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Differences of Biochemical Endotoxin-Related Markers and Faecal Microbiota in Patients with HIV and HCV Infections

Summary of the Doctoral Thesis for obtaining the scientific degree "Doctor of Science (*PhD*)"

Sector Group – Medical and Health Sciences
Sector – Health Sciences
Sub-Sector – Infectious Diseases

Riga, 2023

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The Doctoral Thesis was developed at the Department of Infectology, Rīga Stradiņš University, Latvia, in collaboration with Riga East University Hospital "Gaiļezers"

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The Doctoral Thesis is available in RSU Library and on RSU website: https://www.rsu.lv/en/dissertations

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Abbreviations used in the Thesis

AIDS	Acquired Immune Deficiency syndrome
ALT	Alanine aminotransferase
ART	Antiretroviral therapy
AST	Aspartate aminotransferase
BMI	Body mass index
$CD4^+$	T helper cell
$CD8^+$	Cytotoxic T lymphocyte
$CD38^+$	Activated T lymphocytes
CK18-M30	Cytokeratin 18 neoepitope
DAA	Direct acting antivirals
DNA	Deoxyribonucleic acid
16S rRNA	16S ribosomal ribonucleic acid
ELISA	Enzyme-linked Immunosorbent assay
EndoCAb	Endotoxin core antibodies
FIB-4	Liver fibrosis index
g	Acceleration constant
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCV RNA	Hepatitis C virus Ribonucleic acid
HIV	Human Immunodeficiency virus
HIV RNA	Human Immunodeficiency virus ribonucleic acid
HLA	Human Leukocyte Antigen
IFN-γ	Interferon- γ
IL	Interleukin
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide

mL	Millilitres
MSM	Men who have sex with men
ng	Nanograms
pg	Picograms
PCoA	Principal Coordinate analysis
PCR	Polymerase chain reaction
rpm	Revolutions per minute
sCD14	Soluble CD14 (cluster of differentiation)
TNF	Tumour necrosis factor
TLR	Toll like receptor
U	Units
USA	United States of America
WHO	World Health Organisation
μg	Micrograms
μL	Microlitres

Introduction

Infections caused by human immunodeficiency virus (HIV) and hepatitis C virus (HCV) remain a global and growing healthcare problem, despite the well-known modes of transmission of these viruses and the discovery of new antiviral drugs. They are chronic diseases with a high prevalence and significant impact on human health and quality of life. The World Health Organisation (WHO) estimates that more than 84 million people have been infected with HIV since the beginning of the HIV pandemic. In 2021, 38.4 (33.9–43.8) million people worldwide were infected with HIV and 650 000 died, while HCV-infected approximately 58 million people worldwide and at least 290 000–400 000 people died of complications from HCV infection in 2019. In addition, 1.5 million new cases of both HIV and HCV are detected each year (UNAIDS, 2022; WHO, 2022).

As both infections share similar transmission routes, approximately 25 % of HIV-infected patients are co-infected with HCV (WHO, 2022), which has led to a worldwide focus on the interaction between the two viruses, HIV and HCV. People with HIV/HCV coinfection have a higher risk of cirrhosis and liver complications than people without HIV infection (Lo Re et al., 2014). Liver disease is known to progress more rapidly in HIV-infected patients, cirrhosis develops more rapidly, and liver disease is one of the most common causes of non-AIDS death in HIV-infected patients (Morlat et al., 2014; Abutaleb & Sherman, 2018). The risk of liver-related complications in patients with chronic HCV infection is associated with several factors, including older age at the time of infection, high alcohol consumption, obesity, changes in gut microbiota and human immunodeficiency virus coinfection (Tripathi et al., 2018).

Chronic immune activation is a hallmark of progressive HIV infection, with polyclonal B-cell activation, accelerated T-lymphocyte turnover, and elevated serum levels of inflammatory cytokines and chemokines (Brenchley

et al., 2006; Hunt et al., 2014; Merlini et al., 2021). A decade ago, microbial translocation was described as a possible cause of immune activation in HIV patients (Sandler & Douek, 2012). Currently, the progression of HIV infection is associated with ongoing immune activation, largely due to microbial translocation from the gastrointestinal tract, thereby allowing bacterial products to enter the general blood circulation without clinically and laboratory detectable bacteraemia (Epeldegui et al., 2018). Microbial translocation has been shown to decrease in patients receiving antiretroviral therapy but remains at higher levels compared to HIV-uninfected individuals (Marchetti, Tincati & Silvestri, 2013; Merlini et al., 2021).

Approximately 60–80 % of $CD4^+$ T cells in humans are located in gut-associated lymphoid tissues. The population of these cells and the composition of the gut microbiota are severely affected in HIV infection, but recovery is incomplete even with antiretroviral therapy (Zilberman-Shapira et al., 2016; Mingjun et al., 2022). HIV-infected patients have a lower total amount of bacterial genes in the intestinal tract compared to healthy individuals, as well as lower bacterial diversity (Guillen et al., 2018; Ellis et al., 2021; Mingjun et al., 2022). These changes correlate with various factors, including $CD4^+$ T cell count, comorbidities and HIV treatment (Guillen et al., 2018). In HIV-infected patients, changes in the composition of the gut microbiota at the level of individual bacterial species are also observed, particularly as the disease progresses: the number of pathogenic bacteria increases, while the number of beneficial bacteria in the intestinal tract decreases (Kelley et al., 2017; Neff et al., 2018).

Changes in the composition and function of the gut microbiota of HIV-infected individuals not only indirectly influence the course of HIV infection, but may also directly contribute to various disease-related manifestations. One possible consequence of altered microbiota is the facilitation of impaired gut mucosal barrier function, resulting in increased microbial translocation into the bloodstream, thereby re-stimulating and sustaining immune activation that

continues to maintain a chronic inflammatory state (Zilberman-Schapira et al., 2016; Neff et al., 2018; Mingjun et al., 2022).

The impact of a systemic inflammatory response induced by microbial translocation has also been studied in HCV-infected patients (Sandler et al., 2011; Sacchi et al., 2015), indicating increased markers of microbial translocation and subsequent T cell activation (Sacchi et al., 2015; Lattanzi et al., 2018).

In liver diseases, changes in the diversity and composition of the gut microbiota are also observed, which are in turn maintained by a continuous interaction between the gut-liver axis (Tripathi et al., 2018; Albillos, de Gottardi & Rescigno, 2020). In this context, increased intestinal permeability and dysbiosis, which trigger the translocation of bacterial components, could also be associated with immune activation and may increase liver damage (Marchetti et al., 2012). Thus, gut dysbiosis is not only associated with the progression liver disease, but also with the complications of chronic liver disease (Tripathi et al., 2018). Alterations in the composition of the gut microbiota have also been described among HCV-infected individuals, which vary according to disease severity (Inoue et al., 2018; Heidrich et al., 2018).

In recent years, research has focused on the composition of the gut microbiota and its changes, its impact on microbial translocation and, consequently, on the activation of the immune system.

The role of markers of microbial translocation and various host responses are being investigated in predicting the outcome of HIV infection, with the aim of being able to intervene at specific stages of pathogenesis and stop viral spread in the future. However, it is still not fully understood what stimulates infections to progress more rapidly, and whether and what role the gut microbiota plays in maintaining chronic inflammation, especially in HIV/HCV-coinfected patients. Thus, there is a need to further understand the interplay between the gut

microbiota and microbial translocation and their impact on the course of HIV and HCV infections.

Aim of the study

To identify and analyse differences in endotoxin-related biochemical markers of microbial translocation and faecal microbiota in HIV and HCV-monoinfected and HIV/HCV-coinfected patients, and to assess the correlation between these parameters in the study groups.

Objectives

1) To determine the markers of microbial translocation in the plasma of patients and to evaluate the differences in these parameters between patient groups.
2) To determine markers of liver fibrosis in the plasma of patients and to assess differences in these parameters between patient groups.
3) To analyse differences in faecal microbiota between patient groups by assessing diversity and total bacterial DNA in faeces.
4) To determine the differences in the representation of bacterial species in the faecal microbiota between patient groups.
5) To detect the correlation of the faecal microbiota with markers of microbial translocation and liver fibrosis.

Hypothesis of the study

Biochemical markers of microbial translocation and liver fibrosis are higher in HIV and HCV-coinfected patients compared to HIV and HCV-monoinfected patients and they correlate with variables of faecal microbiota.

Faecal microbiota differs in patients with HIV and HCV monoinfection and patients coinfected with HIV/HCV.

Novelty of the study

The faecal microbiota of HIV-infected patients was first characterised worldwide in 2006, and in the following years, the overall understanding of the role of the microbiota in different diseases has gradually increased as research methodologies have evolved. Alterations in the faecal microbiota have also been described in liver diseases, including viral hepatitis C. However, there are still only a few studies on the characteristics of the faecal microbiota in HIV/HCV-coinfected patients.

Faecal microbiota research is generally new in Latvia, and there are very limited data on microbiota differences in specific patient groups.

To our knowledge, this is the first study in Latvia that compares the faecal microbiota in HIV- and HCV-monoinfected and HIV/HCV-coinfected patients, also in the context of microbial translocation parameters.

Ethical aspects

The study was conducted in accordance with the ethical aspects of the Declaration of Helsinki, and the study was approved by the Riga East University Hospital Support Foundation Medical and Biomedical Research Ethics Committee Opinion No.6-A/16 (05.05.2016.), and by the Riga East University Hospital Science Department Certificate No. AP-ZDA-16/88 (08.06.2016.).

Structure and volume of the Doctoral Thesis

The Doctoral Thesis is written in Latvian. It is presented in 5 chapters: Literature Review, Materials and Methods, Results, Discussion and Conclusions. The Doctoral Thesis comprises 129 pages, including 12 tables and 21 figures. The list of references contains of 235 sources.

1 Materials and methods

1.1 Study design

The cross-sectional study was conducted at Riga East University Hospital "Gaiļezers" from June 2016 to September 2021. The study was approved by the Ethical Committee for Medical and Biomedical Research of the Support Foundation of Riga East University Hospital (No 6-A/16). All patients included in the study were informed about the study and signed a voluntary consent to participate in it.

Inclusion criteria were: age over 18 and under 65 years, confirmed HIV infection with or without HCV coinfection, confirmed chronic HCV infection, no antimicrobial therapy for more than five days in the last three months.

Exclusion criteria: Previous use of specific antiviral therapy for HIV and HCV infection, HBV infection, HIV associated bacterial opportunistic infections, pregnancy, oncological pathologies, diabetes mellitus, chronic heart failure, coronary heart disease, inflammatory bowel disease, organ transplant patients, known chronic non-HCV liver pathology, patients receiving long-term glucocorticosteroids or immunosuppressive drugs, chronic alcoholism, vegetarianism.

The diagnosis of HIV was confirmed by the detection of HIV-specific antibodies in the patients' blood by a 4th generation enzyme-linked immunosorbent assay (ELISA) and confirmatory testing by the detection of HIV ½-specific antibodies (Western blot and Immunoblot methods) and quantitative detection of HIV load (HIV RNA) by polymerase chain reaction. HIV immune status was determined on the basis of $CD4^+$ T cell count.

HCV status was determined by positive anti HCV antibodies (enzyme-linked immunosorbent assay, ELISA) and confirmed by qualitative detection of HCV virus (HCV RNA) by real-time polymerase chain reaction or by detection of HCV Core Ag in plasma (enzyme-linked immunosorbent assay, ELISA).

The following parameters were determined at study entry:

1) HIV load – HIV RNA;
2) Immunological parameters – $CD4^+$ T lymphocyte count, $CD8^+$ T lymphocyte count, CD4/CD8 index. Parameters 1) and 2) were determined only in patients with a positive HIV test;
3) Haematological parameters (leukocyte, erythrocyte and platelet counts, haemoglobin);
4) Blood biochemical parameters, liver enzymes – alanine aminotransferase (ALT), aspartate aminotransferase (AST);
5) Indirect markers of microbial translocation – lipopolysaccharide binding protein (LBP), soluble CD14 receptor (sCD14), antibodies against endotoxin core – IgM and IgG classes;
6) Hepatocyte apoptosis marker – cytokeratin 18 neoepitope M30 (CK18-M30);
7) Analysis of faecal microbiota;
8) Body mass index (BMI).

1.2 Collection and storage of study samples

Collection and storage of blood samples. Blood samples for routine clinical and biochemical (haematological parameters, ALT, AST) laboratory tests were obtained from venous blood. The tests were performed immediately after collection and delivery to the laboratory.

Blood samples for bacterial translocation markers (LBP, sCD14, EndoCAb IgM, IgG) were centrifuged (1500 rpm, 20 min) at room temperature to separate plasma and serum and then frozen at −80 °C upon arrival at the laboratory. Parameters related to microbial translocation were tested in unfrozen plasma or serum samples according to the manufacturers' recommendations.

Collection and storage of faecal samples. In hospitalised patients, faecal samples were obtained using sterile collection cups and after collection were immediately taken to the laboratory where they were frozen at −80 °C until further analysis.

In outpatients, faeces were self-collected using sterile collection cups. Faeces were delivered to the laboratory within one hour, where they were immediately frozen at −80° C and stored for later use.

1.3 Detection of biochemical microbial translocation and apoptosis markers

Detection of lipopolysaccharide binding protein. Lipopolysaccharide binding protein (LBP) levels were determined using the Human LBP (Lipopolysaccharide binding protein) ELISA (*Hycult Biotech*, The Netherlands) reagent kit for the quantification of human LBP in serum. Blood serum obtained by centrifugation of venous blood (1500 rpm) for 20 min at room temperature was used for the analysis. All blood serum samples were frozen at −80 °C until assay. LBP detection range: 4.4–50 ng/mL.

Detection of endotoxin core antibodies (EndoCAb). IgM and IgG levels of endotoxin core antibodies (EndoCAb) were determined in patient sera by enzyme-linked immunosorbent assay (ELISA) (*Hycult*, The Netherlands). Blood serum obtained by centrifugation of venous blood (1500 rpm) for 20 min at room temperature was used for the analysis. All blood serum samples were frozen at −80 °C until assay.

Detection of sCD14. sCD14 levels in blood serum were determined by a solid-phase enzyme-linked immunosorbent assay (*Quantikine ELISA Human CD14 Immunoassay*, *R7D Systems*, USA). Blood serum obtained by centrifugation of venous blood (1500 × g) for 20 min at room temperature was used for the analysis. All blood serum samples were frozen at −80 °C until assay.

