

Dysbiosis of the Duodenal Mucosal Microbiota Is Associated With Increased Small Intestinal Permeability in Chronic Liver Disease

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OBJECTIVES: Chronic liver disease (CLD) is associated with both alterations of the stool microbiota and increased small intestinal permeability. However, little is known about the role of the small intestinal mucosa-associated microbiota (MAM) in CLD. The aim of this study was to evaluate the relationship between the duodenal MAM and both small intestinal permeability and liver disease severity in CLD.

METHODS: Subjects with CLD and a disease-free control group undergoing routine endoscopy underwent duodenal biopsy to assess duodenal MAM by 16S rRNA gene sequencing. Small intestinal permeability was assessed by a dual sugar (lactulose: rhamnose) assay. Other assessments included transient elastography, endotoxemia, serum markers of hepatic inflammation, dietary intake, and anthropometric measurements.

RESULTS: Forty-six subjects (35 with CLD and 11 controls) were assessed. In subjects with CLD, the composition ($P = 0.02$) and diversity ($P < 0.01$) of the duodenal MAM differed to controls. Constrained multivariate analysis and linear discriminate effect size showed this was due to *Streptococcus*-affiliated lineages. Small intestinal permeability was significantly higher in CLD subjects compared to controls. In CLD, there were inverse correlations between microbial diversity and both increased small intestinal permeability ($r = -0.41$, $P = 0.02$) and serum alanine aminotransferase ($r = -0.35$, $P = 0.04$). Hepatic stiffness was not associated with the MAM.

DISCUSSION: In CLD, there is dysbiosis of the duodenal MAM and an inverse correlation between microbial diversity and small intestinal permeability.

TRANSLATIONAL IMPACT: Strategies to ameliorate duodenal MAM dysbiosis may ameliorate intestinal barrier dysfunction and liver injury in CLD.

SUPPLEMENTARY MATERIAL accompanies this paper at <http://links.lww.com/CTG/A68>, <http://links.lww.com/CTG/A69>, <http://links.lww.com/CTG/A70>, <http://links.lww.com/CTG/A71>, <http://links.lww.com/CTG/A72>, <http://links.lww.com/CTG/A73>, <http://links.lww.com/CTG/A74>, <http://links.lww.com/CTG/A75>, <http://links.lww.com/CTG/A76>, <http://links.lww.com/CTG/A77>, <http://links.lww.com/CTG/A78>, <http://links.lww.com/CTG/A79>, <http://links.lww.com/CTG/A80>, <http://links.lww.com/CTG/A81>, <http://links.lww.com/CTG/A82>, and <http://links.lww.com/CTG/A83>

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INTRODUCTION

In chronic liver disease (CLD), liver injury results in progressive fibrosis, culminating in cirrhosis, liver failure, and associated complications. Both the gut microbiota (1–4) and intestinal barrier dysfunction (1) are implicated in the pathogenesis of liver injury. Dysbiosis of the stool microbiota has been reported in CLD (5–9) with a reduction in the abundance of commensal microbiota and a relative increase in potentially pathogenic ones (10). This may in part be due to invasion of the distal gut by “oral type” microbiota (5). The mechanisms by which gut microbial dysbiosis contribute to liver disease in humans are not clear, but it is thought that an increase in intestinal permeability (11,12), leading to the translocation of microbial products, plays a major role.

Although the prevalence of small intestinal bacterial overgrowth is increased in CLD (13), very little is known about the small intestinal mucosal microbiota. Most studies have examined stool microbiota (5–7,14), which are not necessarily representative of small intestinal mucosa-associated microbiota (MAM) due to regional variation in the bacterial populations throughout the gut (15). In animal models, mucosal bacteria are more likely to translocate across the gut wall compared to luminal bacteria (16). The MAM from the proximal small intestine (SI) drains into the portal vein and are well placed to exert an influence on the liver. Emerging data suggest that there may be dysbiosis of the duodenal MAM in cirrhosis (17), although how this relates to the pathogenesis of cirrhosis is unclear. Additionally, the associations between small intestinal dysbiosis, small intestinal permeability, and liver injury in humans have not been examined in detail.

The aims of this study were to characterize the duodenal MAM in patients with CLD in comparison to a well-characterized, disease-free control group, and to examine relationships with both small intestinal permeability and liver disease severity. We hypothesized that CLD would have dysbiosis of the duodenal MAM, which would be associated with increased small intestinal permeability, serum alanine aminotransferase (ALT), and hepatic fibrosis.

MATERIALS AND METHODS

Subject recruitment

Subjects with CLD and a healthy control group, both who underwent routine upper endoscopy, were included. Indications for endoscopy were iron deficiency, surveillance of noninflamed Barrett’s esophagus (both cohorts), or variceal surveillance (CLD subjects only). Subjects were recruited over a 24-month period from a tertiary care liver center (Princess Alexandra Hospital, Brisbane, Australia). CLD was defined as liver injury for greater than 6 months, as determined by clinical history, liver biochemistry, imaging, and/or histology. Etiologies of CLD were hepatitis C virus (HCV) and hepatitis B virus (HBV), non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease (ALD), or autoimmune liver disease. Informed consent was obtained from all participants and ethical clearance was granted by the Metro South Human Research Ethics Committee (HREC/12/QPAH/083) and University of Queensland Medical Research Ethics Committee (Approval number 2013000800) before commencement. The study was performed in accordance with the principles of the Declaration of Helsinki.

