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Original Article

Altered blood microbiome in patients with HCV-related Child-Pugh class B cirrhosis

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ABSTRACT

Background: Altered bacterial translocation is associated with changes in hepatic function and the progression from compensated to decompensated cirrhosis. Child-Turcotte-Pugh (CTP) score is an essential indicator of liver severity. Thus, we aimed to study differences in the blood microbiome together with metabolome profile between HCV-infected patients with CTP class B (CTP-B, significant functional compromise) and patients with CTP class A (CTP-A, well-compensated cirrhosis).
Methods: We conducted a cross-sectional study in patients with advanced HCV-related cirrhosis (n = 88) stratified by CTP-B and CTP-A. Bacterial 16S rRNA sequencing was sequenced by MiSeq Illumina technology and non-targeted metabolomics was performed by GC-MS and LC-MS ESI+ and ESI- to complement the analysis.
Results: Patients with CTP-B had lower levels of richness (Chao1), and alpha diversity (Shannon and Simpson indexes) at phylum level than patients with CTP-A. Likewise, we observed significant differences in beta diversity between groups at phylum, class, and order levels, showing lower diversity in patients with CTP-B. Higher relative abundance of Proteobacteria (p = 0.012), Alphaproteobacteria (p = 0.005), Sphingomonadales (p = 0.012) and *Sphingomonadaceae* (p = 0.016) were significantly associated with CTP-B. The phylum Proteobacteria was positively correlated with ethanolamine and oleic acid (p = 0.005 and p = 0.004, respectively) and negatively with p-cresol (p = 0.006). In addition, the order Sphingomonadales

Abbreviations: aAMR, Adjusted arithmetic mean ratio; AMR, Arithmetic mean ratio; ART, Antiretroviral therapy; CTP, Child-Turcotte-Pugh; CTP-A, CTP class A, well-compensated cirrhosis; CTP-B, CTP class B, significant funtional compromise; CTP-C, CTP class C, decompensated cirrhosis; ESI, Electrospray ionization; FDR, False discovery ratio; GC-MS, Gas chromatography-mass spectrometry; GLM, Generalized linear model; HCC, Hepatocellular carcinoma; HCV, Hepatitis C virus; HIV, Human immunodeficiency virus; HVPG, Hepatic venous pressure gradient; INR, International normalized ratio; LC-MS, Liquid chromatography-mass spectrometry; MANOVA, Multivariate analysis of variance; OTU, Operative taxonomic Unit; PCoA, Principal coordinates analysis; PCR, Polymerase chain reaction

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and the family *Sphingomonadaceae* was also negatively correlated with p-cresol (p = 0.001 and p = 0.001).
Conclusions: Blood microbial diversity was significantly decreased in patients with CTP-B, who presented an enrichment of Proteobacteria, Alphaproteobacteria, Sphingomonadales and *Sphingomonadaceae* compared to patients with CTP-A.

Background

Hepatitis C virus (HCV) infection is considered one of the most notable causes of chronic liver disease worldwide. Without antiviral therapy, about 5–20 % of HCV-infected patients will develop cirrhosis, increasing the risk of alterations in liver function, hepatic decompensation, hepatocellular carcinoma (HCC), and death. Traditionally, non-invasive indexes have been used to triage patients with advanced liver diseases. Among them, the Child-Turcotte-Pugh (CTP) score is an essential indicator of severity, determining the cirrhosis status and predicting morbidity and mortality. Serious adverse events and mortality rates are notably higher in patients with CTP class B (CTP-B; significant functional compromise) and CTP class C (CTP-C; decompensated cirrhosis) compared to those with CTP class A (CTP-A; well-compensated cirrhosis) [1].

The gut microbiota and bacterial translocation may influence liver disease progression in patients with viral hepatitis [2]. Likewise, gut microbiota dysbiosis and sustained bacterial translocation have been observed among people living with human immunodeficiency virus (HIV) despite the use of antiretroviral therapy (ART) [3]. In this setting, bacterial overgrowth, increased intestinal permeability, and defects in gut-associated lymphatic tissue in patients with advanced liver disease promote impaired bacterial translocation. This situation leads to severe liver damage by several mechanisms related to persistent immune activation and inflammation [4]. Thus, altered bacterial translocation is associated with changes in liver function and the progression from compensated to decompensated cirrhosis [5].

In this context, the presence in the blood of certain bacteria or bacterial products, and the finding of specific microbiome patterns, could constitute an innovative approach for a better understanding of the pathogenesis of advanced HCV-related cirrhosis. In addition, as microbial functions are closely reflected by the composition of the metabolome, intestinal or oral dysbiosis is frequently accompanied by metabolic changes. Analyzing these changes, we can gain a better understanding of the intricate interaction between bacteria and metabolites.

So far, scarce studies have investigated the blood microbiome and its relation to the metabolomic profile in patients with liver functional compromise. Traykova *et al.* [6] found a markedly higher number of bacterial species in cirrhotic patients with CTP-B than in control individuals. Alvarez-Silva *et al.* [7] described a relationship between the microbiota composition and systemic inflammation in the blood. However, to our knowledge, none of the previous studies focused on studying the role of the blood microbiome and metabolomic profile in HCV-infected patients across different stages of cirrhosis severity.

