BLOOD MICROBIOME IS ASSOCIATED WITH CHANGES IN PORTAL HYPERTENSION AFTER SUCCESSFUL DIRECT-ACTING ANTIVIRAL THERAPY IN PATIENTS WITH HCV-RELATED CIRRHOSIS

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Abstract

Background: Patients with a significant decrease in hepatic venous pressure gradient (HVPG) have a considerable reduction of liver complications and higher survival after hepatitis C virus (HCV) eradication. We aimed to evaluate the association between the baseline blood microbiome and the changes in HVPG after successful direct-acting antiviral (DAA) therapy in patients with HCV-related cirrhosis.

Methods: We performed a prospective study in 32 cirrhotic patients (21 HIV positive) and clinically significant portal hypertension (HVPG≥10 mmHg). Patients were assessed at baseline and 48 weeks after HCV treatment completion. The clinical endpoint was to decrease ≥20% in HVPG or HVPG <12 mmHg at the end of follow-up. Bacterial 16S ribosomal DNA was sequenced using MiSeq Illumina technology, inflammatory plasma biomarkers by ProcartaPlex immunoassays, and metabolome by GC-MS.

Results: During the follow-up, 47% of patients reached the clinical endpoint. Those patients had at baseline a higher relative abundance of *Corynebacteriales* and *Diplorickettsiales* orders, *Diplorickettsiaceae* family, *Corynebacterium* and *Aquicella* genus, and *Undibacterium parvum* specie; and lower of *Oceanospirillales* and *Rhodospirillales* orders, *Halomonadaceae* family, and *Massilia* genus compared to those who did not achieve the clinical endpoint according to the LEfSe algorithm. *Corynebacteriales* and *Massilia* were consistently found within the 10 bacterial taxa with the highest differential abundance between groups. Additionally, the relative abundance of *Corynebacteriales* order was inversely correlated with IFN-gamma, IL-17A, and TNF-alpha levels and *Massilia* genus with glycerol and lauric acid.

Conclusions: Baseline-specific bacterial taxa are related to HVPG decrease in patients with HCV-related cirrhosis after successful DAA therapy.

Introduction

Globally, an estimated 71·million individuals have hepatitis C virus (HCV) infection,[1], the leading cause of chronic liver disease. In the absence of antiviral therapy, about 5–20% of patients with chronic hepatitis C develop cirrhosis, a disease that supposes a significant risk for liver failure, hepatocellular carcinoma, and death.[2]

The introduction of direct-acting antivirals (DAAs) has revolutionized HCV therapy, with excellent antiviral efficacy and very high cure rates.[3] However, it has been described that portal hypertension does not reverse in a significant number of patients who achieve sustained virological response (SVR), indicating a persistent risk of progression and death.[4] Portal hypertension is a severe complication of chronic liver diseases, responsible for most clinical outcomes of cirrhosis.[5] Hepatic vein catheterization with the hepatic venous pressure gradient (HVPG) measurement is currently the most accurate method to assess the presence and severity of portal hypertension and the best available predictor of liver-related outcomes. In response to therapy, patients with a decrease in HVPG ≥20% or a value of HVPG <12 mmHg are defined as responders,[6] having a considerable reduction of complications and higher survival.

Numerous studies have suggested a relevant influence of bacterial translocation in the development of liver diseases related to various viral hepatitis and the pathogenesis and complications of cirrhosis.[7,8] Likewise, recent data show that bacteria in the blood can play an essential role in the pathogenesis of diseases.[9-13] Concerning liver diseases, compositional and functional changes in the blood microbiome have been described in liver fibrosis and cirrhosis. In this regard, Lelouvier *et al*. found a correlation between blood 16S ribosomal DNA (rDNA) concentration and liver fibrosis in obese patients.[14] Santiago *et al*. described an alteration of the serum microbiome composition in cirrhotic patients with ascites.[15] Traykova *et al*. found a higher number of bacterial species and total bacterial DNA in the blood of a cirrhotic cohort compared to healthy individuals,[16] and Alvarez-Silva *et al*. suggested the existence of a relationship between systemic inflammation and microbiota composition in blood among decompensated cirrhotic patients.[17] However, no previous studies have described the role of the blood microbiome on the development or evolution of HCV-associated liver disease. Furthermore, metabolomics studies looking at pathogen and damage-associated molecular patterns and endotoxins have shown a clear correlation to the microbiota, supporting the relationship between dysbiotic microbiota and systemic inflammation.[18]

