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Microbial translocation and T cell activation are modified by direct-acting antiviral therapy in HCV-infected patients

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Summary

Background: Microbial translocation from the gut lumen has been involved in the pathogenesis of liver damage in hepatitis C virus (HCV) infection.

Aim: To investigate the impact of direct-acting antiviral treatment on microbial translocation and T-cell activation, in patients with hepatitis C-related liver disease.

Methods: We enrolled two groups of HCV-infected patients undergoing direct-acting antiviral treatment: patients with fibrosis ≥F3 according to Metavir (Group ≥F3); patients with hepatitis C recurrence after liver transplantation and Metavir ≥F2 (Group Liver Transplantation + ≥F2). All patients were treated with direct-acting antivirals based on ongoing guidelines. Surrogate biomarkers of microbial translocation (plasma concentrations of soluble-CD14, lipopolysaccharide-binding protein and intestinal fatty acid-binding protein) were evaluated at baseline, at first month, at the end of treatment and 3 months later. T-cell activation was measured by expression of CD38+ HLA-DR at the same time points, only in Group ≥F3.

Results: There were 32 patients in Group ≥F3 and 13 in Group LT + ≥F2. At baseline, levels of soluble-CD14 and lipopolysaccharide-binding protein were significantly higher in both groups vs healthy controls. Baseline soluble-CD14 correlated with glutamic-oxalacetic transaminase (r = 0.384, P = 0.009) and glutamic-pyruvic transaminase (r = 0.293, P = 0.05). A significant decrease in plasma levels of surrogate microbial translocation biomarkers was observed during and after treatment in the two groups although values were not normalised. In Group ≥F3, CD38+ HLADR+ T-cell expression was significantly decreased by direct-acting antiviral treatment. Relapsers (9%) showed higher soluble-CD14 levels at baseline.

Conclusion: Surrogate microbial translocation markers and T cell activation are increased in HCV-infected patients with liver fibrosis and decrease during direct-acting antiviral treatment.

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1 | INTRODUCTION

Hepatitis C virus (HCV) infection is, at present, one of the most frequent causes of cirrhosis in the western world, representing both a social and substantial health burden.[1]

Until recently, the standard of care for treatment of HCV infection was the combination of pegylated interferon alpha and ribavirin, which obtained virus clearance in a substantial fraction of patients, but at a high cost in terms of side effects. The advent of the new direct-acting antivirals, by targeting the hepatitis C viral proteins including NS3/4A, NS5A, and NS5B, changed dramatically the treatment of HCV infection reaching a high rate of HCV eradication with a low frequency of side effects.

Pro-inflammatory status and pro-fibrotic cytokines play an important role in the pathogenesis of liver damage during HCV infection, although the mechanisms involved are not completely clarified.[2] Several authors have reported that chronic inflammation and liver fibrosis in chronic HCV infection, are associated with microbial translocation (MT), from the gut lumen to the systemic circulation.[3-5] Indeed, lipopolysaccharide (LPS), which is a component of the outer layer of Gram-negative bacteria, is able to stimulate liver resident Kupffer and hepatic stellate cells, thereby contributing to the development of liver fibrosis.[6] Several plasma proteins have been validated as biomarkers of microbial translocation. Among these, lipopolysaccharide binding protein (LBP) and soluble CD14 (sCD14), have been used to measure the degree of microbial translocation in the peripheral blood.[7-10] Intestinal fatty acid-binding protein (I-FABP), reflecting enterocyte damage, is an additional biomarker strictly related to microbial translocation.[11,12] Microbial translocation occurs as a consequence of disruption of the intestinal barrier integrity. This condition is associated with a range of diseases such as intestinal ischemia, inflammatory bowel disease, graft-versus-host disease, and chronic viral infections including human immunodeficiency virus (HIV) and hepatitis C virus.[13,14] Microbial translocation products are considered a driving force of systemic immune activation in which increase of CD38+ and HLADR+ expression is one of the main feature.[14]

Few data are available about the role of microbial translocation in patients with chronic hepatitis or cirrhosis related to HCV infection, and during and after HCV clearance. No data are available about the liver transplanted patients with recurrence of HCV infection, who represent a particular population under immunosuppressive treatment.

Changes in microbial translocation markers in subjects with HCV infection treated with pegylated interferon and ribavirin have been reported in two studies.[15,16] Biomarkers of microbial translocation were found to be associated with the treatment outcome; however, in both studies, the results could have been partly influenced by the presence of pegylated interferon alpha in the treatment regimen, which may interfere with plasma levels of sCD14 by activating monocytes.

We therefore, conducted an observational study aiming at evaluating the modifications of microbial translocation and of T cells activation marker, in HCV-infected patients treated with direct-acting antiviral regimens.

2 | MATERIALS AND METHODS

Two groups of patients with chronic HCV infection, undergoing direct-acting antiviral therapy, were included in the study. The first group included patients with HCV infection and advanced fibrosis (F3-F4 according to Metavir) (Group ≥F3). The second group included patients with HCV recurrence after liver transplantation (LT) and fibrosis ≥ F2 according to Metavir (Group LT + ≥F2). Exclusion criteria in both groups were HIV co-infection, inflammatory bowel disease or other chronic intestinal diseases, alcohol abuse in the last 3 months and other concomitant causes of liver disease in addition to HCV infection.

A group of 11 healthy subjects were enrolled as controls for basal microbial translocation surrogate biomarkers.

