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Title: Bile acid homeostasis and intestinal dysbiosis in alcoholic hepatitis

Running Title: Bile acid, microbiota and 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

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3 26 statistical analysis, and wrote the manuscript. CSV designed the project and included
4
5 27 patients. LW designed the project and performed presequencing preparation
6
7 28 procedures. CH performed presequencing preparation procedures. DR and LH
8
9 29 performed bile acids analyses. AMC designed, performed and supervised
10
11 30 experiments and wrote the manuscript. GP designed the project, included patients,
12
13 31 supervised experiments and wrote the paper. All authors discussed the results,
14
15 32 commented and approved the final version of the manuscript.
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35 **Summary**

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

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

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

45 **Results:** Plasma levels of total bile acids (84.6 vs. 6.8 $\mu\text{mol/l}$, $p<0.001$) and total
46 ursodeoxycholic acid (1.3 vs. 0.3 $\mu\text{mol/l}$, $p=0.03$) were higher in Cir_sAH than
47 Cir_noAH whereas faecal total (2.4 vs. 11.3, $p=0.01$) and secondary bile acids (0.7
48 vs. 10.7, $p<0.01$) levels were lower. Cir_sAH patients had a different microbiota than
49 Cir_noAH patients: at the phyla level, the abundance of Actinobacteria (9 vs 1%,
50 $p=0.01$) was higher and that of Bacteroidetes was lower (25 vs 40%, $p=0.04$).
51 Moreover, the microbiota of Cir_sAH patients showed changes in the abundance of
52 genes involved in 15 metabolic pathways, including upregulation of glutathione
53 metabolism, and downregulation of biotin metabolism.

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

58 **Keywords:** 16S sequencing, microbiota, UDCA, biotin, glutathione, Actinobacteria,
59 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 properties 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-

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

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

96 However, while both the bile acids and intestinal microbiota profiles were reported in
97 alcoholic liver disease, these studies focused either only on the bile acids profile
98 (11,18) or intestinal microbiota profile (4) and never on both, in the same cohort of
99 patients or didn't included patients with severe alcoholic hepatitis (12,13). In order to
100 study the relationships between intestinal microbiota modifications and bile acids
101 metabolism, we investigated herein the interplay between bile acids and intestinal
102 microbiota in well phenotyped patients at different stages of alcoholic liver disease by
103 assessing and comparing plasma and faecal bile acids profiles and intestinal
104 microbiota composition and functions in currently drinking alcoholic patients
105 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 $\geq$ 6 with neutrophilic infiltration) (4,20).

- 130 • patients with alcoholic cirrhosis, but without severe alcoholic hepatitis
131 (Cir_noAH, n = 17). As the impact of non-severe alcoholic hepatitis was limited
132 on the parameters that we studied in non-cirrhotic patients, we pooled the
133 patients with mild alcoholic hepatitis with patients with no alcoholic hepatitis in
134 the cirrhotic patients group.
- 135 • patients without alcoholic cirrhosis or alcoholic hepatitis (noCir_noAH, n = 61),
136 • patients without alcoholic cirrhosis, but with alcoholic hepatitis (noCir_AH, n =
137 13). Alcoholic hepatitis was defined by aspartate aminotransferase>50,
138 aspartate aminotransferase/alanine aminotransferase > 1.5, and both values <
139 400 IU/L (20,21) or, if a liver biopsy was available (12/13 patients), an AH
140 score between 3 and 5 (with neutrophilic infiltration) or ≥ 6 (with neutrophilic
141 infiltration and a Maddrey score <32).

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

148 **Biochemical assays**

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

154 **Analysis of the intestinal microbiota by 16S ribosomal RNA sequencing**

155 Faecal samples were available for 96 patients. The composition of the faecal
 156 microbiota was analyzed by high-throughput sequencing with Illumina MiSeq
 157 technology, targeting the 16S ribosomal DNA V3-V4 region in the paired-end mode
 158 (2 x 300 base pairs) (GenoToul, Toulouse), as previously described (23). Data were
 159 processed with the quantitative insights into microbial ecology (QIIME v1.9.0)
 160 pipeline, using its default parameters. Closed reference operation taxonomic
 161 mapping was performed using the Greengenes database (v13.8, 97% sequence
 162 similarity).

