# Linkage of gut microbiome with cognition in hepatic encephalopathy

**Jasmohan S. Bajaj,[1] Jason M. Ridlon,[1,2] Phillip B. Hylemon,[1,2] Leroy R. Thacker,[4] Douglas M. Heuman,[1] Sean Smith,[3] Masoumeh Sikaroodi,[3] and Patrick M. Gillevet[3]**

*Divisions of [1]Gastroenterology, Hepatology and Nutrition, [2]Microbiology, and [4]Biostatistics, Virginia Commonwealth University and McGuire VA Medical Center, Richmond, Virginia; [3]Microbiome Analysis Center, George Mason University, Manassas, Virginia*

Bajaj JS, Ridlon JM, Hylemon PB, Thacker LR, Heuman DM, Smith S, Sikaroodi M, Gillevet PM. Linkage of gut microbiome with cognition in hepatic encephalopathy. *Am J Physiol Gastrointest Liver Physiol* 302: G168–G175, 2012. First published September 22, 2011; doi:10.1152/ajpgi.00190.2011.—Hepatic encephalopathy (HE) has been related to gut bacteria and inflammation in the setting of intestinal barrier dysfunction. We aimed to link the gut microbiome with cognition and inflammation in HE using a systems biology approach. Multitag pyrosequencing (MTPS) was performed on stool of cirrhotics and age-matched controls. Cirrhotics with/without HE underwent cognitive testing, inflammatory cytokines, and endotoxin analysis. Patients with HE were compared with those without HE using a correlation-network analysis. A select group of patients with HE ($n = 7$) on lactulose underwent stool MTPS before and after lactulose withdrawal over 14 days. Twenty-five patients [17 HE (all on lactulose, 6 also on rifaximin) and 8 without HE, age 56 ± 6 yr, model for end-stage liver disease score 16 ± 6] and ten controls were included. Fecal microbiota in cirrhotics were significantly different (higher *Enterobacteriaceae*, *Alcaligeneceae*, and *Fusobacteriaceae* and lower *Ruminococcaceae* and *Lachnospiraceae*) compared with controls. We found altered flora (higher *Veillonellaceae*), poor cognition, endotoxemia, and inflammation (IL-6, TNF-α, IL-2, and IL-13) in HE compared with cirrhotics without HE. In the cirrhosis group, *Alcaligeneceae* and *Porphyromonadaceae* were positively correlated with cognitive impairment. *Fusobacteriaceae*, *Veillonellaceae*, and *Enterobacteriaceae* were positively and *Ruminococcaceae* negatively related to inflammation. Network-analysis comparison showed robust correlations (all $P < 1E\text{-}5$) only in the HE group between the microbiome, cognition, and IL-23, IL-2, and IL-13. Lactulose withdrawal did not change the microbiome significantly beyond *Fecalibacterium* reduction. We concluded that cirrhosis, especially when complicated with HE, is associated with significant alterations in the stool microbiome compared with healthy individuals. Specific bacterial families (*Alcaligeneceae*, *Porphyromonadaceae*, *Enterobacteriaceae*) are strongly associated with cognition and inflammation in HE.

cirrhosis; inflammation; systems biology; multitag pyrosequencing

CIRRHOSIS IS OFTEN COMPLICATED by hepatic encephalopathy (HE), a condition characterized by cognitive impairment and poor survival (2, 8). There is evidence that pathogenic abnormalities in HE are related to the gut flora and their byproducts such as ammonia and endotoxin in the setting of intestinal barrier dysfunction and systemic inflammation (14, 35, 36, 44). Patients with cirrhosis also have widespread derangements of their immune response, which can potentiate insults such as sepsis and result in HE (36, 43). The present treatments for HE rely on manipulation of the gut flora; however, their mechanisms of action as well as prediction of resistance to therapy are not clear (2). In addition, the characterization of gut flora in prior HE studies has been limited by the use of culture-based techniques that do not identify the majority of the intestinal bacteria (23). Because the pathogenesis of HE likely spans several metabolic processes, we proposed that a systems biology approach could be useful to identify novel functional hypothesis and new therapeutic targets for HE. Specifically, we used correlation-network analysis to correlate features within each treatment group to dissect out functionality in the system (27). This analysis provides potential clues to the functionality of the system, leading the way to hypothesis-driven experimental research.

The aims of this study were *1*) to link the gut microbiome with cognition and inflammation in cirrhotic patients with and without HE using a systems biology approach, *2*) to identify differences in the microbiome of healthy controls and cirrhotic patients, and *3*) to define the effect of lactulose withdrawal on microbiome of cirrhotic patients. The a priori hypothesis was that the gut microbiome composition would be correlated with cognition and inflammation in cirrhotic patients with HE and that this association would be different from those who have never developed HE.

## MATERIALS AND METHODS

Patients with cirrhosis and healthy age-matched controls were recruited after providing a written informed consent. We only included controls without liver disease and those who were not taking medications apart from those for hypertension, hyperlipidemia, or gastroesophageal reflux disease. In the case of cirrhotic patients, we excluded those with a current infection (defined by elevated white blood cell count, clinical suspicion, or fever), who had experienced variceal bleeding within the last 4 wk, who were on gut-absorbable antibiotic therapy, or had alcohol or illicit drug intake within 3 mo (checked by drug and alcohol screens). The data collected from their medical record were model for end-stage liver disease (MELD) score, etiology of cirrhosis, complications of cirrhosis in the past, and present medication use. Patients in the no HE group had never had an episode of HE and were not on any therapy for it. Patients in the HE group had suffered at least one HE episode within the last 3 mo and were currently controlled on lactulose alone or lactulose with rifaximin. We did not include patients during an acute HE episode because those patients are often hospitalized, are on systemic antibiotic therapy, and are not able to give consent or perform cognitive testing.

