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Colonic mucosal microbiome differs from stool microbiome in cirrhosis and hepatic encephalopathy and is linked to cognition and inflammation

Jasmohan S. Bajaj,1 Phillip B. Hylemon,1,2 Jason M. Ridlon,1,2 Douglas M. Heuman,1 Kalyani Daita,1,2 Melanie B. White,1 Pamela Monteteih,1 Nicole A. Noble,1 Masoumeh Sikaroodi,3 and Patrick M. Gillevet3

1Division of Gastroenterology, Hepatology and Nutrition; 2Department of Microbiology, Virginia Commonwealth University and McGuire Veterans Affairs Medical Center, Richmond, Virginia; and 3Microbiome Analysis Center, George Mason University, Manassas, Virginia

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Bajaj JS, Hylemon PB, Ridlon JM, Heuman DM, Daita K, White MB, Monteteih P, Noble NA, Sikaroodi M, Gillevet PM. Colonic mucosal microbiome differs from stool microbiome in cirrhosis and hepatic encephalopathy and is linked to cognition and inflammation. Am J Physiol Gastrointest Liver Physiol 303: G675–G685, 2012. First published July 19, 2012; doi:10.1152/ajpgi.00152.2012.—Although hepatic encephalopathy (HE) is linked to the gut microbiota, stool microbiome analysis has not found differences between HE and no-HE patients. This study aimed to compare sigmoid mucosal microbiome of cirrhotic patients to controls, between HE vs. no-HE patients, and to study their linkage with cognition and inflammation. Sixty cirrhotic patients (36 HE and 24 no-HE) underwent cognitive testing, stool collection, cytokine (Th1, Th2, Th17, and innate immunity), and endotoxin analysis. Thirty-six patients (19 HE and 17 no-HE) and 17 age-matched controls underwent sigmoid biopsies. Multitag pyrosequencing (including autochthonous genera, i.e., Blautia, Roseburia, Fecalibacterium, Dorea) was performed on stool and mucosa. Stool and mucosal microbiome differences within/between groups and correlation network analyses were performed. Controls had significantly higher autochthonous and lower pathogenic genera compared with cirrhotic patients, especially HE patients. HE patients had worse MELD (model for end-stage liver disease) score and cognition and higher IL-6 and endotoxin than no-HE. Mucosal microbiota was different from stool within both HE/no-HE groups. Between HE/no-HE patients, there was no difference in stool microbiota but mucosal microbiome was different with lower Roseburia and higher Enterococcus, Veillonella, Megasphaera, and Burkholderia abundance in HE. On network analysis, autochthonous genera (Blautia, Fecalibacterium, Roseburia, and Dorea) were associated with good cognition and decreased inflammation in both HE/no-HE, whereas genera overrepresented in HE (Enterococcus, Megasphaera, and Burkholderia) were linked to poor cognition and inflammation. Sigmoid mucosal microbiome differs significantly from stool microbiome in cirrhosis. Cirrhotic, especially HE patients, mucosal microbiota is significantly different from controls with a lack of potentially beneficial autochthonous and overgrowth of potentially pathogenic genera, which are associated with poor cognition and inflammation.

THE PATHOGENESIS OF CIRRHOSIS and its complications, specifically bacterial translocation, infections, and hepatic encephalopathy, are closely related to changes in the intestinal micro-

Address for reprint requests and other correspondence: J. S. Bajaj, Division of Gastroenterology, Hepatology and Nutrition, Virginia Commonwealth Univ. and McGuire VA Medical Center, 1201 Broad Rock Blvd., Richmond, VA 23249 (e-mail: jsbajaj@vcu.edu).

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G675
Comparison of clinical parameters between patients with and without HE

