luminex Screening Assay were performed to analyze bacterial translocation, inflammation and cardiovascular risk markers from serum and plasma samples of HIV-infected patients and healthy subjects. All the analyses were performed with commercially available kits and according to the manufacturers' instructions. Repeated freeze-thaw cycles were avoided.

4.1.2.3.1. ELISA principle

ELISA is an immunological assay commonly used to measure antibodies, antigens, proteins, peptides, and glycoproteins in biological samples. All ELISAs carried out in this Doctoral Thesis employed the quantitative sandwich enzyme immunoassay. This type of ELISA includes two specific antibodies for the antigen of interest, known as capture and detection antibodies. In this method, a specific antibody for the antigen of interest has been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any antigen of interest present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked antibody specific for antigen was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color was developed in proportion to the amount of antigen bound in the initial step. The color development was stopped and the intensity of the color was measured in a plate reader spectrophotometer at the wavelength indicated by the manufacturer.

Although all assays were sandwich ELISA, they differed in relation to the secondary antibody (detection antibody) between the trading houses. Thus, the ELISAs from R&D (Minneapolis, USA) included a detection antibody that was previously conjugated with the peroxidase enzyme, while in the rest of the assays the peroxidase enzyme was added after adding the detection antibody. Figure 14 illustrates the different steps carried out in each case.

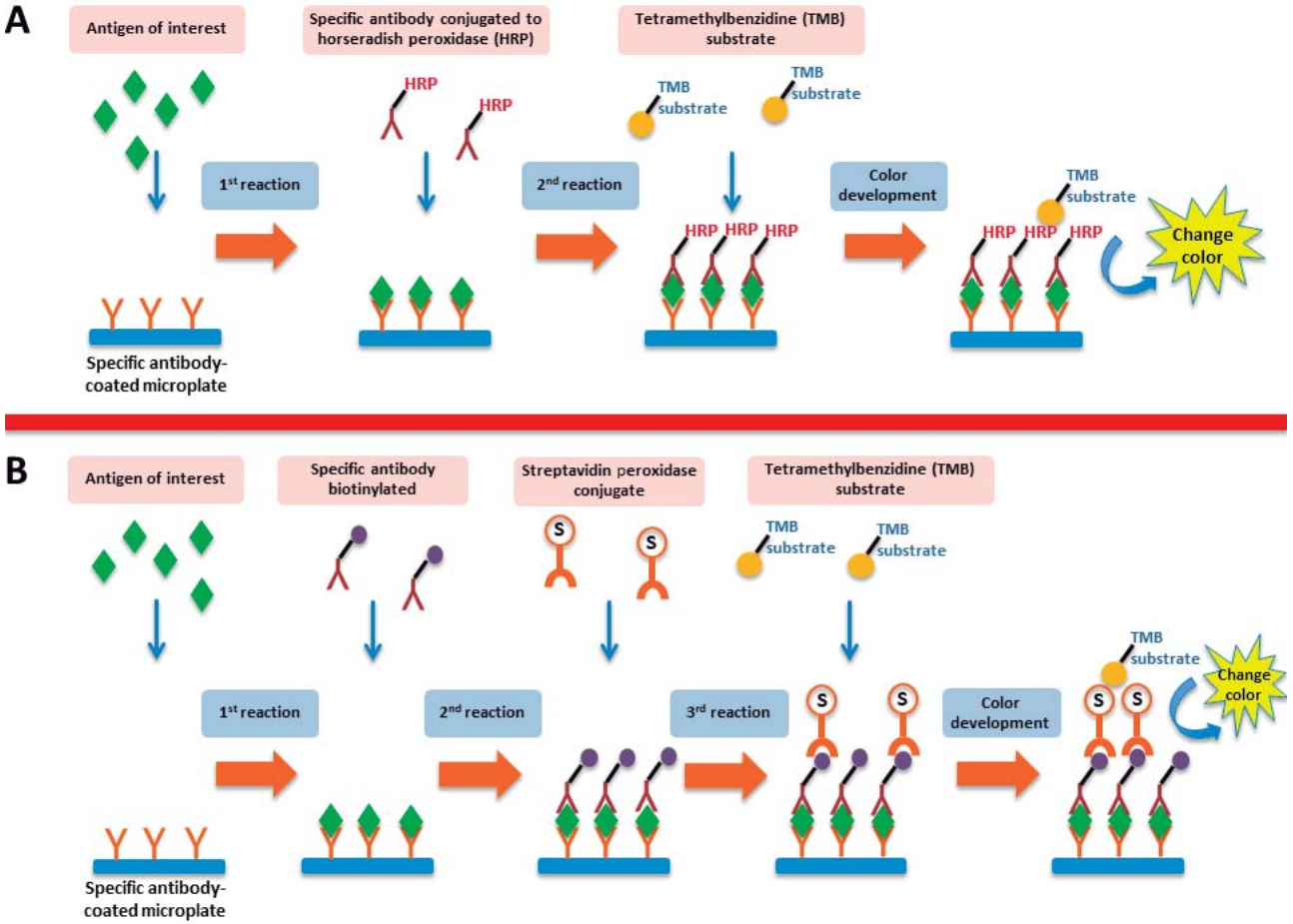


Figure 14. (A) Different steps carried out with the ELISA sandwich from R&D, which included a detection antibody that was previously conjugated with the peroxidase enzyme (Minneapolis, USA). (B) Steps performed with the ELISA kits different from R&D in which the peroxidase enzyme was added after adding the detection antibody.

4.1.2.3.1.1. Parameters measured using ELISA

The lipopolysaccharide-binding protein (LBP), soluble CD14 (sCD14), interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1), and insulin levels were measured by ELISA. Results were measured in a POLARstar Omega plate reader spectrophotometer (BMG LABTECH). The information of the different assays performed is summarized in Table 3.

Table 3. Parameters measured using ELISA from human samples.

	Analyte	Sample	Commercial kit from	Wavelength	Study
Bacterial translocation markers	LBP	Plasma diluted 1:1000	Hycult Biotech (Uden, The Netherlands)	450 nm	- Antiretroviral families study - Coinfection study - Metabolic syndrome study
	sCD14	Serum diluted 1:200	R&D (Minneapolis, USA)	450 - 540 nm	
Inflammation marker	IL-6	Serum without diluting	R&D (Minneapolis, USA)	490 - 690nm	- Antiretroviral families study - Metabolic syndrome study
Cardiovascular risk marker	PAI-1	Plasma without diluting	R&D (Minneapolis, USA)	450 - 540 nm	- Metabolic syndrome study
Glucose metabolism	Insulin	Serum without diluting	EMD Millipore (Massachusetts, USA)	450 nm	- Metabolic syndrome study

4.1.2.3.2. Luminex Screening Assay principle

The Luminex technology allows measuring several parameters in one procedure. This technique is based on polystyrene color-coded microparticles which are pre-coated with antigen-specific capture antibodies. Thus, the antibodies bind to the antigens of interest.

In the procedure, standards and samples were added to a mixture of color-coded beads which were pipetted into wells and the immobilized antibodies bound the analytes. Then, it was necessary to wash any unbound substances before adding to each well a biotinylated antibody cocktail specific to the antigen of interest. Following a wash to eliminate any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds the biotinylated detection antibodies, was added to each well. A final wash eliminated unbound Streptavidin-PE, and, then, a buffer was added to resuspend the microparticles and to read using the Luminex analyzer.

Polystyrene beads are read on a dual-laser; one laser (red) is microparticle-specific and determines which analyte is being detected, whereas the other laser (green) determines the magnitude of the phycoerythrin-derived signal, which is in direct proportion to the amount of analyte bound.

4.1.2.3.2.1. Analytes measured with the Luminex Screening Assay

Serum levels of the cardiovascular risk markers such as: intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM), and the inflammatory marker monocyte chemoattractant protein-1 (MCP-1) were quantified by a Human Premixed Multi-Analyte Kit (Luminex, Minneapolis, USA). The results were measured in a Luminex 200™ analyzer

situated at San Pedro´s Hospital (Logroño). The details of the assay performed are summarized in the Table 4.

Table 4. Parameters quantified with the Luminex Screening Assay from human samples.

	Analyte	Sample	Commercial kit from	Wavelength	Study
Cardiovascular risk markers	ICAM	Serum diluted 1:2	Human Premixed Multi-Analyte Kit (Luminex, Minneapolis, USA)	Red laser: 635 nm Green laser: 532 nm	- Antiretroviral families study
	VCAM				
Inflammation marker	MCP-1				- Metabolic syndrome study

The triglycerides and HDL levels quantified with an AutoAnalyzer were used to calculate the triglycerides-to-HDL ratio as a marker of cardiovascular risk (Eeg-Olofsson *et al.,* 2014;Marotta *et al.,* 2010). Likewise, the values of glucose and insulin levels were used to calculate the "*homeostasis model assessment insulin resistance index* (HOMA-IR)" as follows: HOMA-IR index = fasting insulin (mU/L) × fasting glucose (mg/dL)/405 according to the report by Matthews *et al*., (1985) (Matthews *et al.,* 1985).

4.1.3. Fecal samples

4.1.3.1. Collection of samples

All patients and healthy volunteers received adequate instructions and a sterile tube for collecting fecal samples. The instructions were as follows:

- Urinate before defecating

- Clean the perineal area with a sponge with soap
- Rinse the perineal area with plenty of water
- Dry the perineal area with a clean and unused towel
- Defecate in a urinal or, otherwise, in a clean and dry place
- Open the sterile tube without touching the edges
- Collect a small amount of stool (about the size of a walnut) that has not touched the sides of the urinal with the spoon which is situated on the inside of the tube lid
- Close the tube and stores in a fridge at 4-5ºC until its transportation to the CIBIR (within 24 hours).

4.1.3.2. DNA extraction from fecal samples

Fresh stool samples were received at CIBIR and fecal DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands) following manufacturer's instructions. Then, purity and concentration were subsequently determined by a Nanodrop spectrophotometer 1000 (Thermo Scientific, USA).

The DNeasy Blood & Tissue kit was designed for rapid purification of total DNA from a range of sample sources including fresh or frozen animal tissues and cells, blood, or bacteria. The first step of the procedure was the lysis; hence, fecal sample (25 mg) was mixed by vortexing with proteinase K (enzyme that digests the proteins, including nucleases that might otherwise degrade the DNA) and a lysis buffer (Buffer AL). Samples were then incubated overnight at 56ºC. A tissue lysis buffer (Buffer ATL) was also added to the samples, which were leaved at 56ºC for 10 minutes. Once the tissue was lysed, DNA was purificated by adding ethanol. Subsequently, samples were transferred into columns (DNeasy Mini spin columns) with a silica-based membrane, and during centrifugation, DNA was selectively

bound to the membrane as contaminants passed through. Remaining contaminants and enzyme inhibitors were eliminated in two wash steps by adding two wash buffers (Wash Buffer 1 and 2). Finally, DNA was eluted in an elution buffer (Buffer AE).

4.1.3.3. 16S DNAr sequencing and bioinformatic analysis

Samples were amplified for the 16S rDNA hypervariable sequence V4. Sequencing was performed using the Illumina MiSeq Instrument (two reads of 150 base pairs) with approximately 100,000 reads per sample (Illumina, INC, San Diego, CA, USA).

Computational analysis was externally performed by Era7 Bioinformatics (Granada, Spain). The first step was to assemble the two reads obtained from the Illumina technology. The computational tool FLASh was used to assembly the two reads of each pair to obtain a larger sequence for a more specific taxonomic assignment of the reads. The "not merged reads" had not sufficient quality to be analyzed and were discarded for further analysis steps.

Subsequently, reads were assigned to a taxon based on sequence similarity to 16S rRNA genes extracted from the NCBI nucleotide (nt) database based on the presence of similar sequences in the set of sequences included in the Ribosomal Database Project (RDP) and on the specificity of their taxonomical assignment at the NCBI (all the sequences at NCBI nt database has an taxonomic assignment) (Cole *et al.,* 2014). This database included 902,131 16S sequences (around 1000 Mb). Current version of the db.rna16s database is open and available in GitHub: (*https://github.com/ohnosequences/db.rna16s*).

Massive BLAST (Basic Local Alignment 116 Search Tool) tasks were performed using MG7 system (Alekhin *et al.,* 2015;Jimenez *et al.,* 2015;Ouma *et al.,* 2015). MG7 assigned each read independently and there was not binning or clustering of reads. Each read was independently assigned to a taxon based on BLAST similarity to the sequences in the db.rna16s database, as mentioned. Two different taxonomic assignment approaches were used: BBH (Best Blast Hit: each read was assigned to the taxon corresponding to the Best Blast Hit over a threshold of similarity) and LCA (Lowest Common Ancestor: adopted by advanced tools of metagenomics analysis such as the last version of MEGAN (MEtaGenome ANalyzer)) (Huson and Weber 2013) (Figure 15).

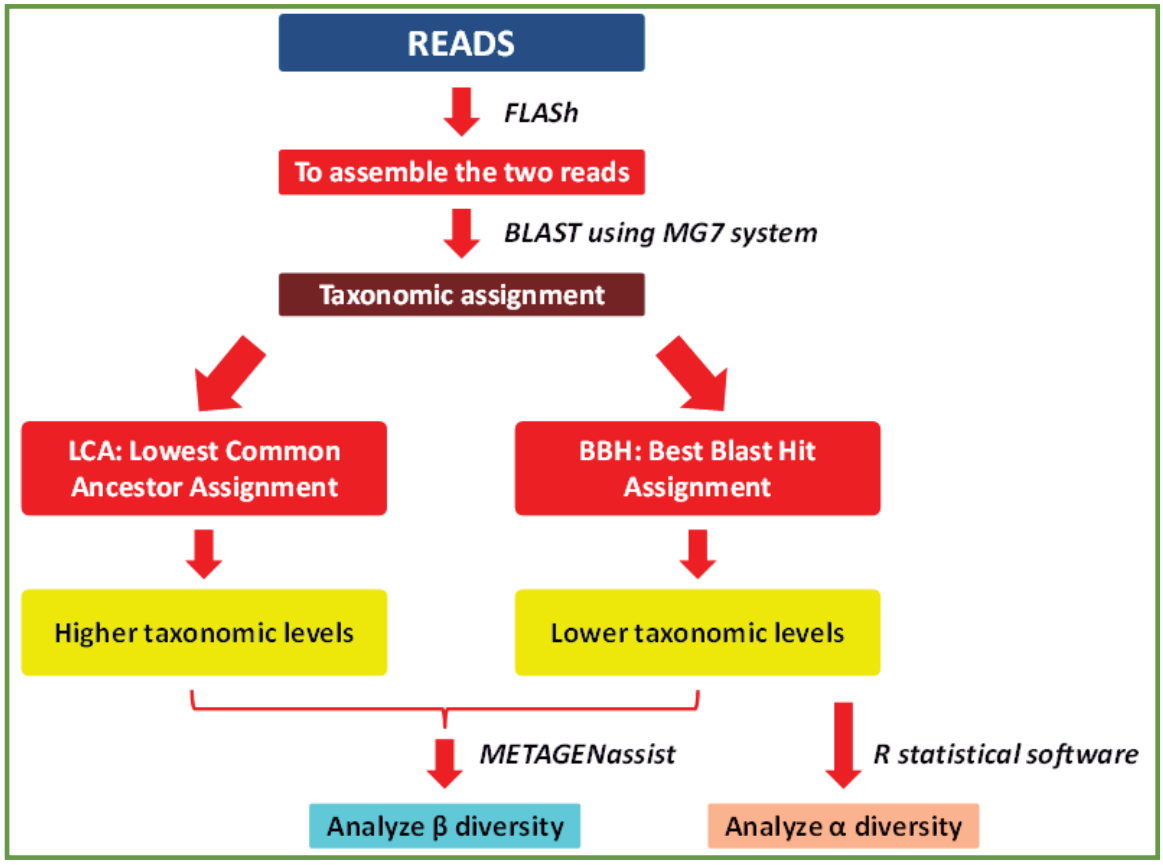


Figure 15. Scheme of the bioinformatic analysis performed in the human studies.

In the BBH method, the taxonomic assignment was based on the Best BLAST Hit obtained in the BLAST of each read against the 16S database over a threshold of similarity ($e<1x10^{-15}$). However, in the LCA method the 10 Best BLAST Hits over a threshold of similarity ($e<1x10^{-15}$) were selected and their taxonomic assignments were obtained. Then, it was searched on the taxonomy tree for the node that includes all the assignments that is the Lowest Common Ancestor taxon for all the 10 hits. In some cases, the reads had not 10 hits over the similarity threshold, thus, the BBH over this threshold were selected (from 1 to 10 hits). Some reads could not find sequences with enough similarity in the database and, then, they were classified as reads with no hits.

The direct assignments and the cumulative assignment frequencies for each taxonomy node were analyzed. Direct assignments were calculated counting reads specifically assigned to a node, not including the reads assigned to the descendant nodes in the taxonomy tree. In contrast, cumulative assignments were calculated including the direct frequencies and also the frequencies of the descendant nodes.

Studies at higher taxonomic levels were carried out by assignment using direct LCA. This method was chosen because it seemed to be the most robust method, since it searches the node that includes all the assignments that is the Lowest Common Ancestor taxon for all the 10 hit, but also because the direct assignment not includes the frequencies of the descendant nodes. Studies at lower levels were performed using the BBH technique. This was because the lower taxonomic ranks did not have an assigned frequency with the LCA method due to its estimation. BBH direct and cumulative had the same values, since there were not descendant nodes at these taxonomic levels.

α-diversity was calculated with R software (version 3.2.2; The R foundation for Statistical Computing, Vienna, Austria) considering the BBH method. The analysis of the β-diversity was carried out using the web server METAGENassist (*www.metagenassist.ca/*) (Arndt *et al.,* 2012), in which the LCA and BBH techniques were used to study the higher and lower taxonomic levels, respectively. β-diversity describes the inter-subject similarity of microbial composition and facilitates the identification of broad differences between samples (Lozupone *et al.,* 2011) β-diversity data was filtered by the robust estimate interquartile range that detect the variables that are near-constant throughout the experiment conditions. Moreover, two types of normalization were performed. Row-wise normalization aims to make each sample comparable to each other (sample *vs*. sample), while the column-wise normalization aims to make each variable comparable to each other within the same sample (taxon *vs*. taxon). Row-wise normalization was performed by sum and column wise normalization was calculated by Pareto Scaling.

The LCA and BBH assigned reads from healthy participants and HIV-infected patients included in the abovementioned studies are available in the repository of bacterial sequences dbBact (http://dbbact.org/main).

4.2. Animal study

Thirty two male C57BL/6 mice were purchased at five weeks of age with an average weight of 18.89 ± 0.18 g from Charles River (Barcelona, Spain). Upon arrival, mice were housed under controlled temperature, humidity, and light. They were randomly assigned (n=8) to the following groups: a) Control: fed with a normal chow (4% wt/wt of lipids commercially available (standard diet RM1A (P); SDS, Essex, UK) and autoclaved water; b) MVC: normal chow diet but receiving 300 mg/L of MVC (Pfizer, New York, NY, USA) in the drinking water. Mouse equivalent drug doses were assessed

in order to get a human equivalent dose (300 mg/day) (Neff *et al.,* 2010;Perez-Martinez *et al.,* 2014); c) HFD: animals fed with a HFD (D12492, Research Diets Inc., NJ, USA) and autoclaved water; and d) HFD+MVC group: HFD but receiving MVC in the drinking water (same concentration than MVC group). All animals had free access to food and water. Mice were weighted and food and water ingestion were recorded every 2-3 days per week. All mice were euthanized by using CO_2 after 16 weeks of treatment. The standard diet contained 79% carbohydrates, 17% proteins and 4% lipids, whereas the HFD was composed of 60% lipids, 20% carbohydrates and 20% proteins (Figure 16).

All procedures were carried out in accordance with the European Communities Council Directive on animal experiments (86/609/CEE and EU Directive 2010/63/EU) and with approval from the ethical committee on animal welfare of our institution (*Comité Ético de Experimentación Animal del CIBIR*).

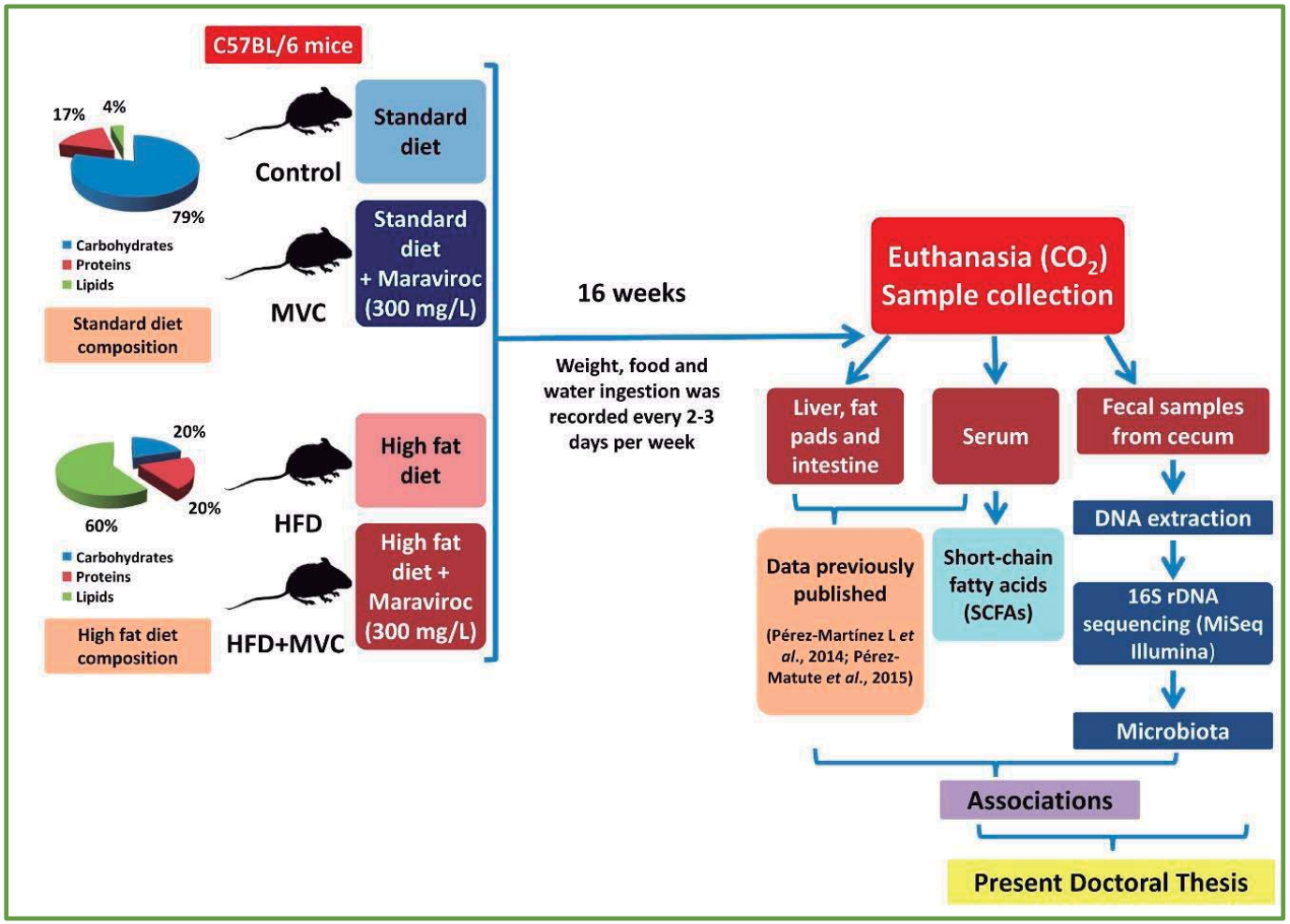


Figure 16. Scheme of the animal study carried out.

4.2.1. Serum, liver, fat pads, intestine and fecal samples collection

Blood samples were obtained under anesthesia by cardiac puncture after a 4 h fast. Samples were collected into tubes and leaved at 4ºC for at least 30 minutes to allow the blood to clot. Then, samples were centrifuged in a refrigerated centrifuge for 10 min at 3,000 xg. Serum was transferred into clean tubes using a micropippette and was stored at -20ºC for subsequent analysis.

Liver, fat pads, intestine and fecal samples from the cecum were also collected and weighted at the time of sacrifice. Once samples were collected, they were introduced immediately in liquid nitrogen, and subsequently stored at -80ºC for further analyses (Figure 17).