Strict exclusion criteria were applied to minimize confounding factors. Exclusion criteria were as follows: A documented history

of gastrointestinal disease (other than noninflamed Barrett’s esophagus) including, where available, endoscopic evidence of esophagitis, gastritis, duodenitis, peptic ulcer disease, coeliac disease, or inflammatory bowel disease; or any of the following within 6 weeks of the study: symptoms of abdominal pain, diarrhea, vomiting; or use of aspirin, nonsteroidal anti-inflammatory drugs, proton pump inhibitors, bile acids or bile acid sequestrants, oral iron therapy, antibiotics, prebiotics or probiotics; or significant alcohol intake. Specifically, subjects with ALD were not drinking for 6 weeks before the study. For the control group, subjects with the metabolic syndrome (MetS, diagnosed by the IDF/AHA/NHLBI 2009 consensus criteria (18)) were excluded, whereas for the CLD group, subjects with ascites were excluded.

Daily macronutrient intake, anthropometric measurements, assessment of hepatic fibrosis, peripheral endotoxemia, and standard blood markers of liver enzymes, fasting blood glucose, insulin, and lipid profiles were measured as outlined in Supplementary Methods (see Supplementary Digital Content 1, <http://links.lww.com/CTG/A68>). Small intestinal permeability was assessed using a dual lactulose: rhamnose (L:R) assay, described in detail in Supplementary Methods (see Supplementary Digital Content 1, <http://links.lww.com/CTG/A68>).

Assessment of duodenal mucosa-associated microbiota

During endoscopy, a 2 × 1 mm specimen of mucosal tissue was collected from the second part of the duodenum opposite the ampulla of Vater using standard biopsy forceps. To minimize oropharyngeal contamination, the endoscope was advanced straight to the duodenum, and mucosal samples were obtained before suction of oropharyngeal or gastric contents. From the duodenal tissue, microbiota profiles were generated for each patient. DNA was extracted from the mucosal biopsies and amplified using barcoded primers targeting the V6–V8 region of the 16S ribosomal ribonucleic acid (rRNA) gene. Resultant libraries were sequenced by MiSeq and bioinformatics analysis performed using Quantitative Insights Into Microbial Ecology. Full details and primers are described in Supplementary Methods (see Supplementary Digital Content 1, <http://links.lww.com/CTG/A68>).

Statistical analysis

Normally distributed variables were presented as mean and standard deviation, and analyzed with Student’s *t* test or analysis of variance (with Tukey’s multiple comparison). L:R ratios were log transformed to normalize their distribution. Liver stiffness measurements were analyzed as a continuous score. Non-normally distributed variables were presented as median and interquartile range, and analyzed with the Mann-Whitney *U*, rank test, or Kruskal-Wallis tests; or Spearman’s rho for correlations. Statistical significance was defined as *P* < 0.05. Statistical analyses were performed using IBM Statistical Package for Social Sciences version 22, 2013.

Microbiota data were analyzed using Calypso web-based program for microbial ecology, Calypso v8.2 (cgenome.net/calypso) (19), as outlined in the Supplementary Methods (see Supplementary Digital Content 1, <http://links.lww.com/CTG/A68>). In brief, alpha diversity was assessed using the Shannon index, and differences in the relative abundance of taxa were analyzed by both differential expression analysis based on the negative binomial distribution and analysis of composition of

microbiomes (ANCOM) statistical tests. Analyses were adjusted for multiple comparisons testing. For both differential expression analysis based on the negative binomial distribution and ANCOM analyses, taxa with a $P < 0.05$ with a false discovery rate q value < 0.05 were highlighted as significant. Beta diversity was assessed by principal co-ordinate analysis (PCoA), using the Bray-Curtis distance metric and adonis non-parametric statistic. To assess the overall contribution of taxa to the patient cohorts, linear discriminant analysis effect size and the constrained multivariate mixOmics method, sparse partial least squares discriminant analysis (20) were used. Predicted function of the genome based on the 16S rRNA sequence data was determined using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (<http://picrust.github.io/picrust>) (21).

RESULTS

Study population characteristics

A total of 46 subjects (35 with CLD and 11 controls) met inclusion and exclusion criteria and had duodenal MAM profiles available for analysis. When comparing CLD and control subjects, age, gender, body mass index, and dietary intake of major macronutrients (fat, protein, and carbohydrate) were similar (Table 1). As expected, CLD subjects had a lower platelet count and serum albumin concentration. Cirrhosis were present in 23 (65%) CLD subjects, 18 (78%) of whom had Child's A cirrhosis. The MetS (an exclusion criteria for controls) was present in 17 (46%) of CLD subjects. Among the different etiologies of CLD, 15 patients had HCV (all HCV RNA positive), 9 NAFLD, 7 ALD, 3 autoimmune liver disease, and 1 patient had HBV. When comparing the 3 most prevalent etiologies (HCV, NAFLD, and ALD), HCV subjects had higher serum ALT, but other baseline characteristics were similar (Table 1). All except for 2 subjects in the entire cohort were Europids (Australian or European ethnicity).

Small intestinal permeability is higher in CLD

Small intestinal permeability was significantly higher in CLD subjects compared to controls (mean \pm SD log [L:R \times 100] 1.41 ± 0.37 vs 1.09 ± 0.26 , $P < 0.05$, Table 1). Among CLD subjects, permeability was highest in those with HCV. Endotoxemia was present in 5 subjects, all with CLD. For these subjects, intestinal permeability did not differ when compared to nonendotoxemic CLD subjects (log [L:R \times 100] 1.32 ± 0.40 vs 1.42 ± 0.34 , $P = 0.6$), or controls ($P = 0.29$).

