Bile acid homeostasis and intestinal dysbiosis in alcoholic hepatitis

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Authorship Statement

Gabriel Perlemuter is the submission's guarantor. Specific author contributions: DC designed the project, performed the post-sequencing data processing and the

statistical analysis, and wrote the manuscript. CSV designed the project and included patients. LW designed the project and performed presequencing preparation procedures. CH performed presequencing preparation procedures. DR and LH performed bile acids analyses. AMC designed, performed and supervised experiments and wrote the manuscript. GP designed the project, included patients, supervised experiments and wrote the paper. All authors discussed the results, commented and approved the final version of the manuscript.

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Summary

Background: Intestinal microbiota plays an important role in bile acid homeostasis.

Aim: We aimed to study the structure of the intestinal microbiota and its function in bile acid homeostasis in alcoholic patients based on the severity of alcoholic liver disease.

Methods: In this prospective study, we included four groups of active alcoholic patients (N=108): two non-cirrhotic, with (noCir_AH, n=13) or without alcoholic hepatitis (noCir_noAH, n=61), and two cirrhotic, with (Cir_sAH, n=17) or without severe alcoholic hepatitis (Cir_noAH, n=17). Plasma and faecal bile acids profiles, and intestinal microbiota composition, were assessed.

Results: Plasma levels of total bile acids (84.6 vs. 6.8 μmol/l, p<0.001) and total ursodeoxycholic acid (1.3 vs. 0.3 μmol/l, p=0.03) were higher in Cir_sAH than Cir_noAH whereas faecal total (2.4 vs. 11.3, p=0.01) and secondary bile acids (0.7 vs. 10.7, p<0.01) levels were lower. Cir_sAH patients had a different microbiota than Cir_noAH patients: at the phyla level, the abundance of Actinobacteria (9 vs 1%, p=0.01) was higher and that of Bacteroidetes was lower (25 vs 40%, p=0.04). Moreover, the microbiota of Cir_sAH patients showed changes in the abundance of genes involved in 15 metabolic pathways, including upregulation of glutathione metabolism, and downregulation of biotin metabolism.

Conclusions: Patients with Cir_sAH show specific changes of the bile acid pool with a shift towards more hydrophobic and toxic species that may be responsible for the specific microbiota changes. Conversely, the microbiota may also alter the bile acid pool by transforming primary to secondary bile acids, leading to a vicious cycle.

Keywords: 16S sequencing, microbiota, UDCA, biotin, glutathione, Actinobacteria, Bacteroidetes

INTRODUCTION

Severe alcoholic hepatitis is a life-threatening complication seen in a subset of patients with alcoholic liver disease with a mortality rate of up to 25% and few therapeutic options (1,2). The causal role of the intestinal microbiota in the development and individual susceptibility to alcoholic hepatitis has only recently been shown (3–6). It has also been suggested that faecal microbiota transplantation may improve gut dysbiosis and clinical outcomes in patients with cortico-resistant severe alcoholic hepatitis in a recent pilot study (7). Nevertheless, the mechanisms related to the role of the intestinal microbiota in alcoholic liver disease are not fully understood.

The relationship between bile acids and the intestinal microbiota is complex. Bile acids have both direct antimicrobial effects on bacteria (8), and indirect effects through their signaling proprieties which allows them to induce antimicrobial peptides production (9). Detergent properties of bile acids, needed for fat digestion, influence the composition of the intestinal microbiota by acting on bacterial cell membranes (8). However, the diversity of the bile acids pool and enterohepatic circulation are dependent on the intestinal microbiota. Indeed, primary bile acids (cholic acid, CA, and chenodeoxycholic acid, CDCA) are synthesized in the liver, but secondary bile acids are produced in the digestive tract. The complex pool of bile acids is then reabsorbed in the portal circulation via a large panel of transporters. In addition, bile acids are signaling molecules involved in regulating hepatic metabolism, inflammation, and their own synthesis through the activation of various nuclear receptors, such as the farnesoid X receptor (FXR) (10).

Chronic alcohol consumption is associated with an impaired bile acids homeostasis (11–14). The level of plasma bile acids positively correlates with the histological severity of AH (11) and is predictive of poor patient survival (15). Moreover, FXR-

specific agonists attenuate chronic alcohol-induced liver injury and steatosis in experimental alcoholic liver disease models (16,17). Conversely, FXR-deficient mice develop more severe liver injury (17). Overall, these results suggest that bile acids-dependent hepatotoxicity may be due, in part, to impaired FXR signaling.

We have shown in a recent work that patients with severe alcoholic hepatitis have a specific dysbiosis that renders their liver more susceptible to alcohol-induced injury (4). This sensitivity was transmissible from patients to mice by intestinal microbiota transplant. In these humanized mice, the bile acids pool was impaired in the feces. Moreover we also showed that alcoholic patients that develop severe alcoholic hepatitis have a different microbial composition as compared to patients that develop other types of complications such as alcoholic pancreatitis (6).

However, while both the bile acids and intestinal microbiota profiles were reported in alcoholic liver disease, these studies focused either only on the bile acids profile (11,18) or intestinal microbiota profile (4) and never on both, in the same cohort of patients or didn't included patients with severe alcoholic hepatitis (12,13). In order to study the relationships between intestinal microbiota modifications and bile acids metabolism, we investigated herein the interplay between bile acids and intestinal microbiota in well phenotyped patients at different stages of alcoholic liver disease by assessing and comparing plasma and faecal bile acids profiles and intestinal microbiota composition and functions in currently drinking alcoholic patients according to the severity of liver lesions.

METHODS

Study subjects

All patients included in this prospective study were admitted to the Hepato-Gastroenterology and Nutrition Department of Antoine-Béclère University Hospital, Clamart, France, for the management of excessive drinking.

Alcoholic patients were eligible for inclusion if they were between 17 and 75 years old and had been consuming at least 50 g of alcohol/day and were negative for hepatitis B surface antigens and hepatitis C. The exclusion criteria were gastrointestinal bleeding, bacterial infection, hepatocellular carcinoma, any other carcinoma, other associated severe diseases, the presence of anti-HIV antibodies, antibiotic intake in the last three months, probiotic drugs use, refusal to undergo a liver biopsy if required (abnormal liver function), use of any hepatoprotective treatment (UDCA, TUDCA). A standardized questionnaire was used to collect information about alcohol consumption (19) and patients' families were also interviewed, when possible.

General demographic and clinical characteristics were recorded for all patients at inclusion. The study was carried out in accordance with the Helsinki Declaration and was approved by the Ile de France VII ethics committee (Bicêtre Hospital, 94270 le Kremlin-Bicêtre, France). All patients provided written informed consent for participation in the study.

Patients were classified into four groups:

- patients with alcoholic cirrhosis and severe alcoholic hepatitis (Cir_sAH, n = 17). Severe alcoholic hepatitis was suspected in patients with a Maddrey score > 32 and was confirmed by a liver biopsy (histological score for AH ≥ 6 with neutrophilic infiltration) (4,20).

- patients with alcoholic cirrhosis, but without severe alcoholic hepatitis (Cir_noAH, n = 17). As the impact of non-severe alcoholic hepatitis was limited on the parameters that we studied in non-cirrhotic patients, we pooled the patients with mild alcoholic hepatitis with patients with no alcoholic hepatitis in the cirrhotic patients group.
- patients without alcoholic cirrhosis or alcoholic hepatitis (noCir_noAH, n = 61),
- patients without alcoholic cirrhosis, but with alcoholic hepatitis (noCir_AH, n = 13). Alcoholic hepatitis was defined by aspartate aminotransferase>50, aspartate aminotransferase/alanine aminotransferase > 1.5, and both values < 400 IU/L (20,21) or, if a liver biopsy was available (12/13 patients), an AH score between 3 and 5 (with neutrophilic infiltration) or ≥ 6 (with neutrophilic infiltration and a Maddrey score <32).

Diagnosis of cirrhosis was made based on clinical examination, laboratory test, imaging and endoscopy studies or by a liver biopsy, when available. As patients with cirrhosis have a different intestinal microbiota profile than those without, and most patients with severe alcoholic hepatitis exhibit histological evidence of micronodular cirrhosis, we did not perform a global comparison between the four groups but separately compared the patients with and without cirrhosis.

Biochemical assays

Bile acids measurements in plasma of 55 patients and feces of 73 patients were performed using high-performance liquid chromatography-tandem mass spectrometry as previously described (22). Serum fibroblast growth factor-19 (FGF-19) was measured for 55 patients using a sandwich ELISA kit (R&D Systems) according to the manufacturer's instructions.

Analysis of the intestinal microbiota by 16S ribosomal RNA sequencing

Faecal samples were available for 96 patients. The composition of the faecal microbiota was analyzed by high-throughput sequencing with Illumina MiSeq technology, targeting the 16S ribosomal DNA V3-V4 region in the paired-end mode (2 x 300 base pairs) (GenoToul, Toulouse), as previously described (23). Data were processed with the quantitative insights into microbial ecology (QIIME v1.9.0) pipeline, using its default parameters. Closed reference operation taxonomic mapping was performed using the Greengenes database (v13.8, 97% sequence similarity).

The mean number of quality-controlled reads was 26535±7840 (mean ± SD) per sample (minimum count: 9833, maximum count: 56968). After rarefaction at 9,000 reads per sample, bacterial alpha diversity was estimated on the basis of the Shannon's index. OTUs with a prevalence < 5% were removed from the analysis.

