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Salivary Microbiota Reflects Changes in Gut Microbiota in Cirrhosis With Hepatic Encephalopathy

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Altered gut microbiome is associated with systemic inflammation and cirrhosis decompensation. However, the correlation of the oral microbiome with inflammation in cirrhosis is unclear. Our aim was to evaluate the oral microbiome in cirrhosis and compare with stool microbiome. Outpatients with cirrhosis (with/without hepatic encephalopathy [HE]) and controls underwent stool/saliva microbiome analysis (for composition and function) and also systemic inflammatory evaluation. Ninety-day liver-related hospitalizations were recorded. Salivary inflammation was studied using T helper 1 cytokines/secretory immunoglobulin A (IgA), histatins and lysozyme in a subsequent group. A total of 102 patients with cirrhosis (43 previous HE) and 32 age-matched controls were included. On principal component analysis (PCA), stool and saliva microbiome clustered far apart, showing differences between sites as a whole. In salivary microbiome, with previous HE, relative abundance of autochthonous families decreased whereas potentially pathogenic ones (*Enterobacteriaceae*, *Enterococcaceae*) increased in saliva. Endotoxin-related predicted functions were significantly higher in cirrhotic saliva. In stool microbiome, relative autochthonous taxa abundance reduced in previous HE, along with increased *Enterobacteriaceae* and *Enterococcaceae*. Cirrhotic stool microbiota demonstrated a significantly higher correlation with systemic inflammation, compared to saliva microbiota, on correlation networks. Thirty-eight patients were hospitalized within 90 days. Their salivary dysbiosis was significantly worse and predicted this outcome independent of cirrhosis severity. Salivary inflammation was studied in an additional 86 age-matched subjects (43 controls/43 patients with cirrhosis); significantly higher interleukin (IL)−6/IL-1β, secretory IgA, and lower lysozyme, and histatins 1 and 5 were found in patients with cirrhosis, compared to controls. *Conclusions:* Dysbiosis, represented by reduction in autochthonous bacteria, is present in both saliva and stool in patients with cirrhosis, compared to controls. Patients with cirrhosis have impaired salivary defenses and worse inflammation. Salivary dysbiosis was greater in patients with cirrhosis who developed 90-day hospitalizations. These findings could represent a global mucosal-immune interface change in cirrhosis. (HEPATOLOGY 2015;62:1260-1271)

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Cirrhosis is associated with a proinflammatory milieu that can potentiate disease progression and complications, such as hepatic encephalopathy (HE) and infections.[1] Dysbiosis or altered gut microbiota, resulting from decreased autochthonous or commensal taxa, has been found in stool and colonic mucosa in patients with cirrhosis, which is, in turn, linked with disease severity and systemic inflammation.[2-4] However, It is not clear whether this dysbiosis-

Abbreviations: AUC, area under the curve; CI, confidence interval; GI, gastrointestinal; HCV, hepatitis C virus; HE, hepatic encephalopathy; IFN-γ, interferon-gamma; IgA, immunoglobulin A; IL, interleukin; MELD, Model for End-Stage Liver Disease; MTPS, multitagged pyrosequencing; NASH, nonalcoholic steatohepatitis; OR, odds ratio; PCA, principal component analysis; PiCRUST, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; PPIs, proton pump inhibitors; QIIME, Quantitative Insights Into Microbial Ecology; SBP, spontaneous bacterial peritonitis; Th, T helper; TNF-α, tumor necrosis factor alpha; VB, variceal bleeding.

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inflammatory state exists only in the gut or is a generalized phenomenon in cirrhosis. The salivary microbiome has been studied in healthy individuals as part of the Human Microbiome project, but not directly in cirrhosis.[5] Though Qin et al. and our earlier studies have shown that microbes presumed to be of oral origin could be present in stool, the direct evaluation of the oral microbiome has not been performed in cirrhosis.[6,7] Patients with cirrhosis are also predisposed to periodontal infections, which necessitates a dental examination before liver transplant listing.[8,9] The study of salivary defenses is important in establishing a global microbiota-immune change given that salivary microbiome could influence the distal gut microbiome.[7,10] Furthermore, if similarities were noted between stool and salivary microbiota, given that saliva is easier to collect compared to stool or mucosal biopsies, this would greatly increase the ease of subject participation in microbiota research. Our aim was to analyze the salivary microbiome composition and function in patients with cirrhosis with and without HE, study their linkage with stool microbiota and outcomes, and also to analyze the impact of cirrhosis on salivary defenses and oral inflammatory response.

Patients and Methods

The study was conducted in two parts: The first was a microbiota-inflammatory milieu analysis of the stool, saliva, and systemic circulation, whereas the second was evaluation of salivary inflammatory response.

Outpatients with cirrhosis diagnosed by histology, radiological evidence of cirrhosis, or endoscopic evidence of varices in the setting of chronic liver disease were recruited prospectively. Because the focus was HE, we divided patients into those with and without previous HE as defined by at least one hospitalization for overt HE within the last year that was currently controlled as an outpatient on lactulose and/or rifaximin. We compared previous HE patients to a compensated age-matched cohort (without HE, ascites, and variceal bleeding [VB]) that was termed no-HE. A group of age-matched healthy controls without chronic diseases were also recruited. A careful smoking history was taken from all groups, and an oral examination was performed in addition to review of the dental records within 6 months. We excluded patients on absorbable antibiotics, tobacco, alcohol, or illicit drug use within 3 months, transjugular intrahepatic portosystemic shunt, periodontal/gingival disease undergoing treatment, or edentulous patients.

Dietary history for the day preceding stool sampling was recorded using recall. All subjects underwent serum, stool, and saliva collection the same day. For saliva collection, all subjects were asked to rinse their mouth with normal saline using published protocols in the presence of the coordinator.[11] This rinse was discarded and saliva collected after that was collected and flash-frozen. Serum endotoxin was evaluated using published Limulus Amebocyte Lysate gel-clot techniques, whereas inflammatory cytokines (interleukin [IL]-6, IL-2, IL-1β, IL-4, IL-10, tumor necrosis factor alpha [TNF-α], and interferon-gamma [IFN-γ]) were analyzed using enzyme-linked immunosorbent assay (Assaygate, Ijamsville, MD).[12] Patients were then followed for 90 days for their first hospitalization resulting from liver-associated conditions or infections (HE, infections, fluid/electrolyte issues, and gastrointestinal [GI] bleeding).