Duodenal MAM composition

Altogether, 109 different operational taxonomic units were identified in the duodenal MAM (at 97% sequence identity threshold) derived from 5 bacterial phyla and differentiated into 9 classes, 11 orders, 20 families, and 25 genera (see Supplementary Table 1, Supplementary Digital Content 2, <http://links.lww.com/CTG/A69>). The average number of quality reads was 13,204 per CLD subject, and 14,480 per control subject.

Overall, Firmicutes and Bacteroidetes were the most abundant phyla, together accounting for 85% of detected bacterial operational taxonomic units. The most abundant genera were *Streptococcus*, followed by *Prevotella*, *Veillonella*, and *Actinomyces* (see Supplementary Table 2, Supplementary Digital Content 3, <http://links.lww.com/CTG/A70>). Rarefaction analysis of the cumulative

sum-scaled data showed that sufficient coverage of the bacterial diversity present in all samples was achieved (see Supplementary Figure 1, Supplementary Digital Content 4, <http://links.lww.com/CTG/A71>).

Both composition and diversity of the duodenal MAM differ between CLD and controls

The duodenal bacterial community in CLD differed to that of healthy controls on PCoA (see Supplementary Figure 2, Supplementary Digital Content 5, <http://links.lww.com/CTG/A72>). Using both a constrained multivariate model (Figure 1a) and linear discriminant analysis effect size (Figure 1b), it was possible to identify those taxa that were most discriminatory of CLD and control subjects. Both these analyses indicated that the MAM of CLD subjects was characterized by *Streptococcus*-affiliated lineages, whereas the control subjects were shaped by smaller contributions from a variety of taxa (*Neisseria*, *Haemophilus*, *Porphyromonas*, *Veillonella*, *Moryella*, and *Prevotella* spp.). The duodenal MAM of CLD subjects also exhibited less alpha diversity than controls (Shannon index, Figure 1c).

Significant differences in the relative abundance of particular taxa were observed between CLD subjects and controls (Figure 2). At the phylum level, CLD subjects had a greater abundance of Firmicutes and lower abundance of Actinobacteria (Figure 2a and see Supplementary Table 2A, Supplementary Digital Content 3, <http://links.lww.com/CTG/A70>). Decreases in the abundance of the genera *Moryella*, *Porphyromonas*, and *Veillonella* (Figure 2b, and Supplementary Table 2B, Supplementary Digital Content 3, <http://links.lww.com/CTG/A70>) and *Actinomyces*-affiliated taxa (see Supplementary Table 2C, Supplementary Digital Content 3, <http://links.lww.com/CTG/A70>) were observed in CLD subjects. These differences were also validated by the ANCOM statistical test, which showed the CLD subjects had a reduction in the relative abundance of *Moryella*, *Porphyromonas*, and *Actinomyces* (see Supplementary Tables 2D and E, Supplementary Digital Content 3, <http://links.lww.com/CTG/A70>), and some *Veillonella*-affiliated taxa (see Supplementary Table 2F, Supplementary Digital Content 3, <http://links.lww.com/CTG/A70>).

Microbial composition or diversity did not differ by sex when examined across all participants ($P = 0.5$ and $P = 0.4$ respectively, see Supplementary Figure 3, Supplementary Digital Content 6, <http://links.lww.com/CTG/A73>), or when this was confined to CLD subjects ($P = 0.3$ and $P = 0.5$ respectively, data not shown).

Functional properties of the microbiota distinguish CLD and control subjects

PICRUSt analysis, to infer functional properties of the microbiota, indicated that the microbial communities of the CLD subjects possessed a greater abundance of pathways related to sugar and carbohydrate metabolism, glycan degradation, and bacterial toxin production. Control subjects had a greater abundance of lipopolysaccharide biosynthesis and amino acid-related pathways (see Supplementary Table 3, Supplementary Digital Content 7, <http://links.lww.com/CTG/A74>, and Supplementary Figure 4, Supplementary Digital Content 8, <http://links.lww.com/CTG/A75>).

Table 1. Characteristics of CLD and control subjects

Variable	Control	CLD	P (control vs CLD)	CLD etiologies ^a			P (between CLD etiologies)
				HCV	NAFLD	ALD	
Number: (%) or median (IQR)	n = 11	n = 35		n = 15	n = 9	n = 7	
Demographic							
Age (yrs)	50 (43–64)	56 (50–64)	NS	52 (49–57)	64 (54–70)	60 (52–67)	NS
Male sex: n (%)	5 (45%)	26 (74%)	NS	13 (87%)	5 (55%)	5 (71%)	NS
Metabolic factors							
BMI (kg/m ²)	25.9 (22.1–31.7)	28 (25.2–29.9)	NS	27.4 (25.3–28.2)	32.2 (28.5–36.7)	25.3 (24.3–26.8)	NS
MetS ^b : n (%)	0 (0%)	16 (46%)	—	9 (60%)	5 (56%)	1 (14%)	NS
Dietary intake (% of EI)	n = 11	n = 27		n = 12	n = 6	n = 5	
Fat	37.3 ± 4.2	40.6 ± 6.0	NS	41.0 ± 3.4	39.2 ± 6.6	44.8 ± 4.2	NS
Carbohydrate	40.4 ± 5.4	39.5 ± 7.1	NS	37.9 ± 4.9	40.6 ± 9.2	37.3 ± 5.9	NS
Protein	23.0 ± 4.3	21.6 ± 4.3	NS	21.9 ± 2.7	21.8 ± 4.1	23.9 ± 7.9	NS
Liver-related							
LSM (kPa)	4.9 (3.5–5.4)	15.7 (7.2–27.3)	**	14.9 (7.0–20.9)	28.0 (8.9–34.0)	19.7 (10.1–43.7)	NS
ALT (U/L)	26 (15–45)	36 (29–78)	NS	80 (56–120)	29 (25–32)	30 (21–30)	**
Albumin (g/L)	42 (41–43)	39 (35–40)	**	39 (38–40)	34 (29–38)	39 (35–42)	NS
Platelet count (×10 ⁹)	225 (217–332)	177 (129–198)	**	181 (166–191)	123 (90–238)	164 (135–211)	NS
Cirrhosis: n (%)	NA	23 (65%)	—	10 (67%)	7 (78%)	5 (71%)	NS
Child A (% of cirrhosis)		18 (78%)	—	9 (90%)	3 (43%)	5 (100%)	NS
Child B		5 (22%)	—	1 (10%)	4 (57%)	0 (0%)	NS
Child C		0 (0%)	—	0 (0%)	0 (0%)	0 (0%)	NS
MELD score (cirrhotics only)	NA	8.6 (7.7–10.6)	—	8.5 (7.5–10.1)	11.0 (10.3–15.8)	9.0 (8.5–9.2)	NS
SI permeability and endotoxemia							
Log (L:R × 100)	1.09 ± 0.26	1.41 ± 0.37	*	1.57 ± 0.27	1.41 ± 0.40	1.12 ± 0.31	*
Endotoxemia (EU/mL)	0	5 (14%)	NS	4	0	1	NS

