Colonic Inflammation and Secondary Bile Acids in Alcoholic Cirrhosis

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Running Head: Alcoholic cirrhosis, bile acids and colonic inflammation

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Abstract

Alcohol abuse with/without cirrhosis is associated with an impaired gut barrier and inflammation. Gut microbiota can transform primary bile acids (BA) to secondary BAs which can adversely impact the gut barrier. **Aim**: Define the effect of active alcohol intake on fecal BA levels, ileal & colonic inflammation in cirrhosis. **Methods**: Five age-matched groups; two non-cirrhotic (control and drinkers) & three cirrhotic [(NAIc: non-alcoholic (non-drinkers), AbsAlc: abstinent alcoholic for >3mths & CurrAlc: (currently drinking)] were included. Fecal and serum BA analysis, serum endotoxin and stool microbiota using pyro-sequencing were performed. A subgroup of controls, NAIc and CurrAlc underwent ileal and sigmoid colonic biopsies on which mRNA expression of TNF-α, IL1β, IL6 and Cox-2 were performed. **Results**: 103 patients (19 healthy, 6 non-cirrhotic drinkers, 10 CurrAlc, 38 AbsAlc and 30 NAIc, age 56 yrs, median MELD: 10.5) were included. Five each of healthy, CurrAlc and NAIc underwent ileal/colonic biopsies. Endotoxin, serum conjugated DCA and stool total BAs and secondary/primary BA ratios were highest in current drinkers. On biopsies, a significantly higher mRNA expression of TNF-α, IL1β, IL6 and Cox-2 in colon but not ileum was seen in CurrAlc compared to NAIc & controls. **Conclusion**: Active alcohol use in cirrhosis is associated with a significant increase in the secondary BA formation compared to abstinent alcoholic cirrhotics and non-alcoholic cirrhotics. This increase in secondary BAs is associated with a significant increase in expression of inflammatory cytokines in colonic mucosa but not ileal mucosa, which may contribute to alcohol-induced gut barrier injury.
Introduction:
Changes in the gut milieu can predispose patients with cirrhosis to increased bacterial translocation and serious systemic infections such as spontaneous bacterial peritonitis (36). Alcohol, with or without accompanying cirrhosis, can negatively impact the gut barrier and potentially propagate the cycle of bacterial translocation and systemic inflammation (22). Bile acids (BAs) are key modulators of the gut microbiota and intestinal permeability and can activate nuclear receptors in the intestinal wall such as FXR which can regulate inflammation and hepatic BA synthesis, and are affected by cirrhosis progression (12-14, 26). There is evidence of increased intestinal inflammation and diminished intestinal barrier function in animal models of alcoholic injury and in patients(22). However, the interaction of BAs and alcohol as co-modulators of intestinal inflammation in human cirrhosis has not been previously studied (31, 33). The aim was to study the interactions between alcohol, microbiota and BAs, which could increase our insight into mechanisms behind intestinal inflammation in cirrhosis and encourage focused therapies directed at ameliorating these negative effects.

Our approach was to determine (i) the effect of current and prior alcohol use in cirrhotic and non-cirrhotic subjects on serum and fecal BA profiles and (ii) effects of current alcohol use in the setting of cirrhosis on expression of specific nuclear receptors (FXR, SHP) and mediators of inflammation that occur downstream (IL-1β, IL-6, TNF-α, MCP, and COX2) in colonic and ileal mucosa.

Materials and Methods:
We prospectively enrolled five groups of subjects: three cirrhotic groups which were: 1) cirrhotics without alcohol as an etiology; 2) those with alcohol as an etiology but who were abstinent for >3 months and 3) those who had an alcoholic etiology and continued to drink and two non-cirrhotic groups, age-matched 4) healthy controls and 5) active drinkers without liver disease. The diagnosis of cirrhosis was made using a combination of clinical (low
platelets, high AST/ALT ratio or frank decompensation in the setting of chronic liver disease), radiological (evidence of cirrhosis and portal hypertension on abdominal imaging), histological (biopsy evidence) or endoscopic (presence of varices) evidence. We excluded subjects who were not able to give informed consent, had alcoholic hepatitis currently or within 6 months of the study or had an unclear diagnosis of cirrhosis. Active drinkers without liver disease had normal AST, ALT, liver function and imaging. Patients underwent written informed consent and a detailed questionnaire regarding their stool habits, medications and past and current alcohol use.