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

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

177 ***Statistical analysis***

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

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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).

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

222 At the phyla level, Cir_sAH patients had a higher abundance of Actinobacteria and
223 lower abundance of Bacteroidetes than Cir_noAH patients. Among Actinobacteria,
224 Cir_sAH patients had a higher abundance of *Actinomyces*, *Rothia*, and
225 *Bifidobacterium* than Cir_noAH patients. Among Proteobacteria, Cir_sAH patients
226 had a higher abundance of *Haemophilus* and *Enterobacteriaceae* and a lower
227 abundance of *Bilophila* than Cir_noAH patients. Cir_sAH patients also had a lower
228 relative abundance of *Parabacteroides* (Bacteroidetes phylum), *Oscillospira*, and
229 *Christensenellaceae* families (Firmicutes phylum) and a higher relative abundance of
230 *Lactobacillus* and *Lactococcus* (Firmicutes phylum) than Cir_noAH patients (Figure
231 1C and Supplemental Table 1).

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

239 ***Functional Intestinal Metagenome Prediction in Alcoholic Hepatitis:***

240 The dysbiosis identified in Cir_sAH patients prompted us to also examine the
241 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 terpenoids 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

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5 267 primary glyco- and tauroconjugated bile acids, total CA (glycocholate (GCA) and
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7 268 taurocholate (TCA)), total CDCA (glycochenodeoxycholate (GCDCA) and
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9 269 taurochenodeoxycholate (TCDCA)), and total UDCA (tauroursodeoxycholate
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11 270 (TUDCA)) than Cir_noAH patients (Figures 2 B-D and Supplementary Table 2).
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13 271 Because of the higher total bile acids levels in Cir_sAH patients, we also studied the
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15 272 relative proportion of each bile acids (bile acids concentration/total bile acids
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17 273 concentration) between the two groups. Cir_sAH patients had a higher relative
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19 274 proportion of total primary bile acids, total CDCA (TCDCA), total primary conjugated
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21 275 bile acids, primary tauroconjugated bile acids, and TUDCA than Cir_noAH patients.
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23 276 They also had a lower relative proportion of total sulphoconjugated bile acids, total
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25 277 secondary bile acids, total secondary conjugated bile acids, secondary
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27 278 glycoconjugated bile acids, total lithocholate (LCA) (LCA, glycolithocholate (GLCA),
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29 279 lithocholate-3-sulfate (LCA3s), taurolithocholate-3-sulfate (TLCA3s), and
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31 280 glycolithocholate-3-sulfate (GLCA3s)), total deoxycholate (DCA) (DCA;
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33 281 glycodeoxycholate (GDCA), and taurodeoxycholate (TDCA)), chenodeoxycholic acid
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35 282 3-sulfate (CDCA3s), and glyoursodeoxycholate-3-sulfate (GUDCA3s) than
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37 283 Cir_noAH patients (Figures 2 B, C, E).
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42 284 We then used PLS-DA to find the plasma bile acids that best discriminate between
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44 285 the two groups. The model showed a significant distinction ($R^2 = 0.6$, $Q^2 = 0.4$,
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46 286 prediction accuracy during training: $p = 0.001$, 1000 permutations) between the
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48 287 Cir_sAH and Cir_noAH groups (Figure 3A). The average AUROC confirmed that the
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50 288 model was able to discriminate Cir_noAH from Cir_sAH patients (0.955, 95% CI:
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52 289 0.763-1, Supplementary Figure 2A). TUDCA was the most discriminant bile acids
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290 between the two groups according to the PLS-DA (variable importance in projection,
 291 VIP = 2.1, Figure 3B).