Detection of CK18-M30. CK18-M30 is produced by apoptosis of epithelial tissue from epithelial interfilament CK18 via activation of caspase 3, 7, or 9. CK18-M30 was quantified in patient serum by a solid-phase enzyme-linked immunosorbent assay (*M30 Apoptosense ELISA*, *PEVIVA*, Sweden). Patient blood serum was obtained by centrifugation of venous blood (1500 rpm) for 20 minutes at room temperature. All blood serum samples were frozen at −80 °C until assay.

1.4 Stool sample analysis

Extraction of the bacterial fraction from faecal samples. Total faecal microbiota DNA was extracted using the *FastDNA Spin Kit for Soil* and *FastPrep-2* (*MP Biomedicals*, USA) according to the manufacturer's protocol.

Additionally, for quality control purposes, DNA was also isolated from the elution buffer and a bacterial mixture of known content, *ZymoBIOMICS Microbial Community Standard D6300* (*ZymoBIOMICS*, USA), which were used as negative and positive controls, respectively.

Preparation and sequencing of 16S rRNA gene amplicons. Sequencing of hypervariable regions of the 16S ribosomal RNA (rRNA) gene was used for microbiota analysis or bacterial taxonomic composition. The data from the V4 region were used for further analysis in the Thesis as they contained the most information on the taxonomic composition of the samples included in the study.

A degenerate primer pair 515F-806R was used to obtain amplicons of the V4 hypervariable region of the 16S rRNA gene (Table 1.1).

Table 1.1

Primer sequences for amplicons of the 16S rRNA gene V4 hypervariable region

Primers	Sequence 5'-3'	Length	Reference
515F	GTGYCAGCMGCCGCGGTAA	19	Parada et al., 2015
806R	GGACTACNVGGGTWTCTAAT	20	Apprill et al., 2015

A two-step amplification method was used to obtain a set of sequencing libraries, or indexed amplicons to be sequenced.

The quality of the DNA fragments obtained from the polymerase chain reaction (PCR) was imaged on a 2 % agarose gel (65 mA, 100 V for 1 hour) with *SYBR Gold* nucleic acid stain (*ThermoFisher*, USA). Successful PCR products were purified and normalised using a combined purification-normalisation plate *SequalPrep* normalisation plate kit (*ThermoFisher*, USA) following the manufacturer's protocol.

The purified first PCR fragments were used in a second step PCR or sequencing adapter and index addition reaction using *Illumina Nextera XT2* combinatorial index sequences (*Illumina*, USA).

In the second PCR step, the obtained indexed amplicons were purified using purification-normalisation plates and 5 µl from each well with different index features were pooled into one well. The average fragment length of the pooled sequencing libraries was determined using the *QIAxcel DNA High Resolution kit* and the *QIAxcel Advanced Capillary Electrophoresis System* (*Qiagen*, Germany) and quantified using the *KAPA Library quantification kit* (*Roche*, Switzerland) real-time PCR sequencing reagents.

The final pooled library was sequenced with paired-end reads (2 × 300 cycles) on an *Illumina MiSeq* using the v3 600 cycle reagent kit (*Illumina*, USA).

Sequence analysis. Sequence analysis was performed at the Sequencing Department of the Laboratory Service of Riga East University Hospital.

The raw data from the sequencing were demultiplexed and purified from indices and adaptors with *cutadapt* and *fastp*. The data were processed for microbiota analysis using *R software* (version 4.2.1). Pre-processing of reads was performed with the *DADA2 package* (version 1.16) (Callahan et al., 2016). Pairs of merged reads were purified from chimeric sequences, yielding sequence amplicon variants that were assigned a taxonomic affiliation using the *Decipher* package (version 2.20.0) (Murali, Bhargava & Wright, 2018) with the *SILVA 138 SSU* database (version 2019) with standard parameters (60 % confidence level). All reads found in the negative controls were removed from the amplicon sequence variants with recognised taxonomic affiliation using the *decontam* (version 1.12.0) package.

1.5 Statistical methods

Statistical data processing was performed using *Statistical Package for Social Sciences IBM (SPSS)* version 23 (*IBM Corporations*, USA). The first step of the analysis aimed to compare the differences in the indicators between the study groups. As the data obtained did not follow a normal distribution, the non-parametric Kruskal-Wallis test was applied to compare the three groups. The Mann-Whitney test was applied to the two-group comparison. In the second step of the analysis, the correlations between the traits were investigated. Spearman's rank correlation coefficient (r_s) was used to analyse the relationships. The closeness of the correlation was interpreted according to the magnitude of the correlation coefficient: very weak correlation if $r_s < 0.19$; weak correlation if $r_s = 0.2–0.39$; moderate correlation if $r_s = 0.4–0.59$; strong correlation if $r_s = 0.6–0.79$ and very strong correlation if $r_s > 0.8$. Results were considered statistically significant if the p value was less than 0.05. Partial correlation was used to exclude group effects from the estimation of correlations between traits. Partial

correlation calculations were performed in the *JASP free software* (*JASP 0.16.3 for Windows*).

Processing of faecal microbiome data. Statistical analysis of the microbiome was performed with *phyloseq* (version 1.40.0), *vegan* (version 2.6-2), *microbiome* (version 1.18.0), *DESeq2* (version 1.36.0) packages and results were visualised with *ggplot2* (version 3.3.6). Internal or α diversity of single sample and diversity between two samples or ß diversity were determined using *phyloseq* and *vegan*. Permutation multivariate ANOVA or PERMANOVA analysis was performed using the *adonis* function from the *vegan* package.

2 Results

2.1 Patient characteristics

The study included 81 patients, 47 men (58 %) and 34 women (42 %), aged from 18 to 65 years (median age 44 years, IQR [37; 50]), who were divided into three groups according to their diagnoses: HIV monoinfection group, HIV/HCV coinfection group and HCV monoinfection group.

The HIV monoinfection group comprised 28 patients: 18 men (64 %) and 10 women (36 %, median age 42.5 years, IQR [36.0; 55.8]). The HIV/HCV coinfection group consisted of 29 patients: 21 men (72 %) and 8 women (28 %), median age 44 years, IQR [39.5; 49.0]. The HCV monoinfection group consisted of 24 patients: 8 men (33 %) and 16 women (67 %), median age 44.5 years, IQR [36.0; 51.5]. No differences in age were found between the groups.

To characterise the HIV and HIV/HCV patient groups, possible differences in immunological parameters ($CD4^+$, $CD8^+$ T lymphocyte count and CD4/CD8 index) and HIV load (HIV RNA) were assessed. Differences between groups were assessed using the Mann-Whitney test (Table 2.1).

Table 2.1

Differences between HIV-monoinfected and HIV/HCV-coinfected patient groups in HIV RNA, $CD4^+$ and $CD8^+$ T lymphocyte counts

Parameters	Groups		Mann-Whitney U test
	HIV (n = 28)	HIV/HCV (n = 29)	
HIV RNA (copies/mL), median (IQR)	6.0×10^5 (2.7×10^4; 2.3×10^6)	1.1×10^5 (4.3×10^3; 5.9×10^5)	269.0*
< 50 copies/mL, number of patients, %	0 %	0 %	–
$CD4^+$ T lymphocytes (cells/mm^3), median (IQR)	78 (33; 214)	131 (58; 270)	327.0
< 200 cells/mm^3	75 %	66 %	–

Table 2.1 continued

Parameters	Groups		Mann-Whitney U test
	HIV (n = 28)	HIV/HCV (n = 29)	
$CD8^+$ T lymphocytes ($cells/mm^3$), median (IQR)	546 (256; 915)	693 (452; 1009)	314.0
CD4/CD8 index	0.19 (0.07; 0.37)	0.18 (0.09; 0.31)	383.0

HIV RNA – HIV load, CD4/CD8 – $CD4^+$ T lymphocyte and $CD8^+$ T lymphocyte ratio.
* – $p < 0.05$.

Patients with HIV monoinfection and HIV/HCV coinfection did not differ in $CD4^+$ and $CD8^+$ T lymphocyte counts or CD4/CD8 index. Of note, $CD4^+$ T lymphocyte counts < 200 cells/μL were found in 75 % of patients in the HIV-monoinfected group and 66 % of patients in the HIV/HCV-coinfected group. However, HIV RNA was higher in patients with HIV monoinfection ($6.0 \cdot 10^5$ copies/mL, IQR [$2.7 \cdot 10^4$; $2.3 \cdot 10^6$]) than in patients with HIV/HCV coinfection ($1.1 \cdot 10^5$ copies/mL, IQR [$4.3 \cdot 10^3$; $5.9 \cdot 10^5$]), Mann-Whitney U (55) = 269.0, $p < 0.029$. No patient in either group was found to have HIV RNA < 50 copies/mL, as all patients in the HIV and HIV/HCV groups were enrolled in the study immediately after diagnosis until ART initiation. Similarly, patients with HCV were also enrolled in the study immediately after diagnosis, so they had not yet received HCV-specific DAA therapy at the time of all assessments. The immunological status of HCV patients was not determined.

As the values did not follow a normal distribution, the Kruskal-Wallis test was used to detect differences in the other parameters analysed. Differences between groups are discussed in more detail below, grouping the parameters into routine clinical parameters (leukocyte, erythrocyte, platelet counts, ALT, AST, BMI), biochemical markers of microbial translocation (LBP, sCD14, IgM and IgG antibodies to endotoxin core), liver apoptosis (CK18-M30) and fibrosis markers (FIB-4), and microbiota parameters (total abundance, Shannon index, inverse Simpson index).

Differences between groups were found in BMI index, erythrocyte count, haemoglobin, ALT and AST, but in contrast, there were no differences between groups in leukocyte and platelet counts.

Body mass index was higher in HCV-monoinfected patients (24.7 kg/m^2, IQR [22.6; 25.6]) than in HIV-monoinfected patients (21.3 kg/m^2, IQR [19.7; 23.8]) and in HIV/HCV-coinfected patients (21.0 kg/m^2, IQR [19.1; 23.4]), $p < 0.01$. In contrast, no differences in BMI were observed between HIV-monoinfected and HIV/HCV-coinfected patients.

Patients with HCV monoinfection had higher erythrocyte counts and haemoglobin levels than patients with HIV monoinfection and HIV/HCV coinfection. Patients with HCV infection also had higher ALT than patients with HIV mono and HIV/HCV coinfection. AST was higher in patients with HCV monoinfection (53 U/L, IQR [33; 75]) than in patients with HIV monoinfection (24 U/L, IQR [15; 43]), but AST did not differ between HCV-monoinfected and HIV/HCV-coinfected patients. Both ALT (28 U/L, IQR [18; 59]) and AST (40 U/L, IQR [29; 53]) values did not differ between HIV/HCV-coinfected and HIV-monoinfected patient groups.

2.2 Microbial translocation, apoptosis and fibrosis markers

When analysing the markers of microbial translocation, a statistically significant difference was found only for the LBP level (Table 2.2).

Table 2.2

Microbial translocation markers differences in patients with HIV monoinfection, HIV/HCV coinfection and HCV monoinfection

Parameters	Groups			Kruskal-Wallis test
	HIV (n = 28)	HIV/HCV (n = 29)	HCV (n = 24)	
	Median (IQR)	Median (IQR)	Median (IQR)	H
LBP, µg/mL	21.6[a] (17.4; 35.2)	23.5[a] (17.9; 39.0)	16.0[b] (14.7; 22.1)	12.12**
sCD14, ng/mL	7253 (5258; 13912)	6483 (5224; 7713)	5216 (4289; 7720)	5.21
IgM EndoCAb, U/mL	0.33 (0.20; 0.58)	0.40 (0.19; 0.68)	0.52 (0.28; 0.76)	3.72
IgG EndoCAb, U/mL	175.6 (100.1; 295.7)	174.8 (107.2; 263.0)	210.8 (124.4; 331.4)	1.50

LBP – lipopolysaccharide binding protein, sCD14 – soluble CD14 receptor, IgM EndoCAb – endotoxin core IgM antibodies, IgG EndoCAb – endotoxin core IgG antibodies.
[a, b] – indexes, showing differences between groups; ** – $p < 0.01$.

Patients with HIV monoinfection and HIV/HCV coinfection had higher LBP than HCV-monoinfected patients, but levels did not differ between HIV-monoinfected and HIV/HCV-coinfected groups (Figure 2.1).

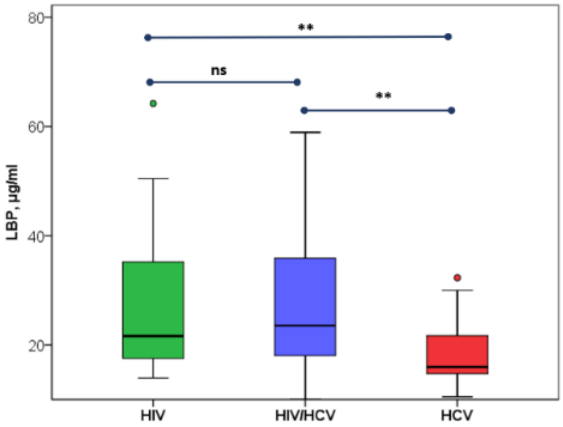

Figure 2.1 **Differences in LBP levels between groups**

**p < 0.01, ns – no significant difference.

However, no differences were found between the groups for sCD14 and IgM and IgG EndoCAb antibodies. Some parameters, such as IgM EndoCAb and IgG EndoCAb, showed a similar trend of differences, i.e. they were higher in patients with HCV than in patients with HIV monoinfection and HIV/HCV coinfection, but the differences did not reach statistical significance. sCD14 was higher in HIV-monoinfected patients than in HCV-monoinfected patients, but again this difference did not reach statistical significance.

When liver apoptosis and fibrosis parameters were assessed, differences between groups were found in the hepatocyte apoptosis marker (CK18-M30).

CK18-M30 was higher in patients with HCV monoinfection (196 U/L, IQR [110; 380]) and HIV/HCV coinfection (138 U/L, IQR [95; 300]) than in patients with HIV (95 U/L, IQR [69; 170]), whereas no differences in CK18-M30 were observed between HCV-monoinfected and HIV/HCV-coinfected patients.

Using a CK18-M30 cut-off level of ≥ 200 U/L (as recommended by the manufacturer), increased hepatocyte apoptosis was observed in 18 % of patients with HIV monoinfection, 35 % of patients with HIV/HCV coinfection and 50 % of patients with HCV infection.

There was no difference in the FIB-4 index between the groups, but HIV/HCV-coinfected patients had a higher median FIB-4 index than HIV and HCV-monoinfected patients.