**Sample collection:** Stool: All subjects underwent stool collection; in those who agreed to undergo a colonoscopy for colonic and ileal biopsies, their stool collection was performed at least 5 days from the colonoscopy. The stool was divided into two aliquots and frozen at -80 °C. One sample was used for the BA determinations and the other for microbiome analysis.

**Blood:** All cirrhotic patients underwent blood collection for analysis of MELD score (validated logarithmic score of serum bilirubin, creatinine and INR) and serum albumin. A portion of the serum was analyzed for BAs (Junshin Clinic Bile Acid Institute, Tokyo) and endotoxin (Assaygate, Ijamsville, MD)(5, 14).

**Ileal and colonic biopsies:** A subset of these eligible subjects (controls, cirrhotics without alcoholic etiology and those who were currently drinking) underwent colonoscopy using a standard colonic preparation which was performed by experienced endoscopists. Consent was obtained to get pinch biopsy samples of the terminal ileum and sigmoid colon while performing the colonoscopy. During the colonoscopy, the pinch biopsy samples were snap frozen on retrieval and stored at -80 °C till the analysis was performed.

**Specimen Analysis: BA analysis:** The fecal BA analysis was performed using previously published HPLC and LC-MS techniques(14) (detailed methods in supplementary information). Specific BAs quantitated included: primary [Cholic (CA), Chenodeoxycholic (CDCA)] and
secondary BAs [lithocholic (LCA) and deoxycholic (DCA)] acids. The median primary,
secondary BAs concentrations and their ratios (CA+CDCA/LCA+DCA) were compared between
groups. Since this was a one-time collection of stool, the ratios were considered more important
compared to the individual BA concentrations. LC-MS using published techniques was also
used to perform serum BA measurements which included conjugated (tauro or glycol N-acyl
amidated), double-conjugated ((N-acyl amidated and C-3 sulphated ) and unconjugated(14).

**Stool microbiome analysis:** We performed multi-tagged pyrosequencing according to
previously published techniques(5) (detailed methods in supplementary information).

**Tissue analysis:** was performed on ileal and colonic biopsy samples to determine mRNA
levels of (A) bile acid modulators: farnesoid X receptor (FXR), SHP and fibroblast growth factor
(FGF-19) and (B) potential mediators of intestinal inflammation: IL-1β, IL-6, TNF-α, monocyte
chemoattractant protein-1 (MCP-1) and COX-2. Total RNA was isolated from samples using
QIAzol lysis Reagent (from QIAGEN). Total RNA (1 ug) was used for the first-strand cDNA
synthesis using the High-Capacity cDNA Archive kit (from Applied Biosystems). The mRNA
levels of TNF-α IL-6, IL-1β, FXRα and COX-2, were quantified using specific primers for each
gene. iQ SYBR Green Supermix was used as a fluorescent dye to detect the presence of
double-stranded DNA and the mRNA levels for each gene were normalized using β-actin as an
internal control. The ratio of normalized mean value for each treatment group to vehicle control
group was calculated.

**Statistical analysis:**
Demographics, cirrhosis severity, medication use, serum inflammatory cytokines and BAs,
including ratios were compared between groups using ANOVA, Chi-square and Kruskall-Wallis
tests where appropriate. Kruskal-Wallis tests and Metastats were used to compare fecal
microbiota abundance between groups and Pearson’s correlation analyses were used to
correlate microbiota, MELD score and BA concentrations(35). Tissue analytes were compared
between the selected controls, drinkers and non-alcoholic cirrhotic patients using Kruskal-Wallis tests.

This protocol was approved by the Institutional Review Board at the Hunter Holmes McGuire VA Medical Center in Richmond.

**Results:**

The study had two parts: (a) Study of the entire group with fecal and serum BA analysis and (b) study of the subgroup undergoing mucosal biopsies

**Analysis of the entire group:**

We enrolled a total of 103 subjects; 19 healthy, 6 drinkers without liver disease and 78 cirrhotic patients of which 10 were currently drinking, 30 had never drank and 38 had an alcoholic etiology but were abstinent for >6 months. The pattern of alcohol abuse was similar amongst the three alcoholic groups with the difference of alcohol intake only occurring within the past 6 months (median 21 years of alcohol intake). The last intake of alcohol was a median of 1 day prior to enrollment in both groups of current drinkers. Groups were statistically similar on median age (controls 49, drinkers without liver disease, 51, NAlec 53, AbsAlec 52, CurrAlec 50 years, p=0.61), percentage of men (controls 74%, drinkers without liver disease 67% NAlec 93%, AbsAlec 92%, CurrAlec 100%, p=0.84) or MELD score (NAlec 8.5, AbsAlec 10.5, CurrAlec 8.5, p=0.24).

