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Original Article

Gut Microbiota and Cytokine Profile in Cirrhosis

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Abstract

Background and Aims: Gut dysbiosis and abnormal cytokine profiles are common in cirrhosis. This study aimed to evaluate the correlations between them. ***Methods:*** In the blood plasma of cirrhosis patients and controls, 27 cytokines were examined using a multiplex assay. The plasma levels of nitrites (stable metabolites of the endothelial dysfunction biomarker nitric oxide) and lipopolysaccharide (LPS) were examined. The fecal microbiota was assessed by 16S rRNA gene sequencing. ***Results:*** Levels of IL-1b, IL-2, IL-6, IL-13, IP-10, IFN-g, TNF-a, LPS, and nitrites were higher in cirrhosis patients than in controls, while levels of IL-4, IL-7, and PDGF-BB were lower. The LPS level was directly correlated with the levels of IL-1b, IL1-Ra, IL-9, IL-17, PDGF-BB, IL-6, TNF-a, and nitrites. The nitrite level was significantly directly correlated with the levels of TNF-a, GM-CSF, IL-17, and IL-12, and inversely correlated with the IL-7 level. TNF-a levels were directly correlated with ascites severity and the abundance of Negativicutes, Enterobacteriaceae, Veillonellaceae, and Klebsiella, while inversely correlated with the abundance of Firmicutes, Clostridia, and Subdoligranulum. IFN-g levels were directly correlated with the abundance of Bacteroidaceae, Lactobacillaceae, Bacteroides, and Megasphaera, and inversely correlated with the abundance of Verrucomicrobiota, Akkermansiaceae, Coriobacteriaceae, Akkermansia, Collinsella, and Gemella. IL-1b levels were directly correlated with the abundance of Comamonadaceae and Enterobacteriaceae and inversely correlated with the abundance of Marinifilaceae and Dialister. IL-6 levels were directly correlated with the abundance of Enterobacteriaceae, hepatic encephalopathy, and ascites severity, and inversely correlated with the abundance of Peptostreptococcaceae, Streptococcaceae, and Streptococcus. ***Conclusions:*** The abundance of harmful gut microbiota taxa and endotoxinemia directly correlates with the levels of proinflammatory cytokines.

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Introduction

Cirrhosis, a natural outcome of chronic liver diseases, significantly contributes to increased disability and mortality in the population.[1–3] It was assumed that disorders in the composition of the gut microbiota (gut dysbiosis) play an important role in the pathogenesis of this disease, in addition to the etiological factors (hepatotropic viruses, alcohol, various metabolic disorders, etc.). This interaction is known as the gut-liver axis.[4–9] Intestinal bacteria are crucial in developing immune responses.[10–14] By penetrating our body through the intestinal epithelial barrier (bacterial translocation), they begin to interact with immune system cells, modulating their production of various cytokines. These cytokines have pro- and anti-inflammatory effects, stimulate and suppress the formation of various cells, and have many other effects.[10–14] Studies have shown that the plasma levels of IL-1b, IL-6, TNF-a, and IFN-g are increased in cirrhosis.[15,16] Moreover, the plasma levels of most of these cytokines are higher in decompensated cirrhosis than in compensated cirrhosis.[17,18] TNF-a levels directly correlate with biomarkers of bacterial translocation (lipopolysaccharide [LPS] and bacterial DNA in the blood plasma) in cirrhosis.[19,20] However, the levels of other cytokines in the plasma in cirrhosis have not been studied enough. It is suggested that the increased formation of pro-inflammatory cytokines in cirrhosis leads to the development of vasodilating endothelial dysfunction (with NO as the main mediator), arterial hypotension, compensatory fluid retention, hyperdynamic circulation, increased blood inflow to the abdominal organs, and outflow through the portal vein system. This aggravates portal hypertension, increasing the severity of ascites, hypoalbuminemia, shunting, and hepatic

Keywords: Endotoxin; Leaky gut; Gut microbiome; Systemic inflammation; Gut-liver axis; Endotoxemia.

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encephalopathy.[4–9]

Direct associations of gut dysbiosis with arterial vasodilation, hyperdynamic circulation, hypoalbuminemia, hepatic encephalopathy, and poor short- and long-term prognosis have been demonstrated.[21–25] Only one study analyzed the association of plasma levels of seven cytokines (IL-6, IL-2, IL-1b, IL-4, IL-10, TNF-a, IFN-g) with the abundance of gut microbiota taxa in cirrhosis.[26] However, this study established correlations between the levels of some cytokines and only one minor gut microbiota taxon, which did not allow verification of the aforementioned model of cirrhosis-associated gut-liver axis disorders in humans. Modern multiplex technologies enable the evaluation of several dozen cytokines in one sample, providing an almost complete cytokine profile. No studies have assessed the association of gut microbiota taxa with the plasma levels of various cytokines in a wide cytokine profile in cirrhosis, which became the aim of our study.

Methods

The study was conducted in accordance with the Declaration of Helsinki and approved by the local ethics committee (No. 03-16). All participants signed informed consent.

Patients and controls

Patients with cirrhosis who came to our clinic for periodic examinations were screened for participation in the study. Inclusion criteria included a diagnosis of cirrhosis, established based on biopsy data or a combination of clinical, laboratory, and instrumental signs, and an age range of 18 to 70 years. Exclusion criteria included significant concomitant diseases (such as cancer, cardiac, renal, or respiratory failure), infections, intestinal diseases, alcohol consumption in the past six weeks, and the use of drugs affecting the gut microbiota (probiotics, prebiotics, synbiotics, antibiotics) in the past six weeks.

Clinically healthy individuals who came to the clinic for periodic preventive examinations served as the control group.

Investigations

All patients underwent standard laboratory, instrumental, physical, and basic neurological examinations. Additionally, they underwent a number of connection tests to diagnose minimal hepatic encephalopathy,[27] and fasting blood was taken for analysis of the cytokine profile, nitrites, and LPS (see *Cytokines, LPS, and Nitrites Analysis* subchapter). The blood was immediately centrifuged and then the plasma was separated and immediately frozen. Patients also provided stool samples for gut microbiota testing in a clean disposable container, which was immediately placed in a refrigerator at −80°C.