The following antiviral treatments were administered: inhibitor of non-structural protein 5B (NS5B) sofosbuvir together with ribavirin for 24 weeks; sofosbuvir + inhibitor of NS4 (simeprivir) for 12 weeks in association with ribavirin (when indicated and in absence of contraindications); sofosbuvir + inhibitor of NS5A (ledipasvir or daclatasvir) for 12 or 24 weeks with or without ribavirin. The choice of treatment for each patient was based on the current guidelines according to the genotype, the characteristics of patients and the availability of drugs in our country/region at that moment.

Chronic HCV infection was defined as persistence of HCV RNA in serum 6 months after infection.[17] Sustained virological response for 12 weeks was defined as undetectable HCV RNA in serum at 12 weeks after completing therapy. Patients were evaluated at baseline (before starting the antiviral therapy) (T = 0), first month after initiation of treatment (T1), at the end of treatment (T2) and 3 months after the end of treatment (T3).

At each time-point clinical and biochemical data (albumin, International Normalised Ratio, cholinesterase, creatinine, urea, Glutamic-Oxalacetic Transaminases, Glutamic-Pyruvic Transaminases, γ-glutamyl transpeptidase, Alkaline Phosphatase, bilirubin, complete blood count, HCV-RNA PCR, and genotype, Model of End stage Liver Disease score) were recorded; microbial translocation markers, such as sCD14, LBP and I-FABP, were measured at the same time-points. The immunophenotypic study on T-cells was performed in Group ≥F3 but not in Group LT + ≥F2, where the chronic administration of immunosuppressive drugs, by reducing T cell activation and T-helper-cell dependent B-cell proliferation could represent a confounding factor.

Non-invasive assessment of liver fibrosis was performed at T0 and T3 through Fibroscan® (Echosens, Paris). At least 10 valid measurements, a 60% success rate, an interquartile range of less than 30% of the median elasticity, were required for eligibility.[18]

The study was approved by the Ethical Committee of La Sapienza University Hospital, Rome (2454/15). All subjects signed a

written informed consent before enrolment in the study. Data and plasma samples were collected respecting donor's confidentiality and privacy.

2.1 | Microbial translocation and enterocytes damage biomarkers analysis

Peripheral blood was drawn from each patient fasting in the morning in EDTA vials and processed within 24 hours. Plasma samples from 11 healthy individuals, were used as controls. Aliquots of plasma samples were stored at −80° until required for analysis.

Soluble CD14 (sCD14, R&D Systems, Minneapolis, MN, USA), Lipopolysaccharide Binding Protein (LBP) (LBP Human, ELISA Hycult biotech, Uden The Netherlands) and Intestinal fatty acid binding protein (I-FABP, Hycult Biotech, Uden,The Netherlands), were measured in plasma according to the manufacturer's instructions. For each biomarker, the optimal dilutions were determined: 1:500 for sCD14; 1:1000 for LBP and 1:2 for IFABP.

2.2 | Flow cytometry analysis

Multi-color flow cytometry analysis was performed on whole blood. Fluorescence intensities were measured with Gallios cytometer and analysed using Kaluza Analysis software version 1.3 (Beckman Coulter, Brea, CA, USA) using cut-offs based on isotype antibody staining. T-cell subpopulations were determined using the following fluorochrome-conjugated antibodies: PerCP-Cy5.5 anti-CD3, PE-Cy7 anti-CD4, FITC anti-CD8, in combination with Brilliant Violet 421 anti-CD38, V500 anti-HLA-DR (all purchased from BD Biosciences, San Diego, CA, USA).

2.3 | Statistical analysis

Statistical analyses and graphical presentation were done using SPSS software, version 24 (IBM, Somers, NY, USA). Results are given as the median, interquartile range (IQR) and percentage. Differences between groups were evaluated using the χ^2 test for categorical variables and by Kruskal-Wallis and Mann-Whitney *U* test for quantitative variables. Spearman's correlation coefficient was used to evaluate correlations between quantitative variables. Differences were considered statistically significant when $P < 0.05$.

3 | RESULTS

3.1 | Study population

Forty-five subjects with chronic HCV hepatitis were included in the study: 32 patients in Group ≥F3 and 13 patients in Group LT + ≥F2. All patients in the latter group were receiving immunosuppressive therapy to prevent graft rejection. The baseline characteristics of the patients are shown in Table 1. Eleven healthy subjects (73% males, median age 48 ± 5.4 years) were utilised as control group.

TABLE 1 Baseline characteristics of the two group of patients at enrolment

	Group ≥ F3	Group LT + ≥F2	*P* value
Patients n	32	13	
Age (y)	59 (52.0-70.2)	65 (55.0-66.5)	0.437
Male Gender n (%)	24 (75)	10 (77)	0.600
HCV genotype n (%)			
1	22 (69)	8 (62)	0.400
2	2 (6)	1 (8)	
3	7 (22)	2 (15)	
4	1 (3)	2 (15)	
SVR12 n (%)	30 (94%)	11 (85%)	0.300
Metavir, n (%)			
F2	0	6 (46)	<0.050
F3	3 (9)	2 (15)	
F4	29 (91)	5 (38)	
MELD score	9.0 (7-11.0)	9.0 (7.0-10.5)	0.400
Gastro-oesophageal varices, n (%)			
None	19 (59)	12 (92)	0.070
F1	7 (22)	0	
≥F2	6 (19)	1 (8)	
Ascites, n (%)	0	0	—
Time from LT (mo)	—	105 (16-225)	—
Immunosuppressive treatment, n (%)			
Tacrolimus	—	8 (62)	—
Everolimus	—	3 (24)	
Sirolimus	—	1 (7)	
Cyclosporine	—	1 (7)	

Values are expressed as medians (IQR) or percentages.
HCV: hepatitis C virus; LT: liver transplantation; MELD: model for end stage liver disease; SVR: sustained virological response.

