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Original Article

Short-term effects of direct-acting antiviral agents on inflammation and gut microbiota in hepatitis C-infected patients

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ABSTRACT

Liver damage is associated with gut dysbiosis. New direct-acting antiviral agents (DAAs) are able to eradicate hepatitis C virus (HCV) from the body. However, the short and medium-term effects of DAAs at gut level before advanced liver damage occurs have not been evaluated yet. Thus, we investigated the impact of HCV and DAAs on gut microbiota composition (GM) and systemic inflammation. To achieve this objective, twenty-three non HCV-infected controls and 22 HCV-infected patients were recruited. Only non-cirrhotic patients (fibrosis stage 0–3) were included to avoid the direct impact of cirrhosis and portal hypertension on gut. The HCV-groups were evaluated before the treatment, after completing DAAs treatment and after 3 months. Fecal bacterial 16S rDNA was ultrasequenced and several biochemical/metabolic/inflammatory parameters were quantified. HCV infection was accompanied by a significant increase in TNFα plasma levels. DAAs were able to reduce this increase, especially in lower fibrosis grades. HCV infection was not accompanied by dramatic changes in α-diversity and was not recovered after HCV negativization, although a complete restoration was observed in lower fibrosis degrees. Six phyla, 15 genera and 9 bacterial species resulted differentially abundant among the groups. These differences were almost blunted with lower fibrosis. In summary, neither the usage of DAAs nor 3 months in sustained viral response were able to counteract the changes induced by HCV at gut level. The partial restoration observed in inflammation and α-diversity was only observed in low fibrosis degrees. Thus, it is urgent to begin treatment with DAAs as soon as possible.

1. Introduction

Hepatitis C (HCV) infection represents one of the major causes of chronic liver disease, cirrhosis and hepatocellular carcinoma (HCC) with large hospital admission rates and increased morbidity/mortality worldwide [1,2]. HCV infection is the most frequent indication for virus-related liver transplantation in the western world and, therefore, it is a major burden to the healthcare systems [3].

Liver is, by far, the most affected organ, but HCV infection is definitely not a liver-limited disease. HCV infection has been associated with other extra-hepatic manifestations [4–6]. Up to 74% of HCV-infected patients experienced some forms of these extra-hepatic manifestations [7], and, this is accompanied with higher mortality rates [8]. In line with this, several studies have demonstrated that HCV may infect other tissues apart from liver [9], such as the intestine. HCV induces liver damage with reduced bile salt production and protein

Abbreviations: DAAs, Direct-acting antivirals; HCV, Hepatitis C virus; SVR, Sustained viral response; LPS, Lipopolysaccharide; LDL, Low-density lipoprotein; HDL, High-density lipoprotein-; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; γ-GT, Glutamil transpeptidase; LBP, Lipopolysaccharide-binding protein; sCD14, Soluble CD14; TNF-α, Tumor necrosis factor-α; IL-6, Interleukin-6; PCA, Principal component analysis

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synthesis, but also infects B-lymphocytes in gut decreasing IgA levels and increasing the intestinal permeability that leads to an increased bacterial translocation (BT) [10]. The result of such BT is the passage of lipopolysaccharide (LPS) and other bacterial molecules into bloodstream. These bacterial products induce a significant increase in proinflammatory cytokine production via toll-like receptors, contributing to immune activation and inflammation. In addition, the increased release of LPS also contributes to liver damage, which indicates a clear and close association among gut and liver, known as "gut-liver axis". Gut-liver axis malfunction is a leading factor in the development and progression of liver pathologies such as hepatitis C-infection and HCC development [10–15]. In this sense, a decrease in gut bacterial diversity/richness has been observed in patients with chronic hepatitis C, with and without cirrhosis, compared to healthy controls [14–17]. Gut microbiota alterations are also strongly related to HCC risk, secondary to HCV direct damage of the liver that also implies the intestinal derangement. Indeed, gut dysbiosis is one of the reversible factors implicated in HCC carcinogenesis and can be a future target for both prevention and treatment of this neoplasia [18].

Both interferon-related treatments and new oral direct-acting antivirals (DAAs) eradicate HCV from the body. Specifically, antiviral therapy for HCV infection along with attainment of sustained virological response (SVR) normalizes liver enzymes, halts the progression of liver disease and reduces the risk of liver failure and hepatocellular carcinoma. However, up to now, there is no clear evidence concerning the short and medium-term effects of eradication of HCV from the body. Recent studies observed that SVR achieved using pegilated interferon + ribavirin does not impact GM or systemic inflammation in cirrhotic patients [19] and the new DAAs did not either improve the intestinal barrier function in HCV cirrhotic patients [17]. However, the effects of the new DAAs on GM before advanced liver damage occurs have not been evaluated yet. This needs to be clarified in order to understand the effects of HCV per se on GM and inflammation without the bias of cirrhosis and portal hypertension impacts on gut. Thus, the aims of this study were i) to investigate the impact of HCV infection on gut bacterial diversity/richness and composition as well as on systemic inflammation and ii) to analyze if the elimination of HCV from the body by DAAs is accompanied in the short term by a restoration of the inflammatory state and gut microbiota composition. In addition, as the severity of the clinical stage has been reported to influence gut bacterial diversity in HCV infection [16], we have also investigated the impact of liver fibrosis on systemic inflammation and GM as well as the ability of DAAs to restore GM depending on the fibrosis degree (pre-cirrhosis stages).

2. Patients and methods

2.1. Patient recruitment

Twenty-two caucasian HCV-infected patients were recruited (January 2016–July 2017) (Infectious Diseases Department at Hospital Universitario San Pedro and from Hospital Universitario Álvaro Cunqueiro, Spain). Degree of liver fibrosis was non-invasively evaluated using FibroScan® method (Echosens, Paris, France). Patients were classified according to METAVIR scoring system. Only non-cirrhotic patients (F0–3) were recruited in this study. All HCV genotypes were included. HCV patients were evaluated before the treatment (group 2), after completing the antiviral treatment (group 3) and 3 months after the end of therapy with SVR (group 4). Sustained virological response (SVR) was defined as undetectable viral load at least 12 weeks after treatment completion. All treatments approved for clinical practice available during 2016 and 2017 were included (Suplementary Table 1). Non-infected volunteers (n = 23) were also included as "reference/control" group. This control population was matched for age and gender with the HCV-infected group. For both HCV patients and controls, the following exclusion criteria were applied: < 18 years old; pregnant women; patients treated with antibiotics, anti-inflammatory drugs, corticosteroids, immunosuppressive drugs, ursodeoxycholic acid, or probiotics in the last 3–5 and 7 months respectively (for groups 2, 3 and 4 respectively); individuals with kidney, coeliac, or inflammatory disease, thyroid disorders, neoplasms, history of intestinal surgery (except appendectomy or cholecystectomy), inflammatory bowel disease (IBD) (even if inactive), chronic pancreatitis, or any syndrome related to an intestinal malabsorption.