Therefore, since there are no data on blood microbiome in patients with HCV-related cirrhosis after HCV eradication by DAA therapy, this study aimed to evaluate the influence of the blood microbiome at baseline on the changes in HVPG after successful DAA therapy in patients with HCV-related cirrhosis. In addition, we integrated microbiome data with the use of metabolomics and inflammatory markers.

Materials and methods

Study subjects

We performed a multicenter prospective study in patients with advanced HCV-related cirrhosis from the cohort ESCORIAL (see **Acknowledgements section**) enrolled in Madrid, Spain, from January 2015 to June 2016. All participants gave written informed consent to participate in the study. This study was conducted following the Declaration of Helsinki and received the approval of the Research Ethics Committee of the Instituto de Salud Carlos III (CEI41_2014, CEI42_2020).

The selection criteria were: 1) active HCV infection confirmed by PCR; 2) advanced cirrhosis defined by any of the following criteria: i) prior history of liver decompensation (ascites, bleeding esophageal varices, hepatic encephalopathy); ii) liver stiffness ≥25 kPa, and iii) Child-Turcotte-Pugh score ≥7); 3) clinically significant portal hypertension defined as an HVPG ≥ 10 mmHg; 4) initiation of all-oral DAA therapy and achieving an SVR. HIV/HCV-coinfected patients had undetectable plasma HIV viral load (<50 copies/mL) and stable antiretroviral therapy for more than six months.

Samples and clinical data

From each patient, 30-40 mL of whole blood was collected in ethylenediaminetetraacetic acid tubes. Aliquots of whole blood and plasma samples obtained by centrifugation were stored frozen (-80°C) in the HIV BioBank (http://hivhgmbiobank.com/?lang=en) until use. Baseline epidemiological, clinical, and virological variables were recorded prospectively using an online form within each center. HVPG was calculated by the difference between wedged hepatic venous pressure and free hepatic venous pressure at baseline and 48 weeks after HCV treatment completion, following well-established recommendations.[19] The clinical endpoint was to achieve a decrease ≥20% in HVPG or an HVPG <12mmHg 48 weeks after completing HCV treatment.

Blood microbiome

As previously described, DNA was extracted from 50 µl of each blood sample using an optimized tissue-specific technique.[20-22] Library preparation was performed by two-step PCR amplification using 16S universal primers targeting the V3–V4 region of the bacterial 16S rDNA, as described previously.[23] For each sample, a sequencing library was generated by adding sequencing adapters (full description in **Supplementary data 1**). The detection of the sequencing fragments was performed using MiSeq Illumina technology. DNA extraction and 16S rDNA sequencing were performed in a strictly controlled environment by Vaiomer (Toulouse, France) with a previously described protocol.[20, 22, 23]

The targeted metagenomic sequences from microbiota were analyzed using the bioinformatics pipeline established by Vaiomer based on the Find, Rapidly, operational taxonomic units (OTUs) with Galaxy Solution guidelines.[24] OTUs were produced via single-linkage clustering using the Swarm algorithm and its adaptive sequence agglomeration.[24] The taxonomic assignment was performed against the Silva v132 database to determine taxonomic profiles (full description in **Additional File 1**). Positive and negative controls were added to ensure the low impact of possible DNA contamination.

Non-targeted metabolomics

Plasma samples were inactivated for viruses by mixing methanol for ultra-performance liquid chromatography supergradient with plasma (3:1, v/v). Next, vortexing (15 seg), maintenance cold for 5 min, centrifugation (16000 g, 20 min, 4°C), and freezing at 80°C were performed before the shipment of samples to the Center for Metabolomics and Bioanalysis (San Pablo-CEU University, Pozuelo de Alarcón, Spain). The samples were processed for the subsequent measurement by gas chromatography-mass spectrometry on the day of analysis. Quality controls samples were prepared by pooling and mixing equal volumes of each corresponding sample. A GC system (Agilent Technologies 7890A) was used to analyze samples injected through a GC-Column DB5-MS with a pre-column (full description in **Supplementary data 1**).