<table>
<thead>
<tr>
<th>Family_Genus, % abundance</th>
<th>Control Mucosa (n = 17)</th>
<th>HE Mucosa (n = 36)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burkholderiaceae_Burkholderia</td>
<td>0.0</td>
<td>0.2</td>
<td>&lt;0.0001</td>
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<tr>
<td>Burkholderiaceae_Ralstonia</td>
<td>0.0</td>
<td>0.1</td>
<td>0.001</td>
</tr>
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<td>Clostridiaceae_Clostridium</td>
<td>0.0</td>
<td>0.2</td>
<td>0.005</td>
</tr>
<tr>
<td>Clostridiaceae_other</td>
<td>0.0</td>
<td>0.2</td>
<td>0.009</td>
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<tr>
<td>Enterobacteriaceae_Proteus</td>
<td>0.0</td>
<td>0.1</td>
<td>&lt;0.0001</td>
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<tr>
<td>Enterococcaceae_Enterococcus</td>
<td>0.0</td>
<td>0.2</td>
<td>0.02</td>
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<tr>
<td>Enterococcaceae_Enterococcus</td>
<td>0.0</td>
<td>0.5</td>
<td>0.008</td>
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<tr>
<td>Lactobacillaceae_Dorea</td>
<td>1.6</td>
<td>0.4</td>
<td>0.02</td>
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<tr>
<td>Lactobacillaceae_Other</td>
<td>20.3</td>
<td>12.0</td>
<td>0.03</td>
</tr>
<tr>
<td>Ruminococcaceae_Nitrosospira</td>
<td>0.0</td>
<td>0.1</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Values are means ± SD, *P < 0.05. As expected, patients with hepatic encephalopathy (HE) have a worse model for end-stage liver disease (MELD) score and cognitive performance and higher venous ammonia, endotoxin, IL-6, asymmetric dimethyl arginine (ADMA), and S100b protein compared with patients without HE. A high score in Digit symbol, Block design, and Targets indicates good cognitive performance whereas a high score in the remaining cognitive tests suggests poor performance. sICAM-1, soluble intravascular adhesion molecule; sVCAM-1, soluble vascular adhesion molecule.

(subjects are required to replicate designs with given blocks in a timed manner), and 3) inhibitory control test [ICT; subjects are instructed to respond to alternating presentations of X and Y on the screen (targets) while inhibiting response when X and Y are not alternating (lures)] (4, 50). The PHEs consists of number connection test-A/B (subjects are asked to “join the dots” between numbers or numbers and alphabets in a timed fashion), digit symbol (subjects are required to copy corresponding figures from a given list within 2 min), line drawing (time) and (errors) [subjects are required to trace a line between two parallel lines with balance speed and accuracy; time required and the number of times the subject’s line strays beyond the marked lines (errors) are recorded], and serial dotting [subjects are asked to dot the center of a group of blank circles]. The PHEs is a validated battery for cognitive dysfunction in cirrhosis and tests for psychomotor speed, visuomotor coordination, attention, and set shifting (40). Block design tests for visuomotor coordination. A high score on block, digit symbol, and ICT targets and a low score on the rest indicate good performance.

Blood was collected from cirrhotic patients for venous ammonia, liver disease severity [using the model for end-stage liver disease (MELD) score; a validated score of international normalized ratio of the prothrombin time (INR), serum bilirubin, and serum creatinine (MELD) score; a validated score of international normalized ratio of the prothrombin time (INR), serum bilirubin, and serum creatinine (MELD)] and pro- and anti-inflammatory cytokines (28). A portion of the serum was stored at −80°C and was subsequently analyzed for innate immunity (IL-1β, IL-6, TNF-α, Th1 (IFN-γ and IL-2), Th2 (IL-4, IL-10, IL-13), and Th17 responses (IL-17 and IL-23), endotoxin, neural function [neuron-specific enolase (NSE) and s100b protein] (53), endotoxin activation [soluble intravascular adhesion molecule (sICAM-1) and soluble vascular adhesion molecule (sVCAM-1)], and asymmetric dimethyl arginine (ADMA). These were analyzed in duplicate by using published techniques by AssayGate (Liamsville, MD) (5, 6).

Interrogation of the microbiome. Stool was collected and DNA was extracted for microbiome analysis by use of published techniques (38). A subset underwent an unsedated, unprepared flexible sigmoidoscopy during which a pinch biopsy of the rectosigmoid mucosa was obtained, snap-frozen, and stored till the analysis at −80°C. We first used length heterogeneity PCR (LH-PCR) fingerprinting of the 16S rRNA to rapidly survey our samples and standardize the community amplification. We then interrogated the microbial tax associated with the gut fecal microbiome using multilag pyrosequencing (MTPS) (17). This technique allows for rapid sequencing of multiple samples at one time, yielding thousands of sequence reads per sample. Microbiome community fingerprinting. LH-PCR was done to standardize the community analysis as previously published. Briefly, total genomic DNA was extracted from tissue by using a Bio101 kit from AJP-Gastrointest Liver Physiol. doi:10.1152/ajpgi.00152.2012 www.ajpgi.org
MP Biomedicals, Montreal, Quebec, Canada as per the manufacturer’s instructions. About 10 ng of extracted DNA was amplified by PCR by using a fluorescently labeled forward primer 27F [5’-6FAM] AGAGTTTGATCCTGGCTCA G-3’ and unlabeled reverse primer 355R’ [5’-GCTGCTCCTCCGTAGGAGT-3’]. Both primers are universal primers for bacteria (29). The LH-PCR products were diluted according to their intensity on agarose gel electrophoresis and mixed with ILS-600 size standards (Promega) and HiDi Formamide (Applied Biosystems, Foster City, CA). The diluted samples were then separated on a ABI 3130xl fluorescent capillary sequencer (Applied Biosystems) and processed using the Genemapper software package (Applied Biosystems). Normalized peak areas were calculated by using a custom PERL script, and operational taxonomic units constituting less than 1% of the total community from each sample were eliminated from the analysis to remove the variable low-abundance components within the communities.