P value calculated using Fisher's exact test for comparisons between proportions, Mann-Whitney U and Kruskal-Wallis for comparisons between medians, and Student's *t* test for comparison of means. NS, not significant; **P* < 0.05; ***P* < 0.01.

ALD, alcoholic liver disease; ALT, alanine aminotransferase; BMI, body mass index; CLD, chronic liver disease; EI, energy intake; EU, endotoxin units; HBV, hepatitis B virus; HCV, hepatitis C virus; IQR, inter-quartile range; L:R, lactulose: rhamnose; LSM, liver stiffness measurement; MELD, model of end stage liver disease; MetS, Metabolic syndrome; NAFLD, non-alcoholic fatty liver disease; NS, not significant.

^aHBV and autoimmune liver disease are not included in subgroup analysis etiology as numbers are too small.

^bMetS was an exclusionary criteria for controls.

Lower microbial diversity correlates with increased small intestinal permeability and serum ALT in CLD

Among the CLD subjects, microbial diversity inversely correlated with both small intestinal permeability ($r = -0.41$, $P = 0.02$, Figure 3a), and with serum ALT ($r = -0.35$, $P = 0.04$, Figure 3b). Among those genera that had lower abundance in CLD, the abundance of *Porphyromonas* inversely correlated with increased small intestinal permeability (see Supplementary Table 4, Supplementary Digital Content 9, <http://links.lww.com/CTG/A76>).

There were no associations observed between the duodenal MAM and hepatic stiffness for either microbial diversity

($r = 0.16$, $P = 0.4$, see Supplementary Figure 5, Supplementary Digital Content 10, <http://links.lww.com/CTG/A77>) or the relative abundance of any taxa (see Supplementary Table 5, Supplementary Digital Content 11, <http://links.lww.com/CTG/A78>). There were no overall differences in community composition between CLD subjects with and without cirrhosis (see Supplementary Figure 6A, Supplementary Digital Content 12, <http://links.lww.com/CTG/A79>), including those with compensated and decompensated cirrhosis (see Supplementary Figure 6B, Supplementary Digital Content 12, <http://links.lww.com/CTG/A79>). In addition, the duodenal MAM was not significantly