There was a significantly higher mean endotoxin level in CurrAlec compared to all groups (CurrAlec 0.75±0.91 vs. NAlec 0.49±0.68, AbsAlec 0.30±0.56, non-cirrhotic drinker 0.33±0.21 and controls 0.0±0.1 EU/ml p=0.021). Within the non-cirrhotic group, drinkers’ endotoxin levels were significantly higher compared to controls (p=0.007).

**Fecal BA analysis:** We combined the fecal BA profile of the two currently drinking groups (CurrAlec cirrhotics and those without liver disease) and compared them against the other groups
using medians due to non-normal BA distributions. The combined current drinkers (cirrhotics and non-cirrhotics) had the highest median total fecal BAs (median 10.5, p<0.0001), total secondary BAs (6.5, p<0.0001), DCA (4.7, p<0.0001) and LCA (2.5, p=0.005) compared to all other groups, including controls. This group also had the highest secondary/primary BA ratio (median 26.4, p=0.002) when compared to the remaining cirrhotics; this ratio was similar to the control values. Primary BAs were not significantly different between cirrhotic groups (Table 1) but were the lowest in the controls. When groups were individually compared, the highest level of total, secondary and secondary/primary BA were in active drinkers without liver disease.

**Serum BA analysis**: There was a significantly higher concentration of conjugated DCA and a lower concentration of conjugated CDCA in current drinkers (both cirrhotic and non-cirrhotic) compared to other groups (Table 2).

**Microbiota, BAs and MELD score**: In the entire group, MELD score was negatively correlated with total fecal BAs (r=-0.4, p=0.004), LCA (r=-0.4, p=0.001), DCA (r=-0.32, p=0.009), total secondary BAs (r=-0.4, p=0.004) and secondary/primary BA ratio (p=-0.4, p=0.002) but not CA or CDCA. Incertae sedis XIV abundance was positively correlated with total BAs (r=0.4, p=0.001), LCA (r=0.2, p=0.05), DCA (r=0.3, p=0.03), CA (r=0.3, p=0.005), CDCA (r=0.2, p=0.04), total primary (r=0.3, p=0.01) and secondary BAs (r=0.2, p=0.03).

*Enterobacteriaceae* abundance was positively correlated with primary BAs (r=0.3, p=0.03) and with CA (r=0.2, p=0.024) but not with other BAs. MELD score was negatively correlated with Incertae Sedis XIV (r=-0.4, p=0.003), *Ruminococcaceae* (r=-0.5, p<0.0001), *Lachnospiraceae* (r=-0.3, p=0.03) and positively with *Porphyromonadaceae* (r=0.3, p=0.01).

**Subgroup analysis of those with mucosal biopsies**: Fifteen subjects, 5 controls, 5 active drinkers and 5 cirrhotics without alcoholic etiology agreed to have biopsies taken. They were
statistically similar in age (median 50 vs. 51 vs 53 years), all were men and there was no significant difference in the median MELD score between the cirrhotic patients (drinkers 8.5 vs. non-alcoholic cirrhosis 8).

Stool microbiome comparison between the five drinkers and the five non-alcoholic cirrhotics showed a significant increase in a taxon from phylum Firmicutes, *Veillonellaceae* (5% vs 2%, p=0.02) and reduction in two Bacteroidetes taxa, *Bacteroidaceae* (4% vs 2%, p=0.03) and *Porphyromonadaceae* reduction trend (2% vs 4%, p=0.09) in drinkers. No changes were seen in components of phyla Actinobacteria, or other taxa of Firmicutes, *Lachnospiraceae* (2 vs 2%), *Ruminococcaceae* (0.7 vs 1%), Incertae Sedis XIV (5 vs 5%), other taxa of Bacteroidetes *Prevotellaceae* (6 vs 6%), *Rikenellaceae* (0.4 vs 1%) or phylum Proteobacteria, *Enterobacteriaceae* (4 vs 3%).