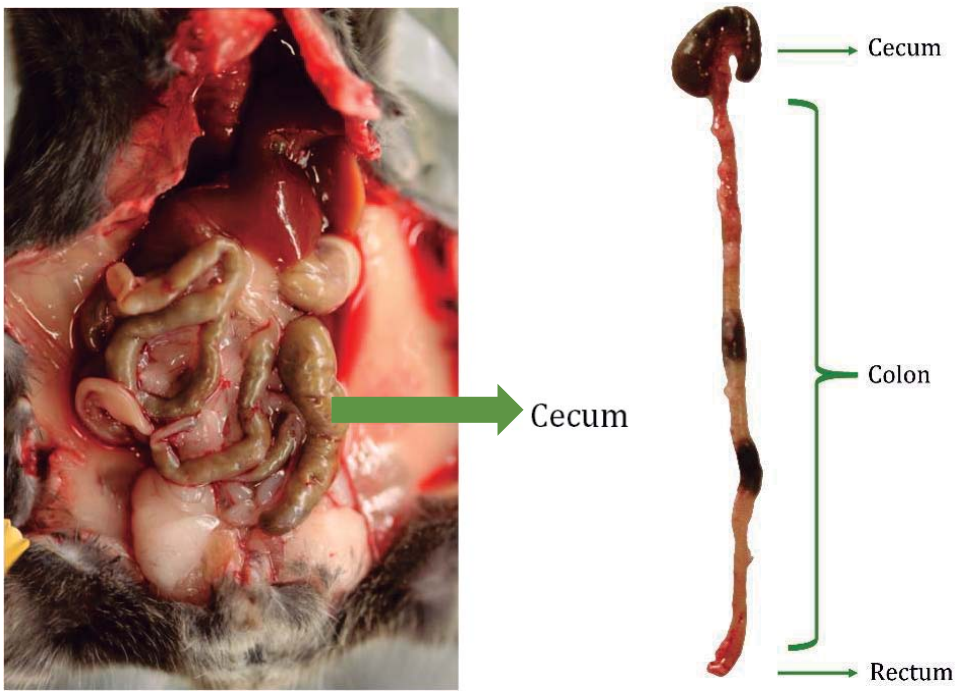


Figure 17. Collection of fecal samples from the cecum.

4.2.1.1. Biochemical parameters

Serum levels of glucose, AST and ALT were measured at the San Pedro´s Hospital (Logrono) using an AutoAnalyzer (Cobas C711, Roche, Madrid, Spain).

4.2.1.2. ELISA

Serum levels of the inflammatory markers IL-6 and tumor necrosis factor alpha (TNFα), as well as insulin were measured by ELISA as explained above. All the analyses were performed with commercially available kits and according to the manufacturers' instructions. Repeated freeze-thaw cycles were avoided. The details of the ELISAs performed are summarized in Table 5.

Table 5. Parameters measured by ELISA from mice samples.

	Analyte	Sample	Wavelength	Commercial kit from
Inflammatory markers	IL-6	Serum without dilution	450 - 540 nm	R&D (Minneapolis, USA)
	TNFα		450 - 540 nm	R&D (Minneapolis, USA)
Glucose metabolism	Insulin		450 - 590 nm	EMD Millipore (Massachusetts, USA)

The HOMA-IR was also calculated, as explained before (Matthews *et al.,* 1985).

4.2.1.3. Hepatic triglyceride content

To determine the hepatic triglyceride content, frozen liver samples (150 mg) were weighed and homogenized by Ultraturrax (IKA-Weke, Staufen, Germany) in 1.5 mL of buffer (150 mM NaCl, 0.1% Triton X-100, and 10 mM Tris pH 8) at room temperature. Then, homogenized samples were centrifuged at 12,000g for 10 minutes. The obtained supernatant was collected (the two different phases, leaving only the pellet) and mixed, and, finally, triglyceride levels were measured using an AutoAnalyzer (Cobas C711, Roche, Madrid, Spain) at San Pedro´s Hospital (Logrono).

4.2.1.4. Analysis of short-chain fatty acids

The short-chain fatty acids acetate, butyrate, propionate and isovalerate were measured from serum samples at the Institute of Grapevine and Wine Sciences (ICVV) (Logrono, Spain).

50 µL of serum were placed in a 12x32 mm glass crimp top vial with 350 µL fused insert containing 20 mg of KCl. Then, 10 µL of HCl 1N and 100 µL of *terc*-Butylethylether (^{t}BuEtO) containing internal standard 6.5 ppm solution (valproic acid) were added. Vials were immediately capped with an aluminium cap with PTFE/Silicon septum and sealed. Samples were vigorously shaken at room temperature for 30 minutes and centrifuged at 4,696 xg for 5 minutes. Upper organic phase was analyzed by gas chromatography-mass spectrometry (GCMS). 7890C Series gas chromatograph coupled to a 7000C Series Triple Quad GC/MS triple quadrupole mass spectrometer (Agilent Technologies Inc., Wilmington, DE, USA) with a MPS automatized liquid sample injection system (Gerstel GmbH & Co. KG, Múlheim an der Ruhr, Germany). Chromatographic separation was performed in a capillary column TG-WaxMS (30m x 0.25 mm int. diam., 0.25

µL film) (Thermo Scientific™). A volume of 3 µL of sample was automatically injected into a split/splitless inlet (in splitless mode) kept at 250ºC. Helium was used as carrier gas at a flow rate of 1 mL/min in constant flow mode. The oven program was set as follows: an initial temperature of 40ºC for 5 min, increased to 150ºC at a rate of 3ºC/min, then increased to 240ºC at a rate of 15ºC/min and held at 240ºC for 10 min. Total analysis time was 57.7 min. Detection was performed with the mass spectrometer operating in SIM mode (dwell time 75 ms), by electronic impact ionisation with 70 eV ionization energy.

GERSTEL Maestro software (Gerstel GmbH & Co. KG) and MassHunter Workstation Software: GCMS Acquisition, Version B.07.02 (Agilent Technologies Inc.) were used for data acquisition. Firstly, peak identification was made by comparison of retention times and ion spectra from fatty acids real standards and spectra from the NIST mass spectral library. Analyte quantification was performed by external calibration comparing the area of each analyte in samples with calibration curves. Calibration curves for each analyte were made from the chromatographical analysis data of standard solutions at different concentrations. MassHunter Workstation Software: GCMS Qualitative Analysis, Version B.07.00 (Agilent Technologies Inc.) was used for data analysis and MassHunter Workstation Software: GCMS Quantitative Analysis, Version B.07.00 (Agilent Technologies Inc.), was used for sample quantification.

4.2.1.5. Fecal samples

4.2.1.5.1. DNA extraction from fecal samples

DNA was extracted from frozen fecal samples from the cecum using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands), as previously

mentioned. Purity and concentration were also determined by a Nanodrop spectrophotometer 1000 (Thermo Scientific, USA).

4.2.1.5.2. 16S DNAr sequencing and bioinformatic analysis

For the metagenomic analysis, fecal samples from 7 controls, 8 MVC mice, 5 mice fed with a HFD and 7 mice from the HFD+MVC group were collected (total 27 samples). The V4 hypervariable region of the gene encoding 16S rDNA was amplified. Metagenomic analysis was performed using the Illumina MiSeq platform (two reads of 250 base pairs) (Illumina, INC, San Diego, CA, USA).

Bioinformatic analysis was developed by the Genomics & Bioinformatics Core Facility at the CIBIR (La Rioja, Spain). In short, quality check of reads and adapter trimming were performed with the quality control tool FastQC and Trim Galore program. Then, reconstruction of full-length V4 16S rRNA region for taxonomic assignment and the determination of operational taxonomic units (OTUs) were carried out through the QIIME program (v1.9.1), following the "pick open reference otus" methodology against the 16S rRNA gene database Greengenes 13.8 at 97% sequence similarity (Caporaso *et al.,* 2010;DeSantis *et al.,* 2006;Edgar 2018). Uclust program was used for the establishment of taxonomy clusters (*http://drive5.com/usearch/manual/uclust_algo.html*).

The meta-analysis of microbiome data was performed with the web-based tool MicrobiomeAnalyst (Dhariwal *et al.,* 2017). The data uploaded to this web was previously filtered by the Genomics & Bioinformatics Core Facility, thus, no filtering was needed for the analysis carried out by this tool. Instead, normalization was carried out by cumulative sum scaling (CSS) (Figure 18).

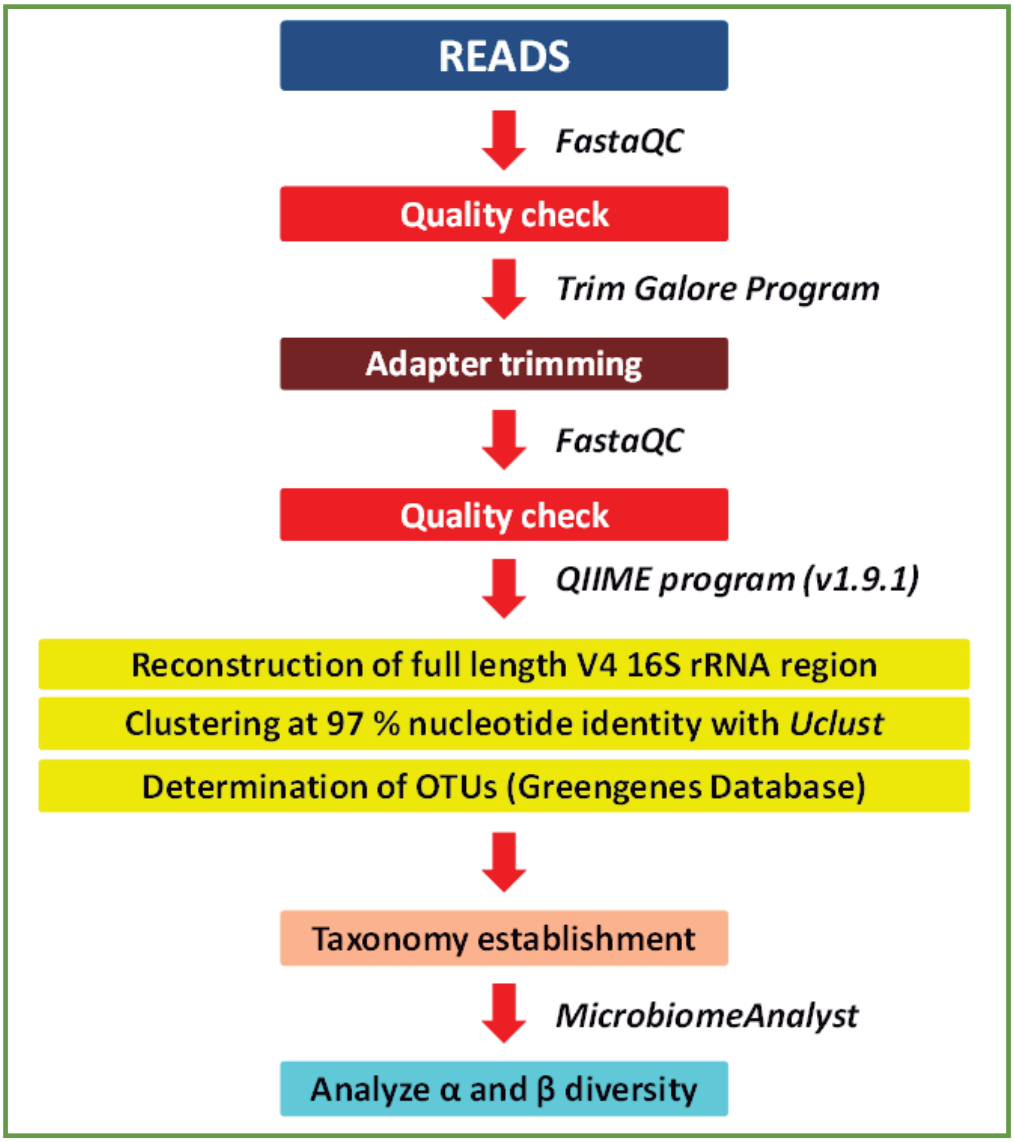


Figure 18: Scheme of the bioinformatic analysis carried out in the animal study.

4.2.2. Antimicrobial susceptibility testing

An antimicrobial test of Maraviroc was carried out by the Molecular Microbiology Area at the CIBIR (Logrono, Spain). *Escherichia coli* ATCC25922, *Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC29213 and *Pseudomonas aeruginosa* ATCC27853 are strains of the

American Type Culture Collection (ATCC) recommended as quality controls in antimicrobial activity studies (Clinical and Laboratory Standards Institute, 2015 (CLSI 2015)). *Listeria monocytogenes* CECT4035 is a Spanish Type Culture Collection strain recommended to be used for UNE-CEN ISO/TS 11133.

Minimal inhibitory concentration (MIC) of MVC was determined by microdilution method (CLSI 2015) in Mueller–Hinton broth. The stock concentration of MVC was 3 mg/ml (dissolved in dimethyl sulfoxide (DMSO)). Two-fold aqueous dilutions of MVC in concentrations ranging from 128 to 0.125 mg/mL were analyzed in order to get a mice equivalent dose. The MIC values were determined against *E. coli* ATCC25922, *E. faecalis* ATCC29212, *S. aureus* ATCC29213, *L. monocytogenes* CECT4032, and *P. aeruginosa* ATCC27853. The initial bacterial inoculum was $5x10^5$ CFU/mL, and after incubation for 24 h at 37 ºC, the MIC was considered as the lowest concentration of MVC that completely visually inhibited the growth of the bacteria.

4.3. Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). P values <0.05 were considered statistically significant. Categorical variables were analyzed using the Chi-square test or Fisher's exact test. Normal distribution of quantitative variables was checked using the Shapiro-Wilk test. Comparisons between three or more groups were analysed by one way ANOVA followed by a Bonferroni post hoc test or by Kruskal-Wallis test followed by Dunns post-tests depending on normality. Comparisons between two groups were performed with Unpaired t test or U Mann-Whitney. Relationships between variables were analyzed by calculating Spearman's rank correlation coefficients. Statistical analysis was performed

using SPSS 19.0 (SPSS® Inc. Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Prism®, La Jolla, California, USA).

4.3.1. Human study

The measure of sample-level species richness, also known as α-diversity, was calculated using R (version 3.2.2; The R foundation for Statistical Computing, Vienna, Austria) and presented as four indices: number of observed species, Alpha index, Margalef's diversity index, and Chao 1 (McHardy *et al.,* 2013). The number of observed species, as well as Margalef's diversity index and Alpha index are estimators of the specific bacterial richness, whereas Chao 1 is a non-parametric method that estimates diversity based on the number of rare species in the sample. β-diversity was assessed using the web server METAGENassist (Arndt *et al.,* 2012). Data obtained from β-diversity were statistically analyzed using the Wilcoxon rank-sum non-parametric test. P values <0.05 were considered statistically significant when the effect of cART and hepatotropic viruses on GM composition were analyzed, whereas a false discovery rate (FDR) <0.1 was considered significant when the effect of MS was studied. A principal component analysis (PCA) was also developed. Results are plotted according to the first two principle components (Arndt *et al.,* 2012).

4.3.2. Animal study

The web server MicrobiomeAnalyst was used to obtain the same α-diversity metrics than those selected in the human study: number of observed species, Alpha index, Margalef's diversity index, and Chao 1. Differences among groups at family, genus and specie level were also assessed using Mann-Whitney non-parametric test with the web server MicrobiomeAnalyst (Dhariwal *et al.,* 2017). Data obtained from β-diversity

were statistically analyzed using the Mann-Whitney non-parametric test. A false discovery rate (FDR) <0.1 was considered significant. To evaluate overall differences in β-diversity, a principal component analysis (PCA) was also performed. Results are plotted according to the first two principle components. A heat map was drawn by hierarchal clustering according to the main genus. The distance measure and the cluster algorithm chosen were Euclidean and the Ward's linkage respectively.

5. Results

5.1. Antiretroviral families study

5.2. Coinfection study

5.3. Metabolic syndrome study

5.4. Maraviroc study

5.1. Bacterial translocation and gut microbiota composition in HIV-infected patients on different cART

5.1.1. Clinical and demographic characteristics of participants

Table 6 shows the main characteristics of the population analyzed to fulfill the first objective of this Doctoral Thesis. All participants were Caucasian. Sixty per cent of the HIV+(naive) patients presented over 500 nadir $CD4^+$ cells/mm^3, whereas only 11.1% of the patients using cART showed nadir $CD4^+$ cells above 500 cells/mm^3 (p=0.024). There were no differences concerning the characteristics of patients on different families of cART, although individuals in the NRTIs+INSTIs group had a higher incidence of AIDS events (p=0.013). More than 50% (51.1%) of the patients using cART presented coinfection with hepatitis C virus (p=0.054), with no differences between the treatments. HIV patients were infected for an average of 16 years. Total time using cART (including the last therapy evaluated in this study) was 13 years. No differences were observed for the total length on treatment, although a slightly longer time on treatment was observed in the INSTI group, showing significant differences when compared with the NRTIs+NNRTIs group (p=0.048).

Table 6. Characteristics of the subjects included in the antiretroviral study.

	Control	HIV+		Overall p value[1]	HIV+(cART)			Overall p value[2]
		HIV+(naive)	HIV+(cART)		NRTIs+PIs	NRTIs+NNRTIs	NRTIs+INSTIs	
No. of patients	21	5	45		15	22	8	
Gender (male)	11/21 (52.38%)	4/5 (80%)	30/45 (66.7%)	0.449	11/15 (73.33%)	13/22 (59.09%)	6/8 (75%)	0.639
Age (years)	48.81 ± 2.65	44.2 ± 4.87	48.68 ± 0.98	0.541	51.27 ± 1.47	47.29 ± 1.62	47.5 ± 1.43	0.166
Body mass index (kg/m²)	27.41 ± 1.2	25.78 ± 2.67	24.33 ± 0.89	0.140	23.09 ± 1.2	25.7 ± 1.65	23.9 ± 1.74	0.697
CD4 nadir count (cells/mm³)	-	<200: 0/5 (0%)	<200: 19/45 (42.22%)	0.142	<200: 9/15 (60%)	<200: 6/22 (27.27%)	<200: 4/8 (50%)	0.135
	-	200–500: 2/5 (40%)	200–500: 21/45 (46.67%)	1	200–500: 6/15 (40%)	200–500: 12/22 (54.55%)	200–500: 3/8 (37.5%)	0.673
	-	>500: 3/5 (60%)	>500: 5/45 (11.11%)	0.024	>500: 0/15 (0%)	>500: 4/22 (18.18%)	>500: 1/8 (12.5%)	0.180
CD4 count (cells/mm³)	-	200–500: 2/5 (40%)	200–500: 12/45 (26.7%)	0.611	200–500: 6/15 (40%)	200–500: 4/22 (18.18%)	200–500: 2/8 (25%)	0.369
	-	>500: 3/5 (60%)	>500: 33/45 (73.3%)	0.611	>500: 9/15 (60%)	>500: 18/22 (81.82%)	>500: 6/8 (75%)	0.361
T4/T8 index	-	0.62 ± 0.09	0.92 ± 0.05	0.068	0.83 ± 0.09	0.94 ± 0.08	1 ± 0.14	0.482
Time since diagnosis of HIV infection (years)	-	3.20 ± 0.73	16.51 ± 1.14	< 0.001	18.53 ± 2.11	13.95 ± 1.55	19.75 ± 1.95	0.111
AIDS	-	0/5 (0%)	20/45 (44.4%)	0.075	9/15 (60%)	5/22 (22.73%) [a]	6/8 (75%) [b]	0.013

	Control	HIV+		Overall p value[1]	HIV+(cART)			Overall p value[2]
		HIV+(naive)	HIV+(cART)		NRTIs+PIs	NRTIs+NNRTIs	NRTIs+INSTIs	
Coinfection with hepatitis B virus	-	0/5 (0%)	1/45 (2.2%)	1	1/15 (6.67%)	0/22 (0%)	0/8 (0%)	0.513
Coinfection with hepatitis C virus	-	0/5 (0%)	23/45 (51.11%)	0.054	10/15 (66.67%)	8/22 (36.36%)	5/8 (62.5%)	0.140
Mode of transmission	-	IVDU: 1/5 (20%)	IVDU: 17/45 (37.78%)	0.642	IVDU: 7/15 (46.67%)	IVDU: 5/22 (22.73%)	IVDU: 5/8 (62.5%)	0.099
	-	HS: 2/5 (40%)	HS: 17/45 (37.78%)	1	HS: 6/15 (40%)	HS: 10/22 (45.45%)	HS: 1/8 (12.5%)	0.285
	-	MSM: 1/5 (20%)	MSM: 2/45 (4.44%)	0.276	MSM: 0/15 (0%)	MSM: 2/22 (9.09%)	MSM: 0/8 (0%)	0.669
	-	IVDU/HS: 0/5 (0%)	IVDU/HS: 1/45 (2.22%)	1	IVDU/HS: 0/15 (0%)	IVDU/HS: 0/22 (0%)	IVDU/HS: 1/8 (12.5%)	0.179
	-	Unknown: 1/5 (20%)	Unknown: 8/45 (17.78%)	1	Unknown: 2/15 (13.33%)	Unknown: 5/22 (22.73%)	Unknown: 1/8 (12.5%)	0.878
Degree of hepatic fibrosis	-	F0–F1: 5/5 (100%)	F0–F1: 29/45 (64.44%)	0.163	F0–F1: 6/15 (40%)	F0–F1: 17/22 (77.27%)	F0–F1: 6/8 (75%)	0.078
	-	F2: 0/5 (0%)	F2: 5/45 (11.11%)	1	F2: 4/15 (26.67%)	F2: 1/22 (4.55%)	F2: 0/8 (0%)	0.096
	-	F3: 0/5 (0%)	F3: 8/45 (17.78%)	0.577	F3: 3/15 (20%)	F3: 4/22 (18.18%)	F3: 1/8 (12.5%)	1
	-	F4: 0/5 (0%)	F4: 3/45 (6.67%)	1	F4: 2/15 (13.33%)	F4: 0/22 (0%)	F4: 1/8 (12.5%)	0.172
Advanced degree of hepatic fibrosis	-	F2–F4: 0/5 (0%)	F2–F4:16/45 (35.56%)	0.163	F2–F4: 9/15 (60%)	F2–F4: 5/22 (22.73%)	F2–F4: 2/8 (25%)	0.079

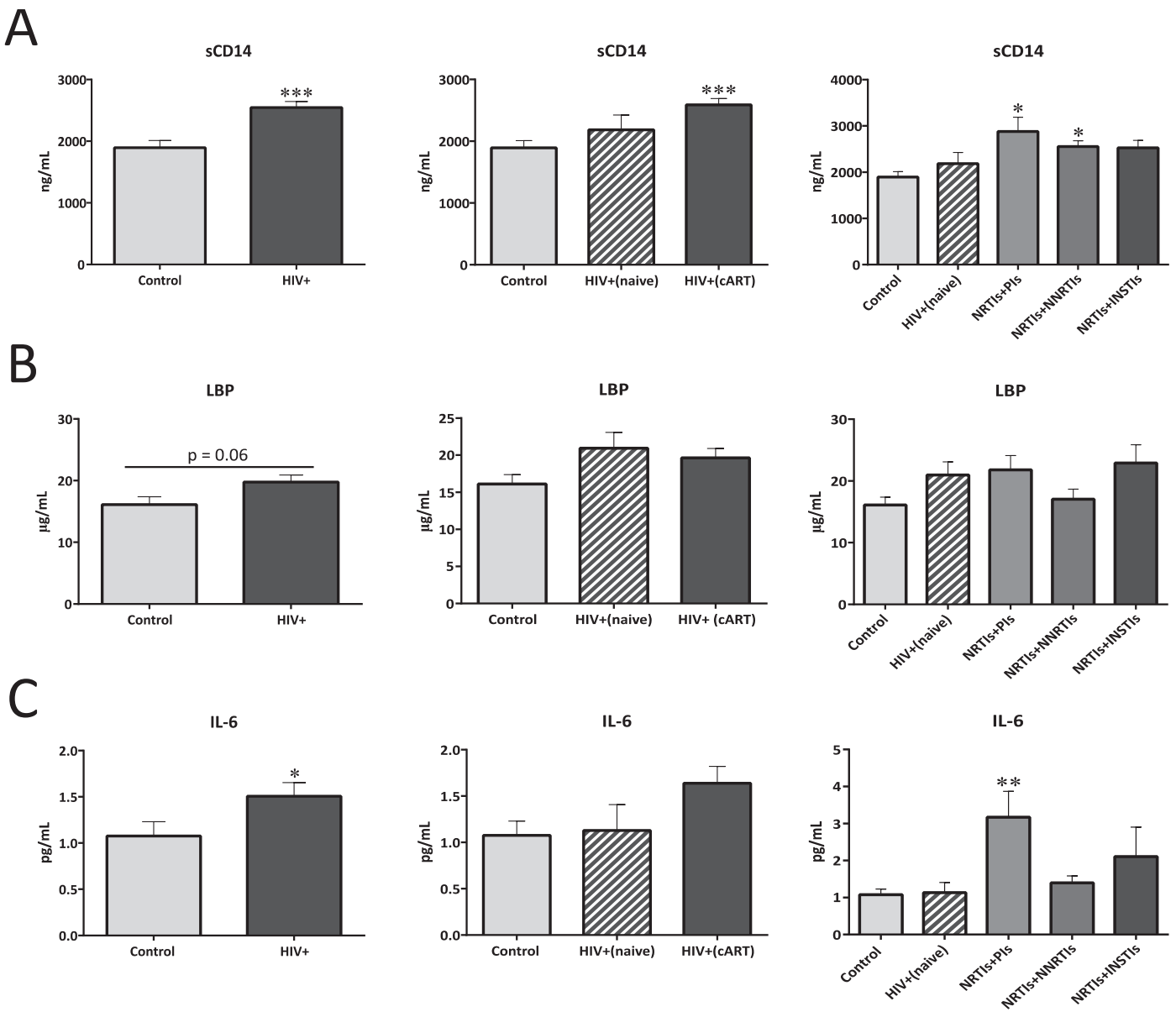
	Control	HIV+		Overall p value[1]	HIV+(cART)			Overall p value[2]
		HIV+(naive)	HIV+(cART)		NRTIs+PIs	NRTIs+NNRTIs	NRTIs+INSTIs	
Time on cART (years)	-	-	13.55 ± 1	-	14.47 ± 1.73	11.29 ± 1.42	17.75 ± 1.71 [b]	0.054
Time on the last cART (years)	-	-	5.31 ± 0.41	-	5.73 ± 0.84	5.32 ± 0.59	4.50 ± 0.68	0.497

F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; F4, cirrhosis (Castera *et al*., 2005); HIV+(cART), HIV-infected patients on combined antiretroviral therapy for at least one year or more and with viral load <20 copies/mL for at least 6 months; HIV+(naive), untreated HIV-infected patients; HS, heterosexual; IVDU, intravenous drug user; IVDU/HS, intravenous drug user and multiple heterosexual contacts; MSM, men who have sex with men; NRTIs+INSTIs, nucleoside reverse transcriptase inhibitors and integrase strand transfer inhibitors; NRTIs+NNRTIs, nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors; NRTIs+PIs, nucleoside reverse transcriptase inhibitors and protease inhibitors.