Gut microbiota analysis

Gut microbiota analysis was performed as previously described.[28–30]

Total DNA was isolated using an AmpliPrime DNA-sorb-AM kit (NextBio, Moscow, Russia) for clinical specimens, according to the manufacturer's protocol. The isolated DNA was stored at −20°C. For qualitative and quantitative assessment of the isolated DNA, we used NanoDrop 1000 equipment (Thermo Fisher Scientific, Waltham, MA, United States). The 16S library preparation was carried out according to the protocol of 16S metagenomic sequencing library preparation (Illumina, San Diego, CA, United States), recommended for Illumina MiSeq sample preparation. The first round of amplification of V3-V4 16S rDNA variable regions was performed using the following primers: forward (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG) and reverse (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC). These primers are aimed at amplifying bacterial (more than 90%) but not archaeal (less than 5%) rRNA genes. The amplification program (Applied Biosystems 2720 Thermal Cycler, Foster City, CA, United States) was as follows: (1) 95°C for 3 m; (2) 30 cycles: 95°C for 30 s; 55°C for 30 s; 72°C for 30 s; (3) 72°C for 5 m; and (4) 4°C.[28]

The derived amplicons were purified using Agencourt AMPure XP (Beckman Coulter, Brea, CA, United States) beads according to the manufacturer's protocol. The second round of amplification was used for double-indexing samples with a combination of specific primers. The amplification program was as follows: (1) 95°C for 3 min; (2) 8 cycles: 95°C for 30 s; 55°C for 30 s; 72°C for 30 s; (3) 72°C for 5 m; and (4) 4°C.[28]

The purification of PCR products was also carried out using Agencourt AMPure XP beads. The concentration of the derived 16S rDNA libraries was measured using a Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA, United States) with the QuantiT™ dsDNA High-Sensitivity Assay Kit. The purified amplicons were mixed equimolarly according to the derived concentration values. The quality of the libraries was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States) and an Agilent DNA 1000 Kit. Sequencing was carried out on a MiSeq machine (Illumina) using the MiSeq Reagent Kit v2 (paired-end reads, 2 × 300 nt).[28]

First, forward and reverse reads were merged using MeFiT 1.0, a wrapper for and CASPER 0.8.2 tool.[28] The merging was performed with the default MeFiT parameters, except for the meep-score threshold (0.4), and default CASPER parameters, except for minimum overlap (30 bp), with a threshold mismatch ratio of 0.5. For most samples, more than 99% of the reads were successfully merged. For reads without overlaps, we included only forward reads that were trimmed with trimmomatic 0.39 (3'-tail trimming quality threshold 28; average quality threshold 24) in the analysis. Next, reads were analyzed with the DADA2 1.22 package (a part of the Bioconductor project) for R 4.2.2[29] to remove primers (cutadapt 3.2; primer error rate threshold 0.1), filter reads (without trimming, since the reads had been pre-merged), correct errors, infer RSV (ribosomal sequence variants), and remove chimeras. Next, a taxonomic annotation of the derived RSVs was performed using the naive RDP classifier algorithm (built-in default DADA2 annotation engine) based on the Silva (version 138.1) 16S reference sequence database.[30]

Cytokines, LPS, and nitrites analysis

The cytokine profile (27 cytokines: IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, bFGF, G-CSF, GM-CSF, IFN-g, IP-10, MCP-1, MIP-1a, MIP-1b, PDGF-BB, RANTES [regulated on activation, normal T cell expressed and secreted], TNF-a, VEGF) was assessed using a multiplex assay (kit 171-304000; BioRad).[31]

The plasma level of nitrites (as a stable metabolite of unstable NO) was assessed using photometric analysis (kit A013-2; Cloud-Clone Corp., China).[32]

The plasma LPS levels were measured by the LAL-test (kit EC64405S; Xiamen Bioendo Technology Co., Xiamen, China).[33]

Statistics

Data are presented as median (interquartile range). Comparisons of continuous variables were made using the Mann-

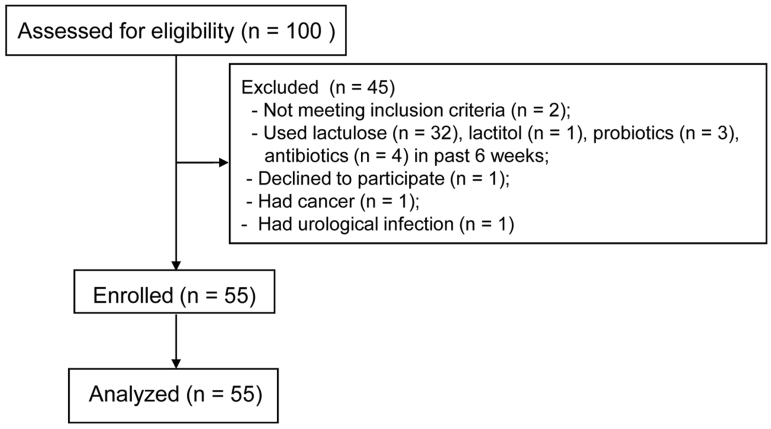

Fig. 1. Flow chart of the study.

Whitney test. Comparisons of categorical variables were made using the Fisher exact test. Correlations were assessed using the Spearman test. Statistical calculations were carried out using STATISTICA 12 (StatSoft; USA) software. Comparison of gut microbiota between cirrhosis patients and control subjects was carried out using linear discriminant analysis effect size with the online service "http://www.bic.ac.cn/BIC/#/". Beta-diversity was calculated using Bray-Curtis distances.

Results

The study included 55 patients with cirrhosis and 15 clinically healthy controls (Fig. 1). These groups did not differ in age, gender, or body mass index (Table 1). The etiology of cirrhosis was viral (n=12), alcohol (n=28), mixed (n=9), and unknown (n=6). The main characteristics of patients and controls are presented in Table 1.

Among the cytokines tested, the levels of IL-15 and VEGF were below the lower limit of detection for all samples. The levels of IL-1b, IL-13, IP-10, IL-2, IL-6, IFN-g, TNF-a, LPS, and nitrites were higher in patients with cirrhosis than in healthy individuals, while IL-4, IL-7, and PDGF-BB levels were lower in patients with cirrhosis than in healthy controls. There was no significant difference in the levels of the other cytokines tested between patients with cirrhosis and healthy controls (Table 2).

The gut microbiome of cirrhosis patients differed from that of healthy controls (Fig. 2). There was no significant difference in gut microbiota alpha-biodiversity between the groups of patients with cirrhosis and healthy individuals (Table 2). However, the control and cirrhosis groups differed significantly in beta-diversity (Fig. 3; p (PERMANOVA)<0.001; p (PERMDISP)=0.003).

The composition of the gut microbiota at the phylum, class, family, and genus levels for both patients with cirrhosis and healthy controls is presented in Supplementary Figures 1–3.

Among the cytokines tested, the plasma LPS level was significantly directly correlated with the levels of IL-1b, IL1-Ra, IL-9, IL-17, PDGF-BB, IL-6, and TNF-a. The plasma nitrite level was significantly directly correlated with the levels of TNF-a, GM-CSF, IL-17, and IL-12, and inversely correlated with the IL-7 level in cirrhosis (Table 3). The plasma levels of LPS and nitrites were directly correlated (r=0.455; p=0.002).