**MPiPS:** We employed the MPiPS process to characterize the microbiome from the fecal and biopsy samples. Specifically, we have generated a set of 96 emulsion PCR fusion primers that contain the 454 emulsion PCR linkers on the 27F and 355R primers and a different eight-base “barcode” between the A adapter and 27F primer. Thus each fecal sample was amplified with unique bar-coded forward 16S rRNA primers, and then up to 96 samples were pooled and subjected to emulsion PCR and pyrosequenced by use of a GS-FLX pyrosequencer (Roche). Data from each pooled sample were “deconvoluted” by sorting the sequences into bins based on the barcodes by using custom PERL scripts. Thus we were able to normalize each sample by the total number of reads from each barcode. We have noted that ligating tagged primers to PCR amplicons distorts the abundances of the communities and thus it is critical to incorporate the tags during the original amplification step.

**Microbiome community analysis.** We identified the taxa present in each sample using the Bayesian analysis tool in Version 10 of the Ribosomal Database Project (RDP10). The abundances of the bacterial identifications were then normalized by using a custom PERL script, and genera present at >1% of the community were tabulated. We chose this cutoff because of our a priori assumption that genera present in <1% of the community vary between individuals and have minimal contribution to the functionality of that community and 2,000 reads per sample will only reliably identify community components that are greater than 1% in abundance (17).

**Statistical analysis.** Cirrhotic patients with HE were compared with those without HE with respect to BMI, inflammatory markers, cognitive performance, and microbiome constituents. Mucosal microbiota constituents were also compared between controls and cirrhotic patients. Unpaired t-tests were used to compare demographics, cognitive tests, and inflammatory markers. Since the microbiome constituents tend to be sparse and nonparametrically distributed, we used Metastats to compare microbiome between stool and mucosa of patients with and without HE (51). Metastats performs statistical analysis (to investigate metagenomic differences) along with biomarker discovery (to evaluate specific features underlying these differences) based on repeated t-statistics and Fisher’s tests on random permutations (42). We also performed Metastats analysis between the mucosal microbiota compared with the stool microbiome in patients with HE and without HE and those with HE on or not on rifaximin, and between controls and cirrhotic patients’ mucosal microbiota. Principal component analysis (PCA) on the abundance tables was also performed (25, 33).

Subsequently, we analyzed the correlations between MELD score, BMI, inflammatory markers, cognitive tests, and microbiome constituents using a correlation network analysis obtained through a customized statistical script in R (6) using a P value cutoff of <0.05 and an R value >0.5 to identify the most significant relationships (3, 17).

This study was approved by the Institutional Review Boards of the McGuire Veterans Affairs Medical Center and the Virginia Commonwealth University Medical Center.

**Table 5. Cirrhosis mucosa vs. cirrhosis stool comparison using Metastats**

<table>
<thead>
<tr>
<th>Family_Genus, % abundance</th>
<th>Mucosa (n = 36)</th>
<th>Stool (n = 36)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incertae Sedis XIV_Blastia</td>
<td>4.1</td>
<td>1.5</td>
<td>0.002</td>
</tr>
<tr>
<td>Vibrio_Glumivaldella</td>
<td>3.1</td>
<td>0.0</td>
<td>0.001</td>
</tr>
<tr>
<td>Propionibacteriaceae_Propionibacterium</td>
<td>1.3</td>
<td>0.0</td>
<td>0.001</td>
</tr>
<tr>
<td>Streptomyces_Spinulosporoides</td>
<td>1.5</td>
<td>0.0</td>
<td>0.001</td>
</tr>
<tr>
<td>Incertae Sedis XI_other</td>
<td>0.5</td>
<td>0.0</td>
<td>0.001</td>
</tr>
<tr>
<td>Verrucomicrobiaceae_Verrucomicrobium</td>
<td>0.5</td>
<td>1.2</td>
<td>0.005</td>
</tr>
<tr>
<td>Veillonellaceae_Veillonella</td>
<td>0.2</td>
<td>2.2</td>
<td>0.01</td>
</tr>
<tr>
<td>Lachnospiraceae_Roseburia</td>
<td>0.0</td>
<td>0.2</td>
<td>0.001</td>
</tr>
<tr>
<td>Lachnospiraceae_Roseburia</td>
<td>0.2</td>
<td>1.0</td>
<td>0.03</td>
</tr>
</tbody>
</table>