Severe fibrosis was almost universal in Group ≥F3 (F4 91%) but less frequent in the transplanted patients at the time of treatment (F4 38% $P < 0.05$). Due to the characteristics of patients (including genotype) and drugs available at the time of treatment, direct-acting antiviral regimens were different in the two groups: most of the patients in Group ≥F3 were treated with sofosbuvir + NS5A inhibitor (ledipasvir or daclatasvir) (53%), followed by those treated with sofosbuvir + simeprevir (38%) and with sofosbuvir alone (9%). On the other hand, patients of Group LT + ≥F2 were treated mostly with sofosbuvir alone (62%), followed by sofosbuvir + simeprevir (38%). Ribavirin was used in all transplanted patients and in 50% of Group ≥F3.

3.2 | Virological outcome

A sustained virological response at 12 weeks was obtained in thirty patients (94%) in Group ≥F3 and in eleven patients (85%) in Group LT + ≥F2. Modifications in liver function tests, viral load and

ultrasonography parameters before, during and after treatment are shown in Table 2. An amelioration in transaminases, serum albumin levels, and liver stiffness was observed in both groups during and after treatment.

3.3 | Basal LBP, sCD14 and I-FABP in HCV patients and healthy controls

Baseline levels of markers of microbial translocation and enterocyte damage are reported in Figure 1. In both groups, plasma levels of LBP, sCD14, and I-FABP were increased when compared to healthy subjects. Plasma values measured in this latter group were consistent with those reported for adult healthy population in the individual package inserts of the relevant ELISA assays.

Specifically, median sCD14 values at baseline in HCV patients of were significantly higher in comparison with healthy controls (1176.0 ng/mL, IQR: 1048.0-1358.0; $P < 0.0001$) with no differences between group ≥F3 and group LT + ≥F2 (1928.5 ng/mL, IQR: 1441.8-2321.5 vs 2117.5 ng/mL, IQR: 1814.5-2161.5 $P = 0.430$). Baseline LBP level, on the other hand, were higher in Group LT + ≥F2, followed by Group ≥F3 (22.5 mg/mL IQR: 18.1-24.9 vs 14.1 mg/mL, IQR: 11.8-17.0, respectively; $P = 0.001$) and controls (8.9 mg/mL, IQR: 7.8-10.4; $P < 0.0001$). Concerning I-FABP, baseline levels were significantly higher in Group LT + ≥F2 in comparison with controls (728.0 pg/mL, IQR: 338.0-1008.0, vs 277.2 pg/mL, IQR: 173.0-374.0, $P = 0.008$). sCD14 levels in both groups significantly correlated with I-FABP ($r = 0.342$; $P = 0.01$) and LBP ($r = 0.430$; $P = 0.001$) (Figure S1).

To better understand the clinical significance of our findings, we analysed potential correlations between baseline sCD14 levels and markers of hepatic necrosis, fibrosis, and liver protein synthesis. We found that sCD14 plasma levels were associated with the increase of glutamic-oxalacetic transaminase ($r = 0.384$, $P = 0.009$) and glutamic-pyruvic transaminases ($r = 0.293$, $P = 0.05$). No association

TABLE 2 Longitudinal changes in hepatic markers during direct-acting antivirals treatment in two group of patients

Group 1	TIME 0 (Baseline)	TIME 1 (1st month)	TIME 2 (End of treatment)	TIME 3 (3rd month after end of treatment)
ALP (U/L)	110 (92.0-149.5)	91.0 (76.8-126.5)**	85.0 (78.5-100.5)*	91.5 (77.3-122.8)
GOT (U/L)	62.5 (40.0-97.8)	30.0 (23.0-37.0)***	25.0 (21.5-32.5)***	27.0 (22.0-32.0)***
GPT (U/L)	62.0 (40.0-117.0)	28.0 (16.0-40.0)***	24.0 (17.5-35.0)***	29.0 (20.0-37.0)***
GGT (U/L)	72.0 (52.3-115.8)	44.0 (20.0-72.0)*	32.0 (20.0-59.0)**	32.0 (21.0-87.0)
MELD score	9.0 (7.0-11.0)	9.5 (8.0-12.0)**	9.0 (7.3-12.0)	8.0 (7.0-11.0)
HCV RNA (detectable n, %)	32 (100%)	5 (15.7%)	0 (0%)	2 (6.3%)
Stifness (kPa)	23.9 (16.3-28.7)	ND	ND	14.7 (8.7-19.0)***
Albumin (gr/dL)	3.75 (3.40-4.10)	3.95 (3.63-4.28)*	4.00 (3.70-4.40)	4.00 (3.80-4.20)**
INR	1.14 (10.8-1.38)	1.20 (1.10-1.30)	1.18 (1.07-1.36)	1.17 (1.09-1.38)
Platelets ($x10^3$)	105 (71.2-128)	113 (70-153)	103 (63-131)	95.5 (59.2-142)
Portal vein diameter (mm)	12.8 (11.8-13.9)	ND	ND	12.9 (11.6-14.1)
Spleen longitudinal diameter (cm)	14.6 (12.7-16.9)	ND	ND	13.8 (12.6-16.6)
Group 2				
ALP (U/L)	106.0 (77.5-116.5)	87.0 (75.0-113.5)*	92.0 (72.0-107.0)	73.5 (62.0-91.0)**
GOT (U/L)	51.0 (30.0-81.0)	19.0 (16.5-30.5)**	21.0 (15.0-22.5)**	16.5 (16.0-28.0)*
GPT (U/L)	72.0 (39.5-100.0)	19.0 (18.0-28.5)***	16.0 (13.0-28.0)**	17.0 (14.0-33.5)*
GGT (U/L)	72.0 (40.5-185.5)	44.0 (18.3-83.8)**	20.0 (12.5-35.0)*	25.5 (16.0-43.0)*
MELD score	9.0 (7.0-10.5)	9.0 (7.5-12.0)	10.0 (7.0 -13.0)	9.5 (7.25-10.8)
HCV RNA (detectable n, %)	13 (100%)	6 (46.2%)	1 (7.7%)	2 (15.4%)
Stifness (kPa)	11.9 (8.1-15.3)	ND	ND	6.9 (6.1-11.6)*
Albumin (gr/dL)	4.30 (4.00-4.40)	4.30 (4.20-4.50)	4.30 (4.05-4.40)	4.50 (4.25-4.65)*
INR	1.07 (1.05-1.15)	1.10 (1.03-1.22)	1.12 (10.5-1.23)	1.09 (1.05-1.10)
Platelets ($x10^3$)	142 (110-175)	165 (131-231)*	145 (128-222)*	153 (118-224)
Portal vein diameter (mm)	12.6 (10.0-16.0)	ND	ND	13.2 (12.1-14.1)
Spleen longitudinal diameter (cm)	13.7 (12.8-15.9)	ND	ND	14.1 (12.0-16.0)