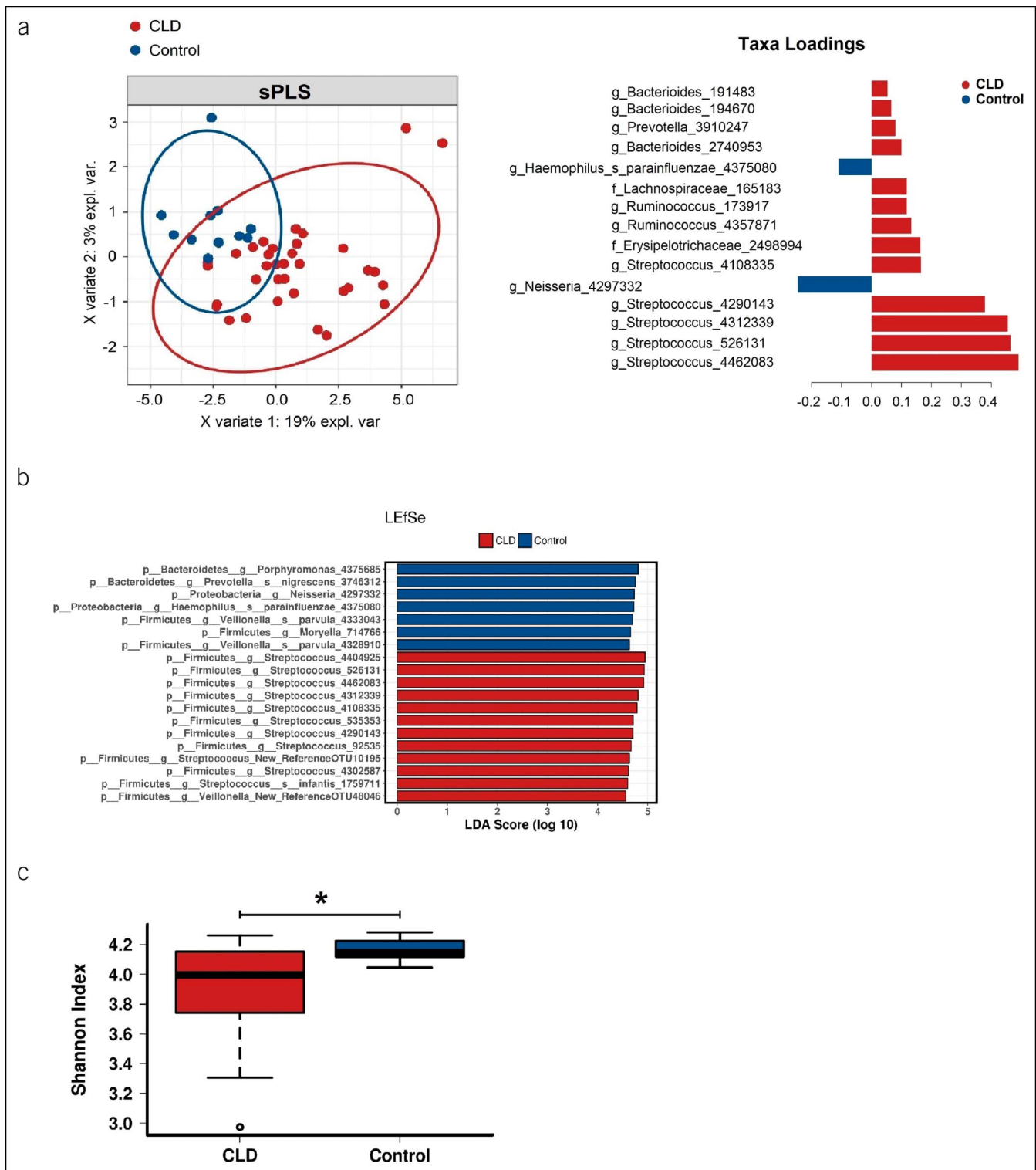


Figure 1. Multivariate analyses and diversity of microbial community composition. **(a)** sPLS-DA showing the effect size of OTUs contributing to the differences between CLD and controls. **(b)** LEfSe plot showing LDA scores for OTUs that differentiated CLD from controls. **(c)** Alpha diversity in CLD MAM compared to controls. Plot shows median Shannon index and IQR, * $P < 0.01$, Mann Whitney U test. CLD, chronic liver disease; expl. var, explained variance; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; MAM, mucosa-associated microbiota; OTU, operational taxonomic unit; PCoA, principal coordinate analysis; sPLS-DA, sparse partial least squares discriminant analysis.

different between CLD subjects with and without the MetS (see Supplementary Figure 7, Supplementary Digital Content 13, <http://links.lww.com/CTG/A80>). We were unable to identify

any associations between dietary macronutrient intake and abundance of any microbial taxa ($P > 0.05$ or false discovery rate q value > 0.1 , data not shown).

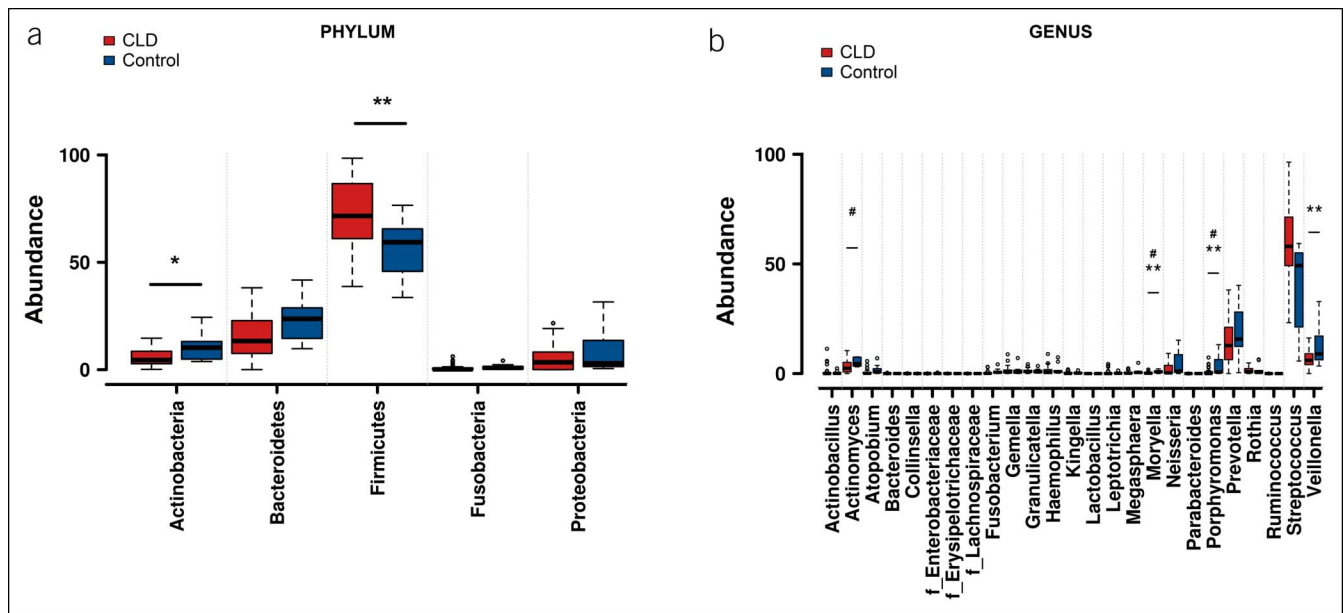


Figure 2. Abundance of microbial taxa in CLD and control subjects at the phylum (a) and genus (b) level. Box plots represent median (IQR) relative abundance. * $P < 0.05$ and FDR $q < 0.1$ on DESeq2. ** $P < 0.01$ and FDR $q < 0.05$ on DESeq2. #Significantly different on ANCOM. ANCOM, analysis of composition of microbiomes; CLD, chronic liver disease; DESeq2, Differential gene expression analysis based on the negative binomial distribution; FDR q , False discovery rate q value; IQR, interquartile range.