There was a significantly higher Blastia, Verrucomicrobiaceae, and Veillonellaceae abundance and lower Incertae Sedis XIV, Veillonella, Bacteroides, and Roseburia in the stool.
Innovative Methodology

G678 COLONIC MUCOSAL MICROBIOME AND HEPATIC ENCEPHALOPATHY

RESULTS

A total of 60 patients with cirrhosis and 17 age-matched healthy controls were included in the study. Ten controls were women, the mean age was 52 ± 6 yr, and all of the controls underwent flexible sigmoidoscopy with biopsy. The distribution of HE and No-HE was relatively uniform with 24 patients without HE and 36 with HE. Of the 36 HE patients, 17 were only on lactulose whereas 19 were on both lactulose and rifaximin therapy. All subjects were nonvegetarians and had similar dietary intake and constituents on recall prior to sample collection (mean intake 2,390 kcal and 16% protein intake). HE patients had a significantly higher MELD score and also, as expected, higher ammonia and worse cognitive performance on all tests compared with patients without HE (Table 1). There was higher endotoxin, s100b, IL-6, and ADMA in the HE patient group. Of the 60 patients, 36 (17 patients without HE and 19 with HE) underwent flexible sigmoidoscopy with biopsy the same day of the stool and sample collection. Patients on rifaximin had a worse cognitive performance compared with those only on lactulose on number connection-A (68.3 vs. 52.25 s, \( P = 0.05 \)) and -B (192.9 vs. 145.8 s, \( P = 0.03 \)), targets (86 vs. 95%, \( P = 0.05 \)), serial dotting (94.2 vs. 84.0 s, \( P = 0.04 \)), line tracing errors (62.5 vs. 44.9, \( P = 0.05 \)) but not line tracing time (118.0 vs. 127.3 s, \( P = 0.41 \)), digit symbol (37 vs. 38 score, \( P = 0.89 \)), block design (17.5 vs. 19.8 score, \( P = 0.56 \)), and lures (15.7 vs. 16.9 responses, \( P = 0.92 \)). Patients currently on rifaximin also had a significantly higher level of IL-6 (51.04 vs. 30.13, \( P = 0.04 \)) and endotoxin (0.43 vs. 0.20, \( P = 0.05 \)), with a trend toward higher MELD (18 vs. 16, \( P = 0.08 \)) compared with those on lactulose alone.

Comparison between control and patient mucosa microbiota.

We found significant differences on Metastats between healthy controls and cirrhotic patients mucosa (Table 2), and when the controls were compared with HE group (Table 3) and comparatively fewer differences between controls and no-HE patients (Table 4). There was a significantly higher abundance of autochthonous genera (Dorea, Subdoliglumum, and Incertae Sedis other) and a lower abundance of potentially pathogenic ones (Enterococcus, Proteus, Clostridium, and Burkholderia) in controls compared with cirrhotic patients’ mucosa. We also found significant clustering of the controls with each other in the PCA (Fig. 1). Additionally, the no-HE patients clustered around the controls whereas HE patients were scattered further from the central control cluster (Fig. 1). These results indicate that there is a greater variance in the microbiome composition between controls and HE patients compared with controls and no-HE patients.

Comparison between all patients’ mucosa to stool microbiome.