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

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

301 ***Faecal bile acids***

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

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5 314 tauroconjugated bile acids, and total DCA (DCA, GDCA, TDCA) (Figures 4 B, C, E).
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8 315 PLS-DA showed a significant distinction ($R^2 = 0.8$, $Q^2 = 0.6$, prediction accuracy
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10 316 during training: $p = 0.001$, 1000 permutations) between the Cir_sAH and Cir_noAH
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12 317 groups (Figure 5A). The average AUROC confirmed that the model was able to
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14 318 discriminate Cir_noAH from Cir_sAH patients (0.977, 95% CI: 0.769-1)
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16 319 (Supplementary Figure 2B). Secondary glycoconjugated bile acids and GDCA were
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18 320 the most discriminant bile acids between the two groups according to the PLS-DA
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20 321 (variable importance in projection, VIP = 2.1 and 2.1, respectively, Figure 5B).
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24 322 Comparison of the faecal bile acids profiles between noncirrhotic patients showed the
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26 323 observed changes (higher tauroolithocholate, TLCA, and GLCA ratios in noCir_AH
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28 324 than noCir_noAH patients) to no longer be significant after correction for multiple
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30 325 comparisons (data not show), as observed in plasma.
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34 326 These results confirm the decrease in bile acids excretion and impaired bile acids
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36 327 transformation in the gut by the intestinal microbiota of Cir_sAH patients relative to
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38 328 that of Cir_noAH patients.
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41 329 ***The relationship between intestinal microbiota and bile acids homeostasis***
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43 330 We assessed the correlation between the bile acids profiles with the bacteria species
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45 331 identified in the feces, as the composition and quantity of the bile acids pool influence
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47 332 the intestinal microbiota, and conversely, the metabolism of bile acids is dependent
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49 333 on intestinal microbiota composition. Primary and secondary plasma bile acids levels
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51 334 positively correlated with most of the taxa in Cir_noAH patients (Figure 6A) while
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53 335 primary plasma bile acids negatively correlated with taxa from Bacteroidetes and
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55 336 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.

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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), *Haemophilus* (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 previously 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

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

408 It has been suggested, in a previous work, that an increase in primary bile acids was
409 associated with the severity of histological lesions in AH (11). A similar result was
410 observed in our study. Of note, in the previous work (11), the authors provided an
411 overall plasma bile acids profile in patients with AH ranging from mild AH to severe
412 alcoholic hepatitis and irrespective of the presence of cirrhosis, that was present in
413 75 % of their patients. As alcoholic cirrhosis is associated with an impaired bile acids
414 profile (12,13), their results might be biased by the mix of cirrhotic and non-cirrhotic
415 patients. Moreover, according to the method used for bile acids assay (HPLC), they
416 could only detect primary and secondary bile acids but neither their conjugated forms
417 nor UDCA. Other studies have investigated plasma bile acids, faecal bile acids and
418 intestinal microbiota in alcoholic patients (12,13). They suggested, that alcohol intake
419 in both cirrhosis and non-cirrhotic patients is associated with a decrease in
420 conjugated CDCA in plasma (12). In our study, CDCA was increased in severe
421 alcoholic hepatitis, suggesting that this increase is due to liver inflammation (ie
422 severe alcoholic hepatitis) independently of the presence of cirrhosis of alcohol
423 intake. We also suggest that perturbation of intestinal microbiota is involved in the
424 specific modifications bile acids metabolism observed in patients with severe
425 alcoholic hepatitis.

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

cholesterol 7 α -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 glycoconjugated 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

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456 smaller ductules (progenitor cells). Here, FGF-19 positively correlated with the MELD
457 score. This is consistent with other studies that reported a correlation between FGF-
458 19 levels and liver disease severity (14,44) and suggests that the increase in FGF-19
459 levels observed in our study has a double origin, hepatic (due to increased plasma
460 bile acids , dominated by CDCA) and intestinal. Indeed, there was a shift of the faecal
461 bile acids pool in the gut toward species with a higher affinity for FXR, as shown by
462 the increase in the proportion of CDCA, although there was an overall decrease in
463 total faecal bile acids. Thus, high levels of FGF-19 could increase CDCA synthesis by
464 directing bile acids synthesis from the classic (neutral) to the alternative (acidic)
465 pathway, due to the blockade of CYP7A1, but not of cholesterol 7 β -hydroxylase
466 (CYP7B1). Therefore, high levels of FGF-19 are probably insufficient to counteract
467 the increased bile acids synthesis induced by alcohol in alcoholic liver disease.
468 Moreover, it promotes the shift of the bile acids pool towards more hydrophobic, toxic
469 species.