The deconvolution and identification were performed using MassHunter Quantitative Unknowns Analysis (B.07.00, Agilent), alignment with MassProfiler Professional software (version 13.0, Agilent), and peak integration using MassHunter Quantitative Analysis (version B.07.00, Agilent).

Cytokines

ProcartaPlexTM multiplex immunoassay (Bender MedSystems GmbH, Vienna, Austria) was used to measure the plasma concentrations of IFN-γ, IL1-β, IL10, IL12p70, IL17A, IL2, IL4, IL6, TNF-α, according to the manufacturer's specifications using a Luminex 200™ analyzer (Luminex Corporation, Austin, TX, United States).

Statistical analysis

For the descriptive study, categorical variables were shown as absolute count (percentage), and quantitative variables were expressed as median (interquartile range). Comparisons between groups were carried out using the Chi-square test or Fisher's exact test for categorical data and the Mann-Whitney U test for continuous variables.

Regarding 16S metagenomic data, we used both univariable and multivariable methods to evaluate differential abundance between groups. As univariable methods, we applied: i) A linear discriminative analysis (LDA) effect size (LEfSe) method algorithm (Galaxy software; https://huttenhower.sph.harvard.edu/galaxy/) to identify the bacterial taxa with the largest differences in relative abundance between groups.[25] Only taxa with an LDA score>2 and a significance of alpha <0.05 were selected; ii) Wilcoxon test: nonparametric test to compare relative abundances of bacterial taxa between groups. Also, we applied statistical methods that allowed adjustment for covariates, including age, sex, and coinfection with HIV in the multivariable models, in order to avoid potential confounders. In this regard, we applied: iii) ALDEx2:[26] the relative abundances were estimated by repeated samplings from a Dirichlet distribution and centered-log ratio transformed before fitting the generalized linear model (via aldex.glm); iv) MetagenomeSeq:[27] a zero-inflated Gaussian model, where the count distribution was modeled as a mixture of two distributions, a point mass at zero and a normal distribution. Since OTUs are usually sparse, the zero counts are modeled with the former, and the rest of the log-transformed counts are modeled as the latter distribution.

Moreover, Spearman correlation was carried out to investigate the relationship between the relative abundance of significant bacterial taxa and inflammatory biomarkers and plasma metabolites. Using the false discovery rate (FDR) with Benjamini and Hochberg procedure, P-values were corrected for multiple testing.

The statistical analysis was carried out with SPSS (SPSS INC, Chicago, IL, USA) and R statistical package (R Foundation for Statistical Computing, Vienna, Austria).

Results

Characteristics of the study population

Table 1 describes the characteristics of 32 patients with advanced HCV-related cirrhosis (11 HCV-monoinfected and 21 HIV/HCV-coinfected). Overall, the median age was 52.9 years, 65.6% were men, 56% were current smokers, and 50% had a prior history of injection drug use. Regarding virological aspects, 50% had previously failed interferon-based therapy, 64.5% were infected with HCV-genotype 1, 65.6% were coinfected with HIV, and 38.1% had AIDS before.

During the follow-up, 47% of patients reached the clinical endpoint (decrease of HVPG ≥ 20% and/or HVPG<12mmHg 48 weeks after completing HCV treatment). Patients who achieved the endpoint showed similar characteristics to those who did not, except for HIV coinfection (p=0.019).