Using FIB-4 cut-off levels (Sterling et al., 2006), potential liver fibrosis was assessed in patient groups. Marked liver fibrosis (FIB-4 ≥ 3.25) was likely in 4 % of patients with HIV monoinfection, 21 % of patients with HIV/HCV coinfection and 21 % of patients with HCV. Correspondingly, significant liver fibrosis (FIB-4 = 1.46–3.24) could be present in 39 % of patients with HIV, 44 % of patients with HIV/HCV and 21 % of patients with HCV coinfection. FIB- 4 ≤ 1.45 was more likely to exclude liver fibrosis in 57 % of patients with

HIV monoinfection, 35 % with HIV/HCV coinfection and 58 % with HCV monoinfection.

2.3 Correlation analysis of parameters

To initially assess the overall trends in parameter correlations, Spearman's correlation coefficient was determined in the entire cohort of patients. The results of the correlation analysis are discussed in separate subsections.

Age showed a positive association with liver fibrosis index FIB-4 ($r_s = 0.25$, $p < 0.05$) and a negative association with IgM EndoCAb ($r_s = -0.27$, $p < 0.05$). Body mass index correlated positively with $CD8^+$ T lymphocyte count ($r_s = 0.43$, $p < 0.001$), erythrocyte count ($r_s = 0.23$, $p < 0.05$), haemoglobin ($r_s = 0.39$, $p < 0.001$) and Shannon index ($r_s = 0.43$, $p < 0.001$).

HIV viral load showed only negative correlations: with $CD4^+$ T lymphocyte count ($r_s = -0.56$, $p < 0.001$), $CD8^+$ T lymphocyte count ($r_s = -0.37$, $p < 0.01$), leukocyte count ($r_s = -0.34$, $p < 0.01$) and haemoglobin ($r_s = -0.42$, $p < 0.01$). $CD4^+$ T lymphocyte count was positively correlated with $CD8^+$ T lymphocyte count ($r_s = 0.62$, $p < 0.001$), leukocyte count ($r_s = 0.58$, $p < 0.001$) and haemoglobin ($r_s = 0.28$, $p < 0.05$) but negatively correlated with IgG antibodies to endotoxin core antigen ($r_s = -0.34$, $p < 0.05$). Similar to $CD4^+$ T lymphocyte count, $CD8^+$ T lymphocyte count correlated positively with leukocyte counts ($r_s = 0.41$, $p < 0.05$) and haemoglobin ($r_s = 0.41$, $p < 0.05$), but negatively with IgG antibodies to endotoxin core antigen ($r_s = -0.26$, $p < 0.05$).

Leukocytes and erythrocytes showed similar correlations with the other parameters in the study, as they were correlated with each other ($r_s = 0.36$, $p < 0.001$). In addition to the above correlations, leukocyte count and erythrocyte count correlated positively with haemoglobin ($r_s = 0.46$, $p < 0.001$ and $r_s = 0.76$, $p < 0.001$, respectively), with ALT ($r_s = 0.49$, $p < 0.001$ and $r_s = 0.56$, $p < 0.001$, respectively), AST ($r_s = 0.28$, $p < 0.05$ and $r_s = 0.34$, $p < 0.01$, respectively) and

CK18-M30 ($r_s = 0.24$, $p < 0.05$ and $r_s = 0.30$, $p < 0.01$, respectively), but negatively with sCD14 ($r_s = -0.36$, $p < 0.05$ and $r_s = -0.35$, $p < 0.01$, respectively). In contrast, leukocyte count was positively associated with platelet count ($r_s = 0.37$, $p < 0.001$) and negatively associated with FIB-4 ($r_s = -0.24$, $p < 0.05$), while erythrocyte count was positively associated with Shannon index ($r_s = 0.46$, $p < 0.001$). Similar to erythrocyte count, haemoglobin, in addition to the above correlations, was positively associated with ALT ($r_s = 0.58$, $p < 0.001$), AST ($r_s = 0.29$, $p < 0.01$) and Shannon index ($r_s = 0.48$, $p < 0.001$), but negatively associated with LBP ($r_s = -0.28$, $p < 0.05$) and sCD14 ($r_s = -0.47$, $p < 0.001$). Platelet count additionally showed only one correlation with FIB-4 ($r_s = -0.64$, $p < 0.001$).

FIB-4 showed a positive association with IgM antibodies to endotoxin core antigen ($r_s = 0.26$, $p < 0.05$) and CK18-M30 ($r_s = 0.56$, $p < 0.001$). The marker of hepatocyte apoptosis, CK18-M30, showed additional positive association with ALT ($r_s = 0.47$, $p < 0.001$), AST ($r_s = 0.56$, $p < 0.001$), IgM EndoCAb antibodies ($r_s = 0.30$, $p < 0.01$) and a negative association with LBP ($r_s = -0.30$, $p < 0.01$). sCD14 correlated positively with LBP ($r_s = 0.30$, $p < 0.01$), but negatively with CK18-M30 and Shannon index ($r_s = -0.31$, $p < 0.01$). In contrast, the Shannon index showed a positive association with ALT ($r_s = 0.35$, $p < 0.01$) and CK18-M30 ($r_s = 0.24$, $p < 0.05$) and a negative association with microbial translocation markers – LBP and sCD14 ($r_s = -0.31$, $p < 0.01$ and $r_s = -0.37$, $p < 0.001$, respectively).

2.3.1 Associations of parameters in patient groups

In patients with **HIV monoinfection**, HIV RNA correlated negatively with $CD4^+$ T lymphocyte count ($r_s = -0.43$, $p < 0.05$) and haemoglobin level ($r_s = 0.61$, $p < 0.01$), and positively with sCD14 ($r_s = 0.45$, $p < 0.05$) and IgG antibody to endotoxin core antigen ($r_s = 0.55$, $p < 0.01$) (Table 2.3).

Haemoglobin levels were associated with sCD14 ($r_s = -0.47$, $p < 0.05$) and Shannon index ($r_s = 0.46$, $p < 0.05$). IgM and IgG antibodies to endotoxin core were associated with each other ($r_s = 0.51$, $p < 0.01$), while IgM antibodies to endotoxin core showed an association with Shannon index ($r_s = -0.38$, $p < 0.05$).

Table 2.3

Spearman correlations of parameters in HIV-monoinfected group (n = 28)

Parameters	1.	2.	3.	4.	5.	6.	7.	8.	9.
1. HIV RNA	–								
2. $CD4^+$ T lymphocytes	−0.43*	–							
3. Hb	−0.61**	0.30	–						
4. FIB-4	0.28	−0.35	−0.17	–					
5. LBP	0.18	−0.10	−0.27	−0.08	–				
6. sCD14	0.45*	−0.21	0.47*	−0.01	0.33	–			
7. IgM EndoCAb	0.28	0.09	−0.07	−0.07	0.22	0.37	–		
8. IgG EndoCAb	0.55**	−0.35	−0.23	0.27	0.32	0.44*	0.51**	–	
9. CK18-M30	0.18	0.05	−0.08	0.36	−0.19	0.15	0.09	0.07	–
10. Shannon index	−0.29	0.07	0.46*	−0.30	−0.31	−0.31	−0.38*	−0.32	−0.04

HIV RNA – HIV load, Hb – haemoglobin, FIB-4 – liver fibrosis index, LBP – lipopolysaccharide binding protein, sCD14 – soluble CD14 receptor, IgM EndoCAb – endotoxin core IgM antibodies, IgG EndoCAb – endotoxin core IgG antibodies, CK18- M30 – cytokeratin 18 neoepitope M30.
* – $p < 0.05$; ** – $p < 0.01$.

In the **HIV/HCV-coinfected group**, only two correlations were found: HIV RNA negatively correlated with $CD4^+$ T lymphocyte count ($r_s = -0.60$, $p < 0.01$) and IgM antibodies positively correlated with IgG antibodies to endotoxin core ($r_s = 0.49$, $p < 0.01$).

In the **HCV-monoinfected group**, there was a correlation between IgM and IgG antibodies to endotoxin core ($r_s = 0.54$, $p < 0.01$), a correlation between IgM EndoCAb and CK18-M30 ($r_s = 0.46$, $p < 0.01$) and negative correlation between CK18-M30 and sCD14 ($r_s = -0.47$, $p < 0.05$).

2.3.2 Partial Spearman correlations in the whole group of patients

Given the differences found between the HIV, HIV/HCV and HCV-infected patient groups and the different trends in the association between each group, the association analysis was adjusted for using the partial Spearman correlation coefficient (Table 2.4). This means that correlations were calculated excluding the effects of group differences. It should be noted that HIV RNA, $CD4^+$ and $CD8^+$ T lymphocyte counts were not used in the correlation analysis as these were only measured in two groups. Given the objective to determine the correlation of the faecal microbiota with markers of microbial translocation and liver fibrosis, the relationship between the Shannon index and other markers was also examined. The amount of bacteria in the faecal sample (Shannon index) was positively associated with patient BMI ($r_s = 0.28$, $p < 0.05$), erythrocyte count ($r_s = 0.35$, $p < 0.01$) and haemoglobin ($r_s = 0.32$, $p < 0.01$) and negatively associated with the microbial translocation marker sCD14 ($r_s = -0.30$, $p < 0.01$). In contrast, sCD14 was positively associated with LPB ($r_s = 0.25$, $p < 0.05$) and negatively associated with blood parameters – leukocytes ($r_s = -0.35$, $p < 0.01$), erythrocytes ($r_s = -0.30$, $p < 0.01$) and haemoglobin ($r_s = -0.42$, $p < 0.001$).

Table 2.4

Partial Spearman correlations between parameters (n = 81)

Parameters	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.
1. Age	–													
2. BMI	0.20	–												
3. Leukocytes	−0.01	0.03	–											
4. Erythrocytes	−0.12	0.12	0.36	–										
5. Haemoglobin	0.03	0.26	0.51	0.72	–									
6. Platelets	−0.07	−0.10	0.36	0.02	−0.03	–								
7. ALT	−0.09	0.02	0.52	0.49	0.47	0.14	—							
8. AST	−0.13	−0.14	0.27	0.24	0.14	0.13	0.70	–						
9. FIB-4	0.25	−0.01	−0.25	−0.10	−0.10	−0.65	−0.01	0.38	–					
10. LBP	0.10	−0.01	0.07	−0.12	−0.17	0.12	0.04	−0.11	−0.14	–				
11. sCD14	−0,04	−0.10	−0.35	−0.30	−0.42	0.15	−0.14	−0.04	−0.06	0.25	–			
12. IgM EndoCab	−0.27	0.12	−0.02	0.11	−0.01	0.01	0.11	0.20	0.00	−0.13	0.11	–		
13. IgG EndoCab	−0.16	−0.08	−0.21	−0.06	−0.06	−0.17	−0.09	−0.03	0.08	−0.05	0.18	0.50	–	
14. CK18-M30	−0.17	−0.18	0.23	0.19	0.01	−0.12	0.34	0.47	0.29	−0.21	−0.04	0.25	0.08	–
15. Shannon ind.	0.02	0.28	0.14	0.35	0.32	−0.03	0.12	−0.05	−0.09	−0.19	−0.30	−0.13	−0.16	0.02

BMI – body mass index, Hb – haemoglobin, ALT – alanine aminotransferase, AST – aspartate aminotransferase, FIB-4 – liver fibrosis index, LBP – lipopolysaccharide binding protein, sCD14 – soluble CD14 receptor, IgM EndoC – endotoxin core IgM antibodies, IgG EndoC – endotoxin core IgG antibodies, CK18-M30 – cytokeratin 18 neoepitope M30.

Positive correlation, $p < 0.05$ Negative correlation, $p < 0.05$

Positive correlation, $p < 0.01$ Negative correlation, $p < 0.01$

Positive correlation, $p < 0.001$ Negative correlation, $p < 0.001$

2.4 Analysis of faecal microbiota

The first metrics compared when assessing the faecal microbiota were diversity parameters – the total number of taxonomic units of faecal bacteria or observed richness, the Shannon index and the inverse Simpson index.

Differences between the study groups were found in these parameters. HCV patients showed higher total faecal bacterial diversity compared to HIV-monoinfected and HIV/HCV-coinfected patients, but no differences were found between HIV-monoinfected and HIV/HCV-coinfected patients.

Similar results were found for the Shannon index (Figure 2.2) and the inverse Simpson index. They were higher in HCV-monoinfected patients than in HIV-monoinfected and HIV/HCV-coinfected patients, but no differences in bacterial gene diversity were found between HIV-monoinfected and HIV/HCV-coinfected patients.

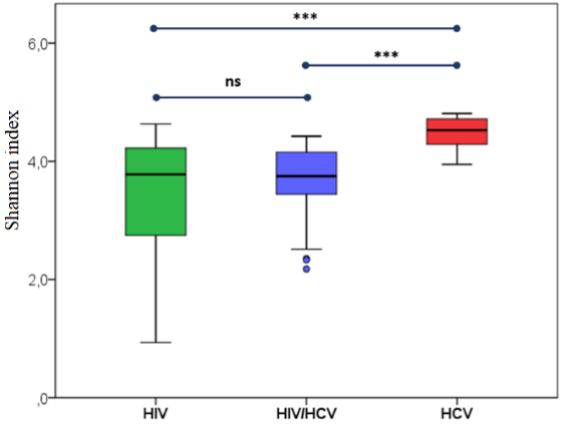

Figure 2.2 **Difference between groups in Shannon index**

*** – p < 0.001, ns – no significant difference.

In order to analyse ß diversity of bacterial composition, a *Principal Coordinate Analysis* (PCoA) based on the Bray-Curtis dissimilarity index was performed. The result showed that the gut microbiota composition

of HCV-monoinfected patients formed a distinct cluster, which was confirmed by a permutational multivariate ANOVA (p = 0.001). HIV patients did not show distinct clusters of specific gut microbiota composition, regardless of the presence of HCV (Figure 2.3).