The study was performed following the 1975 Declaration of Helsinki and was approved by the Committee for Ethics in Drug Research in La Rioja (CEImLAR) (29 January 2016, reference PI-194). All participants provided their written informed consent.

2.2. Biochemical parameters and markers of BT and inflammation

Plasma and serum samples were collected from peripheral blood after 12 h fast. Plasma levels of glucose, triglycerides, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamil transpeptidase (γ-GT), albumin and alkaline phosphatase were measured using an AutoAnalyzer (Cobas C711, Roche, Madrid, Spain). Lipopolysaccharide-binding protein (LBP), soluble CD14 (sCD14), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL6) were quantified by ELISA using commercially kits and according to the manufacturers' instructions (R&D, Minnesota, USA).

2.3. DNA extraction from stool samples and 16S rRNA gene sequencing

Fresh stool samples were collected in the morning from all the participants and frozen at −80 °C. Fecal DNA was extracted from 250 mg of stools using the QIAamp® PowerFecal® DNA kit (Qiagen, Venlo, Netherlands) and purity and concentration were subsequently determined by a Nanodrop spectrophotometer 1000 (Thermo Scientific, USA). Sequencing was carried out by Illumina (MiSeq, 2 × 300 pb, paired-end). Quality check of reads and adapter trimming were performed with the quality control tool FastQC and Trim Galore program (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Then, reconstruction of full-length V3-V4 16S rRNA gene regions for taxonomic assignment and the determination of operational taxonomic units (OTUs) were carried out through the QIIME program (v1.9.1), following the "*pick open reference otus*" methodology against the 16S rRNA gene database Greengenes 13.8 at 97% sequence similarity [20–22]. Uclust program was used for the establishment of taxonomy clusters (http://drive5.com/usearch/manual/uclust_algo.html). Data have been filtered to eliminate all those OTUS with an abundance of < 0.01%, as these OTUS mostly correspond to spurious sequences. The meta-analysis of these data was performed with the web-based tool MicrobiomeAnalyst. Both α- and β-diversity were analyzed: α-diversity is a measure of sample-level species richness, whereas β-diversity describes inter-subject similarity of microbial composition and facilitates identification of broad differences between samples. The measure of sample-level species richness was analyzed using the Fisher and Chao-1 indexes. Differential abundances among the groups were calculated using Univariate analyses at phylum, genus and species taxonomic levels.

2.4. Statistical analysis

Results are expressed as mean ± standard error of the mean. Qualitative variables were analyzed using the χ^2 test or Fisher's exact test. Normal distribution of continuous variables was tested with Shapiro-Wilk test. Comparisons were performed with unpaired t-test/U-Mann Whitney or ANOVA/Kruskall Wallis depending on the normality of the data. Repeated measurements concerning biochemistry, inflammation and bacterial translocation among the three HCV-patients groups were analyzed by a Friedman test. Differential abundance

Table 1
Characteristics of healthy uninfected controls (n = 23) and HCV-infected patients (n = 22), prior treatment, after finishing DAAs and after 3 months with SVR.

	Healthy controls	HCV-infected patients	HCV-infected patients after DAAs	HCV-infected patients after 3 months SVR	P^1	p^2
Age (years)	51.91 ± 11.34	54.18 ± 14.42			0.559	
Gender (% men)	12/23 (52%)	12/22 (54.50%)			0.873	
HCV genotype		1a: 5/22 (22.73%) 1b: 9/22 (40.90%) 2a: 1/22 (4.55%) 2 a/2c: 1/22 (4.55%) 3a: 3/22 (13.64%) 4: 3/22 (13.64%)				
Degree of hepatic fibrosis		F0–1: 3/22 (13.64%) F1: 3/22 (13.64%) F2: 14/22 (63.64%) F3: 2/22 (9.09%)				
Tobacco consumption	Never: 3/23 (13.0%) Past[a]: 16/23 (69.5%) Ongoing: 4/23 (17.4%)	Never: 11/22 (50.0%) Past[a]: 2/22 (9.1%) Ongoing: 9/22 (39.1%)			0.0106 0.0002 0.1075	
Biochemical parameters						
GOT (U/L)	19.68 ± 0.89	50.78 ± 8.43	27.24 ± 2.94*	25.30 ± 2.44**	< 0.0001	0.0006
GPT (U/L)	18.64 ± 1.82	66.43 ± 10.85	22.65 ± 2.50**	20.78 ± 2.32***	< 0.0001	< 0.0001
γGT (U/L)	26 ± 3.55	73.57 ± 18.95	24.85 ± 3.90****	24.57 ± 3.50****	0.0003	< 0.0001
Cholesterol (mg/dL)	191 ± 6.08	181.7 ± 7.78	195.7 ± 10.20	196.9 ± 7.24	0.3337	0.0732
Triglycerides (mg/dL)	86.23 ± 10.92	147.2 ± 30.47	118.7 ± 13.43	152 ± 18.42	0.0121	0.4216
Alkaline Phosphatase	62.32 ± 3.20	84.87 ± 8.20	74.99 ± 5.98	69.43 ± 5.20**	0.0306	0.0087
Albumin (g/dL)	4.61 ± 0.05	4.38 ± 0.07	4.43 ± 0.05	4.49 ± 0.07	0.0612	0.2130

Quantitative data are presented as mean ± SEM; Qualitative data are indicated as percentage. Overall p value[1] was obtained comparing Controls vs. HCV-infected controls using Mann Whitney test. Overall p^2 value was obtained by comparing HCV-infected people before treatment, after DAA treatment and 3 months with SVR using Friedman test for paired measurements. The results of multiple comparisons followed the Friedman tests are presented as: *p < 0.05; **p < 0.01; ***p < 0.001 and ****p < 0.0001 vs. HCV-infected patients.

[a] Past tobacco consumption: at least one year with no smoking habits.

analysis was carried out by univariate analysis. Data obtained from β-diversity were statistically analyzed using the Wilcoxon rank-sum non-parametric test. A false discovery rate (FDR) < 0.05 was considered significant. FDR values were calculated by the web-tool *MicrobiomeAnalyst*. It is the Benjamini-Hochberg method for P-value adjustment. FDR was obtained comparing the four groups using Kruskal Wallis test (Controls vs. HCV-infected-patients prior treatment, after finishing DAAs and after 3 months with SVR after DAAs) or using Mann-Whitney test when comparing the controls vs. HCV-infected-patients' prior treatment. A Principal Coordinate Analysis (PCoA) was also developed. Results are plotted according to the first two principle components. Statistical analysis was carried out using SPSS 19·0 (SPSS® Inc. Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Prism®, La Jolla California USA). P values < .05 were considered statistically significant.