Data are expressed as median (IQR).
ALP: alkaline phosphatase; GGT: γ-glutamyl transpeptidase; GOT: glutamic-oxalacetic transaminase; GPT: glutamic-pyruvic transaminase; HCV: hepatitis C virus; INR: international normalised ratio; MELD: model for end-stage liver disease; ND: not done.
Asterisks indicate statistical differences with respect to baseline values *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$.

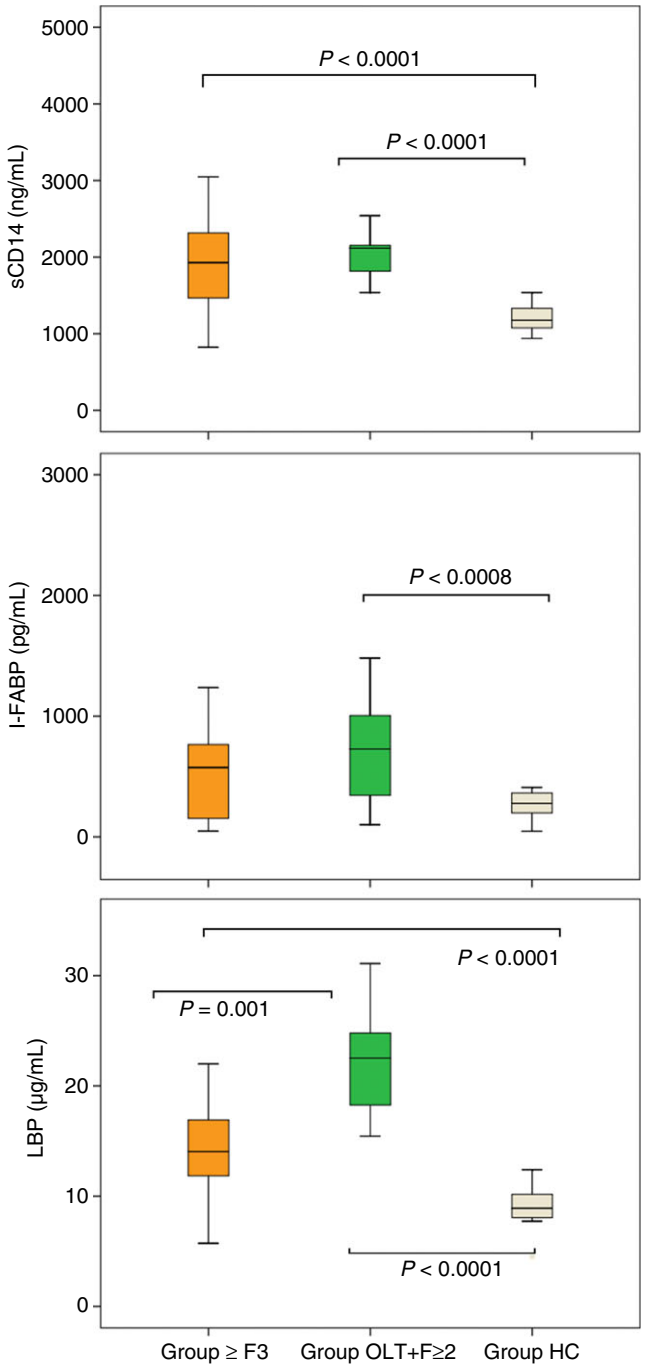

FIGURE 1 Baseline levels of soluble-CD14 (sCD14), intestinal fatty acid-binding protein (I-FABP) and lipopolysaccharide binding protein (LBP) in hepatitis C virus positive patients eligible for direct-antiviral agents treatment. Group ≥F3 (orange; n = 32 patients with HCV infection and advanced fibrosis), group LT + ≥F2 (green; n = 13 patients with HCV recurrence after liver transplantation) and Group HC (beige; n = 11 healthy controls)

was found between sCD14 plasma levels and albumin or INR plasma levels and indirect signs of portal hypertension (spleen diameter, the presence of oesophageal varices). No correlation was found between sCD14, LBP or I-FABP levels and liver stiffness (kPa) ($P = 0.876$; $P = 0.091$; $P = 0.813$ respectively) nor with baseline viral load. A weak correlation was found between I-FABP and MELD score ($r = 0.303$; $P = 0.045$, Figure 2).