Functional composition of the intestinal metagenome was predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (24). This is a computational approach that accurately predicts the abundance of gene families in the microbiota and thus provides information about the functional composition of the microbial community. Linear discriminant analysis (LDA) effect size (LEfSe) analysis was performed to identify the taxa and functions displaying the largest differences in abundance in the microbiota between groups (25). Only taxa and functions with an LDA score > 2 and a significance of < 0.05, as determined by Wilcoxon signed-rank tests, are shown. LEfSe and PICRUSt were accessed online (http://huttenhower.sph.harvard.edu/galaxy/).

Statistical analysis

The results are expressed as the means ± SD for normally distributed data or median [min, max] for non-normally distributed data. Data normality was tested for each

parameter using the Shapiro-Wilk test ($p > 0.05$). Unpaired t-tests or Mann–Whitney U-tests were used to compare continuous data between groups, depending on the data distribution. Chi2 or Fisher's exact tests were used to compare discrete parameters between groups. The Spearman correlation test was used to find correlations between bile acids and intestinal microbiota. Benjamini–Hochberg false discovery rate (FDR) correction was used to correct for multiple hypothesis testing, when applicable. A p-value < 0.05 was considered to be statistically significant. The comparisons were performed with R software v2.14.1 unless stated otherwise.

Bile acids data were processed and analyzed in MetaboAnalyst (http://www.metaboanalyst.ca) (26) using supervised and unsupervised methods: Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA). Data were log transformed and pareto-scaled and the results validated using leave-one-out cross-validation procedures.

RESULTS

Demographic and laboratory data

A total of 108 patients were included in the study. We classified patients into four groups: patients with alcoholic cirrhosis and severe alcoholic hepatitis (Cir_sAH, n = 17); patients with alcoholic cirrhosis, but without severe alcoholic hepatitis (Cir_noAH, n = 17); patients without alcoholic cirrhosis or alcoholic hepatitis (noCir_noAH, n = 61); and patients without alcoholic cirrhosis, but with alcoholic hepatitis (noCir_AH, n = 13).

The demographic and laboratory data are summarized in Table 1. There was no difference in age, sex, body mass index (BMI), or duration of alcohol intake between the groups. As expected, patients with noCir_AH had higher aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, gamma-glutamyl transferase (GGT), and C-reactive protein levels than noCir_noAH patients. Cir_sAH patients had lower alcohol consumption than the Cir_noAH patients and higher total bilirubin and C-reactive protein levels, a higher MELD score, and lower albumin levels.

Intestinal microbiota profiles

We first studied interindividual bacterial diversity (beta diversity). Cir_sAH patients had a different intestinal microbiota structure (proportion of bacteria) than Cir_noAH patients (weighted UNIFRAC distances, R = 0.09, p = 0.04) (Figure 1A). There was no difference in the overall bacterial composition of the intestinal microbiota between these two groups (unweighted UNIFRAC distances, R = 0.05, p = 0.1) (Figure 1B). This result suggests that the two groups have an intestinal microbiota with similar bacterial species, but with different relative abundances. There was no difference in the beta diversity between noCir_noAH and noCir_AH patients (data not shown).

There was also no difference in the intra-individual bacterial diversity (alpha diversity) either between the noCir_noAH and noCir_AH groups, nor the Cir_sAH and Cir_noAH groups, measured by various indices (observed OTUs, Shannon index, Chao index, and PD whole tree index, data not shown).

At the phyla level, Cir_sAH patients had a higher abundance of Actinobacteria and lower abundance of Bacteroidetes than Cir_noAH patients. Among Actinobacteria, Cir_sAH patients had a higher abundance of *Actinomyces*, *Rothia*, and *Bifidobacterium* than Cir_noAH patients. Among Proteobacteria, Cir_sAH patients had a higher abundance of *Haemophilus* and *Enterobacteriaceae* and a lower abundance of *Bilophila* than Cir_noAH patients. Cir_sAH patients also had a lower relative abundance of *Parabacteroides* (Bacteroidetes phylum), *Oscillospira*, and *Christensenellaceae* families (Firmicutes phylum) and a higher relative abundance of *Lactobacillus* and *Lactococcus* (Firmicutes phylum) than Cir_noAH patients (Figure 1C and Supplemental Table 1).

Although there was no difference in the overall composition of the intestinal microbiota between noCir_AH and noCir_noAH patients, LEFsE analysis showed that noCir_AH patients had a higher abundance of *Dorea* (Firmicutes phylum), *Wolbachia* (Proteobacteria phylum) and *Rothia* (Actinobacteria phylum) than noCir_noAH patients (Figure 1D). These results suggest that a specific dysbiosis is associated with hepatic inflammation in AH in both patients with and without cirrhosis and independently of alcohol consumption.

Functional Intestinal Metagenome Prediction in Alcoholic Hepatitis:

The dysbiosis identified in Cir_sAH patients prompted us to also examine the metabolic pathways associated with this specific intestinal microbiota. The intestinal

microbiota of the Cir_sAH group had a higher proportion of metabolic pathways containing gene functions, such as glutathione metabolism, membrane transport (phosphotransferase system), and nucleotide metabolism than that of the Cir_noAH group. The intestinal microbiota of Cir_sAH patients also had a lower proportion of genes for energy metabolism (methane metabolism and carbon fixation pathways in prokaryotes), amino acid metabolism (arginine, proline and histidine metabolism), lipid metabolism (lipid biosynthesis proteins), glycan biosynthesis and metabolism, metabolism of cofactors and vitamins (biotin metabolism), metabolism of terpenoïds and polyketides (polyketide sugar unit biosynthesis), biosynthesis of other secondary metabolites (streptomycin biosynthesis), and of the transcription machinery than that of Cir_noAH patients (Figure 1E). There was no difference between the two groups in the secondary bile acids biosynthesis pathway.

These results indicate that the dysbiosis observed in patients with severe alcoholic hepatitis is also associated with a shift in the bacterial metabolic pathways.

Bile acids

Bile acids can shape the intestinal microbiota, and in turn, the intestinal microbiota alters the bile acids pool. Thus, we studied plasma bile acids and faecal bile acids profiles and their relationship with the dysbiosis observed in alcoholic patients with and without alcoholic cirrhosis.

Plasma bile acid profile

We first studied the plasma bile acids profile associated with liver inflammation in alcoholic patients. Cir_sAH patients had a different plasma bile acids profile than Cir_noAH patients, as shown by PCA (Figure 2A) and on a heatmap (Supplementary figure 1A). Cir_sAH patients had higher levels of total bile acids, total primary bile

acids, total conjugated bile acids (total glyco- and tauroconjugated bile acids), primary glyco- and tauroconjugated bile acids, total CA (glycocholate (GCA) and taurocholate (TCA)), total CDCA (glycochenodeoxycholate (GCDCA) and taurochenodeoxycholate (TCDCA)), and total UDCA (tauroursodeoxycholate (TUDCA)) than Cir_noAH patients (Figures 2 B-D and Supplementary Table 2). Because of the higher total bile acids levels in Cir_sAH patients, we also studied the relative proportion of each bile acids (bile acids concentration/total bile acids concentration) between the two groups. Cir_sAH patients had a higher relative proportion of total primary bile acids, total CDCA (TCDCA), total primary conjugated bile acids, primary tauroconjugated bile acids, and TUDCA than Cir_noAH patients. They also had a lower relative proportion of total sulphoconjugated bile acids, total secondary bile acids, total secondary conjugated bile acids, secondary glycoconjugated bile acids, total lithocholate (LCA) (LCA, glycolithocholate (GLCA), lithocholate-3-sulfate (LCA3s), taurolithocholate-3-sulfate (TLCA3s), and glycolithocholate-3-sulfate (GLCA3s)), total deoxycholate (DCA) (DCA; glycodeoxycholate (GDCA), and taurodeoxycholate (TDCA)), chenodeoxycholic acid 3-sulfate (CDCA3s), and glycoursodeoxycholate-3-sulfate (GUDCA3s) than Cir_noAH patients (Figures 2 B, C, E).

We then used PLS-DA to find the plasma bile acids that best discriminate between the two groups. The model showed a significant distinction (R2 = 0.6, Q2 = 0.4, prediction accuracy during training: p = 0.001, 1000 permutations) between the Cir_sAH and Cir_noAH groups (Figure 3A). The average AUROC confirmed that the model was able to discriminate Cir_noAH from Cir_sAH patients (0.955, 95% CI: 0.763-1, Supplementary Figure 2A). TUDCA was the most discriminant bile acids

between the two groups according to the PLS-DA (variable importance in projection, VIP = 2.1, Figure 3B).

Comparison of the bile acids profile of noncirrhotic patients showed higher levels of TUDCA in noCir_AH patients than noCir_noAH patients. Moreover, noCir_AH patients had higher proportions of TUDCA and total conjugated bile acids and a lower proportion of DCA than noCir_AH patients (data not show). However, these changes were no longer significant after correction for multiple comparisons.

These results indicate an increase in the pool of bile acids in Cir_sAH patients Moreover, the increase in TUDCA levels, a bile acids produced exclusively by the intestinal microbiota from CDCA, and that of primary bile acids s are consistent with a shift in bile acids transformation in the gut.