Quantitative data are presented as mean ± SEM, whereas qualitative data are indicated as percentage. Overall p value[1] was obtained comparing controls *vs*. HIV+(naive) and HIV+(cART). Overall p value[2] was obtained by comparing the three treatments. [a]p<0.05 *vs.* NRTIs+PIs, [b]p<0.05 vs. NRTIs+NNRTIs.

5.1.2. Bacterial translocation, inflammation and endothelial markers

sCD14 plasma levels were significantly increased in HIV+ patients compared with controls (p=0.0003), and especially in those patients using cART (NRTIs+PIs, p=0.034 and NRTIs+NNRTIs, p=0.011 *vs.* controls, respectively (Figure 19A)). However, patients using NRTIs+INSTIs presented similar sCD14 plasma levels to the controls, although no significant differences were observed between the cART regimens. No changes were observed in LBP plasma levels (Figure 19B). IL-6, ICAM and VCAM plasma levels were significantly increased in HIV+ patients ($p<0.05$, $p<0.001$ and $p<0.001$, respectively), especially in those on NRTIs+PIs treatment compared with the controls (p=0.005, p=0.005 and $p<0.001$, respectively). ICAM values were also significantly increased in patients using NRTIs+NNRTIs (p=0.011 *vs.* controls) and NRTIs+INSTIs (p=0.020 *vs.* controls), although the increase was less potent than that observed with the NRTIs+PIs regimen (Figure 19C–E). A positive association was found between sCD14 and IL-6 and also between sCD14 and VCAM in all HIV-infected patients (r=0.409; p = 0.006; r=0.343; p = 0.023, respectively).


A
sCD14
ng/mL
3000
2000
1000
0
***
Control
HIV+
sCD14
ng/mL
3000
2000
1000
0
***
Control
HIV+(naive)
HIV+(cART)
sCD14
ng/mL
4000
3000
2000
1000
0
*
*
Control
HIV+(naive)
NRTIs+PIs
NRTIs+NNRTIs
NRTIs+INSTIs
B
LBP
µg/mL
30
20
10
0
p = 0.06
Control
HIV+
LBP
µg/mL
25
20
15
10
5
0
Control
HIV+(naive)
HIV+ (cART)
LBP
µg/mL
30
20
10
0
Control
HIV+(naive)
NRTIs+PIs
NRTIs+NNRTIs
NRTIs+INSTIs
C
IL-6
pg/mL
2.0
1.5
1.0
0.5
0.0
*
Control
HIV+
IL-6
pg/mL
2.0
1.5
1.0
0.5
0.0
Control
HIV+(naive)
HIV+(cART)
IL-6
pg/mL
5
4
3
2
1
0
**
Control
HIV+(naive)
NRTIs+PIs
NRTIs+NNRTIs
NRTIs+INSTIs

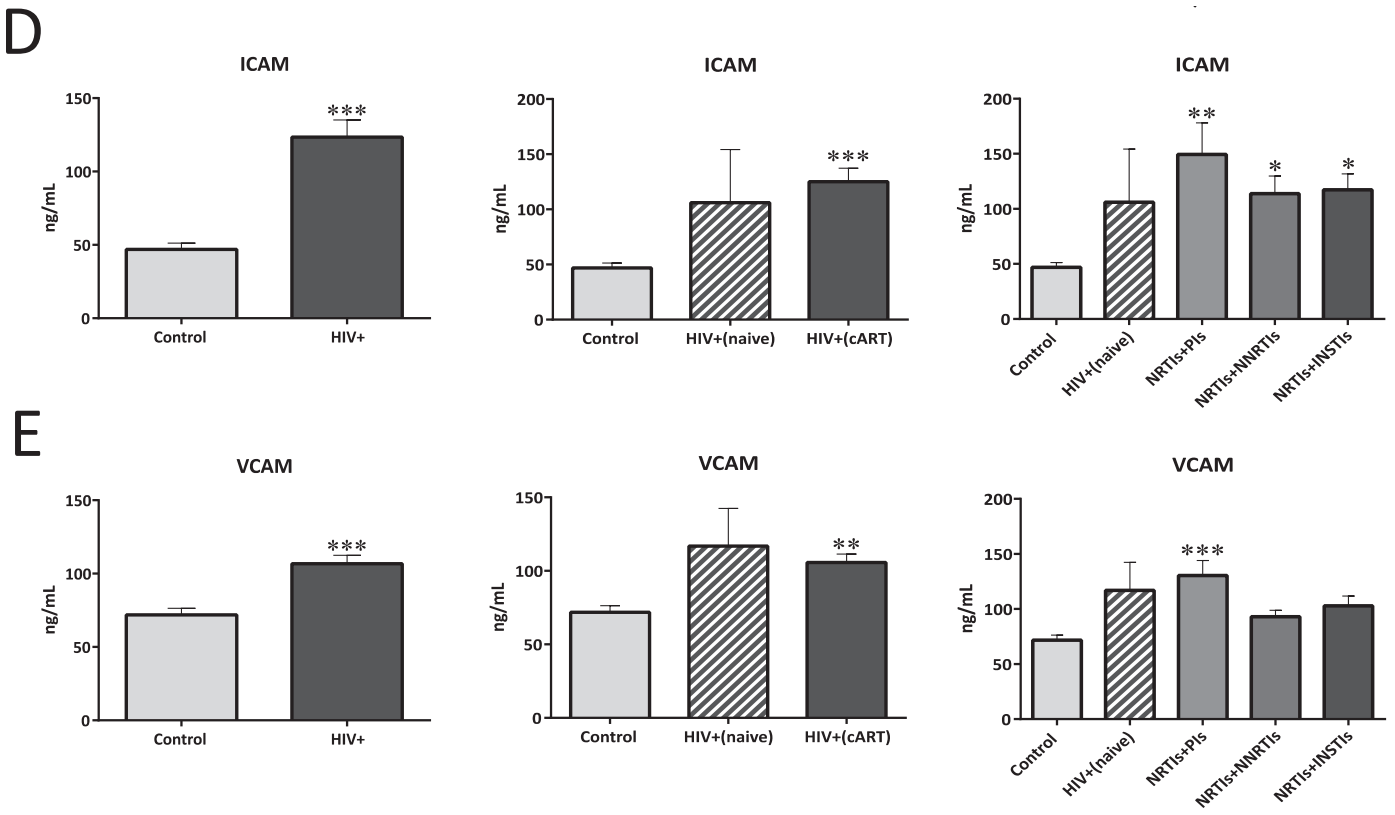


Figure 19. Markers of bacterial translocation, inflammation and endothelial damage in control group compared with untreated HIV infected patients and using different cART.

(A) Soluble CD14 (sCD14). (B) Lipopolysaccharide binding protein (LBP). (C) Interleukin 6 (IL-6). (D) Intercellular adhesion molecule (ICAM). (E) Vascular cell adhesion molecule (VCAM). Each bar represents the mean ± SEM. $p<0.05$ was considered significant. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ *vs.* control (uninfected patients)

5.1.3. Gut microbiota diversity and composition

HIV infection dramatically decreased α-diversity, as observed with all the indexes analyzed and as presented in Figure 20 (p=0.0006–p=0.003). Patients in the NRTIs+INSTIs group showed a similar α-diversity profile to the controls and a significant increase compared with the HIV+(naive) group (p=0.006–p=0.008). The combination of NRTIs+NNRTIs was also able to partially restore the decreased observed in α-diversity due to HIV infection (p=0.026–p=0.033 *vs*. naive).

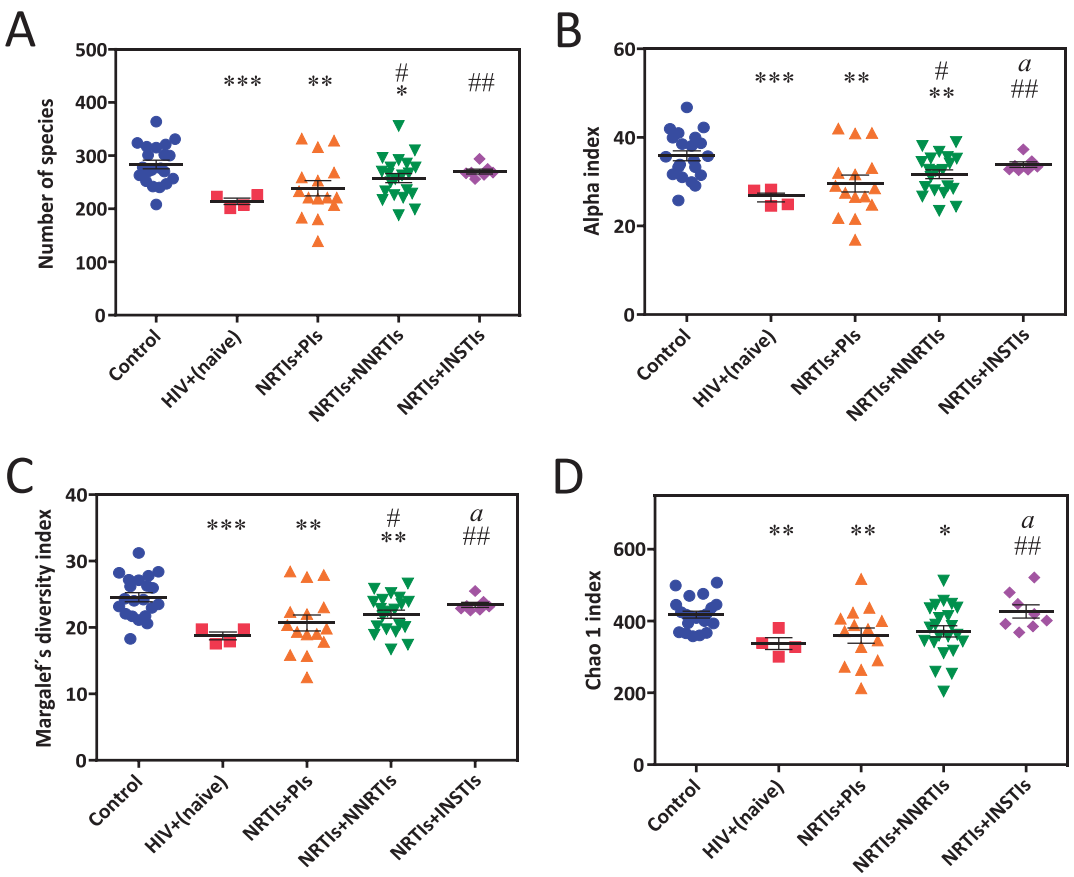


Figure 20. α-diversity measurements in control group compared with untreated HIV infected patients and using different cART.
(A) Number of species. (B) Alpha index. (C) Margalef's diversity index. (D) Chao 1 index. Each bar represents the mean ± SEM. $p<0.05$ was considered significant. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ *vs*. control, $^{\#}p<0.05$; $^{\#\#}p<0.01$ *vs*. HIV+(naive), $^{a}p<0.05$ *vs*. NRTIs+PIs.

Concerning GM composition (β-diversity), the most abundant phyla in gut were Bacteroidetes and Firmicutes. Thus, approximately 73% of the bacteria detected in gut belong to these phyla (74.11%, 73.14% and 74.2% of abundance for control, HIV+(naive) and HIV+(cART) respectively) (Table 7). No significant differences were observed in the most abundant phyla in gut when HIV patients were compared with controls. Only an increase in the

abundance of Proteobacteria and, consequently, in the ratio Proteobacteria/Firmicutes was observed in HIV-infected patients on cART (p=0.021; p=0.023 *vs*. control, respectively). A significant decrease (p=0.049) in the abundance of Firmicutes was observed in HIV-infected patients using NRTIs+PIs compared with controls. Lentisphaerae, Euryarchaeota, Synergistetes, and Fusobacteria represented around 0.18% of the bacteria. The differences observed in the abundance of these phyla are shown in Table 7.

At the class level, a significant increase was observed in the relative abundance of δ-Proteobacteria in HIV+(cART) (p=0.025 *vs*. controls). This increase was more evident in the NRTIs+INSTIs group (p=0.013 *vs*. control). A decrease in the abundance of α-Proteobacteria was observed in naive patients (p=0.028 *vs*. control) and cART was able to restore the abundance of this bacterial class (Table 7).

Table 7. Relative abundance of major phyla present in the gut in control and HIV-infected patients.

	Control	HIV+		Overall p value[1]	HIV+(cART)			Overall p value[2]
		HIV+ (naive)	HIV+ (cART)		NRTIs + PIs	NRTIs + NNRTIs	NRTIs + INSTIs	
Firmicutes	42.86 ± 1.56	36.04 ± 5.88	39.17 ± 1.47	0.281	36.72 ± 2.78	40.54 ± 2.02	40.02 ± 3.40	0.505
Bacteroidetes	31.25 ± 2.46	37.10 ± 2.81	35.03 ± 1.68	0.347	37.68 ± 2.71	34.80 ± 2.49	30.69 ± 4.02	0.513
Proteobacteria	2.96 ± 0.35	4.69 ± 1.56	5.15 ± 0.54 *	0.068	4.05 ± 0.65	5.92 ± 1.04	5.95 ± 1.11	0.386
*** α-Proteobacteria**	0.43 ± 0.12	0.02 ± 0.01 *	0.29 ± 0.07	0.086	0.34 ± 0.09	0.18 ± 0.08	1.63 ± 0.76 [b]	0.028
*** β-Proteobacteria**	0.79 ± 0.2	1.33 ± 0.47	1.28 ± 0.2	0.478	1.56 ± 0.41	1.10 ± 0.24	1.28 ± 0.49	0.837
*** γ-Proteobacteria**	0.10 ± 0.02	0.06 ± 0.04	0.12 ± 0.03	0.179	0.06 ± 0.02	0.34 ± 0.14	0.17 ± 0.09	0.470
*** δ-Proteobacteria**	0.49 ± 0.07	0.51 ± 0.14	0.91 ± 0.10 *	0.023	0.70 ± 0.14	0.96 ± 0.17	1.15 ± 0.14 [a]	0.176
Actinobacteria	0.96 ± 0.17	1.50 ± 0.09	1.44 ± 0.22	0.229	3.44 ± 0.94	1.24 ± 0.21	1.11 ± 0.23	0.419
Lentisphaerae	0.05 ± 0.02	0.02 ± 0.02	0.08 ± 0.04 *	0.037	0.01 ± 0.007	0.11 ± 0.08	0.11 ± 0.08	0.167
Euryarchaeota	0.08 ± 0.05	0.0004 ± 0.0002	0.02 ± 0.01 **	0.006	0.01 ± 0.009	0.03 ± 0.02	0.04 ± 0.04	0.426
Synergistetes	0.03 ± 0.01	0.0004 ± 0.0004	0.04 ± 0.03 **	0.023	0.10 ± 0.09	0.005 ± 0.004	0.02 ± 0.02	0.615
Fusobacteria	0.002 ± 0.0008	0.002 ± 0.001	0.04 ± 0.03	0.850	0.007 ± 0.006	0.02 ± 0.01	0.15 ± 0.15	0.266
Ratio Bacteroidetes /Firmicutes	0.76 ± 0.09	1.21 ± 0.29	0.97 ± 0.07	0.124	1.06 ± 0.11	0.83 ± 0.09	0.85 ± 0.16	0.268
Ratio Proteobacteria /Firmicutes	0.08 ± 0.01	0.14 ± 0.04	0.13 ± 0.01 *	0.068	0.09 ± 0.01	0.12 ± 0.02	0.16 ± 0.04 [a]	0.245
Ratio Actinobacteria /Firmicutes	0.02 ± 0.004	0.04 ± 0.01	0.05 ± 0.007	0.094	0.10 ± 0.02	0.04 ± 0.007	0.03 ± 0.004	0.388

Data are presented as mean ± SEM. Overall p value[1] was obtained by comparing controls *vs.* HIV+(naive) and HIV+(cART). Overall p value[2] was obtained by comparing the three treatments. *p<0.05; **p<0.01 *vs.* control (non-infected patients). #p<0.05 *vs.* HIV+(naive), [a]p<0.05 *vs.* NRTIs + PIs, [b]p<0.05 *vs.* NRTIs + NNRTIs.

One of the goals of this study was to test whether different families of ART used in clinical practice were able to restore the gut dysbiosis induced by HIV infection. Thus, we compared GM composition (at lower taxonomic units) of the different cART families against uninfected subjects (controls). A combination of NRTIs+INSTIs produced a pronounced increase in bacteria belonging to Desulfovibrionales and Selenomonadales orders, Desulfovibrionaceae and Lachnospiraceae families and *Desulfovibrio* genus, whereas only a significant depletion in the abundance of unclassified Clostridiales order was observed. Patients using NRTIs+NNRTIs showed an increased abundance of Coriobacteriales order, Coriobacteriaceae and Lachnospiraceae bacterial families, as well as *Pseudomononas* genus, whereas a lower abundance of Bacteroidales order, Bacteroidaceae family and *Streptococcus* genus was observed. Finally, patients using NRTIs+PIs showed a significant increase in the presence of Clostridiales order, Lachnospiraceae family and Eggerthella genus, and a significant reduction in Actinomycetales, Pseudomonadales, and Sphingomonadales orders, Eubacteriaceae and Prevotellaceae families, and *Prevotella*, *Pseudomonas* and *Solobacterium* genera (Table 8). At the lowest taxons, NRTIs+INSTIs and NRTIs+PIs patients showed a higher abundance of *Blautia* sp. 3. In addition, a significant increase in the abundance of *Flavonifractor plautii* was observed in NRTIs+PIs patients in comparison with controls, whereas an increase in the levels of *Parabacteroides merdae* was observed in NRTIs+INSTIs patients compared to healthy volunteers. In summary, 10 bacterial species were reduced in HIV patients using NRTIs+PIs, whereas patients using NRTIs+NNRTIs or NRTIs+INSTIs regimens showed a lower abundance of only six bacterial species when compared with controls (Table 8).

Table 8. Taxonomic ranks (order, family, genus, species) modified by combined antiretroviral therapy compared with uninfected-controls subjects.

	CONTROLS *vs.*								
	NRTIs+PIs			NRTIs+NNRTIs			NRTIs+INSTIs		
	Taxonomic group	Category	p value	Taxonomic group	Category	p value	Taxonomic group	Category	p value
↑	**Clostridiales bacterium**	Order	0.016	**Coriobacteriales**	Order	0.011	**Desulfovibrionales**	Order	0.032
↑	**Lachnospiraceae**	Family	0.021	**Coriobacteriaceae**	Family	0.005	**Selenomonadales**	Order	0.032
↑	***Eggerthella***	Genus	0.020	**Lachnospiraceae**	Family	0.039	**Lachnospiraceae**	Family	0.011
↑	***Blautia sp.* 3**	Species	0.018	***Pseudomonas***	Genus	0.039	**Desulfovibrionaceae**	Family	0.012
↑	***Flavonifractor plautii***	Species	0.009				***Desulfovibrio***	Genus	0.009
↑							***Blautia sp.* 3**	Species	0.003
↑							***Parabacteroides merdae***	Species	0.047
↓	**Actinomycetales**	Order	0.021	**Bacteroidales bacterium**	Order	<0.001	**Unclassified Clostridiales**	Order	<0.001
↓	**Pseudomonadales**	Order	0.016	**Bacteroidaceae bacterium**	Family	0.014	***Clostridium sp. 29***	Species	0.032
↓	**Sphingomonadales**	Order	0.021	***Streptococcus***	Genus	0.028	***Clostridium sp.* Clone-24**	Species	0.028
↓	**Prevotellaceae**	Family	0.025	***Alistipes senegalensis***	Species	0.028	***Clostridium sp.* Clone-33**	Species	0.010

	CONTROLS *vs.*								
	NRTIs+PIs			NRTIs+NNRTIs			NRTIs+INSTIs		
	Prevotella	Genus	0.033	***Blautia sp.***	Species	<0.001	***Desulfovibrio sp. 6***	Species	<0.001
	Pseudomonas	Genus	0.046	***Coriobacterium sp.* AUH-Julong21**	Species	<0.001	***Eubacterium eligens***	Species	0.041
	Solobacterium	Genus	0.036	***Eubacterium eligens***	Species	<0.001	***Ruminococcus flavefaciens***	Species	0.041
	Alistipes senegalensis	Species	0.009	***Papillibacter cinnamivorans***	Species	0.007			
	Bacillus sp.	Species	0.003	***Ruminococcus flavefaciens***	Species	0.003			
	Clostridium sp.6	Species	0.044						
	***Clostridium sp.* Clone-24**	Species	0.015						
	Eubacterium eligens	Species	0.002						
	Faecalibacterium prausnitzii	Species	0.002						
	Paludibacter sp. 2	Species	0.002						
	Roseburia inulinivorans	Species	<0.001						
	Roseburia intestinalis	Species	0.001						
	Ruminococcus flavefaciens	Species	0.046						

Red represents a significant increase in the relative abundance of the taxonomical groups compared with the control group, whereas blue represents a significant decrease.

Figure 21 shows a PCA where the NRTIs+INSTIs cluster is represented inside the control cluster, in contrast with the diagrams obtained for the other combinations of cART compared with the controls. The clustering of samples was represented by their respective 95% confidence interval ellipse and results were plotted according to the first two principle components. The PCA of the HIV-infected patients using NRTIs+INSTIs combination *vs*. non-infected subjects accounting for 28.3% of the total variation (Component 1 = 15.4% and Component 2 = 12.9%)) (Figure 21A), the PCA of the HIV-infected patients using NRTIs+NNRTIs combination *vs*. non-infected subjects represents 33.1% of the total variation (Component 1 = 17.9% and Component 2 = 15.2%)) (Figure 21B), whereas the PCA of the HIV-infected patients using NRTIs+PIs combination *vs*. non-infected subjects accounting for 27.7% of the total variation (Component 1 = 14.4% and Component 2 = 13.3%)) (Figure 21C).