The levels of TNF-a were directly correlated with the abundance of Negativicutes, Enterobacteriaceae, Veillonellaceae, and Klebsiella, and inversely correlated with the abundance of Firmicutes, Clostridia, and Subdoligranulum. The levels of IFN-g were directly correlated with the abundance of Bacteroidaceae, Lactobacillaceae, Bacteroides, and Megasphaera, and inversely correlated with the abundance of Verrucomicrobiota, Akkermansiaceae, Coriobacteriaceae, Akkermansia, Collinsella, and Gemella. The levels of IL-1b were directly correlated with the abundance of Comamonadaceae and Enterobacteriaceae, and inversely correlated with the abundance of Marinifilaceae and Dialister. The levels of IL-6 were directly correlated with the abundance of Enterobacteriaceae, and inversely correlated with the abundance of Peptostreptococcaceae, Streptococcaceae, and Streptococcus. The levels of IL-2 were directly correlated with the abundance of Negativicutes, Veillonellaceae, and Oscillibacter. The levels of IL-4 were inversely correlated with the abundance of Enterobacteriaceae and Micrococcaceae (with Rothia genus). The levels of IL-7 were directly correlated with the abundance of Firmicutes and Clostridia, and inversely correlated with the abundance of Actinobacteriota, Bacteroidota, Acidaminococcaceae, Erysipelotrichaceae, and Holdemanella. These and other significant correlations between gut microbiota taxa and the studied cytokines are presented in Figures 4 and 5.

Patients with clinically significant ascites (grade 2–3 ascites according to the International Ascites Club) had higher levels of LPS, nitrites, TNF-a, and IL-6, and lower levels of IL-10 and IL-4 compared to patients without clinically significant ascites (Fig. 6). There was no significant difference in the levels of other tested cytokines between patients with and without clinically significant ascites.

The abundances of Enterobacteriaceae, Veillonellaceae, and Peptostreptococcus were increased, and the abundance of Defluviitaleaceae was decreased in patients with clinically significant ascites compared to patients without this complication of cirrhosis (Fig. 7).

Patients with hepatic encephalopathy (overt + covert) had higher plasma levels of IL-8 (13.9 [6.0–33.5] vs. 11.2 [3.9–12.8] pg/mL; p=0.041), IL-6 (9.6 [5.9–18.6] vs. 4.2 [2.5–5.1] pg/mL; p=0.002), and LPS (0.03 [0.01–0.18] vs. 0.00 [0.00–0.02] EU/mL; p=0.049) compared to patients without cognitive impairment. There was no significant difference in the levels of other tested cytokines and nitrites between these groups of patients.

The Child-Pugh score directly correlated with the levels of LPS (r=0.438; p=0.003), TNF-a (r=0.33199; p=0.019), and IL-6 (r=0.705; p=0.001), as well as with Enterobacteriaceae abundance (r=0.272; p=0.044). It inversely correlated with the levels of IL-5 (r=−0.399; p=0.011) and IL-4 (r=−0.366; p=0.016), and with the abundances of

Table 1. The main characteristics of included patients with cirrhosis and healthy controls

	Cirrhosis (*n*=55)	Healthy controls (*n*=15)	*p*-value
Age, year	48 (43–56)	46 (39–54)	0.306
Gender, M/F	21/34	7/8	0.380
Body mass index, kg/m^2	25.4 (23.7–28.4)	25.0 (23.7–25.8)	0.356
Child–Pugh score	8 (7–9)	Not evaluated	
Child–Pugh class, B+C/A	37+10/8		
Esophageal varices (grade 2–3 + grade 1/absent)	31+19/5		
Hepatic encephalopathy (overt + minimal/absent)	4+35/16	0/15	<0.001
Ascites (grade 2 + grade 1/absent)	16+20/19	0/15	<0.001
Serum total protein, g/L	71 (65–75)	Not evaluated	
Serum albumin, g/L	34 (31–38)		
Serum glucose, mmol/L	4.8 (4.4–5.5)	5.0 (4.6–5.2)	0.524
Serum cholesterol, mmol/L	4.3 (3.3–5.1)	4.8 (4.6–5.2)	0.023
Serum total bilirubin, μmol/L	44 (26–65)	Not evaluated	
ALT, U/L	28 (19–47)		
AST, U/L	48 (36–68)		
GGT, U/L	86 (40–126)		
Alkaline phosphatase, U/L	242 (194–342)		
Cholinesterase, kU/L	3.8 (2.9–4.9)		
Iron, μmol/L	14.8 (7.3–22.4)		
Sodium, mmol/L	141 (140–142)		
Creatinine, μmol/L	77 (71–97)		
Red blood cells, 10^{12} cell/L	3.85 (3.35–4.20)	4.67 (4.32–4.78)	<0.001
White blood cells, 10^9 cell/L	4.1 (3.1–5.6)	6.5 (5.8–7.2)	<0.001
Platelets, 10^9 cell/L	95 (75–113)	265 (208–298)	<0.001
International normalized ratio	1.52 (1.38–1.71)	Not evaluated	
Fibrinogen, g/L	2.4 (2.0–3.0)		
Splenic length, cm	15.0 (13.2–17.0)		

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl aminotransferase.

Firmicutes (r=−0.269; *p*=0.047), Clostridia (r=−0.288; *p*=0.033), Clostridiaceae (r=−0.338; *p*=0.012), and Peptococcaceae (r=−0.350; *p*=0.009.

The IL-2 levels inversely correlated with serum iron levels (r=−0.306; *p*=0.035).

The level of IL-4 directly correlated with the levels of albumin (r=0.357; *p*=0.018) and sodium (r=0.430; *p*=0.004).

The level of IL-5 directly correlated with the cholinesterase level (r=0.381; *p*=0.031) and inversely with the total bilirubin level (r=−0.365; *p*=0.021).

The level of IL-6 directly correlated with the severity of ascites (r=0.661; *p*<0.001) and hepatic encephalopathy (r=0.598; *p*=0.002), the international normalized ratio (r=0.475; *p*=0.016), and inversely correlated with serum levels of albumin (r=−0.471; *p*=0.018), cholesterol (r=−0.441; *p*=0.028), and iron (r=−0.510; *p*=0.009). The level of IL-8 directly correlated with the severity of hepatic encephalopathy (r=0.303; *p*=0.026), the levels of white blood cells (r=0.297; *p*=0.029), and GGT (r=0.344; *p*=0.010).

The level of IL-9 inversely correlated with the creatinine level (r=−0.311; *p*=0.045). The level of IL-12 inversely correlated with the iron level (r=−0.310; *p*=0.020). The level of IL-13 directly correlated with the GGT level (r=0.305; *p*=0.030) and inversely correlated with body mass index (r=−0.282; *p*=0.045). The level of PDGF-BB directly correlated with the white blood cell count (r=0.392; *p*=0.003) and platelet count (r=0.300; *p*=0.028).

The level of MIP-1a inversely correlated with the level of IgM (r=−0.337; *p*=0.019). The level of IP-10 directly correlated with the severity of esophageal varices (r=0.352; *p*=0.008) and the levels of iron (r=0.272; *p*=0.044), albumin (r=0.272; *p*=0.045), and ALT (r=0.270; *p*=0.044).

There were no other significant correlations between plasma cytokine levels and clinical parameters of cirrhosis. The number of healthy controls was too small to adequately assess the correlation between the composition of their gut microbiota and cytokine profile.