Microbiome data

We evaluated the differences at all taxonomic levels between endpoint groups using the LEfSe algorithm (**Figure 1A**). The cladogram shows the differences at various taxonomic levels starting from phylum levels at the inner circle to class, order, family, genus, and species levels toward the periphery (**Figure 1B**). In this analysis, *Corynebacteriales* (LDA=3.53, p=0.010), *Diplorickettsiaceae* (LDA=3.07, p=0.014), *Diplorickettsiales* (LDA=3.06, p=0.014), *Corynebacterium* (LDA=2.96, p=0.025), *Aquicella* (LDA= 2.89, p=0.005) and *Undibacterium parvum* (LDA=2.60, p=0.025) had higher relative abundance in patients who reached the endpoint; while *Halomonadaceae* (LDA=2.50, p=0.049), *Oceanospirillales* (LDA=2.56, p=0.049), *Rhodospirillales* (LDA=2.60, p=0.049), and *Massilia* (LDA=3.38, p=0.031) had higher relative abundances in patients who not reached the endpoint. Of them, *Corynebacteriales* and *Massilia* were consistently found within the 10 bacterial taxa with the highest differential abundance between groups according to all the statistical methods used (LEfSe, ALDEx2, MetagenomeSeq, and Wilcoxon) and also after adjustment for age, sex, and HIV coinfection in multivariate methods (ALDEx2 and MetagenomeSeq), indicating that its abundances were associated with the endpoint, regardless of age, sex, and HIV coinfection (**Figure 2, Table S1**).

Correlation analysis

Table 2 shows the statistically significant correlations found between the bacterial taxa in the blood and the plasma markers of inflammation and metabolites at baseline. Relative abundance of *Corynebacteriales* order was inversely correlated with IFN-γ, IL17A and TNF-α

(r=-0.743, q=0.041; r=-0.430, q=0.042; r=-0.454, q=0.041, respectively) and *Massilia* genus abundance was inversely correlated with glycerol and lauric acid (r=-0.541, q=0.047; r=-0.585, q=0.030).

Discussion

We assessed for the first time the association of blood microbiome with changes in HVPG after successful DAA therapy in patients with HCV-related liver cirrhosis. We found a higher relative abundance of *Corynebacteriales* order at baseline in patients achieving a decrease ≥20% in HVPG or HVPG<12mmHg 48 weeks after completing HCV treatment. In contrast, a higher relative abundance of *Massilia* genus at baseline was observed in those not achieving this endpoint.

The presence of bacterial DNA in blood has been previously described. Specifically, blood bacterial DNA has been detected in about 90% of cirrhotic patients without clinical evidence of infection, suggesting that the blood microbiome could stimulate inflammatory signaling pathways,[16] and contribute to the pathogenesis of cirrhosis. Our study shows a higher relative abundance of *Corynebacteriales* order at baseline in those patients who reached the portal pressure endpoint. The *Corynebacteriales* order encompasses a group of Gram-positive bacteria widely distributed in nature, including corynebacteria, nocardia, mycobacteria, rhodococci, and other related microorganisms.[28] Our results agree with previous studies, where a greater abundance of the *Corynebacteriales* order was found in patients with ulcerative colitis that improved significantly after treatment of the disease,[29] decreasing the levels of proinflammatory cytokines. However, the role of *Corynebacteriales* in liver diseases is still unknown. To further explore its potential role in patients with HCV-related cirrhosis, we studied its correlation with inflammatory and metabolic markers. In this context, an inverse correlation between the relative abundance of *Corynebacteriales* order and plasma levels of several inflammation-related biomarkers was found, being IFN-γ, IL17A, and TNF-α the most significantly associated markers. Among them, elevated levels of IFN-γ are associated with hepatic dysfunction in fibrosis, cirrhosis, and hepatocellular carcinoma.[30] Likewise, the Th17/IL17 axis is involved in fibrogenesis, activation of stellate cells, and increased expression of profibrotic factors in patients with HCV liver disease.[31] Additionally, higher levels of TNF-α are associated with liver disease progression and hepatocellular carcinoma.[32] Therefore, the favorable role of the higher relative abundance of *Corynebacteriales* order at baseline could be explained by its inverse correlation with inflammatory markers previously linked to liver disease progression. This supposes a highly relevant finding, but additional studies would be interesting to corroborate these findings.