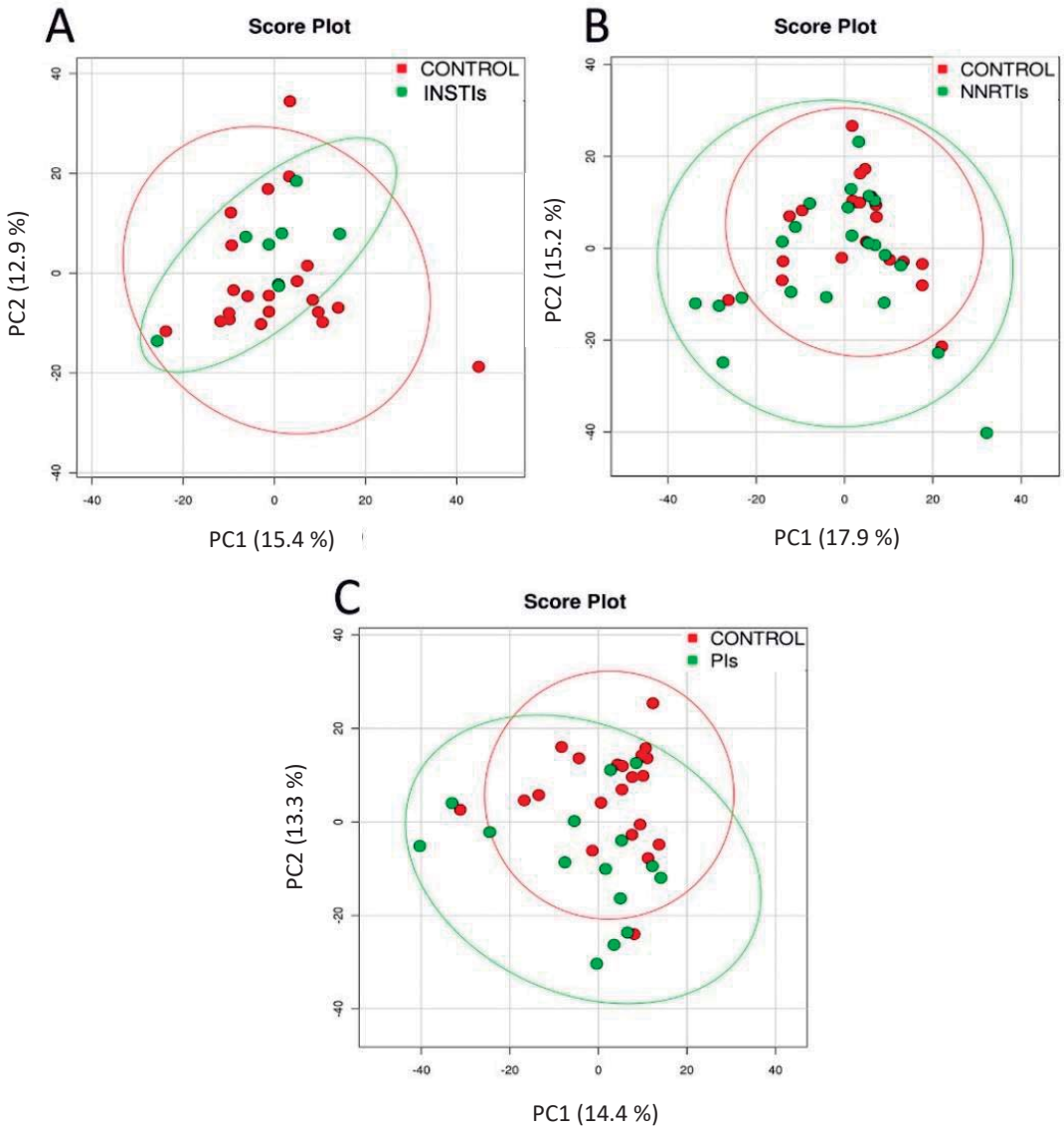


Figure 21. Principal component analysis of different combined antiretroviral therapy compared with the control/uninfected individuals.

Results are plotted according to the first two principle components. Each circle represents a sample: red circles represent the uninfected volunteers, while green circles represent the HIV-infected patients using a combined antiretroviral therapy. (A) PCA of the HIV-infected patients using NRTIs+INSTIs combination *vs*. non-infected subjects. (B) PCA of the HIV-infected patients using NRTIs+NNRTIs combination *vs*. non-infected subjects. (C) PCA of the HIV-infected patients using NRTIs+PIs combination *vs*. non-infected subjects.

5.2. Bacterial translocation and gut microbiota composition on HIV-infected patients in presence or absence of coinfection with hepatotropic viruses

5.2.1. Bacterial translocation markers

A significant increase was observed in LBP plasma levels in coinfected patients compared with non-coinfected participants (p=0.0007) and also compared with controls (p=0.005). No significant differences were observed between both HIV-infected groups when sCD14 was quantified (Figure 22), although an increase (p<0.01) was observed in both HIV-infected groups when compared to the controls.

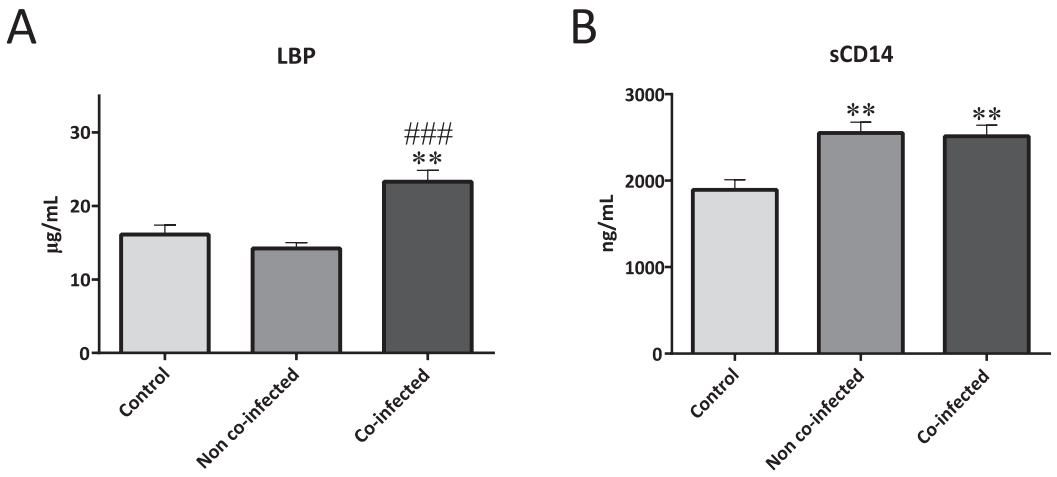


Figure 22. Bacterial translocation markers regarding coinfection with hepatothropic viruses.
(A) Lipopolysaccharide binding protein (LBP). (B) Soluble CD14 (sCD14). Each bar represents the mean ± SEM. **p<0.01 *vs*. Control (uninfected patients); ###p<0.001 *vs*. non-coinfected patients.

5.2.2. Gut microbiota diversity and composition

Coinfected patients showed a lower α-diversity than the controls (p=0.011–p=0.025), although significant differences were not observed among coinfected and non-coinfected patients with all indexes used (Figure 23). Non-coinfected patients had also lower bacterial richness in comparison with control subjects when the alpha (p=0.036) and Margalef's diversity indexes (p=0.040) were measured (Figure 23B, C), while similar diversity than the controls were observed with the number of species and the Chao-1 index. Coinfected patients with advanced hepatic fibrosis (F2–F4) showed a trend towards reduced α-diversity compared with patients with mild hepatic fibrosis (F0–F1) (p=0.098–p=0.078, Figure 24).

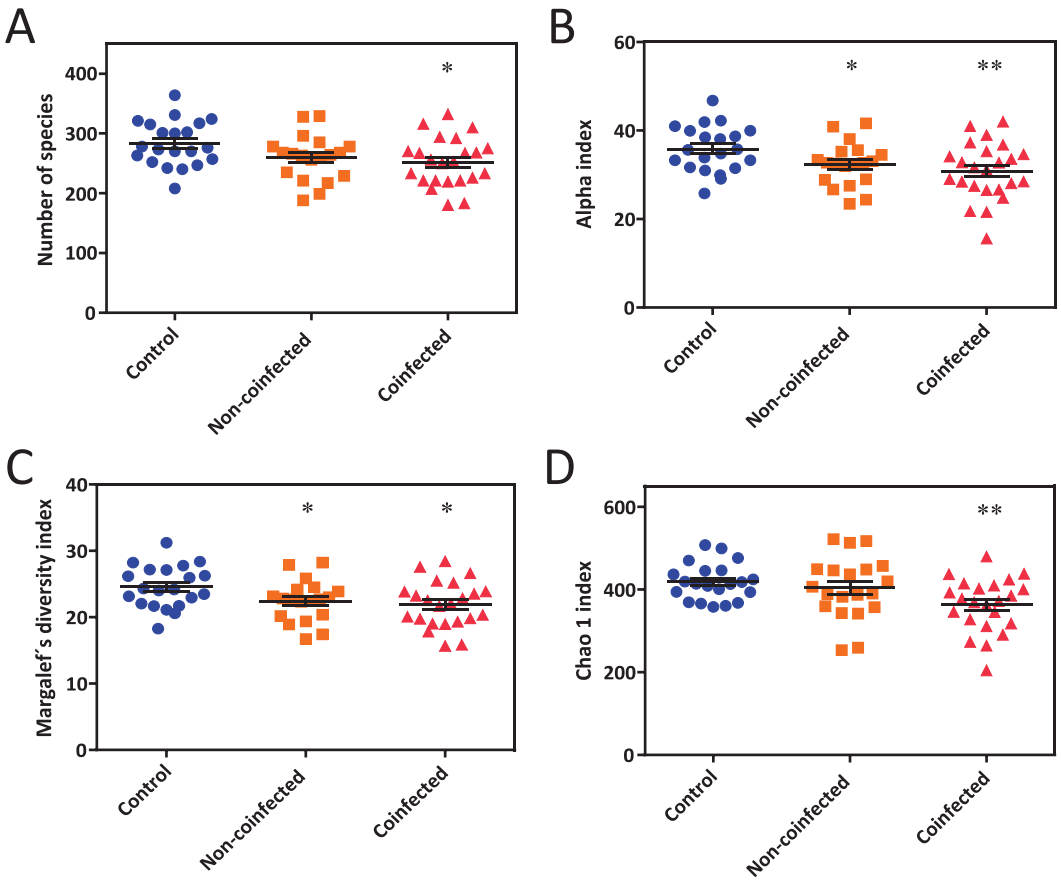


Figure 23. α-diversity measurements in healthy volunteers compared to HIV-infected patients coinfected and non-coinfected with hepatotrophic viruses.
(A) Number of species. (B) Alpha index. (C) Margalef's diversity index. (D) Chao 1 index. Each bar represents the mean ± SEM. $p<0.05$ was considered significant. *$p<0.05$; **$p<0.01$ *vs.* control.

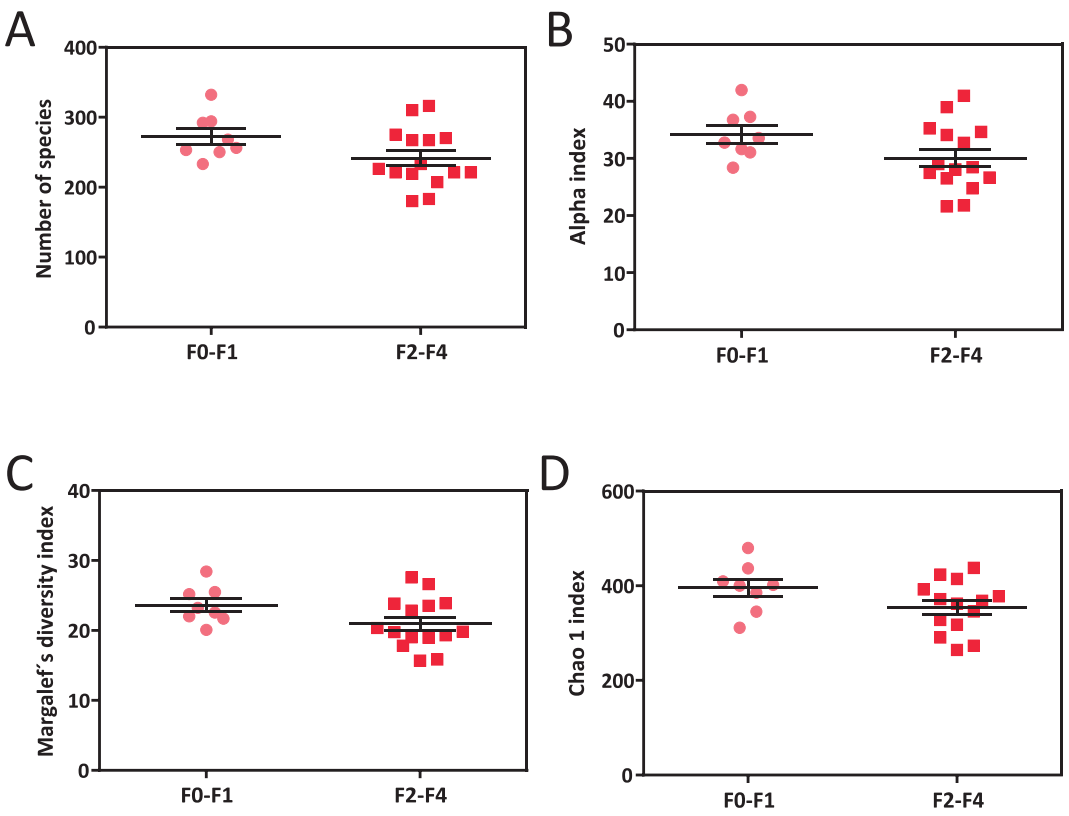


Figure 24. Bacterial richness of HIV patients coinfected with hepatotrophic viruses with advanced hepatic fibrosis compared to patients with mild hepatic fibrosis. (A) Number of species. (B) Alpha index. (C) Margalef's diversity index. (D) Chao 1 index. Each bar represents the mean ± SEM. $p<0.05$ was considered significant.

Concerning GM composition at phylum and class level, HIV coinfected patients showed an increase in the relative abundance of Proteobacteria phylum, δ-Proteobacteria class and, consequently, the ratio of Proteobacteria/Firmicutes in comparison with the control group ($p=0.015$; $p = 0.006$; $p=0.030$, respectively). Likewise, an increase was observed in the abundance of Actinobacteria phylum and the Actinobacteria/Firmicutes ratio in coinfected patients compared to controls ($p=0.004$; 0.002, respectively) and also compared to non-coinfected patients ($p=0.004$; $p=0.003$, respectively). A significant decline in the relative

abundance of the γ-Proteobacteria class was observed in non-coinfected patients in comparison with healthy subjects (p=0.002) and in comparison with coinfected patients (p=0.039) (Table 9).

Table 9. Relative abundance of major phyla present in gut in control group compared to coinfected and non-coinfected HIV patients.

	CONTROL	HIV non-coinfected patients	HIV coinfected patients	Overall p value
Firmicutes	42.86 ± 1.56	38.93 ± 2.22	39.38 ± 2.00	0.329
Bacteroidetes	31.25 ± 2.46	36.86 ± 2.40	33.43 ± 2.34	0.271
Proteobacteria	2.96 ± 0.35	4.40 ± 0.64	4.89 ± 0.61 *	0.094
*** α-Proteobacteria**	0.43 ± 0.12	0.34 ± 0.13	0.34 ± 0.10	0.319
*** β-Proteobacteria**	0.79 ± 0.20	0.80 ± 0.19	1.44 ± 0.29	0.298
*** γ-Proteobacteria**	0.10 ± 0.02	0.03 ± 0.008 **	0.40 ± 0.13 #	0.014
*** δ-Proteobacteria**	0.49 ± 0.07	0.75 ± 0.12	0.90 ± 0.12 **	0.029
Actinobacteria	0.96 ± 0.17	0.87 ± 0.13	3.28 ± 0.65 ** ##	0.004
Ratio Bacteroidetes/Firmicutes	0.76 ± 0.09	1.07 ± 0.12	0.88 ± 0.09	0.169
Ratio Proteobacteria/Firmicutes	0.08 ± 0.01	0.11 ± 0.02	0.13 ± 0.02 *	0.085
Ratio Actinobacteria/Firmicutes	0.02 ± 0.004	0.02 ± 0.003	0.08 ± 0.02 ** ##	0.002

Data are presented as mean ± SEM. Results with superscript are significantly different: *p<0.05; **p<0.01 *vs.* control (non-infected patients). #p<0.05; ##p<0.01 *vs.* HIV non co-infected patients.

Coinfection was also accompanied by some slightly changes in GM at genera and species levels compared with non-coinfected patients. Thus, coinfected patients showed an increase in the *Bacteroides* and *Parabacteroides* genera, as well as in the bacterial specie *Parabacteroides merdae*. In contrast, a decrease in the relative abundance of five bacterial

genera and two species was observed in HIV coinfected patients in comparison with those non-coinfected. Specifically, a significant reduction was observed in the abundance of *Clostridium*, *Coriobacterium*, *Pseudomonas*, *Roseburia*, and *Ruminococcus* genera, and in the bacterial species *Roseburia inulinivorans* and *Sinorhizobium* sp (Table 10).

Table 10. Abundance of lower taxonomic levels (genus and species) which were significantly increased or decreased in faeces from coinfected patients compared with non-coinfected patients.

Coinfected patients *vs.* Non-coinfected					
	Phylum	**Taxonomic group**	**Category**	**p value**	**Coinfected**
↑	Bacteroidetes	***Bacteroides***	Genus	0.007	Increased
↑	Bacteroidetes	***Parabacteroides***	Genus	<0.001	Increased
↑	Bacteroidetes	***Parabacteroides merdae***	Species	0.003	Increased
↓	Firmicutes	***Clostridium***	Genus	0.038	Decreased
↓	Actinobacteria	***Coriobacterium***	Genus	0.043	Decreased
↓	Proteobacteria	***Pseudomonas***	Genus	<0.001	Decreased
↓	Firmicutes	***Roseburia***	Genus	<0.001	Decreased
↓	Firmicutes	***Ruminococcus***	Genus	<0.001	Decreased
↓	Firmicutes	***Roseburia inulinivorans***	Species	<0.001	Decreased
↓	Proteobacteria	***Sinorhizobium sp.***	Species	<0.001	Decreased

Red represents a significant increase in the relative abundance of the taxonomical groups in coinfected patients in comparison with those non-coinfected, whereas blue represents a significant decrease.

PCA showed that clusters representing non-infected subjects and HIV-infected patients with and without coinfection with hepatotropic viruses

were overlapped. However, there was a higher dispersion in HIV patients in comparison with the healthy volunteers. This dispersion was even greater in those coinfected in comparison with the other groups. The results were plotted according to the first two principle components and the clustering of samples was represented by their respective 95% confidence interval ellipse. The PCA accounting for 33.4% of the total variation (Component 1 = 18.1% and Component 2 = 15.3%)) (Figure 25).

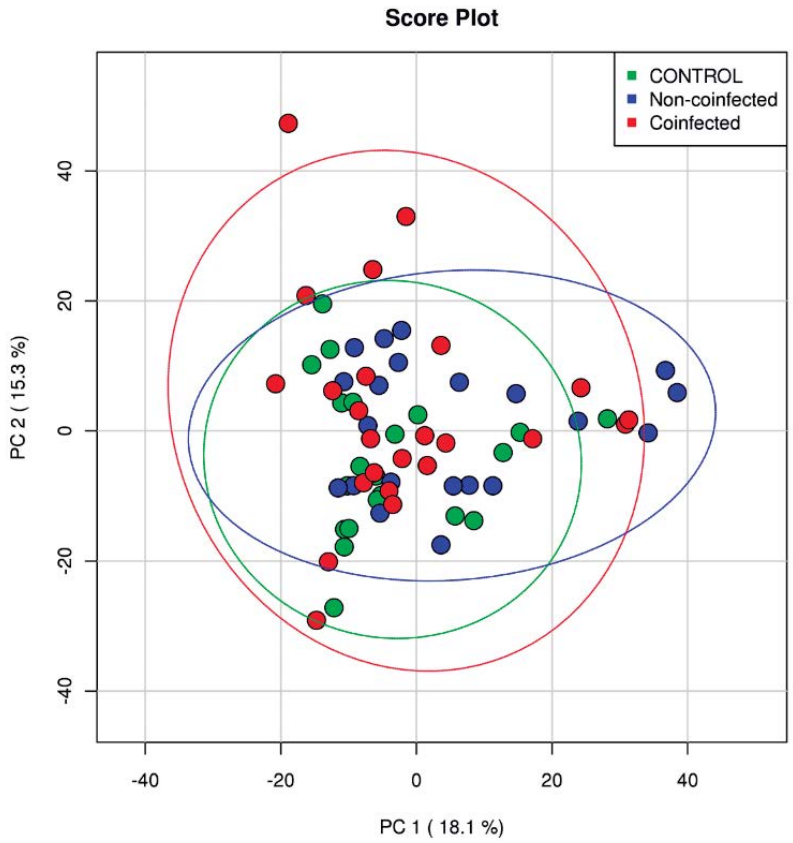


Figure 25. Principal component analysis of non-infected subjects and HIV-infected subjects in presence or absence of coinfection with hepatotropic viruses.
Results are plotted according to the first two principle components. Each circle represents a sample: green circles represent the uninfected volunteers, blue circles represent the HIV-infected patients without coinfection with hepatotropic viruses, whereas those coinfected are represented by red circles.

5.3. Bacterial translocation and gut microbiota composition on HIV-infected patients with or without MS

5.3.1. Characteristics of the participants

Table 11 shows the main characteristics of the studied population. The prevalence of MS in the HIV-infected patients recruited in our Department along eight months and according to the NCEP-ATP III criteria was 21.57%. HIV patients with MS were significantly older than those without MS ($p=0.035$). As expected, patients with MS showed a significant increase in body weight ($p=0.018$), body mass index ($p=0.007$), systolic and diastolic blood pressure ($p=0.027$; $p=0.002$, respectively), triglycerides ($p<0.0001$) and total cholesterol ($p=0.008$) compared to the group without MS. Insulin levels and HOMA-IR index were also significantly increased in patients with MS ($p=0.021$; $p=0.003$, respectively). No statistical differences were observed when comparing the different ART combinations among both HIV-groups ($p=0.920$).

Table 11. Cohort characteristics of HIV infected patients according to the presence of metabolic syndrome.

	HIV+MS-	HIV+MS+	p value
No. of patients	40	11	
Gender (male)	27/40 (67.5%)	7/11 (63.64 %)	1
Age (years)	48.38 ± 0.89	52.30 ± 1.10	0.035
Body weight (kg)	66.95 ± 1.63	76.05 ± 3.98	0.018
Body mass index (kg/m²)	22.78 ± 0.74	27.81 ± 2.04	0.007
Systolic blood pressure (mmHg)	127 ± 2.31	137.8 ± 4.29	0.027
Diastolic blood pressure (mmHg)	80.43 ± 1.80	92.78 ± 1.93	0.002
CD4 nadir count (cells/mm³)	<200: 17/40 (42.5%)	<200: 5/11 (45.45%)	0.898
	200-500: 18/40 (45%)	200-500: 4/11 (36.36%)	
	>500: 5/40 (12.5%)	>500: 2/11 (18.18%)	
CD4 count (cells/mm³)	200-500: 10/40 (25%)	200-500: 2/11 (18.18%)	1
	>500: 30/40 (75%)	>500: 9/11 (81.82%)	
T4/T8 index	0.93 ± 0.05	0.77 ± 0.09	0.106
Time since diagnosis of HIV infection (years)	15.82 ± 1.12	19 ± 2.47	0.206
AIDS	18/40 (45%)	4/11 (36.36%)	0.737
Coinfection with hepatitis B Virus	1/40 (2.5%)	0/11 (0%)	1
Coinfection with hepatitis C Virus	22/40 (55%)	5/11 (45.45%)	0.574
Mode of transmission	IVDU: 16/40 (40%)	IVDU: 4/11 (36.36%)	0.090
	HS: 17/40 (42.5%)	HS: 2/11 (18.18%)	
	MSM: 2/40 (5%)	MSM: 0/11 (0%)	
	Vertical: 1/40 (2.5%)	Vertical: 0/11 (0%)	
	IVDU/HS: 0/40 (0%)	IVDU/HS: 1/11 (9.09%)	

		HIV+MS-	HIV+MS+	p value
		Unknown: 4/40 (10%)	Unknown: 4/11 (36.36%)	
Degree of hepatic fibrosis		NO: 18/40 (45%)	NO: 7/11 (63.63%)	0.939
		F1: 7/40 (17.5%)	F1: 1/11 (9.09%)	
		F2: 5/40 (12.5%)	F2: 1/11 (9.09%)	
		F3: 7/40 (17.5%)	F3: 2/11 (18.18%)	
		F4: 3/40 (7.5%)	F4: 0/11 (0%)	
Antiretroviral treatment		NRTIs+PIs: 11/40 (27.5%)	NRTIs+PIs: 4/11 (36.4%)	0.920
		NRTIs+NNRTIs: 18/40 (45%)	NRTIs+NNRTIs: 4/11 (36.4%)	
		NRTIs+INSTIs: 6/40 (15%)	NRTIs+INSTIs: 2/11 (18.2%)	
		Others: 5/40 (12.5%)	Others: 1/11 (9.1%)	
Advanced degree of hepatic fibrosis		10/40 (25%)	2/11 (18.18%)	1
Time on ART (years)		12.28 ± 0.97	14.64 ± 2.29	0.287
Time on the last ART (years)		6.03 ± 2.90	3.36 ± 2.25	0.006
Biochemical tests	**Plasma triglycerides (mg/dL)**	103.6 ± 5.78	210.7 ± 24.74	<0.0001
	Plasma total cholesterol (mg/dL)	181.2 ± 5.71	214.7 ± 7.91	0.008
	Plasma LDL (mg/dL)	113.6 ± 5.58	132.9 ± 13.68	0.154
	Plasma HDL (mg/dL)	52.77 ± 2.53	42.90 ± 4.41	0.069
	Plasma triglycerides/HDL	3.01 ± 0.17	6.06 ± 0.75	<0.0001
	Plasma AST (U/L)	20.84 ± 1.07	19.89 ± 2.03	0.678

		HIV+MS-	HIV+MS+	p value
	Plasma ALT (U/L)	24.94 ± 1.89	23.67 ± 3.15	0.946
	Plasma glucose (mg/dL)	89.83 ± 1.63	93.27 ± 3.23	0.335
	Serum insulin (µU/mL)	12.38 ± 0.54	16.69 ± 1.83	0.021
	HOMA-IR index	2.74 ± 0.13	3.81 ± 0.43	0.003

Quantitative data are presented as mean values ± SEM, whereas qualitative data are indicated as percentage. A p value of <0.05 was considered significant.
AIDS, acquired immunodeficiency syndrome; ALT, alanine transaminase; ART, antiretroviral therapy; AST, aspartate transaminase; F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; F4, cirrhosis or advanced fibrosis; HDL, high-density lipoprotein; HIV+MS-, HIV-infected patients without metabolic syndrome; HIV+MS+, HIV-infected patients with metabolic syndrome; HOMA-IR, homeostasis model assessment for insulin resistance; HS, heterosexual; IVDU, intravenous drug user; IVDU/HS, intravenous drug user and multiple heterosexual contacts; LDL, low-density lipoprotein; MSM, men who have sex with men; NRTIs+INSTIs, nucleoside reverse transcriptase inhibitors and integrase strand transfer inhibitors; NRTIs+NNRTIs, nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors; NRTIs+PIs, nucleoside reverse transcriptase inhibitors and protease inhibitors.