Faecal bile acids

We further studied the bile acids profile in Cir_sAH patients. Cir_sAH patients had a different faecal bile acids profile than Cir_noAH patients (Figure 4A and Supplementary Figure 1B). Cir_sAH patients had lower total faecal bile acids, total unconjugated bile acids, total glycoconjugated bile acids, total secondary bile acids, secondary unconjugated bile acids, secondary glyco- and tauroconjugated bile acids, total LCA (LCA), and total DCA (DCA, GDCA, TDCA, deoxycholate 3-sulfate: DCA3s) than Cir_noAH patients (Figures 4B-D and Supplementary Table 3). We also examined the relative amount of each bile acids (faecal bile acids/total faecal bile acids). Cir_sAH patients had a higher percentage of total primary bile acids, total CDCA (CDCA, TCDCA), primary unconjugated bile acids, and CA than Cir_noAH patients and a lower percentage of total secondary bile acids, total secondary

unconjugated bile acids, secondary glycoconjugated bile acids, secondary tauroconjugated bile acids, and total DCA (DCA, GDCA, TDCA) (Figures 4 B, C, E).

PLS-DA showed a significant distinction (R2 = 0.8, Q2 = 0.6, prediction accuracy during training: p = 0.001, 1000 permutations) between the Cir_sAH and Cir_noAH groups (Figure 5A). The average AUROC confirmed that the model was able to discriminate Cir_noAH from Cir_sAH patients (0.977, 95% CI: 0.769-1) (Supplementary Figure 2B). Secondary glycoconjugated bile acids and GDCA were the most discriminant bile acids between the two groups according to the PLS-DA (variable importance in projection, VIP = 2.1 and 2.1, respectively, Figure 5B).

Comparison of the faecal bile acids profiles between noncirrhotic patients showed the observed changes (higher taurolithocholate, TLCA, and GLCA ratios in noCir_AH than noCir_noAH patients) to no longer be significant after correction for multiple comparisons (data not show), as observed in plasma.

These results confirm the decrease in bile acids excretion and impaired bile acids transformation in the gut by the intestinal microbiota of Cir_sAH patients relative to that of Cir_noAH patients.

The relationship between intestinal microbiota and bile acids homeostasis

We assessed the correlation between the bile acids profiles with the bacteria species identified in the feces, as the composition and quantity of the bile acids pool influence the intestinal microbiota, and conversely, the metabolism of bile acids is dependent on intestinal microbiota composition. Primary and secondary plasma bile acids levels positively correlated with most of the taxa in Cir_noAH patients (Figure 6A) while primary plasma bile acids negatively correlated with taxa from Bacteroidetes and Firmicutes phyla in Cir_sAH patients (Figure 6B). Total UDCA, TUDCA, and

glycoursodeoxycholate (GUDCA) positively correlated with most of the taxa in Cir_noAH patients while it was negatively correlated with taxa from the Cir_sAH patients (Figure 6A and 6B).

Faecal primary bile acids were mostly negatively correlated with most of the taxa in Cir_noAH patients (Figure 6C) while in Cir_sAH patients, primary faecal bile acids were negatively correlated and secondary faecal bile acids were positively correlated with most of the taxa (Figure 6D)

These correlations suggest that the abundance of bacteria carrying the enzymes needed for bile acids deconjugation and transformation into secondary bile acids and UDCA is reduced in the intestinal microbiota of Cir_sAH patients.

FXR-FGF-19 in severe alcoholic hepatitis

As signaling molecules, bile acids activate ileal FXR and induce the production of FGF19. Cir_sAH patients had higher plasma levels of FGF19 than Cir_noAH patients (282 ± 431 *vs.* 55 ± 75 pg/mL, p = 0.03, Table 1). In patients without cirrhosis, plasma levels of FGF19 were higher in patients with AH patients than those without, but did not reach statistical significance (154 ± 368 *vs*. 69 ± 74 pg/mL, p = 0.5). However, FGF-19 positively correlated with the MELD score (r = 0.49, p = 0.04), but not the Maddrey or AH histological scores.

These results suggest that FXR is activated in Cir_sAH patients independently of the faecal bile acids concentration in the gut.

DISCUSSION

In this study, we characterized the intestinal microbiota, its functions, and its relationship with bile acids homeostasis in well phenotyped alcoholic liver disease patients, in order to overcome potential confounders such as alcoholic liver disease stage and previous or concomitant treatments. Moreover, as alcohol induces a specific dysbiosis in both animal models of alcoholic liver disease (4,23,27) and humans (12,13,28), including higher levels of some members of Proteobacteria and lower Bacteroidaceae, Lachnospiraceae, and Prevotellaceae, we only compared patients with ongoing alcohol consumption. This allowed us to identify specific changes related only to the liver disease, independently on the amount and duration of alcohol consumption.

Alcoholic hepatitis did not modify the overall composition of the intestinal microbiota in patients without cirrhosis. A similar result has been recently reported in a mouse model of acute-on-chronic alcohol feeding (29). However, we observed an increase in the abundance of *Dorea, Wolbachia* and *Rothia* in noCir_AH patients. Among patients with alcoholic cirrhosis, severe alcoholic hepatitis patients had higher abundance of bacteria of the Actinobacteria phylum including the *Actinomyces*, *Rothia*, and *Bifidobacterium* genus. The abundance of *Lactobacillus* (Firmicutes phylum), *Haemophylus* (from the Pasteurellaceae family, Proteobacteria phylum), and an unidentified member of the Enterobacteriaceae family (Proteobacteria phylum) was also higher. Conversely, the abundance of bacteria of the Bacteroidetes phylum was lower. Interestingly, these changes are consistent with data from other studies that investigated the intestinal microbiota in alcoholic liver disease and in other liver diseases such as NAFLD (28–32), suggesting that these changes may be related to cirrhosis and impaired liver function rather than to the cause of liver

disease. These results also confirms the increase seen in *Bifidobacterium* genus in severe alcoholic hepatitis patients that we previousely reported in a smaller sample of severe alcoholic hepatitis patients (4). Furthermore, by increasing the number of patients included in the present study we also identifies new taxa associated with severe alcoholic hepatitis as compared to our previous study.

We further explored the role of the intestinal microbiota in Cir_sAH using PICRUSt to predict the metagenomic profile of the intestinal microbiota. We observed a switch in the functions of the intestinal microbiota in Cir_sAH patients, including a decrease in the biotin metabolic pathway. Biotin is a member of the vitamin-B family of vitamins and acts as a cofactor for several carboxylases in mitochondria. Exogenous biotin is obtained from dietary sources or intestinal biotin-producing bacteria (33). Plasma biotin levels in chronic alcohol patients are reduced due to inhibition of carrier-mediated biotin transport in the jejunum and colon (34). Thus, reduced production in the gut and decreased absorption in alcoholic patients could lead to the dysfunction of mitochondria, which could impair the hepatic response to inflammation in severe alcoholic hepatitis.

We also observed altered glutathione metabolism in the intestinal microbiota of Cir_sAH patients. Glutathione is a powerful antioxidant and patients with alcoholic liver disease have low hepatic and plasma glutathione levels (35). In this context, it has been shown that the bile salt hydrolase (BSH) gene from *Bifidobacterium longum* is co-transcribed with the gene encoding glutamine synthetase adenylyltransferase (glnE), a component of the nitrogen regulation cascade (36). Thus, the increase in the abundance of *Bifidobacterium* in Cir_sAH patients could be responsible, at least partially, for the increased proportion of primary unconjugated faecal bile acids, as a

result of BSH activity and increased glutathione metabolism of the intestinal microbiota of these patients.

It has been suggested, in a previous work, that an increase in primary bile acids was associated with the severity of histological lesions in AH (11). A similar result was observed in our study. Of note, in the previous work (11), the authors provided an overall plasma bile acids profile in patients with AH ranging from mild AH to severe alcoholic hepatitis and irrespective of the presence of cirrhosis, that was present in 75 % of their patients. As alcoholic cirrhosis is associated with an impaired bile acids profile (12,13), their results might be biased by the mix of cirrhotic and non-cirrhotic patients. Moreover, according to the method used for bile acids assay (HPLC), they could only detect primary and secondary bile acids but neither their conjugated forms nor UDCA. Other studies have investigated plasma bile acids, faecal bile acids and intestinal microbiota in alcoholic patients (12,13). They suggested, that alcohol intake in both cirrhosis and non-cirrhotic patients is associated with a decrease in conjugated CDCA in plasma (12). In our study, CDCA was increased in severe alcoholic hepatitis, suggesting that this increase is due to liver inflammation (ie severe alcoholic hepatitis) independently of the presence of cirrhosis of alcohol intake. We also suggest that perturbation of intestinal microbiota is involved in the specific modifications bile acids metabolism observed in patients with severe alcoholic hepatitis.