Objective

We aimed to study differences in the blood microbiome and its relation to metabolomic profile between HCV-infected patients with CTP-B (significant functional compromise) and patients with CTP-A (well-compensated cirrhosis).

Methods

Study subjects

We performed a cross-sectional study in patients with advanced HCV-related cirrhosis with or without HIV, recruited at four tertiary referral hospitals in Madrid (Spain) between January 2015 and June 2016. The study received the approval of the Research Ethics Committee of the Instituto de Salud Carlos III (CEI42_2020, CEI41_2014) and was carried out following the Declaration of Helsinki. All participants of the study gave their written informed consent.

The selection criteria were: 1) demonstrable active HCV infection by polymerase chain reaction (PCR); 2) advanced cirrhosis defined by any of the following criteria: i) prior history of ascites, bleeding esophageal varices, or hepatic encephalopathy; ii) liver stiffness ≥ 25 kPa; iii) CTP ≥ 7); and/or iii) clinically significant portal hypertension defined as a hepatic venous pressure gradient (HVPG) ≥ 10 mmHg; 3) available CTP score; 4) available blood sample for microbiome analysis. HIV/HCV-coinfected patients had a stable ART for over six months and undetectable plasma HIV viral load (< 50 copies/mL).

Samples

Approximately 30–40 mL of whole blood was collected from each patient in EDTA tubes, which were sent to the HIV Biobank (http://hivhgmbiobank.com/?lang=en), where an aliquot of whole blood was directly stored at −80 °C until use and plasma was separated by centrifugation and stored at −80 °C. Samples were sent to National Center of Microbiology for further processing. The patients did not have fever or signs of infection at the time of sampling. The epidemiological and clinical variables were collected using an online form within each center, which fulfilled data confidentiality requirements.

Outcome variable

CTP score was calculated from five routine laboratory parameters and clinical measures of liver disease (serum albumin, total bilirubin, international normalized ratio (INR), ascites, and hepatic encephalopathy) (https://www.hepatitisc.uw.edu/page/clinical-calculators/ctp). The outcome was the presence of significant functional compromise, defined as a CTP-B (range: 7 to 9), compared to patients with well-compensated cirrhosis, defined as CTP-A (range: 5 to 6). There were no patients with CTP-C.

Blood microbiome

After thawing, 50 µl of whole blood samples were used for DNA extraction using an optimized tissue-specific technique, as previously described [8,9]. The quality and quantity of extracted nucleic acids were controlled by gel electrophoresis and NanoDrop 2000 UV spectrophotometer (Thermo Scientific). Subsequent 16S-targeted metagenomic sequencing was performed in a strictly controlled environment at Vaiomer (Toulouse, France), a biotech company expert in tissue and blood microbiota, which uses a rigorous

contamination-aware approach, described and discussed elsewhere [8–10].

Library preparation was performed by two-step PCR amplification using 16S universal primers targeting the V3–V4 region of the bacterial 16S ribosomal DNA (rDNA), as described previously [10]. The resulting amplicon of approximately 467 base pairs was sequenced using 2 × 300 paired-end MiSeq kit V3. For each sample, a sequencing library was generated by the addition of sequencing adapters. The detection of the sequencing fragments was performed using MiSeq Illumina technology.

The targeted metagenomic sequences from microbiota were analyzed using the bioinformatics pipeline established by Vaiomer based on the Find, Rapidly, OTUs with Galaxy Solution guidelines. After demultiplexing the barcoded Illumina paired reads, single-read sequences were independently cleaned and paired for each sample into longer fragments. Operational taxonomic units (OTUs) were produced via single-linkage clustering using the Swarm algorithm and its adaptive sequence agglomeration [11]. The taxonomic assignment was performed against the Silva v132 database to determine taxonomic profiles. The following specific filters were applied for this analysis to obtain the best results: (1) the last ten bases of reads R1 were removed; (2) the last 40 bases of reads R2 were removed; (3) amplicons with a length of < 350 or > 500 nucleotides were removed; (4) OTUs with abundance < 0.005 % of the whole dataset abundance were removed. Negative controls were incorporated throughout the 16S rRNA gene sequencing pipeline to minimize the impact of potential DNA contamination from the environment, particularly from reagents. Thus, negative controls were performed for the DNA extraction and amplification steps with molecular-grade water as the starting material. The sequencing library procedure also included positive controls with a mock community.

Non-targeted metabolomics

Firstly, methanol was mixed with plasma samples (3:1, v/v) for viruses' inactivation. Then, samples were vortexed (15 s), maintained cold for 5 min, centrifuged (16000 g, 20 min, 4 °C), and frozen (−80 °C). After this pre-processing, samples were sent to the Center for Metabolomics and Bioanalysis (CEU-San Pablo University, Pozuelo de Alarcón, Spain) to perform gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) with positive and negative electrospray ionization (ESI) on each sample. Quality controls were prepared by pooling and mixing equal volumes of each corresponding sample independently for each analytical platform (full description in **Supplementary File 1**).