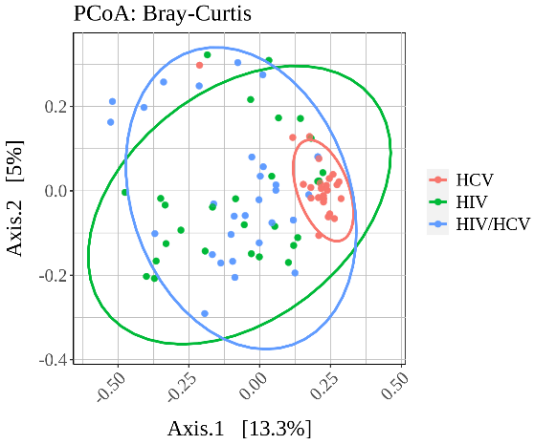

Figure 2.3 **Differences in ß diversity of the faecal microbiota between patient groups**

2.4.1 Composition of faecal microbiota

A total of 1651 taxonomic units were detected in the faecal samples, belonging to 12 bacterial phyla, 15 classes, 40 orders and 71 bacterial families. More than 98 % of all bacterial entities belonged to 10 bacterial phyla: *Firmicutes*, *Bacteroidota*, *Actinobacteriota*, *Proteobacteria*, *Desulfobacteriota*, *Fusobacteriodota*, *Verrucomicrobiota*, *Campilobacteriota*, *Spirochaetota*, *Cyanobacteriota*.

To assess differences in bacterial composition, taxonomic units were then analysed by study groups, looking at differences in relative abundance of bacteria at the level of phylum, classes, orders and families (Cavalier-Smith, 1998).

At the **phylum level**, the following bacteria dominated in all study groups: *Firmicutes*, *Bacteroidota*, *Actinobacteriota* and *Proteobacteria*, with the relative abundance of *Firmicutes* occupying more than 50 % of all bacterial sequences. Of the total bacterial relative abundance, their median and IQR [interquartile range] were 66.2 % [63.3–73.6 %] for HCV-monoinfected, 62.8 % [53.6– 71.6 %] for HIV-monoinfected and 64.4 % [51.4– 72.9 %] for HIV/HCV-coinfected patients.

Subsequently, in HCV-monoinfected were found *Bacteroidota* 17.9 % [13.4–22.9 %], *Actinobacteriota* 10.2 % [7.1–13.2 %] and *Proteobacteria* 0.8 % [0.57–1.8 %]. In HIV-monoinfected group: *Bacteroidota* 17 % [11.5–25.9 %], *Actinobacteriota* 4.5 % [0.3–9.1 %] and *Proteobacteria* 0.8 % [0.1–7.3 %], but in HIV/HCV-coinfected patients: *Bacteroidota* 15.4 % [7.1–26.6 %], *Actinobacteriota* 6.9 % [3.9–10.8 %], and subsequently *Proteobacteria* 3.1 % [0.7–9.3 %] (Figure 2.4).

The *Firmicutes-Bacteroidota* ratio was not statistically significantly different between groups.

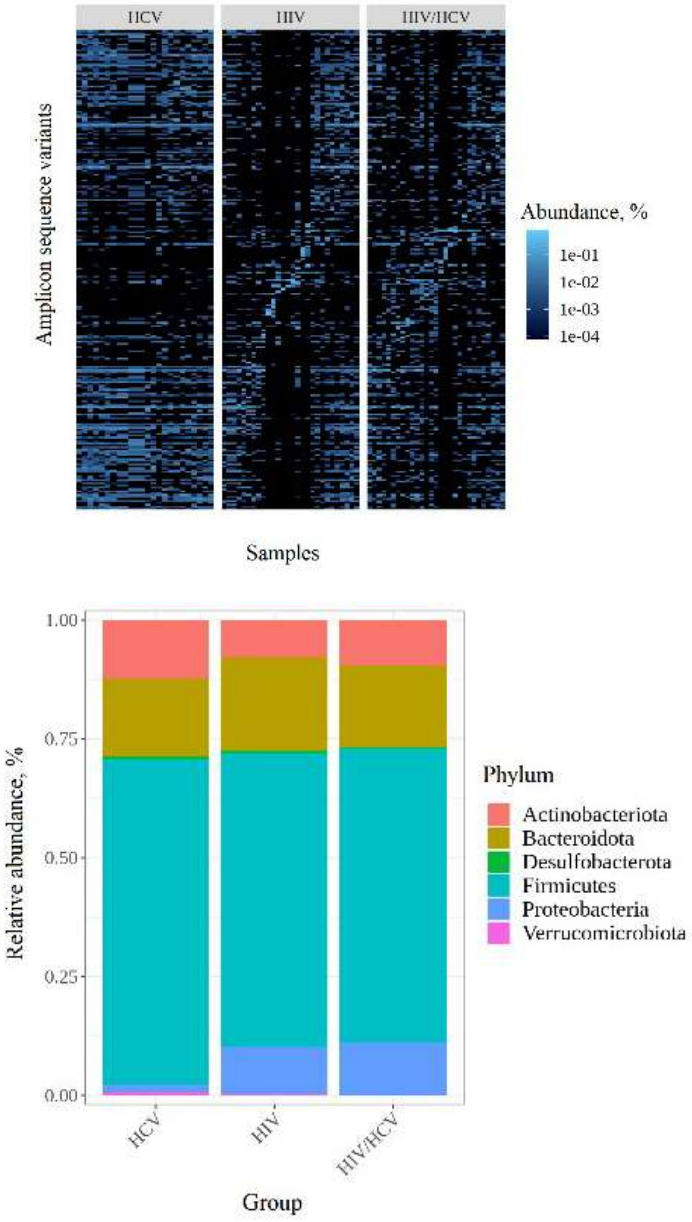

Figure 2.4 **Overview of differences between groups in relative abundance at the phylum level**

At the level of *dominant phyla*, the relative abundance of *Firmicutes* and *Bacteriodata* did not differ significantly between groups, while the relative abundance of *Actinobacteria* was higher in HCV-monoinfected patients compared to HIV-monoinfected ($p < 0.01$) and HIV/HCV-coinfected ($p < 0.05$) patients, but did not differ between HIV-monoinfected and HIV/HCV-coinfected patients. The relative abundance of *Verrucomicrobiota* phylum showed similar results: it was higher in HCV-monoinfected patients compared to HIV-monoinfected ($p < 0.05$) and HIV/HCV-coinfected ($p < 0.05$) patients but did not differ between HIV mono and HIV/HCV-coinfected patients. The relative abundance of *Desulfobacteriota* was lower in HCV-monoinfected patients compared to HIV-monoinfected ($p < 0.05$) and HIV/HCV-coinfected ($p < 0.05$) patients, but no differences were observed between HIV mono and HIV/HCV-coinfected patients. Significantly higher relative abundances of *Proteobacteria* phylum ($p < 0.0001$) were observed in the HIV/HCV-coinfected group compared to HCV-monoinfected patients (Figure 2.5).

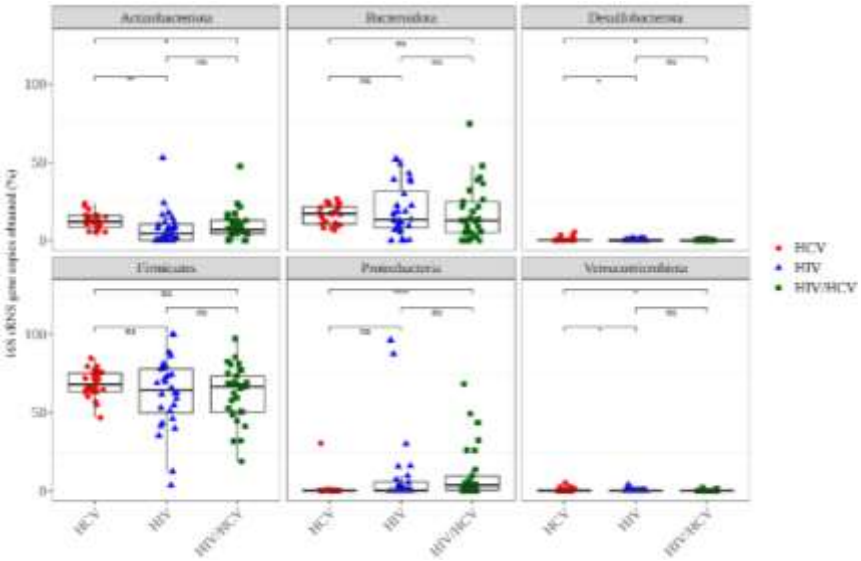

Figure 2.5 **Differences between groups in relative abundance at the phylum level**

* – p < 0.05; ** – p < 0.01; **** – p < 0.0001; ns – no significant difference.

Further analysis of the differences in bacteria at the **class level** revealed the following most frequent bacterial classes: the most frequent class was *Clostridia* – 60.2 % [57.0–64.2 %] in HCV-monoinfected, 52.6 % [25.5–52.7 %] in HIV-monoinfected and 35.6 % [27.5–44.1 %] in HIV/HCV-coinfected patients. In HCV-monoinfected patients, *Bacteroidia* class was observed in 18.2 % [13.5–23.4 %], *Actinobacteria* in 5.1 % [2.5–7.7 %], *Bacilli* in 6.1 % [4.7–8.4 %], *Coriobacteria* in 4.7 % [3.9–5.7 %], *Desulfovibrionia* in 0.3 % [0.1–0.7 %] and *Gammaproteobacteria* in 0.8 % [0.6–1.8 %]. In HIV-monoinfected: *Bacteroidia* 18.2 % [13.5–23.4 %], *Actinobacteria* 1.4 % [0.0– 4.6 %], *Bacilli* 15.3 % [14.9–33.5 %], *Coriobacteria* 1.7 % [0.2–4.1 %], *Desulfovibrionia* 0.0 % [0.0–0.6 %] and *Gammaproteobacteria* 0.8 % [0.1– 7.4 %]. In contrast, in HIV/HCV-coinfected patients, the *Bacteroidia* class was observed in 15.5 % [7.1–26.7 %], *Actinobacteria* in 3.6 % [1.1–5.8 %],

Bacilli in 22.2 % [14.9–33.5 %], *Coriobacteria* in 3.7 % [0.6–5.8 %], *Desulfovibrionia* in 0.2 % [0.0–0.5 %] and *Gammaproteobacteria* in 3.1 % [0.7– 9.3 %] (Figure 2.6).

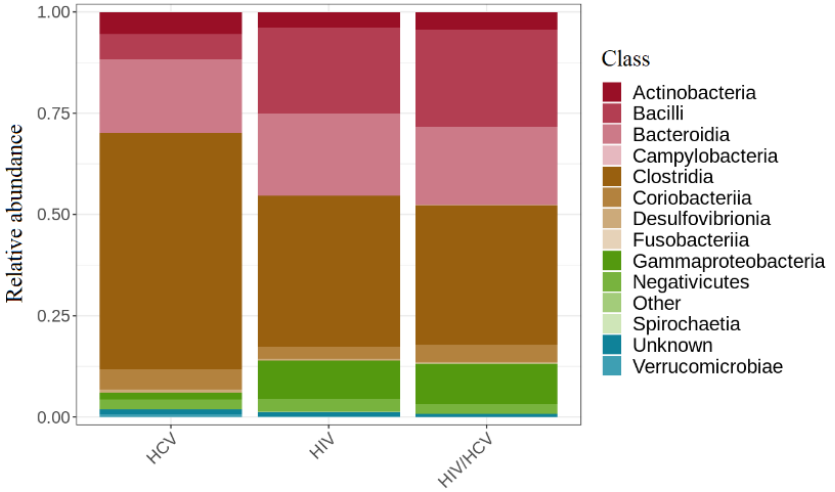

Figure 2.6 **Differences between groups in relative abundance at the faecal bacteria class level**

No differences in relative abundance between classes were found in the *Bacteroidia* class. Significant differences were found for the *Bacilli* class, with HCV-monoinfected patients having lower relative abundances than HIV-monoinfected and HIV/HCV-coinfected patients ($p < 0.001$). However, the opposite trend was observed for the *Clostridia* class, with higher abundance in the HCV group compared to the HIV-monoinfected group ($p < 0.001$). *Actinobacteria* were higher in HCV-monoinfected compared to HIV-monoinfected patients ($p < 0.01$), but did not differ between the other groups. In contrast, *Gammaproteobacteria* were lower in HCV-monoinfected compared to HIV/HCV-coinfected patients ($p < 0.05$), while *Desulfivibrionia* differed between HCV and HIV-monoinfected and HCV-monoinfected and HIV/HCV-coinfected patients ($p < 0.05$).

At the bacterial **family level**, the most frequent bacterial families in HCV-monoinfected patients were: *Lachnospiraceae* 45.5 % [39.6–54.5 %], *Ruminococcaceae* 14.2 % [11.9–15.8 %], *Bifidobacteriaceae* 10.9 % [5.6– 13.7 %], *Bacteroidaceae* 5.8 % [2.7–13.4 %], *Coriobacteriaceae* 7.5 % [6.4–9.3 %], *Prevotellaceae* 0.0 % [0.0–2.0 %], *Enterobacteriacae* 0.24 % [0.0–0.4 %]. *Enterococcaceae* in HCV patients were not found in detectable amounts. In HIV-monoinfected patients: *Lachnospiraceae* 23.4 % [8.8–33.5 %], *Ruminococcaceae* 8.5 % [0.0–18.6 %], *Bacteroidaceae* 4.6 % [2.8–20.1 %], *Coriobacteriaceae* 0.3 % [0.0–6.1 %], *Bifidobacteriaceae* 2.2 % [0.0–7.5 %], *Prevotellaceae* 0.5 % [0.0–3.8 %] and *Enterobacteriaceae* 0.2 % [0.0–13.2 %] and *Enterococcaceae* 0.0 % [0.0–3.5 %], while in HIV/HCV-coinfected patients: *Lachnospiraceae* 27.2 % [19.1–34.1 %], *Ruminococcaceae* 5.34 % [1.7–13.5 %], *Bacteroidaceae* 4.2 % [1.5–15.9 %], *Coriobacteriaceae* 3.8 % [0.0–9.9 %], *Bifidobacteriaceae* 3.8 % [0.0–6.8 %], *Prevotellaceae* 0.0 % [0.0– 0.4 %], *Enterobacteriaceae* 3.8 % [0.1–10.9 %] and *Enterococcaceae* 0.6 % [0.0–5.9 %].

