Research Article

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Altered profile of human gut microbiome is associated with cirrhosis and its complications

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Background & Aims: The gut microbiome is altered in cirrhosis; however its evolution with disease progression is only partly understood. We aimed to study changes in the microbiome over cirrhosis severity, its stability over time and its longitudinal alterations with decompensation.

Methods: Controls and age-matched cirrhotics (compensated/decompensated/hospitalized) were included. Their stool microbiota was quantified using multi-tagged pyrosequencing. The ratio of autochthonous to non-autochthonous taxa was calculated as the cirrhosis dysbiosis ratio (CDR); a low number indicating dysbiosis. Firstly, the microbiome was compared between controls and cirrhotic sub-groups. Secondly, for stability assessment, stool collected twice within 6 months in compensated outpatients was analyzed. Thirdly, changes after decompensation were assessed using (a) longitudinal comparison in patients before/after hepatic encephalopathy development (HE), (b) longitudinal cohort of hospitalized infected cirrhotics MELD-matched to uninfected cirrhotics followed for 30 days.

Results: 244 subjects [219 cirrhotics (121 compensated outpatients, 54 decompensated outpatients, 44 inpatients) and 25 age-matched controls] were included. CDR was highest in controls (2.05) followed by compensated (0.89), decompensated (0.66), and inpatients (0.32, p <0.0001) and negatively correlated with endotoxin. Microbiota and CDR remained unchanged in stable outpatient cirrhotics (0.91 *vs.* 0.86, p = 0.45). In patients studied before/after HE development, dysbiosis occurred post-HE (CDR: 1.2 to 0.42, p = 0.03). In the longitudinal matched-cohort, microbiota were significantly different between infected/uninfected cirrhotics at baseline and a low CDR was associated with death and organ failures within 30 days.

Conclusions: Progressive changes in the gut microbiome accompany cirrhosis and become more severe in the setting of decompensation. The cirrhosis dysbiosis ratio may be a useful quantitative index to describe microbiome alterations accompanying cirrhosis progression.

Introduction

The investigation of the gut microbiome in cirrhosis is important because of the key role in bacterial translocation and their products such as endotoxin in the pathogenesis of complications, including hepatic encephalopathy (HE), spontaneous bacterial peritonitis (SBP), and other infections [1–3]. These infections are the leading cause of multi-organ failure, acute-on-chronic liver failure (ACLF), and death in cirrhosis [4–6]. Prior outpatient-centered studies have demonstrated changes in the cirrhotic stool microbiome but these are only partly understood due to the small sample sizes and considerable inter-person variability [7–11]. Therefore there is a need to evaluate larger populations of cirrhotics ranging from compensated to pre-terminal in their severity in conjunction with bacterial products to delineate the role of the microbiome in cirrhosis.

The aims of this study were to (a) define changes in the stool microbiome over the entire disease spectrum in a large population of cirrhotic patients, (b) investigate the stability of microbiota composition over time in cirrhosis, (c) evaluate changes in microbiome longitudinally with advancing cirrhosis with infections and HE development.

Keywords: Microbiota; Hepatic encephalopathy; Decompensation; Infections; Acute-on-chronic liver failure; Endotoxin; MELD score; Cirrhosis dysbiosis ratio.
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Abbreviations: HE, hepatic encephalopathy; ACLF, acute-on-chronic liver failure; SBP, spontaneous bacterial peritonitis; RDP10, ribosomal data project; PCO, principle component analysis; MELD, model for end-stage liver disease; SBP, spontaneous bacterial peritonitis; CDR, cirrhosis dysbiosis ratio; NASH, non-alcoholic steatohepatitis; MELD, model for end-stage liver disease; BMI, body mass index; HCV, hepatitis C virus; TIPS, transjugular intra-hepatic porto-systemic shunt.

Patients and methods

This prospective study was carried out in the Virginia Commonwealth University and McGuire VA Medical centers. We enrolled patients with cirrhosis (diagnosed histologically, endoscopic/radiological evidence or signs of decompensation) after informed consent. All cirrhotic patients underwent blood draw for MELD score and endotoxin (using published techniques) [11]. Subsequently we enrolled age-matched healthy controls that were free of liver disease and were not on any medications apart from non-steroidal analgesics or antihypertensives. Detailed demographic, cirrhosis-severity characteristics and medications were recorded. We excluded patients with an unclear cirrhosis diagnosis, other end-organ disease prior to admission, hospitalized for >48 h before enrollment, or transferred from another hospital. We collected stool from patients at the time of enrollment, either as an outpatient or within 48 h of hospitalization. All subjects' dietary history for the day prior to stool sampling was recorded.

Stool was analyzed using published multi-tagged pyrosequencing techniques and ribosomal data (RDP10) taxa analysis [12,13] was performed. Data was analyzed using Metastats [14], standard non-parametric tests (Kruskal-Wallis test) and principle component (PCO) analyses. Unifrac PCO analysis was performed using the Qiime package [15]. Multiple comparison adjustments were performed as part of these techniques (Supplementary data).