5.3.2. Markers of bacterial translocation, inflammation and cardiovascular risk

As previously demonstrated in this Doctoral Thesis, HIV-infected subjects presented higher levels of sCD14 in comparison with a general population. These results were obtained when patients with and without MS were analyzed together. However, no differences were observed when the bacterial translocation markers LBP and sCD14 were analyzed comparing both HIV-groups (Figure 26A, B). Higher levels of IL-6 and MCP-1 that approached the threshold of statistical significance were observed in

HIV+MS+ subjects when compared to non-MS HIV patients (p=0.069; p=0.067, respectively) (Figure 26C, D). HIV-infected patients with MS showed higher levels of the cardiovascular risk marker PAI-1 in comparison with those HIV-infected patients without MS (p=0.007) (Figure 26E). Likewise, HIV patients with MS showed significantly higher values of the triglycerides-to-HDL ratio, which is a marker of cardiovascular risk, compared to the HIV+MS- group (p<0.0001). Similarly, patients with MS showed significantly higher values of the triglycerides-to-HDL ratio, which is a marker of cardiovascular risk, compared to the HIV+MS- group (p<0.0001) (Figure 26F).

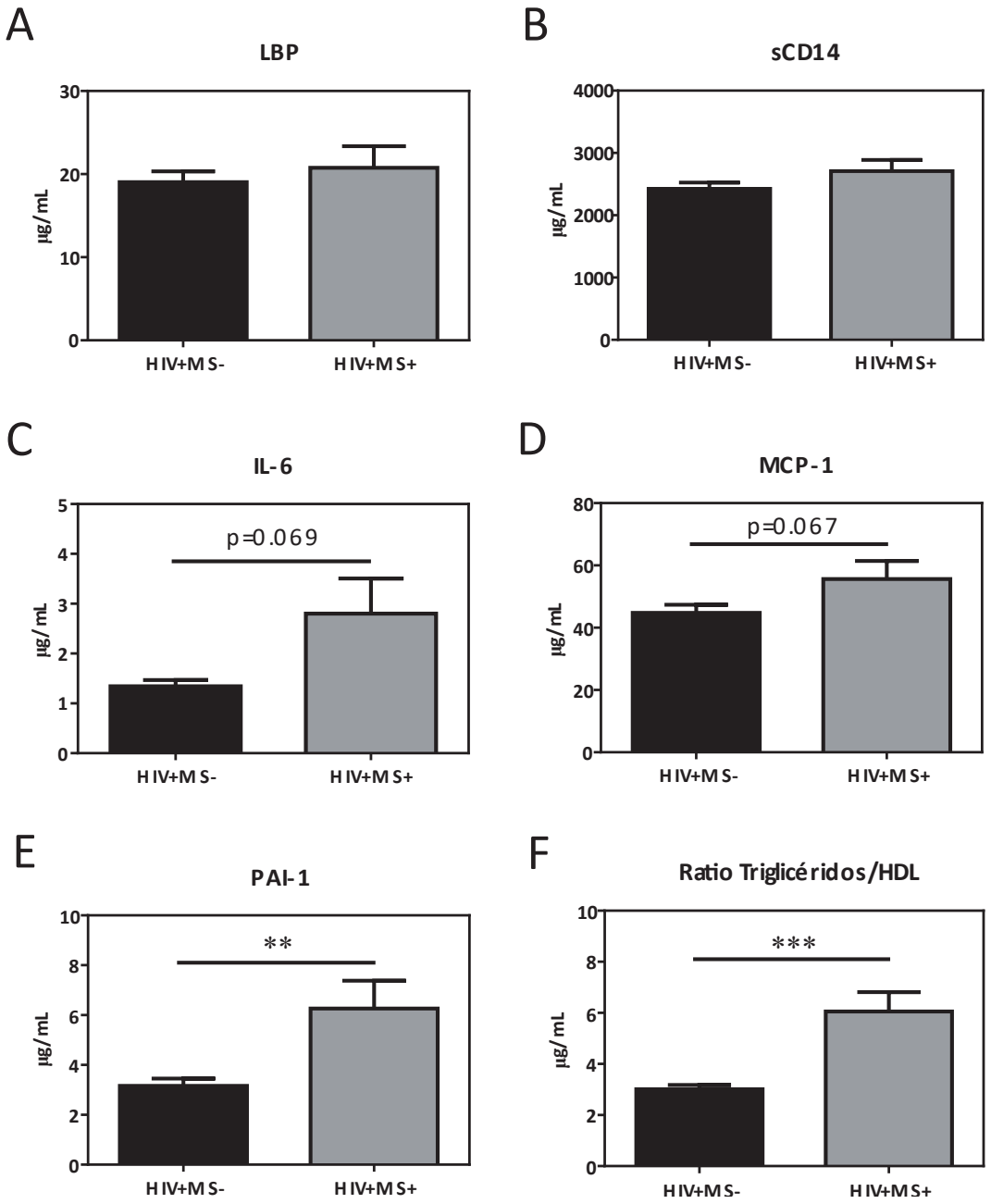


Figure 26. Bacterial translocation, inflammation and cardiovascular risk markers according to the presence of metabolic syndrome.
(A) LBP. (B) sCD14. (C) IL-6. (D) MCP-1. (E) PAI-1. (F) Triglycerides-to-HDL ratio. Each bar represents the mean ± SEM. $p < 0.05$ was considered significant.

5.3.3. Bacterial diversity and gut microbiota composition

α-diversity was assessed using the number of observed species, alpha-index, the Margalef and Chao-1 indexes. No differences were found when comparing HIV-infected patients with and without MS with none of these indexes (Figure 27).

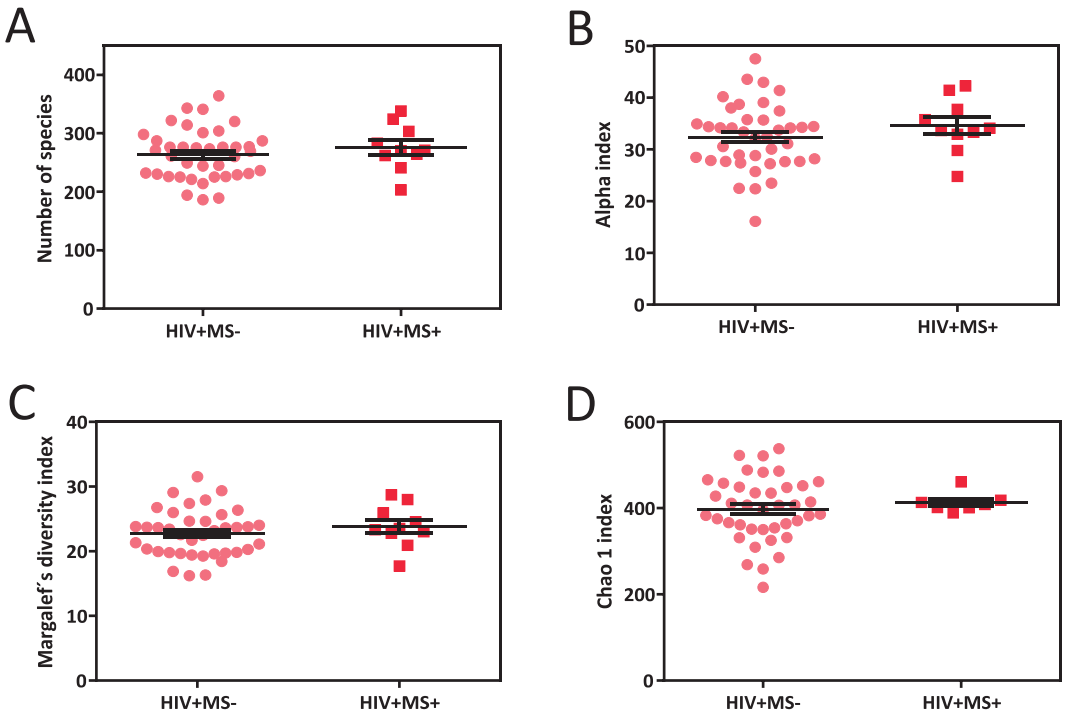


Figure 27. α-diversity measurements in HIV-infected patients with and without metabolic syndrome.
A) Number of species. (B) Alpha index. (C) Margalef's diversity index. (D) Chao 1 index. Each bar Each bar represents the mean ± SEM. $p < 0.05$ was considered significant.

Regarding GM composition, phylum-level composition was dominated by bacteria belonging to the Firmicutes and Bacteroidetes phyla. Thus, HIV+MS- and HIV+MS+ groups showed a relative abundance of 74.32% and 71.22% respectively of these two phyla. When comparing the relative

abundance of the main phyla among the two HIV-groups only a significant decrease in the abundance of Firmicutes phylum was observed in HIV+MS+ patients in comparison with HIV+MS- subjects (p=0.046) (Table 12).

Table 12. Relative abundance (%) of the major bacterial phyla and Proteobacteria classes present in gut in HIV-infected patients with and without metabolic syndrome.

Taxonomic group (relative abundance (%))	HIV		p value
	HIV+MS-	HIV+MS+	
Firmicutes	40.40 ± 1.53	33.77 ± 1.91	0.046
Bacteroidetes	33.92 ± 1.73	37.45 ± 3.74	0.362
Proteobacteria	4.46 ± 0.39	5.97 ± 1.40	0.503
*** α-Proteobacteria**	0.22 ± 0.06	0.25 ± 0.14	0.644
*** β-Proteobacteria**	1.45 ± 0.22	0.73 ± 0.26	0.159
*** γ-Proteobacteria**	0.15 ±0.05	0.23 ± 0.13	0.854
*** δ-Proteobacteria**	0.91 ± 0.10	0.71 ± 0.14	0.378
Actinobacteria	1.37 ± 0.24	2.42 ± 0.71	0.111
Ratio Bacteroidetes/Firmicutes	0.87 ± 0.07	1.04 ± 0.13	0.161
Ratio Proteobacteria/Firmicutes	0.10 ± 0.008	0.14 ± 0.03	0.100
Ratio Actinobacteria/Firmicutes	0.04 ± 0.008	0.05 ± 0.02	0.310

Data are presented as mean values ± SEM. A p value of <0.05 was considered significant.

At lower taxonomic levels, HIV-infected patients with MS showed a decrease in the relative abundance of seven bacterial genera and seven species, whereas no increase was observed in any genera or species in these patients compared to those without MS. Within the Firmicutes phylum, four genera and six species were reduced in the HIV+MS+ group, including the genera *Eubacterium*, *Roseburia*, *Ruminococcus* and *Subdoligranulum*, and the bacterial species *Eubacterium eligens*, *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, *Roseburia inulinivorans*, *Ruminococcus flavefaciens* and

Subdoligranulum sp. The genera *Desulfovibrio* and *Sutterella* and the specie *Sutterella wadsworthensis*, which belong to Proteobacteria phylum, were also reduced, while only the *Bifidobacterium* genus of the Actinobacteria phylum was decreased (Table 13). A principal component analysis (PCA) was performed and clusters representing both groups were overlapped; thus, very small differences in GM profile were observed among HIV-infected patients with and without MS despite the differential abundance described before (Fig. 28). The clustering of samples was represented by their respective 95% confidence interval ellipse and results were plotted according to the first two principle components accounting for 54.6 % of the total variation (Component 1 = 30.4 % and Component 2 = 24.2%).

Table 13. The presence of metabolic syndrome in HIV-infected patients was associated with a decrease in the relative abundance of several bacterial genera and species in comparison with HIV patients without metabolic syndrome.

Phylum	Taxonomic group	Category	FDR
Firmicutes	***Eubacterium***	Genus	0.012
Firmicutes	***Eubacterium eligens***	Specie	0.002
Firmicutes	***Faecalibacterium prausnitzii***	Specie	0.037
Firmicutes	***Roseburia***	Genus	7.47×10^{-4}
Firmicutes	***Roseburia intestinalis***	Specie	0.002
Firmicutes	***Roseburia inulinivorans***	Specie	8.85×10^{-4}
Firmicutes	***Ruminococcus***	Genus	3.59×10^{-4}
Firmicutes	***Ruminococcus flavefaciens***	Specie	0.002
Firmicutes	***Subdoligranulum***	Genus	0.012
Firmicutes	***Subdoligranulum sp.***	Specie	0.002
Proteobacteria	***Desulfovibrio***	Genus	0.019
Proteobacteria	***Sutterella***	Genus	0.002
Proteobacteria	***Sutterella wadsworthensis***	Specie	0.002
Actinobacteria	***Coriobacteriales bacterium***	-	0.002
Actinobacteria	***Bifidobacterium***	Genus	0.009

A false discovery rate (FDR) <0.1 was considered significant.

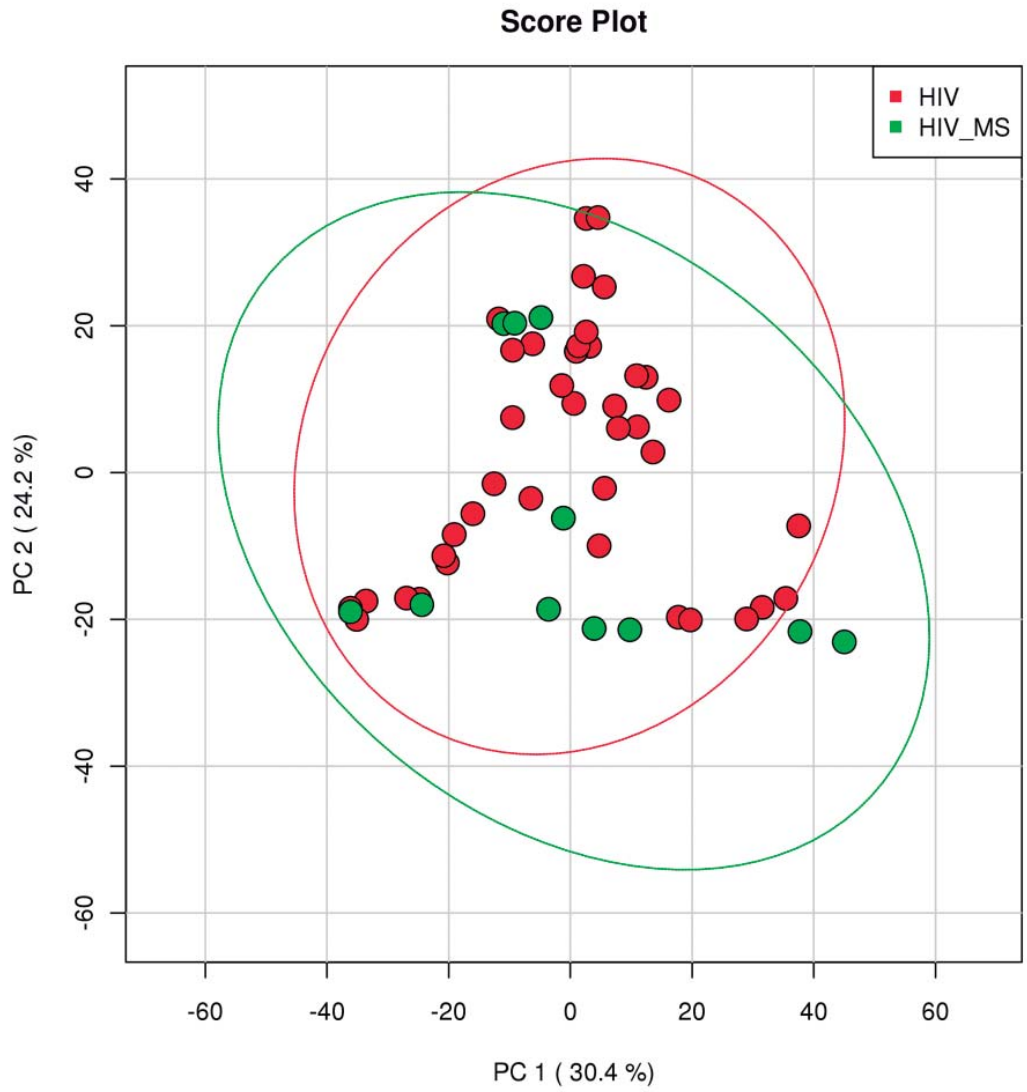


Figure 28. Principal component analysis (PCA) of HIV-infected patients according to the presence of metabolic syndrome.

Each circle represents a sample: red circles represent the HIV-infected patients without metabolic syndrome (HIV) and green circles represent the HIV-infected patients with metabolic syndrome (HIV_MS).

5.3.4. Associations between the reduced bacteria observed in HIV-patients with metabolic syndrome and several physiological and biochemical parameters

The relative abundance of *Coriobacteriales bacterium* was positively associated with serum glucose levels (r=0.362, p=0.009), whereas it was negatively associated with systolic and diastolic blood pressures (r=−0.365, p=0.013; r=−0.336, p=0.023, respectively). Negative correlations between *F. prausnitzii* and the bacterial translocation marker sCD14 (r=−0.398, p=0.007) and triglyceride levels (r=−0.466, p=0.001) were detected. A significant negative correlation was also observed between *R. intestinalis* and LDL levels (r=−0.332, p=0.032). The abundance of *R. flavefaciens* was negatively correlated with the cardiovascular risk marker PAI-1 (r=−0.345, p=0.020) and also with LDL levels (r=−0.374, p=0.015) (Table 14).

Table 14. Associations (Spearman's rank correlation coefficients) found between the abundance of the decreased bacterial groups with the presence of metabolic syndrome in HIV-infected patients and several biochemical and physiological parameters.

	Marker	Spearman Rho	Significance (two tail)
Coriobacteriales bacterium	**Glucose**	0.362	0.009
	SBP	-0.365	0.013
	DBP	-0.336	0.023
Faecalibacterium prausnitzii	**Triglycerides**	-0.466	0.001
	sCD14	-0.398	0.007
Roseburia intestinalis	**LDL**	-0.332	0.032
Ruminococcus flavefaciens	**LDL**	-0.374	0.015
	PAI-1	-0.345	0.020

A p value of <0.05 was considered significant.

5.4. Effect of Maraviroc on gut microbiota composition in a mouse model of diet-induced obesity/fatty liver

5.4.1. *In vitro* antibacterial activity of Maraviroc

No antibacterial activity of MVC was observed against the tested strains: *E. coli* ATCC25922, *E. faecalis* ATCC29212, *S. aureus* ATCC29213, *L. monocytogenes* CECT4032, and *P. aeruginosa* ATCC27853. All the MIC values were ≥128 mg/mL.

5.4.2. Bacterial richness/diversity

Mice fed with a HFD showed a lower bacterial richness with the four indexes analyzed compared to the control group (p=0.003-0.006). The addition of MVC to the HFD was also accompanied by a lower α-diversity in all cases compared to the control (p=0.001-0.002) but also compared to HFD groups (p=0.008-0.009). MVC supplementation with a control diet was not associated with differences in α-diversity compared to control animals (Figure 29).

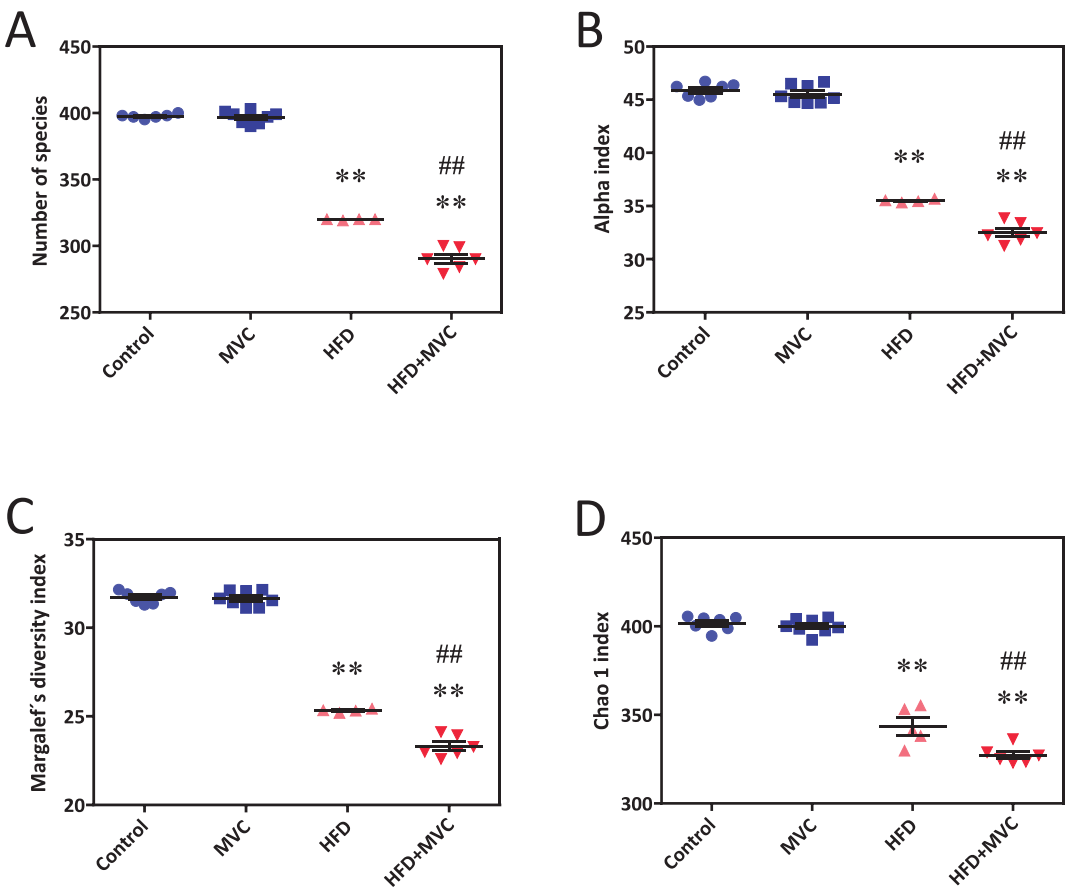


Figure 29. Alpha diversity measurements in control and HFD–fed mice.
A) Number of species. (B) Alpha index. (C) Margalef's diversity index. (D) Chao 1 index. Each bar represents the mean ± SEM. $p < 0.05$ was considered significant.

5.4.3. Gut microbiota composition

Concerning GM composition at phylum level, the cecum bacterial composition of the four groups of mice was dominated by the Firmicutes and Bacteroidetes phyla (both phyla represented the 93%, 94%, 93% and 92% for control, MVC, HFD and HDF+MVC groups respectively).

No differences were observed among the two groups of animals fed with a control/standard diet at the phylum level. The HFD was associated

with an increase in the Proteobacteria phylum (p=0.006) and in the Proteobacteria/Firmicutes ratio (p=0.006) in comparison with control mice. The HFD+MVC group showed a reduction in Firmicutes phylum (p=0.001 *vs*. control; p=0.003 *vs*. HFD) and an increase in Bacteroidetes (p=<0.0001 *vs.* control; p=0.003 *vs.* HFD) and Proteobacteria phyla (p=<0.0001 *vs.* control; p=0.006 *vs.* HFD). HFD+MVC group had also higher values in the ratio Bacteroidetes/Firmicutes (p<0.0001 *vs*. control; p=0.003 *vs.* HFD) and in the ratio Proteobacteria/Firmicutes (p<0.0001 *vs*. control; p=0.006 *vs.* HFD). The ratio Actinobacteria/Firmicutes was also significantly higher in HFD+MVC animals compared to control mice (p=0.026) (Table 15).

Table 15. Effects of MVC supplementation during 16 weeks on major phyla and their respective ratios in mice fed a control/standard or HFD.