Discussion

The aim of our study was to evaluate the correlations between the abundance of gut microbiota taxa and the levels of

Table 2. The plasma levels of the cytokines, nitrites, and LPS and gut microbiota diversity indices in patients with cirrhosis and healthy controls

	Cirrhosis (*n*=55)	Healthy controls (*n*=15)	*p*-value
bFGF, pg/mL	5.0 (3.4–10.1)	6.4 (4.8–7.0)	0.934
Eotaxin, pg/mL	41 (17–93)	46 (36–54)	0.736
G-CSF, pg/mL	5.8 (3.4–14.8)	6.4 (5.0–7.0)	0.611
GM-CSF, pg/mL	3.3 (1.9–8.4)	3.8 (3.3–4.2)	0.546
IFN-g, pg/mL	***2.7 (1.5–4.2)***	***1.9 (1.7–1.9)***	***0.040***
IL-1b, pg/mL	***2.7 (1.6–4.5)***	***1.8 (1.4–1.9)***	***0.040***
IL1-ra, pg/mL	97 (39–317)	203 (96–250)	0.518
IL-2, pg/mL	***0.34 (0.17–0.68)***	***0.20 (0.15–0.20)***	***0.031***
IL-4, pg/mL	***3.9 (2.3–6.6)***	***5.7 (5.2–6.0)***	***0.024***
IL-5, pg/mL	77 (24–159)	79 (57–88)	0.622
IL-6, pg/mL	***5.9 (4.0–13.3)***	***4.2 (2.5–5.1)***	***0.033***
IL-7, pg/mL	***50 (15–95)***	***145 (91–163)***	***0.001***
IL-8, pg/mL	11.2 (5.7–26.2)	11.2 (3.9–12.8)	0.333
IL-9, ng/mL	0.93 (0.17–1.43)	1.10 (0.99–1.17)	0.452
IL-10, pg/mL	2.6 (1.8–5.9)	3.8 (3.0–4.0)	0.461
IL-12, pg/mL	24 (13–35)	27 (25–29)	0.338
IL-13, pg/mL	***3.9 (1.9–5.9)***	***2.4 (2.2–2.8)***	***0.048***
IL-17, pg/mL	11.6 (5.6–21.5)	12.6 (10.2–13.4)	0.920
IP-10, pg/mL	***558 (287–895)***	***419 (184–483)***	***0.045***
MCP-1, pg/mL	15 (6–43)	18 (15–21)	0.745
MIP-1a, pg/mL	1.8 (1.0–3.6)	2.0 (2.5–3.0)	0.156
MIP-1b, pg/mL	174 (75–252)	214 (180–221)	0.161
PDGF-BB, pg/mL	***77 (35–193)***	***225 (180–249)***	***0.004***
RANTES, ng/mL	18.3 (2.5–25.0)	20.8 (20.7–21.1)	0.886
TNF-a, pg/mL	***27 (24–43)***	***26 (9–29)***	***0.048***
Nitrites, µmol/L	***137 (80–168)***	***101 (93–118)***	***0.043***
LPS, EU/mL	***0.02 (0.01–0.09)***	***0.00 (0.00–0.02)***	***0.002***
Shannon index	2.41 (1.96–2.72)	2.36 (2.16–2.56)	0.920
Chao index	173 (105–493)	208 (196–231)	0.283

Significant differences are shown in bold italics. LPS, lipopolysaccharide.

various cytokines in cirrhosis. To measure the latter, we used a modern multiplex method, which allows us to determine several analytes in one sample. We attempted to test the hypothesis that gut dysbiosis, leading to bacterial translocation, changes the cytokine profile to a pro-inflammatory status, which is associated with several manifestations of cirrhosis. We were able to establish direct correlations between the abundance of harmful Enterobacteriaceae, which have endotoxin (LPS) and are capable of bacterial translocation, and the levels of the main pro-inflammatory cytokines (IL-6, TNF-a, IL-1b). Additionally, similar direct correlations are found between these cytokines and endotoxemia. A direct correlation the levels of TNF-a and LPS with nitrite levels is also established. The lack of correlation between IL-6 and nitrite levels, despite its correlation with the severity of ascites, may indicate that this cytokine has less of a hemodynamic effect and more of an effect promoting fluid accumulation in the abdominal cavity. This effect can be due to its role in suppressing albumin formation in the liver, leading to hypoalbuminemia, which, along with hemodynamic disturbances, plays an important role in ascites development. The anti-inflammatory cytokine IL-4 reduces IL-6 production, which may explain why the development of ascites is associated with a decrease in the concentration of IL-4. In accordance with the hypothesis of gut-liver axis disorder, our study demonstrates that patients with clinically significant ascites (grade 2–3) have higher levels of endotoxin, nitrites, and the main pro-inflammatory cytokines (TNF-a and IL-6), and lower levels of anti-inflammatory cytokines (IL-10 and IL-4) compared to patients without ascites or those with ascites detected only by instrumental methods.

In addition to this primary finding of our study, we observed that while the levels of some cytokines, such as IL-13, IL-2, and IFN-g, were increased in cirrhosis, others (IL-7 and PDGF-BB) were decreased, and the levels of most cytokines did not significantly differ from those in healthy individuals.

IL-2 is produced by T-cells in response to antigenic stimulation and stimulates the development of an immune re-