3.4 | Changes of LBP, I-FABP, and sCD14 during direct-acting antiviral treatment

In group ≥F3, following the sharp decrease of viral load during the first month of treatment, plasma levels of sCD14 and LBP showed a rapid and significant decline. Three months after the end of the treatment sCD14 and LPB plasma levels remained significantly lower with respect to baseline ($P = 0.016$ and $P = 0.04$, respectively). I-FABP plasma levels showed a minor non-significant decline during direct-acting antiviral treatment (Figure 3).

Patients in group LT + ≥F2 experienced a less pronounced decrease in surrogate markers of microbial translocation when compared with patients in group ≥F3. sCD14, I-FABP, and LBP plasma level did not show significant variations with respect to baseline values during the first month of direct-acting antiviral treatment. Only LBP levels showed a significant decrease at T3 ($P = 0.016$) with respect to baseline levels. However, at T3 all markers of microbial translocation in Group LT + ≥F2 patients were still more elevated than in controls (Figure 3).

No significant difference was observed in microbial translocation biomarkers changes with respect to different types of direct-acting antiviral treatments.

3.5 | Changes in T-cells activation markers expression in Group 1 during direct-acting antiviral treatments

With the aim of exploring the impact of direct-acting antivirals on T-cell activation, we measured the variations in the CD38+HLADR+ expression on CD4+ and CD8+ T-cells. As expected, the percentage of total CD4+ (median: 63.5%, IQR: 58.4-68.6) and CD8+ cells (median: 30.7%, IQR: 24.5-34.1) remained stable during the direct-acting antiviral treatment, but in both lymphocyte populations a significant decrease was seen in the subset of cells expressing the marker of activation CD38+HLADR+, paralleled by the decrease of sCD14. At T3 the percentage of CD4+ and CD8+ expressing CD38+HLADR+ receptors were reduced by 40.8% ($P = 0.001$) and 64%, $P = 0.004$) respectively, respect to baseline (Figure 4).

3.6 | Factors associated with HCV relapse

Only four patients (two in each group), experienced HCV relapse after treatment. Age, gender, viral genotype, and viral load, type of treatment, baseline stiffness (kPa), baseline LBP and I-FABP levels were similar in patients reaching SVR 12 and in those with relapse. On the other hand, we observed that in both groups, the sCD14 levels at baseline were significantly higher in the relapsers with respect to patients with SVR12 (2582.5 ng/mL vs 1777.5 ng/mL, $P = 0.019$). Nevertheless, the trend in sCD14 decrease during treatment was similar in patients with and without SVR.