	Control	MVC	HFD	HFD+MVC	Overall p value
Firmicutes	0.677 ± 0.001	0.678 ± 0.009	0.680 ± 0.004	0.606 ± 0.005 $^{**\,\#\#}$	0.002
Bacteroidetes	0.263 ± 0.004	0.261 ± 0.006	0.254 ± 0.007	0.313 ± 0.003 $^{***\,\#\#}$	0.002
Proteobacteria	0.019 ± 0.0006	0.019 ± 0.0009	0.023 ± 0.0001 **	0.031 ± 0.0008 $^{***\,\#\#}$	0.0002
Actinobacteria	0.015 ± 0.0008	0.014 ± 0.0003	0.017 ± 0.001	0.016 ± 0.001	0.143
Ratio Bacteroidetes/Firmicutes	0.390 ± 0.007	0.386 ± 0.01	0.374 ± 0.01	0.517 ± 0.009 $^{***\,\#\#}$	0.002
Ratio Proteobacteria/Firmicutes	0.029 ± 0.0008	0.028 ± 0.002	0.034 ± 0.0001 **	0.050 ± 0.002 $^{***\,\#\#}$	0.0004
Ratio Actinobacteria/Firmicutes	0.022 ± 0.001	0.021 ± 0.0009	0.025 ± 0.001	0.027 ± 0.002 *	0.009

$p<0.05$ was considered significant. Data are presented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control group; $^{\#\#}$ $p<0.01$ *vs.* HFD group.

Concerning lower taxonomical levels (family, genus and bacterial species), HFD mice showed different abundances in several family, genus and bacterial species compared to standard/control diet fed-mice. Concretely, HFD mice presented an increase in the relative abundance of 13 bacterial families (Bacteroidaceae, Deferribacteraceae, Dehalobacteriaceae, Desulfomicrobiaceae, Desulfovibrionaceae, Enterobacteriaceae, Enterococcaceae, Lachnospiraceae, Lactobacillaceae, Peptococcaceae, Porphyromonadaceae, Ruminococcaceae, and Streptococcaceae), 16 genera (*Anaerotruncus*, *Anaerovorax*, *Bacteroides*, *Bilophila*, *Butyricimonas*, *Butyrivibrio*, *Dehalobacterium*, *Desulfomicrobium*, *Desulfovibrio*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Mucispirillum*, *Oscillospira*, *Parabacteroides*, and *Ruminicoccus*) and three species (*Bacteroides acidifaciens*, *Mucispirillum schaedleri*, and *Ruminicoccus gnavus*), whereas these mice showed a decrease in the relative abundance of 8 bacterial families (Alcaligenaceae, Anaeroplasmataceae, Bifidobacteriaceae, Clostridiaceae, Eubacteriaceae, Oxalobacteraceae, Prevotellaceae, and Propionibacteriaceae), and 10 genus (*Anaerofustis*, *Anaeroplasma*, *Bifidobacterium*, *Clostridium*, *Coprobacillus*, *Oxalobacter*, *Prevotella*, *Propionibacterium*, *Roseburia*, and *Sutterella*) compared to controls (Table 16).

Table 16. Effects of a HFD intake during 16 weeks on gut microbiota composition of mice at bacterial family, genus and specie level compared to mice fed with a standard diet.

	Phylum	Taxonomic group	Category	FDR
	Bacteroidetes	**Bacteroidaceae**	Family	0.009
	Deferribacteres	**Deferribacteraceae**	Family	0.009
	Firmicutes	**Dehalobacteriaceae**	Family	0.009
	Proteobacteria	**Desulfomicrobiaceae**	Family	0.014
	Proteobacteria	**Desulfovibrionaceae**	Family	0.009
	Proteobacteria	**Enterobacteriaceae**	Family	0.009
	Firmicutes	**Enterococcaceae**	Family	0.010
	Firmicutes	**Lachnospiraceae**	Family	0.009
	Firmicutes	**Lactobacillaceae**	Family	0.041
	Firmicutes	**Peptococcaceae**	Family	0.009
	Bacteroidetes	**Porphyromonadaceae**	Family	0.009
	Firmicutes	**Ruminococcaceae**	Family	0.009
↑	Firmicutes	**Streptococcaceae**	Family	0.009
	Firmicutes	***Anaerotruncus***	Genus	0.011
	Firmicutes	***Anaerovorax***	Genus	0.011
	Bacteroidetes	***Bacteroides***	Genus	0.011
	Proteobacteria	***Bilophila***	Genus	0.011
	Bacteroidetes	***Butyricimonas***	Genus	0.011
	Firmicutes	***Butyrivibrio***	Genus	0.012
	Firmicutes	***Dehalobacterium***	Genus	0.011
	Proteobacteria	***Desulfomicrobium***	Genus	0.015
	Proteobacteria	***Desulfovibrio***	Genus	0.015
	Firmicutes	***Enterococcus***	Genus	0.012
	Firmicutes	***Lactobacillus***	Genus	0.042
	Firmicutes	***Lactococcus***	Genus	0.011
	Deferribacteres	***Mucispirillum***	Genus	0.011
	Firmicutes	***Oscillospira***	Genus	0.011

	Phylum	Taxonomic group	Category	FDR
↑	Bacteroidetes	***Parabacteroides***	Genus	0.011
	Firmicutes	***Ruminococcus***	Genus	0.042
	Firmicutes	***Streptococcus***	Genus	0.011
	Bacteroidetes	***Bacteroides acidifaciens***	Specie	0.017
	Deferribacteres	***Mucispirillum schaedleri***	Specie	0.017
	Firmicutes	***Ruminococcus gnavus***	Specie	0.017
↓	Proteobacteria	**Alcaligenaceae**	Family	0.009
	Tenericutes	**Anaeroplasmataceae**	Family	0.014
	Actinobacteria	**Bifidobacteriaceae**	Family	0.013
	Firmicutes	**Clostridiaceae**	Family	0.094
	Firmicutes	**Eubacteriaceae**	Family	0.062
	Proteobacteria	**Oxalobacteraceae**	Family	0.020
	Bacteroidetes	**Prevotellaceae**	Family	0.009
	Actinobacteria	**Propionibacteriaceae**	Family	0.062
	Firmicutes	***Anaerofustis***	Genus	0.063
	Tenericutes	***Anaeroplasma***	Genus	0.014
	Actinobacteria	***Bifidobacterium***	Genus	0.013
	Firmicutes	***Clostridium***	Genus	0.014
	Firmicutes	***Coprobacillus***	Genus	0.063
	Proteobacteria	***Oxalobacter***	Genus	0.013
	Bacteroidetes	***Prevotella***	Genus	0.011
	Actinobacteria	***Propionibacterium***	Genus	0.063
	Firmicutes	***Roseburia***	Genus	0.013
	Proteobacteria	***Sutterella***	Genus	0.011

Red represents a significant increase in the relative abundance of several families, genera and bacterial species in mice fed a high fat diet compared to standard diet fed-mice, whereas blue represents a significant decrease.
A false discovery rate (FDR) <0.1 was considered significant.

MVC supplementation was only associated with an increase in *Anaerotruncus* genus and a decrease in the *Dorea* genus when compared with mice fed a chow diet (Table 17). No differences in the relative abundance of other genera, families or bacterial species were observed.

Table 17. Effects of MVC supplementation in drinking water during 16 weeks on gut microbiota composition of mice at bacterial family, genus and specie level compared to mice fed a standard diet without MVC.

Phylum	Taxonomic group	Category	FDR	Effect of MVC
Firmicutes	***Anaerotruncus***	Genus	0.065	Increase
Firmicutes	***Dorea***	Genus	0.086	Decrease

Red represents a significant increase in the relative abundance in mice fed a standard diet with Maraviroc (MVC) in drinking water in comparison with mice fed a standard diet without treatment, whereas blue represents a significant decrease.
A false discovery rate (FDR) <0.1 was considered significant.

MVC supplementation with a HFD was accompanied by several disturbances in GM composition. Thus, HFD+MVC mice showed an increase in the abundance of 4 bacterial families (Alcaligenaceae, Bacteroidaceae, Porphyromonadaceae, and Verrumicrobiaceae), as well as 7 genera (*Akkermansia*, *Anaerotruncus*, *Bacteroides*, *Dorea,* *Parabacteroides*, *Sutterella*, and *Coprobacillus*), and 3 species (*Akkermansia muciniphila*, *Bacteroides acidifaciens*, and *Parabacteroides distasonis*). By contrast, these mice showed a reduction in the abundance of 6 bacterial families (Clostridiaceae, Coriobacteriaceae, Lachnospiraceae, Lactobacillaceae, Peptococcaceae, and Ruminococcaceae), and 4 genera (*Adlercreutzia*, *Lactobacillus*, *Oscillospira*, and *Ruminococcus*) in comparison with mice fed a HFD (Table 18).

Table 18. Effects of MVC supplementation in drinking water of mice fed a HFD during 16 weeks on gut microbiota composition at bacterial family, genus and specie level compared to HFD-fed mice without MVC.

	Phylum	Taxonomic group	Category	FDR
↑	Proteobacteria	**Alcaligenaceae**	Family	0.018
	Bacteroidetes	**Bacteroidaceae**	Family	0.018
	Bacteroidetes	**Porphyromonadaceae**	Family	0.018
	Verrumicrobia	**Verrucomicrobiaceae**	Family	0.018
	Verrumicrobia	***Akkermansia***	Genus	0.014
	Firmicutes	***Anaerotruncus***	Genus	0.014
	Bacteroidetes	***Bacteroides***	Genus	0.014
	Firmicutes	***Coprobacillus***	Genus	0.023
	Bacteroidetes	***Parabacteroides***	Genus	0.014
	Proteobacteria	***Sutterella***	Genus	0.014
	Verrumicrobia	***Akkermansia muciniphila***	Specie	0.024
	Bacteroidetes	***Bacteroides acidifaciens***	Specie	0.048
	Bacteroidetes	***Parabacteroides distasonis***	Specie	0.024
↓	Firmicutes	**Clostridiaceae**	Family	0.031
	Actinobacteria	**Coriobacteriaceae**	Family	0.043
	Firmicutes	**Lachnospiraceae**	Family	0.031
	Firmicutes	**Lactobacillaceae**	Family	0.063
	Firmicutes	**Peptococcaceae**	Family	0.018
	Firmicutes	**Ruminococcaceae**	Family	0.018
	Actinobacteria	***Adlercreutzia***	Genus	0.023
	Firmicutes	***Dorea***	Genus	0.014
	Firmicutes	***Lactobacillus***	Genus	0.066

	Phylum	Taxonomic group	Category	FDR
↓	Firmicutes	***Oscillospira***	Genus	0.014
	Firmicutes	***Ruminococcus***	Genus	0.066

Red represents a significant increase in the relative abundance of several families, genera and bacterial species in mice fed a high fat diet with MVC compared to mice fed the same diet without MVC in drinking water, whereas blue represents a significant decrease.
A false discovery rate (FDR) <0.1 was considered significant.

A PCA was assessed to better understand the differences in GM composition between the groups herein analyzed. Figure 30 showed a PCA according to the first two principles components accounting for 74.5% of the total variation (Component 1 = 64.3% and Component 2 = 10.2%). Each group is represented by one cluster; thus, the control and MVC clusters were completely overlapped, while the HFD and HFD+MVC groups were represented in two clusters well-differenced. Thus, control groups showed a similar GM profile regardless MVC supplementation. In contrast, HFD and HFD+MVC groups showed marked differences in GM composition between them and also when compared to the control groups.

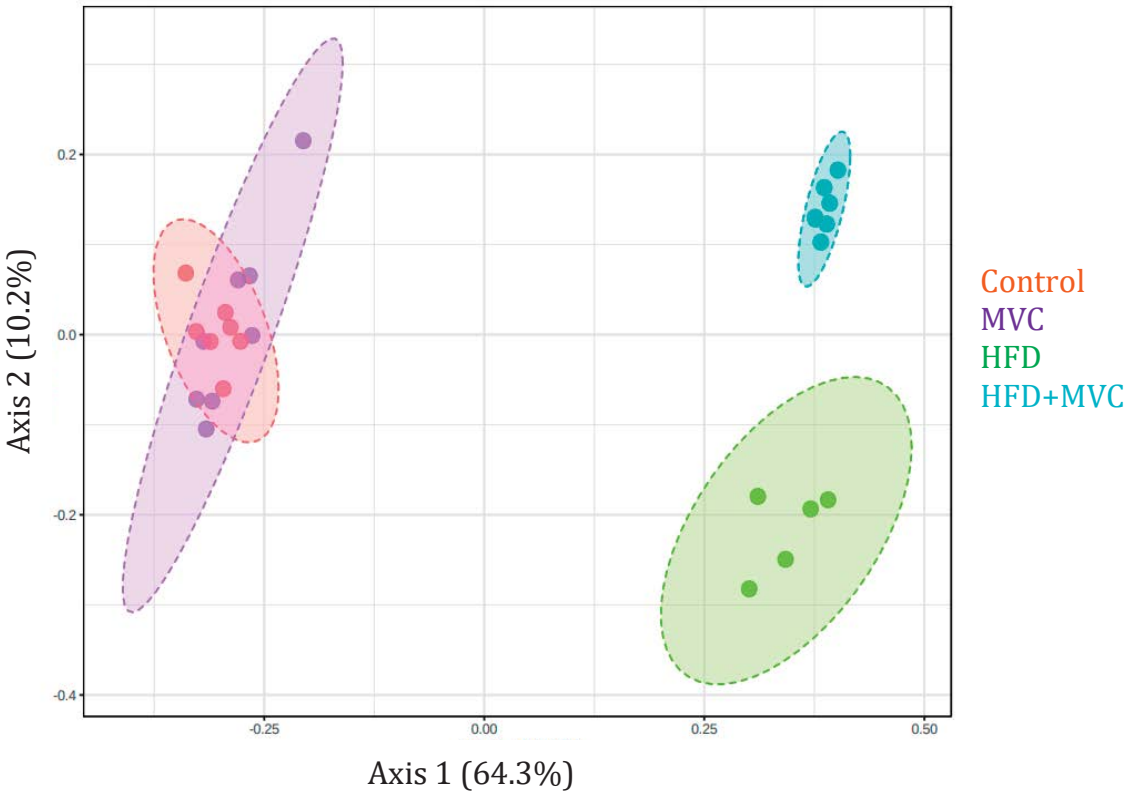


Figure 30. Principal component analysis showing the effects of MVC supplementation during 16 weeks on gut microbiota profile in mice fed a control/standard or HFD.
Each symbol represents a sample: orange circles represent the control group, purple circles represent MVC mice, gr een circles represent HFD group, whereas blue circles represent HFD+MVC group.

5.4.4. Analysis of short-chain fatty acids in serum

The consumption of a HFD was accompanied by a significant decrease in the serum levels of propionate ($p=0.05$) and butyrate, although it did not reach statistical significance ($p=0.158$). In contrast, the HFD-feeding was accompanied by a significant increase in acetate serum levels ($p<0.05$) and isovalerate ($p<0.05$). MVC supplementation did not exert any additional effect on the serum concentrations of theses fatty acids, with the exception of butyrate, as a significant increase was observed in the MVC

group when compared with the control animals ($p<0.05$). A non-significant increase was also observed in HFD+MVC mice in comparison with the HFD-animals (Figure 31).

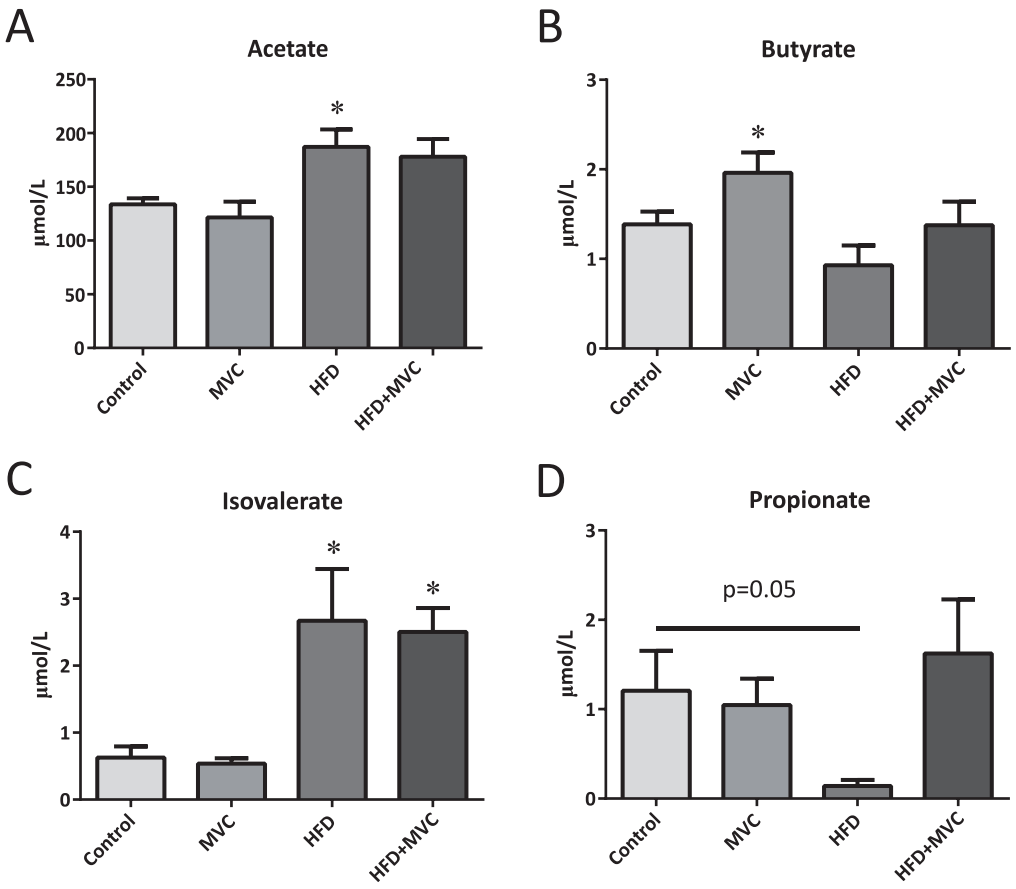


Figure 31. Effects of MVC supplementation during 16 weeks on serum levels of short-chain fatty acids from control and high fat fed mice.
A) Acetate. (B) Butyrate. (C) Isovalerate. (D) Propionate. Each bar represents the mean ± SEM. $p < 0.05$ was considered significant.

5.4.5. Associations between gut microbiota composition and body-weight-related measurements, serum inflammation and biochemical parameters in mice fed with a HFD

Spearman's rank correlation tests were performed to analyze the possible associations between the genera and bacterial species differentially present in both HFD groups and the body-weight-related measurements, serum inflammation and biochemical parameters of these mice (data previously published by our group, (Perez-Martinez *et al.,* 2014;Perez-Matute *et al.,* 2015)). Among all the genera and species differently found in the cecum of mice of the HFD+MVC *vs*. HFD, only 5 genera and 1 specie were found significantly associated with several physiological and biochemical parameters. Thus, the genera *Bacteroides*, *Parabacteroides* and the bacterial specie *Bacteroides acidifaciens* were positively associated with body weight gain, liver weight, total adipose tissue weight, epididymal adipose tissue weight, hepatic triglyceride content, transaminases levels (ALT, AST), the insulin resistance index (HOMA) and the inflammation marker IL-6, whereas the genera *Sutterella* and *Ruminococcus* were negatively associated with all these parameters. In addition, the *Sutterella* genus was also negatively associated with the inflammation marker TNF-α, while a significant positive correlation was observed between this marker and the *Lactobacillus* genus. The genus *Ruminococcus* was positively associated with butyrate plasma levels (r=0.516; p=0.017) (Table 19).

Table 19. Spearman's rank correlation coefficient between body-weight-related measurements, and serum inflammation and biochemical parameters and the differential genera and species found in the cecum of mice when comparing the HFD+MVC and HFD groups.

Parameters	Pearson (r)	p value
GENUS	***Bacteroides***	
Body weight gain (g)	0.594	0.002
Liver weight (g)	0.496	0.014
Adipose Tissue (g)	0.633	0.001
Epididymal Adipose Tissue (g)	0.558	0.005
Hepatic Triglyceride Content (mg/g tissue)	0.596	0.002
ALT (U/L)	0.596	0.002
AST (U/L)	0.596	0.002
HOMA index	0.610	0.002
Il-6 (pg/mL)	0.755	<0.0001
GENUS	***Parabacteroides***	
Body weight gain (g)	0.757	<0.0001
Liver weight (g)	0.594	0.002
Adipose Tissue (g)	0.601	0.002
Epididymal Adipose Tissue (g)	0.601	0.002
Hepatic Triglyceride Content (mg/g tissue)	0.676	<0.0001
ALT (U/L)	0.676	<0.0001
AST (U/L)	0.676	<0.0001
HOMA index	0.704	<0.0001
Il-6 (pg/mL)	0.639	0.002

Parameters	Pearson (r)	p value
GENUS	***Sutterella***	
Body weight gain (g)	-0,701	<0.0001
Liver weight (g)	-0.776	<0.0001
Adipose Tissue (g)	-0.810	<0.0001
Epididymal Adipose Tissue (g)	-0.841	<0.0001
Hepatic Triglyceride Content (mg/g tissue)	-0.793	<0.0001
ALT (U/L)	-0.793	<0.0001
AST (U/L)	-0.793	<0.0001
HOMA index	-0.703	<0.0001
TNFα (pg/mL)	-0.501	0.013
Il-6 (pg/mL)	-0.675	0.001
GENUS	***Lactobacillus***	
TNFα (pg/mL)	0.439	0.032
GENUS	***Ruminococcus***	
Body weight gain (g)	-0.447	0.028
Liver weight (g)	-0.476	0.019
Adipose Tissue (g)	-0.452	0.027
Epididymal Adipose Tissue (g)	-0.441	0.031
Hepatic Triglyceride Content (mg/g tissue)	-0.529	0.008
ALT (U/L)	-0.529	0.008
AST (U/L)	-0.529	0.008
HOMA index	-0.497	0.013
Il-6 (pg/mL)	-0.584	0.005

Parameters	Pearson (r)	p value
Butyrate (µmol/L)	0.516	0.017
SPECIE	***Bacteroides acidifaciens***	
Body weight gain (g)	0.685	<0.0001
Liver weight (g)	0.554	0.005
Adipose Tissue (g)	0.649	0.001
Epididymal Adipose Tissue (g)	0.621	0.001
Hepatic Triglyceride Content (mg/g tissue)	0.578	0.003
ALT (U/L)	0.578	0.003
AST (U/L)	0.578	0.003
HOMA index	0.744	<0.0001
Il-6 (pg/mL)	0.721	<0.0001

A p value of <0.05 was considered significant.

6. Discussion

6.1. Antiretroviral study

6.2. Coinfection study

6.3. Metabolic syndrome study

6.4. Maraviroc study

This discussion has been performed taking into account the different studies carried out.

6.1. Bacterial translocation and gut microbiota composition in HIV-infected patients on different cART

HIV infection has been associated with an increased BT and alterations of the GM composition that contribute to immune activation and inflammation (Dillon *et al.,* 2014;Dinh *et al.,* 2015;Mutlu *et al.,* 2014). The questions are: Can cART reverse this situation? Have all ARVs the same effects on GM?

A potential impact of cART on inflammation markers cannot be ruled out as Hileman *et al*., (2015) demonstrated that patients who switch from EFV-based to EVG-based regimens showed a decrease in the BT translocation marker sCD14, which may result in an improvement in immune activation, and, consequently, in vascular inflammation (Hileman *et al.,* 2015). In our study, sCD14 levels were still raised despite cART, as previously described (Boulassel *et al.,* 2012;Chege *et al.,* 2011;D'Amico *et al.,* 2005;Dinh *et al.,* 2015;Merlini *et al.,* 2011). However, concerning the actions of different cART combinations on GM composition and bacterial translocation, only two research groups have evaluated this up to now. Nowak *et al*., (2015) analyzed the role of NNRTIs *vs*. PIs on GM composition and they did not found differences among treatments (Nowak *et al.,* 2015). However, the study of Pinto-Cardoso *et al*., (2017) observed that patients on PIs had increased levels of the BT marker sCD14 compared to uninfected participants (Pinto-Cardoso *et al.,* 2017). In our study, an increase in sCD14 levels was also observed in patients on PIs and NNRTIs. Moreover, patients on PIs also showed increased inflammation markers (IL-6 and others) compared to healthy volunteers. The sCD14 increase observed in both studies could be

due to the fact that all patients on PIs included in the study of Pinto-Cardoso *et al*., (2017) and most of patients of our study were on ART regimens based on RTV-boosted. In fact, it has been demonstrated that RTV induce apoptosis and decrease barrier function in human intestinal epithelial cells, although it is important to note that these effects were observed at antiretroviral doses, and not at boost concentrations. In addition, these effects could explain, at least in part, the leak-flux diarrhea and the increase in microbial translocation observed in patients on PIs (Bode *et al.,* 2005). In fact, RTV-boosted protease inhibitors show a range of gastrointestinal side effects, such as noninfectious diarrhea, nausea or vomiting (Elperin and Sax 1996;Hill and Balkin 2009).