Additional Subset for Salivary Inflammation Analysis. We subsequently recruited an additional group of age-matched healthy controls and patients with cirrhosis in a case-control–based approach (with identical inclusion/exclusion criteria as described above) that underwent analysis of saliva for IL-6, IL-1β, histatin 1, 3, and 5, lysozyme, and secretory immunoglobulin A (IgA).

Additional Supporting Information may be found at http://onlinelibrary.wiley.com/doi/10.1002/hep.27819/suppinfo.

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Statistical Analysis. Stool and salivary microbial DNA was extracted, which was analyzed using published multitagged pyrosequencing (MTPA) techniques (Supporting Information).[13] Microbiota results were analyzed using Metastats, standard nonparametric tests (Kruskal-Wallis' test), and UNIFRAC QIIME (Quantitative Insights Into Microbial Ecology) principle component analyses (PCA)[14] with multiple comparison adjustments. Functionality of microbiota was assessed using PiCRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States),[15] and results were compared between groups.[16]

We compared controls to patients with cirrhosis and those with/without previous HE using analysis of variance and Kruskal-Wallis' tests. Based on earlier studies, MTPS results were expressed as relative abundances between groups and compared for saliva and stool between groups. Correlation networks were created between microbiota and inflammatory cytokines for saliva and stool separately. Differences in correlations were evaluated and visualized using Cytoscape.[17,18] We compared dysbiosis ratios created separately for stool and saliva within the cirrhosis group. In order to account for potential baseline differences between those who were hospitalized, a univariate and multivariable logistic regression with disease severity indices, age, diabetes, and salivary dysbiosis ratio was performed. In addition, a sensitivity analysis for outcome prediction using the dysbiosis ratio alone and with other significant variables was performed using receiver operating characteristic curves using the Youden's index.

Results

Initial Study of the Microbiota-Inflammatory Analysis

We considered 167 patients with cirrhosis; 11 were edentulous, 15 had current periodontitis/gingival disease or were undergoing dental treatment, 13 were using alcohol/illicit drugs, and 26 refused participation. We ultimately enrolled 102 patients with cirrhosis. We also enrolled 32 age-matched healthy controls without any chronic systemic or oral diseases (age 54 ± 5 years, 21 males; median daily calories: 2,201 ± 124). None of the controls were on proton pump inhibitors (PPIs). There was no significant difference in tobacco use between controls (18 never used tobacco, 14 had a remote history [>3 months ago], with none current users) and patients with cirrhosis (56 never used, 46 remote use, and none current users). Review of dental history and oral examination did not reveal active gingival or periodontal disease in any of the included subjects. The leading etiologies of cirrhosis were hepatitis C virus (HCV; 47%), alcohol alone (17%), alcohol+HCV (21%), and nonalcoholic steatohepatitis (NASH; 16%). Most subjects were Caucasian (53%) followed by African American (43%) and Hispanic (4%). Eight-five percent of subjects were male. Forty-two percent of patients with cirrhosis had previous HE (median HE episodes: 1 [range, 1-5]; last HE episode: median, 3 months before [range, 2-11]) before sample collection. All previous HE patients were alert and oriented with a Mini–Mental Status Exam of >25 and were able to give informed consent. These patients were adherent on lactulose and 24% were on additional rifaximin; both medications were prescribed for at least 2 months. Patients on rifaximin had a nonsignificant trend toward a higher Model for End-Stage Liver Disease (MELD) score (18 ± 10 vs. 16 ± 7; $P = 0.09$). Previous HE patients had a higher MELD score with evidence of a systemic proinflammatory milieu and endotoxemia, compared to no-HE patients (Table 1). None of the previous HE patients were on antibiotics, patients with ascites were controlled on diuretics, and 4 patients had previous VB more than 1 year before with obliterated varices at the time of sample collection. No-HE patients had no ascites determined by imaging and physical examination, were not on any antibiotics for any current/past infections, and did not have a history of VB.

Table 1. Comparison Between Previous HE and No-HE Patients

	No-HE (n = 59)	Previous HE (n = 43)
Age, years	54 ± 13	56 ± 16
Gender, male/female	50/9	34/9
Tobacco use, none/remote/current	35/24/0	21/22/0
Use of PPIs (%)	21 (35.6)	19 (44.2)
Type 2 diabetes (%)	16 (27.1)	15 (34.9)
Etiology (HCV, alcohol, HCV+alcohol, NASH, others)	27/14/7/7/4	20/6/9/7/1
Calories in the past day (mean ± SD)	2,304 ± 732	2,245 ± 920
MELD score (mean ± SD)*	8.6 ± 2.5	17.2 ± 7.2
Child-Pugh score (mean ± SD)*	6.2 ± 5.1	9.7 ± 4.4
Serum Albumin (mg/dL, mean ± SD)*	3.5 ± 1.7	3.3 ± 1.1
Ascites (%)**	0	16 (37)
IL-6, pg/mL (median, IQR)**	2.4 (8.3)	4.5 (33.5)
IL-2, pg/mL (median, IQR)**	0.0 (3.0)	1.9 (10.4)
IL-1β, pg/mL (median, IQR)*	0.0 (1.8)	0.7 (8.4)
IL-4, pg/mL (median, IQR)	0.6 (2.3)	0.6 (15.5)
TNF-α, pg/mL (median, IQR)**	4.2 (2.7)	5.4 (10.8)
IFN-γ, pg/mL (median, IQR)*	0.9 (10.5)	0.5 (3.2)
IL-10, pg/mL (median, IQR)	1.9 (6.2)	1.6 (6.0)
Endotoxin EU/ml (mean±SD)**	0.05 ± 0.01	0.34 ± 0.21

*$P < 0.001$; **$P < 0.01$; *$P < 0.05$; previous HE patients had a significantly worse systemic inflammation, compared to patients without HE.
Abbreviations: SD, standard deviation; IQR, interquartile range.