MassHunter Quantitative Unknown analysis (B.07.00, Agilent) was used for the deconvolution and identification process in GC-MS. Alignment was carried out with MassProfiler Professional software (version 13.0, Agilent), and Masshunter Quantitative Analysis (version B.07.00, Agilent) was used for peak integration. In LC-MS, the Molecular Feature Extraction and the Recursive Feature Extraction algorithms in the MassHunter Profinder software (B.08.00, Agilent) were used for deconvolution, peak integration, and alignment of the raw data (more details are available in **Supplementary File 1**).

Statistical analysis

For the descriptive study, quantitative variables were expressed as median (interquartile range) and categorical variables as absolute count (percentage). When comparing data between CTP-B vs. CTP-A groups, we used Fisher's exact test for categorical and unpaired Wilcoxon rank-sum test for continuous variables.

Regarding the metagenomics data, richness (Chao1 estimator), alpha diversity (Shannon and Simpson index), and beta diversity (Weighted Unifrac distance, Bray-Curtis dissimilarity, Jaccard index) of bacterial communities were calculated (Vegan package V. 2.5–7). The differences in richness and alpha diversity indexes between patients with CTP-B and patients with CTP-A were analyzed by the Wilcoxon rank-sum test and multivariable generalized linear model (GLM) with a gamma distribution (diversity indexes as dependent variables, and CTP class as independent), unadjusted and adjusted by HIV coinfection (included as covariate in the model). Besides, we compared beta diversity between groups using principal coordinates analysis (PCoA) plots and permutational MANOVA. These analyses were also adjusted by HIV coinfection.

To analyze differences in the relative abundance of OTUs between groups, we carried out univariable and multivariable analyses. Univariable analysis was performed using the Wilcoxon rank-sum test, a non-parametric test comparing bacterial taxa's relative abundances between groups. The false discovery rate (FDR) was applied for multiple comparisons, and adjusted p-values (q-values) were calculated according to the Benjamini-Hochberg method. Those taxa with $p < 0.05$ and $q < 0.150$ were included in the multivariable models. Multivariable analyses were carried out using the aldex.glm function from the ALDEx2 package (v. 1.22.0) and HIV coinfection was included as a covariate.

Finally, the bacterial taxa significantly different in the relative abundance between groups, were correlated with plasma metabolomic data from different platforms (GC-MS, LC-MS ESI+, and LC-MS ESI-) using the Spearman rank order correlation test. The correlations were considered relevant when $r > 0.3$ or $r < -0.3$ and $q < 0.150$.

All the analyses were carried out by using the R statistical package (R Foundation for Statistical Computing, Vienna, Austria, v. 4.0.5).

Results

Characteristics of the study population

A total of 88 patients were recruited, and their characteristics, stratified by the CTP class (CTP-B vs. CTP-A) are shown in Table 1. Patients with CTP-B (n = 13) had a mean age of 52.1 years, and 92.3 % were male. Among patients with CTP-A (n = 75), the mean age was 53.1 years, and 65 % were male. Of note, CTP-B patients were less frequently coinfected with HIV ($p = 0.019$).

Microbiome analysis

Richness and alpha diversity

CTP-B patients showed lower richness at phylum (Chao1, p = 0.032) and class levels (Chao1, p = 0.020) than CTP-A patients (Fig. 1). Regarding alpha diversity, CTP-B patients showed lower levels of Shannon (p = 0.005) and Simpson indexes (p = 0.003) at the phylum level than CTP-A patients (Fig. 1).

After adjustment for HIV coinfection in a gamma GLM model, we found that CTP-B patients had lower levels of Chao1 estimator at the phylum (adjusted arithmetic mean ratio (aAMR) = 0.85, p = 0.021) and class level (aAMR = 0.85, p = 0.019) than CTP-A. Likewise, after adjustment for HIV coinfection, CTP-B patients showed lower levels of Shannon (aAMR = 0.80, p = 0.005) and Simpson indexes (aAMR = 0.83, p = 0.006) at the phylum level than CTP-A patients (**Supplementary File 2).** Thus, a significant association between CTP-B and richness and alpha diversity was observed regardless of HIV coinfection. In addition, association data for the covariate HIV coinfection are presented in **Supplementary File 3**. HIV coinfection showed no significant association with the different richness and alpha diversity indexes.

Table 1
Clinical and epidemiological characteristics of patients with HCV-related advanced cirrhosis.