An increase in sCD14 levels was also observed in patients on NNRTIs, which contrasts with Pinto-Cardoso *et al*., (2017). The discrepancy could be explained as the patients included in Pinto-Cardoso *et al*., (2017) study were on EFV-based regimen, while the patients included in this work were on different NNRTIs, not only EFV (50%) (Pinto-Cardoso *et al.,* 2017).

INSTI-based regimens are recommended as initial therapy in the current guidelines, but to our knowledge, no studies have addressed the effect of INSTI-based regimens on BT and GM composition. This study has demonstrated, for the first time, that the effects of INSTI-based regimens on GM and BT can be "good". Our study has demonstrated that INSTI-based ART is associated with levels of systemic inflammation and sCD14 similar to uninfected controls. Furthermore, NRTIs+INSTIs patients also showed similar levels of IL-6, VCAM and ICAM to the controls, suggesting that this regimen is able to counteract the increased BT induced by HIV infection and also diminish systemic inflammation, potentially reducing future HIV related complications (such as cardiovascular events) triggered by BT and

inflammation. In this context, several studies have previously demonstrated that starting treatment with INSTIs and/or the intensification or switching to this regimen is associated with a favourable effect on HIV-related immune activation and also with inhibition of CD4-T cell depletion (Asmuth *et al.,* 2012;Cummins and Badley 2014;Hileman *et al.,* 2015;Martinez *et al.,* 2010;Vallejo *et al.,* 2012) which is in line with the findings of this study. It is also important to note than although an increase in BT and inflammation was observed in HIV-infected patients compared with the controls, such difference was only significant in HIV-patients using cART. This could be due to the low number of naive patients recruited, which makes difficult to reach statistical significance.

Although HIV infection has been associated with a reduced bacterial diversity (McHardy *et al.,* 2013;Mutlu *et al.,* 2014;Nowak *et al.,* 2015), others have observed a significant increase in HIV+(naive) patients compared with HIV+(cART) (Lozupone *et al.,* 2013). In our study, a significant and clear collapse was observed in α-diversity in HIV+(naive) compared with the controls, as occurs in other pathologies such as obesity (Villanueva-Millan *et al.,* 2015). Compared with controls, a more pronounced decrease was observed in untreated patients than in those using cART, suggesting that cART is able to partially restore the bacterial diversity in gut. A study by Lozupone *et al*., (2013) also observed that long-term cART is able to partially restore α-diversity to the values obtained in HIV-negative individuals; however, in contrast with the findings of the present study, they observed a significant increase in α-diversity in chronic HIV+(naive) patients compared with controls (Lozupone *et al.,* 2013). These discrepancies could be due to the different indices used to compare α-diversity. Concerning the effects of different antiretrovirals, this study demonstrates that NRTIs+INSTIs patients present a microbial diversity similar to the controls, which

highlights the ability of this regimen to counteract the actions of HIV infection on gut bacterial richness. The superior capacity of cART with INSTIs to restore GM diversity may be due to the fact that INSTIs induce a greater reduction in proviral DNA, which could lead to rapid immunologic reconstitution (Hoenigl *et al.,* 2016;Llibre and Martinez-Picado 2008;Pallikkuth *et al.,* 2013).

HIV infection and usage of different cART did not translate into significant changes at higher taxonomic levels, suggesting that HIV infection could be more closely related to changes in lower taxonomic units and diversity rather than at the phylum level, as occurs in other metabolic pathologies (Villanueva-Millan *et al.,* 2015). These findings contrast with other studies that observed changes in some of the most abundant phyla when HIV-infected patients (both naive and using cART) were compared with healthy individuals (Dillon *et al.,* 2014;McHardy *et al.,* 2013). Thus, McHardy *et al*., (2013) showed a significant decrease in Firmicutes phylum in HIV+(naive) patients and intermediate depletion in HIV+(cART) compared with controls, whereas only a slightly depletion was observed in this phylum in both HIV+(naive) patients and patients using NRTIs+PIs compared with the controls (McHardy *et al.,* 2013). In contrast, a significant increase in the relative abundance of Proteobacteria was observed in HIV+(cART) with no differences among the different ART used, whereas others have not reported any differences (Vujkovic-Cvijin *et al.,* 2013). A potential explanation for these discrepancies could be the type of sample used (rectal mucosal biopsies *vs*. faeces). There is no agreement regarding the best sample to use for these determinations, and, therefore, this is an issue that needs further investigation. However, it is worth mentioning that the majority of the changes observed at lower taxonomic levels in this study were detected in taxonomic groups belonging to Firmicutes phylum and,

especially, to the Clostridiales class. In the study, seven bacterial species were found to be depleted in NRTIs+PIs patients, four were depleted in NRTIs+NNRTIs patients, and five depleted in NRTIs+INSTIs patients. In contrast, within the Clostridiales class, Lachnospiraceae family, one of the major taxonomic groups of the human GM known to degrade complex polysaccharides to SCFAs to be used as energy by the host, was significantly increased with cART, suggesting that these patients are more efficient from an energetic point of view (Meehan and Beiko 2014). *Eubacterium eligens* and *Ruminococcus flavefaciens* were two species whose abundance was significantly depleted by all treatments used. Nowak *et al*., (2015) also observed a decrease in *Eubacterium* genus in HIV-infected patients compared with healthy subjects, especially after introducing cART (Nowak *et al.,* 2015). Of interest, a decrease was observed in the butyrate-producing specie *Faecalibacterium prausnitzii* in NRTIs+PIs patients. *F. prausnitzii* is a beneficial intestinal commensal bacterium with known anti-inflammatory properties; thus, the decrease observed could imply loss of protection and persistent inflammation (Hatano 2013;Quevrain *et al.,* 2016). These findings are in agreement with the increased BT observed in these patients. In addition, a reduction in the abundance of the butyrate-producing bacterial species, normally present in gut, *Roseburia inulinivorans* and *Roseburia intestinalis* has also been observed in the patients on this regimen, corroborating the loss of richness observed compared with the controls. In addition, it is important to mention that the loss of butyrate-producing bacteria is related to several disorders, including IBD and Crohn's disease (Geirnaert *et al.,* 2017;Morgan *et al.,* 2012). In fact, butyrate is important to gut homeostasis, being the preferred energy source for the colon epithelial cells, thus, contributing to the maintenance of the gut barrier functions (Riviere *et al.,* 2016). Moreover, butyrate has anti-inflammatory properties

(Segain *et al.,* 2000). Finally, patients using NRTIs+INSTIs showed a lesser reduction in bacteria from the Clostridiales class. In addition, a reduction in *Desulfovibrio sp*. 6 was observed. This bacterium belongs to the *Desulfovibrio* genus, known to produce hydrogen sulphide, a compound that can be toxic to human cells. Surprisingly, this genus was found to be increased in patients on INSTIs-based regimen. Similarly, higher frequencies of this genus have been found in other pathologies such as IBD and also in HIV infection (Christophersen *et al.,* 2011;Lozupone *et al.,* 2013;Verma *et al.,* 2010). In contrast, Nowak *et al*., (2015) found a significant increase in viraemic patients compared with controls, although the relative abundance of this genus decreased after introduction of ART (Nowak *et al.,* 2015). These discrepancies could be due to the fact that Nowak *et al*., (2015) did not include patients on the NRTIs+INSTIs regimen (Nowak *et al.,* 2015).

It is important to note that a higher number of patients on INSTIs presented AIDS definition compared with the other groups. The usage of INSTIs in these patients was considered a very effective rescue therapy and, in fact, similar CD4 counts were observed when compared with the other groups. Therefore, differences in GM composition could not be due to the CD4 counts. Moreover, only RAL-users were included in this group as it was the first INSTI approved for clinical practice and the only one available when patients were recruited for this study. Several studies have reported some beneficial effects of RAL. Masiá *et al*., (2013) showed that administration of RAL was associated with a beneficial impact on lipid profile in patients previously-treated with PI boosted with RTV (PI/r) with virological suppression (Masia *et al.,* 2013). In the same line, Macías *et al*., (2017) demonstrated that switch from EFV to RAL led to a decline in the degree of hepatic steatosis in HIV-infected patients with nonalcoholic fatty liver disease (NAFLD) compared with HIV-patients continuing with EFV (Macias

et al., 2017). In addition, our group has demonstrated that RAL exerts neutral actions on adipogenesis and glucose and lipid metabolism in adipocytes, which could explain, at least partly, the neutral metabolic effects of this drug in clinical trials (Perez-Matute *et al.,* 2011). This Doctoral Thesis also demonstrates that HIV-infected patients under RAL presented a GM composition similar to uninfected volunteers, which highlights the interest of such findings. However, it is unknown whether EVG or DTG would have similar effects and, therefore, this deserves further investigation.

This study has several limitations. Important aspects that could have an impact in GM composition have not been controlled in this study, such as the exact composition of the diet, stress conditions and HIV acquisition (heterosexual, MSM, intravenous drug user, etc.) (Bailey *et al.,* 2011;Noguera-Julian *et al.,* 2016;Ursell *et al.,* 2012;Yatsunenko *et al.,* 2012). Moreover, it would be interesting to compare the GM composition in the HIV+(naive) patients with different CD4 count ranges; however, this comparison and others could not be performed because of the small sample size and because patients with a CD4 count <400 cells/mm^3 were not included in this study. Finally, this study also included a very limited number of untreated HIV-infected patients. However, the comparison among controls and HIV+(naive) has been previously described and the purpose of this study was to analyse the effects of different cART. In fact, this work includes more patients on cART than others published to date (Dinh *et al.,* 2015;Lozupone *et al.,* 2013;McHardy *et al.,* 2013;Mutlu *et al.,* 2014;Nowak *et al.,* 2015;Pinto-Cardoso *et al.,* 2017;Vujkovic-Cvijin *et al.,* 2013).

To sum up, the present study provides a clear description of GM composition in HIV-infected patients compared with a healthy population and specifically investigates in-depth the impact of different cART, in order

to better understand which regimen is able to restore GM composition and, therefore, resist the actions of HIV infection on BT and subsequent immune activation, disease progression and future complications. In general terms, HIV infection is closely associated with changes in lower taxonomic units and diversity rather than at the phylum level. The NRTIs+PIs regimen showed the highest reduction in bacterial species, which could suggest a significant loss of diversity and increased dysbiosis. In contrast, NRTIs+INSTIs increased the abundance of several bacterial orders, families, genera, and bacterial species and induced a minor loss of bacterial species suggesting a healthier gut and potentially fewer HIV-related complications, which, in turn, could contribute to the lower inflammation and BT observed.

6.2. Bacterial translocation and gut microbiota composition in HIV-infected patients in presence or absence of coinfection with hepatotropic viruses

As it is well known, HIV infection induces a marked $CD4^+$ T cell depletion in GALT, which is associated with damage of the intestinal barrier, increased microbial translocation, dysbiosis and, subsequently, systemic inflammation with development of non-AIDS events (Dillon *et al.,* 2014;Dinh *et al.,* 2015;Hsu *et al.,* 2013;Lozupone *et al.,* 2013;Nowak *et al.,* 2015). Alterations in GM composition and gut barrier, that leads to increased BT and inflammation, have also been observed in HBV- and HCV-infected patients (Aly *et al.,* 2016;Bajaj *et al.,* 2016;Heidrich *et al.,* 2018;Lu *et al.,* 2011;Sandler *et al.,* 2011;Wei *et al.,* 2013). Thus, it would be expected that HIV patients coinfected with hepatotropic viruses have a higher BT and altered GM composition compared to HIV non-coinfected patients.

Our study demonstrates that HIV coinfection with hepatotropic viruses, especially with HCV, is associated with very slight effects on BT and major phyla abundance compared to non-coinfected patients. However, at lower taxonomic levels, HIV coinfected patients have a different GM composition compared to non-coinfected patients with a reduction of some butyrate-producing bacteria. It is important to emphasize that in the present study only one patient within the coinfected group was coinfected with the HBV, whereas the rest of the patients were coinfected with the HCV. Thus, it is considered that most of the effects on GM composition were mainly induced by HCV, and it is for this reason that this discussion is primarily focused on HCV.

Similarly to the results found in our study, the work carried out by Tudesq *et a*l., (2017) observed that coinfected patients had higher levels of the BT

markers LBP and sCD14 compared to non-infected individuals (Tudesq *et al.,* 2017). It seems that BT could be further increased by the presence of HCV. However, in our study, these differences were only observed with LBP levels and not with sCD14. The lack of differences in sCD14 levels could be due to the fact that the levels of this marker are influenced by the liver fibrosis degree (Marchetti *et al.,* 2013;Marchetti *et al.,* 2014) and, in our coinfected group, all fibrosis grades were analyzed together. Moreover, sCD14 is also a known marker of disease progression in HBV/HCV/HIV, thus, as both groups were HIV-infected patients, sCD14 levels could be elevated by HIV itself and coinfection may not be accompanied by higher levels of this marker, although more studies are needed to confirm this (reviewed by Kotsounas et al., 2015) (Koutsounas *et al.,* 2015).

Concerning GM profile, several studies have showed a disturbed GM composition in patients infected with HCV and HBV compared to non-infected individuals (Aly *et al.,* 2016;Bajaj *et al.,* 2016;Heidrich *et al.,* 2018;Lu *et al.,* 2011;Wei *et al.,* 2013). In this context, the studies carried out by Chen *et al*., (2011) and Heidrich *et al*., (2018) showed a decrease in bacterial richness in HBV- and HCV-infected patients in comparison with healthy controls, suggesting that HBV and/or HCV infection is associated with depletion in bacterial richness (Chen *et al.,* 2011;Heidrich *et al.,* 2018). Similarly, in our study, a lower bacterial richness was observed in coinfected patients when compared to uninfected subjects, although no differences were observed when compared to non-coinfected patients. Thus, the decrease in bacterial richness seems to be due to the significant reduction induced by HIV infection rather than HCV, and not additional effects have been observed. Heidrich *et al*., (2018) found that the decrease was more pronounced in the presence of cirrhosis, being α-diversity associated with the stage of hepatic fibrosis as well as with the virus (Heidrich *et al.,* 2018).

However, in our study, coinfected patients with advanced hepatic fibrosis did not show a lower bacterial richness compared to patients with mild hepatic fibrosis and only a trend towards reduced α-diversity was observed. The lack of differences in α-diversity according to liver fibrosis could be due to the fact that only 12.5 percent of the patients enrolled in this study presented cirrhosis.

Coinfection was associated with mild effects on major phyla abundance as only an increase was observed in the Proteobacteria and Actinobacteria phyla compared to uninfected patients. In contrast, the work performed by Aly *et al*., (2016) showed that healthy individuals had higher abundance of Firmicutes, Proteobacteria, and Actinobacteria compared to HCV mono-infected patients. Differences among studies could be attributable to the fact that the study of Aly *et al*., (2016) included only seven patients, all of them with cirrhosis, whereas our study included a higher number of patients with different degrees of hepatic fibrosis, and only 12.5 percent of the coinfected patients recruited by our study presented cirrhosis. Moreover, it is important to note that the study carried out by Aly *et al*., (2016) were performed in HCV-mono-infected patients and not in HIV coinfected patients. Thus, the effect of both viruses on bacterial richness was not addressed by these authors (Aly *et al.,* 2016). To sum up, due to the different characteristics of the patients recruited, it is very difficult to compare the results among both studies.

Generally, few differences were observed at lower taxonomic levels among the different groups analyzed in the present study. Thus, PCA showed that all groups were overlapped, although a higher dispersion of the GM profile of coinfected patients compared to those non-coinfected and uninfected subjects was observed. The presence of cirrhosis among the

coinfected group is a variable that could contribute to such higher dispersion. In fact, it has been demonstrated by previous works that patients with liver cirrhosis showed an altered GM composition compared to healthy subjects (Bajaj *et al.,* 2014;Chen *et al.,* 2011;Liu *et al.,* 2012), as a close association exists among liver and intestine in what is called as liver-gut axis.

Regarding changes at genus and specie level, an increase was observed in the *Bacteroides* and *Parabacteroides* bacterial genera, as well as in the specie *Parabacteroides merdae* in coinfected patients compared to those non-coinfected. *Bacteroides* and *Parabacteroides* species represent approximately 25% of the GM, being normally commensals. Nakano *et al*., (2013) have associated these genera with a higher morbidity and mortality (Nakano *et al.,* 2013). In fact, even though the genus *Bacteroides* become part of the normal human microbiota from the earliest stages of life, it was demonstrated that when members of this genus escape the gut, they can cause different pathologies including abscess formation in multiple body sites as well as bacteremia (Wexler 2007). As it is well-known, both HIV and HCV are associated with damage to the epithelial barrier of the gut, thus, the higher relative abundance of this genus in coinfected patients compared to those non-coinfected could be related with the higher BT observed, which, in turn, may be associated with pathogenic effects of this genus. In the same line, the higher abundance observed of *Parabacteroides* in coinfected patients could also have deleterious effects, since can represent opportunistic pathogens in infectious diseases (Chiu *et al.,* 2014). Thus, the role of these bacteria in coinfected patients needs to be researched further but points towards an unhealthy state.

Coinfected patients showed a decrease compared to non-coinfected patients in the relative abundance of the butyrate-producing bacteria

Roseburia and *Ruminococcus* and in the genus *Clostridium* as well as in the bacterial specie *Roseburia inulinivorans*. The reduction of these bacteria could have deleterious effects in these patients, as butyrate plays an important role in the metabolism and normal development of colonic epithelial cells, as previously mentioned (Barcenilla *et al.,* 2000;Pryde *et al.,* 2002). The study performed by Aly *et al*., (2016) also observed a decrease in the relative abundance of *Ruminococcus* and *Clostridium* genera in HCV mono-infected patients compared to uninfected individuals (Aly *et al.,* 2016). Coinfected patients also showed a reduction in the abundance of the genus *Pseudomonas*, which includes species known to cause disease in humans (Iglewski 1996). However, the role of this genus in coinfection needs to be further investigated. Finally, it is interesting to mention the decrease observed in *Coriobacterium* in coinfected patients since it has been demonstrated that this genus plays a role in the early development of the newborn infant's GM, being present in faeces of healthy individuals (Harmsen *et al.,* 2000). Thus, whether the reduction of this bacterial genus in coinfected patients could have a detrimental role needs to be further investigated.

To sum up, coinfection with hepatotropic viruses was associated with very mild effects on BT and major phyla abundance compared to non-coinfected patients. At lower taxonomic levels, a different microbiota composition was observed in HIV coinfected patients when compared with non-coinfected patients, and for this reason the impact of such coinfection could not be discarded and should be taken into account in these types of studies.

6.3. Bacterial translocation and gut microbiota composition In HIV-infected patients with or without metabolic syndrome

In this study we analyzed what happen in gut in HIV-infected patients that suffer from MS in comparison with those not suffering from such syndrome. But, first of all, we calculated the prevalence of MS in our HIV cohort. Thus, the prevalence of MS in HIV-infected patients recruited from San Pedro´s Hospital for this study according to the NCEP-ATP III criteria was 21.57%, which was similar to the overall prevalence observed in HIV-infected population worldwide and in Europe using the same criteria (24.6% and 24.1%, respectively) (Nguyen *et al.,* 2016). When comparing this prevalence with previous studies carried out in HIV-people in Spain, similar rates were also observed (10-25%) (Cubero *et al.,* 2011;Jerico *et al.,* 2005;Palacios *et al.,* 2007). In addition, the prevalence observed in our Hospital was also within the range of the 17-46% observed in the general population with no infection of HIV, which indicates that the prevalence of this syndrome is not higher in HIV-infected people than in the general population. However and even though the prevalence is similar, the metabolic changes begin at early ages in HIV-infected people, due to, among other factors, the chronic inflammatory state caused by HIV infection ("inflammaging") and the prolonged use of antiretroviral drugs that could lead to the development of different metabolic alterations, as described in the introduction section of the present Doctoral Thesis (Nakhla and Ruble 2010;Nguyen *et al.,* 2016;Smith *et al.,* 2012;Swami 2016).

As previously described, our HIV cohort showed higher plasma levels of sCD14 when compared to a control/healthy population. When these patients were split out depending on the presence of MS, both groups showed higher plasma levels of sCD14 in comparison with the controls,

however, no differences were observed among them, despite the fact that an increase of BT has been reported in non-HIV people with MS or its separated components (Lassenius *et al.,* 2011;Sato *et al.,* 2014). These results could suggest that HIV-infection is the responsible for such increase and the presence of MS in these patients does not potentiate such actions.

Analysis of GM composition using PCA revealed that HIV+MS- and HIV+MS+ groups clustered together, indicating very little differences in GM profile. In fact, no differences were observed in α-diversity among groups and, at phylum level, only a reduction was observed in Firmicutes phyla in HIV patients with MS. Conversely, the study carried out by Lim *et al*., (2017) found that the presence of MS in uninfected subjects was accompanied by a reduction in bacterial diversity (Lim *et al.,* 2017). The differences could be due to the fact that HIV by itself induces a strong decrease in α-diversity (as demonstrated above) and MS does not translate into a higher reduction.

At lower taxonomic levels, the presence of MS was accompanied by a lower relative abundance of *Desulfovibrio* genus, which is known as a hydrogen sulfide producer and associated with damage in the gut barrier (Beerens and Romond 1977). This is the first study where a reduction in the abundance of this genus has been reported in HIV-infected patients with MS in comparison with those not suffering from such syndrome. Only the study from Zhang *et al*., (2010) found a higher number of bacteria belonging to the Desulfovibrionaceae family in fresh faecal samples from animal models of diet-induced MS, specifically in Apoa-I knockout mouse and in wild-type C57BL/6J mice (Zhang *et al.,* 2010). However, these discrepancies could be due to the fact that animal's models not always mimicks what happen in humans. In addition and more importantly, the HIV-infection impact on gut microbiota composition can not be compared among both studies. Thus, the

decline observed in this endotoxin producer in HIV-infected patients with MS and its clinical implications needs to be further investigated.

The vast majority of the deplected bacteria observed in HIV-infected patients with MS were commensal and belong to Firmicutes phylum, including *R. intestinalis*, *R. inulinivorans*, *F. prausnitzii*, and some members of the *Subdoligranulum* genus, all of them butyrate producers (Vital *et al.,* 2013;von Engelhardt *et al.,* 1998). As previously mentioned, butyrate plays an important role at the intestinal level by contributing to intestinal mobility, epithelial defense barrier and reduction of inflammation (Canani *et al.,* 2011). Accordingly, low levels of butyrate has been associated with some diseases in which inflammatory processes are implicated, such as ulcerative colitis (Rios-Covian *et al.,* 2016). A reduction in colonic butyrate-producing bacteria was found by Dillon *et al*., (2017) in HIV-infected patients, which was associated with increased BT and immune activation (Dillon *et al.,* 2017). Although in our study butyrate has not been specifically measured, the reduction in butyrate-producing bacteria in HIV patients with MS is clear and could suggest an unhealthier gut and increased gut inflammation compared to HIV patients without MS. In the same line, it is of great interest the reduction observed in the abundance of *F. prausnitzii*. This bacterial specie presents anti-inflammatory properties and it was demonstrated that can improve intestinal barrier function in animal models with low grade or acute inflammation, being recognized as a biomarker of intestinal health (Carlsson *et al.,* 2013;Laval *et al.,* 2015;Miquel *et al.,* 2013). Therefore, a lower abundance of *F. prausnitzii* could suggest that HIV patients with MS have a greater inflammatory state, which, in turn, could be associated with a greater cardiovascular risk. Overall, these results underline the need to monitor these patients even after immunological control with cARTs in order to avoid, if possible, the deleterious effects derived from loss of protection at

gut level. The genus *Bifidobacterium* (Actinobacteria phylum) includes bacteria with known beneficial effects. In fact, several probiotics are based on different bacterial strains belonging to this genus (Riviere *et al*., 2016). Interestingly, the presence of MS was associated with a decrease in *Bifidobacterium* when intestinal microbiota of both uninfected subjects (Lim et al., 2017) and HIV-patients of our study were analyzed. Thus, these patients could benefit from these probiotics in order to reduce the incidence of future events associated to inflammation.

In general terms, the presence of MS was associated with a decrease in the relative abundance of several bacteria with known anti-inflammatory roles and, therefore, could suggest loss of protection against future cardiovascular events (Kasselman *et al*., 2018). Thus, the increased levels of PAI-1 and the triglycerides-to-HDL ratio observed in HIV-infected patients with MS could be due, at least in part, to the reduction of these bacteria. Thus, these bacteria could constitute reliable markers of future cardiovascular events in HIV-infected people with MS. However, more metabolic/functional studies are needed to corroborate such hypothesis.