More hydrophobic bile acids (CA, CDCA, DCA) rapidly induce apoptosis (37), whereas less hydrophobic bile acids (UDCA) are less toxic (38). Moreover, total plasma bile acids and primary plasma bile acids (CA and CDCA) levels have been shown to positively correlated with the AH severity and steatosis (11,18,39). Several mechanisms may explain the increase of the bile acids pool, including upregulation of

cholesterol 7α-hydroxylase (Cyp7A1) induced by both chronic and acute alcohol consumption (40) and/or a decrease in bile acids excretion in the bile and subsequent release in the plasma in the context of AH. Moreover, it has also been suggested that the intestinal microbiota can contribute to biliary inflammation (41), which could impair bile acids circulation. These hypotheses are supported by the increased levels of primary and conjugated plasma bile acids in the Cir_sAH patients and decrease in the proportion of secondary plasma bile acids and total faecal bile acids. We can thus hypothesize that the excess plasma bile acids do not reach the gut where they could be deconjugated and transformed into secondary bile acids. Moreover, conjugation of either taurine or glycine to bile acids decreases their hydrophobicity and thus their toxicity. There was also a trend towards switching the plasma bile acids pool from gylcoconjugated forms towards tauroconjugated forms, which are less toxic.

CDCA is the most potent FXR agonist capable of inducing FGF19 expression (42). Here, plasma FGF-19 levels were higher in Cir_sAH than Cir_noAH patients. FGF-19 is produced in the ileum by FXR activation. Faecal bile acids activates FXR and acts in a negative feedback loop by blocking CYP7A1 and bile acid synthesis (classical pathway). FGF-19 is absent from primary, non-activated hepatocytes, but bile acids - activated hepatic FXR can induce FGF19 secretion *in vitro* (43) and *in vivo* in patients with cholestasis (44) by an autocrine/paracrine mechanism, independently of SHP (43,44). However, a recent study found that FGF19 levels were significantly elevated in patients with alcoholic hepatitis while serum 7-alpha-hydroxy-4-cholesten-3-one (C4) levels, a bile acids synthesis marker for de novo synthesis was decreased suggesting a prominent role of cholestasis (14). Moreover, the authors showed that in alcoholic hepatitis FGF-19 originates in cholangiocytes and ductular cells from

smaller ductules (progenitor cells). Here, FGF-19 positively correlated with the MELD score. This is consistent with other studies that reported a correlation between FGF-19 levels and liver disease severity (14,44) and suggests that the increase in FGF-19 levels observed in our study has a double origin, hepatic (due to increased plasma bile acids , dominated by CDCA) and intestinal. Indeed, there was a shift of the faecal bile acids pool in the gut toward species with a higher affinity for FXR, as shown by the increase in the proportion of CDCA, although there was an overall decrease in total faecal bile acids. Thus, high levels of FGF-19 could increase CDCA synthesis by directing bile acids synthesis from the classic (neutral) to the alternative (acidic) pathway, due to the blockade of CYP7A1, but not of cholesterol 7β-hydroxylase (CYP7B1). Therefore, high levels of FGF-19 are probably insufficient to counteract the increased bile acids synthesis induced by alcohol in alcoholic liver disease. Moreover, it promotes the shift of the bile acids pool towards more hydrophobic, toxic species.

There was also an increase in total plasma UDCA and TUDCA levels in Cir_sAH patients relative to Cir_noAH patients. These bile acids have hepatoprotective effects. However, TUDCA exerts this effect by replenishing hepatic mitochondrial glutathione (45). Thus, the increase in glutathione metabolism of the intestinal microbiota, which may decrease its bioavailability to the mitochondria, combined with an intestinal microbiota-associated decrease in the levels of biotin, an essential cofactor of mitochondrial metabolism, could explain why the increased TUDCA levels seen in Cir_sAH patients does not have a hepatoprotective effect. This is supported by the fact that UDCA showed hepatoprotective effects in *in vitro* studies and early stages of alcoholic liver disease, but not in severe alcoholic hepatitis patients with cholestasis (46).

Specific bacteria may be involved in the production of UDCA in severe alcoholic hepatitis patients. Plasma UDCA, that was increased in severe alcoholic hepatitis patients, and that is only produced by bacteria in the gut from CDCA, positively correlated with the abundance of Actinobacteria and Proteobacteria phyla. The abundance of these phyla was increased in severe alcoholic hepatitis patients, as was the abundance of the *Bifidobacteria* and *Clostridium* genera. Administration of *Bifidobacteria animalis*, as a bile salt-hydrolysing bacteria, and *Clostridium absonum*, as a CDCA to UDCA epimerizing bacteria, result in increased levels of faecal UDCA in pigs (47).

An increased level of faecal bile acids was reported in cirrhotic patients with ongoing alcohol consumption but, in these patients, the consequences of a potential liver inflammatory process (ie alcoholic hepatitis) is unknown (12,13). We now show that total faecal bile acids levels probably decreased in Cir_sAH patients due to decreased excretion of plasma bile acids in the bile, as discussed earlier. Faecal bile acids shape the intestinal microbiota as deconjugation provides cellular carbon, nitrogen, and sulfur for some bacterial species, especially *Bacteroides and Bilophila* (48). Thus, the decrease in faecal bile acids levels in Cir_sAH patients may be responsible for the decrease in the abundance of Bacteroidetes and *Bilophila*. Moreover, primary faecal bile acids levels have been shown to increase intestinal permeability (49), which can increase PAMP release into the systemic circulation, participating in the higher levels of endotoxemia observed in alcoholic liver disease patients. Furthermore, bile acids bactericidal activity is related to their hydrophobicity, which increases their affinity for the phospholipid bilayer of the bacterial cell membrane, and unconjugated bile acids are week acids with strong bactericidal activities. Among the bile acids, DCA is extremely toxic and inhibits the growth of

many intestinal bacteria, including *Clostridium perfringens*, *Bacteroides fragilis,* *Lactobacilli*, and *Bifidobacteria* (50,51). In our study we observed a decrease in unconjugated bile acids, total secondary bile acids and DCA in the feces, that are highly hydrophobic. This may be responsible for the increase levels of *Lactobacillus* and *Bifidobacterium* seen in the microbiota of these patients. It has also been suggested that a decreased level of faecal bile acids stimulated the growth of gram-negative and conversely decreases the growth gram-positive bacteria (52). Indeed, we observed in severe alcoholic hepatitis patients an increase in several taxa that are gram-negative (eg Gammaproteobacteria) that could be secondary to the decrease faecal bile acids level. Moreover, gram-negative bacteria produce LPS that was related to increases alcoholic liver necrosis and inflammation (53). We also observed in our study a decrease in several taxa from the gram-positive Firmicutes phylum (Christensenellaceae, Oscillospira) that 7α-dehydroxylate primary bile acids to toxic secondary bile acids. Thus, the decrease and shift of the bile acids pool in the feces could be responsible for the increase in LPS-producing bacteria and for the decrease of gram-positive members of Firmicutes able to transform primary bile acids into secondary bile acids. This hypothesis may explain the decrease in secondary bile acids in severe alcoholic hepatitis patients observed in our study.

A limit of our study was a potential lack of power related to the small number of patients, which did not allow us to identify changes in taxa with low counts. However, the recruitment of severe alcoholic hepatitis patients for intestinal microbiota studies is challenging, as most are rapidly treated (often by antibiotics to prevent or treat complications). This bias did not occur in our patients as they were included before any specific treatment for severe alcoholic hepatitis . Moreover, we did not exclude patients with proton-pump inhibitors intake which was shown to alter the IM

composition (54). However, there was no difference in the use of proton-pump inhibitors between groups, suggesting that they are not responsible for the results observed in our study.

In conclusion, severe alcoholic hepatitis is associated with specific alterations of the bile acids homeostasis and of the intestinal microbiota. These changes are characterized by an increased level of hydrophobic bile acids and of Actinobacteria and a decrease of Bacteroidetes. The increase and shift in the bile acids pool towards hydrophobic and toxic species could be responsible for the specific intestinal microbiota changes, including an increase in the LPS-producing gram-negative bacteria such as Gammaproteobacteria and a decrease in certain gram-positive bacteria capable to transform primary into secondary bile acids. Furthermore, the changes in the intestinal microbiota were associated with a shift in its functions, especially decreased biotin metabolism and increased glutathione metabolism, which could play a role in the initiation and progression of severe alcoholic hepatitis, through impairment of the protective effects of UDCA on mitochondrial metabolism. Our study provides a new hypothesis for future studies to address bile acids and the intestinal microbiota as new therapeutic targets to improve the management of alcoholic liver disease patients.