Characteristic	CTP-A	CTP-B	P-value
No.	75	13	
Age (years)	53.1 [50.5 −56.1]	52.1 [46.6 −56.4]	0.301
Gender (male %)	49/75 [65.33 %]	12/13 [92.3 %]	0.058
BMI (kg/m^2)	24.2 [22.1 −27.7]	26.7 [23.9 −27.9]	0.198
Smoker			0.389
Never	16/75 [21.3 %]	1/13 [7.7 %]	
> 6 months	17/75 [22.7 %]	5/13 [38.5 %]	
Currently	42/75 [56 %]	7/13 [53.9 %]	
Alcohol intake			0.376
Never	39/75 [52.0 %]	5/13 [38.5 %]	
> 6 months	31/75 [41.3 %]	6/13 [46.2 %]	
Currently	5/75 [6.7 %]	2/13 [15.4 %]	
IVDU	44/75 [58.7 %]	8/13 [61.5 %]	0.999
HCV genotype 1	51/75 [68.0 %]	7/11 [57.1 %]	0.743
HCV viral load (log10)	6.2 [5.7 −6.6]	5.79 [5.2 −6.2]	0.053
Previous anti-HCV treatment	36/75 [48.0 %]	7/13 [53.9 %]	0.770
Statins	9/75 [12.0 %]	0/13 [0 %]	0.345
Coinfection (HIV)	56/75 [74.7 %]	5/13 [38.5 %]	**0.019**
CD4$^+$/mm^3	446.5 [239.3 −719.5]	378.0 [164.0 −685.0]	0.502
Nadir CD4 cells	119.0 [63.0 −232.5]	207.0 [144.0 −300.0]	0.131
AIDS	37/56 [66.1 %]	3/5 [60.0 %]	0.999

Statistics: Continuous variables were expressed as median [interquartil range], and p-values were calculated by the unpaired Wilcoxon rank-sum test. Categorical variables were expressed as absolute count [percentage], and p-values were calculated by the Fisher exact test.
Abbreviations: BMI, Body mass index; IVDU, Intravenous drugs user; HCV, Hepatitis C virus; HIV, Human immunodeficiency virus; AIDS, Acquired immune deficiency syndrome, CTP-A: Child-Pugh class A, CTP-B, Child-Pugh class B.

Beta diversity

Beta-diversity was significantly different in CTP-B patients compared to those with CTP-A, showing lower beta-diversity at the phylum level (Jaccard: p = 0.049), class level (Weighted Unifrac: p = 0.040, Bray-Curtis: p = 0.017, Jaccard: p = 0.028), and order level (Weighted Unifrac: p = 0.016, Bray-Curtis: p = 0.047) in CTP-B patients, as determined using PCoA plots and permutational MANOVA. In addition, these significant differences were maintained when models were adjusted by HIV coinfection at the phylum level (Jaccard: adjusted p = 0.046), class level (Weighted Unifrac: adjusted p = 0.033, Bray-Curtis: adjusted p = 0.018), and order level (Weighted Unifrac: adjusted p = 0.019, Bray-Curtis: adjusted p = 0.048) (Fig. 2**, Supplementary File 4),** which means that CTP-B was significantly associated with changes in beta diversity regardless of HIV coinfection. In addition, note that the association data for the covariate HIV coinfection showed only association with the Jaccard index (p = 0.046) at the phylum level and Bray-Curtis (0.043) and Jaccard (p = 0.005) at the class level (**Supplementary File 5).**

Relative abundances

We assessed the differences in the relative abundance at all taxonomic levels between CTP-B and CTP-A patients. CTP-B patients had a higher relative abundance of the phylum Proteobacteria (p = 0.004), the class Alphaproteobacteria (p ≤ 0.001), the orders Sphingomonadales (p ≤ 0.001) and Oceanospirillales (p = 0.047), families *Sphingomonadaceae* (p = 0.002), *Paenibacillaceae* (p = 0.038) and *Microbacteriaceae* (p = 0.043), and genus *Bradyrhizobium* (p = 0.001), *Sphingomonas* (p = 0.009) and *Rhodococcus* (p = 0.027) than CTP-A patients. However, only the phylum Proteobacteria (q = 0.037), the class Alphaproteobacteria (q = 0.006), the order Sphingomonadales (q = 0.036), family *Sphingomonadaceae* (q = 0.090) and genus *Bradyrhizobium* (q = 0.087) were considered relevant after adjusting for multiple comparisons (FDR, q < 0.150) (Table 2**, Supplementary File 7**). When HIV coinfection was taken into account for adjusting the models by ALDEx2, a higher relative abundance of Proteobacteria (p = 0.012), Alphaproteobacteria (p = 0.005), Sphingomonadales (p = 0.012) and *Sphingomonadaceae* (p = 0.016) remained significantly associated with CTP-B, regardless of HIV coinfection. Boxplots for the relative abundances of these significant bacterial taxa are shown in **Supplementary File 6**. In these adjusted models, we found no significant association for HIV coinfection (**Supplementary File 7**).

Correlation analysis with metabolomic data

The correlation between bacterial taxa significantly related to CTP-B and metabolomic data from different platforms was explored. Regarding GC-MS data, we observed a significant positive correlation of the phylum *Proteobacteria* with ethanolamine and oleic acid (r = 0.330, p = 0.005, q = 0.115, and r = 0.302, p = 0.004, q = 0.115, respectively) and a significant negative correlation with p-cresol (r = −0.308, p = 0.006, q = 0.115). In addition, the order *Sphingomonadales* and the family *Sphingomonadaceae* were negatively correlated with p-cresol (r = −0.354, p = 0.001, q = 0.112, and r = −0.358, p = 0.001, q = 0.096, respectively) (Fig. 3**, Supplemental File 8**). Regarding LC-MS data (LC-MS ESI+, and LC-MS ESI-), we found a significant negative correlation between the order *Sphingomonadales* and the family *Sphingomonadaceae* with p-cresol (r = −0.367, p = 0.001, q = 0.122, and r = −0.380, p = 0.001, q = 0.080, respectively). (Fig. 3**, Supplementary File 8**).