All subjects underwent a mini-mental status exam, and only those scoring above 25 were included in the full study (11). Participants then underwent a 24-h dietary recall. Subsequently, a recommended cognitive battery consisting of the following tests was administered to the cirrhotic patients: *1*) psychometric HE score (PHES), *2*) block design test (BDT: subjects are required to replicate standardized designs with given blocks in a timed manner. The score is based on

the designs correctly copied), and *3*) inhibitory control test [ICT, this is a 15-min computerized test. 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)] (3, 41). The PHES consists of five tests: number connection test-A/B (subjects are asked to "join the dots" between numbers or numbers and alphabets in a timed fashion, and the number of seconds required is the outcome), digit symbol test (DST: subjects are required to copy corresponding figures from a given list within 2 min, and the number correctly copied is the result), line drawing test [LDTt (time) and LDTe (errors): subjects are required to trace a line between two parallel lines and balance between 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 test (subjects are asked to dot the center of a group of blank circles, and the time required is the outcome). The PHES is a validated battery for cognitive dysfunction in cirrhosis and tests for psychomotor speed, visuomotor coordination, attention, and set shifting (32). The BDT tests for visuomotor coordination. The ICT is a validated computerized test of attention, psychomotor speed, response inhibition, and working memory. A high score on BDT, DST, and ICT targets and a low score on the rest of the tests indicate good cognitive performance.

Cirrhotic patients also underwent serum collection for inflammatory cytokines testing for innate immunity (IL-1b, IL-6, TNF-$\alpha$), Th1 response (IFN-$\gamma$ and IL-2), Th2 response (IL-4, IL-10, IL-13), Th17 response (IL-17 and IL-23), and endotoxin. These were analyzed in duplicate by multiplex bead-based sandwich ELISA and Limulus Amebocyte Lysate assay for endotoxin using published techniques by Assay Gate, Ijamsville, MD (4, 17, 45).

*Prospective study.* A selected group of seven cirrhotic patients in the HE group currently only on lactulose (who were also included in the cross-sectional study) were systematically withdrawn from therapy. Their diet was controlled over the study period. Their stool microflora was analyzed while on lactulose and then 14 days following discontinuation of lactulose therapy. *Day 14* was chosen because prior culture-based studies have shown a change in fecal flora after lactulose initiation within that time frame (31).

*Interrogation of the microbiome.* Stool was collected and DNA extracted for microbiome analysis within 24 h of collection from patients and controls (29). Stool (0.2 mg) was suspended in buffer argininosuccinate lyase to which 0.75-g 0.1-mm zirconia/silica beads (Biospec Products) were added, and cells were disrupted twice at 4,800 revolution/min for 1 min. Tubes were placed in a 90°C water bath for 10 min, and the remainder of the protocol was followed according to the manufacturer. We first routinely use 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 taxa associated with the gut fecal microbiome using multitag pyrosequencing (MTPS). This technique allows the rapid sequencing of multiple samples at one time, yielding thousands of sequence reads per sample (12).

*Microbiome community fingerprinting.* LH-PCR was done to standardize the community analysis as previously published (21). Briefly, total genomic DNA was extracted from tissue using Bio101 kit from MP Biomedicals per the manufacturer's instructions. About 10 ng of extracted DNA was amplified by PCR using a fluorescently labeled forward primer 27F [5′- (6FAM) AGAGTTTGATCCTGGCTCAG-3′] and unlabeled reverse primer 355R′ (5′-GCTGCCTCCCG-TAGGAGT-3′). Both primers are universal primers for bacteria (22). 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). The diluted samples were then separated on an ABI 3130xl fluorescent capillary sequencer (Applied Biosystems) and processed using the Genemapper software package (Applied Biosystems). Normalized peak areas were calculated 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.

*MTPS.* We employed the MTPS process to characterize the microbiome from the fecal 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 barcoded forward 16S rRNA primers, and then up to 96 samples were pooled and subjected to emulsion PCR and pyrosequenced using a GS-FLX pyrosequencer (Roche). Data from each pooled sample were "deconvoluted" by sorting the sequences into bins based on the barcodes 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 (12). Several groups have employed various barcoding strategies to analyze multiple samples, and this strategy is now well accepted (38).

*RDP10 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 using a custom PERL script, and taxa present at >1% of the community were tabulated. We chose this cutoff because of our a priori assumption that taxa present in <1% of the community vary between individuals and have minimal contribution to the functionality of that community and that 2,000 reads per sample will only reliably identify community components that are >1% in abundance (13).

This study was approved by the Institutional Review Boards of the McGuire VA Medical Center and the Virginia Commonwealth University Medical Center in Richmond.

*Statistical analysis.* Clinical and microbiome features of controls were compared with patients with cirrhosis with Metastats using the *P* value and the false discovery rate (*q* value) for nonnormal distributions (42). A principal coordinate analysis was also used to show differences between the two groups. Only taxa with average abundances >1%, *P* values <0.05, and low *q* values (i.e., low risk of false discovery) were considered significant.

The cirrhosis group was divided into those with and without HE and were compared. Data from the significant variables between HE and non-HE groups were combined in a mutivariate ANOVA model. Within patients with HE, comparison was made between those on lactulose alone to those with lactulose and rifaximin. Microbiome abundance comparisons between groups were made at a family level using nonparametric tests. A comparison was performed between patients on and withdrawn from lactulose therapy using the Wilcoxon matched-pair signed rank tests. All values are presented as means ± SD unless mentioned otherwise.