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Table 1: Patient's characteristics

	Without alcoholic cirrhosis		Alcoholic cirrhosis	
	noCir_noAH	noCir_AH	Cir_noAH	Cir_sAH
	(n=61)	(n=13)	(n=17)	(n=17)
Age (years)	51±8.5	46±8.1	58±9.8	56±12.3
Sex: male/female (%)	50/10(83/17)	11/2(85/15)	15/3 (83/17)	14/4(78/22)
BMI (kg/m2)	23.4 [16.4-31.2]	23.9 [20-41.2]	23.7 [15.9-31.2]	24.2 [19.4-39]
Alcohol intake (g/day)	150 [50-500]	200 [60-400]	140 [50-360]	80 [50-240]*
Alcohol time (years)	15 [0.5-40]	10 [1-40]	22.5 [3-50]	20 [6-40]
Smoking (%)	48 (81)	9 (70)	13 (72)	13 (72)
PPIs use (%)	5 (8)	1 (8)	7 (41)	7 (41)
Diabetes	5 (8)	1 (8)	4 (22)	2 (11)
AST (U/L)	47 [15-240]	175 [58-511]***	64 [38-252]	88 [18-2657]*
ALT (U/L)	40.5 [8-224]	59 [32-217]*	40.5 [11-143]	41.5 [19-481]
Total bilirubin (µmol/L)	12 [6-34]	22 [10-186]**	34.5 [4-110]	143 [44-751]***
GGT (U/L)	141 [18-1641]	928 [79-3970]**	382 [58-3923]	185.5 [45-990]
Platelets (x10^9/L)	189.5 [36-455]	193 [71-459]	98.5 [53-378]	94 [33-475]
Prothrombine Time (%)	100 [67-100]	91 [79-100]	66 [25-100]	35 [25-64]***
Glucose (mmol/L)	4.9 [3.8-11.8]	4.8 [4.3-7.5]	5.2 [4.4-13.6]	5.2 [4.4-8.9]
Albumin (g/L)	37.8 [27.6-46.7]	39.5 [23.1-47.5]	36.1 [21.5-40.8]	30 [22.3-36]***
Creatinin (µmol/L)	74 [50-107]	73 [57-118]	71 [52-130]	73 [57-207]
CRP (mg/L)	5 [5-54]	8 [5-91]*	5 [5-34]	22 [5-127]**
Maddrey Score	1.15 [0.35-30.9]	5.5 [0.59-16.5]	19 [0.2-60]	48.9 [32-101]***
FGF-19 (pg/mL)	86 [0-600]	36.18 [4.8-1258]	66 [16-541]	166 [24-2450]
MELD Score			13.7±7.2	24.33±6.8***
Liver Biopsy (%)	24 (39)	11 (85)*	12 (71)	17 (100)*

The data are expressed as the mean ± SD for continuous variables with a normal distribution, median and min and max for data with a non-normal distribution, and *n* (%) for discrete variables. Comparisons between noCir and AH patients, and alcoholic cirrhosis (Cir) and severe alcoholic hepatitis (sAH) patients in Mann-Whitney tests or independent *t*-tests for continuous data and χ^2 tests or Fisher's exact tests for discrete data. **p*<0.05; ***p*<0.01; ****p*<0.001. BMI, body mass index; PPIs, proton-pump inhibitors, AST, alanine aminotransferase; ALT, aspartate aminotransferase; GGT, gammaglutamyltransferase; CRP, C-reactive protein; MELD, Model for End-stage Liver Disease, FGF-19, fibroblast growth factor-19 .

Figure legends

Figure 1: Intestinal microbiota profiles and its metabolic functions. (**A**) Weighted UniFrac distances (quantitative method reflecting the structure of the intestinal microbiota) and (**B**) Unweighted UniFrac distances (qualitative method reflecting the composition of the intestinal microbiota) showing a difference in the structure of the intestinal microbiota only between Cir_sAH patients (blue) and Cir_noAH patients (red, $p < 0.05$ for Weighted UniFrac distances). Each point represents a subject and the distance between the points is proportional to the similarity of the intestinal microbiota. Cladogram showing the taxa with the largest differences in abundance between (**C**) Cir_sAH patients (green) and Cir_noAH patients (red) and (**D**) noCir_AH patients (red) and noCir_noAH patients. The size of the circle in the cladogram plot is proportional to bacterial abundance. From inside to outside, the circles represent phylum, class, order, family, and genus. Only taxa with a LDA score > 2 and $p < 0.05$, determined by the Wilcoxon signed rank test, are shown. (**E**) LDA Effect Size (LEfSe) for the predicted metagenome metabolic pathways (KEGG modules) increased in Cir_sAH (green) and Cir_noAH patients (red) (LDA score > 2.0, $p < 0.05$ determined by the Wilcoxon signed rank test).

Figure 2: Plasma bile acids profiles in patients with alcoholic liver disease. (**A**) PCA ordination plot with 95% confidence ellipse for all plasma bile acids s in cir_sAH and cir_noAH patients showing clustering of patients according to the liver complication. The first two components of the PCA explained 64% of the total variance (component 1 = 42.4%; component 2 = 21.5%). (**B**) Total plasma bile acids , primary, total conjugated, glyco-conjugated and tauro-conjugated levels of plasma bile acids . (**C**) and (**D**) plasma bile acids composition (% of total plasma bile acids). (**E**) Individual plasma bile acids levels.*$p < 0.05$, **$p < 0.01$. CA: cholic acid;

CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; LCA: lithocholic acid; GCA, glycocholic acid: GCDCA: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid; sAH: severe alcoholic hepatitis; TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid; TLCA: taurolithocholic acid; TUDCA: tauroursodeoxycholic acid; UDCA: ursodeoxycholic acid; _3s: sulfated forms.

Figure 3: Specificity of the plasma bile acids profile depending on alcoholic-induced liver inflammation. (**A**) PLS-DA score plot of plasma bile acids concentrations in Cir_sAH *vs.* Cir_noAH patients with the 95% confidence ellipse showing a significant difference between the two groups (R2 = 0.6, Q2 = 0.4, p = 0.001). (**B**) Variable importance in projection (VIP) of PLS-DA showing the plasma bile acids that discriminate Cir_sAH from Cir_noAH patients (VIP score >1). The colored boxes on the right indicate the relative concentrations of the corresponding plasma bile acids in each group.

Figure 4: Faecal bile acids profiles in alcoholic liver disease. (**A**) PCA ordination plot with 95% confidence ellipse for all faecal bile acids showing clustering of patients according to the liver complication. The first two components of the PCA explained 57% of the total variance (component 1 = 39.3%; component 2 = 17.9%). (**B**) Total faecal bile acids, total unconjugated, secondary and secondary unconjugated levels of faecal bile acids. (**C**) and (**D**) faecal bile acids composition (% of total faecal bile acids). (**E**) Individual faecal bile acids levels. *p < 0.05, **p < 0.01. CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; LCA: lithocholic acid; GCA: glycocholic acid; GCDCA: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid; sAH: severe alcoholic hepatitis; TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid;

TDCA: taurodeoxycholic acid; TLCA: taurolithocholic acid; TUDCA: tauroursodeoxycholic acid; UDCA: ursodeoxycholic acid; _3s: sulfated forms.

Figure 5: Specificity of faecal bile acids profiles depending on alcoholic-induced liver inflammation. (**A**) PLS-DA score plot of faecal bile acids concentrations of Cir_sAH *vs.* Cir_noAH patients with 95% confidence ellipse showing a significant difference between the two groups (R2 = 0.8, Q2 = 0.6, p = 0.001). (**B**) Variable importance in projection (VIP) of PLS-DA showing the faecal bile acids that discriminate Cir_sAH from Cir_noAH patients (VIP score >1). The colored boxes on the right indicate the relative concentrations of the corresponding faecal bile acids in each group.

Figure 6: Heatmap representation of the Spearman's r correlation coefficient between bacterial taxa (phylum and genus level) and bile acids profiles in plasma of Cir_noAH (**A**) and Cir_sAH (**B**) patients and feces of Cir_noAH (**C**) and Cir_sAH (**D**). Only the bacteria for which at least one significant correlation with bile acids was found are displayed (p, phyla; g, genus). CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; LCA: lithocholic acid; GCA: glycocholic acid; GCDCA: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid; TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid; TLCA: taurolithocholic acid; TUDCA: tauroursodeoxycholic acid; UDCA: ursodeoxycholic acid; A: Actinobacteria, B: Bacteroidetes, F: Fusobacteria; P: Proteobacteria. *Adjusted p value < 0.05. Red: negative correlation, blue: positive correlation.

Figure 1

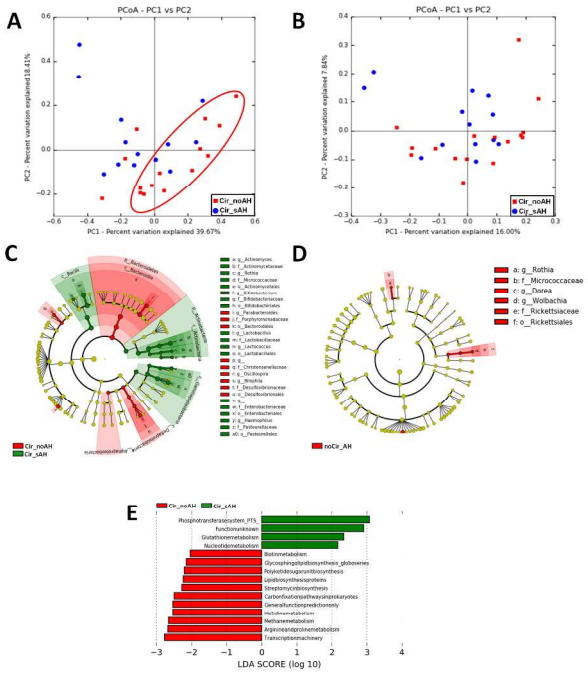

Figure 1: Intestinal microbiota profiles and its metabolic functions. (A) Weighted UniFrac distances (quantitative method reflecting the structure of the intestinal microbiota) and (B) Unweighted UniFrac distances (qualitative method reflecting the composition of the intestinal microbiota) showing a difference in the structure of the intestinal microbiota only between Cir_sAH patients (blue) and Cir_noAH patients (red, $p < 0.05$ for Weighted UniFrac distances). Each point represents a subject and the distance between the points is proportional to the similarity of the intestinal microbiota. Cladogram showing the taxa with the largest differences in abundance between (C) Cir_sAH patients (green) and Cir_noAH patients (red) and (D) noCir_AH patients (red) and noCir_noAH patients. The size of the circle in the cladogram plot is proportional to bacterial abundance. From inside to outside, the circles represent phylum, class, order, family, and genus. Only taxa with a LDA score > 2 and $p < 0.05$, determined by the Wilcoxon signed rank test, are shown. (E) LDA Effect Size (LEfSe) for the predicted metagenome metabolic pathways (KEGG modules) increased in Cir_sAH (green) and Cir_noAH patients (red) (LDA score > 2.0, $p < 0.05$ determined by the Wilcoxon signed rank test).