We found a significant change in the microbiome of the mucosa compared with stool in the entire group by use of Metastats (Table 5). This change persisted when the comparison between stool and mucosa was performed for the HE and the no-HE group. The composition of the mucosal microbiome in the entire population differed considerably from the corresponding stool microbiome. Prominent bacterial genera found at a higher abundance in the mucosa belonged to Firmicutes (Blautia, Incertae Sedis XI, Actinobacteria (Propionibacterium and Streptomyces), and Proteobacteria (Vibrio). Interestingly most bacteria found in higher abundances in stool were Firmicutes (Leuconostoc, Roseburia, Veillonella, and Incertae Sedis XIV). These differences persisted when the group was divided into HE

Table 6. HE mucosa vs. HE stool

<table>
<thead>
<tr>
<th>Family_Genus, % abundance</th>
<th>HE Mucosa (n = 19)</th>
<th>HE stool (n = 19)</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incertae Sedis XIV, Blautia</td>
<td>5.2</td>
<td>1.6</td>
<td>0.004</td>
</tr>
<tr>
<td>Vibriostraceae_Vibrio</td>
<td>4.4</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>Propionibacteriaceae_Propionibacterium</td>
<td>1.1</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>Incertae Sedis XI_other</td>
<td>0.8</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>Vibriostraceae_other</td>
<td>0.6</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>Incertae Sedis XIV_other</td>
<td>0.3</td>
<td>1.4</td>
<td>0.005</td>
</tr>
<tr>
<td>Fusobacteriaceae_other</td>
<td>0</td>
<td>1.1</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 7. No HE mucosa vs. No HE stool

<table>
<thead>
<tr>
<th>Family_Genus, % abundance</th>
<th>No-HE Mucosa (n = 17)</th>
<th>No-HE Stool (n = 17)</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leuconostocaceae_Leuconostoc</td>
<td>0</td>
<td>1.0</td>
<td>0.002</td>
</tr>
<tr>
<td>Bacteroidales_incertae_sedis_other</td>
<td>0</td>
<td>1.0</td>
<td>0.001</td>
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<tr>
<td>Alcaligenaceae_other</td>
<td>0</td>
<td>0.8</td>
<td>0.001</td>
</tr>
<tr>
<td>Streptomycesaceae_Streptomyces</td>
<td>2.5</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>Propionibacteriaceae_Propionibacterium</td>
<td>1.8</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>Vibriostraceae_Vibrio</td>
<td>1.3</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>Burkholderiaceae_Ralstonia</td>
<td>0.5</td>
<td>0</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Comparison between control and patient mucosa microbiota.

Comparison between all patients’ mucosa to stool microbiome.

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COLONIC MUCOSAL MICROBIOME AND HEPATIC ENCEPHALOPATHY

G679

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Table 8. Comparison between mucosal microbiome abundances between HE and no-HE groups using Metastats

<table>
<thead>
<tr>
<th>Family_Genus</th>
<th>HE Mucosa (n = 17)</th>
<th>No-HE Mucosa (n = 19)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lachnospiraceae_Roseburia</td>
<td>0.5</td>
<td>2.5</td>
<td>0.002</td>
</tr>
<tr>
<td>Veillonellaceae_Veillonella</td>
<td>0.7</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>Burkholderiaceae_other</td>
<td>0.8</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>Veillonellaceae_Megasphaera</td>
<td>2.4</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>Streptomycesaceae_Streptomyces</td>
<td>2.7</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>Fusobacteriaceae_other</td>
<td>3.5</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>Bifidobacteriaceae_Bifidobacterium</td>
<td>3.8</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>Enterococcaceae_Enterooccus</td>
<td>7.7</td>
<td>0</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 9. Comparison of the mucosal microbiome between patients on lactulose alone compared with those on lactulose and rifaximin using Metastats

<table>
<thead>
<tr>
<th>Family_Genus</th>
<th>Lactulose Alone</th>
<th>Lactulose and Rifaximin</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incertae Sedis XIV_Blautia</td>
<td>4.2</td>
<td>1.5</td>
<td>0.008</td>
</tr>
<tr>
<td>Lachnospiraceae_Roseburia</td>
<td>1.9</td>
<td>0</td>
<td>0.005</td>
</tr>
<tr>
<td>Propionibacteriaceae_Propionibacterium</td>
<td>1.1</td>
<td>2.3</td>
<td>0.03</td>
</tr>
<tr>
<td>Veillonellaceae_Other</td>
<td>1.1</td>
<td>0</td>
<td>0.03</td>
</tr>
<tr>
<td>Rikenellaceae_Alistipes</td>
<td>1.8</td>
<td>0</td>
<td>0.03</td>
</tr>
</tbody>
</table>

and no-HE (Tables 6 and 7). Propionibacterium and Vibrio genera were significantly more abundant in the mucosa than in the stool in both HE and no-HE.