*Correlation-network models.* Groups were divided into HE or no HE, and they were analyzed separately. The microbiome features along with the presence of HE, cirrhosis severity, serum markers, and cognitive function tests were correlated using a Pearson's correlation function and then filtered for correlations >0.90. These correlates were calculated using a custom R module, and the correlations and corresponding attributes were imported into Cytoscape for visualization of the network models (34). We then compared the network topology of the two disease classes, HE and no HE, to identify which subnetworks were present in one and not the other, giving us clues on system functionality. It is assumed that correlations present in one treatment group that are missing in another not only differentiate the groups but indicate potential clues to the functionality of the system, leading the way to hypothesis-driven experimental research.

**RESULTS**

Twenty-five cirrhotic patients (MELD score $16 \pm 6$) and ten healthy controls were included. HE was present in 17 patients (68%; 11 were on lactulose alone, 6 were on both lactulose and rifaximin). None of the patients with HE were on rifaximin alone. All patients who were on rifaximin were started on it because of difficulties in tolerating lactulose alone. The mean age of the controls was $54 \pm 5$ yr with 6 men and 4 women and 60% Caucasian, 30% African-American, and 10% Hispanic ethnicity. The ages of cirrhotics with and without HE ($56 \pm 3$ and $55 \pm 5$ yr) as well as the sex (16 men in HE and 7 men in no HE group) were similar to controls. The ethnicity distribution in the HE group (64% Caucasian, 29% African-American, and 6% Hispanic) and without HE group (63% Caucasian, 25% African-American, and 13% Hispanic) was statistically similar to controls. There was no significant difference in the body mass index between the groups (controls $25 \pm 3$, cirrhotics with HE $26 \pm 5$, and those without HE $25 \pm 3$).

All patients and controls were nonvegetarians and had similar dietary intake and constituents on recall before sample collection (mean intake 2,470 Kcal and 16% protein intake). Patients had been abstinent of alcohol and illicit drugs for at least 3 mo, confirmed by serum alcohol and urine drug screens. At the time of sample collection, none of the subjects had systemic infections as evidenced by normal white blood cell counts, normal body temperature, and physical examination unremarkable for infections. The majority of patients and none of the controls were on proton pump inhibitor (PPI) therapy (92%) (Table 1). Thirteen (52%) had alcoholic liver disease; the remainder had hepatitis C (40%) or cryptogenic cirrhosis (8%); eight (32%) had both alcoholic and hepatitis C virus
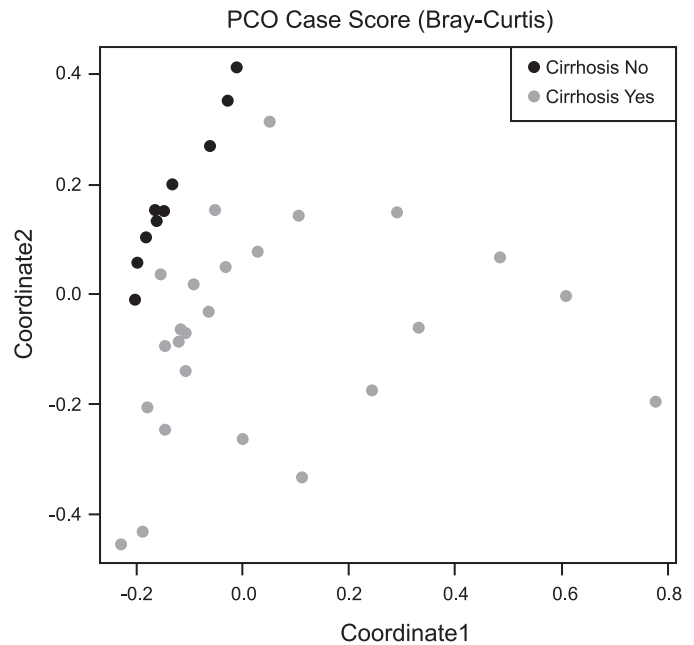


Fig. 1. Principal coordinate analysis of the fecal microbiome of controls and cirrhotic patients. This graph shows the variation in fecal microbiome plotted on a principal coordinate analysis plot. Points that are closer to each other are similar with respect to their stool microbiota. The healthy controls represented by the black dots are clustered together, whereas the cirrhotic patients represented by the gray dots are distant from the controls. This indicates a difference in the stool microbiome of healthy controls compared to cirrhotic patients.