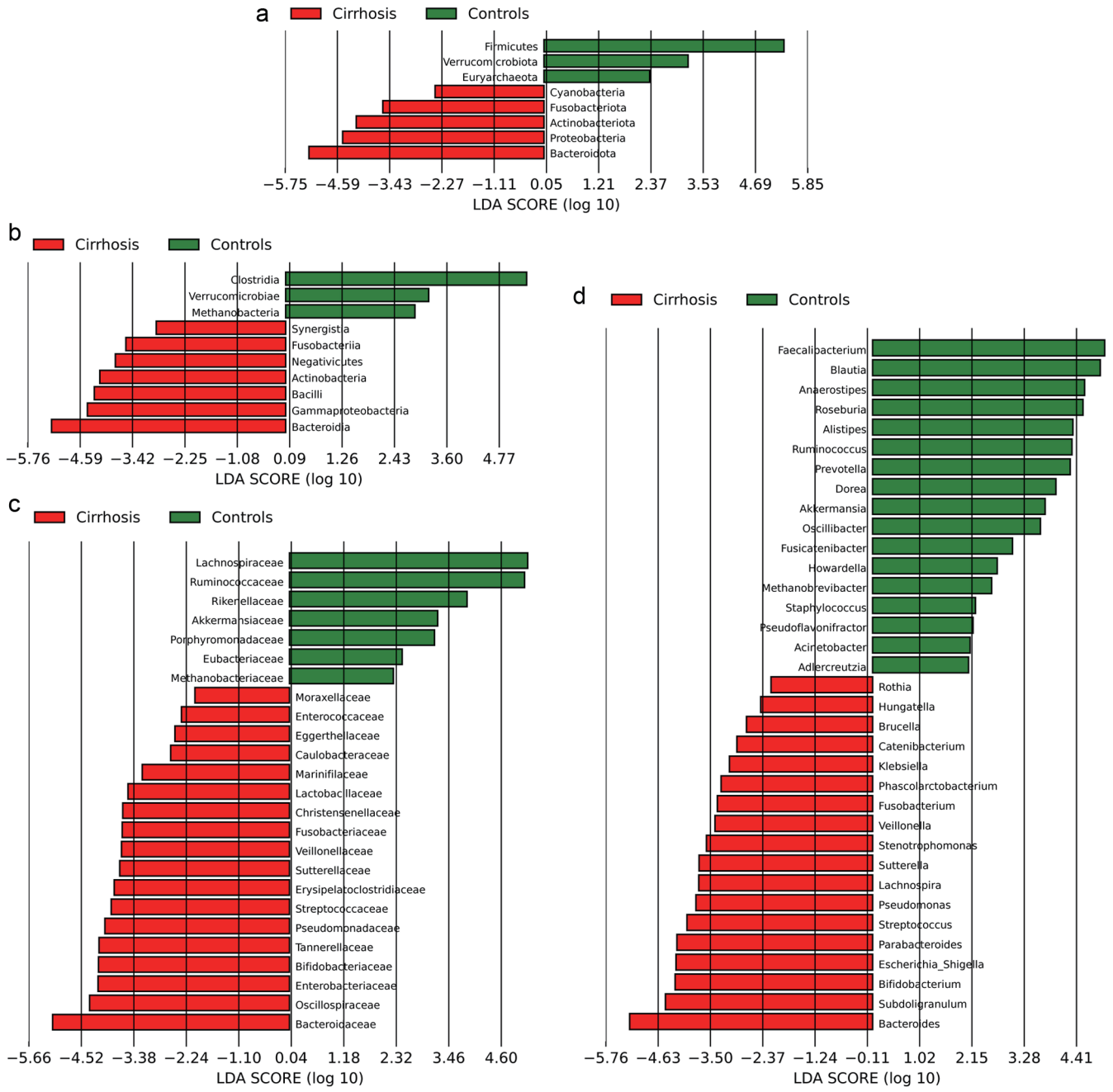

Fig. 2. Comparison of the intestinal microbiota of patients with cirrhosis and healthy controls at the level of phyla (a), classes (b), families (c), and genera (d). LDA, Linear discriminant analysis.

sponse.[34] Although its level was increased in cirrhosis, it did not significantly correlate with endotoxemia levels, nitrites, or cirrhosis manifestations. Among gut microbiota taxa, its level significantly correlated only with the abundance of Veillonellaceae within the Negativicutes class, suggesting a limited role of this cytokine in cirrhosis pathogenesis and the gut-liver axis.

IFN-g is one of the main cytokines that stimulate cellular immunity.[35] While its level is increased in cirrhosis, our study did not find a significant correlation with cirrhosis manifestations or endotoxemia. Direct correlations of this cytokine with the levels of Bacteroidaceae, which have many positive and negative properties,[36] were obtained, as well as negative correlations with the abundance of beneficial Akkermansiaceae[37] within the Verrucomicrobiota phylum. Further research is needed to understand these connections.

Although IL-13 is a major cytokine in allergic inflammation with demonstrated profibrotic effects, its role in the liver remains unclear.[38] Our study found higher IL-13 levels in cirrhosis than in healthy individuals, without a correlation with endotoxemia severity. IL-13 levels correlated with the abundance of minor families Acidaminococcaceae and Sutterel-

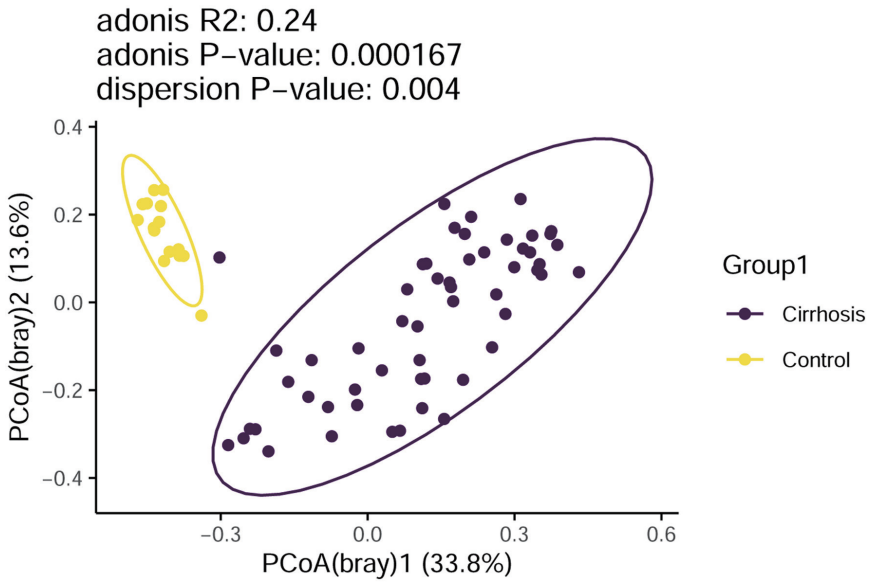

Fig. 3. Principal Coordinates Analysis (PCoA) using the Bray-Curtis distance. The gut microbiota of patients with cirrhosis and healthy controls.

laceae, highlighting the need for further investigation into their role in the gut-liver axis in cirrhosis.

IP-10 is secreted by monocytes, endothelial cells, and fibroblasts in response to IFN-g stimulation.[39] This cytokine acts as a chemoattractant for monocytes, macrophages, and T cells, and also inhibits angiogenesis among other functions. In our study, IP-10 levels were higher in cirrhosis compared to healthy individuals but did not correlate with the severity of endotoxemia or the abundances of the main gut microbiota taxa. Its role in the gut-liver axis in cirrhosis appears limited.

IL-7 is a cytokine with diverse functions,[40] including an antifibrotic effect through the blockade of cell production of the profibrotic molecule transforming growth factor beta.[41] Our study found lower IL-7 levels in cirrhosis compared to healthy individuals, suggesting a profibrotic status in these individuals that may contribute to the development of liver fibrosis leading to cirrhosis. IL-7 levels did not significantly correlate with endotoxemia levels in our study but showed an inverse correlation with nitrite levels, a biomarker of endothelial dysfunction. IL-7 levels positively correlated with the abundance of beneficial Clostridia within the Firmicutes phylum, and negatively correlated with Actinobacteriota and Bacteroidota levels, which increase significantly in cirrhosis compared to healthy individuals. These findings suggest that IL-7 may play an important, endotoxin-independent role in the gut-liver axis, potentially preventing profibrogenic disorders in chronic liver diseases. Further research is needed to clarify these interactions.