3. Results

3.1. Clinical characteristics of the participants

Table 1 shows the main characteristics of the studied population. HCV patients did not present any co-infections with HIV or other hepatotrophic viruses. The most prevalent genotype was 1b (40.90%). 13.64% presented grade F0–1, 13.64% showed grade F1, 63.64% grade F2 and 9.09% grade F3. All treatments approved for clinical practice (2016 and 2017) were included (Supplementary Table 1). The length of DAAs averaged 2.8 months. SVR was achieved in all patients after DAAs and also 3 months after finishing the treatment.

No differences were observed in age and gender when the uninfected/control group was compared with HCV-infected subjects. More past-smokers were found in the control-group, although all of them were non-smokers at the moment of the study for at least one year. No statistical significances were observed among smokers in both groups (p = 0.107). In addition, significant increases were observed on hepatic transaminases (GOT, GTP and γGT) (p = 0.0003-p < 0.0001), plasma triglycerides and alkaline phosphatase concentrations (p < 0.05). DAA treatment was accompanied by a significant improvement in GOT, GTP and γGT plasma levels (p = 0.0006-p < 0.0001) and also in alkaline phosphatase plasma levels (p = 0.0087).

3.2. Markers of inflammation and BT

HCV infection was accompanied by a significant increase in TNFα plasma levels (p < 0.001) (Fig. 1A). DAAs were able to significantly reduce this increase, especially in lower grades of fibrosis (p < 0.05 SVR vs. HCV-infection) (Fig. 1B). A significant increase in IL-6 plasma levels were only observed in HCV-infected patients with higher degree of fibrosis and it was not reduced despite the usage of DAAs (Fig. 1C–D).

Two markers of BT were analyzed. No effects were observed on sCD14 plasma levels (data not showed). Concerning LBP, no differences were observed when uninfected/control subjects were compared against those infected with HCV (Fig. 1E). However, when the analysis was carried out taking into account the fibrosis degree, a significant increase in LBP plasma levels were observed in HCV-patients with higher degree of fibrosis (p < 0.05) and SVR achieved by DAAs were able to reduce this increase up to similar levels than those observed in uninfected-control subjects (Fig. 1F).

3.3. Bacterial diversity/richness's

HCV infection was not accompanied by dramatic changes in α-diversity, and only a slight-non significant decrease was observed in HCV-infected patients when compared with uninfected individuals with Chao-1 index (Fig. 2A). A significant reduction was observed using the Fisher index (p = 0.027) (Fig. 2B). This decrease observed in the Fisher index was completely abolished in lower degrees of fibrosis (p = 0.1743, HCV-patients vs. controls) but persisted in higher degrades (F2–3) (p = 0.043, HCV-patients vs. controls).

α-diversity did not recover after negativization of the virus by DAAs nor after 3 months in SVR (Fig. 2 A-B). When this analysis was carried

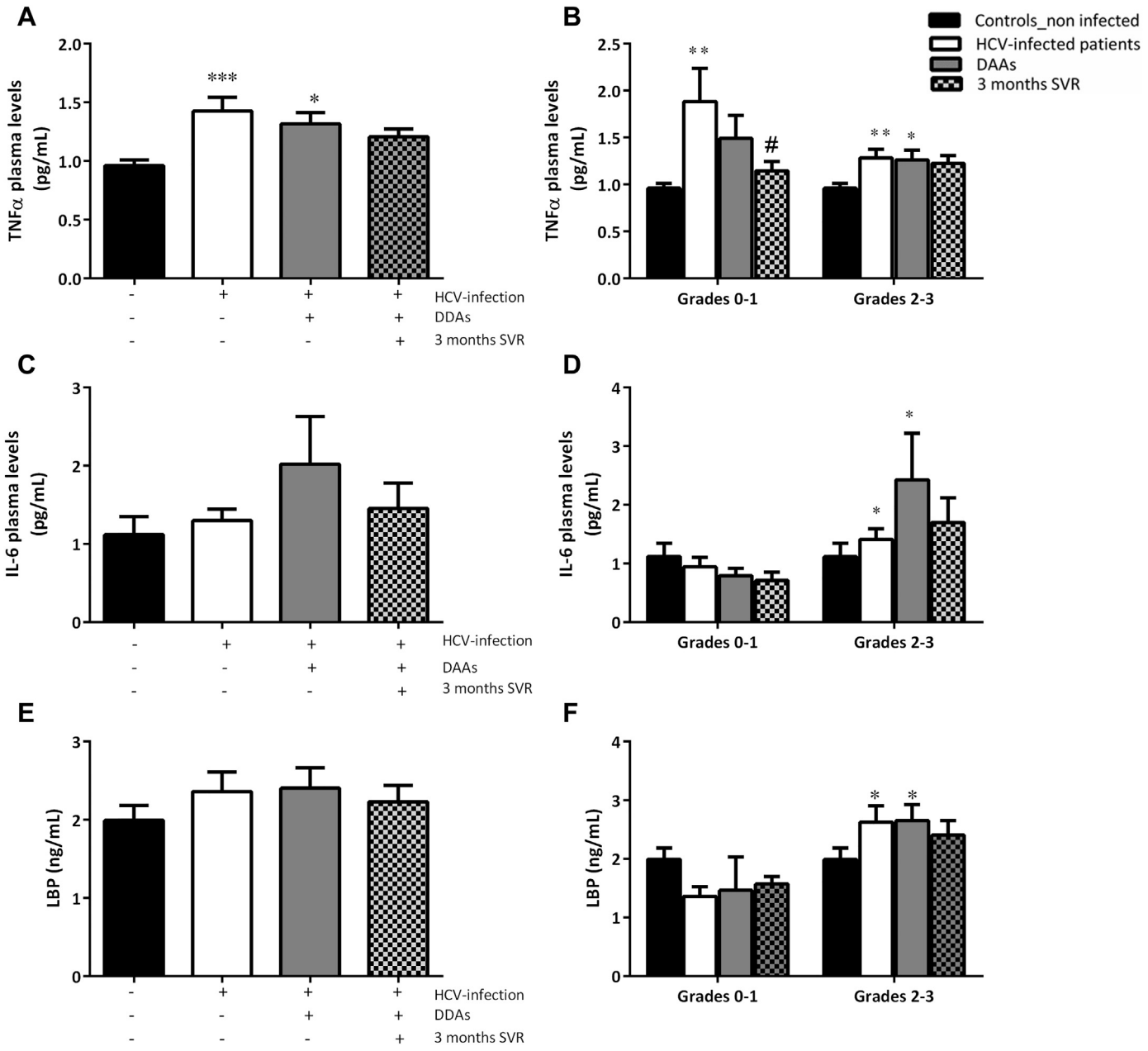

Fig. 1. Effects of HCV infection, DAAs and SVR on systemic inflammation. Effects on plasma levels of TNFα (A,B), IL-6 (C,D) and on bacterial translocation measured by LBP plasma levels (E,F). *P < 0.05; **P < 0.01; ***P < 0.001 vs. uninfected subjects. #P < 0.05 vs. HCV-infected patients.

out taking into account the fibrosis degree, a complete restoration of α-diversity was observed in patients after 3 months in SVR in lower degrees of fibrosis, especially with the Chao-index (Fig. 2 C-D) but not in higher degrees (F2–3) (Fig. 2 E–F).