Discussion

We compared the blood microbiome between HCV-infected patients with CTP-B and CTP-A, finding lower richness, alpha, and beta diversity in the first ones. We also found that the relative abundance of Proteobacteria, Alphaproteobacteria, and Sphingomonadales was higher in CTP-B patients. The dysbiosis of the blood microbiome was accompanied by changes in metabolites related to liver reparation processes and microbiota fermentation.

Although several studies have previously investigated the blood microbiome in cirrhosis, few articles have described its role among HCV-infected cirrhotic patients. Recently, Gedgaudas *et al.* found that circulating microbiome profiles in cirrhotic patients with portal hypertension were distinct from those of healthy individuals, with enrichment of the genera *Bacteroides, Escherichia/Shigella,* and *Prevotella* [12]. However, this study analyzed patients with cirrhosis of different etiologies and examined plasma instead of whole blood [12]. Traykova *et al.* studied the blood microbiome in nine patients with CTP-B patients compared to controls, finding that the bacterial microbiome is related to changes in systemic vascular resistance and cardiac outcomes [6]. However, they only screened for 53 specific bacteria from the gut in the blood [6]. Moreover, our group previously found an association between specific bacterial taxa before HCV therapy, such as *Corynebacteriales* and *Massilia,* and decreased HVPG in patients with HCV-related cirrhosis after direct-acting antiviral therapy for HCV [13]. Thus, to our knowledge, this is the first study in which the circulating microbiome from HCV-infected patients with CTP-B and CTP-A has been compared, providing new insight into the potential mechanisms underlying this liver injury status.

In our study, cirrhotic patients with CTP-B had lower richness and alpha diversity for different taxonomic ranks than patients with CTP-A. These findings are consistent with previous data supporting that less diverse gut microbiome ecosystems are associated with poorer body health status, probably because reduced species diversity leads to lower efficient systems [14]. Regarding viral hepatitis and gut microbiome, a less diverse microbiome has been previously described in the gut of cirrhotic patients with hepatitis B and C compared to healthy controls, finding significant differences in the