One of the limitations of the present study was the difference in age among both HIV-groups, despite the similarities observed in all other parameters (time under stable antiretroviral treatment, families of ART used…). In fact, GM composition can be modulated by age (Odamaki *et al.,* 2016). For this reason, stratification by age was carried out in the HIV-subjects (median age 49 years) in order to evaluate whether the changes observed in GM composition in patients with MS were due to age or by the presence of MS itself. As no changes in GM composition were observed at all the levels analyzed (genera and bacterial species), the reduction observed in the abundance of several bacteria seems to be associated with the presence

of MS itself and not secondary to age. Other potential limitation of the present study could be the small number of HIV-infected patients with MS included, although this sample size was similar to other metagenomic studies (Graessler *et al.,* 2013;Medina *et al.,* 2017).

In summary, the presence of MS in HIV-infected patients was not accompanied by major changes in GM composition and only minimal deviations with potentially clinical impact have been observed when compared with HIV patients without MS. The reduced relative abundance observed in some relevant bacterial species, such as *F. prausnitzii* suggests a greater inflammatory state at intestinal level that could underline the higher CVD risk observed in these patients. In addition, our study highlights the potential usage of some bacteria as markers of future cardiovascular events in HIV-infected patients with MS, although more studies are needed to confirm such results.

6.4. Effects of Maraviroc on gut microbiota composition in a mouse model of diet-induced obesity/fatty liver

Obesity has been defined as one of the 21st Century epidemics associated with different pathologies such as diabetes, hypertension, heart disease and osteoarthritis, which increases morbidity and mortality (Rossner 2002). This condition affects the general population (13%) (WHO: *http://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight*, date accessed: April 2018) but also HIV-infected people. In fact, the proportion of HIV-infected patients with overweight/obesity under antiretroviral treatment has significantly increased in recent years (Tate *et al.,* 2012). These patients have an increased cardiovascular risk and metabolic abnormalities compared with non-obese and non-HIV infected patients (Shah *et al.,* 2012). Thus, antiretroviral therapy-based molecules able to modulate obesity and other metabolic alterations are of great interest.

Previous studies from our group have demonstrated that the CCR5 antagonist MVC exerts beneficial effects against a murine model of obesity and fatty liver (Perez-Martinez *et al.,* 2014;Perez-Matute *et al.,* 2017). It has also been demonstrated in a very preliminary study that MVC modulates microbiota (Perez-Matute *et al.,* 2015). In this context, a deeper investigation of the effects of this antiretroviral on GM are of interest. For this purpose, three different approaches have been carried out in this Doctoral Thesis: (i) the analysis of the *in vitro* actions of MVC on cultured strains, (ii) the investigation of the *in vivo* effects of MVC supplementation in gut in mice fed a control or a high fat diet using next sequencing technologies, and finally, (iii) the determination of MVC actions on SCFAs serum levels.

Concerning the *in vitro* actions of MVC, our study has clearly demonstrated for the first time that MVC did not exert any *in vitro*

bacteriostatic effect in the tested strains. This could suggest that MVC does not directly interfere with the microorganisms themselves. In the same line, our *in vivo* work highlights the lack of actions of MVC in gut microbiota composition when administered along with a control diet, since no differences in the microbiota profile was observed when control mice where compared to mice fed with the same diet but supplemented with MVC. Likewise, Vitomirov *et al*., (2015) demonstrated that this antiretroviral drug did not either induce changes in GM composition, at least at order level, in HIV-infected patients (Vitomirov *et al.,* 2015). However, in our study, it is remarkable the increase observed in butyrate levels observed in those animals supplemented with MVC. As only an increase in the presence of *Anaerotruncus* was observed in mice fed with a control diet supplemented with MVC in comparison with the controls, it is tempting to suggest that such increase could be produced by *Anaerotruncus,* a known butyrate producer from the Ruminococcaceae family (Esquivel-Elizondo *et al.,* 2017). However, more functional/transcriptomic studies are needed in order to deeply understand the clinical role of increased production of butyrate induced by MVC.

Although no significant effects of MVC were found when administered with a control diet, several and interesting actions were observed when administered with a HFD. This could suggest that HFD induces a significant increase in the expression of CCR5 at gut level, and, therefore, MVC could exert different actions. We have not directly measured CCR5 mRNA levels in gut, but previous studies observed a significant increase in the expression of this co-receptor in adipose tissue of obese human, but also in genetically obese (ob/ob) and HFD-induced obese mice (Huber *et al.,* 2008;Kitade *et al.,* 2012;Wu *et al.,* 2007), which makes plausible that this could also happen at gut level. Anyway, MVC actions on GM seem to

be dependent on physiological status (more effective after an "input" such as induction of obesity) and, therefore, should be taken into account when prescriptions are made.

Concerning the effects of MVC on GM composition when administered along with a HFD, it is important to mention that a decrease in Firmicutes and an increase in the Bacteroidetes phyla were observed in comparison with mice fed a HFD. These changes could be beneficial since Rabot *et al*., (2016) associated a GM rich in Bacteroidetes with a protective role against high fat-feeding induced glucose intolerance (Rabot *et al.,* 2016). In fact, our previous studies observed a partial improvement in insulin resistance after MVC administration and despite the ingestion of a HFD (Perez-Martinez *et al.,* 2014;Perez-Matute *et al.,* 2015). The addition of MVC along with a HFD was able to restore most of the effects induced by a HFD (Alcaligenaceae, Lachnospiraceae, Lactobacillaceae, Peptococcaceae and Ruminococcaceae families, and *Coprobacillus*, *Sutterella*, *Lactobacillus*, *Oscillospira* and *Ruminococcus* genera), but, in contrast, MVC also potentiates the effects that a HFD exerts in the abundance of other taxa (Bacteroidaceae, Clostridiaceae and Porphyromonadaceae families, *Anaerotruncus*, *Bacteroides* and *Parabacteroides* genus and *Bacteroides acidifaciens* specie). Finally, MVC showed other effects different from those observed with the HFD (such as those observed on Coriobacteriaceae and Verrumicrobiaceae families and *Akkermansia* and *Adlercreutzia* genera). In fact and as represented by the PCA, the majority of the changes induced by MVC in these HFD-animals were independent of those exerted by the diet. Thus, we can not conclude that MVC could be a promising drug to counteract the HFD effects on gut as other actions can not be discarded and should be taken into account.

In accordance with the present study, several authors have associated a HFD feeding with the reduction of the *Sutterella* genus, while dietary interventions that improve host metabolism increase the abundance of this genus, just as the treatment with MVC (Duparc *et al.,* 2017). In agreement with these statements, *Sutterella* genus was negatively associated with body weight gain, liver and adipose tissue weight, hepatic triglycerides content, HOMA index and levels of transaminases and the inflammatory markers measured in our study. Thus, the increase of this bacterial genus after MVC supplementation could have a beneficial impact at liver and adipose tissue level, though more studies are needed in this regard.

MVC was able to counteract the increase observed in *Ruminococcus* genus in mice fed with a HFD. The study carried out by Boursier *et al*., (2016) found that *Ruminococcus* accumulation was correlated with liver fibrosis stage in patients with NAFLD (Boursier *et al.,* 2016). Then, since MVC decreased the relative abundance of this genus and was negatively associated with liver weight, hepatic triglycerides transaminases levels and the inflammatory marker IL-6, it is tempting to suggest that MVC actions on *Ruminococcus* could underline the beneficial effects of this ARV on liver, as previously reported by our group (Perez-Martinez *et al.,* 2014).

The role of *Lactobacillus* is not clear since it has been traditionally believed to exert beneficial actions and a recent study indicated that could even reduce the endotoxin level, endotoxin-induced inflammation and regulation of the immunity (Zhou *et al.,* 2017) but an increase of *Lactobacillus* in obese NAFLD patients compared to healthy controls has also been observed in previous works (Nobili *et al.,* 2018;Raman *et al.,* 2013). In this Doctoral Thesis, mice fed with a HFD showed an increase in this genus while the administration of MVC decreased the abundance of *Lactobacillus*. These

results are quite intriguing, but it is important to mention that even though some members of *Lactobacillus* genus are used as probiotics for its beneficial role, it has been demonstrated that other *Lactobacillus* species are associated with different effects on weight change (*L. acidophilus*, *L. fermentum*, *L. ingluviei L* and *reuteri*) (Million *et al.,* 2012a;Million *et al.,* 2012b). Hence, the increase in this genus should be further investigated before making nutritional recommendations as several species belonging to this genus are actually used as probiotics.

The ingestion of a HFD was also associated with increased presence of *Oscillospira* genus, despite the fact that other studies have associated these bacteria with leanness and health in humans (Gophna *et al.,* 2017). However, the study performed by Krych *et al*., (2015) in non-obese diabetic mice suggested that this genus promotes the development of diabetes (Krych *et al.,* 2015). MVC supplementation reduced the abundance of this genus and this could underline the partial improvement observed in insulin resistance in these mice, although more studies are needed in this regard.

Coprobacillus is a genus that belongs to the Firmicutes phylum. It is known as a bacterial genus characteristic of mice, however, its role in gut is still being explored (Lagkouvardos *et al.,* 2016;Meehan *et al.,* 2015). Thus, the lower abundance observed after the ingestion of a HFD and the subsequent increase observed after MVC supplementation needs to be further investigated.

Concerning the effects of MVC that are in the same way than those observed with a HFD, the genus *Anaerotruncus* was increased by a HFD intake but also when MVC was administered in both mice fed a HFD or a control diet, as previously mentioned. Intriguingly, a previous work has observed that *Anaerotruncus* is a dominant genus in GM in control mice as

well as in healthy children and adolescents in comparison with obese subjects (Hou *et al.,* 2017). These apparent contradictory results compared to our results need to be clarified but what is clear is that *Anaerotruncus* is a target of MVC, independently of the metabolic status of the animals.

Xiao *et al*., (2017) observed a decrease in the relative abundance of *Bacteroides* and *Parabacteroides* genera and in the bacterial family Bacteroidaceae in response to high fat feeding, which contrasts with our findings. These differences can be attributed to the difference in the length of HFD feeding, since Xiao *et al*., (2017) performed the treatment for 6 weeks, while the present study lasts longer, concretely 16 weeks (Xiao *et al.,* 2017). Instead, and corroborating our results, other authors showed an increase in the abundance of *Bacteroides spp*. (an endotoxin producer) in mice with liver steatosis compared to control mice and an increase in the relative abundance of *Parabacteroides* in non-alcoholic steatohepatitis (NASH) patients compared to healthy subjects (Bashiardes *et al.,* 2016;Xie *et al.,* 2016). In the same line, Xie *et al*., (2016) showed a positive association between the *Bacteroides acidifaciens* with LPS plasma levels. Moreover, an association between increased *Bacteroides* concentrations and NASH development was also observed (Bashiardes *et al.,* 2016;Xie *et al.,* 2016). In addition, Lecomte *et al*., (2015) also observed an increased in *Bacteroides* and *Parabacteroides* genera in rats fed a HFD, which were positively associated with body weight gain (Lecomte *et al.,* 2015). The function of these bacteria in HFD mice could be linked to a higher metabolic activity or could indicate an induced state of inflammation. Our present study is in line with these former studies and showed a significant increase in the abundance of *Parabacteroides*, *Bacteroides* and *Bacteroides acidifaciens* in HFD-mice when compared to controls but also in MVC-supplemented mice when compared to the HFD group. Furthermore, a positive correlation of *Parabacteroides*, *Bacteroides*

and *Bacteroides acidifaciens* with body weight gain, liver and adipose tissue weight, hepatic triglycerides content, HOMA index, transaminases levels and the inflammatory marker IL-6 was observed. Therefore, these results should be deeply analyzed from a clinical point of view before MVC is prescribed.

Finally, MVC supplementation exerts its own actions on GM, independently of HFD-feeding actions. Thus, MVC along with a HFD was associated with an increase in Verrumicrobiaceae family and *Akkermansia* genus, and a decrease in Coriobacteriaceae family and *Adlercreutzia* genus.

Several studies have associated an increase in *Akkermansia* genus (belongs to Verrumicrobiaceae family), with a reduction in fat accumulation and an improvement in the metabolic profile of mice with diet-inducing obesity (Axling *et al.,* 2012;Shin *et al.,* 2014). Moreover, it is worth mentioning that *Akkermansia muciniphila*, which belongs the abovementioned genus, showed a beneficial role in several metabolic disorders, such as obesity, diabetes, cardiometabolic diseases and low-grade inflammation. In fact, this specie has been proposed as a potential candidate to improve these metabolic disorders (reviewed by Cani and de Vos (2017)) (Cani and de Vos 2017). Therefore, the increase observed of *Akkermansia* in HFD-fed mice treated with MVC seems to suggest a protective role of this drug, which is of great interest and deserves further investigation.

The reduction of the Coriobacteriaceae family is not clear. This taxon includes important constituents of the host physiology, but some members (such as *Enterorhabdus*, *Collinsella*, and *Eggerthella lenta*) have also been implicated in different pathologies, such as IBD or type-2 diabetes (Clavel *et al.,* 2014;Chen *et al.,* 2012). The abundance of the genus *Adlercreutzia,* which belongs to the abovementioned family, was reduced in those mice fed a HFD and supplemented with MVC. A recent study found an increase of this genus

in mice fed a HFD, and it was associated with an increase in body weight gain and increased intestinal permeability (Hamilton *et al.,* 2017). Thus, MVC could be exerting a beneficial role in body weight gain by decreasing the relative abundance of these taxa in mice fed a HFD, although its role needs further functional investigation.

In summary, this study has clearly demonstrated, for the first time, that MVC did not exert any *in vitro* bacteriostatic effect in the tested strains, suggesting that MVC is not able to interfiere with the microorganisms themselves. In addion, minimal significant effects of MVC were either found in gut microbiota composition when administered with a control diet, while several and interesting actions were observed when administered with a HFD. This could suggest that MVC maximal actions on gut microbiota are only observed when a challenge is carried out, such the induction of obesity and adiposity after a HFD. This opens the opportunities to investigate if its immunological actions could also be potentiated if administered along with a high fat diet. This, for sure, deserves further investigation.

7. Conclusions

Conclusiones

1. HIV infection decreases alpha-diversity and is closely associated with changes in lower taxonomic units rather than at the phylum levels when compared with uninfected people.

2. Combined antiretroviral therapy is associated with changes in bacterial translocation. In fact, HIV-infected patients under treatment showed higher plasma levels of the microbial translocation marker sCD14 and also increased levels of the cardiovascular risk markers ICAM and VCAM when compared with uninfected subjects.

3. Combined antiretroviral therapy is also associated with alterations in gut microbiota composition.

4. Patients on PI-based antiretroviral therapy showed the highest loss of alpha-diversity and increased dysbiosis.

5. Patients on INSTI-based antiretroviral therapy showed levels of systemic inflammation, sCD14 serum levels and alpha-diversity similar to uninfected controls.

6. INSTI-based therapy is accompanied by increased abundance of several bacterial orders, families, genera and species and, in contrast, a minor loss of bacterial species in comparison with the other combined antiretroviral therapies tested.

7. HIV/HCV coinfection results in a further increase of bacterial translocation, since higher levels of LBP is observed in HIV/HCV coinfected subjects compared to those non-coinfected.

8. Coinfection with hepatotropic viruses does not produce further changes in alpha-diversity, and only an increase in Actinobacteria phylum was observed when compared to non-coinfected patients.

9. At lower taxonomic levels, HIV/HCV-coinfected patients have a different gut microbiota profile compared to non-coinfected patients, showing a reduction of some butyrate-producing bacteria.

10. The presence of metabolic syndrome is not accompanied by an increase in bacterial translocation or changes in alpha-diversity and only minimal deviations with potentially clinical impact have been observed in gut microbiota.

11. The reduced abundance of *F. prausnitzii* in HIV-infected patients with metabolic syndrome suggests a greater inflammatory state at intestinal level which could underline the greater cardiovascular risk observed in these patients in comparison with those HIV-infected patients not suffering from metabolic syndrome.

12. Metabolic syndrome is associated with a reduction in the butyrate-producing bacteria *Eubacterium eligens*, *Roseburia intestinalis*, *Roseburia inulinivorans* and *Subdoligranulum* sp., which could constitute, along with *F. prausnitzii*, reliable markers of future cardiovascular events in HIV-infected patients.

13. Maraviroc did not exert any *in vitro* bacteriostatic effect in the tested strains.

14. Minimal effects of Maraviroc were found in gut microbiota composition when administered to mice fed a control diet, while several and interesting actions were observed when administered to high-fat diet-fed mice. Thus, Maraviroc maximal actions on gut microbiota are only observed when a challenge is carried out, such the induction of obesity and adiposity after a high-fat diet.

15. The effects of Maraviroc on gut microbiota composition of high-fat diet-fed mice are very diverse. It can restore the effects induced by a high-fat diet in some bacteria but, in contrast, Maraviroc also can potentiate the effects of the high-fat diet in the abundance of other bacteria. Maraviroc treatment is also associated with other effects on other bacterial taxa different from those observed with the high-fat diet.

16. All the results and conclusions arisen above should be taken into account in future investigations and when an antiretroviral treatment is being prescribed.

Conclusiones

1. La infección por el VIH reduce la riqueza de especies (alfa-diversidad) y está asociada con cambios en la abundancia de unidades taxonómicas inferiores a los filos bacterianos cuando se compara con sujetos no infectados.

2. La terapia antirretroviral está asociada con cambios en la translocación bacteriana. De hecho, los pacientes infectados por el VIH bajo tratamiento antirretroviral mostraron niveles incrementados del marcador de translocación bacteriana sCD14, y también de los marcadores de riesgo cardiovascular ICAM y VCAM al compararlos con los sujetos no infectados.

3. La terapia antirretroviral también se asocia con alteraciones en la composición de la microbiota intestinal.

4. Los pacientes bajo tratamiento basado en inhibidores de la proteasa mostraron una mayor reducción de la alfa-diversidad y una mayor disbiosis.

5. Los pacientes bajo tratamiento basado en inhibidores de la integrasa mostraron niveles de inflamación sistémica, niveles séricos de sCD14 y de alfa-diversidad similar a los sujetos no infectados.

6. La terapia basada en inhibidores de la integrasa se acompaña por un incremento en la abundancia de varios órdenes, familias, géneros y

especies bacterianas y, en cambio, una menor pérdida de especies bacterianas en comparación con las demás terapias testadas.

7. La coinfección por VIH/VHC se asocia con un mayor incremento de la translocación bacteriana, ya que los pacientes coinfectados por VIH/VHC presentaron niveles incrementados de LBP en comparación con aquellos no coinfectados.

8. La coinfección por virus hepatotropos no produce mayores cambios en la alfa-diversidad, y solamente se observó un incremento en la abundancia del filo Actinobacteria al compararse con los pacientes no coinfectados.

9. A niveles taxonómicos inferiores, los pacientes coinfectados mostraron un perfil de la microbiota intestinal diferente al de los pacientes VIH no coinfectados, mostrando además una reducción en la abundancia de algunas bacterias productoras de butirato.

10. La presencia de síndrome metabólico no se acompaña de un incremento en la translocación bacteriana o de grandes cambios en la alfa-diversidad al compararse con los pacientes VIH sin síndrome metabólico. Caben destacar algunos cambios observados en la composición de la microbiota intestinal con potencial impacto clínico.

11. La menor abundancia de *F. prausnitzii* en pacientes infectados por el VIH con síndrome metabólico podría sugerir un mayor estado inflamatorio a nivel intestinal. Este hecho podría justificar el mayor

riesgo cardiovascular observado en estos pacientes en comparación con aquellos sin síndrome metabólico.

12. El síndrome metabólico se asocia con una reducción de las bacterias productoras de butirato *Eubacterium eligens*, *Roseburia intestinalis*, *Roseburia inulinivorans* y *Subdoligranulum spp.*, especies bacterianas que, junto con *F. prausnitzii*, podrían convertirse en marcadores de riesgo de eventos cardiovasculares en los pacientes infectados por el VIH.

13. Maraviroc no ejerce ningún efecto bacteriostático *in vitro* en las cepas bacterianas testadas.

14. La administración de Maraviroc se asocia con cambios mínimos en la composición de la microbiota intestinal cuando se administra a ratones alimentados con una dieta estándar, mientras que al administrarse junto con una dieta alta en grasa se pudieron apreciar modificaciones importantes en la composición de la microbiota intestinal. Por tanto, los efectos de Maraviroc sobre la microbiota intestinal se observan únicamente cuando los animales son sometidos a un "*challenge*", en este caso, la inducción de la obesidad y adiposidad tras la ingesta de una dieta alta en grasa.

15. Los efectos de Maraviroc sobre la composición de la microbiota intestinal de ratones alimentados con una dieta alta en grasa es muy diversa. Este fármaco puede restaurar los efectos inducidos por la dieta alta en grasa en la abundancia de algunas bacterias, aunque este fármaco también puede potenciar los efectos de la dieta alta en

grasa en la abundancia de otras bacterias. Finalmente, el tratamiento con Maraviroc también se asocia con efectos sobre taxones bacterianos diferentes de aquellos alterados por la ingesta de una dieta alta en grasa.

16. Todos los resultados y conclusiones presentados anteriormente deben tenerse en cuenta en futuras investigaciones y a la hora de plantear un plan de tratamiento antirretroviral personalizado.

8. Publications and Conferences

SCIENTIFIC ARTICLES PUBLISHED AND CONFERENCES IN WHICH THE PhD STUDENT HAVE PARTICIPATED DURING THIS DOCTORAL THESIS

Published articles regarding the present Doctoral Thesis

- Villanueva-Millán MJ, Pérez-Matute P, Recio-Fernández E, Lezana Rosales JM, Oteo JA. Differential effects of antiretrovirals on microbial translocation and gut microbiota composition of HIV-infected patients. J Int AIDS Soc 2017; 20:21526.
- Villanueva-Millán MJ, Pérez-Matute P, Oteo JA. Gut microbiota: a key player in health and disease. A review focused on obesity. J Physiol Biochem. 2015; 71(3):509-25.

Published articles carried out in collaboration

- Martínez-Herrero S, Larrayoz IM, Narro-Íñiguez J, Villanueva-Millán MJ, Recio-Fernández E, Pérez-Matute P, Oteo JA, Martínez A. Lack of Adrenomedullin Results in Microbiota Changes and Aggravates Azoxymethane and Dextran Sulfate Sodium-Induced Colitis in Mice. Front Physiol. 2016; 7:595.

- Hijona E, Aguirre L, Pérez-Matute P, Villanueva-Millán MJ, Mosqueda-Solis A, Hasnaoui M, Nepveu F, Senard JM, Bujanda L, Aldámiz-Echevarría L, Llarena M, Andrade F, Perio P, Leboulanger F, Hijona L, Arbones-Mainar JM, Portillo MP, Carpéné C. Limited beneficial effects of piceatannol supplementation on obesity complications in the obese Zucker rat: gut microbiota, metabolic, endocrine, and cardiac aspects. J Physiol Biochem. 2016; 72(3):567-82.

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Book chapters:

- Pérez-Matute P, Villanueva-Millán MJ, Oteo JA. Cambios en el Microbioma en VIH/SIDA. In: Microbioma, Disbiosis, Probióticos y Bacterioterapia. Joel Faintuch. (In press).

- Pérez-Matute P, Íñiguez M, Villanueva-Millán MJ, Oteo JA. The oral, digestive and vaginal microbiome in HIV infection. Elsevier. Submitted.

Conferences

- Pérez-Matute P, Morano LE, Íñiguez M, Recio-Fernández E, Villanueva-Millán MJ, Suarez-Santamaría M, Prado-González E, Oteo JA. *¿Qué sucede en la microbiota intestinal tras la eliminación del VHC?*. Oral communication. SEIMC. Bilbao, Spain. May 2018.

- Villanueva-Millán MJ, Pérez-Matute P, Recio-Fernández E, Lezana Rosales JM, Oteo JA. *Caracterización de la composición de la microbiota intestinal y del estado inflamatorio en pacientes infectados por el VIH con síndrome metabólico*. Poster. SEIMC. Bilbao, Spain. May 2018.

- Martínez-Herrero S, Larráyoz IM, Narro-Íñiguez J, Villanueva-Millán MJ, Recio-Fernández E, Pérez-Matute P, Rubio-Mediavilla S, Oteo JA, Martínez A. Lack of adrenomedullin aggravates colitis symptoms in mice through microbiota changes and altered expression of toll-like receptor 4. Poster. SEHIT. Santiago de Compostela, Spain. September 2017.

- Villanueva-Millán MJ, Pérez-Matute P, Lezana Rosales JM, Oteo JA. *Maraviroc modifica la composición de la microbiota intestinal en un modelo murino de obesidad*. Poster. SEIMC. Málaga, Spain. May 2017.

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- Pérez-Matute P, Villanueva-Millán MJ, Recio-Fernández E, Ibarra V, Oteo JA. Microbiota y Tratamiento Antirretroviral en el paciente VIH. *¿Son fiables los marcadores clásicos de translocación bacteriana en una población VIH con y sin síndrome metabólico?* En busca del marcador definitivo. Oral communication. SEIMC. Sevilla, Spain. May 2015.

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