Table 3. Correlation matrix of significant correlations of plasma levels of various cytokines with plasma levels of LPS and nitrites in cirrhosis

	LPS	Nitrites
IL-1b	0.410; 0.047	NS
IL1-ra	0.306; 0.046	NS
IL-6	0.566; 0.018	NS
IL-7	NS	−0.335; 0.032
IL-9	0.380; 0.020	NS
IL-12	NS	0.384; 0.005
IL-17	0.293; 0.048	0.306; 0.029
PDGF-BB	0.400; 0.007	NS
GM-CSF	NS	0.336; 0.021
TNF-a	0.495; 0.001	0.300; 0.043

NS, not significant; LPS, lipopolysaccharide.

PDGF-BB is formed and released into the blood plasma by platelets.[42] Therefore, a decrease in its plasma level in cirrhosis, characterized by thrombocytopenia, is logical. Its plasma level correlated directly with platelet and white blood cell counts. Furthermore, our study found a direct correlation between plasma PDGF-BB levels and endotoxemia, suggesting that LPS may stimulate platelet formation and PDGF-BB secretion in cirrhosis.

IL-17 is a recently discovered pro-inflammatory cytokine.[43] Although its level in our study did not differ significantly between patients with cirrhosis and healthy controls, it showed a significant correlation with the level of endotoxemia and vasodilating endothelial dysfunction. The plasma level of this cytokine correlated with several taxa of the gut microbiota, most of which are minor and have unknown functions. Further studies are required to determine the role of IL-17 in the disordered gut-liver-heart axis in cirrhosis.

IL-4 is mainly produced by T-helpers and has anti-inflammatory effects by blocking the activation of macrophages and their cytokine production, as well as the acute-phase response of hepatocytes to IL-6.[44] The IL-4 level decreases in cirrhosis but does not depend on the degree of endotoxemia and is not associated with endothelial nitric oxide formation. Moreover, it inversely correlates with the abundance of harmful Enterobacteriaceae in the gut microbiota. This suggests that these bacteria may inhibit IL-4 formation through an LPS-independent pathway. The direct correlation between IL-4 and albumin levels can be easily explained as IL-4 antagonizes IL-6 in the development of the acute-phase inflammatory response of hepatocytes, which includes a decrease in albumin production. Hypoalbuminemia is a factor in ascites progression, which sequesters sodium, potentially

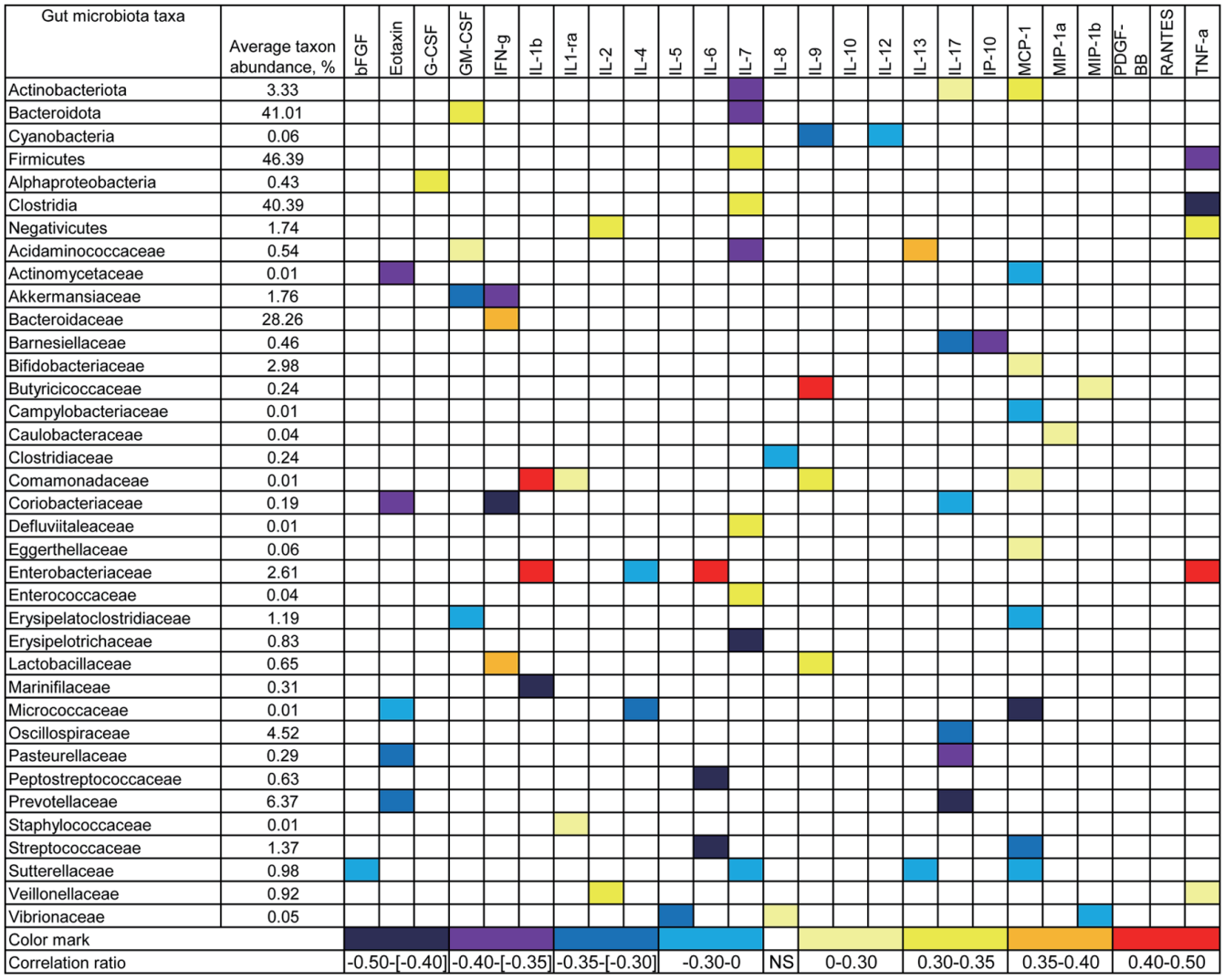

Fig. 4. Correlation between tested cytokines and gut microbiota taxa at supragenus level. NS, not significant.

explaining the association of low IL-4 levels with low sodium levels.

GM-CSF is a growth factor for progenitor cells of neutrophils and monocytes, which together constitute the largest group of white blood cells.[44] The plasma level of this cytokine did not change in cirrhosis and did not correlate with a reduced number of white blood cells, suggesting a minimal role in the pathogenesis of cirrhotic pancytopenia. GM-CSF levels directly correlated with the abundance of Bacteroidota, which have a pluripotent effect on the macroorganism, and inversely correlated with the levels of beneficial Akkermansia and Brautia. The level of this cytokine did not significantly correlate with the severity of any cirrhosis manifestation. Probably, the role of GM-CSF in cirrhosis pathogenesis is minimal, and its correlations with gut microbiota taxa reflect its general pro-inflammatory effect (Bacteroidota having weak endotoxin) and anti-inflammatory effect (Akkermansia and Brautia), which increase in cirrhosis due to weakened intestinal barrier. Interestingly, the level of this cytokine did not correlate with the levels of endotoxemia and the abundance in the gut microbiota of main producers of active LPS, such as Enterobacteriaceae. Possibly, GM-CSF production by intestinal intraepithelial lymphocytes is stimulated in response to increased LPS levels in intestinal contents, primarily dependent on the abundance of Bacteroidota, which significantly dominate over Enterobacteriaceae in gut microbiota. In contrast, Enterobacteriaceae are the main source of LPS among translocated gut bacteria. Further research is needed to clarify the nature of the relationships between gut microbiota and GM-CSF production under normal and pathological conditions.