No differences in relative abundance were observed between groups for the following families – *Bacteroidaceae*, *Prevotellaceae*, *Streptococcaceae*, *Erysipelotrichaceae* and *Tannerellaceae* (Figure 2.7). The relative abundance of *Lachnospiraceae* and *Bifidobacteriaceae* families was significantly lower in HIV-monoinfected and HIV/HCV-coinfected patients compared to HCV-monoinfected patients ($p < 0.0001$; $p < 0.01$, respectively), but did not differ between HIV-monoinfected and coinfected groups. Several bacterial families showed similar results, with lower levels in HIV/HCV-coinfected and HIV-monoinfected compared to HCV-monoinfected patients: *Oscillospiraceae* ($p < 0.001$), *Ruminococcaceae* ($p < 0.01$), *Peptostreptococcaceae* ($p < 0.05$; $p < 0.001$, respectively) and *Coriobacteriaceae* ($p < 0.05$; $p < 0.001$, respectively). Contrary results were found for the relative abundance of *Enterobacteriaceae* and *Enterococcaceae*, which were least represented in the

HCV-monoinfected group compared to HIV/HCV-coinfected patients ($p < 0.01$; $p < 0.001$, respectively). The relative abundance of *Lactobacillaceae* was highest in the HIV/HCV-coinfected group compared to the HIV-monoinfected ($p < 0.01$) and HCV-monoinfected patient groups ($p < 0.001$), but did not differ between the HIV and HCV-monoinfected groups.

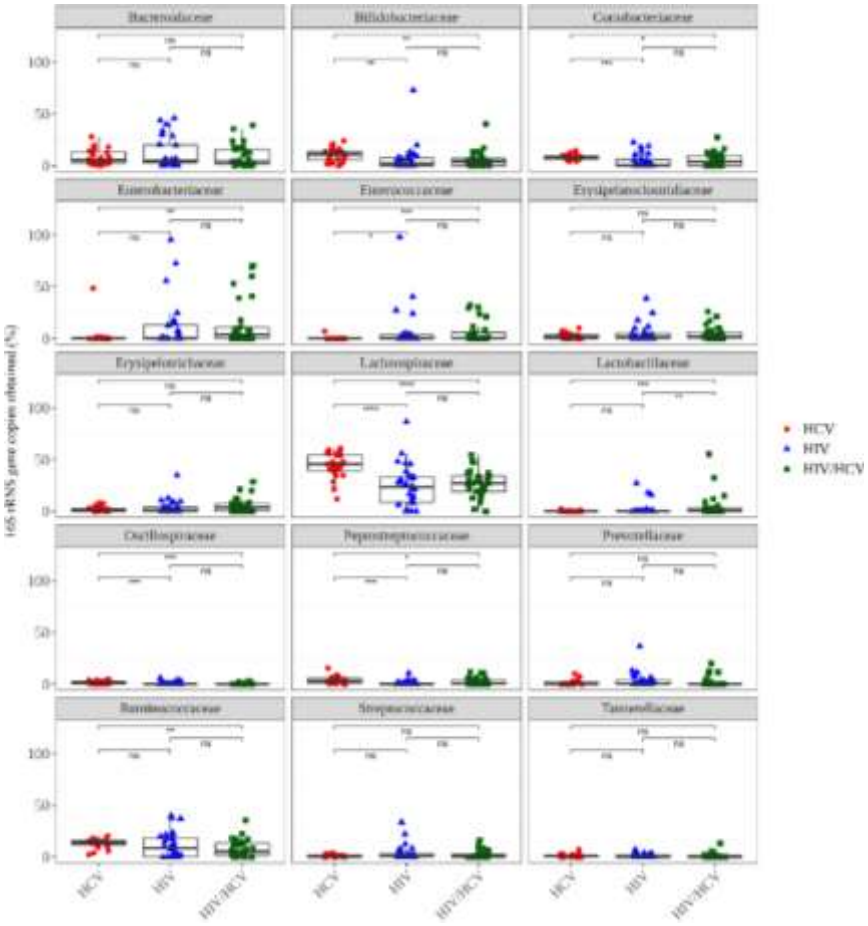

Figure 2.7 **Differences between groups in relative abundance at the family level**

* – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$; **** – $p < 0.0001$; ns – no significant difference.

At the level of **bacterial genus**, differences between groups were observed. The HCV-monoinfected compared to HIV/HCV-coinfected and HIV-monoinfected patients group had a lower relative abundance of the following bacterial genera: *Clostridium innocuum* in HIV/HCV-coinfected patients ($p < 0.001$) and HIV-monoinfected patients ($p < 0.01$), *Enterococcus* in

HIV/HCV-coinfected patients ($p < 0.01$) and HIV-monoinfected patients ($p < 0.05$), *Erysipeloclostridium* in HIV/HCV-coinfected patients ($p < 0.001$) and HIV-monoinfected patients ($p < 0.01$), *Streptococcus* in HIV/HCV-coinfected patients ($p < 0.05$), HIV-monoinfected patients ($p < 0.01$), while the relative abundance of *Lactobacillus* genus did not differ between HCV and HIV-monoinfected patients, but differences were observed between HIV/HCV-coinfected and HCV-monoinfected patients ($p < 0.01$), and HIV/HCV-coinfected and HIV-monoinfected patients ($p < 0.05$).

However, the opposite trend, where HCV-monoinfected individuals had higher relative amounts of a genus compared to HIV-infected individuals, was observed in the following genera: *Bifidobacterium* in HIV/HCV-coinfected patients ($p < 0.05$), in HIV-monoinfected patients ($p < 0.001$), *Collinsella* in HIV/HCV-coinfected patients ($p < 0.01$), in HIV-monoinfected patients ($p < 0.0001$), *Anaerostipes* in HIV/HCV-coinfected patients ($p < 0.01$), in HIV-monoinfected patients ($p < 0.0001$), *Eubacterium halli* in HIV/HCV-coinfected patients ($p < 0.01$), in HIV-monoinfected patients ($p < 0.001$), *Faecalibacterium* – HIV/HCV-coinfected patients ($p < 0.01$), HIV-monoinfected patients ($p < 0.05$), *Romboutsia* – HIV/HCV-coinfected patients ($p < 0.0001$), HIV-monoinfected patients ($p < 0.0001$), *Subdoligranalum* in HIV/HCV-coinfected patients ($p < 0.0001$), and HIV-monoinfected patients ($p < 0.001$).

2.4.2 Association of faecal microbiota diversity and biochemical markers

To assess the association of individual bacterial classes with microbial translocation and liver fibrosis parameters correlation analyses were subsequently performed in the study groups. Associations between $CD4^+$, $CD8^+$ T lymphocytes and HIV RNA were only examined in HIV-monoinfected and HIV/HCV-coinfected patients (Figure 2.8). The class level was chosen for the

analysis to better assess potential correlations and to reduce data fragmentation. Spearman's correlation test was used for the analysis.

The following correlations were found in **bacterial classes of the *Actinobacteriota phylum***: *Actinobacteria* class correlated positively with $CD8^+$ T lymphocyte count in HIV patients ($r_s = 0.560$, $p < 0.01$), while *Coriobacteria* class showed negative correlations with LBP in HIV patients ($r_s = -0.426$, $p < 0.05$), sCD14 ($r_s = -0.02$, $p < 0.05$), EndoCAB IgM ($r_s = -0.501$, $p < 0.01$) and EndoCAb IgG antibodies ($r_s = -0.456$, $p < 0.05$).

In the ***Firmicutes phylum***: in HIV-monoinfected patients, the *Bacilli* class correlated negatively with $CD8^+$ T lymphocytes ($r_s = -0.444$, $p < 0.05$) and positively with HIV RNA ($r_s = 0.378$, $p < 0.05$), LBP ($r_s = 0.48$, $p < 0.05$) and FIB-4 ($r_s = 0.424$, $p < 0.05$), while in HCV patients correlated negatively with CK18-M30 ($r_s = -0.433$, $p < 0.05$). The *Negativicutes* class correlated positively with EndoCAb IgM ($r_s = 0.526$, $p < 0.01$) and CK18-M30 ($r_s = 0.409$, $p < 0.05$) in HCV-monoinfected patients. The *Clostridia* class, one of the most represented bacterial classes in the gut microbiota, was negatively correlated with age ($r_s = -0.436$, $p < 0.05$) only in the HCV-monoinfected group.

In the ***Bacteriodota phylum*** relative amounts of the *Bacteroidia* class correlated positively with sCD14 ($r_s = 0.535$, $p < 0.05$) and FIB-4 index ($r_s = 0.379$, $p < 0.05$) in HIV-monoinfected patients, but negatively correlated with EndoCAb IgG antibodies ($r_s = -0.417$, $p < 0.05$) in the HIV/HCV-coinfected group.

Gammaproteobacteria class of the ***Proteobacteria phylum*** showed the following correlations: HIV-monoinfected group had a negative correlation with FIB-4 ($r_s = -0.408$, $p < 0.05$), HIV/HCV-coinfected group had a negative correlation with $CD4^+$ T lymphocytes ($r_s = -0.554$, $p < 0.01$), but positive correlations with EndoCAb IgG antibodies ($r_s = 0.40$, $p < 0.05$) and sCD14 ($r_s = 0.369$, $p < 0.05$).

In contrast, the *Desulfovibrionia* class, which is one of the least represented classes and belongs to the ***Desulfobacteriota phylum***, correlated positively with $CD8^+$ T lymphocyte count in HIV patients ($r_s = 0.544$, $p < 0.01$), but negatively with FIB-4 index ($r_s = -0.455$, $p < 0.05$), HIV RNA ($r_s = -0.476$, $p < 0.05$), EndoCAb IgM ($r_s = -0.474$, $p < 0.05$), EndoCAb IgG antibodies ($r_s = -0.554$, $p < 0.01$) and sCD14 ($r_s = -0.495$, $p < 0.01$). In the HIV/HCV-coinfected group, there was a positive correlation with BMI ($r_s = 0.377$, $p < 0.01$) and CK18-M30 ($r_s = 0.386$, $p < 0.01$), and in the HCV-monoinfected group a positive correlation with sCD14 ($r_s = 0.471$, $p < 0.01$).

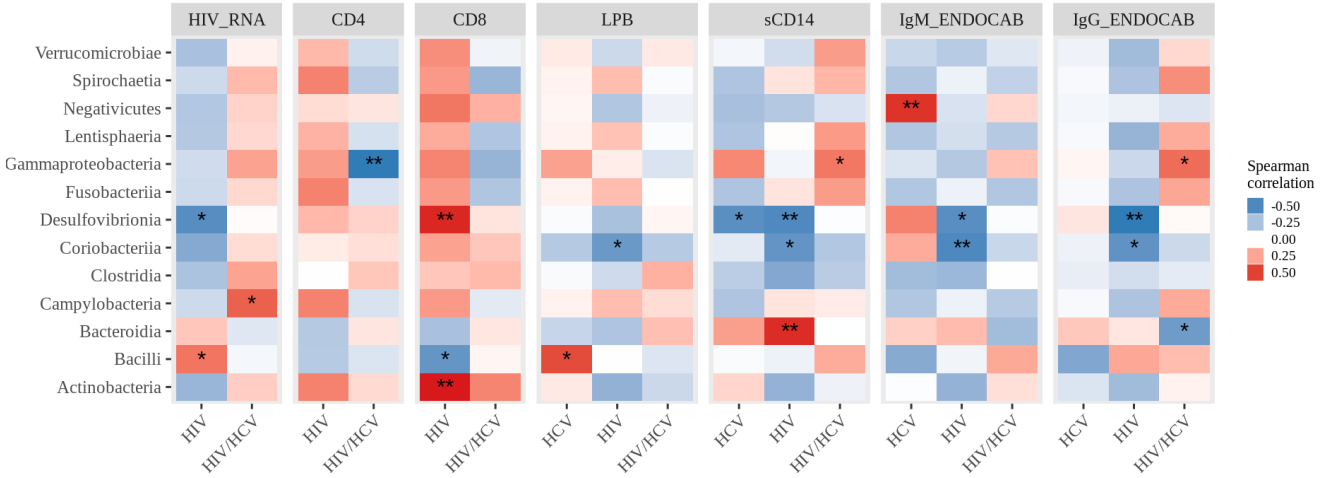

Figure 2.8 **Spearman correlations between study groups for bacterial classes, HIV infection characteristics and microbial translocation parameters**

HIV – Human Immunodeficiency virus, HCV – hepatitis C virus, HIV RNS – HIV viral load, CD4 – $CD4^+$ T lymphocytes, CD8 – $CD8^+$ T lymphocytes, IgG Endo CAB – endotoxin core IgG antibodies, IgM EndoCAB – endotoxin core IgM antibodies, LBP – lipopolysaccharide binding protein, sCD14 – soluble CD14 receptor.
* – $p < 0.05$; ** – $p < 0.01$; red colour – positive correlation; blue colour – negative correlation.

3 Discussion

The Doctoral Thesis was devoted to study the composition and diversity of the gut microbiota in HIV and HCV-monoinfected and HIV/HCV-coinfected patients, differences in microbial translocation parameters in these groups of patients, as well as possible correlation between microbiota parameters and microbial translocation markers.

Previous studies have demonstrated the role of microbial translocation in the maintenance of immune activation in HIV infection. As HIV infection damages the gastrointestinal tract, causing structural damage to the epithelial barrier and disrupting intestinal homeostasis, the changes in the gut microbiota induced by HIV infection, its mechanism of interaction with mucosal immune cells and its impact on microbial translocation have received increasing attention (Mingjun et al., 2022). Some studies have also shown the negative effects of HCV on microbial translocation and microbiota composition (Caradonna et al., 2002; Medrano et al., 2021). However, there are very few studies comparing HIV/HCV-coinfected patients with HIV and HCV-monoinfected patients and, although some studies have described an increase in microbial translocation in HIV patients, it is unclear whether and how microbial translocation interacts with changes in gut microbiota composition.

Microbial translocation markers. Our study included HIV-infected patients who have not received ART and HCV patients not received DAA. The endotoxin-related biochemical markers of microbial translocation selected for our study were LBP, sCD14 and EndoCAb antibodies, which are also the most studied and significant markers of microbial translocation, in both – HIV and HCV-infected patients (Lien et al., 1998; Lopez-Cortez et al., 2018; Ouyang et al., 2020). Studies have also used LPS or endotoxin levels in peripheral blood to measure microbial translocation, but some researchers have recognised that not the level of endotoxemia itself, but the body's responses to microbial

translocation, such as sCD14, EndoCAb antibodies and LBP, are more accurately reflecting microbial translocation and its consequences (Brechley, Price & Douek et al, 2006; Sandler et al., 2011), plus LPS correlates with blood LBP levels, but LBP is a more reliable marker due to its technically simpler detection methodology and longer half-life (Abad-Fernandez et al., 2013; Marquez, Fernandez Gutierrez del Alamo & Giron-Gonzalez et al., 2016).

In our study, the markers of microbial translocation, **LBP** and **sCD14**, were higher in patients with HIV infection; however, only LBP levels were statistically significantly different between HIV and HCV-monoinfected patients and HIV/HCV-coinfected and HCV-monoinfected patients. There was no significant difference between HIV-monoinfected and coinfected patients.