Ileal and colonic tissue mRNA expression: We found a significantly higher expression of mRNA of FXRα and COX-2 in the ileal and sigmoid colonic mucosa in drinkers compared to non-alcoholic cirrhotics and controls (Primers in Table 2). There was also a significantly higher expression of TNF-α, IL-1β, IL-6, MCP-1 and FGF-19 in drinkers compared to the other groups in the colon but not in the ileal mucosa. SHP expression was not different between groups in the colon or ileum. While there was a trend towards an increasing FGF-19, TNF-α, MCP-1, IL-6 and IL-1β expression in the ileum in drinkers more than in non-alcoholic cirrhotics more than controls, it did not reach statistical significance.
Discussion:

Patients with cirrhosis are prone to a pro-inflammatory milieu which has consequences in the form of altered inflammatory responses to infections and complications of cirrhosis such as hepatic encephalopathy (29). This systemic inflammation often portends a worse prognosis and the role of alcohol intake, ongoing or past, is important to delineate since alcohol is associated with a higher rate of infections and worse outcome in cirrhotic patients (27). However, the role of alcohol intake on fecal BA levels and their effect on intestinal and colonic inflammation is not completely understood (14). Our data show a highly significant increase in total and secondary fecal BA concentrations in current drinkers, regardless of cirrhosis status, compared to other cirrhotic patients and healthy controls that are not consuming alcohol. While the abstinent alcoholics had relatively advanced cirrhosis although statistically similar, which could partly explain the lower total BA, the non-alcoholics and active alcoholic cirrhotics had similar MELD scores; therefore, it is unlikely that the BA profile results would be a function of cirrhosis severity alone. This was accompanied by dysbiosis that was linked with liver disease severity.

There are a number of reports in the literature that alcohol increases the rate of synthesis of bile acids in humans and animal models (2, 9, 19, 37). This was corroborated by the highest fecal BA levels of total, secondary and secondary/primary BA ratio in current drinkers without cirrhosis compared to the rest. There was also a significant increase in the mRNA levels of genes encoding pro-inflammatory cytokines (TNFα, IL-6, IL-1β, MCP-1) in colonic, but not ileal, tissue in cirrhotic patients consuming alcohol. Cox 2 was highly elevated in cirrhotic patients consuming alcohol in both ileum and colon. However, Cox2 mRNA levels were higher in the colon. Cirrhotic patients and healthy controls not consuming alcohol did not have a significant increase in the mRNA levels of pro-inflammatory cytokines in either ileal or colonic tissue samples. The dichotomy between ileal and colonic tissue changes is interesting and may be due to the higher abundance of gut microbiota in the colon which could modulate the BA 7α-dehydroxylation. This could also potentiate the impaired intestinal barrier function that has been
shown to occur largely in the colon and not the small bowel, in humans with compensated cirrhosis (similar to our population) (21). A potential functional test of this barrier function is serum endotoxemia, which was increased in currently drinking cirrhotics compared to the others and in non-cirrhotics, was increased in drinkers compared to non-drinkers (39). This replicates prior studies and extends this functional gut barrier dysfunction to actively drinking cirrhotic patients (3).