**Table 1.** *Features of patients with and without hepatic encephalopathy*

|  | HE ($n = 17$) | No HE ($n = 8$) | P value |
|---|---|---|---|
| Alcoholic etiology | 58% | 37% | 0.41 |
| Prior variceal bleeding | 23% | 0% | 0.07 |
| Prior SBP | 0% | 0% | 1.0 |
| Renal insufficiency | 0% | 18% | 0.09 |
| Clinically evident ascites | 29% | 47% | 0.65 |
| Median daily bowel movements | 2 | 1 | 0.02 |
| Proton pump inhibitor therapy | 94% | 86% | 0.51 |
| MELD score | $17 \pm 6$ | $12 \pm 5$ | 0.048 |
| Venous ammonia | $52 \pm 28$ | $31 \pm 21$ | 0.148 |
| WBC count, /mm$^3$ | $5.2 \pm 2$ | $5 \pm 3$ | 0.33 |
| Endotoxin | $0.27 \pm 0.24$ | $0.059 \pm 0.012$ | 0.002 |
| IL-1b, pg/ml | $6.2 \pm 11.1$ | $1.07 \pm 0.55$ | 0.06 |
| IFN-$\gamma$, pg/ml | $11.3 \pm 26.6$ | $1.6 \pm 1.8$ | 0.148 |
| IL-10, pg/ml | $8.21 \pm 8.70$ | $2.9 \pm 1.5$ | 0.022 |
| IL-23, pg/ml | $1842 \pm 4873$ | $317 \pm 359$ | 0.205 |
| IL-17, pg/ml | $32.1 \pm 81.3$ | $4.53 \pm 5.27$ | 0.107 |
| IL-6, pg/ml | $67.8 \pm 72.2$ | $9.3 \pm 7.8$ | 0.004 |
| IL-2, pg/ml | $48 \pm 91$ | $2.7 \pm 2$ | 0.04 |
| TNF-$\alpha$, pg/ml | $7.01 \pm 4.09$ | $4.33 \pm 2.33$ | 0.05 |
| IL-13, pg/ml | $32.0 \pm 17.2$ | $0.80 \pm 0.02$ | 0.0001 |

Applicable values are means $\pm$ SD. There were significant differences at baseline between those with and without HE with respect to endotoxemia and inflammation, all of which were significantly worse in patients with hepatic encephalopathy (HE). As expected, patients with HE had a significantly higher model for end-stage liver disease (MELD) score and a higher number of daily bowel movements because they were on lactulose. SBP, spontaneous bacterial peritonitis; WBC, white blood cells.

disease. All patients in the HE group had residual cognitive impairment or minimal HE at the time of the testing (5, 30).

*Cross-sectional microbial analysis between controls and patients with cirrhosis.* There were significant differences in stool microbiome between cirrhotic patients and controls (Fig. 1, Table 2). There was a significantly higher abundance of *Lachnospiraceae* and *Ruminococceae* in the control group, whereas *Enterobacteriaceae, Fusobacteriaceae, Alcaligenaceae, Lactobacillaceae,* and *Leuconostocaceae* were significantly lower in the controls compared with cirrhotic patients. These differences persisted and widened when controls were compared with patients with and without HE (Tables 3 and 4). The differences existed for *Leuconostocaceae,* Clostridiales_Incertae Sedis XIV, *Fusobacteriaceae, Lachnospiraceae,* and *Ruminococcaceae* in both groups of cirrhotic patients (with or without HE). Interestingly, however, the HE group differed from controls on several additional bacterial families compared with cirrhotics without HE in that they had a significantly higher concentration of *Enterobacteriaceae, Alcaligenaceae, Lactobacilaceae,* and *Streptococcaceae.*

*Comparison within the HE group.* There was no significant difference between the clinical, inflammatory, or cognitive profile between HE patients on lactulose alone compared with those on lactulose and rifaximin (Table 5). Additionally, no differences in the microbiome components were identified using classic multivariate analysis. Specifically the normalized abundances at the family level of *Alcaligenaceae* ($12.6 \pm 9\%$ vs. $10 \pm 13\%$, $P = 0.8$), *Enterobacteriaceae* ($26 \pm 40\%$ vs. $24 \pm 40\%$, $P = 0.7$), *Bacteroidaceae* ($39 \pm 35\%$ vs. $58 \pm 39\%$, $P = 0.35$), *Porphyromonadaceae* ($14 \pm 21\%$ vs. $8 \pm 15\%$, $P = 0.23$), *Prevotellaceae* ($18 \pm 25\%$ vs. $10 \pm 19\%$,

Table 2. *Differences in bacterial abundances between controls and cirrhotic patients*

| | Control | | Cirrhosis | | | |
|---|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | *P* value | *Q* value |
| *Leuconostocaceae* | 0.00 | 0.00 | 2.02 | 0.70 | 0.0009 | 0.009 |
| Clostridium Incertae sedis XIV | 7.35 | 1.59 | 1.08 | 0.18 | 0.0009 | 0.008 |
| *Lachnospiraceae* | 23.44 | 2.24 | 10.40 | 2.60 | 0.0009 | 0.008 |
| *Ruminococcaceae* | 17.72 | 1.89 | 6.75 | 1.28 | 0.001 | 0.008 |
| *Enterobacteriaceae* | 0.00 | 0.00 | 7.60 | 2.89 | 0.001 | 0.008 |
| *Fusobacteriaceae* | 0.00 | 0.00 | 1.80 | 1.06 | 0.0059 | 0.0408 |
| *Alcaligenaceae* | 0.89 | 0.34 | 2.76 | 0.73 | 0.032 | 0.1723 |

A comparison between controls and cirrhotic patients' microbial flora was performed using Metastats, and only significantly different and values with around 1% are shown; the rest were nonsignificant. *Q* value indicates that the false discovery rate and that a lower value is generally preferred to avoid a false positive result. There was a significantly higher abundance of *Lachnospiraceae* and *Ruminococceae* in the control group, whereas *Enterobacteriaceae, Fusobacteriaceae, Alcaligenaceae, Lactobacillaceae*, and *Leuconostocaceae* were significantly lower in the controls compared to patients with cirrhosis. Incertae sedis: uncertain placement.

$P = 0.45$), *Veillonellaceae* ($15 \pm 13\%$ vs. $15 \pm 17\%$, $P = 0.8$), *Ruminococcaceae* ($17 \pm 22\%$ vs. $17 \pm 19\%$, $P = 0.6$), *Streptococcaceae* ($4 \pm 10$ vs. $2 \pm 3$, $P = 0.55$), and *Lactobacillaceae* ($2 \pm 1\%$ vs. $1 \pm 1\%$, $P = 0.42$) between the two groups were not statistically significant.