Etiologies of CLD

Among the 3 main etiologies of CLD, the alpha diversity for both the HCV and NAFLD subjects was significantly less than the control group ($P < 0.01$ and $P < 0.05$ respectively, see Supplementary Figure 8A, Supplementary Digital Content 14, <http://links.lww.com/CTG/A81>). Notably, the duodenal MAM of the HCV group was the least diverse, whereas this group also had the highest intestinal permeability and serum ALT. On PCoA, the microbiota profiles of HCV and NAFLD, but not ALD, were different to the control subjects ($P < 0.01$ and $P < 0.05$, respectively; see Supplementary Figure 8B, Supplementary Digital Content 14, <http://links.lww.com/CTG/A81>); however, CLD etiologies did not differ to each other ($P = 0.4$). HCV RNA viral loads were not associated with taxa abundance (see Supplementary Table 6, Supplementary Digital Content 15, <http://links.lww.com/CTG/A82>), microbial diversity (see Supplementary Figure 9A, Supplementary Digital Content 16, <http://links.lww.com/CTG/A83>), or small intestinal permeability (see Supplementary Figure 9B, Supplementary Digital Content 16, <http://links.lww.com/CTG/A83>).

DISCUSSION

To date, most of the evidence for intestinal microbial dysbiosis in CLD is based on the stool samples in cirrhotic patients. We have demonstrated that the duodenal mucosal microbiota of CLD subjects is not only different to controls, but this dysbiosis is associated with increased small intestinal permeability. The CLD MAM was less diverse overall, and specifically had a reduction in the abundance of *Moryella*, *Porphyromonas*, and *Veillonella* and *Actinomyces* taxa. Certain *Streptococcus*-affiliated lineages were increased in CLD, and these largely accounted for the differences in separation between the duodenal MAM communities of CLD and control subjects. In CLD, lower microbial diversity correlated

with both increased small intestinal permeability, and serum ALT, suggesting an association with gut barrier dysfunction and hepatic inflammation.

Previous studies demonstrate that the stool, distal colonic mucosal, and salivary microbiomes of cirrhotic subjects have different profiles (10,22), but also share some common disease-associate signatures—primarily overabundance of potentially pathogenic endotoxin-producing *Enterobacteriaceae* (7,10,22), *Enterococcaceae* (10,22), and *Streptococcaceae* (5,7) as well as lower abundance of the autochthonous taxa *Ruminococcaceae* and *Lachnospiraceae* (7,10,22). *Streptococcaceae* in particular are a key component of the oral microbiome (10), and Qin et al. (5) also reported an overrepresentation of *Streptococci* in the stool of cirrhotic subjects compared to healthy controls, inferring that oral commensals appear to “invade” the gut in cirrhosis. Our observation that Streptococcal-affiliated lineages were largely contributory to the distinct CLD MAM supports this notion. Qin et al. (5) also showed an overall reduction in microbial gene richness of stool microbiota in cirrhotic subjects, in line with our findings of overall decreased diversity and abundance of *Moryella*, *Porphyromonas*, and *Veillonella* and *Actinomyces* taxa in CLD.

Duodenal dysbiosis in CLD could occur as a result of disturbances in the small intestinal niche caused by alterations in bile acid flow (23) and motility (24). The number of human studies in CLD that focus specifically on the duodenal MAM is very limited. Chen et al. (17) observed dysbiosis in a study of duodenal MAM from cirrhotic subjects with chronic HBV and primary biliary cholangitis. Taxonomic differences in the CLD subjects between their study and ours are likely explained by demographic differences between the study populations, and variations based on the 16S rRNA gene variable region selected for amplification. In our study, HCV subjects had the most pronounced dysbiosis. This may be linked to overall disease severity, with the higher ALT levels implying a greater degree of hepatic inflammation.