Comparison between HE and No-HE patients' microbiome. Next we compared the stool and mucosal microbiome of the HE and No-HE groups. We again found no appreciable difference in the stool microbiome between patients with and without HE despite the higher sample size in this study. However, there was a significant difference in the mucosal microbiome between HE and no-HE patients (Table 8). Specifically, Firmicutes such as members of genera Veillonella, Megasphaera, Bifidobacterium, and Enterococcus were higher in HE whereas Roseburia was more abundant in the no-HE group.

Comparison between patients on lactulose alone compared with those on lactulose and rifaximin. As found between HE and no-HE patients, there was no difference in the stool microbiome of patients on rifaximin and lactulose compared with those on lactulose alone. The mucosal microbiome in rifaximin-treated patients, however, was significantly different (Table 9). There was a significantly decreased abundance of autochthonous bacteria (Roseburia and Blautia) and Veillonellaceae but an increased abundance of Propionibacterium in the rifaximin group.

Correlation network analysis. We performed a Spearman correlation using a custom R package to analyze linkages between the cognitive performance and inflammatory markers and the mucosal microbiome in HE and No-HE patients. We did not perform the analysis with the stool microbiome since there was no significant difference between the two groups' stool microbiome when using Metastats. The overall view of the two networks shows a distinct increase in the connectivity within the HE network (Fig. 2A) compared with the No-HE network (Fig. 3A). Certain bacterial genera were negatively correlated with inflammation and endothelial activation and linked to good cognitive performance across both networks. These were Fecalibacterium, Roseburia, other Lachnospiraceae, and Blautia. We also found a significant dense correlation network surrounding IL-17 and MELD with other inflammatory markers and cognitive performance in both networks. Replicating our prior experience, we found members of the Alcaligenaceae and Porphyromonadaceae families associated with poor cognitive performance in the No-HE network (6). What was interesting is that the genera present in higher abundance in the HE patients' mucosa (Tables 5–7) was associated with higher inflammation, worse cognition, and worse endothelial activation in the correlation network (Fig. 2A). Specifically, the subnetworks centered on Megasphaera, Veillonella, Burkholderia, and Bifidobacterium showed that they were associated with poor cognition, higher MELD, higher inflammation, and endothelial activation. These genera were not present in the no-HE network. In contrast, Roseburia, which was higher in the no-HE group, was associated with beneficial effects, i.e., less inflammation and endothelial activation and better cognition in both networks. Figures 2A and 3A are the correlation networks for the HE and no-HE groups' mucosal microbiome, respectively.

DISCUSSION

We found a significant alteration in the colonic mucosal microbiome compared with stool in cirrhosis. We did not find any significant change in the stool microbiome between patients with or without HE but found a dramatic change in the mucosal microbiome between the two groups and with healthy controls.

Specifically, we found a higher abundance of the beneficial genus Roseburia in patients without HE whereas a higher abundance of members of the genera Enterococcus, Veillonella, Megasphaera, Bifidobacterium, and Burkholderia was found in the HE patients' mucosa. An increase in members of the genus Enterococcus have been previously reported in the fecal microbiome of patients with liver cirrhosis compared with control patients (31). In the present study, an increase in the levels of Bifidobacterium genera in HE patients was unex-
expected since members of this genus have been used as probiotics and the presence of this bacterium is correlated with suppression of inflammation (37). This could be partly explained by the use of lactulose as a carbon and energy source by *Bifidobacterium* species (which only the cirrhosis group was on) and also by the relatively greater species diversity within this genus, some of which may be not related to overall benefit (11, 49). The correlation network linking the mucosal microbiome to cognition, endothelial activation, inflammation, and disease severity was richer in connectivity in the HE group, suggesting functional shifts in the microbial community that correlate with disease.

The differences between the mucosa and stool microbiome has been shown in several disease conditions such as Crohn’s disease as well as in healthy volunteers (46). Prior studies have also shown that the influence of the fecal microbes may be less than that of the mucosal microbiome on immunity and overall health (18, 30). The intestinal barrier has a strong immunological interface comprising mucus, epithelium, and the mucosa-associated immune cells. The bacterial biofilm is usually restricted to the outer mucus layer (24, 35). However, there is evidence of cross talk between the mucosal immune system and the gut bacterial species that can usually differentiate between commensals and pathogens (8). We replicated prior noncirrhotic studies by showing a significant difference between the mucosal and stool microbiome in the overall population and when divided into HE and no-HE. The study of the mucosal microbiome in cirrhosis is relevant because most premortal events in cirrhosis, such as spontaneous bacterial peritonitis and spontaneous bacteremia, are related to intestinal bacterial translocation (1, 47). Alteration in permeability, bacterial overgrowth, and poor motility, along with deficiency of antimicrobial peptides, further increases the risk of bacterial translocation in cirrhosis (41, 48, 52). The underlying suppression of the mucosal immunity in cirrhosis with the resultant proinflammatory milieu leads to endotoxemia and complications of cirrhosis and HE (47, 52).