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Figure 2

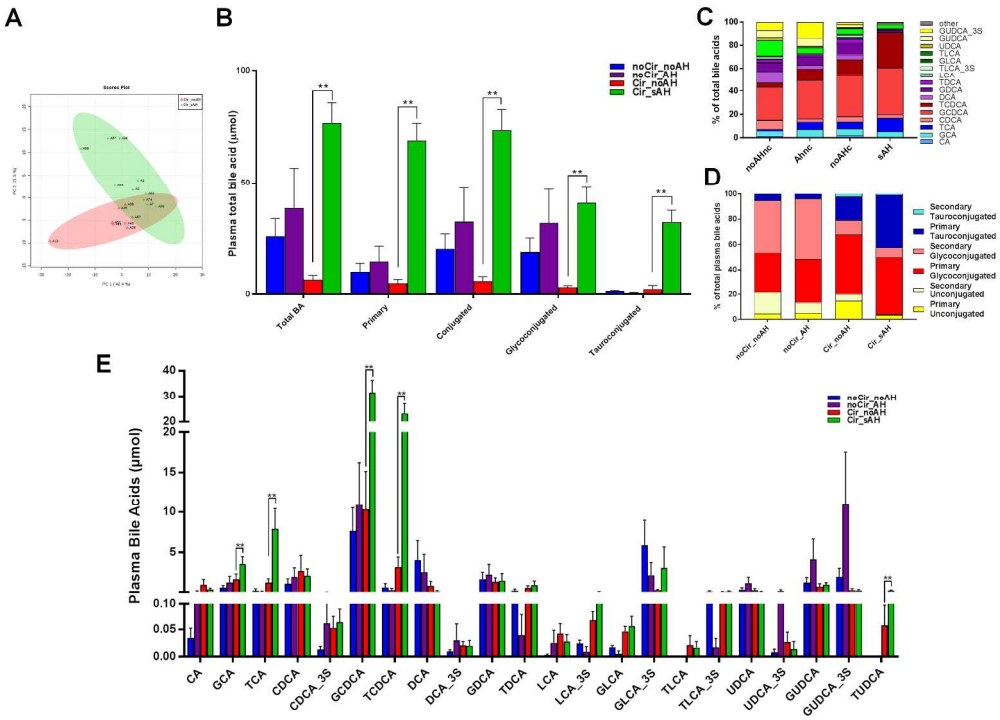

Figure 2: Plasma bile acids profiles in patients with alcoholic liver disease. (A) PCA ordination plot with 95% confidence ellipse for all plasma bile acids s in cir_sAH and cir_noAH patients showing clustering of patients according to the liver complication. The first two components of the PCA explained 64% of the total variance (component 1 = 42.4%; component 2 = 21.5%). (B) Total plasma bile acids , primary, total conjugated, glyco-conjugated and tauro-conjugated levels of plasma bile acids . (C) and (D) plasma bile acids composition (% of total plasma bile acids). (E) Individual plasma bile acids levels.*p < 0.05, **p < 0.01. CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; LCA: lithocholic acid; GCA, glycocholic acid: GCDCA: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid; sAH: severe alcoholic hepatitis; TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid; TLCA: taurolithocholic acid; TUDCA: tauroursodeoxycholic acid; UDCA: ursodeoxycholic acid; _3s: sulfated forms.

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Figure 3

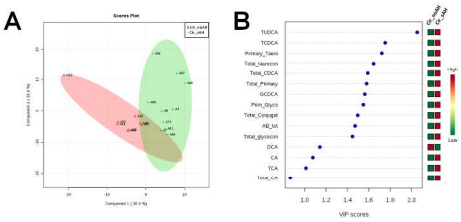

Figure 3: Specificity of the plasma bile acids profile depending on alcoholic-induced liver inflammation. (A) PLS-DA score plot of plasma bile acids concentrations in Cir_sAH vs. Cir_noAH patients with the 95% confidence ellipse showing a significant difference between the two groups (R2 = 0.6, Q2 = 0.4, p = 0.001). (B) Variable importance in projection (VIP) of PLS-DA showing the plasma bile acids that discriminate Cir_sAH from Cir_noAH patients (VIP score >1). The colored boxes on the right indicate the relative concentrations of the corresponding plasma bile acids in each group.

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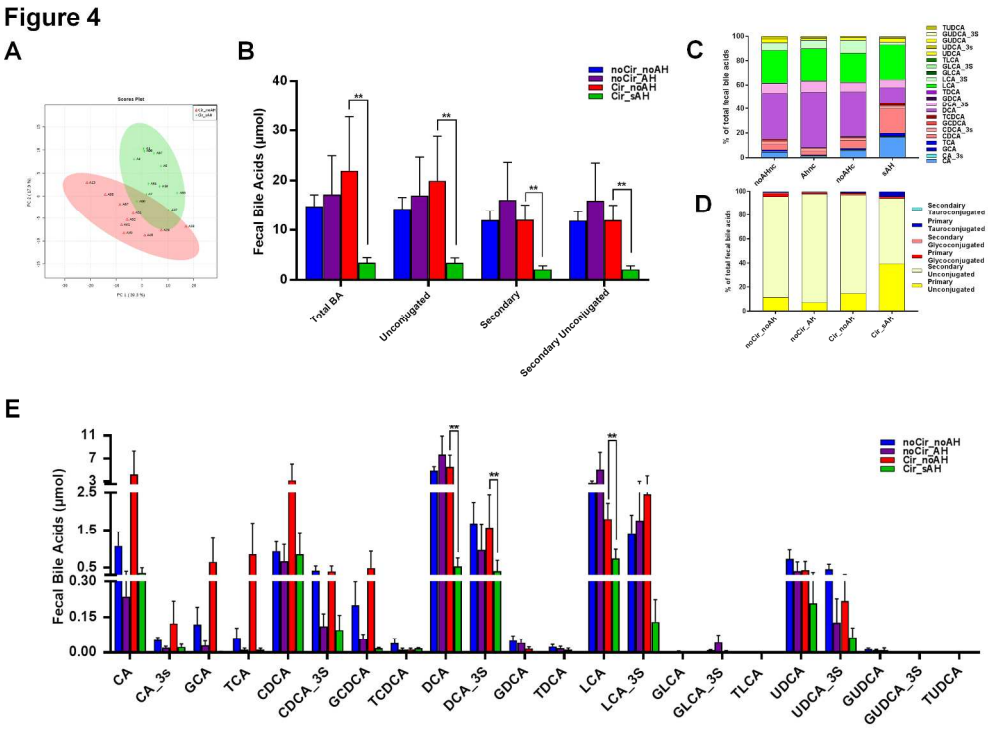

Figure 4: Faecal bile acids profiles in alcoholic liver disease. (A) PCA ordination plot with 95% confidence ellipse for all faecal bile acids showing clustering of patients according to the liver complication. The first two components of the PCA explained 57% of the total variance (component 1 = 39.3%; component 2 = 17.9%). (B) Total faecal bile acids, total unconjugated, secondary and secondary unconjugated levels of faecal bile acids. (C) and (D) faecal bile acids composition (% of total faecal bile acids). (E) Individual faecal bile acids levels. *p < 0.05, **p < 0.01. CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; LCA: lithocholic acid; GCA: glycocholic acid; GCDCA: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid; sAH: severe alcoholic hepatitis; TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid; TLCA: taurolithocholic acid; TUDCA: tauroursodeoxycholic acid; UDCA: ursodeoxycholic acid; _3s: sulfated forms.

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Figure 5

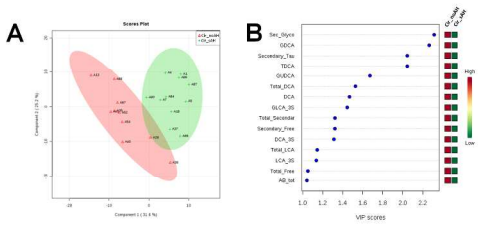

Figure 5: Specificity of faecal bile acids profiles depending on alcoholic-induced liver inflammation. (A) PLS-DA score plot of faecal bile acids concentrations of Cir_sAH vs. Cir_noAH patients with 95% confidence ellipse showing a significant difference between the two groups (R2 = 0.8, Q2 = 0.6, p = 0.001). (B) Variable importance in projection (VIP) of PLS-DA showing the faecal bile acids that discriminate Cir_sAH from Cir_noAH patients (VIP score >1). The colored boxes on the right indicate the relative concentrations of the corresponding faecal bile acids in each group.