Outcomes

Within 90 days, 38 patients required a hospitalization for liver-related conditions a median of 39 days (range,

Table 2. Median Relative Abundances of Bacterial Families Between Groups

	Saliva			Stool		
Bacterial Family	Controls (%)	No-HE (%)	HE (%)	Controls (%)	No-HE (%)	HE (%)
Clostridiales XIV‡	7.8	5.6	2.7*	9.2	6.6	4.5*
Lachnospiraceae‡	20.2	15.0	9.5*	27.0	21.3	16.0*
Ruminococcaceae‡	7.0	4.9	3.7*	13.4	8.7	7.4*
Fusobacteriaceae	0.0	1.6	1.6†	0.0	0.0	1.0
Prevotellaceae	4.2	7.3	7.4†	5.4	5.3	5.0
Enterococcaceae	0.0	2.5	3.1†	0.0	0.0	1.0
Enterobacteriaceae	2.2	5.5	5.8†	0.0	3.0	3.1†
Erysipelotrichaceae	3.5	1.2	1.3†	5.3	1.9	0.6*
Bacteroidaceae	3.4	3.8	4.6	19.9	24.5	24.9
Streptococcacae	33.0	29.1	33.3	2.4	4.4	1.9

*Significant differences between controls, HE, and no-HE patients.
†Significant differences between controls and patients with cirrhosis, but no difference within HE and no-HE patients,
‡Autochthonous taxa.

12-85) after sample collection. None of the patients were started on antibiotics, HE therapy, or underwent non-liver-related hospitalizations between enrollment and this hospitalization. Those who were hospitalized had a higher MELD score (15.6 ± 8.4 vs. 10.6 ± 4.8; $P = 0.003$) and included a higher proportion of previous HE (67% vs. 45%; $P = 0.001$) at the time of sample collection, compared to those free of hospitalization. Twelve hospitalizations were the result of HE without infection (dyselectrolytemia in 5, lactulose noncompliance 6, and 1 spontaneous), 4 for HE with infection (3 spontaneous bacterial peritonitis [SBP]/spontaneous bacteremia and 1 pneumonia), 6 additional patients were admitted for infections without HE (4 SBP/spontaneous bacteremia and 2 urinary tract infection), 16 for other liver issues (9 fluid/electrolyte management, 3 VB, 1 peptic ulcer bleeding, and 3 hepatic hydrothorax). Median time to hospitalization was not significantly different between HE/infections versus other liver-related conditions (36 vs. 42 days; $P = 0.5$).

Overall Microbiome Analysis

The specific families differing between patients with cirrhosis and controls are shown in Table 2. When clustering of the microbiota within groups was performed, stool and saliva clustered separately in patients with cirrhosis as well as in controls, demonstrating that microbiota in saliva is significantly different from the stool, regardless of the presence of cirrhosis (Fig. 1A,B).

Stool Microbiome

Patients with cirrhosis had a significantly lower relative abundance of autochthonous taxa (*Lachnospiraceae*, *Ruminococcaceae*, and Clostridiales XIV),[2] compared to controls, and this was further reduced in previous HE versus no-HE patients. The cirrhosis dysbiosis ratio[3] (*Lachnospiraceae* + *Ruminococcaceae* + Clostridiales Incertae Sedis XIV + *Veillonelllaceae*/ *Enterobacteriaceae* + *Bacteroidaceae*) was significantly lower (indicates dysbiosis) in patients with cirrhosis, compared to controls (3.4 ± 6 vs. 6.7 ± 9.0; $P = 0.03$) and significantly worse in previous HE (previous HE: 2.0 ± 3.3; no-HE: 4.4 ± 6.0; $P = 0.04$). The predominant enterotype was *Bacteroides*, although, as shown above, *Ruminococcus* was significantly lower in previous HE patients with cirrhosis (Supporting Fig. 1).[19] On PCA, clustering of stool microbiota between controls and patients with cirrhosis were not as marked as between site differences (Fig. 1C).

Salivary Microbiome

The salivary microbiome in controls and patients with cirrhosis showed significant differences, which was accentuated in previous HE. We found that the relative abundance of *Streptococcaceae* in saliva was significantly higher than that in the stool in both groups. There was a reduction in autochthonous taxa, even in saliva in patients with cirrhosis, especially in previous HE. Given the different composition of microbiota in saliva, we created a salivary microbiota ratio (*Lachnospiraceae* + *Ruminococcaceae* + Clostridiales Incertae Sedis XIV/*Streptococcaceae*), which was significantly lower (indicates dysbiosis) in patients with cirrhosis, compared to controls (2.0 ± 6.0 vs. 0.4 ± 1.0; $P = 0.04$), although changes within the cirrhosis group using this ratio were not significant (previous HE 0.34 ± 0.9 vs. no-HE 0.45 ± 1.0; $P = 0.6$). There was relatively weak clustering between controls, compared to patients with cirrhosis on PCA (Fig. 1D).

There was no additional change in dysbiosis (represented by changes in dysbiosis ratios) in previous HE patients with or without ascites either in the stool (ascites 2.7 ± 3.6 vs. no ascites 2.9 ± 4.1; $P = 0.44$) or salivary microbiota (0.29 ± 1.2 vs. 0.34 ± 0.92; $P = 0.44$). A similar lack of effect was observed in those with/without rifaximin on saliva (0.41 ± 1.3 vs. 0.37 ± 0.9; $P = 0.6$) or stool (2.7 ± 2.9 vs. 2.9 ± 6.7; $P = 0.43$). We did not find a significant change in those with or without diabetes on the salivary dysbiosis ratio (diabetes 0.43 ± 0.81 vs. 0.37 ± 1.0; $P = 0.38$) and a trend toward decreased dysbiosis on the stool dysbiosis ratio (diabetes 5.9 ± 8.7 vs. 3.4 ± 6.2; $P = 0.08$). A nonsignificant pattern was also observed for PPI use in saliva (PPI 0.56 ± 0.75 vs. 0.53 ± 1.01; $P = 0.57$) or stool dysbiosis ratios (PPI 3.9 ± 7.3 vs. 4.2 ± 9.1; $P = 0.43$).

Predicted Microbial Function Results

We found significant changes in bacterial functionality in saliva and stool between patients with cirrhosis