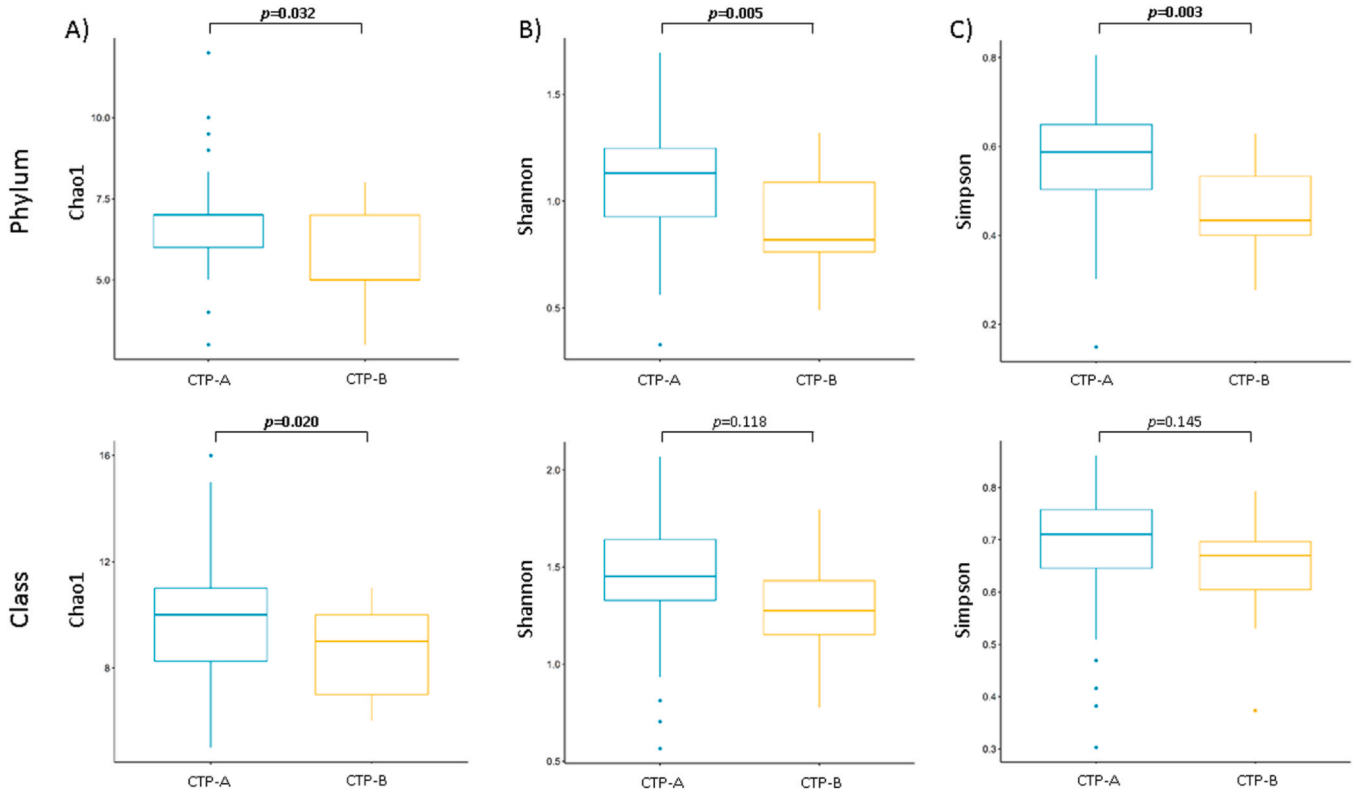

Fig. 1. Representation of richness and alpha-diversity indexes for patients with CTP-A and CTP-B. A) Chao1 estimator: it is calculated by taking the number of different species and also considering as relevant the number of species that were detected once (singletons) or twice (doubletons). B) Shannon index: it is based on the formula to describe entropy and how difficult it is to predict the next microbe detection when the diversity is high. C) Simpson index: it measures the probability that two individuals randomly selected from a sample will belong to the same taxa. Differences between groups were calculated by using Wilcoxon signed-rank test and expressed as p-values. **Abbreviations:** CTP-A: Child Pugh class A, CTP-B: Child Pugh class B.

microbiota community between groups [15,16]. In this line, while changes in the diversity of gut microbiome are being deeply studied, there is scarce data about diversity changes in blood microbiome among patients with viral hepatitis. A recent study described a trend towards lower bacterial diversity with worse disease stages in the context of hepatitis B and D infection in serum [17], consistent with our results in patients with HCV-related cirrhosis.

Regarding the beta diversity, which represents the number of overlapping taxa between samples, microbiome signature differences between CTP-A and CTP-B patients were consistently found for phylum, class, and order taxonomic ranks and corroborated by several indexes, with PCoA helping us to visualize these differences. In previous studies, separate clustering between patients with liver disease induced by viral hepatitis C and healthy controls was described in the gut [18]. However, similar to alpha diversity, information in the literature is sparse, and to our knowledge, this is the first report studying differences in beta diversity between different cirrhosis stages in blood.

We also detected an association of a higher relative abundance of Proteobacteria, Alphaproteobacteria, Sphingomonadales and *Sphingomonadaceae* with CTP-B. Proteobacteria has been described as a potential microbial marker of dysbiosis and disease risk [19]. A study by Sun *et al.* documented that the phylum Proteobacteria, among others, was increased in patients with chronic hepatitis B cirrhosis[16]. In addition, although Wei *et al.* found no significant difference in the fecal microbiota of cirrhotic patients with CTP-A compared to control individuals, they reported an elevated abundance of this phylum in those with CTP-B compared to control subjects [20], which is consistent with the higher Proteobacteria abundance in CTP class B compared to class A observed in blood in our study. Alphaproteobacteria, a class rank belonging to the phylum Proteobacteria, is classified as one of the most diverse bacterial subdivisions influencing host-cell proliferation [21]. We found that the order Sphingomonadales, which is part of the class Alphaproteobacteria, appears to be relatively more abundant in patients with CTP-B. Bacteria from the Sphingomonadales order are known to have lipoxygenases that may have a vital role in bacteria-host signaling [22]. In addition, the class Alphaproteobacteria and the order Sphingomonadales have recently been documented as an important indirect mediator affecting the degree of liver steatosis through histone acetyltransferase activity [23]. All these findings in the literature support the significant association with CTP-B observed in our study. Regarding the *Sphingomonadaceae* family, we found an association between higher relative abundance of this family and CTP-B. In this context, cirrhosis has recently been linked to infections with these organisms [24], which is consistent with our results. However, further studies would be needed to confirm our findings.

Dysbiosis in environments such as the gut or oral cavity often goes hand in hand with metabolic changes and disease progression [25]. Regarding the blood, we observed that metabolic changes also accompanied microbiome dysbiosis. The phylum Proteobacteria was directly associated with two metabolites in this study, ethanolamine and oleic acid. Firstly, ethanolamine, a source of nitrogen and carbon by diverse bacteria, has been associated with liver reparation processes in the damaged liver [26]. Secondly, oleic acid is a monounsaturated fatty acid with numerous beneficial properties in various animal and vegetable sources. In liver injuries, oleic acid recruitment from other tissues has been described as one of the rescue systems [27]. Some studies have observed its role in inhibiting chemotaxis and attenuating inflammation [28]. Additionally, we found an inverse correlation of the relative abundance of the phylum Proteobacteria, the order Sphingomonadales, and the