Although there were no statistically significant differences in **sCD14** between the groups, the median sCD14 was higher in both groups of HIV-infected patients. This could be explained by influence of different immunological processes on sCD14 values (Lien et al., 1998) and, as a result, they may not fully reflect the true situation. One reason for this is the duration of HIV infection. Although the HIV patients in our study had not received ART before inclusion in the study, they were at different clinical stages of HIV infection and the duration of their disease was unknown and probably varied widely, up to 10 years. In HCV-infected patients, sCD14 scores may also be influenced by HCV genotype (Meiler et al., 2005), which was not addressed in our study. These factors may cause relatively important variations in disease-specific parameters, including microbial translocation rates.

However, our results are broadly in line with several other researchers. Most studies found elevated markers of microbial translocation in HIV patients compared to HIV uninfected individuals, indicating greater microbial translocation and consequently more pronounced immune activation (Lien et al., 1998; Balagopal et al., 2008; Villanueva-Millan et al., 2017; Lopez-Cortez et al.,

2018; Ouyang et al., 2020). Similar results to our study were described by *Balagopal et al*. in their study that included both HIV-monoinfected and HIV/HCV-coinfected patients, with a proportion of the HIV-infected group having pre-HIV blood samples available and used as a control group. Higher LBP and LPS levels but lower EndoCAb IgM antibody titres were observed in HIV-infected patients compared to HIV uninfected subjects. Interestingly, similar to our study, higher sCD14 levels were observed in the HIV group, but without a statistically significant difference. Moreover, differences in sCD14, LPS and antibody levels were increased in HIV-infected compared to uninfected individuals when $CD4^+$ T cell counts were < 350 cells/μL (Balagopal et al., 2008). *Villanueva-Millan* and colleagues compared HIV-infected patients with and without antiviral therapy with healthy people and found that sCD14 was higher in the HIV-infected group, whereas in 50 % of treated HIV patients it was at the level of healthy people. LBP levels were also significantly higher in the HIV-infected group, but did not differ between patients receiving or not receiving ART. Interestingly, a proportion of patients (51.1 %) receiving ART were found to be coinfected with HCV. These patients had higher LBP rates compared to HIV-monoinfected patients receiving ART and to healthy controls (Villanueva-Millan et al., 2017). Our study did not include patients who had received ART, but work by other investigators has found that markers of microbial translocation may remain elevated in HIV patients receiving ART even after successful antiviral therapy (Sandler et al., 2011; Villanueva-Millan et al., 2017). This may indicate persistent immune activation despite the treatment received.

EndoCAb antibodies are produced in response to increased levels of endotoxins in the blood. In our study, there were no differences in EndoCAb antibody levels between groups, but the mean levels of both IgM and IgG EndoCAb antibodies tended to be lower in the HIV-monoinfected group. These results may be explained by what other researchers have described: higher

endotoxin levels may result in lower antibody presence, as antibodies bind to circulating LPS and clear them from the body (Balagopal et al., 2008; Vassallo et al., 2012). Also in the study by *Sandler et al*., lower levels of EndoCAb antibodies were observed in a group of HIV-infected patients compared to healthy controls, while other markers of microbial translocation, such as LBP and sCD14, were higher in HIV-infected patients (Sandler et al., 2011).

In our study, a statistically significant difference was found only between HIV-infected (both mono- and coinfected) and HCV-infected patients. HIV-infected patients, irrespective of the presence of HCV, generally showed more pronounced microbial translocation compared to HCV-monoinfected patients, characterised by increased LBP, while the lower EndoCAb antibody count than HCV-monoinfected patients could be explained by increased antibody utilisation for endotoxin binding and elimination in HIV-infected patients. The findings suggest that HCV infection does not cause a significant increase in microbial translocation in our sample compared to HIV-infected patients. Contrary to the hypothesis, HIV/HCV-coinfected patients did not show more pronounced microbial translocation than HIV-monoinfected patients. Such results were also found in the works of other investigators, where markers of microbial translocation were higher in HIV patients compared to healthy controls, but HIV/HCV-coinfected patients did not differ from HIV-monoinfected patients (Marquez, Fernandez Gutierrez del Alamo & Giron-Gonzalez, 2016; Villanueva-Millan et al., 2017). Given the differences in the markers of microbial translocation found, it can be said that HIV patients, regardless of HCV coinfection, showed a more pronounced microbial translocation compared to HCV-monoinfected patients.

HIV infection is associated with a chronic inflammatory state that can be characterised by parameters such as IL-6 and markers of T cell activation ($CD38^+$ and HLA-DR expression), as well as by persistent immune activation, which are

important factors in the decline of $CD4^+$ T cells and loss of immune function that can gradually lead to AIDS (Lopez-Cortez et al., 2018). Regarding the impact of chronic hepatitis C on immune activation and inflammation in HIV/HCV-coinfected patients, evidence for increased levels of $CD4^+$ and $CD8^+$ T cell immune activation has been found (Lopez-Cortez et al., 2018). However, few studies have found no differences in immune activation rates between HIV-monoinfected and HIV/HCV-coinfected patients (Shmagel et al., 2014). Despite similar rates of immune activation, markers of microbial translocation have been found to be higher in the coinfected group in most studies (Nystrom et al., 2015; Shmagel et al., 2016; Lopez-Cortez et al., 2018). The clinical relevance of these markers has also been studied in relation to the outcome of HIV and HCV infections, even in treatment-naive groups. Thus, *Sandler* and colleagues showed that sCD14 correlates with mortality related indicators of HIV infection, such as BMI, lipid profile and insulin resistance (Sandler et al., 2011), while HCV eradication in HIV/HCV-coinfected patients results in less microbial translocation and subsequent immune activation (Lopez-Cortez et al., 2018).

To better understand the interactions between the different factors in both infections, **Spearman correlations** were calculated. They were examined both – in the total sample and separately for each patient group.

In the overall sample of our study, no correlation was found between HIV RNA and microbial translocation and liver fibrosis parameters. However, when patients were stratified by study group, HIV RNA was positively correlated with sCD14 and EndoCAb IgG antibody levels in the HIV-monoinfected group. Interestingly, no such correlations were found in the HIV/HCV-coinfected group. On the other hand, the number of $CD4^+$ T lymphocytes in the total HIV sample correlated negatively with EndoCAb IgG antibodies, but this correlation disappeared when the patients were divided into study groups. A reciprocal positive correlation was observed between sCD14 and LBP levels in the total

sample, but this correlation disappeared when the results were analysed by group. It is possible that increasing the number of patients and reducing their differences in the clinical spectrum of HIV and HCV infections could lead to more significant and statistically reliable associations between different parameters.

This finding does not contradict data from other authors and supports the fact that the extent of microbial translocation increases as the amount of HIV RNA in the blood increases and the number of $CD4^+$ T cells decreases (Balagopal et al., 2008; Marchetti et al., 2011). For example, *Marchetti* and colleagues analysed data from 379 HIV patients and found that HIV RNA in the blood correlated positively with sCD14, while sCD14 correlated negatively with peripheral blood $CD4^+$ T cell count, and sCD14 correlated negatively with IL-6 and TNF-α, confirming that increasing depth of immunosuppression and higher levels of HIV RNA in the blood increase microbial translocation and consequent immune activation. Furtheermore, observing these patients dynamically, it was found that HIV patients with higher sCD14 levels had a 35 % increased risk of disease progression (Marchetti et al., 2011).

This suggests that as HIV infection progresses and the HIV RNA load in the blood increases, microbial translocation becomes more pronounced, which in turn could sustain immune activation and a chronic inflammatory state in HIV-infected patients. Therefore, early initiation of treatment for HIV infection can significantly reduce microbial translocation and immune activation by reducing HIV RNA load, but hepatitis C virus was not associated with an additional factor for increased microbial translocation in our study sample.

Indicators of liver fibrosis. Liver disease has surpassed HIV-associated indicator diseases as a major cause of death in HIV-infected patients (Bica et al., 2001). Assessment of liver status is therefore essential in this population, but liver biopsy is not always feasible in patients with HIV/AIDS, and in patients with HIV monoinfection, even with elevated liver enzymes but without proven

viral hepatitis, biopsies are not routinely performed because they do not influence the choice of HIV therapy. However, HIV is also known to affect liver cells both directly and indirectly, and HIV RNA has been found in liver biopsy material from HIV-infected patients (Blackard, 2011).

In addition to markers of microbial translocation, indirect markers of liver fibrosis, the **FIB-4 index** and **CK18-M30**, were measured in our study. No differences in the FIB-4 index between groups were observed in our study, but a higher number of patients with potential liver fibrosis was observed in HIV/HCV-coinfected and HCV-monoinfected patients compared to HIV-monoinfected patients using FIB-4 cut-off levels (Sterling et al., 2006).

In 2006, *Sterling* and colleagues were the first to describe the FIB-4 index in HIV/HCV-coinfected patients. FIB-4 index has also been validated in HCV-infected patients (Sterling et al., 2006). ALT, AST, platelet count and patient age are used to calculate FIB-4. In our study, the correlations between these markers were also analysed and in the pooled sample FIB-4 was positively correlated with patient age and AST, but negatively correlated with platelet count. The importance of these parameters is supported by the fact that both HIV and HCV infections in isolation can affect these parameters, for example – a reduced platelet count itself is a characteristic feature of HIV infection, and studies have described a negative correlation of platelet count with blood HIV RNA levels (Blackard et al., 2011). The advanced stage of chronic HCV infection is also characterised by a decrease in platelet count and an increase in ALT and AST.

Other researchers have also described conflicting results regarding the FIB-4 index. For example, in a study on the severity of liver fibrosis, *Rohrbach* and colleagues found no significant difference in the FIB-4 index between HIV-monoinfected and HIV/HCV-coinfected patients (Rohrbach et al., 2014), which is similar to the results of our study, which found no statistical difference in the FIB-4 index. In contrast, *Blackard* and colleagues found a higher FIB-4 index in

the HIV/HCV group compared to HIV or HCV-monoinfected patients when analysing liver fibrosis in 1227 patients (Blackard et al., 2011). However, a positive correlation between the FIB-4 index and CK18-M30 was significant, confirming a previously described association between apoptosis and fibrosis in the liver (Parfieniuk-Kowerda et al., 2014). Moreover, this association was calculated using the partial correlation coefficient, indicating the presence of this association in all three patient groups.

In our study, **CK18-M30** levels differed between groups, with the lowest levels in HIV-monoinfected patients and the highest levels in HCV-monoinfected patients. A similar trend in group differences was also observed for ALT and AST, suggesting that HCV patients included in the study sample had a more active chronic HCV course than HIV coinfected patients. Also, the percentage of patients with CK18-M30 above 200 U/L, i.e. (according to the established CK18-M30 test manufacturer's recommendations) reaching levels consistent with enhanced hepatocyte death, was highest in the HCV-monoinfected group and lowest in the HIV-monoinfected group. Significantly, the partial Spearman correlation coefficient showed a positive correlation between CK18-M30, ALT, AST and FIB-4 indices regardless of patient group, confirming hepatocellular damage in all three patient groups.

Similar results were described by *Rohrbach* and colleagues in a study in which HIV/HCV-coinfected patients had higher CK18-M30 than HIV-monoinfected patients, and elevated CK18-M30 levels correlated with AST values but not ALT values (Rohrbach et al., 2014). This may be explained by the different localisation of ALT and AST in hepatocytes, and since apoptosis is associated with mitochondrial membrane destruction, the more apoptotic CK18-M30 may correlate more significantly with AST values.

In the patients included in our study, ALT and AST levels were higher in HCV-monoinfected patients and, although not statistically significant, platelet counts were lower in the HIV/HCV-coinfected group. As other known liver and metabolic diseases were exclusion criteria in our study, it can be suggested that the liver status of the patients included in our study was more significantly influenced by the presence of HCV and to a lesser extent by the effect of HIV on liver cells, but additional factors not considered in the study could include, for example, alcohol consumption patterns beyond previously confirmed chronic alcoholism.

An interesting correlation between CK18-M30 and markers of microbial translocation was found in the overall sample of our study: CK18-M30 correlated positively with IgM EndoCAb antibodies, but negatively with the LBP levels. However, when the results were analysed in individual patient groups, statistically significant correlations remained only in HCV-monoinfected patients, where CK18-M30 correlated positively with IgM EndoCAb antibodies but negatively with sCD14. As CK18-M30 is a marker of cell death or apoptosis and can only be used indirectly as a marker of liver fibrosis, these results suggest that increasing cell apoptosis in certain patient groups may lead to decreased microbial translocation. This may also be based on the association between CK18-M30 and IgM EndoCAb using the partial correlation coefficient. However, the presence of these correlations only in the HCV-monoinfected group may partly explain the less pronounced microbial translocation compared to HIV-infected patients. However, it would be premature to make such a statement on the basis of these results alone. Further studies would be needed to better assess the possible relevance of the correlation between these parameters.

Microbiota analysis. For microbiota analysis, most studies now use the 16S rRNA gene assays, which give an idea of the diversity and composition of the microbiota but, unlike metagenomic methods, do not provide information on microbiome function. In our study, the 16S rRNA methodology was used to determine the faecal microbiota, which allowed us to determine the microbiota composition down to the family or even genus level for most bacteria, and to compare differences in the composition of the faecal microbiota between the study groups. To assess differences, we used total gene abundance, diversity indices, and differences in richness and abundance of different bacterial taxonomic units.

Diversity of microbiota. Our study compared HIV-monoinfected, HCV-monoinfected and HIV/HCV-coinfected patients. Such a comparison of groups in terms of microbiota has not been studied before. Initially, we looked at differences between groups in **α diversity** parameters: total observed richness, Shannon diversity index and inverse Simpson index. α diversity is used to describe the average diversity of species within a sample. All three α diversity parameters showed similar results for differences between groups. In the pooled microbiota analysis, α diversity was higher in HCV-infected patients than in HIV-monoinfected and HIV/HCV-coinfected patients, but no differences were observed between HIV-monoinfected and HIV/HCV-coinfected patients. Our results describing the differences between HIV and HCV monoinfections are in agreement with most investigators, but comparative data for coinfected patients are virtually non-existent, as mentioned above, which prevents an adequate comparative assessment.