3.4. Microbial communities in healthy controls compared to HCV-infected patients prior treatment, after treatment and 3 months on SVR

The most abundant phyla in gut were Firmicutes and Bacteroidetes. Approximately 90% of the bacteria detected in faeces from the subjects recruited in this study belong to these phyla (Supplementary Table 2).

Four phyla (Actinobacteria, Firmicutes, Tenericutes and Verrucomicrobia) resulted significantly different among the control and HCV-group (Table 2). When HCV-patients were classified according to fibrosis, the same results were observed in higher degrees of fibrosis (2–3) (Table 2), while only Actinobacteria were significantly increased in HCV-infected subjects with lower degrees of fibrosis (Table 2). When comparing the four groups of subjects, six phyla (Actinobacteria, Firmicutes, Bacteroidetes, Tenericutes, Verrucomicrobia and Cyanobacteria) resulted differentially abundant among the groups (Table 3). Only Actinobacteria and Bacteroidetes remained differentially present when HCV-subjects with low fibrosis were compared. All the aforementioned phyla were differentially abundant among the groups with higher fibrosis degrees (Table 3). No differences were observed at phyla level when the HCV-infected patients were compared prior treatment, after DAAs and 3 months on SVR.

At lower taxonomic levels, 15 bacterial genera were found differentially present in gut when the four groups of subjects were compared (Table 4). All genera, with the exception of *Lachnospira*, were more abundant in HCV-infected subjects than in controls. Of those, only 6 genera along with *Adlercreutzia* resulted significantly different in patients with low degree of fibrosis. In contrast, 15 genera resulted different again with higher levels of hepatic damage (Table 4). DAAs were only able to restore the abundance of *Lachnospira* (FDR: 0.031,

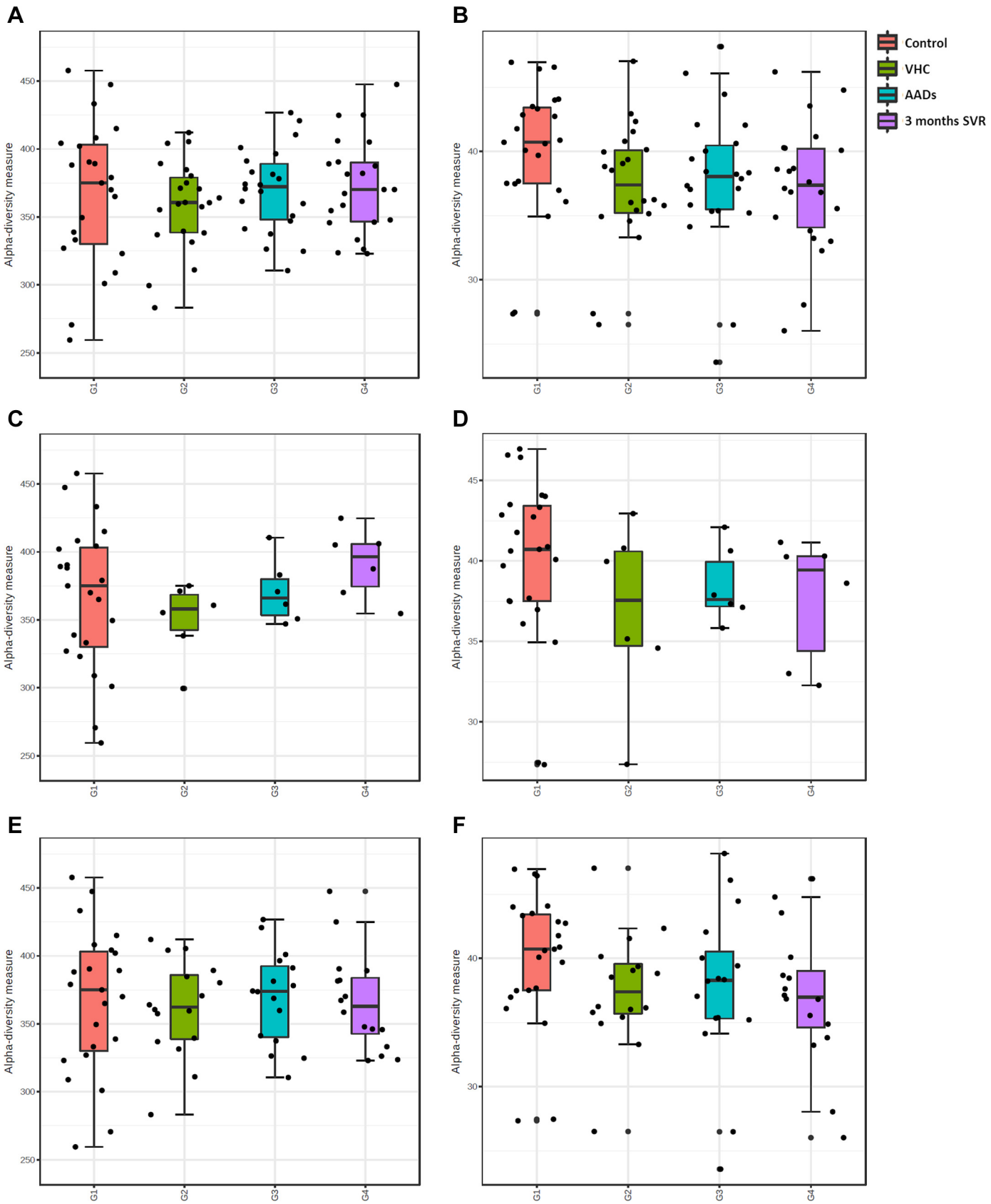

Fig. 2. α-diversity in control/uninfected individuals, HCV-infected subjects prior treatment, after DAAs treatment and after 3 months of SVR using Chao-1 (A) and Fisher indexes (B). C, D: α-diversity in control/uninfected individuals and HCV-infected subjects with low fibrosis degrees (F = 0–1) prior treatment, after DAAs treatment and after 3 months of SVR using the Chao-1 (C) and Fisher indexes (D). E, F: α-diversity in control/uninfected individuals, HCV-infected subjects with high fibrosis degrees (F = 2–3) prior treatment, after DAAs treatment and after 3 months of SVR using the Chao-1 (E) and Fisher indexes (F).