*Cross-sectional analysis within the cirrhosis group.* MELD was not correlated with endotoxin, inflammatory cytokines, or cognition. We also did not find any differences in the inflammatory cytokines or endotoxemia between cirrhotic patients of differing etiologies using ANOVA, possibly attributable to the sample size, dual etiologies, and probable effect of HE overwhelming the underlying etiologies (data not shown). Interestingly, MELD score was positively correlated with *Enterobacteriaceae* ($r = 0.61$, $P = 0.001$) and negatively with *Ruminococcaceae* ($r = -0.38$, $P = 0.05$) with a trend toward lower *Prevotellaceae* ($r = -0.36$, $P = 0.056$). *Enterobacteriacae* were also associated with TNF-$\alpha$ ($r = 0.5$, $P = 0.03$). *Veillonellaceae* and *Fusobacteriaceae* were also associated with worsening inflammation (IL-13, IL-6) and endotoxemia ($P < 0.05$). *Ruminococcaceae*, importantly, were negatively correlated with endotoxemia ($r = -0.5$, $P = 0.02$). The presence of *Alcaligeneceae* and *Porphyromonadaceae* was associated with poor cognition on individual tests (Table 6).

*Multivariate analysis of the HE and no HE groups.* HE patients, as expected had a higher MELD score and bowel movement frequency compared with those without HE (Table 1). The rate of PPI use and other complications of cirrhosis were not different between the groups. Although the major families were present in both sample classes, there were observable abundance differences in some of the taxa; there

was a significantly higher abundance of *Veillonellaceae* in the HE group ($14 \pm 12\%$ vs. $4 \pm 9\%$, $P = 0.046$) compared with the no HE group. There were no significant differences in the other microbiome families between the HE and no HE groups. The multivariate ANOVA performed using *Veillonellaceae*, IL-13, IL-6, MELD score, and endotoxin demonstrated a *P* value of 0.002 using the Lawley-Hotelling test statistic of 2.25672 with an *F* statistic of 5.481.

*Correlation-network analysis.* In patients with HE, in contrast to the multivariate analysis above, several significantly strong correlations were found between features within the HE group with the correlation coefficients (Fig. 2*A*). IL-23 was an important correlate with several bacterial families across different phyla, such as *Leuconostocaceae*, *Eubacteriaceae*, *Erysipelotrichaceae*, *Moraxellaceae*, *Streptophyta*, and *Streptococcaceae* within the HE group. All *P* values for this correlation were below 8.2E-05, indicating a highly robust linkage. The correlation of immune function with bacterial families was further illustrated by the highly significant correlation (*P* values <3.5E-0.5) between inflammatory cytokines IL-2 and IL-13 with *Fusobacteriaceae* and *Prevotellaceae*. The correlation between *Porphyromonadacae* and *Alcaligenacae* with poor performance on cognitive tests was observed in this group accompanied by very significant *P* values ($P < 1E-05$). In patients without HE, in sharp contrast, relatively few correlations that reached the stringent threshold we had set for this analysis were seen in patients without HE and markers of inflammation, cognition, and microbial families. MELD score was negatively correlated with *Ruminococcaceae*, whereas there was positive correlation between *Porphyromonadaceae*

Table 3. *Differences in bacterial abundances between controls and cirrhotic patients with HE*

| Name | Control Mean | Control SE | HE Mean | HE SE | *P* value | *Q* value |
|---|---|---|---|---|---|---|
| *Leuconostocaceae* | 0.00 | 0.00 | 2.19 | 1.08 | 0.0009 | 0.007 |
| Clostridiales_Incertae Sedis XIV | 7.35 | 1.59 | 0.99 | 0.21 | 0.0009 | 0.007 |
| *Ruminococcaceae* | 17.72 | 1.89 | 5.68 | 1.42 | 0.0009 | 0.007 |
| *Lachnospiraceae* | 23.44 | 2.24 | 9.54 | 3.72 | 0.0039 | 0.022 |
| *Enterobacteriaceae* | 0.00 | 0.00 | 10.02 | 4.13 | 0.0049 | 0.026 |
| *Streptococcaceae* | 0.62 | 0.26 | 4.05 | 1.98 | 0.0239 | 0.099 |
| *Fusobacteriaceae* | 0.00 | 0.00 | 1.36 | 0.95 | 0.0369 | 0.146 |
| *Alcaligenaceae* | 0.89 | 0.34 | 2.61 | 0.74 | 0.048 | 0.169 |

This table shows the differences between bacterial abundances in stool of controls with patients with HE; only those bacteria whose abundances were >1% and were significantly different between groups are shown. There was a significantly higher abundance of *Enterobacteriaceae*, *Fusobacteriaceae*, *Leuconostocaceae*, *Streptococcaceae*, and *Alcaligenaceae* in patients with HE, whereas the rest of the bacteria listed were lower in the HE group.