HIV-infected patients have been found to have a lower total number of bacterial genes when comparing the faecal microbiota of HIV-infected patients with healthy individuals (Li et al., 2016; Guillen et al., 2018; Mingjun et al., 2022). Also, α diversity indices were lower in HIV-infected patients compared

to healthy individuals (Li et al., 2016; Guillen et al., 2018; Ellis et al., 2021; Mingjun et al., 2022). Studies comparing HCV-monoinfected patients with healthy subjects also describe lower α diversity (Aly et al., 2016; Inoue et al., 2018; Heidrich et al., 2018). However, some researchers have found opposite changes in α diversity in both – HIV-infected (Taylor et al., 2020) and HCV-infected patients (Sultan et al., 2021) compared to healthy subjects. Although most studies found lower α diversity in both HIV and HCV-monoinfected patients, the overall findings of the studies are now conflicting.

For HIV/HCV-coinfected patients, there are only a few publications describing changes in the microbiota of HIV/HCV-coinfected patients, and HIV/HCV-coinfected patients have only been compared to HCV-monoinfected (Chuaypen et al., 2020) or HIV-monoinfected patients (Taylor et al., 2020). Interestingly, *Chuaypen et al*. found no differences in α diversity between HCV and HIV/HCV-coinfected groups (Chuaypen et al., 2020), while *Taylor et al*. found lower α diversity in coinfected patients compared to HIV-monoinfected patients and healthy controls, but no differences between HIV-monoinfected patients and healthy controls (Taylor et al., 2020). It should be noted that the study by *Chuaypen et al*. was conducted in Thailand, where microbiota diversity and composition may be significantly influenced by differences in race and diet. In addition, the HIV patients included in this study had also received ART, which may influence microbiota diversity, but compared to healthy individuals α diversity was lower in both HCV and HIV/HCV groups (Chuaypen et al., 2020). In contrast, the study by *Taylor et al*. was conducted in the USA, where only half of the participants were of European race, the study did not have data on patients' BMI, and some of the HIV patients had received ART (Taylor et al., 2020). These factors, together with the dietary habits of the US population, may have a significant impact on α diversity, as it may also be reduced in adipose individuals (Tavakoli et al., 2021).

The human microbiota is known to be influenced by several factors, including age, race, BMI, geographic location, diet, medications, and various diseases, resulting in a microbiome unique to each individual (Lazupone et al., 2013; Hou et al., 2022). Increasingly, the impact of the microbiome on various processes in the body, both its role in maintaining health and in the development of various diseases, is being studied worldwide (Ursell et al., 2012; Hou et al., 2022). A decrease in α diversity is also associated with a more severe course of several diseases, including inflammatory bowel disease (Tavakoli et al., 2021), metabolic disease (Ursell et al., 2021), and HIV infection. In Latvia, research on the microbiota, its composition and its importance has started relatively recently; there are relatively few data on the microbiota of the Latvian population, especially in the context of individual diagnoses or health conditions; therefore, it is currently not possible to compare HIV-infected patients living in Latvia.

Further in our study, we identified differences in microbiota **ß diversity**, which describes differences between multiple samples. In our study, HCV-monoinfected patients showed the most distinct clustering of microbiota composition, whereas HIV patients did not show the formation of distinct specific clusters regardless of the presence of HCV. In their study, *Chuaypen et al*. found distinct clustering of microbiota of HIV and HCV-infected patients and healthy individuals, confirming that both infections result in changes in the diversity of the gut microbiota, but they found no differences between HCV-monoinfected and HIV/HCV-coinfected patients (Chuaypen et al., 2020). In contrast, *Taylor et al*. found differences in ß diversity between HIV-monoinfected and HIV/HCV-coinfected patients and between healthy individuals (Taylor et al., 2020). Both of these studies observed differences between healthy and infected individuals, demonstrating a significant effect of HIV and HCV on microbiota diversity.

Our study findings suggest that in our study sample, HIV had more significant negative impact on gut microbiota diversity than HCV, and the addition of HCV to HIV infection did not further significantly impair overall gut microbiota diversity.

Changes in microbiota composition. To further analyse the composition of the microbiota and its differences between groups, we carried out analysis according to taxonomic groups – bacterial phyla, classes, families and genera. The 16S rRNA approach used was uninformative at the species level. Works by other researchers has also compared changes in the relative abundance of bacteria at different levels of bacterial taxonomic groups similar to our study.

The first study on changes in the composition of the faecal microbiota in HIV patients was published in 2008 (Gori et al., 2008). With the gradual development of technology, more and more information becomes available on the composition of the microbiota and its relationship to microbial translocation and other parameters. A common feature in HIV patients compared to healthy individuals is an increase in the relative abundance of Gram-negative bacteria and a decrease in the relative abundance of certain Gram-positive bacterial species (Ellis et al., 2011; Lazupone et al., 2013; Mutlu et al., 2014; Dinh et al., 2015). Some studies have found an increase in *Campylobacter* and *Desulfovibrio* species compared to HIV uninfected people (Lazupone et al., 2013; Mutlu et al., 2014; Lazupone et al., 2014).

Bacterial composition at the **phylum** level in our study revealed 3 predominant bacterial phyla: in HIV-infected patients – *Firmicutes*, *Bacteroidota* and *Proteobacteria*, similar to other researchers (Dillon et al., 2014), while in HCV-monoinfected patients – *Firmicutes*, *Bacteroidota* and *Actinobacteriota* were predominant. When analysing differences between groups, difference in the *Proteobacteria* phylum was found between HCV-monoinfected and HIV/HCV-coinfected patients, but no difference between HIV and HCV-monoinfected and

HIV-mono- and coinfected patients. Relative abundance of the *Desulfobacteriota* phylum was higher in HCV-monoinfected compared to HIV and HIV/HCV-coinfected patients, but there was no difference between HIV-mono- and coinfected patients. Relative abundance of *Actinobacteriota* phylum was higher in HCV-monoinfected patients compared to HIV-monoinfected patients, but not in HIV/HCV-coinfected patients. In contrast, *Bacteroidota* did not differ between groups, and further analysis at the class and family levels showed no differences between groups. Interestingly, the relative abundances of the *Firmicutes* phylum also did not differ between groups, although their mean abundances were lower in HIV-infected patients (both mono- and coinfected) compared to HCV-monoinfected patients. This finding is supported by the fact that further analysis of the bacterial classes and families of the *Firmicutes* phylum showed a significant reduction of individual families in the HIV-infected groups compared to HCV-monoinfected patients.

In our study, in the **Firmicutes** phylum ***Clostridia class*** was predominant, showing a significant difference of the relative abundance between HCV-monoinfected compared to HIV-monoinfected and HIV/HCV-coinfected patients, but no difference was seen between the two groups of HIV-infected patients. When further analysed at the **family level**, similar findings of differences were found in the following bacteria – *Lachnospiraceae*, *Oscillospiraceae*, P*eptospteptococcacae* and *Ruminococcacae*. The same trends were also observed at the bacterial **genus** level, where higher relative abundances of the genera *Anaerostipes*, *Eubacterium*, *Faecalibacterium*, *Romboutsia*, *Subdoligranulum* were found in HCV-monoinfected patients.

Dillon et al. compared the faecal microbiota of HIV-infected ART naive patients to healthy subjects using the V4 region of the 16S rRNA gene assay, similar to our study, and found that HIV-infected patients had higher levels of *Proteobacteria*, but lower *Firmicutes* abundance, while *Bacteroidota* relative

abundances did not differ between groups and, similar to our study, *Firmicutes* phylum had reduced relative abundances of *Lachnospiraceae* and *Ruminococcacae* (Dillon et al., 2014). Similar data were described by *Vujkovic-Cvijin* and colleagues, whose study of HIV-infected patients found a reduction in *Lachnospiraceae* and *Ruminococcacae*, but higher amounts of *Gammaproteobacteria,* including *Enterobacteriacae* (Vujkovic-Cvijin et al., 2013). *Ruminococcus*, *Coprococcus*, *Eubacteria*, which are reduced in HIV-infected patients, have been described to play a role in maintaining overall gut health and are involved in the production of butyrate and other short-chain fatty acids, resulting in a positive effect on inflammatory processes in the gut and reducing gut wall permeability (McHardy et al., 2013; Mutlu et al., 2014).

The less represented class in the ***Firmicutes*** **phylum** in our study was ***Bacilli***, where lower amounts were observed in HCV-monoinfected patients compared to HIV-infected patients. Further analysis at the **family level** revealed higher relative abundances of *Lactobacillaceae* and *Enterococcaceae* in HIV/HCV-coinfected and HIV-monoinfected patients compared to HCV-monoinfected patients. Similar differences between groups persisted at the **genus** level, with higher relative abundances of bacteria found in HIV/HCV-coinfected compared to HCV-monoinfected patients, and included the following genera: *Enterococcus*, *Erysipelatoclostridium*, *Lactobacillus*, *Streptococcus* and *Clostridium innocuum*. Several studies have also described an increase in bacteria of the genera *Lactobacillacae* and *Enterococcacae* in HIV-infected patients (Lazupone et al., 2014; Dinh et al., 2015).

The relative amount of ***Proteobacteria*** **phylum** was higher in HIV/HCV-coinfected compared to HCV-monoinfected patients in our study, but did not differ between HIV mono- and coinfected patients. Further analysis at class (*Gammaproteobacteria*) and family (*Enterobacteriaceae*) level maintained the same differences. In HIV-infected patients, an increase in Gram-negative

bacteria has also been observed by other researchers, which may play a role in the subsequent increase in microbial translocation and immune activation. Similar to our study, an increase in *Enterobacteriacae* has been found in HIV-infected patients compared to healthy subjects (Gori et al., 2008; Ellis et al., 2011; Dillon et al., 2014; Dihn et al., 2015; Chuaypen et al., 2020). Interestingly, in *Chuaypen et al*. who compared HIV/HCV-coinfected, HCV-monoinfected and healthy individuals, *Proteobacteria* phylum had higher abundance in the HIV/HCV group compared to HCV-monoinfected or healthy individuals, whereas an increase in *Enterobacteriaceae* was observed only in comparison to healthy individuals but not between the infected groups (Chuaypen et al, 2020).

The relative abundances of bacteria in the ***Bacteriodota*** **phylum** did not differ between groups in our study, although several investigators have described the potential role of individual families of this compartment, such as *Prevotellaceae* and *Bacteroidaceae*, in predicting progression to HIV infection (Dillon et al., 2014; Noguera-Julian et al., 2016). An increase in the genus *Prevotella* and a decrease in *Bacteroides* have been observed in HIV-infected patients compared to healthy individuals, and changes in the *Bacteroides: Prevotella* ratio have been specifically evaluated, with a decrease observed in HIV patients (Lazupone et al., 2013; Dillon et al., 2014). However, a decrease in this ratio has also been described in homosexual men, regardless of HIV status (Noguera-Julian et al., 2016; Kelley et al., 2017). In our study, infected patients were compared with each other and not with healthy individuals, which may explain, at least in part, the lack of differences between the groups, as similar trends, a decrease in the *Bacteroides: Prevotella* ratio, can also be observed in HCV-infected patients compared to healthy individuals (Sultan et al., 2021).

The relative abundance of ***Actinobacteriota*** **phylum**, as mentioned above, was higher in HCV-monoinfected compared to HIV-monoinfected but not HIV/HCV-coinfected patients in our study, but further analysis at **class** level

(*Actinobacteria* and *Coriobacteria*) and subsequently at **family** level revealed significant differences between groups in the relative abundances of *Bifidobacteriaceae* and *Coriobacteriaceae* with higher levels in the HCV-monoinfected group compared to HIV-monoinfected and coinfected patients, but they did not differ between the HIV-infected groups. Lower levels of *Bifidobacteriaceae* in HIV-infected patients compared to healthy subjects have been previously described by other authors (Gori et al., 2008). These findings indicate a reduction in the number of potentially beneficial bacteria, including *Clostridia* and *Actinobacteria* classes, in HIV-infected patients. *Clostridia* and *Actinobacteria* classes have been described to have anti-inflammatory activity, while reduction in their numbers helps to maintain chronic inflammation in HIV patients, as previously described by *Rocafort et al*. whose study in a European population also described a reduction in the relative abundance of these bacteria, which may play a role in maintaining the chronic inflammatory process in HIV patients (Rocafort et al., 2019).

Also, in the ***Desulfobacteriota*** phylum, and subsequently in the ***Desulfovibrionia class***, we found differences – higher numbers of these bacteria in HCV-monoinfected compared to HIV-mono- and coinfected patients, but no differences in the HIV-infected groups, even though these bacteria represent a very small proportion of the total patient microbiota in our study. There is very little information in the literature on the role of this phylum in the microbiota of HIV and HCV-infected patients. For example, *Lazupone et al*. found higher levels of these bacteria in HIV-infected patients compared to healthy controls (Lazupone et al., 2014), while *Chuaypen et al*. described the presence of these bacteria in both HCV mono and HIV/HCV-coinfected patients (Chuaypen et al., 2020). Interestingly, *Villanueva-Millan et al*. found both an increase in the *Desulfobacteriaceae* family and a decrease in one *Desulfovibrio* species in HIV-infected patients receiving ART (Villanueva-Millan et al., 2017). *Desulfovibrio*

bacteria are known as hydrogen sulphide producing bacteria. Hydrogen sulphide is toxic to epithelial cells and is thought to contribute to the microbiome's ability to cause damage to the intestinal epithelium and reduce the integrity of the intestinal mucosa. Thus, in the context of HIV infection, this increase in hydrogen sulphide from gut microbes may contribute to loss of epithelial and mucosal integrity, microbial translocation and immune activation (Vujkovic-Cvijin et al., 2013; Villanueva-Millan et al., 2017). However, hydrogen sulphide at low concentrations in the intestinal tract can also have positive effects: it has a cytoprotective effect, allows the maintenance of mucosal integrity and inhibits pathological fragmentation of microbiota biofilms (Buret et al., 2022).

Changes in the gut microbiota in **HCV patients** compared to healthy individuals have also been described previously and include increased relative abundances of *Bacterioidota* and *Firmicutes* phylum bacteria, increased relative abundances of *Prevotellacae*, *Faecalibacterium* and *Veilonella* at genus level, and decreased abundances of *Ruminococcus*, *Clositridium* and *Bifidobacterium* (Aly et al., 2016; Sultan et al., 2021). Most of the studies published to date have compared HCV patients with healthy subjects, and therefore an adequate comparison with these studies is not possible.