We found significant differences between controls and cirrhotic mucosal microbiota that were more pronounced when HE patients were compared with controls. These results showed a significantly higher autochthonous genera abundance (*Dorea, Subdoligranulum, Incertae Sedis XIV, Blautia, Roseburia, Faecalibacterium*) and lower abundance of pathogenic genera (*Enterococcus, Burkholderia, Proteus*) in controls compared with cirrhotic patients.

The reason for marked changes in the mucosal gut microbiome between control and patients with cirrhosis and hepatic encephalopathy is not clear. A potential explanation could be the altered synthesis and secretion of bile acids and other biliary components as shown in a preliminary study with worsening cirrhosis severity that could have a significant effect on the gut mucosal physiology, microbiome function, and systemic inflammation (26). A decrease in bile acids, antibacterial peptides, and mucins in the colon may allow for the selection of potentially pathogenic bacteria to adhere and grow in association with the colonic mucosa of patients with cirrhosis allowing for increased translocation of bacterial toxic products (21, 23). This hypothesis is consistent with an increase in serum endotoxin and proinflammatory cytokines (i.e., IL-6) observed in HE patients compared with those without. Certain species of *Fecalibacterium*, along with other bacteria belonging to Clostridium cluster XIV, have dampening effects on inflammation, in some cases due to NF-κB suppression (10, 19). Interestingly, there was a significantly higher abundance of butyrate-producing genera such as *Roseburia, Fecalibacterium, Lachnospiraceae*, and *Subdoligranulum* in less-affected patients (controls and no-HE cirrhotic patients) compared with HE patients. This distribution has been seen in controls when compared against patients with inflammatory bowel disease and colon cancer and may be related to butyrate being the preferred energy source for colonocytes (7, 32). There is also a redundancy in the function of the human microbiome in which several bacterial classes can perform essential metabolic functions, i.e., carbohydrate metabolism. Specifically, *Ruminococcus*, with its cellulosytic activity, has been shown to ferment plant-based material in the gut and is beneficial from an energy standpoint to the host (15). The high abundance of key genera in controls and in no-HE patients reflects the “healthy human microbiome” in which there is consistently the presence of the families *Lachnospiraceae* and *Ruminococcaceae* in healthy volunteers compared with any disease. Further research into the metabolomics of these microbiota is needed to evaluate the functional consequences of these changes (3).

We confirmed our prior study demonstrating that there was no appreciable difference in the fecal bacterial composition of patients with and without HE, including those on rifaximin or not (6). This was intriguing because patients with HE were significantly different from those without HE from a standpoint of liver disease, clinical severity, inflammation, and cognitive function. Therefore the changes in the mucosal bacterial composition were sought and were found to be significantly different between the groups and relatively similar to the ones between controls and HE patients. Autochthonous bacteria such as *Roseburia* have evolved to survive in the mucosal niches without eliciting a host immune reaction despite the abundant antimicrobial peptides (36). In contrast, genera such as *Enterococcus* are usually present in the fecal stream, not the mucosa (36). Interestingly we found an increase in abundance of potentially pathogenic genera, *Enterococcus, Burkholderia*, and *Veillonellaceae* constituents, in HE patients. Prior stool studies have shown an increased abundance of *Veillonellaceae* in cirrhosis compared with noncirrhotic patients (12). This shift in HE patients’ mucosa with higher *Enterococcus, Veillonellaceae*, and *Burkholderia* abundance may reflect a disease-associated reduction in the normally present autochthonous bacteria that would allow the growth of these potentially pathogenic genera in the mucosa. Patients on rifaximin had worse cognition and higher endotoxin and IL-6 compared with those without rifaximin, which is to be expected since rifaximin is initiated in those whose HE is not controlled with lactulose. It is likely that the cross-sectional nature of our patients was the reason behind the poor mitigation of endotoxemia by rifaximin as shown in other studies (27). Therefore, their mucosal microbiome also reflected the worse underlying disease, i.e., a significantly decreased abundance of the autochthonous bacteria. Some of these genera are associated with severe infections in cirrhotic and noncirrhotic patients (34, 54). Furthermore, these genera were only seen in HE patients’ network and were associated with a higher MELD score, worse endothelial activation, worse cognitive performance (lures, serial dotting, and digit symbol tests), and higher systemic inflammation (IL-17) in the HE group. We found differences