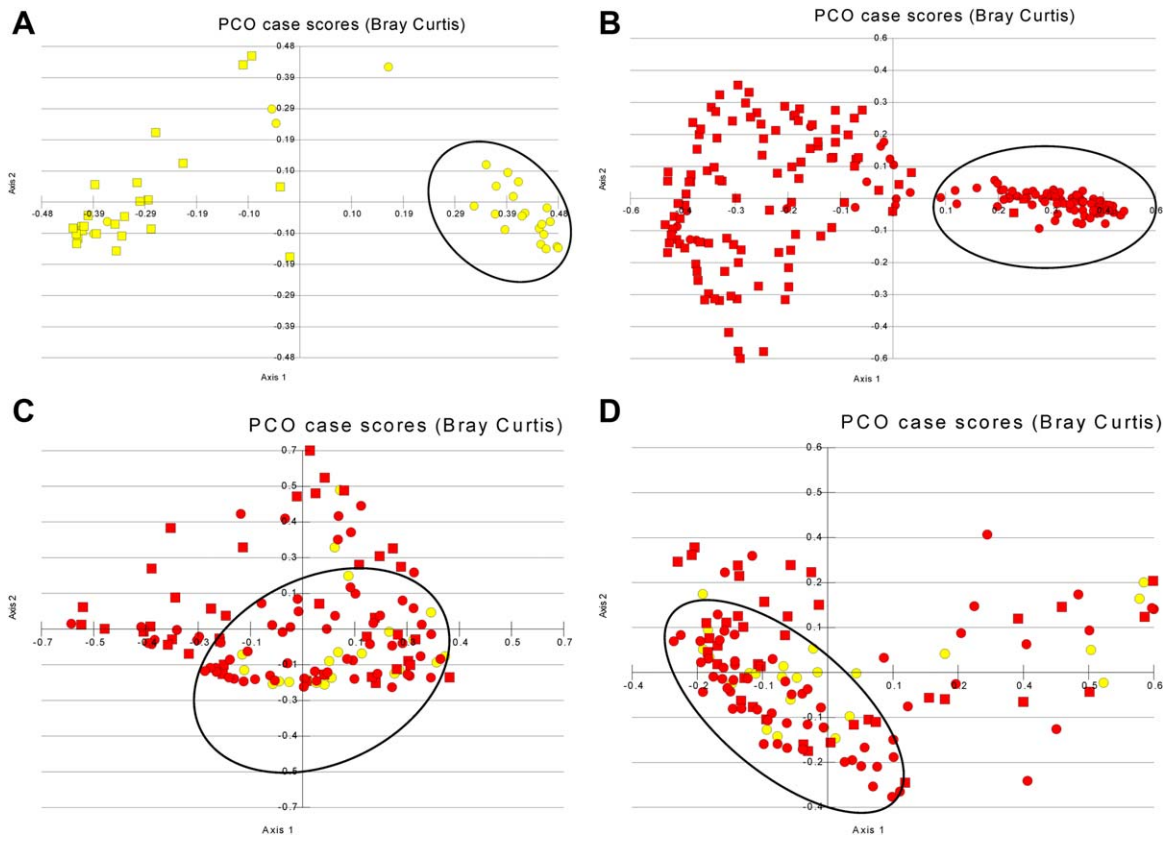

Fig. 1. PCAs of microbiota change. (A) Salivary microbiota (black oval) cluster far apart from the stool in controls (squares = stool; circles = saliva). (B) Salivary microbiota (black oval) cluster far apart from the stool in cirrhosis (squares = stool; circles = saliva). (C) Stool microbiota showing clustering of control and no-HE (black oval), compared to those with previous HE, that is not as apparent as between the control/cirrhosis comparisons (yellow circles = control; red circles = no-HE; red squares = previous HE). (D) Salivary microbiota showing clustering of control and no-HE (black oval), compared to those with previous HE, that is not as apparent as between the control/cirrhosis comparisons (yellow circles = control; red circles = no-HE; red squares = previuos HE).

and controls. Microbiota with greater relative abundance in patients with cirrhosis' saliva had functions related to endotoxin and endotoxin-protein biosynthesis and purine/nucleotide metabolism. In contrast, those in control saliva were more likely to have functionality related to amino acids, phenolic/benzoate, and fatty acid metabolism (Fig. 2A). In stool, there was a similar difference with amino acid metabolism, including branched-chain amino acid synthesis and carbohydrate metabolism being more prominent among control microbiota, compared to cirrhosis. Microbiota found in cirrhotic stool was likely to have functions related to vitamin and oxidant metabolism, especially related to riboflavin and glutathione (Fig. 2B).

Correlation Differences Between Groups

When correlation networks between microbiota and inflammatory cytokines in saliva and stool were compared to one another, significant differences emerged.

Control Saliva Compared to Cirrhosis Saliva. T helper (Th)1 inflammatory cytokines were correlated with one another positively in both groups, but more in patients with cirrhosis. In patients with cirrhosis, there was a negative correlation between the anti-inflammatory cytokine, IL-10, and *Enterobacteriaceae* (Fig. 3A).

Control Stool Compared to Cirrhosis Stool. Similar to saliva, there was higher correlation in cirrhosis within inflammatory cytokines whereas the autochthonous *Ruminococcaceae* was more negatively correlated with *Enterobacteriaceae* in patients with cirrhosis, compared to controls. In addition, *Ruminococcaceae* was positively correlated with other autochthonous taxa only in patients with cirrhosis. *Porphyromonadaceae* was negatively correlated between the anti-inflammatory cytokine, IL-10, and positively with IL-13 in patients with cirrhosis (Fig. 3B).

Correlation Differences Within Groups

Significant differences were also observed when saliva and stool correlation networks were compared within groups.

Control Stool Compared to Control Saliva. As expected, saliva-predominant taxa, such as *Streptococcaceae*,