Table 4. *Differences in bacterial abundances between controls and cirrhotic patients without HE*

| | Control Mean | Control SE | No HE Mean | No HE SE | P value | Q value |
|---|---|---|---|---|---|---|
| *Leuconostocaceae* | 0.00 | 0.00 | 1.69 | 0.95 | 0.0001 | 0.001 |
| Clostridiales_Incertae Sedis XIV | 7.35 | 1.59 | 1.29 | 0.35 | 0.0001 | 0.001 |
| *Fusobacteriaceae* | 0.00 | 0.00 | 2.75 | 2.75 | 0.0001 | 0.001 |
| *Lachnospiraceae* | 23.44 | 2.245 | 12.08 | 2.47 | 0.003 | 0.002 |
| *Ruminococcaceae* | 17.72 | 1.89 | 9.04 | 2.62 | 0.019 | 0.008 |

This table shows the differences between bacterial abundances in stool of controls with cirrhotic patients without HE; only those bacteria whose abundances were >1% and were significantly different between groups are shown. There was a significantly higher abundance of *Fusobacteriaceae* and *Leuconostocaceae* in cirrhotic patients without HE, whereas the rest of the bacteria listed were lower in the cirrhotic no HE group.

and *Veillonellaceae* (Fig. 2*B*). We did not find any significant correlations between inflammation and cognitive function that were abundant in the HE group correlation network.

*Prospective study after lactulose withdrawal.* Seven male cirrhotic patients with HE (age 53 ± 8 yr) controlled on lactulose underwent a systematic withdrawal of lactulose. All patients had alcoholic liver disease, while five also had chronic hepatitis C. None of the patients had clinically recurrent HE at *day 14*. A significant (>1%) abundance was present for only 13 taxa at baseline on lactulose in those seven patients. These were mainly from the phylum Bacterioidetes (*Bacteroides* 35%, *Prevotella* 13%, *Hallella* 4%, *Alistipes* 3%, *Parabacteroides* spp. 2%, *Porphyromonadaceae* 1.7%) and Firmicutes (*Faecalibacterium* 6%, *Lachnospira* 5%, *Roseburia* 3%, *Veillonella* 2%, *Dialister* 2%, and *Succinispira* spp. 2%) with little contribution of Proteobacteria (*Alcaligenaceae* 2.6%, *Hafnia* 2%, and *Sutterella* spp. 1%) and none from *Actinobacter* spp. or Fusobacteria. There was <1% abundance on *Lactobacillus* spp., *Clostridium* spp., *Streptococcus* spp., *Shigella* spp., and *Ruminococcus* spp. After lactulose withdrawal, a decrease in *Faecalibacterium* spp. (abundance on lactulose 6% to 1% postwithdrawal, *P* = 0.026) and a trend toward decrease in *Veillonella* spp. (2% to 0%, *P* = 0.07) were seen. No other significant relative abundance change was identified, including *Porphyromonadacae* and *Alcaligenacae*.

**DISCUSSION**

This study demonstrates that a systems biology approach (correlation-network analysis) can be used to identify key

Table 5. *Comparison within the HE group*

| | On Lactulose Alone (n = 11) | On Lactulose and Rifaximin (n = 6) | P value |
|---|---|---|---|
| MELD score | 16.5 ± 7.6 | 18.2 ± 3.3 | 0.53 |
| Venous Ammonia | 52.8 ± 26.3 | 36.3 ± 32.6 | 0.23 |
| Endotoxin | 0.21 ± 0.21 | 0.41 ± 0.26 | 0.13 |
| IL-6, pg/ml | 47.8 ± 56.4 | 108.0 ± 94.3 | 0.20 |
| IL-2, pg/ml | 61 ± 108 | 21.6 ± 22.4 | 0.25 |
| TNF-α, pg/ml | 6.7 ± 4.2 | 7.7 ± 4.22 | 0.67 |
| IL-13, pg/ml | 31.9 ± 84.2 | 32.1 ± 49.7 | 0.99 |
| Number connection-A, s | 55.2 ± 34.1 | 79.4 ± 49.9 | 0.27 |
| Number connection-B, s | 189 ± 127 | 231 ± 105 | 0.51 |
| Digit symbol score | 36.5 ± 14.0 | 34.8 ± 12.3 | 0.82 |
| Block design, score | 24.9 ± 24.8 | 60.0 ± 53.4 | 0.23 |
| Serial dotting, s | 88.7 ± 29.2 | 124.2 ± 36.5 | 0.10 |
| Line drawing error score | 28.0 ± 18.6 | 43.0 ± 42.8 | 0.49 |
| Line drawing time, s | 106.9 ± 54.5 | 79.4 ± 72.1 | 0.48 |
| ICT targets, % | 90.6 ± 8.64 | 83.7 ± 21.0 | 0.52 |
| ICT lure number | 17.4 ± 10.8 | 19.6 ± 10.6 | 0.71 |

All applicable values are presented as means ± SD. There was no significant difference in any variable tested between patients with HE on lactulose alone compared to those on lactulose and rifaximin. ICT, inhibitory control test.

linkages between the microbiome, inflammatory milieu, endotoxemia, and cognition in patients with HE. The IL-23 system was highly correlated with several bacterial families in patients within the HE group, and there was a direct correlation between cognition, *Porphyromonadaceae*, and *Alcaligeneceae*. We found significant differences between the microbial flora of age-matched healthy controls to the cirrhotic population with a higher degree of difference in patients with HE. The study showed that there was no significant difference in the stool flora between patients with HE on lactulose compared with those additionally on rifaximin. The results also indicate that a systematic withdrawal of lactulose therapy had minimal effect on the gut microflora abundance.

To date, it has been difficult to identify significant differences between control and disease groups using classic multivariate approaches (27). Specifically, the composition of the human gut microbiome has been shown to vary significantly between individuals, and this is a fundamental problem in associating the microbiome with diseases (12). Furthermore, most microbial abundance matrices derived from sequence data are both sparse and nonparametric. A microbial ecological interpretation of these issues is that different phylogenetic taxa play the same functional role in the complex nonlinear interactions between the human host and gut microbiome. It should be noted that these interactions are not static but form a nonlinear complex dynamic network that further confounds classic multivariate analysis methods. Unlike these methods, correlation network analysis allows the interrogation of these nonlinear dynamics to correlate phylogenetically defined taxa with function and disease phenotype. This was leveraged in our study where we found that HE was significantly correlated with microbiome components and inflammatory cytokines.