Older studies in non-cirrhotic humans, human hepatocytes and animal models all report that alcohol increases rates of bile acid synthesis (2, 19). Animal model studies (mice and rats) suggest that both chronic and acute alcohol consumption increases bile acid synthesis and up-regulates cholesterol 7α-hydroxylase (Cyp7A1). In non-cirrhotic rats consuming ethanol, there was a decrease in intestinal fibroblast growth factor 15 (FGF-15) possibly accounting for the up-regulation of Cyp7A1 and bile acid synthesis (37). Recent studies in mice suggest that alcohol increases the expression of Cyp7A1 and Cyp27A1 via activation of hepatic cannabinoid receptor type 1 and CREBH in the liver (9). Our study found an increase in FXR and FGF-19, but not SHP, in ileum and sigmoid colon in drinkers but not in non-alcoholic cirrhotics and controls. This suggests that while the feedback mechanism of a high FGF-19 mRNA expression due to a high intestinal BA load is intact, it is probably ineffective in reducing the accelerated BA synthesis induced by alcohol in the liver in cirrhosis. This is different from the findings in non-cirrhotic rodents that were fed alcohol, in which there was a lower FGF-15 that could in turn allow stimulation of BA synthesis in the liver. This is also supported by the increased level of conjugated serum DCA in current drinkers which indicates that a higher proportion of DCA was absorbed through the entero-hepatic cycle and reconjugated in the liver, but despite that these subjects continued to have higher fecal BA levels. We speculate that in the setting of cirrhosis with ongoing alcohol consumption, there could be a differential regulation of the entero-hepatic cycle i.e. that CYP7A1 is not down-regulated by FGF-19. If the cannabinoid receptor type 1 is
activated by alcohol in the human liver it might override the suppressive effects of FGF-19 on Cyp7A1 allowing for increased bile acid synthesis. The increase in pro-inflammatory cytokine mRNA expression in the setting of FXR activation is interesting since prior animal studies and ileal biopsy analysis in inflammatory bowel disease showed that FXR activation can reduce inflammation by suppressing NF-kB (10, 32, 34). Our results however, indicate a significantly higher inflammatory gene expression in the colon, but not the ileum, in actively drinking cirrhotics despite FXR activation in both locations of the gastrointestinal tract. The interactions of FXR with inflammation vary with disease and tissue and FXR, is associated with higher inflammation in Barrett’s esophagus (6). Therefore our results indicate an inflammatory mechanism in the colon in alcoholic cirrhotics that is not suppressed by FXR activation. A strong candidate species for this could be the secondary BAs that were found in actively drinking cirrhotics and non-cirrhotic subjects in concentrations even higher than healthy controls. Secondary BAs, especially, deoxycholic acid, are found in high concentrations in fecal water and can cross epithelial cell membranes by simple diffusion. DCA also has been reported to activate various cell signaling pathways (EGFR, AKT, ERK 1/2, PKC, β-catenin, Cox-2) in primary cells, intestinal cell lines and animal models at lower concentrations than CDCA (7, 8, 17, 20, 23, 24). Moreover, secondary bile acids activate cellular pathways producing ROS (NADPH oxidase, PLA2, mitochondria) that may activate stress pathways leading to the activation of NF-κB and pro-inflammatory cytokine synthesis (30).

The current results strongly suggest that an increased level of fecal secondary bile acids are associated with increased inflammation and COX-2 induction in colonic tissue. This may be due to the direct effects of secondary bile acids on colonic epithelial cells and/or to changes in the colonic microbiota. Increased levels of bile acids are known to cause changes in the composition of the intestinal microbiota (25). Chronic inflammation is highly associated with an increased risk of colon cancers including patients with ulcerative colitis and Crohn’s disease (11). In cirrhotic patients, this intestinal inflammation could lead to impaired gut-liver axis
through barrier dysfunction, endotoxemia and bacterial translocation (18). This has been shown
to result in hepatic inflammation which can reduce BA secretion from the liver, and potentiate
negative survival outcomes (1, 15, 16, 22, 38). Indeed we found the highest serum endotoxin
level in CurrAlc patients. There could be several mechanisms for this increase in secondary BA
ratio that presents itself to the colon including; increased conversion of primary BAs to
secondary BAs by alteration in microbiome, a decrease in colonic transit time caused by current
alcohol intake or an increased “spillover” of secondary BAs into the colon because of increased
total BA presentation into the small bowel as a result of the heightened BA synthesis by alcohol
intake. When germ-free mice were compared to conventionally-raised ones, they had a
significantly lower fecal BA concentration which corresponded to the colonic tissue BA profile, a
reduced whole body BA pool and no secondary BA due to absence of microbiota (28). Another
study showed that supplementing mice diet with 1% cholate increased their 7α-dehydroxylation
capacity by several fold, indicating that the function of gut microbiota is modulated by the
amount of BAs presenting to the intestine (25). In our study, when the two cirrhotic groups who
ultimately had the biopsies taken (the active drinkers and those who did not have alcohol as an
etiology) were compared, there was only a modest difference in the stool microbiome, especially
in Veillonellaceae and Bacteroidaceae, which are not associated with bile acid 7α-
dehydroxylation capacity but may promote growth of bacteria that do. Veillonellaceae
specifically is linked with higher systemic inflammation and endotoxemia in cirrhotics; the
association with secondary BAs in alcoholic cirrhotics could potentially be a mechanism (4).
Despite these modest changes in bacterial composition, we ultimately found that the microbiota
function, i.e. 7α-dehydroxylation demonstrated by the highest fecal secondary BAs, was
significantly different in the current drinkers (cirrhotic and not). Therefore it is plausible that a
higher total BA pool in alcoholic cirrhotics could lead to a higher substrate for microbiota that
can perform 7α–dehydroxylation and can survive in an environment of relatively higher
secondary BA concentration or that alcohol use could lead to an enhanced 7α-dehydroxylating
capacity of the microbiota. Further metagenomic studies are required to explore these mechanisms.