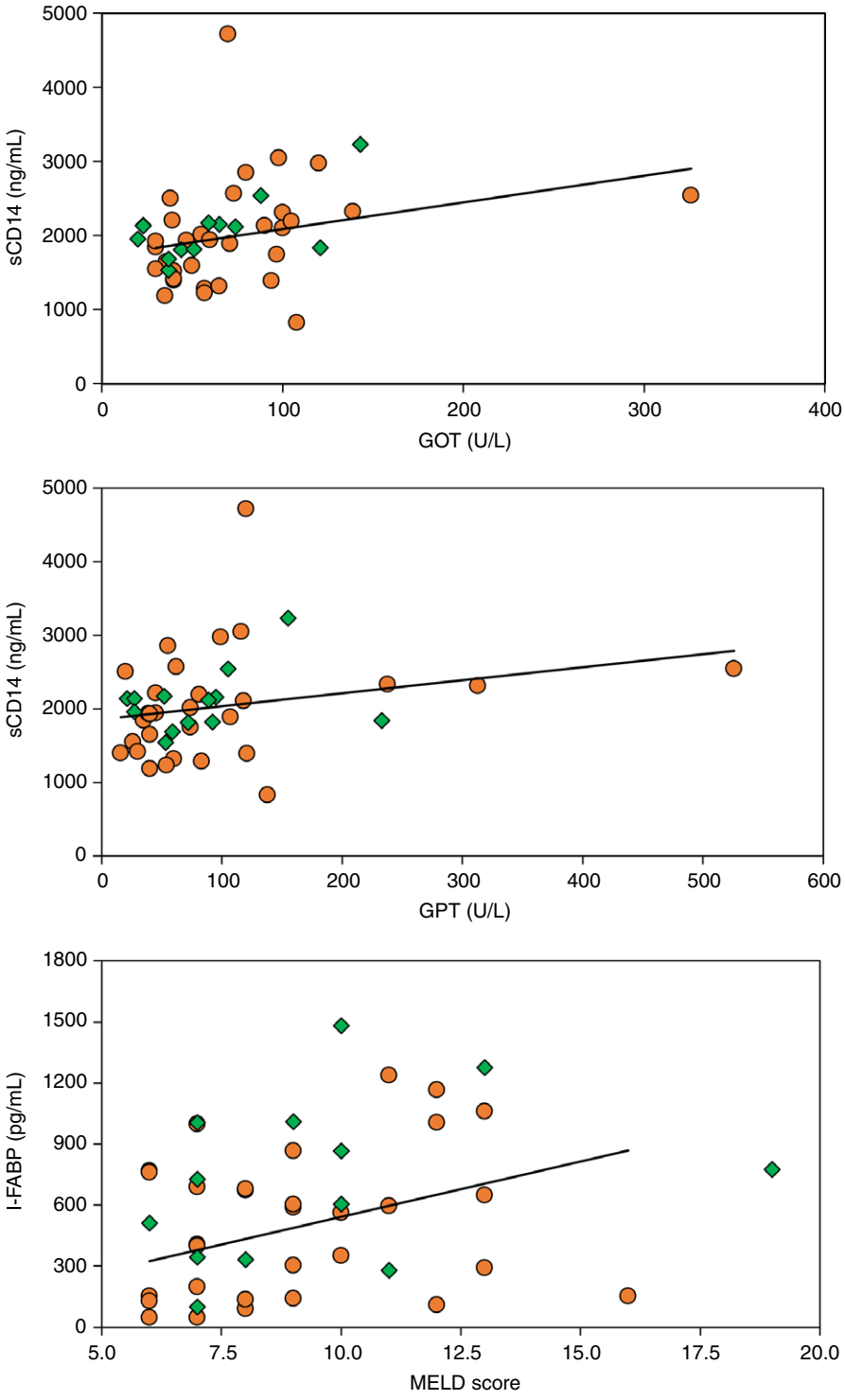

FIGURE 2 Correlations between microbial translocation markers and transaminases or disease severity (model of end stage liver disease - MELD-) at baseline. Group ≥F3 (orange circles) (n = 32 patients with HCV infection and advanced fibrosis), Group LT + ≥F2 (green diamonds) (n = 13 patients with HCV recurrence after liver transplantation). Top panel: soluble-CD14 (s-CD14) and glutamic-oxalacetic transaminases (GOT) (r = 0.384, P = 0.009); Mid panel: soluble-CD14 (s-CD14) and glutamic-pyruvic transaminases (GPT) (r = 0.293, P = 0.050); Bottom panel: intestinal fatty acid-binding protein (I-FABP) and MELD (r = 0.303, P = 0.045)

4 | DISCUSSION

HCV infection represents one of the most important causes of liver disease in the western world. In recent years, direct-acting antiviral therapy has produced a dramatic change in the outcome of HCV infected patients and in the epidemiology of liver diseases.[19]

Microbial translocation and chronic inflammation have been found to be associated with the progression of liver damage, but few studies have explored the modifications induced by direct-acting antiviral treatments on microbial translocation and T-cell activation in HCV patients.

The main findings of our study were the following: (a) plasma levels of sCD14 and LBP, surrogate markers of microbial

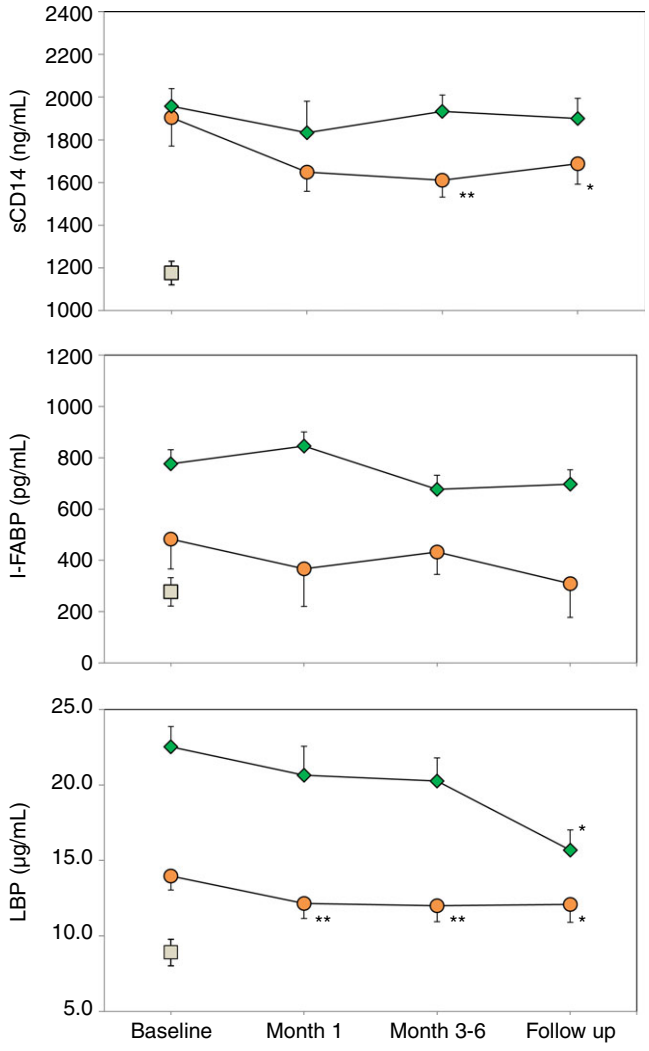

FIGURE 3 Longitudinal changes of soluble-CD14 (sCD14), intestinal fatty acid-binding protein (I-FABP) and lipopolysaccharide binding protein (LBP) plasma levels during and after direct-antiviral agents treatment in group ≥F3 (orange circles) (n = 32 patients with HCV infection and advanced fibrosis), and group LT + ≥F2 (green diamonds) (n = 13 patients with HCV recurrence after liver transplantation). Values for healthy controls (n = 11) are reported only at baseline (beige square). Values are expressed as median and SE. Asterisks indicate a significant change vs baseline levels (*$P < 0.05$; **$P < 0.01$)

translocation, are significantly higher in untreated HCV infected patients than in healthy subjects; (b) the elevated levels of sCD14 correlate with transaminases levels; (c) Direct-acting antiviral treatments induced a significant decrease in microbial translocation surrogate markers and a significant decrease of CD38+HLADR+ expression on T lymphocytes in patients in Group ≥F3. Furthermore, viral relapse after treatment was associated with higher plasma levels of sCD14 at baseline in our population.