In our study, at the family level, we observed 3 families with **higher** relative abundance in HIV-infected patients compared to HCV-monoinfected patients: *Enterobacteriaceae*, *Enterococcaceae* and *Lactobacillaceae*. Although the mean relative abundances of *Prevotellaceae* and *Erysipelotrichacae* were higher in the HIV-infected groups, they did not reach statistical significance. In contrast, **lower** relative abundances in HIV-infected patients were found in the *Clostridia* class families – *Lachnospiraceae*, *Oscillospiraceae*, *Peptostreptococcaceae* and *Ruminococcaceae*. Overall, our results suggest that most findings in the HIV-infected group are not significantly different from those of other investigators; however, it should be noted that the microbiota of HIV or

HCV-infected patients has been compared with that of healthy subjects in most investigators' papers. Interestingly, when comparing the work of other authors, certain bacterial phyla and families emerge with a similar direction of change in relative abundance, while changes in individual families are variable between studies. Although microbiota studies mostly use the same methodology to determine the microbiota by 16S rRNA, individual discrepancies can be explained by different factors such as patient race, geographical location, diet, age and presence of comorbidities.

In our study, most of the differences between bacterial orders, families and genera were found between HIV-infected and HCV-monoinfected patients, but not between HIV-monoinfected and HIV/HCV-coinfected patients. This was further supported by the ß diversity scores, where we found a distinct cluster formation of microbiota only in HCV-monoinfected patients, while the microbiota of the two groups of HIV-infected patients did not differ significantly and overlapped with each other. This suggests that differences in microbiota composition could be observed between HCV and HIV-infected patients in the present study sample, while the microbiota of HIV patients did not change significantly with HCV coinfection. Thus, HIV infection was the most important determinant of microbiota changes in our sample. To better understand the role of HCV and the possible changes in the microbiota it may cause, it would be necessary to compare the microbiota of healthy subjects to see whether the microbiota of healthy subjects is significantly different from the possible changes caused by HCV infection.

Associations of microbiota and markers of microbial translocation. To investigate the possible relationship between gut microbiota and microbial translocation, correlations were calculated. The Shannon index, one of the measures of α diversity, was negatively correlated with LBP and sCD14 in the total study sample, but these correlations disappeared in the individual study

groups, whereas in the HIV-monoinfected group, a negative correlation of α diversity with EndoCAb IgM antibodies was observed. In contrast, α diversity was significantly lower in the HIV-infected groups compared to HCV-monoinfected patients. Taken together, these findings suggest an association of gut microbiota diversity with microbial translocation: as microbiota diversity decreases, microbial translocation from the intestinal tract to the peripheral circulation increases, and in our sample, particularly in HIV-infected patients.

The taxonomic class level was chosen for the analysis of microbiota **correlations**. Our study revealed some interesting correlations, with the most in the group of HIV-monoinfected patients. The relative abundance of ***Bacilli*** was positively correlated with higher blood levels of LBP and HIV RNA, and with lower $CD8^+$ T cell counts, respectively. As we found higher relative abundances of certain families of this class of bacteria in the HIV-infected group, we can suggest that in HIV infection, the abundance of *Bacilli* class bacteria in the intestinal tract increases. Also, with the increase of relative abundance of *Bacilli* class in the gut there is an increase in the level of HIV RNA in the blood, and consequently increases microbial translocation. In contrast, the decrease in *Coriobacteria* was associated with an increase in all established markers of microbial translocation, including LBP, sCD14, EndoCAb IgM and IgG antibodies. *Desulfivibriobacteria* class also had a more significant effect on microbial translocation markers in HIV patients, with negative correlations with sCD14, EndoCAb IgM and IgG antibodies, and a negative correlation with HIV RNA load but a positive correlation with BMI in this group.

This finding supports a more important involvement of certain bacterial species in microbial translocation and maintenance of immune activation in HIV patients, in particular for the more abundant bacterial classes in the microbiota, such as *Coriobacteria* and *Bacilli*. The *Desulfivibrionia* class of bacteria are also referred to as sulphate-reducing bacteria and, although previously considered

commensal, studies confirming the association of this class bacteria with inflammatory bowel disease have recently emerged (Rowan et al., 2010). In a study by *Lazupone et al*. *Desulfovibrio* bacteria were described in higher abundance in HIV-infected patients compared to healthy individuals (Lazupone et al., 2013; Lazupone et al., 2014).

In our study, although the relative abundance of this phylum was low and was even slightly lower in HIV-monoinfected and HIV/HCV-coinfected patients compared to HCV-monoinfected patients, the relative abundances of this class was negatively correlated with both blood HIV RNA levels and markers of microbial translocation, but only in HIV-monoinfected patients. No such correlation was observed in HCV-monoinfected patients. Thus suggests, that *Desulfobacteriota* phylum bacteria play an important role in microbial translocation, especially in HIV infection, which is not observed in HCV-monoinfected patients. Despite the low abundance of these bacteria in the intestinal tract, microbial translocation is also increased in HIV-infected patients. Thus, there may be conditions under which *Desulfibrionia* class bacteria potentiate microbial translocation. One of these is the level of HIV RNA in the blood, which in combination with a higher body mass index and older age, may have a negative impact on the microbial translocation from the gut into the bloodstream. Of course, given the low relative abundance of the *Desulfivibrionia* class in the gut microbiota, its true impact is debatable and larger scale studies would be needed to draw definitive conclusions.

Interestingly, in the HIV/HCV-coinfected cohort, the only bacterial class that showed a reliable association with microbial translocation markers (sCD14, EndoCAb IgG) was the *Gammaproteobacteria* class, of which *Enterobacteriaceae* are the most important members at the family level. Moreover, higher relative abundances of these bacteria were observed in the coinfected group compared to HIV and HCV-monoinfected patients. In addition,

this class of bacteria was negatively correlated with $CD4^+$ T cell count, which was not observed in the HIV-monoinfected group. This finding suggests that microbial translocation increases significantly in HIV/HCV-coinfected patients, especially as HIV infection progresses and $CD4^+$ T lymphocyte counts decline, with a significant increase in *Gammaproteobacteria* in the intestinal tract.

Other researchers have also found correlations at the level of individual bacterial families and species when comparing HIV-infected patients with healthy controls. For example, *Mingjun* and colleagues described higher relative abundances of *Bacilli* and *Gammaproteobacteria* in patients with lower $CD4^+$ T cell counts, while *Clostridia* class bacteria were higher in patients with higher $CD4^+$ T cell counts (Mingjun et al., 2022). In our study, we did not determine $CD4^+$ T cell counts in HCV-monoinfected patients, but assuming that they might be higher or normal in patients without severe immunosuppression compared to HIV-infected people, we can say that these findings are similar. Similar to our study, *Dihn* and colleagues found a positive correlation of microbial translocation markers (sCD14) and certain inflammatory cytokines (Il-1β, IFN-ɣ) with *Enterobacteriaceae*, supporting a greater role of certain bacteria in maintaining microbial translocation and subsequently immune activation (Dihn et al., 2015). However, to date, the number of publications addressing the direct relationship between microbiota and markers of microbial translocation is small, and the work of other researchers is also based on rather small samples of participants and conducted in patients in different geographical locations.

Overall, our findings support the differences in gut microbiota between HIV-infected and HCV-infected individuals. Changes in the diversity of the overall microbiota composition and also in the relative abundance of individual bacterial families have important implications for microbial translocation, which in turn may influence immune activation and maintain a chronic inflammatory process in HIV-infected patients. In contrast, microbial translocation is more

pronounced in HIV-infected patients and is not significantly enhanced by the presence of HCV in HIV infection.

Changes in the gut microbiota in chronic HIV infection suggest that reducing gut inflammation and improving gut barrier function may be effective in improving the prognosis of HIV-infected patients. People living with HIV and/or HCV are also exposed to the long-term use of various medications to treat their disease. Understanding the link between the gut microbiota and chronic immune activation in patients with these diseases may pave the way for microbiome-based interventions (e.g. use of probiotics or prebiotics, faecal transplantation or nutritional interventions). Similar interventions have also been proposed for inflammatory bowel disease, although it must be acknowledged that the results are mixed. However, the observations from this study may also be useful to better understand and develop new microbiome-based adjunctive therapies for HIV infection.

Limiting factors for the study. This study also had some limiting factors that could have influenced the results. One of the factors was the methodology used for collection of faecal samples. The HCV-monoinfected patients' faecal samples were self-collected and transported to the laboratory, therefore it cannot be completely excluded that the faecal samples were stored for more than one hour, which may affect the overgrowth of individual species as well as the total amount of bacterial DNA (Tang et al., 2020). Similarly, the microbiota may be affected by various other factors, such as certain dietary characteristics (Hou et al., 2022) or alcohol consumption (Bajaj et al., 2016), which were not analysed in this study.

The small total number of patients included in the study did not always allow a full assessment of differences between groups with statistical significance. Increasing the number of patients in the groups could have yielded more statistically significant results, especially in the microbiota part of the study

where large individual differences and a large number of detectable microorganisms are present.

Adequate effective and well-tolerated therapy has long been unavailable for HCV patients both in Latvia and worldwide. Direct antivirals were introduced into clinical practice in 2015, so the patients included in the study were likely to have a relatively advanced stage of the disease and more extensive liver damage. HCV patients generally had a higher BMI than HIV-positive patients. Increased BMI may further contribute to the development of non-alcoholic steatohepatitis, which may consequently affect various biochemical parameters as well as the faecal microbiota (Mauzaki et al., 2013; Ramana et al., 2013; Hansen et al., 2015).

Conclusions

1. Microbial translocation is higher in HIV-monoinfected and HIV/HCV-coinfected patients compared to HCV-monoinfected patients. In contrast, no differences in parameters were observed between HIV-mono and HIV/HCV-coinfected groups, suggesting a more significant effect of HIV infection on microbial translocation compared to HCV infection.
2. Indirect marker of liver fibrosis (CK18-M30) is lower in HIV-monoinfected compared to HCV-monoinfected and HIV/HCV-coinfected patients, suggesting a more important role of HCV infection in the development of liver fibrosis.
3. Faecal microbiota diversity and total number of bacterial genes identified are lower in HIV-monoinfected and HIV/HCV-coinfected patients compared to HCV-infected patients, but do not differ between HIV patient groups. This indicates a more significant negative impact of HIV infection on the gut microbiota, and the addition of HCV infection in HIV-infected patients does not impair the overall diversity of the gut microbiota.
4. HIV-monoinfected and HIV/HCV-coinfected patients have different gut microbiota composition from HCV-monoinfected patients, characterised by changes in the relative abundance of individual bacterial taxonomic units. Higher relative abundances of the potentially unfavourable families *Enterobacteriaceae*, *Enterococcaceae* and *Lactobacillaceae* and lower relative abundances of the potentially favourable families *Lachnospiraceae*, *Oscillospiraceae*, *Peptostreptococcaceae* and *Ruminococcaceae* were observed in both HIV-infected patient groups compared to HCV-monoinfected patients.
5. Changes in the relative abundance of certain bacterial classes are associated with greater microbial translocation in HIV-monoinfected patients. In contrast, the association of the relative abundances of individual bacterial

classes with parameters characterising HIV infection suggests that the number of potentially harmful bacteria in the gut increases as HIV infection progresses.

Publications

Scientific papers:

1. **Madelāne, M.,** Krūmiņa, A., Sīmanis, R., Šķenders, Ģ., Ivanovs, A., Stūre, G., Vīksna, L. 2019. Difference in markers of microbial translocation and cell apoptosis in HIV-monoinfected and HIV/HCV-coinfected patients. *Proceedings of the Latvian Academy of Sciences. Section B.* 73 (4), 304–311.

2. Vīksna, L., Ekšteina I., **Madelāne, M.,** Zeltiņa, I., Krūmiņa, A., Koļesovs, A., Sīmanis, R. 2019. Markers of liver fibrosis and apoptosis in patients with HIV mono-infection and HIV/HCV co-infection. *HIV & AIDS Review*. 18 (1), 33–39.

3. Koļesova, O., Eglīte, J., Koļesovs, A., Krūmiņa, A., Ekšteina, I., **Madelāne, M**., Vīksna, L. 2019. Association of non-invasive markers of liver fibrosis with HCV coinfection and antiretroviral therapy in patients with HIV. *Proceedings of the Latvian Academy of Sciences. Section B.* 73 (4), 400–402.

4. Koļesova, O., **Madelāne, M**., Ekšteina, I., Koļesovs, A., Krūmiņa, A., Vīksna, L. 2020. The level of cytokeratin 18 in patients with HIV and viral hepatitis C co-infection in Latvia. *Proceedings of the Latvian Academy of Sciences. Section B.* 74 (2), 94–99.

Abstracts and presentations in international conferences:

1. **Madelāne, M.,** Koļesova, O., Koļesovs, A., Rudzīte, D., Šķenders, Ģ., Vangravs, R., Vīksna, L. 2023. Differences in faecal microbiota diversity and liver related markers in patients with HCV, HIV and HIV/HCV infections. In: *Riga Stradiņš University Scientific Conference 2023*, Riga, Latvia, 2023. (oral presentation).

2. **Madelāne, M.,** Šķenders, Ģ., Rudzīte, D., Ivanovs, A., Vīksna, L. 2019. Microbial translocation markers in HIV and HCV patients. In: *Rīga Stradiņš University Scientific Conference 2019*, Riga, Latvia, 2019, abstract book: 221. (oral presentation).

3. **Madelāne, M**., Stūre, G., Ekšteina, I., Ivanovs, A., Vīksna, L. 2018. Plasma levels of bacteria; LPS and endotoxin antibodies in HIV-monoinfected and coinfected with HCV patients. In: *ISHEID congress 2018*, Marseille, France,abstract book: P 19. (poster presentation).

4. **Madelāne, M**., Stūre, G., Ekšteina, I., Ivanovs, A., Vīksna, L. 2018. Plasma levels of bacteria; LPS and endotoxin antibodies in HIV-monoinfected and coinfected with HCV patients. *Journal of Virus Eradication. Abstracts of the 2018 International Symposium on HIV and Emerging Infectious Diseases (ISHEID)*. 4 (1), 25, P19.

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6. Ekšteina, I., **Madelāne, M**., Koļesovs, A., Vīksna, L. 2018. The levels of hyaluronic acid in different HIV-infected patient groups. *Journal of Virus Eradication. Abstracts of the 2018 International Symposium on HIV and Emerging Infectious Diseases (ISHEID).* 4 (1), 25, P18.

7. **Madelāne, M**., Stūre, G., Ivanovs, A., Vīksna, L. 2018. Serum markers of apoptosis and liver fibrosis in HIV and HCV-coinfected patients. 2018. In: *ECCMID congress 2018*, Madrid, Spain. (electronic poster presentation E0203).

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