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Figure 6

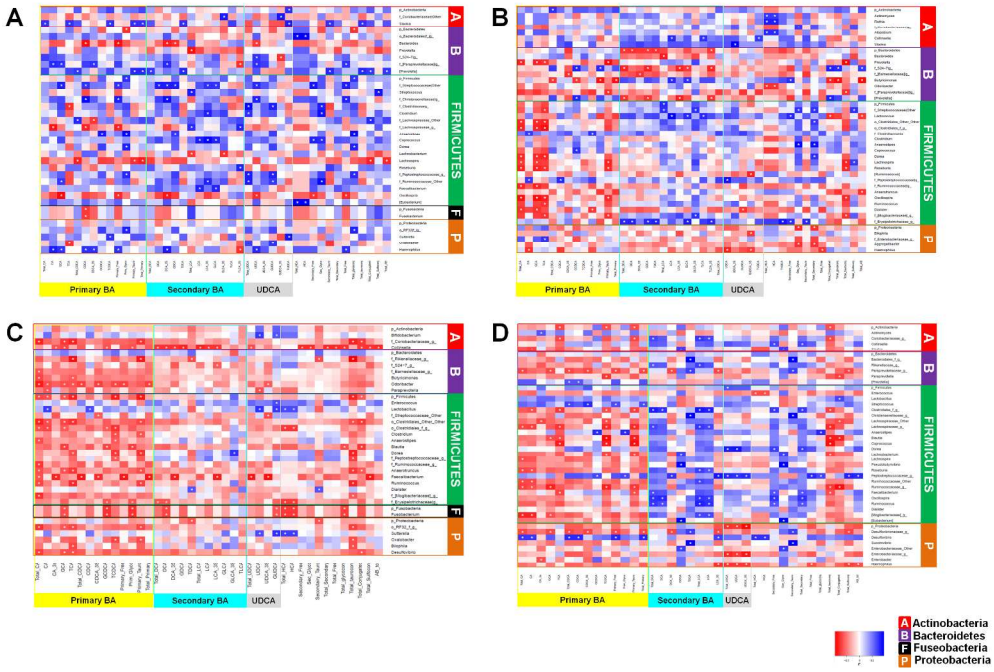

Figure 6: Heatmap representation of the Spearman's r correlation coefficient between bacterial taxa (phylum and genus level) and bile acids profiles in plasma of Cir_noAH (A) and Cir_sAH (B) patients and feces of Cir_noAH (C) and Cir_sAH (D). Only the bacteria for which at least one significant correlation with bile acids was found are displayed (p, phyla; g, genus). CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; LCA: lithocholic acid; GCA: glycocholic acid; GCDCA: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid; TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid; TLCA: taurolithocholic acid; TUDCA: tauroursodeoxycholic acid; UDCA: ursodeoxycholic acid; A: Actinobacteria, B: Bacteroidetes, F: Fusobacteria; P: Proteobacteria. *Adjusted p value < 0.05. Red: negative correlation, blue: positive correlation.

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Title: Bile acids homeostasis and intestinal dysbiosis in alcoholic hepatitis

Running Title: Bile acid, microbiota and alcoholic hepatitis

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Supplementary Table 1: Differences in intestinal microbiota at the genus level between patients with alcoholic cirrhosis, with (Cir_sAH) and without severe alcoholic hepatitis (Cir_noAH).

Phyla	Family	Genus	Increased in	Relative abundance		Mann Whitney		LEfSe	
				Cir_noAH	Cir_sAH	*p*	FDR	LDA score	*p*
Actinobacteria	Actinomycetaceae	Actinomyces	Cir_sAH	$18*10^{-5}$	$79*10^{-5}$	0.03	0.23	3.06	0.03
	Micrococcaceae	Rothia	Cir_sAH	$1*10^{-5}$	$22*10^{-5}$	0.01	0.20	3.29	0.01
	Bifidobacteriaceae	Bifidobacterium	Cir_sAH	$1200*10^{-5}$	$8373*10^{-5}$	0.02	0.23	3.84	0.02
Bacteroidetes	Porphyromonadaceae	Parabacteroides	Cir_noAH	$1665*10^{-5}$	$555*10^{-5}$	0.03	0.23	3.14	0.03
Firmicutes	Streptococcaceae	Lactococcus	Cir_sAH	$68*10^{-5}$	$79*10^{-5}$	0.04	0.27	2.87	0.04
	Christensenellaceae	g_	Cir_noAH	$172*10^{-5}$	$3*10^{-5}$	0.03	0.23	2.73	0.03
	Ruminococcaceae	Oscillospira	Cir_noAH	$1247*10^{-5}$	$421*10^{-5}$	0.00	0.14	3.00	<0.01
	Lactobacillaceae	Lactobacillus	Cir_sAH	$1294*10^{-5}$	$12397*10^{-5}$	0.00	0.14	4.09	<0.01
Proteobacteria	Pasteurellaceae	Haemophilus	Cir_sAH	$165*10^{-5}$	$503*10^{-5}$	0.01	0.22	2.88	0.01
	Desulfovibrionaceae	Bilophila	Cir_noAH	$217*10^{-5}$	$26*10^{-5}$	0.01	0.20	2.97	0.01
	Enterobacteriaceae	g_	Cir_sAH	$3601*10^{-5}$	$8156*10^{-5}$	0.02	0.23	3.79	0.02

Supplementary Table 2: Plasma bile acids concentrations between groups.

Plasma Bile Acids (n=56)	Without cirrhosis						Cirrhosis					
	noCir_noAH (n=29)			noCir_AH (n=8)			Cir_noAH (n=8)			Cir_sAH (n=10)		
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
CA	0	0	0.51	0.00	0	0.90	0	0	0.33	0.19	0	1.38
GCA	0.07	0	7.10	0.26	0.002	5.36	**0.36**	**0**	**0.54**	**3.11**	**0**	**9.30**
TCA	0.03	0	6.44	0.01	0	0.74	**0.16**	**0**	**2.78**	**4.48**	**0**	**25.46**
Total CA	0.16	0	13.54	0.32	0.003	6.25	**0.59**	**0**	**3.05**	**8.46**	**0**	**34.82**
CDCA	0.07	0	17.95	0.03	0	8.37	0.02	0	0.63	1.49	0	9.15
CDCA_3S	0	0	0.16	0.00	0	0.28	0.02	0	0.07	0.02	0	0.22
GCDCA	1.05	0	56.08	1.49	0.01	32.55	**2.49**	**0.002**	**4.10**	**34.89**	**7.36**	**57.37**
TCDCA	0.06	0	12.81	0.05	0	1.83	**0.48**	**0**	**9.05**	**21.97**	**5.02**	**45.01**
Total CDCA	1.73	0.001	68.89	1.85	0.01	42.94	**4.00**	**0.002**	**11.39**	**61.35**	**12.38**	**81.23**
Total Primary	1.90	0.002	82.43	2.30	0.01	43.24	4.74	0.002	14.44	70.67	16.57	99.42
DCA	0.11	0	67.94	0.24	0	17.99	0.16	0.001	0.55	0.01	0	0.88
DCA_3S	0	0	0.07	0	0	0.24	0	0	0.05	0	0	0.10
GDCA	0.21	0	23.50	0.75	0	10.59	0.50	0.002	1.19	0.16	0	6.98
TDCA	0.02	0	6.30	0.00	0	0.31	0.14	0	2.01	0.17	0	4.51
Total DCA	0.47	0	70.94	1.15	0	28.58	1.02	0.002	2.51	0.36	0	12.19
LCA	0	0	0.03	0	0	0.19	0.02	0	0.08	0.01	0	0.11
LCA_3S	0	0	0.11	0	0	0.07	0.06	0	0.23	0.04	0	0.48
GLCA	0	0	0.07	0	0	0.04	0.05	0	0.10	0.04	0	0.17
GLCA_3S	0.18	0	74.05	0.007	0	12.83	0.27	0	0.68	0.19	0	26.48
TLCA	0	0	0	0	0	0	0	0	0.20	0	0	0.10
TLCA_3S	0	0	3.49	0	0	0.13	0.09	0	0.66	0.11	0	0.76
Total LCA	0.29	0	74.05	0.01	0	13.02	0.66	0.001	1.25	0.47	0	26.48
UDCA	0.03	0	7.97	0.00	0	5.94	0.01	0	0.06	0.05	0	0.73
UDCA_3S	0	0	0.13	0	0	1.01	0	0	0.13	0	0	0.14
GUDCA	0.17	0	13.26	0.32	0.000	20.54	0.13	0	0.27	0.76	0	2.49
GUDCA_3S	0.08	0	22.34	0.24	0	49.30	0.09	0	0.15	0.13	0	1.16
TUDCA	0	0	0.01	0	0	0.00	**0**	**0**	**0.02**	**0.26**	**0**	**0.66**
Total UDCA	0.37	0	42.49	0.56	0.001	56.73	**0.28**	**0**	**0.44**	**1.30**	**0**	**4.09**
Total Secondary	1.68	0.002	96.84	2.32	0.00	66.00	2.12	0.004	3.93	2.47	0.58	26.48
Total Unconjugated	0.64	0	85.89	0.37	0	26.49	0.27	0.001	1.74	1.89	0	12.02
Total Glycoconjugated	2.32	0.002	96.49	3.56	0.01	93.90	**3.90**	**0.005**	**6.58**	**39.98**	**9.11**	**83.85**
Total Tauroconjugated	0.16	0	19.25	0.36	0.003	1.83	**0.90**	**0**	**14.71**	**32.17**	**8.19**	**60.33**
Total Sulfoconjugated	0.50	0	74.05	0.84	0.001	54.38	0.79	0.001	1.06	0.65	0.06	26.48
Total Conjugated	2.60	0.002	100	4.26	0.01	93.98	**5.89**	**0.005**	**18.19**	**81.77**	**17.30**	**100**
Total Bile Acids	3.60	0.003	100	4.62	0.01	100	**6.84**	**0.01**	**18.36**	**84.57**	**17.37**	**100**