Previous studies have reported an association of increased IL-6 levels with hepatic encephalopathy,[45–49] however, our study was the first to show direct associations between gut microbiota (Enterobacteriaceae), endotoxemia, systemic inflammation (IL-6), and hepatic encephalopathy.

In our study, many other interesting correlations were found among the levels of other cytokines studied, gut microbiota taxa, levels of endotoxemia and vasodilating endothelial dysfunction, and several manifestations of cirrhosis. The significance of these findings in the disordered gut-liver axis in cirrhosis needs to be established in future studies.

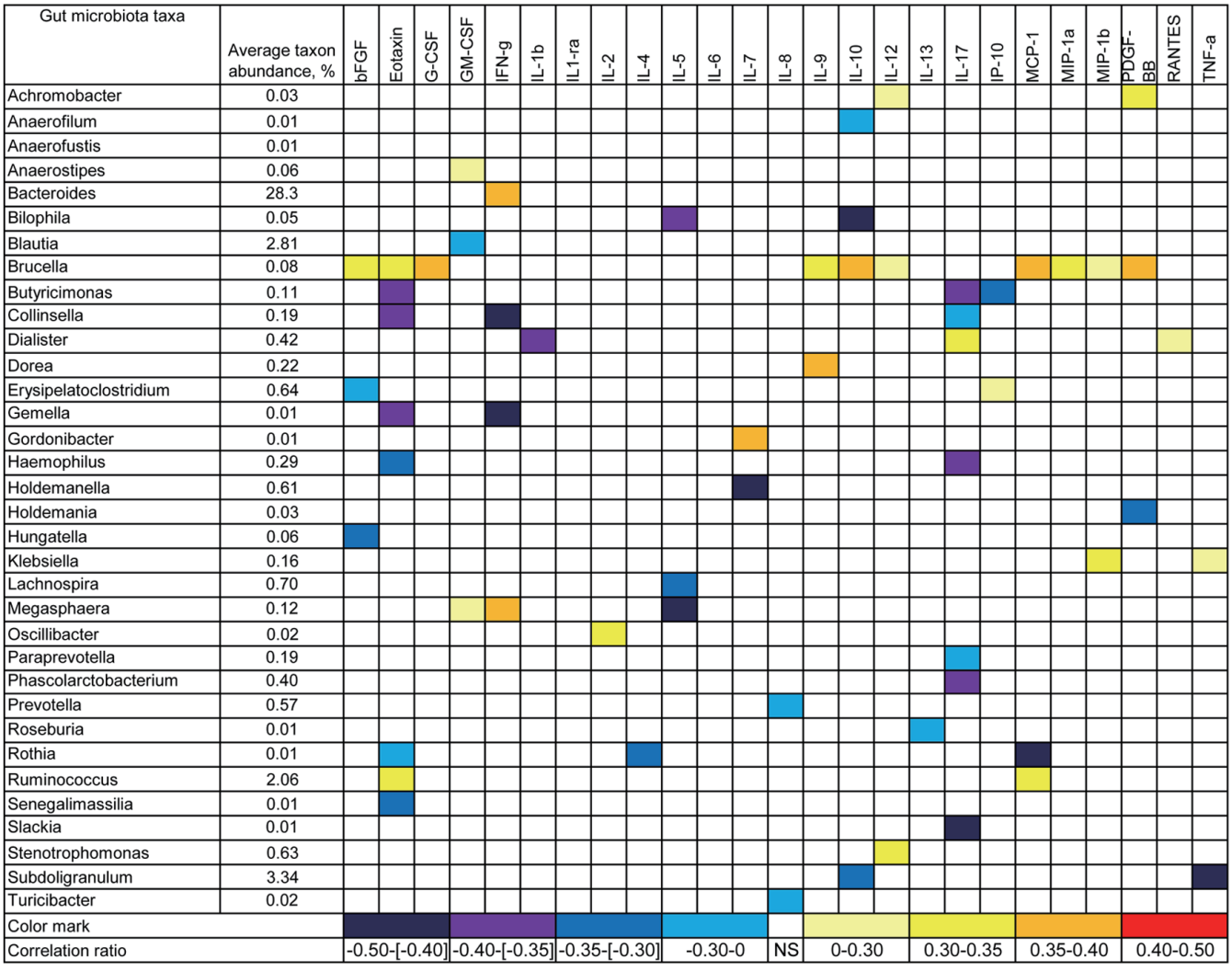

Fig. 5. Correlation between tested cytokines and gut microbiota taxa at the genus level. NS, not significant.

The mechanisms through which the gut microbiota can influence the cytokine profile are diverse. There is an abundance of bacterial pathogen-associated molecular patterns, such as LPS, flagellin, teichoic acids, peptidoglycan, and others. By interacting with various pattern recognition receptors of the cells of the innate and adaptive immune system, these molecules are able to enhance and inhibit the production of various cytokines by these cells. This appears to be the primary mechanism. However, other pathways also appear to exist. For example, the role of bile acids in modulating the response to exogenous interferon has recently been demonstrated, indicating that these molecules may also influence cytokine production.[50] Bile acids are produced by the liver and metabolized by intestinal bacteria. Consequently, secondary bile acids are formed, which, having different affinities for specific receptors, modulate the function of the immune system.[51] In addition, different bacteria in the gut microbiota produce various short-chain fatty acids, which have also shown immunomodulatory effects through their receptors. Intestinal bacteria also form active metabolites of tryptophan and other amino acids. All of these compounds actively interact with immune system cells, modulating cytokine production, which can have various effects, including those associated with worsening cirrhosis.[51] Our study was the first to comprehensively examine a broad spectrum of cytokines in cirrhosis and their association with specific gut microbiota taxa and cirrhosis features. This represents its novelty and strength. However, we acknowledge that we have only begun to explore the gut-liver-immune axis, with a more detailed study being an important challenge for future research.

The limitation of our study was the small number of participants, which nevertheless allowed us to obtain significant results. Additionally, subgroup analysis could not account for the etiology of cirrhosis due to the small size of the subgroups. It should be noted that we have established associations, not causations. Further studies are needed to establish whether changes in the composition of the gut microbiota indeed lead to changes in the cytokine spectrum in cirrhosis. Further studies are also required to identify specific mechanisms by which gut microbiota taxa influence the levels of various cytokines and how these cytokines influence the pathogenetic pathways of cirrhosis.