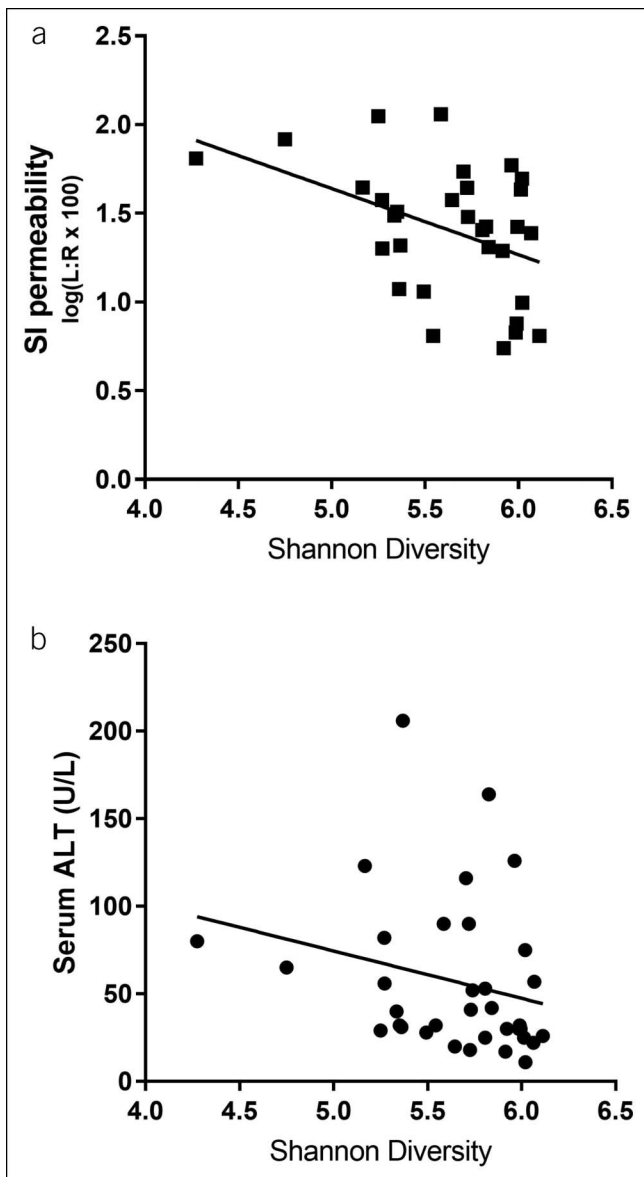


Figure 3. Correlations between microbial diversity with small intestinal permeability and serum ALT in CLD subjects. (a) Alpha diversity and small intestinal permeability, Spearman $r = -0.41$, $P = 0.02$. (b) Alpha diversity and serum ALT, Spearman $r = -0.35$, $P = 0.04$. ALT, alanine aminotransferase; CLD, chronic liver disease; L:R, lactulose: rhamnose ratio; SI, small intestine.

Although the virus itself may influence B and T lymphocyte function (25,26), there was no association between HCV viral load and duodenal MAM or permeability.

Increased permeability of the SI renders the liver susceptible to the effects of the intestinal microbiota or their products. We demonstrated that reduced microbial diversity was associated with increased small intestinal permeability and hepatic inflammation (based on the serum ALT). Collectively, these data suggest that enriched microbial diversity in the SI may be a protective factor, and loss of this may compromise intestinal homeostasis and integrity. Notably, endotoxemia, which is a recognized feature of advanced liver disease, was not associated with small intestinal permeability. This may be explained by the

low overall abundance of Gram-negative bacilli in the duodenal MAM. It is likely that a variety of microbial metabolites in addition to endotoxin may be important in disease processes in CLD (27) and that endotoxemia is not necessarily driven by the small intestinal microbiota.

The duodenal MAM of CLD subjects displayed a greater abundance of pathways related to carbohydrate metabolism and glycan degradation. Dietary carbohydrate intake was similar between CLD and controls, implicating an alternative carbohydrate source such as gut mucins is being metabolized by these bacteria. Primary biliary cholangitis spp., which shaped the CLD MAM, are highly functioning in the SI and are dependent on their capacity to utilize carbohydrates, for which they compete with the host (28). Gut mucins are a key factor in maintaining intestinal barrier function (29), and primary biliary cholangitis spp. produce mucin-degrading enzymes (30). Although we did not find a correlation between the abundance of primary biliary cholangitis and small intestinal permeability, more robust metabolomics and metabolomics analyses are required to interrogate this relationship more fully.

Although there was a clear association between microbial diversity and serum ALT, there was no association between liver stiffness and the microbiota. This may be a limitation of the small sample size, particularly among noncirrhotic subjects, and the heterogeneity of the population. As our primary aim was to evaluate the microbiota in CLD compared to non-CLD subjects, there may have been insufficient power for detailed analysis by grouped stages of hepatic fibrosis, and disease etiology. Hepatic fibrosis and liver disease severity are also a product of disease duration, and longitudinal studies are needed to interrogate this more robustly. However, the novelty of this study is the analysis of microbiota derived from a very limited resource, namely duodenal tissue, from subjects who have no other duodenal pathology, in relation to intestinal permeability. To our knowledge, this has not been done before.

In summary, this study demonstrates that CLD has dysbiosis of the duodenal mucosal microbiota, characterized by a distinct community clustering and reduced bacterial diversity. Of significance, reduced duodenal bacterial diversity is associated with increased small intestinal permeability, and serum ALT. These findings prompt for further mechanistic and longitudinal studies, rather than cross-sectional studies, which would enable the effects of differences in disease pathogenesis on duodenal MAM to be interrogated further.

CONFLICTS OF INTEREST

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Specific author contributions: A.S.R.: Study concept and design, patient recruitment, data acquisition and analysis, writing and editing of manuscript. G.A.M.: Study concept and design, data analysis, writing and editing of manuscript. E.R.S.: Methodology, bioinformatics analysis, data analysis, writing and editing manuscript. C.D.T.: Methodology (high-performance liquid chromatography), data analysis, review of manuscript. P.B.: Supervision of methodology, review of manuscript. D.A.V.: Methodology (endotoxin assay), review of manuscript. L.M.F.: Supervision of methodology, review of manuscript. M.M.: Supervision of methodology, data analysis, editing of manuscript. G.J.H.: Study concept, review of manuscript.

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Study Highlights

WHAT IS KNOWN

- ✓ CLD is associated with dysbiosis of the stool microbiota.
- ✓ Little is known about the small intestinal mucosal microbiota of CLD and how it relates to intestinal permeability and disease severity.

WHAT IS NEW HERE

- ✓ The duodenal mucosa-associated microbiota of CLD differs from that of healthy controls, with distinct community clustering and lower diversity.
- ✓ In CLD, lower microbial diversity correlates with increased small intestinal permeability and serum ALT.

TRANSLATIONAL IMPACT

- ✓ Strategies to ameliorate duodenal MAM dysbiosis could improve intestinal barrier dysfunction and liver injury in CLD.
- ✓ The duodenal MAM warrants further attention as a potential therapeutic target to restore intestinal barrier dysfunction in CLD.