Table 2
Relative abundance of major phyla present in gut when HCV-infected patients were compared to controls.

	All fibrosis degrees		F0–1 fibrosis degree		F2–3 fibrosis degree	
Phyla	FDR	Abundance in HCV-patients	FDR	Abundance in HCV-patients	FDR	Abundance in HCV-patients
Actinobacteria	$5.88e^{-9}$	↑	$2.94e^{-4}$	↑	$2.69e^{-7}$	↑
Firmicutes	$1.87e^{-4}$	↑	0.4516	–	$8.359e^{-5}$	↑
Tenericutes	0.0174	↓	0.5793	–	0.0149	↓
Verrucomicrobia	0.0291	↓	0.7298	–	0.0149	↓

A false discovery rate (FDR) < 0.05 was considered significant. FDR was obtained comparing the four groups using Kruskal Wallis test (Controls vs. HCV-infected patients prior treatment, after finishing DAAs and after 3 months on SVR).

Table 3
Relative abundance of major phyla present in gut when control/uninfected subjects were compared to HCV-infected patients prior treatment, after finishing DAAs and after 3 months with SVR.

	All fibrosis degrees		F0–1 fibrosis degree		F2–3 fibrosis degree	
Phyla	FDR	Abundance in HCV-patients	FDR	Abundance in HCV-patients	FDR	Abundance in HCV-patients
Actinobacteria	$8.34e^{-8}$	↑	0.0015	↑	$2.09e^{-7}$	↑
Firmicutes	0.0017	↑	0.0616		0.0023	↑
Bacteroidetes	0.0151	↑ (*)	0.0376	↑ (*)	–	
Tenericutes	0.0235	↓	–		0.0095	↓
Verrucomicrobia	0.0235	↓	–		0.0095	↓
Cyanobacteria	0.0298	↑	–		0.0551	↑

(*) Increase especially evident in the groups of HCV-treated (DAAs) and 3 months in SVR.
A false discovery rate (FDR) < 0.05 was considered significant. FDR was obtained comparing the four groups using Kruskal Wallis test (Controls vs. HCV-infected patients prior treatment, after finishing DAAs and after 3 months on SVR).

p = 0.0006) and a tendency was also observed with *Dorea* (FDR: 0.576, p = 0.04). At species level, 9 bacterial species resulted differently abundant in gut when the four groups of participants were compared, although only 2 bacterial species (*Collinsella aerofaciens* and *Bifidobacterium longum*) resulted different when patients with a low degree of fibrosis were compared (Table 5). Only *Bacteroides coprophilus* and *Akkermansia muciniphila* resulted less abundant in HVC-infected subjects than in the controls, while the other 7 species were more abundant in HCV-patients.

Taking into account Bray-Curtis Index, a higher dispersion in HCV-infected patients was observed in comparison with the control/uninfected subjects. The results were plotted according to the first two principle components and the clustering of samples was represented accounting for 24.1% of total variation (Component 1 = 14.8% and Component 2 = 9.3%). Fig. 3A shows the PCA where controls and HCV-patients were represented in different clusters (PERMANOVA r^2: 0.0923; p < 0.001). When the four groups were represented, the control cluster was represented inside the others (Fig. 3B), especially evident in lower degrees of fibrosis (p < 0.012) (25.6% of total variation: component 1: 16.6%, component 2 = 9%) (Fig. 3C), whereas a more pronounced dispersion was observed in higher degrees of fibrosis (p < 0.001) (25.4% of total variation: component 1: 15.7%, component 2 = 9.7%) (Fig. 3D). The three HCV-groups overlapped, thus, no statistical differences were observed despite DAAs and 3 months on SVR (Supplementary Fig. S1). In fact, when the three HCV-groups were analyzed together, no significant differences were observed at phyla, genera or bacterial species levels.

4. Discussion

To our knowledge, this is one of the first studies carried out in non-cirrhotic HCV-infected patients where the short-term effects of DAAs on GM have been studied. Thus, in an effort to clarify the natural course of HCV infection once the virus has been eliminated from the organism and before advanced liver damage occurs, it is appropriate to deeply understand the short/mid-term effects of its eradication. Our results show that neither the usage of DAAs nor 3 months in SVR are able to counteract the changes observed in GM after HCV infection. The partial restoration observed in inflammation, α-diversity and some bacterial genera were only observed in very low degrees of fibrosis.

IL-6 and TNFα, markers of inflammation, play a pivotal role in both HCV viral persistence and in the extent of liver damage [23]. More specifically, TNFα is an inflammatory cytokine involved in the apoptotic signaling pathway of hepatocytes infected by HCV [24] and IL-6 is produced by Kupffer cells in liver and induces the production of acute phase proteins [25]. Serum IL-6 levels have been reported to be increased in patients with liver diseases such as chronic viral hepatitis [26], although controversial results have emerged concerning this issue. Our results have demonstrated that HCV-infection is associated with increased inflammation, especially evident with TNFα, while no significant changes were observed in IL-6. DAAs were able to counteract this inflammatory state, particularly in low degrees of fibrosis and, again, only in TNFα. The apparent discordant results obtained in TNFα and IL-6 are in line with previous studies reported in cirrhotic and non-cirrhotic HCV-patients treated with DAAs where no changes were observed on IL-6 plasma levels whereas significant reductions were observed on TNFα plasma levels after DAAs [17,27]. In fact, our results show that TNFα reduction induced by DAAs depends on HCV clearance while IL-6 levels seem to be more associated to the presence of advanced liver fibrosis, as suggested by the study of Fuster et al., (2013) performed in HIV-infected patients with liver fibrosis [28]. In addition, these actions seem to be specific of DAAs [17,27], since no changes on

Table 4
Relative abundance of major genera present in gut when control/uninfected subjects were compared to HCV-infected patients prior treatment, after finishing DAAs and after 3 months with SVR.