We conclude that the consumption of alcohol in non-cirrhotic and cirrhotic subjects increases the levels of total fecal bile acids and secondary bile acids as compared to other cirrhotic patients and healthy controls. This was accompanied by a significant increase in serum endotoxin, serum conjugated secondary BAs and several markers of colonic inflammation in cirrhotic patients consuming alcohol that were not suppressed by concomitant FXR activation. The fecal total and secondary BA increase in actively drinking alcoholics was also not susceptible to the increase in FGF-19 expression in the colon and ileum and may represent a new dimension of the pathogenesis of inflammation and gut barrier injury in alcoholic liver disease.
Table 1: Median fecal bile acid concentrations between groups

<table>
<thead>
<tr>
<th>Median fecal concentrations (µmol/g)</th>
<th>Cirrhosis</th>
<th>Non-cirrhotic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not currently drinking</td>
<td>Currently drinking</td>
</tr>
<tr>
<td>Total BA***</td>
<td>2.9</td>
<td>2.2</td>
</tr>
<tr>
<td>Cholic (CA)</td>
<td>0.1</td>
<td>0.12</td>
</tr>
<tr>
<td>Chenodeoxycholic (CDCA)*</td>
<td>0.1</td>
<td>0.21</td>
</tr>
<tr>
<td>Lithocholic (LCA)*</td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>Deoxycholic(DCA)***</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Total Primary BAs*</td>
<td>0.23</td>
<td>0.46</td>
</tr>
<tr>
<td>Total Secondary BAs***</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Secondary/Primary BA Ratio*</td>
<td>7.4</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Ratios were calculated between CDCA+CA and LCA+DCA.

Medians were used due to the non-normal distribution of Bile acid (BA) profile which were compared using Kruskal-Wallis test;

***p<0.001, *p<0.05 between all five groups,
†p<0.05 in Currently drinking groups (cirrhotics and non-cirrhotics) compared to the rest of the groups.
‡p<0.05 in currently drinking non-cirrhotic patients compared to the other groups.
There was a significantly higher glyco-conjugated deoxycholic acid in the serum of current drinking alcoholic non-cirrhotics and highest tauro-conjugated deoxycholic acid in the currently drinking cirrhotics’ serum compared to the rest. As expected, conjugated and 3-sulphate conjugated CDCA was the highest in cirrhotic’s serum compared to non-cirrhotics and relatively lower in actively drinking cirrhotics compared to the other cirrhosis groups. No difference in unconjugated serum BAs or any form of UDCA was seen between groups and lithocholic acid was not detected in serum. G-glycine conjugated, T-taurine conjugated, -3S: sulphated forms, CA: cholic acid, CDCA: chenodeoxycholic acid, DCA: deoxycholic acid, UDCA: ursodeoxycholic acid.