Clarification of these relationships will enable the justifica-

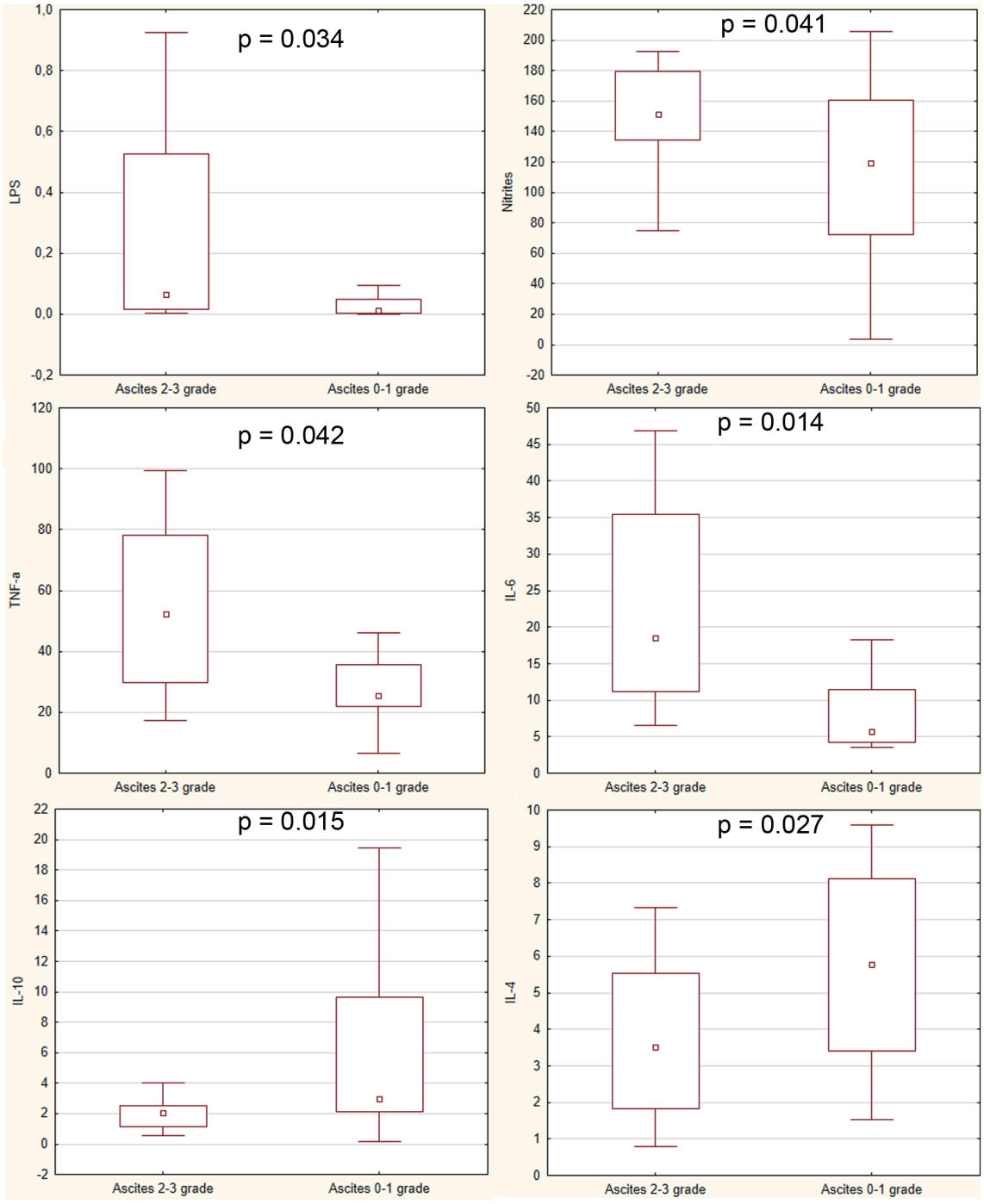

Fig. 6. Differences in plasma levels of lipopolysaccharide (LPS) (EU/mL), nitrite (μmol/L), TNF-a (pg/mL), IL-6 (pg/mL), IL-10 (pg/mL) and IL-4 (pg/mL) between patients with clinically significant ascites (grade 2–3) and those without this complication of cirrhosis. Median, interquartile range, and range without outliers are presented.

tion of new treatment approaches for cirrhosis: by influencing the gut microbiota through antibiotics, probiotics, prebiotics, fecal transplantation, and other methods, it may be possible to change the cytokine profile in cirrhosis, thereby reducing the severity of its manifestations. For example, suppressing the development of harmful Enterobacteriaceae may reduce the formation of TNF-a and IL-6, potentially alleviating the severity of ascites and hepatic encephalopathy. This clinical and translational significance of our study underscores the importance of our study.

Conclusion

The abundance of the major harmful taxon of the gut micro-

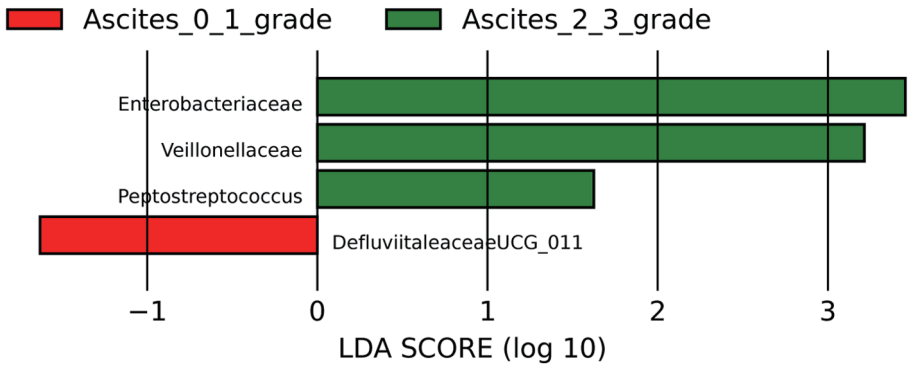

Fig. 7. Differences in gut microbiota taxa between patients with clinically significant ascites (grade 2–3) and those without this complication of liver cirrhosis. LDA, Linear discriminant analysis.

biota, Enterobacteriaceae, correlates directly with the levels of the main pro-inflammatory cytokines (IL-6 and TNF-a), which are higher in patients with clinically significant ascites compared to those without this complication. Additionally, numerous other correlations exist between the abundance of different gut microbiota taxa and the wide range of cytokines studied, though the exact role of these correlations in the disrupted gut-liver axis in cirrhosis remains to be determined.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (VI, RM), acquisition of data (all authors), analysis and interpretation of data (VI, RM), drafting of the manuscript (RM), critical revision of the manuscript for important intellectual content (all authors). All authors have made significant contributions to this study and have approved the final manuscript.

Ethical statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the local ethics committee of Sechenov University (protocol #22-21 dated 9 December 21). Informed consent was obtained from all subjects involved in the study.

Data sharing statement

The data used to support the findings of this study are available from the corresponding author at mmmm00@yandex.ru upon request.

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