Another bacterial taxa that showed a relevant role in HVPG after achieving SVR was the *Massilia* genus since a higher relative abundance of *Massilia* in blood at baseline was

associated with a worse evolution of portal hypertension. This bacterium was described in 1998 in the blood of an immunocompromised individual with cerebellar lesions.[33] However, its pathogenicity was described as unknown. Since then, *Massilia* genus has been found in different human fluids, such as blood, cerebrospinal fluid, bone, and eye.[33-35] Regarding its role in infectious diseases, it has been observed that the nonpathogenic bacterium *Massilia* sp. can efficiently reactivate HIV-reservoir by secreting an HIV-1-reactivating protein factor (HRF) in HIV-infected patients.[36] In our study, although HIV/HCV-coinfected patients had stable antiretroviral therapy and undetectable plasma HIV viral load, the presence of *Massilia* genus and the HRF secretion could have an additional unknown role on the liver disease, contributing to a worse recovery after HCV eradication in this subset of patients. In addition, HRF leads to a high nonsustained peak in nuclear factor kappa B (NF-kB); however, it has not been associated with increased expression of proinflammatory genes induced by NF-kB.[36] This evidence agrees with our results since we also observed an absence of correlation between the relative abundance of *Massilia* genus and inflammation-related biomarkers.

When we explored the relationship between *Massilia* and metabolic molecules, we found that increased relative abundance of *Massilia* genus was correlated with decreased plasma levels of glycerol and free fatty acids. In this setting, a metabolic effect of *Massilia* was previously described. Some *Massilia* genus members have shown the capacity to degrade aromatic compounds and the ability to accumulate polyhydroxyalkanoates (PHAs) as intracellular granules.[37] Thus, the decreased level of glycerol could be due to its use for PHA production,[38] which is involved in collagen deposition and enhanced fibrosis.[39] On the other hand, *Massilia* genus was inversely correlated with several fatty acids, such as lauric acid, palmitic acid, and palmitoleic acid. However, only lauric acid remained significant after adjustment for multiple comparisons. Interestingly, the monoester formed from glycerol and lauric acid, called glycerol monolaurate, has been significantly associated with improved hepatic lipid metabolism and antimicrobial and anti-inflammatory properties.[40,41] In this regard, the decreased levels of glycerol and lauric acid associated with a higher relative abundance of *Massilia* could be contributing to the unfavorable evolution of patients with HCV-related cirrhosis after successful DAA therapy.

Remarkably, it is essential to note that this study was carried out using a rigorous contamination-aware approach. On the one hand, the high volume of blood withdrawn prevents any potential contamination by needle with the skin microbiome. On the other hand, negative and positive controls were added and carried over throughout the sequencing

pipeline to prevent any significant impact of the environment on the results. Additionally, due to the variability found among different statistical methods when analyzing massive data, we replicate the analysis by several statistical methods, some of them adjusting for the most relevant covariates. All of these approaches provide robustness to our data.

Other points should be considered for a correct data interpretation. The sample size was limited, which could restrict the statistical power to detect more relevant bacterial taxa involved in improving portal hypertension after DAA therapy. Besides, we could not study the associations 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. Additionally, an important strength of this study is the availability of the HVPG measurement in a prospective design, with a relatively long interval from the end of the treatment to the determination of the follow-up portal hemodynamic study. Besides, our research adheres to the STROBE recommendations (see **Table S2**).

In conclusion, the higher relative abundance of *Corynebacteriales* at baseline was consistently associated with achieving a decrease ≥20% in HVPG or HVPG <12 mmHg at 48 weeks after HCV treatment completion. In contrast, the increased abundance of *Massilia* was associated with worse evolution in the measurement of HVPG. These findings are the first evidence that blood-specific bacterial taxa could reduce portal hypertension after achieving SVR in patients with advanced HCV-related cirrhosis.

Declarations

Transparency declarations

None to declare.

Originality

The authors confirm that the material contained herein is entirely original.

Availability of data and materials

Datasets used and analyzed during the current study may be available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authorship contribution

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Statistical analysis and interpretation of data: OBK, AVB, MR, MAJS.

Writing of the manuscript: MAJS.