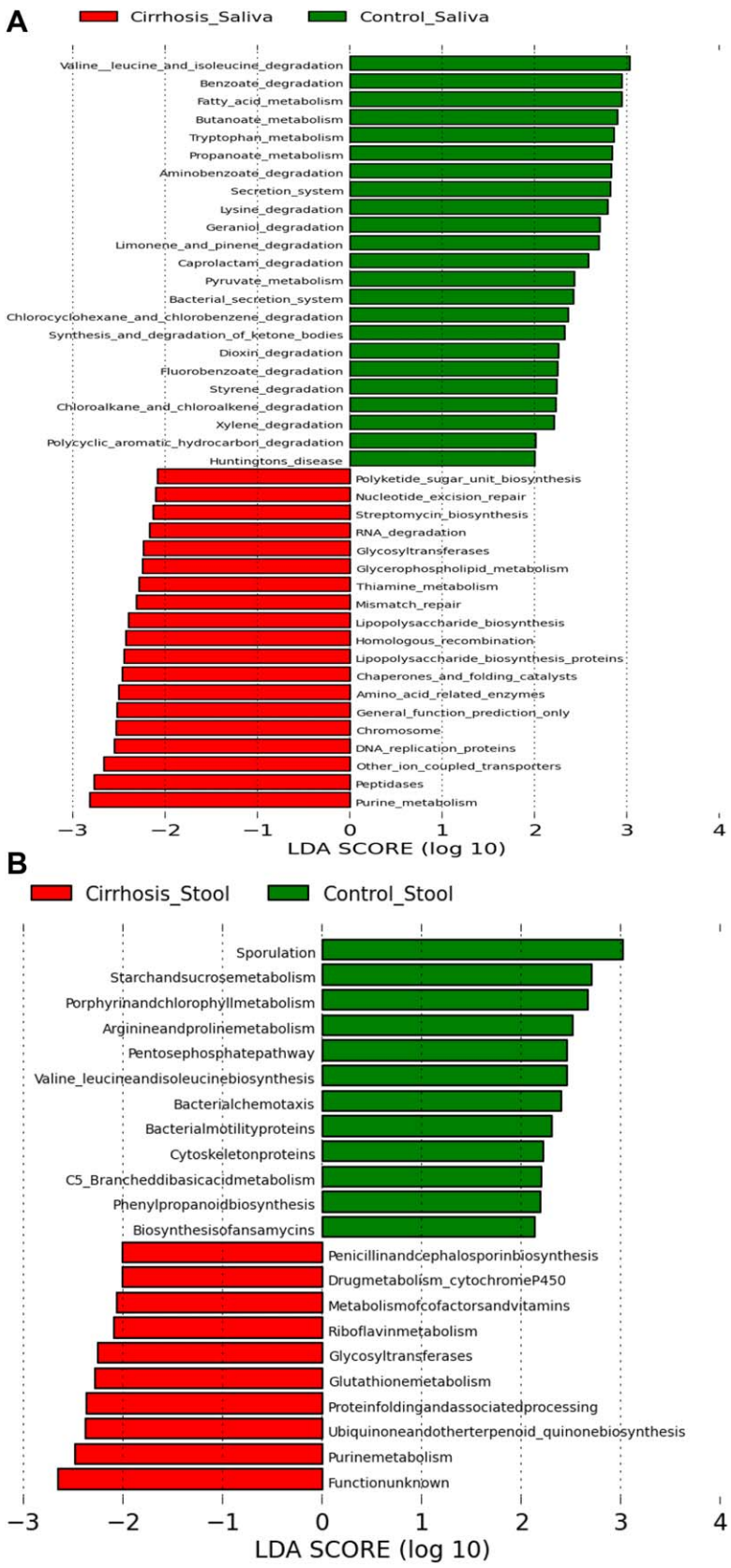

Fig. 2. Predicted metabolic functions of microbiota in saliva and stool between groups. LDA score represents log changes in relative gene expression predicted function between groups. Bars in the green indicate higher activity in controls whereas those in red represent higher activity in cirrhotic saliva or stool. (A) Salivary predicted microbiota functional changes in controls is centered on amino acid and phenolic metabolism whereas a higher expression of genes related to lipopolysaccharides and purine/pryrimidine metabolism was observed in patients with cirrhosis' saliva. (B) Stool predicted microbiota functional changes showing differences in cirrhotic and control microbiota. There was a higher expression of genes related to vitamins, cofactors, and oxidant metabolism in cirrhosis whereas controls had a significantly higher expression of carbohydrate and amino acid metabolism. Abbreviation: LDA, linear discriminant analysis.