		All fibrosis degrees	Abundance in HCV-patients	Do DAAs restore?	F0–1 fibrosis degree	F2–3 fibrosis degree
Phylum	Genera	FDR			FDR	FDR
Actinobacteria	*Collinsella*	2.04e-6	↑	No	0.0286	2.25e-6
Firmicutes	*Blautia*	2.04e-6	↑	No	0.0034	2.32e-5
Actinobacteria	*Actinomyces*	1.09e-4	↑	No	0.0276	2.07e-4
Actinobacteria	*Bifidobacterium*	1.09e-4	↑	No	0.0093	5.67e-4
Firmicutes	*Lachnospira*	2.43e-4	↓	Yes	–	1.12e-4
Firmicutes	*Coprococcus*	1.09e-4	↑	No	–	1.17e-4
Firmicutes	*Lactobacillus*	2.43e-4	↑	No	–	2.32e-5
Firmicutes	*Megasphaera*	1.64e-4	↑	No	–	9.91e-5
Firmicutes	*Acidaminococcus*	1.091e-4	↑	No	0.0342	1.04e-4
Firmicutes	*Streptococcus*	0.0012	↑	No	0.0462	0.0055
Firmicutes	*Turicibacter*	0.0016	↑	No	–	0.0056
Firmicutes	*Dorea*	0.0046	↑	Yes	–	0.0058
Firmicutes	*Clostridium*	0.0267	↑	No	–	0.0146
Firmicutes	*Veillonella*	0.0274	↑	No	–	–
Verrucomicrobia	*Akkermansia*	0.0426	↑	No	–	0.0076
Actinobacteria	*Adlercreutzia*	–		–	0.0342	–
Proteobacteria	*Klebsiella*	–		–	–	0.0257

A false discovery rate (FDR) < 0.05 was considered significant. FDR was obtained comparing the four groups using Kruskal Wallis test (Controls vs HCV-infected patients prior treatment, after finishing DAAs and after 3 months with SVR).

Table 5
Relative abundance of major bacterial species present in gut when control/uninfected subjects were compared to HCV-infected patients prior treatment, after finishing DAAs and after 3 months with SVR.

		All fibrosis degrees	Abundance in HCV-patients	Do DAAs restore?	F0–1 fibrosis degree	F2–3 fibrosis degree
Phylum	Bacterial species	FDR			FDR	FDR
Actinobacteria	*Collinsella aerofaciens*	2.47e-6	↑	No	0.024486	3.61e-6
Firmicutes	*Ruminococcus gnavus*	9.87e-4	↑	Partially	–	0.0010
Firmicutes	*Lactobacillus ruminis*	0.0035	↑	No	–	9.48e-4
Firmicutes	*Streptococcus anginosus*	0.0057	↑	No	–	0.0022
Actinobacteria	*Bifidobacterium adolescentes*	0.0057	↑	No	–	0.0067
Bacteroidetes	*Bacteroides coprophilus*	0.0115	↓	No	–	–
Actinobacteria	*Bifidobacterium longum*	0.0115	↑	No	0.024486	0.0478
Firmicutes	*Eubacterium dolichum*	0.0386	↑	No	–	0.0281
Verrucomicrobia	*Akkermansia muciniphila*	0.0419	↓	No	–	0.0087
Bacteroidetes	*Bacteroides fragilis*	–			–	0.0464

A false discovery rate (FDR) < 0.05 was considered significant. FDR was obtained comparing the four groups using Kruskal Wallis (Controls vs HCV-infected patients prior treatment, after finishing DAAs and after 3 months with SVR).

TNFα plasma levels were observed in cirrhotic patients that had achieved SVR using pegylated interferon + ribavirin [19]. In general, these effects could be very positive to reduce the frequency and development of extrahepatic manifestations caused by on-going inflammation and immune activation [29], although more studies are needed in this regard since a previous study demonstrated a rapid reduction of inflammation but also increased VEGF serum levels after DAAs treatment [27].

Despite the increase observed in systemic inflammation after HCV-infection, we did not find a significant increase in BT in HCV-infected patients, at least with the two markers analyzed (sCD14 and LBP). Only some increases in LBP were observed in grades 2–3 of fibrosis that were completely abolished after 3 months in SVR. Similarly, serum LBP levels decreased at SVR in chronic HCV-infection [30]. In addition, BT does not either seem to play an important role in clinical progression in early stages of cirrhosis of HIV/HCV-coinfected patients [31]. The study by