REFERENCES

- Gäbele E, Dostert K, Hofmann C, et al. DSS induced colitis increases portal LPS levels and enhances hepatic inflammation and fibrogenesis in experimental NASH. *J Hepatol* 2011;55:1391–9.
- Seki E, De Minicis S, Osterreicher CH, et al. TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med* 2007;13:1324–32.
- Le Roy T, Llopis M, Lepage P, et al. Intestinal microbiota determines development of non-alcoholic fatty liver disease in mice. *Gut* 2013;62:1787–94.
- Chen P, Torralba M, Tan J, et al. Supplementation of saturated long-chain fatty acids maintains intestinal eubiosis and reduces ethanol-induced liver injury in mice. *Gastroenterology* 2015;148:203–14.e16.
- Qin N, Yang F, Li A, et al. Alterations of the human gut microbiome in liver cirrhosis. *Nature* 2014;513:59–64.
- Mouzaki M, Comelli EM, Arendt BM, et al. Intestinal microbiota in patients with nonalcoholic fatty liver disease. *Hepatology* 2013;58:120–7.
- Chen Y, Yang F, Lu H, et al. Characterization of fecal microbial communities in patients with liver cirrhosis. *Hepatology* 2011;54:562–72.
- Boursier J, Mueller O, Barret M, et al. The severity of nonalcoholic fatty liver disease is associated with gut dysbiosis and shift in the metabolic function of the gut microbiota. *Hepatology* 2016;63:764–75.
- Bajaj JS, Sterling RK, Betrapally NS, et al. HCV eradication does not impact gut dysbiosis or systemic inflammation in cirrhotic patients. *Aliment Pharmacol Ther* 2016;44:638–43.
- Bajaj JS, Betrapally NS, Hylemon PB, et al. Salivary microbiota reflects changes in gut microbiota in cirrhosis with hepatic encephalopathy. *Hepatology* 2015;62:1260–71.
- Pijls KE, Jonkers DM, Elamin EE, et al. Intestinal epithelial barrier function in liver cirrhosis: An extensive review of the literature. *Liver Int* 2013;33:1457–69.
- Miele L, Valenza V, La Torre G, et al. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. *Hepatology* 2009;49:1877–87.
- Shah A, Shanahan E, Macdonald GA, et al. Systematic review and meta-analysis: Prevalence of small intestinal bacterial overgrowth in chronic liver disease. *Semin Liver Dis* 2017;37:388–400.
- Bajaj JS, Heuman DM, Hylemon PB, et al. Altered profile of human gut microbiome is associated with cirrhosis and its complications. *J Hepatol* 2014;60:940–7.
- Aguirre de Cárcer D, Cuív PO, Wang T, et al. Numerical ecology validates a biogeographical distribution and gender-based effect on mucosa-associated bacteria along the human colon. *ISME J* 2011;5: 801–9.
- Wang L, Fouts DE, Starkel P, et al. Intestinal REG3 lectins protect against alcoholic steatohepatitis by reducing mucosa-associated microbiota and preventing bacterial translocation. *Cell Host Microbe* 2016;19:227–39.
- Chen Y, Ji F, Guo J, et al. Dysbiosis of small intestinal microbiota in liver cirrhosis and its association with etiology. *Sci Rep* 2016;6:34055.
- Alberti KG, Eckel RH, Grundy SM, et al. Harmonizing the metabolic syndrome: A joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation* 2009;120:1640–5.
- Zakrzewski M, Proietti C, Ellis JJ, et al. Calypso: A user-friendly web-server for mining and visualizing microbiome-environment interactions. *Bioinformatics* 2017;33:782–3.
- Lê Cao KA, Costello ME, Lakis VA, et al. MixMC: A multivariate statistical framework to gain insight into microbial communities. *PLoS One* 2016;11:e0160169.
- Langille MG, Zaneveld J, Caporaso JG, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol* 2013;31:814–21.
- Bajaj JS, Hylemon PB, Ridlon JM, et al. 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* 2012;303:G675–85.
- Kakiyama G, Pandak WM, Gillevet PM, et al. Modulation of the fecal bile acid profile by gut microbiota in cirrhosis. *J Hepatol* 2013;58:949–55.
- Chang CS, Chen GH, Lien HC, et al. Small intestine dysmotility and bacterial overgrowth in cirrhotic patients with spontaneous bacterial peritonitis. *Hepatology* 1998;28:1187–90.
- Yao ZQ, Prayther D, Trabue C, et al. Differential regulation of SOCS-1 signalling in B and T lymphocytes by hepatitis C virus core protein. *Immunology* 2008;125:197–207.
- Douam F, Bobay LM, Maurin G, et al. Specialization of hepatitis C virus envelope glycoproteins for B lymphocytes in chronically infected patients. *J Virol* 2015;90:992–1008.
- Krautkramer KA, Dhillon RS, Denu JM, et al. Metabolic programming of the epigenome: Host and gut microbial metabolite interactions with host chromatin. *Transl Res* 2017;189:30–50.
- Zoetendal EG, Raes J, van den Bogert B, et al. The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. *ISME J* 2012;6:1415–26.
- McGuckin MA, Lindén SK, Sutton P, et al. Mucin dynamics and enteric pathogens. *Nat Rev Microbiol* 2011;9:265–78.
- Derrien M, van Passel MWJ, van de Bovenkamp JHB, et al. Mucin-bacterial interactions in the human oral cavity and digestive tract. *Gut Microbes* 2010;1:254–68.

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