We found a significant difference in the bacterial composition of patients with cirrhosis compared with healthy controls that intensified when the cirrhotic group was divided into HE and those without HE. *Ruminococcaceae* and Clostridium incertae sedis XIV were overrepresented in controls similar to prior studies in inflammatory bowel disease and cirrhosis (7, 18, 19, 25, 46). The findings are also similar to those published by Chen et al. (7) in cirrhotic patients despite differences in cirrhosis etiology and diet (7). However, their study did not evaluate HE specifically, and they did not perform a systems biology analysis.

*Alcaligenecaeae* and *Enterobacteriaceae* were among the bacterial taxa that were differentially detected in cirrhotics with HE compared with controls but not different between controls and cirrhotics without HE. Increased *Alcaligenaceae* abundance was significantly associated with poor cognitive performance, whereas *Enterobacteriaceae* were associated with

Table 6. *Correlation between poor cognitive performance and presence of Alcaligeneceae and Porphyromonadaceae in the entire group*

| Cognitive Tests | Alcaligeneceae | | Porphyromonadaceae | |
|---|---|---|---|---|
| | R | P value | R | P value |
| Number connection-A, s | **0.68** | **0.001** | **0.63** | **0.002** |
| Number connection-B, s | **0.445** | **0.04** | 0.28 | 0.24 |
| Serial dotting, s | **0.52** | **0.018** | **0.41** | **0.05** |
| Line drawing error number | **0.58** | **0.009** | **0.59** | **0.008** |
| Line drawing time, s | 0.24 | 0.31 | 0.17 | 0.48 |
| ICT lure number | 0.26 | 0.26 | 0.29 | 0.19 |
| Digit symbol score | **−0.63** | **0.003** | **−0.46** | **0.04** |
| Block design score | −0.27 | 0.25 | −0.21 | 0.37 |
| ICT targets, % | **−0.51** | **0.019** | **−0.57** | **0.007** |

A high score on digit symbol, block design, and ICT targets indicates good cognition (i.e., lower value indicates poor cognition); for the rest of the tests, a high score indicates poor cognitive performance. Significant correlations are in bold text. Therefore, we found a significant correlation between impairment on most cognitive tests and relative abundance of *Alcaligeneceae* and *Porphyromonadaceae*.

worsening inflammation and MELD score in the cirrhosis group. The correlation between the MELD score, HE, and *Enterobacteriaceae* is not surprising because these bacteria are responsible for most of the life-threatening infections associated with advanced cirrhosis (35, 37). Also, the negative correlation of *Ruminococcaceae* with endotoxemia and MELD score and reduction in this class in cirrhotics overall could indicate a protective role.

The striking finding was the direct correlation between specific bacterial taxa and cognitive function. To our knowledge bacterial taxa have not been previously related to cognitive and inflammatory markers in cirrhosis using culture-independent techniques. *Porphyromonadaceae* and *Alcaligeneceae* were associated with poor cognition in almost all tests (Table 6). It is unlikely that this is merely a manifestation of worsening liver disease because the MELD score was not significantly correlated either with cognitive performance or with these bacteria. *Alcaligeneceae* are Proteobacteria that are typically associated with opportunistic infections; interestingly they degrade urea to produce ammonia, which may explain part of this association (28). Porphyromonas are gram-negative anaerobes, whose fecal presence may be attributed to the deficient stomach acid and bile barrier function in cirrhosis (6, 33, 40). Interestingly in animal studies, gut microbial colonization with specific bacteria has been shown to influence neuronal circuitry involved in motor control and behavior (9, 15). The correlation of these bacterial families with cognition in humans is a novel finding that needs further study.