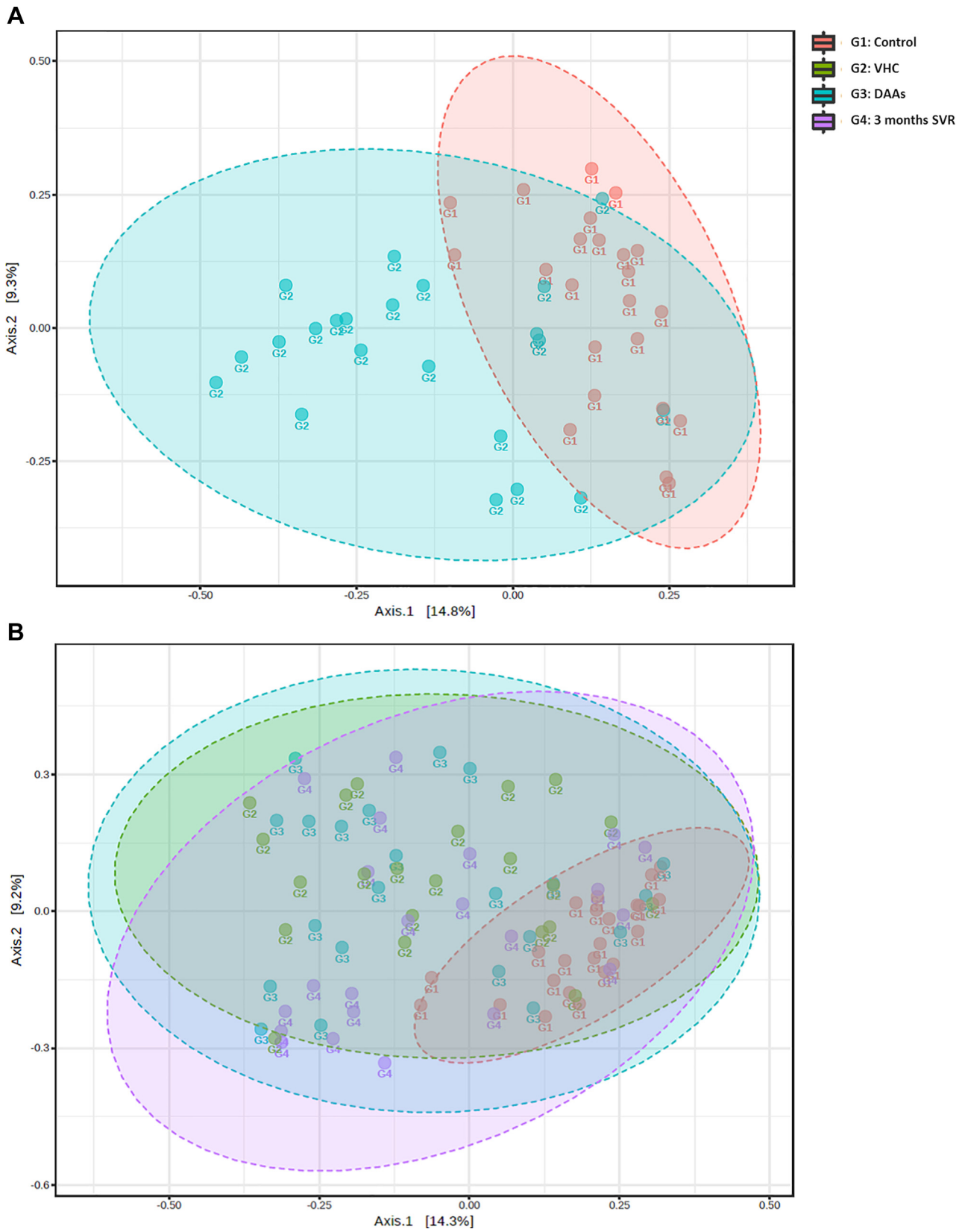

Fig. 3. Principal Coordinate Analysis (PCoA) of the groups included in the study. A: PCoA of control/uninfected individuals and HCV-infected subjects' prior treatment. B: PCoA of control/uninfected individuals, HCV-infected subjects prior treatment, after DAAs treatment and after 3 months of SVR with all hepatic fibrosis degrees analyzed together (B) or classified according to low (F = 0–1) (C) or high (F = 2–3) (D) fibrosis degree.

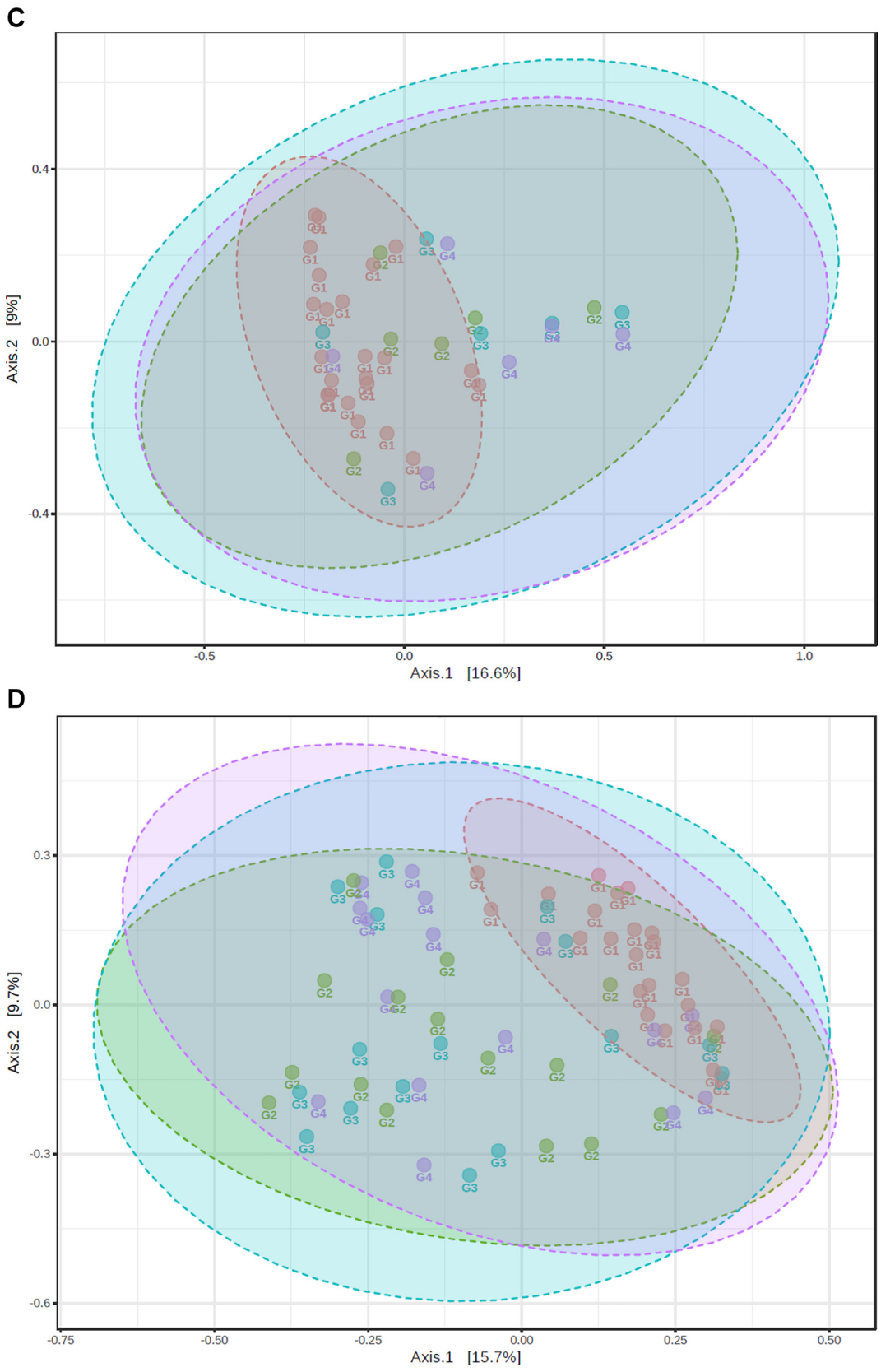

Fig. 3. (*continued*)

Munteanu et al., (2014) contrasts with our present data [32]. However, there are some differences among Munteanu's work and ours, as they use LPS as indicator of BT instead of sCD14 or LBP. They recognize that LPS evaluation may not be the best technique to assess BT. We agree with their observation since detection of LPS can be complicated by its rapid clearance and by inhibitory plasma proteins. In addition, not all bacteria produce bioactive LPS or LPS detectable by the Limulus assay. Thus, sCD14 or LBP may be more relevant biomarkers of BT as they

reflect the host response to products of BT [33].

Decreased bacterial diversity has been observed in chronic hepatitis C patients with cirrhosis compared to healthy persons and regardless of the metric used [14,15,17]. We also observed a mild reduction of bacterial diversity/richness in non-cirrhotic HCV-infected subjects. Similarly, the study of Heidrich et al., (2018) observed a reduction of α-diversity in non-cirrhotic patients [14], which suggests that altered microbiota and liver fibrosis is gradual [14]. The usage of DAAs was not able to counteract the reduced bacterial richness, and only a complete restoration was observed after 3 months on SVR in those patients with a lower fibrosis degree (F0–1). Our data also highlight the strong relationship among liver and intestine and suggests that under lower hepatic damage, the changes at intestinal level are mild and, therefore, can be more easily reversed with appropriate drugs. These results underline the need to treat patients as soon as possible (even after liver damage occurs) in order to reverse the actions caused by HCV infection on GM diversity and the future clinical consequences.

Regarding GM, HCV-infection was associated with changes at both high (phylum level) and lower taxonomic levels (genera and bacterial species), while DAAs and SVR did not translate into significant modifications. These results corroborate those previously observed with former treatments in cirrhotic patients [19].