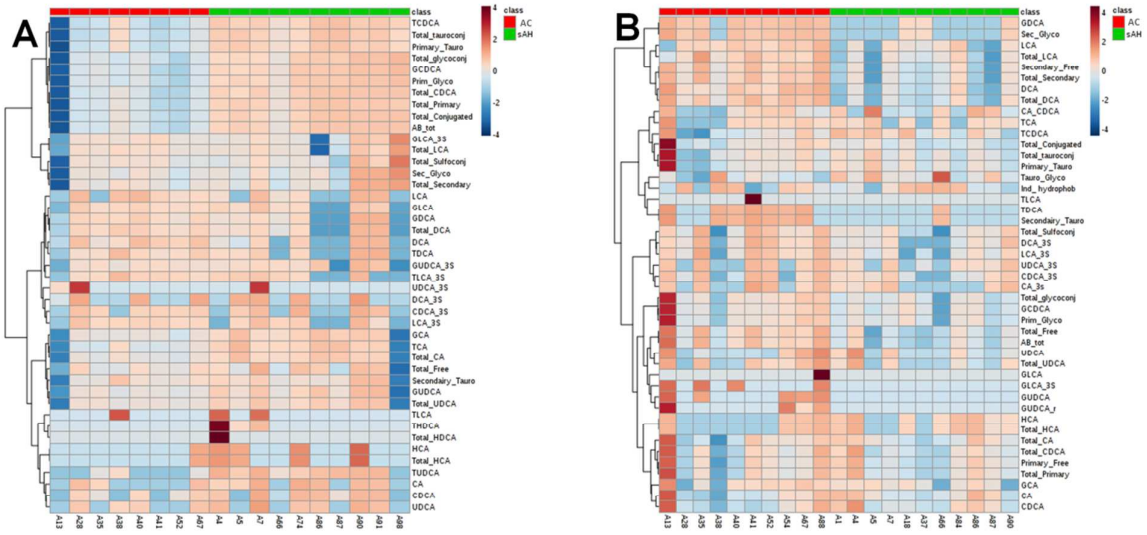
In bold bile acids that were different between the groups ($p<0.05$ using a Mann Whitney test with a FDR correction for multiple comparaissons).

Supplementary Table 3: Fecal bile acids concentrations between groups.

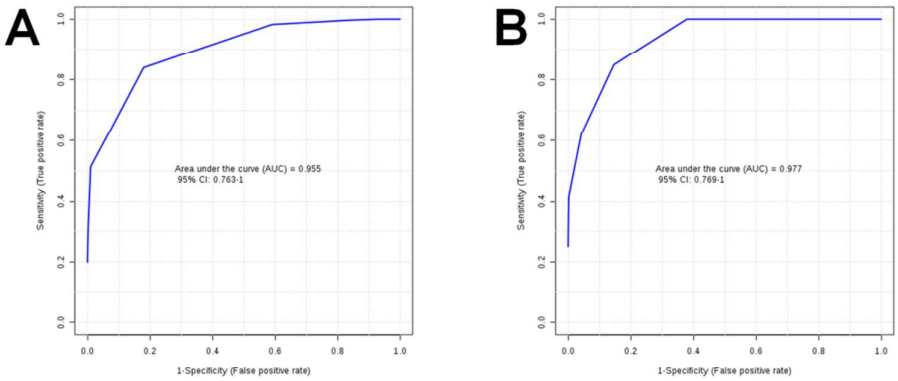
Fecal Bile Acids (n=73)	Without cirrhosis						Cirrhosis					
	noCir_noAH (n=46)			noCir_AH (n=6)			Cir_noAH (n=10)			Cir_sAH (n=11)		
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
CA	0.07	0	11.66	0.10	0	1.02	0.03	0	49.09	0.09	0.01	1.56
CA_3s	0	0	0.32	0.02	0.001	0.05	0.02	0	1.14	0.001	0	0.08
GCA	0.01	0.001	3.17	0.01	0.01	0.13	0.01	0	7.62	0	0	0.01
TCA	0.01	0	1.90	0	0	0.04	0.01	0	10.07	0.01	0	0.08
Total CA	0.17	0.003	12.13	0.14	0.02	1.07	0.23	0	66.82	0.10	0.01	1.58
CDCA	0.16	0.01	6.82	0.08	0.01	2.73	0.05	0.005	34.24	0.11	0.04	6.77
CDCA_3S	0.02	0	4.16	0.07	0.003	0.36	0.32	0	1.30	0.01	0	0.18
GCDCA	0.05	0	4.35	0.05	0.01	0.13	0.02	0.002	5.44	0.01	0.001	0.04
TCDCA	0.01	0	0.94	0.01	0	0.03	0.01	0.001	0.05	0.02	0.004	0.03
Total CDCA	0.45	0.05	9.61	0.33	0.11	2.93	0.64	0.01	40.34	0.31	0.05	6.84
Total Primary	0.78	0.07	20.72	0.53	0.13	4.00	0.89	0.01	107.16	0.38	0.08	7.75
DCA	3.78	0.01	24.12	5.61	0.28	22.36	**3.76**	**0.62**	**25.27**	**0.13**	**0.01**	**2.61**
DCA_3S	0.14	0.002	22.06	0.30	0.01	4.45	**0.07**	**0**	**9.86**	**0**	**0**	**1.24**
GDCA	0.02	0	1.05	0.03	0	0.12	**0.01**	**0**	**0.10**	**0**	**0**	**0.003**
TDCA	0.01	0	0.41	0.01	0	0.04	**0.01**	**0**	**0.08**	**0**	**0**	**0.002**
Total DCA	4.54	0.05	46.49	7.43	0.36	22.74	**6.49**	**0.63**	**25.55**	**0.13**	**0.01**	**2.61**
LCA	1.96	0.003	8.74	1.96	0.38	20.00	**1.95**	**0.02**	**5.43**	**0.58**	**0.005**	**2.93**
LCA_3S	0.17	0	18.64	0.60	0.004	7.82	0.21	0	18.31	0	0	0.25
GLCA	0	0	0.003	0	0	0.02	0.00	0	0	0	0	0
GLCA_3S	0	0	0.06	0.01	0	0.21	0.00	0	0.03	0	0	0
TLCA	0	0	0	0.00	0	0.001	0.00	0	0	0	0	0
Total LCA	2.53	0.05	22.91	2.93	0.44	28.04	**2.69**	**0.38**	**20.15**	**0.58**	**0.01**	**2.93**
UDCA	0.10	0	7.18	0.06	0	1.47	0.01	0.002	2.38	0.01	0.003	1.94
UDCA_3S	0.04	0	4.09	0.04	0	0.63	0.09	0	0.90	0	0	0.23
GUDCA	0	0	0.15	0.01	0	0.02	0	0	0.11	0	0	0
GUDCA_3S	0	0	0.08	0	0	0	0	0	0	0	0	0
TUDCA	0	0	0.04	0	0	0.001	0	0	0	0	0	0
Total UDCA	0.30	0.01	11.31	0.39	0.02	1.54	0.41	0.002	3.11	0.02	0.003	1.94
Total Secondary	8.40	0.21	63.85	10.24	0.83	52.40	**10.68**	**1.80**	**27.92**	**0.72**	**0.02**	**5.68**
Total Unconjugated	9.33	0.40	83.97	10.54	0.93	53.27	**11.17**	**1.80**	**111.58**	**2.30**	**0.19**	**10.97**
Total Glycoconjugated	0.10	0.002	7.67	0.10	0.03	0.51	**0.05**	**0.002**	**13.30**	**0.02**	**0.001**	**0.05**
Total Tauroconjugated	0.02	0	2.86	0.02	0	0.11	0.02	0.001	10.20	0.02	0.01	0.10
Total Sulfoconjugated	0.46	0.01	31.88	1.23	0.05	8.36	0.71	0	25.13	0.04	0	1.99
Total Conjugated	0.12	0.002	10.54	0.16	0.03	0.53	0.08	0.02	23.50	0.03	0.02	0.12
Total BA	9.87	1.21	84.57	10.77	0.96	53.80	**11.32**	**1.82**	**135.08**	**2.36**	**0.31**	**11.03**

In bold bile acids were different between the groups ($p<0.05$ using a Mann Whitney test with a FDR correction for multiple comparaissons).

Supplemental Figures



Supplemental Figure 1: Bile acids profiles in plasma (**A**) and feces (**B**). The relative amounts of bile acids are displayed as a heatmap (values are pareto and log2 scaled).



Supplemental figure 2: ROC curves showing that plasma (**A**) and feces (**B**) bile acids are able to discriminate Cir_sAH patients from Cir_noAH patients (average AUROC = 0.955 and 0.977 respectively).