Table 2: Serum Bile Acid Concentrations between Groups

<table>
<thead>
<tr>
<th>µmol/L (mean±SD)</th>
<th>Cirrhosis</th>
<th>Non-cirrhotic</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-alcoholic</td>
<td>Abstinent alcoholic</td>
<td>Currently drinking</td>
</tr>
<tr>
<td>Conjugated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GDCA</td>
<td>0.6±1.2</td>
<td>1.3±2.1</td>
<td>1.2±2.2</td>
</tr>
<tr>
<td>TDCA</td>
<td>0.2±0.5</td>
<td>0.4±0.6</td>
<td>1.0±2.6</td>
</tr>
<tr>
<td>GCA</td>
<td>6.1±7.5</td>
<td>5.2±5.3</td>
<td>4.3±4.3</td>
</tr>
<tr>
<td>TCA</td>
<td>5.0±6.4</td>
<td>5.2±6.5</td>
<td>3.3±5.8</td>
</tr>
<tr>
<td>GCDCA</td>
<td>17.1±21.9</td>
<td>20.2±25.2</td>
<td>12.4±15.1</td>
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<tr>
<td>TCDCA</td>
<td>18.4±23.6</td>
<td>23.4±33.3</td>
<td>11.5±18.1</td>
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<tr>
<td>GUDCA</td>
<td>11.8±44.2</td>
<td>0.8±1.0</td>
<td>0.4±0.4</td>
</tr>
<tr>
<td>TUDCA</td>
<td>1.4±4.7</td>
<td>0.2±0.21</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Double conjugated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GDCA3S</td>
<td>0.3±0.6</td>
<td>0.1±0.2</td>
<td>0.3±0.3</td>
</tr>
<tr>
<td>TDCA3S</td>
<td>0.02±0.06</td>
<td>0.01±0.04</td>
<td>0.1±0.2</td>
</tr>
<tr>
<td>TCDCA3S</td>
<td>1.1±1.8</td>
<td>0.8±1.1</td>
<td>1.1±1.8</td>
</tr>
<tr>
<td>GCDCA3S</td>
<td>1.4±1.4</td>
<td>0.9±1.1</td>
<td>1.8±3.0</td>
</tr>
<tr>
<td>TUDCA3S</td>
<td>0.3±1.1</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>GUDCA3S</td>
<td>1.6±5.8</td>
<td>0.1±0.1</td>
<td>0.6±0.9</td>
</tr>
<tr>
<td>Unconjugated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA</td>
<td>0.4±0.8</td>
<td>1.2±2.9</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>CDCA</td>
<td>0.9±1.4</td>
<td>1.9±3.9</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>UDCA</td>
<td>1.0±4.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>DCA</td>
<td>0.4±0.8</td>
<td>0.7±1.3</td>
<td>0.2±0.2</td>
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</tbody>
</table>
Table 3: Primers used for mRNA analysis

<table>
<thead>
<tr>
<th></th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>CAGATTTGAGAGTAGTGAGGAAC</td>
<td>CGCAGAATGAGATGAGTTGTC</td>
</tr>
<tr>
<td>FGF-19</td>
<td>CTCGGAGGAAGACTGTGC</td>
<td>GAAATGAGAGACTGGAAGAAAGC</td>
</tr>
<tr>
<td>FXR-α</td>
<td>ATGAACCTAGGGCTATGC</td>
<td>CCACAAACAACACACACAGC</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TGGCTTATTACAGTGCGCAATG</td>
<td>GTGGTGCTCGGAGAGTTG</td>
</tr>
<tr>
<td>SHP</td>
<td>ACTGGGTGCTGTAAG</td>
<td>GGTTCGAATGGAATGGAGG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CGAGTCTGGGCGAGTCTAC</td>
<td>GGGAGGCGTTTGGAAG</td>
</tr>
<tr>
<td>COX-2</td>
<td>CCCCTGACATCTTACGGTTTG</td>
<td>TCGCATACTCTTGTGTGGTCC</td>
</tr>
<tr>
<td>MCP-1</td>
<td>CCCACGTACCTGCTGTTAT</td>
<td>TGGCTGCTGTTGATTCTTTCT</td>
</tr>
</tbody>
</table>
Figure legends

Figures 1 through 8 show a comparison of the fold-change in the mRNA expression levels between the controls (control) compared to non-alcoholic cirrhotics (cirrhotic) and actively drinking cirrhotic patients (drinker). Significant differences are marked with asterisks.

Figure 1: IL-6, Figure 2: TNF-α, Figure 3: MCP-1, Figure 4: IL-1β, Figure 5: COX-2,
Figure 6: FXR-α, Figure 7: FGF-19, Figure 8: SHP
References:


Figure 1: Relative mRNA levels of IL-6 in sigmoid colon and ileum.
Figure 2: Relative mRNA levels of TNF-α in sigmoid colon and ileum
Figure 3: Relative mRNA levels of MCP-1 in sigmoid colon and ileum
Figure 4: Relative mRNA levels of IL-1β in sigmoid colon and ileum
Figure 5: Relative mRNA levels of COX-2 in sigmoid colon and ileum
Figure 6: Relative mRNA levels of FXR-α in sigmoid colon and ileum.
Figure 7: Relative mRNA levels of FGF-19 in sigmoid colon and ileum.

**Colon**

**Intestine**

Relative mRNA levels (FGF19)
Figure 8: Relative mRNA levels of SHP in sigmoid colon and ileum.

- **Colon**
  - Control: 2.0
  - Cirrhotics: 1.5
  - Drinker: 1.0

- **Intestine**
  - Control: 2.0
  - Cirrhotics: 1.5
  - Drinker: 1.0