Critical revision of the manuscript for relevant intellectual content: JB, JGG, DR, CB, AFR, MR, SR.

Supervision and visualization: MR, SR, MAJS.

All authors approved the final version of the article.

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Tables

Table 1. Clinical and epidemiological characteristics of patients with advanced HCV-related cirrhosis stratified by reaching a decrease in HVPG ≥ 20% and/or HVPG<12mmHg 48 weeks after completing HCV treatment.

Variables	All patients	Endpoint not achieved	Endpoint achieved	*p*
No.	32	17 (53.1%)	15 (46.9%)	-
Age (years)	52.9 (49.9-55.2)	53.4 (52.1-59.7)	52.2 (47.9-55.0)	0.136
Gender (male)	21 (65.6%)	11 (64.7%)	10 (66.7%)	0.907
BMI (kg/m^2)	25.1 (22.9-27.9)	25.4 (22.9-28.4)	24.5 (23.4-27.4)	0.550
Smoker				
Never	8 (25%)	4 (23.5%)	4 (26.7%)	0.952
Previous (>6m)	6 (18.8%)	3 (17.6%)	3 (20.0%)	
Current	18 (56,3%)	10 (58.8%)	8 (53.3%)	
High alcohol intake (>50 g/day)				
Never	18 (56.3%)	11 (64.7%)	7 (46.7%)	0.250
Previous (>6m)	12 (37,5%)	6 (35.3%)	6 (40.0%)	
Current	2 (6.3%)	0 (0%)	2 (13.3%)	
History of IDU	16 (50%)	6 (35.3%)	10 (66.7%)	0.077
Previous anti-HCV therapy	16 (50%)	10 (58.8%)	6 (40.0%)	0.288
Treatment with statins	4 (12.5%)	1 (5.9%)	3 (20.0%)	0.319
Other liver markers				
CPT score	5 (5-6)	6 (5-7)	5 (5-5)	0.095
MELD score	8 (7-12)	10 (7-12)	8 (7-10)	0.658
LSM	34.8 (20.8-48.4)	34.3 (21.1-48.8)	35.3 (16.9-48.0)	0.734
HVPG	16.5 (14.8-18.0)	17.0 (15.0-18.0)	16.0 (13.5-17.5)	0.315
HCV markers				
HCV genotype (n=31)				
1	20 (64.5%)	12 (75.0%)	8 (53.3%)	0.329
2	1 (3.2%)	1 (6.3%)	0 (0%)	
3	4 (12.9%)	1 (6.3%)	3 (20.0%)	
4	6 (19.4%)	2 (12.5%)	4 (26.7%)	
Log_{10} HCV-RNA (IU/mL)	5.9 (5.5-6.6)	5.8 (5.4-6.2)	6.5 (5.7-6.8)	0.057
HCV-RNA > 850,000 IU/mL	16 (50.0%)	7 (41.2%)	9 (60.0%)	0.288
HIV markers				
HIV coinfection	21 (65.6%)	8 (47.1%)	13 (86.7%)	**0.019**
Previous AIDS (n=21)	8 (38.1%)	5 (62.5%)	3 (23.1%)	0.164
Nadir $CD4^+$ T cells (n=19)	130 (54-319)	83 (38-152)	207 (70-382)	0.148
Nadir $CD4^+$ T cells<200 cells/mm^3 (n=19)	12 (63.2%)	7 (87.5%)	5 (45.5%)	0.147
Baseline $CD4^+$ T cells	490 (234-719)	288 (238-491)	712 (233-827)	0.218
Baseline $CD4^+$ T cells<500 cells/mm^3	12 (57.1%)	7 (87.5%)	5 (38.5%)	0.067
Antiretroviral therapy				
NRTI+NNRTI	2 (9.5%)	1 (12.5%)	1 (7.7%)	0.763
NRTI+II	11 (52.4%)	3 (37.5%)	8 (61.5%)	
NRTI+PI	3 (14.3%)	1 (12.5%)	2 (15.4%)	
PI+II+NNRTI/MVC	2 (9.5%)	1 (12.5%)	1 (7.7%)	
Others	3 (14.3%)	2 (25.0%)	1 (7.7%)	