Microbiome changes across cirrhosis severity

A cross-sectional study of healthy controls with compensated outpatients (without current or prior ascites, HE or variceal hemorrhage), decompensated outpatients ($\geqslant$1 of HE, ascites with/without SBP prophylaxis, history of variceal hemorrhage) and inpatients with cirrhosis and infections as previously defined was performed [5]. We found in our prior studies that cirrhosis and HE were accompanied by reduced relative abundance of taxa considered benign and autochthonous, including *Lachnospiraceae*, *Ruminococcaceae*, and Clostridialies Incertae Sedis XIV (from now on called Clostridialies XIV) and a relatively higher abundance of others, particularly *Enterobacteriaceae* and *Bacteroidaceae* [7,11,16]. This ratio of "good *vs.* bad" taxa abundance was termed the cirrhosis dysbiosis ratio (CDR), which was used to compare groups going forward. Statistical analysis of demographics, cirrhosis details, endotoxin and microbiota composition was performed between groups. A post-hoc analysis of patients with/without an alcoholic etiology or with/without NASH cirrhosis was also performed.

Stability of the microbiome over time

We collected stool from a group of cirrhotic outpatients at set intervals within 6 months of their prior collection without any interim changes in their cirrhosis natural history. Correlations of the microbiota and comparison of microbiota, CDR and endotoxemia was performed between the initial and second collection.

Longitudinal study of microbiota after decompensation

After HE development

We analyzed changes in the microbiome in a group of compensated cirrhotics who had stool collection before and 1 month after development of their first episode of HE precipitated without infections, TIPS or upper GI bleeding. Microbiota correlations and comparison of dysbiosis, CDR and endotoxemia was performed between the two samples.

Infections and changes in microbiome

We performed a longitudinal cohort study of cirrhotics admitted with infections matched to cirrhotics without infections on MELD score, SBP prophylaxis, rifaximin, and PPI use. The groups were followed for 30 days and development of death, organ failures [defined as (a) grade III/IV HE, (b) dialysis, (c) shock or (d) mechanical ventilation] or ACLF ($\geqslant$2 organ failures during the admission) were recorded [17]. We studied the microbiota and endotoxin between infected/non-infected patients and those who developed organ failures, ACLF and death within 30 days using UNIFRAC QiiME, Metastats and non-parametric tests with corrections for multiple comparisons.

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

Results

Change in cirrhosis microbiome with disease severity

We enrolled 244 subjects; 25 controls, 175 outpatients with cirrhosis (group A: 121 and group B: 54) and 44 cirrhotic inpatients (38 of them had infections; the rest were admitted for non-infectious reasons). Within the cirrhosis group, inpatients and decompensated patients had significantly higher MELD scores, endotoxin, lactulose, beta-blocker and rifaximin use compared to the compensated outpatients. Within the two advanced groups (infected inpatients and decompensated outpatients), the rate of rifaximin, beta-blocker and SBP prophylaxis was similar (Table 1). There was a non-significant trend towards lower caloric intake in inpatients.

Relationship of endotoxin, MELD score and bacterial taxa

MELD score was negatively correlated with Clostridiales XIV, *Lachnospiraceae* and *Ruminococcaceae* ($r = -0.3$, $p < 0.0001$ for all) and with *Rikenellaceae* ($r = -0.2$, $p < 0.0001$) and positively with potentially pathogenic taxa; *Staphylococcae* ($r = 0.2$, $p = 0.03$), *Enterococceae* ($r = 0.4$, $p < 0.0001$) and *Enterobacteriaceae* ($r = 0.3$, $p = 0.001$). There was also a significant correlation of the CDR with MELD score ($r = -0.3$, $p = 0.005$) and endotoxin ($r = -0.3$, $p = 0.001$). Endotoxin was negatively linked to Clostridiales XIV (-0.3, $p < 0.001$), *Lachnospiraceae* ($r = -0.4$, $p < 0.0001$), *Ruminococcaceae* ($r = -0.4$, $p < 0.0001$) and positively with MELD score ($r = 0.5$, $p < 0.0001$), *Enterobacteriaceae* ($r = 0.2$, $p = 0.002$) and *Bacteroidaceae* ($r = 0.2$, $p = 0.001$). No other significant correlations between taxa, MELD score and endotoxin were found.

Microbiome comparison between groups

When controls were compared to outpatients with and without HE and inpatients, there was a significant reduction in autochthonous taxa, Clostridiales XIV, *Ruminococcaceae* and *Lachnospiraceae* with a significant increase in pathogenic taxa such as *Enterococcaeae*, *Staphylococcaceae*, and *Enterobacteriaceae*. We also found a reduction in *Veillonellaceae*, and *Porphyromonadaceae* with worsening liver disease compared to healthy controls (Table 1). The CDR for controls was significantly higher compared to all cirrhotic patients (2.05 *vs.* 0.74, $p < 0.0001$). These comparisons remained consistent when subjects without rifaximin, beta-blockers, SBP prophylaxis or PPIs were compared (Supplementary Tables 1–4). There was significant clustering in the Unifrac PCOs of healthy controls with each other compared to all cirrhotics (Fig. 1A) and to cirrhotics who were inpatient *vs.* outpatient (Fig. 1B). There was no significant difference in the microbiota between patients with and without rifaximin on any level.

NASH and alcoholic etiology sub-analysis

On a post-hoc analysis, alcoholic cirrhotics had a significantly higher abundance of *Enterobacteriaceae* and *Halomonadaceae*, lower *Lachnospiraceae*, *Ruminococcaceae*, and Clostridialies XIV, high endotoxin and lower CDR despite statistically similar MELD score and BMI compared to those without alcoholic etiology (Table 2). We found a higher abundance of *Porphyromonadaceae*, *Bacterioidaceae*, and lower *Veillonellaceae* in NASH patients than the non-NASH counterparts; CDR and endotoxin levels were similar (Table 3).