To our knowledge, this is one of the first studies in which changes in plasma levels of microbial translocation markers during direct-acting antiviral treatments are analysed and no study to date evaluated this aspect in a population with the recurrence of HCV after liver transplantation.

High levels of surrogate microbial translocation markers in HCV patients confirm the observation by other authors[3,16,20] who also suggested that microbial translocation may contribute to persistent inflammation and development of fibrosis in chronic HCV infection.[16] Even if the mechanism underlying the link between HCV infection and microbial translocation was beyond the purpose of our study, a possible explanation could be related to gut epithelial damage, as was observed in a study[21] conducted in HIV/HCV coinfected patients, in whom HIV is known to colonise enterocytes. However, I-FABP, which is a marker of enterocyte damage,[12] was not increased in our series, suggesting that the pathogenetic role of gut epithelial damage is unlikely in our patients. Portal hypertension, derived from liver fibrosis and portosystemic shunting, has also been claimed to cause an alteration of intestinal permeability mainly in cirrhotic patients[22]; this could partially contribute to explain the increase in microbial translocation surrogate markers in our series of compensated advanced liver disease (Group ≥F3) with presence of oesophageal varices in 41% of patients.

Immunological impairment can also lead to a less efficient intestinal barrier and increased microbial translocation as is the case in other conditions of immunodeficiency.[23-25] This may be supported by the observation that liver transplanted patients (group LT + ≥F2), receiving chronic immunosuppressive therapy, showed higher levels of microbial translocation respect to group ≥F3, in the absence of portal hypertension in the majority of them. Indeed, in group LT + ≥F2, sCD14, as well as other surrogate biomarkers of microbial translocation, tended to be higher than in group ≥F3. This is consistent with previous reports that have shown a persistent status of immune activation, likely due to subclinical infections associated with lifelong immunosuppressive therapy, in liver transplant patients.[26,27] Increased levels of LBP have also been reported during acute and chronic rejections,[25] our patients, however, had no signs of rejection.

In both groups, the levels of sCD14 were elevated when compared to healthy subjects and correlated with transaminases levels. Similar findings have been described in a cohort of HCV patients by Sandler et al[16] who also found a significant correlation between microbial translocation and the degree of liver fibrosis. Although these patients were rather similar to those in our cohort, we failed to find an association between microbial translocation surrogate markers and Metavir. Indeed, sCD14 and I-FABP levels were similar in patients with different degree of fibrosis. The different methods utilised for the assessment of liver fibrosis (liver biopsy vs Fibroscan) could have also influenced the results.

Moreover, despite portal hypertension is known to cause an alteration of intestinal permeability, we failed to find this association in our study. This was probably due to the mild degree of portal hypertension in our series. In fact, as shown in Table 1, none of our patients had experienced ascites and only 7 patients (15%) had varices ≥ F2. In the contest of compensated liver disease, it is likely that other mechanisms, such as hepatic inflammation, intestinal damage and alteration in immune system, could have been more relevant as a cause of microbial translocation.

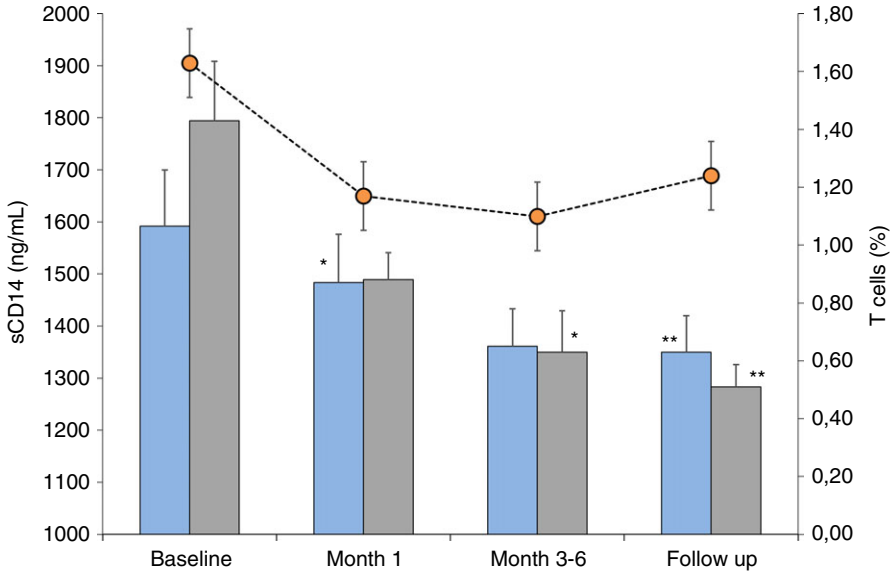

FIGURE 4 Longitudinal variations of CD38+HLDR+ expression on CD4+ (blue bars), and CD8+ (grey bars) T-cells in 30 patients of Group F ≥ 3 during and after direct-antiviral agents treatment. The line shows the changes of sCD14 plasma levels. (*$P < 0.05$, **$P < 0.001$)

To support the association between monocyte activation and the release of bacterial translocation products in our series we found that sCD14 levels were directly correlated with LBP.