**Innovative Methodology**

**COLONIC MUCOSAL MICROBIOME AND HEPATIC ENCEPHALOPATHY**

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studies that with those without HE. We confirmed the results of prior cognition, inflammation, and endothelial activation compared more robust interaction was seen between the microbiome, and endothelial phenotypes in the direction that was expected groups, whereas pathogenic ones were overrepresented in the HE group and were correlated with cognitive, inflammatory, and endothelial phenotypes in the direction that was expected (25, 42).

In the correlation network for patients with HE, a richer and more robust interaction was seen between the microbiome, cognition, inflammation, and endothelial activation compared with those without HE. We confirmed the results of prior studies that Alcaligenaceae and Porphyromonadaceae were associated with poor cognitive performance (6). One interesting finding was that the autochthonous bacteria belonging to Lachnospiraceae, Ruminococcaceae, and Incertae Sedis XIV had similar beneficial linkages, regardless of the setting. This means that the presence of these bacteria is associated with better cognitive function, indicated by a decrease in inflammation and endothelial activation regardless of the early or advanced disease stage. This replicates studies showing that Fecalibacterium and Lachnospiraceae spp. are associated with reduced intestinal inflammation in Crohn’s disease and extends this finding into cirrhosis (45, 46). There is also evidence that these bacteria are correlated with markers for reduced inflammation of Th-17 cells in the colon (2). Prior studies have shown that intestinal inflammation can initiate the IL-17/IL-23 system, which is upregulated in Crohn’s disease (8, 14, 22). Correspondingly, we found correlations with markers for the IL-17/IL-23 inflammatory response system in cirrhosis, in both HE and non-HE patients. There was a negative correlation between autochthonous bacteria and IL-17, and proinflammatory cytokines, indicating that the gut-based inflammation may be modulated in the presence of these bacteria. Prior studies have also shown that HE is associated with significantly worse systemic inflammation that can potentially improve with therapy (5, 44). Interestingly, we also found that levels of the anti-inflammatory cytokine, IL-10, were correlated with poor outcomes similar to the proinflammatory cytokines; this may reflect a mixed scenario between the systemic inflammatory response and the compensatory anti-inflammatory response syndrome that can be seen in cirrhosis (9). It can therefore be speculated that modulation of both pro- and anti-inflammatory cytokines could be a potential mechanism behind the microbiome-associated changes in brain function in HE.

The present study only relied on the presence of bacteria, but it is also possible that their end-products, such as the beneficial short-chain fatty acids or the relatively toxic indoles and phenols, may influence clinical outcomes (20, 39). A study of the functional component of the microbes would be important to analyze these effects (20). The present analysis is also only correlative; therefore no conclusions regarding causality can be made, but this generates hypotheses to be tested in subsequent studies. It is also possible, given the high MELD score in HE patients, that this was related simply to severity of the liver disease in HE patients.

We conclude that there is a significant difference in sigmoid mucosal microbiome of the cirrhotic patients compared with healthy controls. There are also differences between the colonic and stool microbiome in cirrhosis, which persists even when patients are subdivided into those with and without HE. We also found that the colonic mucosal microbiome of HE patients (who were also more advanced from a liver disease perspective) is significantly different from patients without HE but the stool microbiome was essentially the same. There is a lower abundance of autochthonous bacterial genera coupled with a higher level of potentially pathogenic bacteria such as Enterococcus and Burkholderia in the HE patients’ colonic mucosa. Autochthonous bacteria, Lachnospiraceae, Ruminococcaceae, and Incertae Sedis XIV, are associated with better cognition, lower severity of liver disease, and decreased inflammation and endothelial activation in both HE and no-HE groups. However, genera overrepresented in the HE patients’ mucosa were associated with a proinflammatory milieu, higher MELD score, and poor cognition. Therefore, the colonic mucosal microbiome of patients with HE is significantly different from patients without HE and is associated with the proinflammatory milieu, endothelial activation, and poor cognitive performance that is inherent in this patient population. Further studies are needed to determine the longitudinal effect of interventions on both the colonic and stool microbiome in patients with cirrhosis, accounting for liver disease severity so that newer and specific therapeutic targets can be evaluated.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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Innovative Methodology

COLONIC MUCOSAL MICROBIOME AND HEPATIC ENCEPHALOPATHY