Statistics: Values are expressed as absolute number (percentage) and median (25th percentile –75th percentile). *P*-values were calculated by Chi-square test or Fisher exact test for categorical variables and the Mann-Whitney U test for continuous variables. The clinical outcome was to decrease ≥20% in HVPG or HVPG <12 mmHg at the end of follow-up. Significant differences are shown in bold. **Abbreviations**: BMI, body mass index; 6m, 6 months; IDU, injection drug use; HCV, hepatitis C virus; HCV-RNA, HCV plasma viral load; HIV, human immunodeficiency; CPT, Child-Turcotte-Pugh; MELD, model for end-stage liver disease; LSM, liver stiffness measurement; HVPG, hepatic venous pressure gradient; AIDS, acquired

491 immune deficiency syndrome; NNRTI, non-nucleoside analogue HIV reverse transcriptase inhibitor;
492 NRTI, nucleoside analogue HIV reverse transcriptase inhibitor; PI, protease inhibitor; II, integrase
493 inhibitor, MVC, maraviroc.
494

495

Table 2. Correlation between blood significant bacterial taxa and plasma markers of inflammation and metabolites at baseline.

Phylum	Taxa	Plasma marker	rho	p-value	q-value
Actinobacteria	*Corynebacteriales*	IFNgamma	-0.473	0.006	**0.041**
		IL12p70	-0.363	0.041	0.092
		IL17A	-0.430	0.014	**0.042**
		TNFalpha	-0.454	0.009	**0.041**
		Oxoproline	0.364	0.041	0.782
		Aminomalonic.acid	0.354	0.047	0.782
		Tryptophan	0.471	0.006	0.445
		Tyrosine	0.357	0.045	0.782
Proteobacteria	*Massilia*	Glycerol	-0.541	0.001	**0.047**
		Fructose/Psicose/ Sorbose/Tagatose	0.385	0.029	0.427
		Lauric acid	-0.585	<0.001	**0.030**
		Palmitic acid	-0.382	0.031	0.427
		Palmitoleic acid	-0.490	0.004	0.102

Statistics: P-values were calculated by Spearman correlation; q-values represent p-values corrected for multiple testing using the False Discovery Rate (FDR). Significant differences are shown in bold.

Abbreviations: IFN, interferon; IL, interleukin; p-value, level of significance; q-value, corrected level of significance.

Figures

Figure 1. Different abundances of bacterial communities in blood between those patients who reached a HVPG decrease ≥20% or HVPG <12mmHg and those who did not 48 weeks after completing HCV therapy by using LEfSe analysis. (A) Taxa enriched in patients who reached the endpoint are indicated with a positive LDA score (green), and taxa enriched in patients who did not get the endpoint with a negative LDA score (red). Only taxa meeting an LDA significant threshold two are shown. (B): Bacterial taxa that were differentially abundant in the blood microbiome using a cladogram generated from LEfSe analysis.