We confirmed the proinflammatory milieu and endotoxemia in patients with HE (36) and further demonstrated that specific microbial families, *Enterobacteriacae*, *Veillonellaceae*, and *Fusobacteriaceae*, were associated with inflammation (44). Specifically, in patients with HE, inflammatory markers IL-23, IL-1b, IL-2, IL-4, and IL-13 were highly correlated with gut microbiome components, possibly indicating a synergy between inflammation and cognition with microbiome changes (20, 26). It is interesting that IL-13, which, in addition to being an inflammatory mediator with IL-4, also mediates allergic reactions, would be increased in cirrhotic patients with HE. Although none of our patients had an allergic diatheses, the increased IL-13 concentration may also represent its profibrotic potential and the widespread immunomodulatory disturbances that are prevalent in cirrhosis (24). We did not find a difference in the inflammatory cytokines across etiologies, which is likely
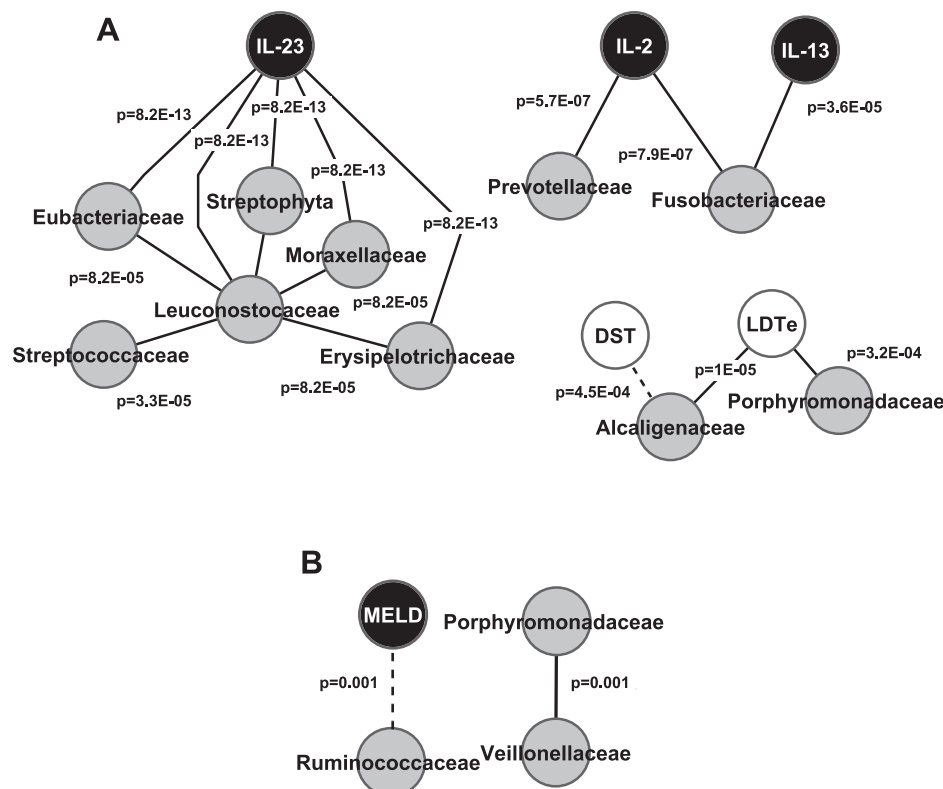


Fig. 2. Correlation-network analysis of cirrhotic patients with and without hepatic encephalopathy (HE). Only correlations with a coefficient >r = 0.90 are displayed. Gray nodes indicate microbiome families; white nodes indicate cognitive tests; and black nodes are serum inflammatory markers. A black line connecting nodes indicates positive correlation, and a dashed line indicates negative correlation >0.90. The P values for the correlations are displayed on the lines connecting the nodes in A and B. MELD, model for end-stage liver disease score; DST, digit symbol test; LDTe, line drawing test errors. A: patients with HE (n = 17) have a high number of significant correlations. There are significant positive correlations between IL-23 and several bacterial families. *Prevotellaceae* and *Fusobacteriaceae* are positively correlated with inflammation. Because a low score on DST and high one on LDTe indicate poor performance, *Alcaligenaceae* and *Porphyromonadaceae* were correlated with poor cognition. The P values for all these correlations are less than the 4th decimal place, indicating a very high significance. B: patients without HE have very few significant correlations (n = 8). There was a significant negative correlation between MELD score and *Ruminococcaceae* and a positive correlation between *Veillonellaceae* and *Porphyromonadaceae*.

attributable to the limited sample size and patients with dual etiologies. The IL-23/IL-17 pathway is triggered by exposure to infectious agents in the intestine, which releases a cascade of proinflammatory cytokines (10). IL-23 functions as a stimulant of IL-17 production, and its role in inflammatory bowel disease has been well described (1, 16). The correlation between IL-23 and several bacterial families indicates that IL-23/IL-17 cytokine pathway may be an important mechanism behind intestinal inflammation in HE and cirrhosis.

Strengthening these correlations was the minimal effect that lactulose withdrawal had on the stool flora composition after 14 days; this replicates prior nonculture-based experience with lactulose in healthy individuals (39). We did not replicate prior culture-based studies in which lactulose therapy resulted in with higher lactobacillus or reduction in *Escherichia coli* and *Staphyloccoci* after symbiotic supplementation (23, 31). Our results are probably different because of the increased depth of the interrogation of the microbial community by MTPS rather than culture methodology. It is, however, possible that lactulose may act through change in bacterial functionality rather than change in abundances, which were measured in this study. These results suggest that these microbial abundances are reflective of HE and cirrhosis rather than just lactulose therapy.

The correlation-network model only indicates the coordinate changes in the features without implying causality, which is a limitation of this and all cross-sectional studies. The study is also limited by the small sample size; however, the correlations seen in the analysis were highly significant ($P$ values $<0.001$). Network modeling indicates that robust analysis can be obtained with small sample sizes if the correlations are high as reflected in the high $P$ values for the correlations in this study. In addition, age, socioeconomic status, medication use, cirrhosis complications, recent alcohol use, and dietary habits were similar between the groups. The current sample size did not allow for etiology-based differentiation in patients with cirrhosis, including inflammatory cytokines, and further research needs to be performed to elucidate those differences. We also could not study the effect of PPIs because most cirrhotic patients seen in our clinic are on PPI therapy, which could limit the generalizability of these results. This study was designed as a proof-of-concept experiment for generation of hypotheses for further definitive studies linking the cognitive impairment in cirrhosis with inflammation and the gut microbiome.

Collectively our data indicate that the gut microbiome components are significantly different between healthy controls and cirrhotic patients, especially those with HE, and are associated with cognition in cirrhosis. Additionally, markers of the Th17 and innate immune response were associated with *Alcaligeneceae*, *Porphyromonadaceae*, and *Enterobacteriaceae* in patients with HE. The IL-17/IL-23 pathway forms an important inflammatory link in this association. We anticipate that these findings could be a starting point for designing novel hypothesis-driven research and therapies such as targeted prebiotics and probiotics aimed at enhancing cognition through modulation of these microbiome components.

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