Direct-acting antiviral treatment induced HCV clearance in the large majority of patients (91%) and caused improvement of liver function, as indicated by the amelioration of all liver function tests.

Viral eradication was paralleled by a significant decline of sCD14 and LBP in group ≥F3 and a significant reduction of LBP in group LT + ≥F2 patients. The decrease of sCD14 and LBP levels, although not reaching normal values, persisted 3 months after the end of antiviral treatment. Previous studies on the impact of direct-acting antivirals on systemic immune activation in HCV patients have reported controversial results; while some studies highlighted a reduction of the markers of immune activation during direct-acting antiviral treatment,[28] others did not find similar changes.[20]

The rapid decline of microbial translocation surrogate markers after direct-acting antiviral treatment suggests that viral clearance may affect microbial translocation. This makes unlikely a relevant role of portal hypertension, mainly in group ≥F3, as the amelioration of portal hypertension, when reversible, is known to take longer than 3 months after eradication.[29]

The evidence that direct-acting antiviral therapy, through HCV clearance, can modify the immune system is provided by studies about HCV-cryoglobulinemia vasculitis in which direct-acting antivirals effectively normalises many of the abnormalities in peripheral B- and T-lymphocytes homeostasis.[30] Specific restoration of proliferative CD8+ T-cell responses was observed, when lymphocytes were stimulated by HCV antigens, following HCV elimination by an interferon-free direct-acting antiviral regimens[31] and it has been reported that successful interferon-free therapy prevented interferon-mediated intrahepatic immune activation and resulted in normalisation of Natural Killer cell functions.[32,33]

T regulatory cells play an important role in HCV infection, both in the maintenance of chronicity by inhibiting anti-HCV immune responses and in attenuating the intrahepatic tissue-damaging response to infection.[34] Literature data exist also indicating that in chronic HCV infection colonic T regulatory cells are higher than in controls[35] and this finding may lead to alteration in the gut barrier and increased microbial translocation, potentially reversible after therapy.

In our study, after direct-acting antiviral treatment, we found a significant decline in markers of T-cell activation. A previous study also reported a decline of T-cells CD38+HLADR+ expression in chronic HCV patients, after interferon-free treatment.[36]

The immunophenotypic analysis was performed only in Group ≥F3, since it is known that the chronic immunosuppressive therapy, as administered in patients in Group LT + ≥F2, could impact on the CD38 expression on T-cells. Indeed, it has been reported that CD38 expression on T-cells is under-expressed after anti-rejection therapy, and its determination has been proposed as assay to be used routinely to quantify the level of immunosuppression in clinical practice.[37]

Overall, our results suggest that direct-acting antiviral treatment is associated to a reduction in inflammation and generalised immune activation. It can be speculated that, this will lead to a reduction in cirrhosis-related complications, including arterial vasodilatation, hyperdynamic circulation and bacterial infections.[38] Further studies, with longer follow up are needed to confirm this hypothesis.

As expected, the relapse rate was very low in our patient population, occurring only in 4 subjects (2 in Group ≥F3 and 2 in Group LT + ≥F2). Interestingly, these patients, independently from groups, showed baseline sCD14 levels significantly higher when compared to patients who achieved an SVR. The pattern of decrease of microbial translocation markers during treatment was similar to other patients. A possible explanation derives from the observation that all these patients initially responded to the therapy and cleared HCV, with the relapse occurring later on, after the end of treatment. Although the number of cases does not allow any further speculations, it is noteworthy that in a previous study the high baseline sCD14 level in patients receiving antiviral treatment (interferon or

PEG-interferon with or without ribavirin) was significantly associated with treatment failure.[16]

Our study, to our knowledge, is one of the first studies where changes of microbial translocation markers during direct-acting antiviral treatment were evaluated in a population of liver transplanted patients with HCV infection recurrence. The study has, however, some limitations: first, the limited number of patients; second, we did not measure directly LPS plasma levels. However, measuring LPS concentration is a technically complex process that can be further complicated by its rapid clearance and by the presence of inhibitory plasma proteins.[39-41] In this view, LPS-binding protein (LBP), a protein with a relatively long half-life synthesised by the liver in response to bacteraemia,[42,43] can be considered a reasonable surrogate biomarker for assessing LPS-induced inflammation in humans. At the same time, as for many other acute phase proteins, the liver is directly responsible for LBP synthesis,[44] and the LBP upregulation may be compromised in case of an impaired capacity of the liver,[45] making the interpretation of results sometimes difficult. Moreover, it is important to underline that this study refers to first generation direct-acting antivirals.

In conclusion, our findings suggest that direct-acting antiviral therapy can significantly reduce plasma levels of microbial translocation biomarkers and proportion of activated T-cells in HCV patients with advanced fibrosis. These effects persist at 3 months after the end of therapy. Further studies, conducted in larger cohorts of patients, with longer follow up, will allow to understand the possible clinical consequences of the reduction/normalisation of systemic inflammation.

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SUPPORTING INFORMATION

Additional supporting information will be found online in the Supporting Information section at the end of the article.

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