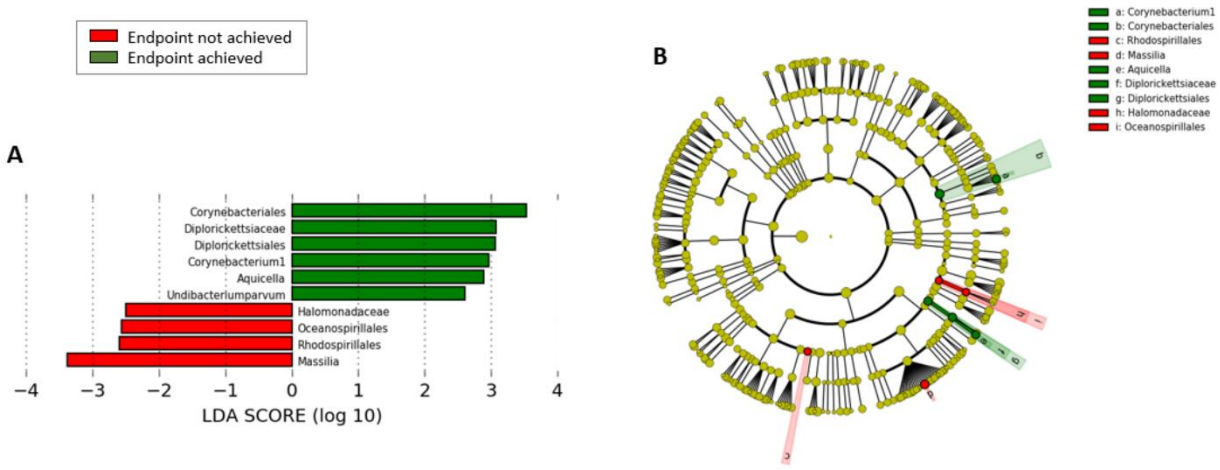
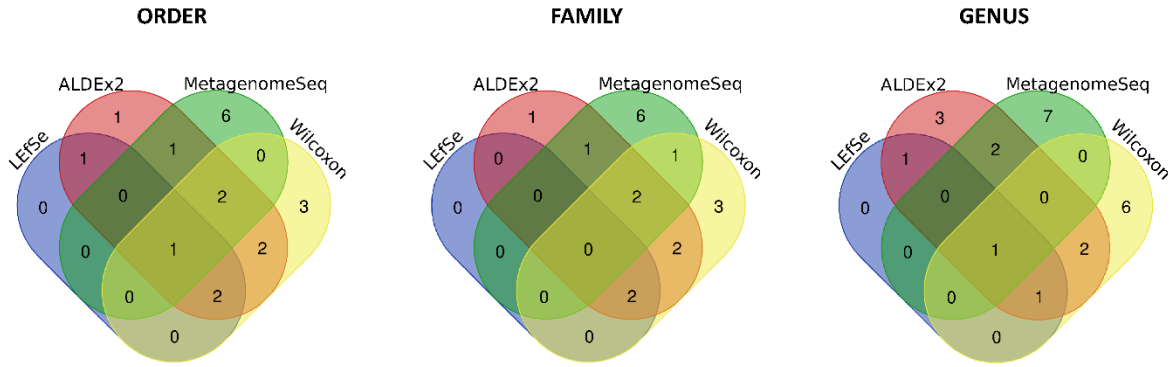


Figure 2. Overlapping of the ten most differentially abundant bacterial taxa identified for each statistical method using Venn diagrams and according to order, family and genus. The overlapping numbers correspond to bacterial taxa shared between different methods. The colors of the ovals correspond to the different statistical methods used: LEfSe (blue), ALDEx2 (red), MetagenomeSeq (green), and Wilcoxon test (yellow).



Methods	Order	Family	Genus
LEfSe ALDEx2 MetagenomeSeq Wilcoxon	Corynebacteriales	-	Massilia
LEfSe ALDEx2 Wilcoxon	Diplorickettsiales	Diplorickettsiaceae	Aquicella
	Rhodospirillales	Halomonadaceae	
LEfSe ALDEx2	Oceanospirillales	-	Corynebacterium1

Supplementary data

Supplementary data 1. Additional description of the methods section.

Table S1. The ten most differentially abundant bacterial taxa obtained using ALDEx2, MetagenomeSeq and Wilcoxon test, according to the taxonomic ranks of order family and genus. Statistics: i) ALDEx2: estimates were obtained of differential abundance analyses accounting for potential confounders (age, gender, and HIV coinfection). The relative abundances were estimated by repeated samplings from a Dirichlet distribution and centered-log ratio (clr) transformed before fitting the GLM model; ii) MetagenomeSeq: Estimates were obtained after fitting a zero-inflated Gaussian (ZIG) model, where the count distribution is modeled as a mixture of two distributions, a point mass at zero and a normal distribution. Since OTUs are usually sparse, the zero counts are modeled with the former, and the rest of the log-transformed counts are modeled as the latter distribution; iii) Wilcoxon test: nonparametric test to compare relative abundances of bacterial taxa between groups.

Table S2. STROBE Statement—checklist of items that should be included in reports of observational studies.