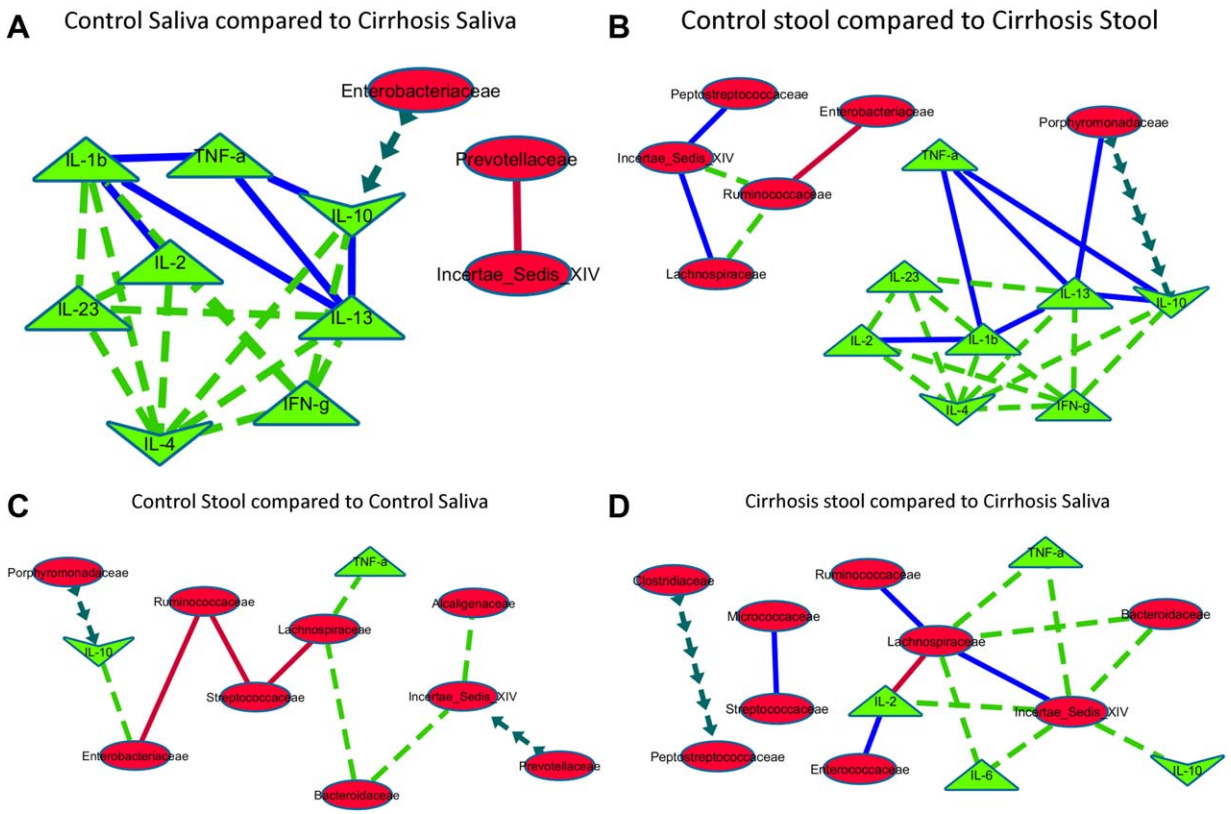

Fig. 3. Correlation network differences. Panels represent differences between correlation networks created for microbial families and inflammatory cytokines in saliva and stool. In all the subsequent panels, the light green nodes represent systemic inflammatory cytokines whereas red ones are microbial families. If the correlations are negative in both compared networks, the connecting line is red, if positive in both compared networks it is dark blue, if negative in one and turns to positive in the other, the line is dark green with arrows whereas if the correlations are positive in one and changes to negative in the other, the line is bright green with dashes. (A) Control saliva compared to cirrhosis saliva networks. The correlation network of salivary microbiota and inflammation and a similar network in cirrhotic saliva was compared to evaluate differences that were $P < 0.001$ and $r > 0.6$ or <-0.6. Relationships between microbiota and inflammatory markers that were different are shown and explained below. Negative correlations in cirrhosis and controls both, but more significant in cirrhosis: between Incertae sedis XIV and *Prevotellaceae*; negative/no correlation in control saliva, but positive correlation in cirrhosis: inflammatory cytokines with one another; positive/no correlation in control saliva, but negative correlation in cirrhosis: *Enterobacteriaceae* with IL-10. This shows that cirrhosis saliva has more robust changes with systemic inflammation and within bacteria in saliva, compared to controls. (B) Control stool compared to cirrhosis stool. The correlation network of control stool microbiota and inflammation and a similar network in cirrhotic stool was compared to evaluate differences that were $P < 0.001$ and $r > 0.6$ or <-0.6. Relationships between microbiota and inflammatory markers that were different are shown and explained below. Negative correlations in cirrhosis and controls both but more significant in cirrhosis: *Enterobacteriaceae* and *Ruminococcaceae*; positive in both groups, but more in cirrhosis: Incertae sedis XIV and *Peptostreptococcaceae*, *Porphyromonadaceae* with IL-13. Negative/no correlation in control stool but positive correlation in cirrhosis: inflammatory cytokines with one another, Incertae sedis XIV with *Ruminococcaceae* and *Lachnospiraceae*; positive/no correlation in control stool, but negative correlation in cirrhosis: *Porphyromonadaceae* with IL-10. The results demonstrate a higher correlation intensity in cirrhosis stool between autochthonous families and between nonautochthonous families and systemic inflammation. (C) Control stool compared to control saliva. The correlation network of control stool microbiota and inflammation and to control saliva microbiota and inflammation was compared to evaluate differences that were $P < 0.001$ and $r > 0.6$ or <-0.6. Relationships between microbiota and inflammatory markers that were changed significantly are shown and explained below. Negative in both control saliva and stool, but stronger negativity in saliva: *Streptococcaceae* with *Ruminococcaceae* and *Lachnospiraceae*; negative in both control saliva and stool but stronger negative correlation in stool: *Enterobacteriaceae* with *Ruminococcaceae*; negative in control stool without significant relationship/positive in control saliva: autochthonous taxa with *Bacteroidaceae*, *Alcaligenaceae*, and with inflammatory cytokines; positive in control stool without significant relationship/negative in control saliva: *Porphyromonadaceae* and IL-10, *Prevotellaceae* and Incertae sedis XIV. These results show that the strength of most correlations between microbial families (positive or negative) is higher in stool, compared to saliva, even within the same control group. (D) Cirrhosis stool compared to cirrhosis saliva. The correlation network of cirrhosis stool microbiota and inflammation and to cirrhosis saliva microbiota and inflammation was compared to evaluate differences that were $P < 0.001$ and $r > 0.6$ or <-0.6. Relationships between microbiota and inflammatory markers that were changed significantly are shown and explained below. Positive in both cirrhosis saliva and stool but stronger positivity in saliva: *Micrococcaceae* and *Streptococcaceae*; positive in both cirrhosis saliva and stool but stronger positivity in stool: *Enterococcaceae* with IL-2, autochthonous taxa with one another; negative in both cirrhosis saliva and stool but stronger negative correlation in saliva: *Lachnospiraceae* with IL-2; negative in cirrhosis stool without significant relationship/positive correlation in cirrhosis saliva: autochthonous taxa with *Bacteroidaceae* and with inflammatory cytokines; positive in cirrhosis stool without significant relationship/negative correlation in saliva: *Clostridiaceae* with *Peptostreptococcaceae*. These results show that within the cirrhosis group, salivary correlations of autochthonous families and systemic inflammation and between predominant salivary microbes (*Streptococcaceae*) were higher than in stool, whereas the relationship with predominantly stool microbiota (*Bacteroidaceae)* with inflammation was higher in stool.

were more negatively correlated with *Ruminococcaceae* and *Lachnospiraceae* in saliva than in stool, whereas the reverse was true for *Enterobacteriaceae* with *Ruminococcaceae* (Fig. 3C).

In control stool, autochthonous taxa were negatively correlated with *Bacteroidaceae*, *Alcaligenaceae*, and inflammatory cytokines, but not in saliva, whereas stool *Lachnospiraceae* and targets, *Porphyromonadaceae* and IL-10, *Prevotellaceae*, and Incertae sedis XIV were positively correlated, but not in saliva.

Cirrhosis Stool Compared to Cirrhosis Saliva. Similar to controls, taxa present in a higher abundance in saliva were related to one another more in saliva (positive between *Micrococcaceae* with *Streptococcaceae* and negative between *Lachnospiraceae* with IL-2), compared to stool, whereas there was a higher correlation in stool with *Enterococcaceae* with IL-2 and within autochthonous taxa (Fig. 3D). In patients with cirrhosis, there were several relationships that were found in stool that were not significant in saliva. Negative stool-only correlations were autochthonous taxa with *Bacteroidaceae* and with inflammatory cytokines, whereas positive stool-only correlations were *Clostridiaceae* with *Peptostreptococcaceae*.