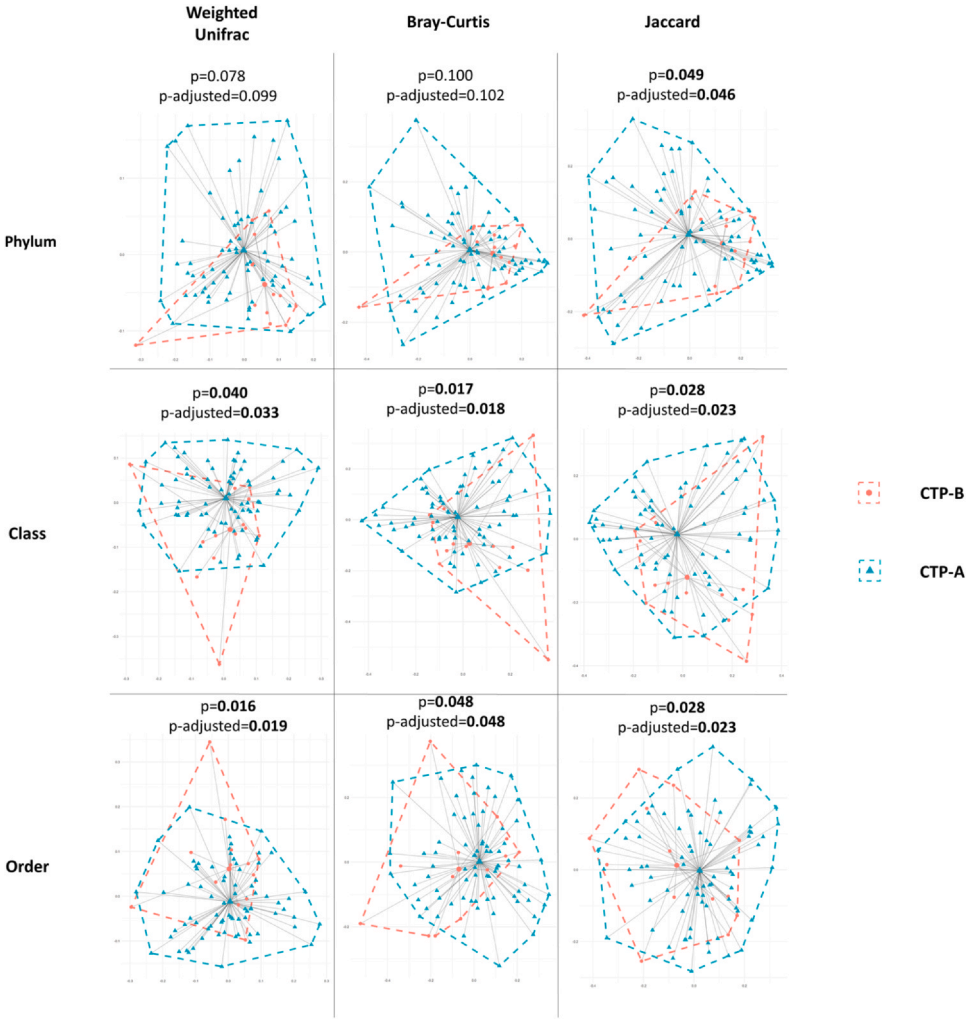

Fig. 2. Principal coordinates analysis (PCoA) representing the beta diversity for patients with CTP-A and CTP-B. Statistics: P-values were calculated from a permutational univariate and multivariate analysis of variance adjusted for HIV-coinfection. **Abbreviations:** CTP-A: Child Pugh class A, CTP-B: Child Pugh class B.

Table 2
Association between the relative abundance of blood bacterial taxa and HCV-related Child-Pugh class B cirrhosis.

Bacterial taxa		Univariate		Multivariate	
Taxonomic rank	**Taxa**	**p-value**	**FDR**	**Estimate**	**p-value**
Phylum	Proteobacteria	**0.004**	**0.037**	1.36	**0.012**
Class	Alphaproteobacteria	**≤ 0.001**	**0.006**	2.48	**0.005**
Order	Sphingomonadales	**≤ 0.001**	**0.036**	4.73	**0.012**
	Oceanospirillales	**0.047**	0.810	-	-
Family	*Sphingomonadaceae*	**0.002**	**0.090**	4.54	**0.016**
	Paenibacillaceae	**0.038**	0.759	**-**	**-**
	Microbacteriaceae	**0.043**	0.759	**-**	**-**
Genus	*Bradyrhizobium*	**0.001**	**0.087**	2.81	0.103
	Sphingomonas	**0.009**	0.377	-	**-**
	Rhodococcus	**0.027**	0.623	-	**-**

Statistics: P-values were calculated using univariate (Wilcoxon rank-sum test) and multivariate models (ALDEx2 package), adjusted by HIV coinfection as a covariate. FDR was estimated by Benjamini and Hochberg's correction.
Abbreviations: FDR, false discovery ratio; HCV, hepatitis C virus.

family *Sphingomonadaceae* with the p-cresol, found by different analytical platforms (GC-MS and LC-MS). P-cresol originates from fermentation in the microbiota. Based on previous microbiological studies, since bacteria of the phylum Proteobacteria are Gram-negative, their cell envelope is more sensitive to p-cresol, so it is consistent that in the absence of this metabolite, these taxa could grow freely in the blood and promote dysbiosis [29]. Besides, Ikematsu *et al.*, in forensic autopsy cases, suggested that low levels of p-cresol in blood could be explained by an accumulation of p-cresol in the liver in cases of liver diseases [30].