Interestingly, the modifications observed at phylum level in HCV infection in our study were completely abolished in low degrees of fibrosis (with the exception of Actinobacteria), which highlights the intimal connection among liver and intestine and highlights the assumption that gut dysbiosis and liver damage are gradual. Thus, with severe liver damage, a greater impact on GM is observed, and, therefore, there are more difficulties to counteract such changes with antivirals.

The increase observed in Firmicutes in HCV-infected patients is a bit intriguing since the study from Aly et al., (2016) demonstrated a mild decrease in the abundance of these bacteria [15]. One explanation for these conflicting results could be the different degree of fibrosis of the patients included in each study (stage 4 vs 0–3 in our study). In fact, there is evidence that the microbiota of pre-cirrhotic patients is significantly different from the microbiota of the cirrhotic ones [34].

We observed a lower abundance of *Lachnospira* in HCV-infected patients, which could suggest loss of protection and persistent inflammation since this genus is a short chain fatty acids (SCFA)-producing taxa. SCFAs are metabolic products of gut microbiota metabolism with beneficial effects on health [35]. Among them, butyrate has been considered among the top targets since depletion of this microbe-derived metabolite is linked to several diseases and seems to facilitate establishment of enteric pathogens by disrupting colonization resistance [36]. The lower abundance of *Lachnospira* observed in HCV-infected people was completely abolished after 3 months under SVR, which could suggest increased production of butyrate and, therefore, a healthier gut. More functional/metabolomics studies are needed to corroborate such findings.

Blautia, Coprococcus and *Dorea genera* were increased in our HCV-infected patients which contrasts with the lower presence observed in stage 4-HCV patients [15]. The different fibrosis degree of the patients included in each study could be responsible for such opposite findings. However, since not only liver damage but also HCV infection is involved in gut dysbiosis, other factors such as HCV genotype should be taken into account. Unfortunately, this comparison could not be performed in our study because of the small sample size.

Veillonella was more abundant in HCV-infected patients than in the controls and treatment with DAAs did not restore this increased abundance. However, no differences in the abundance of this bacterial genus were observed when patients were split out depending on the fibrosis degree. Several studies have demonstrated an over-representation of *Veillonella* in cirrhotic patients (both HBV and HCV infected subjects) [37–39]. Interestingly, *Veillonella* showed a positive association with serum ALT or AST levels in these cirrhotic patients; however, the role of this genus before liver damage occurs is unknown. Thus, future studies are necessary to better characterize the role of *Veillonella* in non-cirrhotic HCV-infected patients. In contrast, a decreased presence of *Veillonella* has been found in colonic mucosal microbiome in cirrhosis and hepatic encephalopathy. However, these differences could be attributed to the fact that colonic mucosal microbiome differs from stool microbiome [40].

This is the first study where an increased abundance of *Dorea*, a SCFA-producing genus was observed in HCV-infected patients (especially in higher fibrosis degrees). A high abundance was also seen in other infectious diseases such as HIV-infection [41]. Its increased abundance was completely abolished after 3 months of SVR, however, its physiological impact needs to be further investigated to analyze the role of this bacterial genus in HCV-infection and recovery after treatment.

The increased presence of *Lactobacillus* in HCV-infected patients (independently of treatment), agrees with the study from Heidrich et al., (2018) [14]. However, the role of this genus and some bacterial species belonging to it (*L. ruminis*) in the evolution of HCV-infection needs to be specifically addressed, since some probiotics are based on different bacterial strains belonging to this genus [42] and its impact in HCV-infection has not been evaluated yet.

The decrease observed in *Akkermansia* was also found in non-cirrhotic patients in the study from Heidrich et al., (2018), although it was not observed in cirrhotic patients [14] which corroborates the fact that liver fibrosis degree is a key factor that contributes to a distinct gut profile. We also found a lower abundance of *A. municiphila* in HCV-infected patients, but only with higher fibrosis degrees (still non-cirrhotic patients). A lower abundance of this bacterial specie has also been observed in other pathologies such as psoriasis, obesity and type 2 diabetes and its administration has been demonstrated to improve health [43–46]. In fact, it has been proposed as a next-generation candidate for developing novel food or pharma supplements with beneficial effects [47]. Thus, *A. municiphila* could have a significant role in HCV-infection evolution and liver damage. The fact that DAAs are not able to preserve its abundance opens the opportunities to develop novel probiotics to potentiate the DAAs actions at this level.

One of the limitations of this study could be the small number of patients included in each group. However, 22 and 23 subjects per group could afford adequate statistical power to detect variation in community structure or composition between groups [48,49], and with potential to understand what is happening in GM in HCV-infection, and, more importantly, after DAAs treatment. In addition, the size of these groups is comparable and even higher than others carried out in the field of microbiota [15–17,19,39]. We are aware that our study is descriptive but provides a clear photo that could serve to understand the short-term effects of DAAs and SVR on GM and inflammation and that could lead to relevant clinical decisions (“treat as soon as possible”). However, more studies are needed in order to investigate other aspects that have not been specifically addressed in this study such as the impact of different DAAs regimens on GM, as previously carried out with antiretrovirals in HIV-infection [50]. Thus, this issue deserves further investigation with larger cohorts.

Finally, important aspects that could have an impact in GM composition have not been controlled in this study, such as the exact composition of the diet, alcohol consumption, smoking or other medications. Current smokers were included in both the control and HCV-infected population. Thus, we could suggest that the potential effects of nicotine in GM are similar in both groups and, therefore, the changes observed in our study are mainly due to the infection per se. Thus, we performed a metagenomic analysis separating HCV-infected patients depending on tobacco consumption (and independently of the fibrosis degree) and no differences were observed in α or β-diversity. Similar results were observed when HCV-infected patients were classified depending on the usage of proton pump inhibitors. Thus, only HCV-infection itself and the severity of the clinical stage (fibrosis degree) seem

to impact GM composition in non-cirrhotic patients, although other factors need to be deeply investigated.

To sum up, our results show that neither the usage of DAAs nor 3 months in SVR are able to counteract the major changes induced by HCV in non-cirrhotic patients. Only mild improvements were observed in the abundance of *Lachnospira* and *Dorea* genera. In addition, the partial restoration observed in inflammation (TNFα levels) and α-diversity was only observed in low degrees of fibrosis. These results underline the urgent need to begin treatment as soon as possible and the need for monitoring patients even after HCV eradication. In fact, it would be of great interest to carry out a follow-up study that could corroborate a significant reduction in the development of extrahepatic clinical events in those subjects with improvements in inflammation and intestinal dysbiosis. Finally, the development of novel probiotics to potentiate the DAAs actions emerges as a very interesting option.

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