Outcome and Microbiota Changes

We found a significantly higher dysbiosis, that is, a lower stool dysbiosis ratio (5.5 ± 8.3 vs. 2.9 ± 4.6; $P = 0.04$) and lower salivary dysbiosis ratio (0.15 ± 0.24 vs. 0.52 ± 1.2; $P = 0.016$) in those that were hospitalized, compared to those who remained free of hospitalization at 90 days. There was a nonsignificant trend toward worse dysbiosis in patients admitted because of HE/infections, compared to others in saliva (0.09 ± 0.15 vs. 0.22 ± 0.3; $P = 0.08$) and stool (2.1 ± 2.4 vs. ± 4.1 vs. 7.2; $P = 0.12$). Given the differences in baseline in disease severity between those with/without 90-day hospitalizations, we fit an univariate binary logistic regression model with age, diabetes, MELD score, HE or no-HE, and the salivary dysbiosis ratio as predictors of 90-day hospitalization. Variables significant on univariate analysis were HE (odds ratio [OR]: 4.7; 95% confidence interval [CI]: 1.93-12.1; $P = 0.006$), MELD (OR, 1.12; 95% CI: 1.05-1.22), and salivary dysbiosis ratio (OR, 0.4; 95% CI: 0.00-0.95; $P = 0.03$). Once adjusted for salivary dysbiosis and HE, MELD score was not an independent predictor of 90-day hospitalization. We then fit a second multivariable logistic model that included only HE and salivary dysbiosis, which showed both variables to be independent significant predictors of 90-day hospitalization (HE OR: 4.4; 95% CI: 1.7-11.5; $P = 0.001$ and salivary dysbiosis ratio OR: 0.5; 95% CI: 0.1-0.9; $P = 0.04$). A further sensitivity analysis was performed using salivary dysbiosis alone; a value >0.18 had an area under the curve (AUC) of only 0.59; however, sensitivity was 84% with specificity of 36% for 90-day hospitalizations. When the second independent variable, HE, was added to this, the threshold of the probability equation (–1.27+1.48 [HE yes/no]–0.78 [salivary dysbiosis ratio]) above which hospitalizations were predicted was >0.44 with a 0.72 AUC, 67% sensitivity, and 73% specificity.

Table 3. Comparison Between Controls and Patients With Cirrhosis on Salivary Immunological Measures

	Controls (n = 43)	Cirrhosis (n = 43)
Age, years	53 ± 15	57 ± 18
Gender, male/female	29/14	34/9
Tobacco use, none/remote/current	38/5/0	31/11/0
Calories in the past day, mean ± SD	$2{,}213 \pm 834$	$2{,}109 \pm 689$
IL-6, pg/mL**	2.9 ± 0.6	7.9 ± 1.8
IL-1β, pg/mL*	21 ± 10	117 ± 40
Secretory IgA, μg/mL*	159 ± 48	281 ± 26
Lyzozyme, pg/mL*	19.2 ± 3.0	12.3 ± 2.5
Histatin 1, μg/mL*	12.3 ± 2.0	5.7 ± 2.8
Histatin 3, μg/mL	158 ± 12	187 ± 18
Histatin 5, μg/mL***	6.3 ± 0.2	5.1 ± 0.1

Data presented as mean ± standard error of the mean.
Abbreviation: standard deviation.
***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

Salivary Inflammatory Analysis

Given these changes in salivary microbiota, we subsequently enrolled an age-matched group of patients with cirrhosis and healthy controls (n = 43 each; Table 3) to study the inflammatory milieu in the saliva. All subjects fulfilled the same inclusion/exclusion criteria as the microbiome analysis study. None of the controls were on PPIs or had diabetes or other chronic diseases. Of the 43 patients with cirrhosis, 21 were previous HE (age 56 ± 4 years; MELD 12 ± 3; 67% HCV; 24% alcoholic cirrhosis; 10 on PPI; 4 with type 2 diabetes; last HE episode: median 2 months before [range, 1-11]) and 22 were no-HE (age 55 ± 6 years; MELD 9 ± 5; 55% HCV; 32% alcoholic cirrhosis; 10 on PPI; and 6 with type 2 diabetes). All HE patients were on lactulose and 2 were on additional rifaximin. None of these patients had ascites, current alcohol/tobacco use, and underwent the same protocol for salivary collection. We found a significantly higher inflammatory response in patients with cirrhosis, compared to controls, as shown by a significantly higher IL-1β, IL-6, and secretory IgA. Interestingly, this was accompanied by a significant decrease in lysozyme and all histatins, except histatin-3, in the cirrhosis group. We did not find a change in all above values in patients with cirrhosis with and without HE. No changes were

observed in oral inflammatory markers between patients with/without diabetes and with/without PPI.

Discussion

The data show that there is evidence of pervasive immune-microbiota interface change in patients with cirrhosis in saliva that is similar to that found in stool. This widespread dysbiosis in patients with cirrhosis' stool and saliva is associated with inflammation, changes in bacterial defenses, and subsequent liver-related hospitalizations.

As shown in earlier studies, there was significant inflammation related to Th1 and Th17 system activation in the systemic circulation in patients with cirrhosis, especially those with previous HE.[20] The microbiota in both saliva and stool were related to the systemic inflammatory milieu, although the linkage with stool microbiota was stronger. Our study shows that, as expected, stool and saliva had different microbiota in both controls and patients with cirrhosis. The major family in the salivary microbiota was *Streptococcaceae* whereas the predominant family in stool was *Bacteroidaceae*; however, neither of these families' relative abundances was different between patients with cirrhosis and controls in saliva or stool. We confirmed previous analysis that stool dysbiosis was greatest in previous HE.[3] Our study extended this onto saliva in patients with cirrhosis with an increase in *Enterobacteriaceae* and reduction in autochthonous microbiota and *Erysipelothricaceae* in HE, compared to no-HE and controls. We found that a similar clustering between microbiota from controls and no-HE, compared to previous HE, patients in saliva and stool. The salivary microbiota showed a significantly higher relative abundance of *Prevotellaceae*, *Fusobacteriaceae*, and *Enterococcaceae* in patients with cirrhosis, compared to controls. Though *Prevotellaceae* and *Fusobacteriaceae* contain species that can cause oral and periodontal infections, the increase in *Enterococcaceae* is intriguing.[9] Species of this family have been recently isolated from saliva and root canals of patients.[21] However, genetic studies suggest that salivary *Enterococcus* is likely exogenous and is unrelated to the species that reside in the lower GI tract. In earlier studies, these organisms are typically cleared from the mouth, but can often persist in patients with deficient immune responses, which could be a potential reason for their detection in patients with cirrhosis.[22] Although Qin et al. and our group's evaluation of acid suppression in patients with cirrhosis have suggested that microbiota of oral origin might be present in the stool through comparisons with a standard microbial database; they did not directly measure salivary microbiota. The presence of these bacteria in the stool in these studies is likely an epiphenomenon of impaired bile and gastric acid output in cirrhosis.[6,7,23] Our results are novel because they directly measure bacterial presence in the saliva of patients with cirrhosis and then relate them to stool bacteria.