Additionally, it is crucial to note that a strict contamination-aware approach was used in this study. Firstly, any potential contamination by needle with the skin microbiome was prevented by the high volume of blood withdrawn. Secondly, positive and negative controls were used throughout the sequencing pipeline in order to control any impact of the environment on the results. Thirdly, to

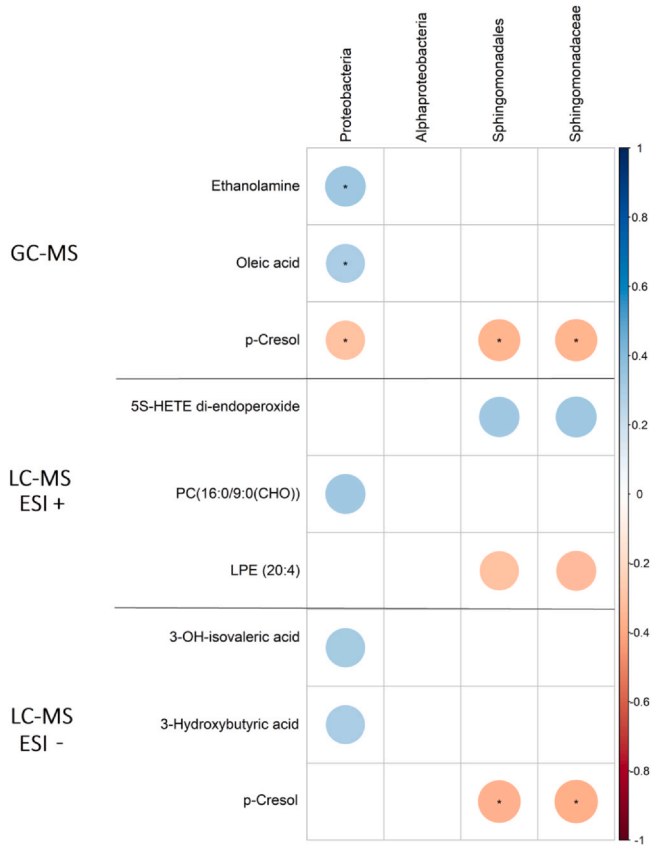

Fig. 3. Correlation between blood significant bacterial taxa and plasma metabolites. Statistics: Direct correlations are shown in blue while inverse correlations are shown in red. Asterisks indicate those correlations with a value of p-value < 0.050 and q-value ≤ 0.150 (q-values (FDR) were calculated from Spearman correlation p-values).

avoid possible confusion with HIV coinfection, multivariate models including HIV coinfection as a covariate were carried out, controlling the potential confounding effects of HIV coinfection. Finally, adjustment for multiple comparisons was performed, avoiding false significant associations. All of these approaches provide robustness to our data.

The following considerations should be considered for a correct interpretation of the results. Firstly, this study had a cross-sectional design; therefore, it does not allow us to determine the causal relationship of the findings. Further studies with follow-up data on the prognosis of the patients would be needed for investigating the long-term clinical relevance of blood microbiome. Secondly, the sample size was limited, which could restrict the statistical power to detect other significant differences in bacterial taxa between groups. Moreover, we could not carry out the statistical analysis separately for HCV-monoinfected and HIV/HCV-coinfected patients due to the limited sample size. Nevertheless, possible confusion with HIV coinfection was ruled out by including HIV status as a covariate in multivariate models. Thirdly, although 16S sequencing has great benefits and is commonly used in microbiome analysis, comparing the obtained results with those deposited in repositories from previous studies using different technologies would have enhanced the robustness of the findings and helped validate the findings functionally. Unfortunately, our study is the first one studying the circulating microbiome from HCV-infected patients with CTP-B and those with CTP-A, and thus, data from similar studies are not available. However, metabolomic analysis has been performed by different platforms (GC-MS and LC-MS) to capture biologically meaningful links.

Conclusions

In conclusion, blood microbial diversity was significantly decreased in HCV-related cirrhotic patients with CTP-B, who presented an enrichment of Proteobacteria, Alphaproteobacteria, Sphingomonadales and *Sphingomonadaceae*, compared to patients with CTP-A. The dysbiosis of the blood microbiome was accompanied by metabolomic changes. Further studies are needed to decipher the impact that blood microbial dysbiosis could have on these patients and strategies to correct it.

Ethical approval statement

The study received the approval of the Research Ethics Committee of the Instituto de Salud Carlos III (CEI42_2020, CEI41_2014).

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Declaration of Competing Interest

The authors declare that they have no competing interests. The funding sources played no role in the study's design, collection, analysis, interpretation of the data, or manuscript writing.

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Consent for publication

Not applicable.

Authorship contribution

Funding body: MAJS, SR; Study concept and design: MAJS; Patients' selection and clinical data acquisition: CD, VH, JB, JGG, LIS, ELH, AO, LPL.; Sample preparation, and biomarker analysis: OBK, AVB, AFR, DR, CB.; Statistical analysis and interpretation of data: OBK, MR, MAJS.; Writing of the manuscript: OBK, MAJS.; Critical revision of the manuscript for relevant intellectual content: JB, JGG, DR, CB, AFR, MR, SR.; Supervision and visualization: MAJS. All authors read and approved the final manuscript.

Appendix

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jiph.2024.102524.

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