This similar trend also continued when changes in salivary microbiota were associated with liver-related hospitalizations over the next 90 days. This builds upon an earlier study that showed that stool microbiota can predict 30-day outcomes in infected patients with cirrhosis and extends it onto outpatients without infections and into salivary microbiota.[3] Of interest, there remained a nonsignificant trend toward worse dysbiosis in those ultimately hospitalized with conditions likely related to the microbiota (i.e., HE and infections), adding biological plausibility to this association. Although the exact mechanism is not clear, it is likely that changes in the oral microbiota follow a systemic proinflammatory milieu that, in turn, is associated with worse outcomes. Given that stool microbiota is relatively stable over 6 months, it is likely that enrollment may provide a window as to what may occur subsequently.[3] Despite the underlying differences in cirrhosis severity, we were able to define a threshold independent of MELD score and HE status that could predict hospitalizations within 90 days using salivary microbiota. This association with poor prognosis gives these microbiota changes a "real-world" connotation. However, it is unlikely that they will replace clinical or laboratory prognosticators at this time, but rather can be developed as potential biomarkers in further validation studies.

Interestingly, the predicted functional analysis showed that the patients with cirrhosis' saliva was enriched with genes pertaining to endotoxin and endotoxin synthesis proteins, as well as nucleic acid and vitamin metabolism. Our results and earlier studies have shown that endotoxemia worsens with, and is associated with, cirrhosis progression and is assumed to be the result of intestinal bacterial overgrowth.[1,3] However, the increased relative abundance of *Enterobacteriaceae* in saliva of patients with cirrhosis coupled with functions related to endotoxin may suggest a role of oral microbiota toward the overall endotoxemia in cirrhosis. Genes related to phenolic and amino acid metabolism were more common in control saliva, compared to patients with cirrhosis. Phenolic compounds are breakdown products of dietary constituents that have putative host beneficial effects.[24] Similar to cirrhosis saliva, patients with cirrhosis' stool microbiota were more likely to be related to nucleic acid and

vitamin metabolism. The bacterial contribution to vitamin metabolism, such as thiamine, riboflavin, and glutathione, could be important in modulating intestinal barrier integrity and oxidative stress that is present in cirrhosis.[25,26]

There were interesting differences in correlations between microbiota and Th1 inflammatory cytokines in both biofluids. Patients with cirrhosis salivary Clostridiales Incertae Sedis XIV were negatively correlated with *Prevotellaceae*, and *Enterobacteriaceae* was significantly negatively correlated with the anti-inflammatory cytokine, IL-10, compared to controls, whereas there was a stronger relationship with systemic inflammation. This indicates that the relatively dysbiotic cirrhotic microbiota was significantly more related to the systemic inflammatory milieu than the otherwise healthy control salivary microbiota. This trend was also replicated in the stool correlation differences in which cirrhotic stool *Enterobacteriaceae* were negatively linked with the autochthonous taxa and there was a strong linkage within those taxa, compared to controls. Interestingly, when saliva correlations were compared to stool correlations within groups, significantly higher correlations were observed with stool microbiota. This points to the changes in gut microbiota being relatively more important than salivary microbiota in determining the overall inflammatory milieu. The relationship between intestinal and oral inflammation has also been explored in inflammatory bowel disease and celiac disease, which showed changes that were commensurate with intestinal findings.[27,28] However, evaluation of oral microbiota after probiotic supplementation did not lead to changes in oral ecology.[29] This points again to a systemic impact that shapes oral microbiota in these diseases and, potentially, in cirrhosis.

As expected, we found significant systemic inflammation related to Th1 activation in cirrhosis, especially in previous HE.[30] The patients with cirrhosis group as a whole also exhibited a proinflammatory milieu in saliva with higher salivary IL-1β and IL-6 concentration and a resultant increase in secretory IgA.[31] This was accompanied by evidence of impaired innate local defenses with reduced histatins 1 and 5 and lysozyme.[32] This extends a study of increased fecal secretory IgA into saliva in cirrhosis and points to an overall activation of systemic inflammation, potentially through contributors in the gut and oral cavity.[33] Interestingly, whereas there clearly were differences in systemic inflammatory response in previous HE, compared to no-HE, we did not find similar changes in salivary inflammatory response between these subgroups. This may point toward a greater contribution of gut dysbiosis toward systemic inflammation, compared to salivary changes. This is not surprising given the quantum difference in the number of bacteria between the two sites. An underlying reason for increased inflammation could be the reduced histatin 1 and 5 and lyzozymes that promote wound healing and prevent bacterial colonization.[34-36] Only histatin 1 and 3 are gene coded, whereas histatin 5 is a cleavage product of histatin 3 that has its own antibacterial properties; therefore, the similarity in histatin 3 may be owing to a reduced cleavage to histatin 5 in patients with cirrhosis.[35] Lysozyme in particular has been associated with anti-inflammatory effects, particularly related to Gram-negative bacteria, which could explain the overabundance of these families in the patients with cirrhosis' saliva.[37,38] This reduced generation of lysozyme and histatins are likely permissive of oral cavity dysbiosis that may lead to local and potential systemic inflammation.[30,39]

Our study is limited by the analysis of associations, which do not prove causation or mechanisms. We focused on previous HE, but it is likely that similar dysbiosis in saliva might be present in those with other forms of decompensation, which requires further study. All our previous HE patients, as per standard of care, were on lactulose and/or rifaximin. However, earlier studies have not shown a significant change in bacterial composition after this therapy.[40-42] Therefore, the changes are likely to be owing to underlying disease process. It is interesting that although there were differences between controls and patients with cirrhosis in salivary microbiota composition and inflammatory markers, the relative differences between previous HE and no-HE patients were not as prominent as they were in the stool. This could be owing to the inherent higher bacterial number and the proximity of the gut bacteria to the cirrhotic liver. However, this warrants further investigation. The study also excluded patients with periodontitis who may actually have even higher dysbiosis. Despite this exclusion, we were still able to demonstrate significant changes in the microbiota in cirrhotic saliva. We also did not find changes in microbiota related to PPI therapy that replicates our earlier cross-sectional analysis.[3] We also did not find an appreciable change with diabetes. These findings may be owing to background dysbiotic state of the cirrhotic microbiota that diluted any potential impact of diabetes. Given that diet and tobacco can influence oral microbiota, we carefully controlled for these issues.

We conclude that dysbiosis, represented by reduction in autochthonous bacterial abundance and change in bacterial function, is also present in saliva, in addition to

stool, in patients with cirrhosis, compared to controls. The alteration in bacterial composition in saliva is associated with a higher risk of further hospitalization owing to liver-related conditions. This could reflect a global mucosal-immune interface change in patients with cirrhosis and represent a target for future microbiota research into the prognostication of cirrhosis.

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