470 There was also an increase in total plasma UDCA and TUDCA levels in Cir_sAH
471 patients relative to Cir_noAH patients. These bile acids have hepatoprotective
472 effects. However, TUDCA exerts this effect by replenishing hepatic mitochondrial
473 glutathione (45). Thus, the increase in glutathione metabolism of the intestinal
474 microbiota, which may decrease its bioavailability to the mitochondria, combined with
475 an intestinal microbiota-associated decrease in the levels of biotin, an essential
476 cofactor of mitochondrial metabolism, could explain why the increased TUDCA levels
477 seen in Cir_sAH patients does not have a hepatoprotective effect. This is supported
478 by the fact that UDCA showed hepatoprotective effects in *in vitro* studies and early
479 stages of alcoholic liver disease, but not in severe alcoholic hepatitis patients with
480 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 weak acids with strong bactericidal activities. Among the bile acids, DCA is extremely toxic and inhibits the growth of

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3 506 many intestinal bacteria, including *Clostridium perfringens*, *Bacteroides fragilis*,
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5 507 *Lactobacilli*, and *Bifidobacteria* (50,51). In our study we observed a decrease in
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7 508 unconjugated bile acids, total secondary bile acids and DCA in the feces, that are
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9 509 highly hydrophobic. This may be responsible for the increase levels of *Lactobacillus*
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11 510 and *Bifidobacterium* seen in the microbiota of these patients. It has also been
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13 511 suggested that a decreased level of faecal bile acids stimulated the growth of gram-
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15 512 negative and conversely decreases the growth gram-positive bacteria (52). Indeed,
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17 513 we observed in severe alcoholic hepatitis patients an increase in several taxa that are
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19 514 gram-negative (eg Gammaproteobacteria) that could be secondary to the decrease
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21 515 faecal bile acids level. Moreover, gram-negative bacteria produce LPS that was
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23 516 related to increases alcoholic liver necrosis and inflammation (53). We also observed
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25 517 in our study a decrease in several taxa from the gram-positive Firmicutes phylum
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27 518 (Christensenellaceae, Oscillospira) that 7 α -dehydroxylate primary bile acids to toxic
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29 519 secondary bile acids. Thus, the decrease and shift of the bile acids pool in the feces
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31 520 could be responsible for the increase in LPS-producing bacteria and for the decrease
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33 521 of gram-positive members of Firmicutes able to transform primary bile acids into
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35 522 secondary bile acids. This hypothesis may explain the decrease in secondary bile
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37 523 acids in severe alcoholic hepatitis patients observed in our study.
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42 524 A limit of our study was a potential lack of power related to the small number of
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44 525 patients, which did not allow us to identify changes in taxa with low counts. However,
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46 526 the recruitment of severe alcoholic hepatitis patients for intestinal microbiota studies
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48 527 is challenging, as most are rapidly treated (often by antibiotics to prevent or treat
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50 528 complications). This bias did not occur in our patients as they were included before
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52 529 any specific treatment for severe alcoholic hepatitis . Moreover, we did not exclude
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54 530 patients with proton-pump inhibitors intake which was shown to alter the IM
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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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728 **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 .

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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 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;

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3 755 CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; LCA: lithocholic acid; GCA,
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5 756 glycocholic acid; GCDCA: glycochenodeoxycholic acid; GDCA: glycodeoxycholic
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7 757 acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid; sAH: severe
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9 758 alcoholic hepatitis; TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid;
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11 759 TDCA: taurodeoxycholic acid; TLCA: tauroolithocholic acid; TUDCA:
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13 760 tauroursodeoxycholic acid; UDCA: ursodeoxycholic acid; _3s: sulfated forms.
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17 761 **Figure 3: Specificity of the plasma bile acids profile depending on alcoholic-**
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19 762 **induced liver inflammation. (A)** PLS-DA score plot of plasma bile acids
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21 763 concentrations in Cir_sAH vs. Cir_noAH patients with the 95% confidence ellipse
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23 764 showing a significant difference between the two groups ($R^2 = 0.6$, $Q^2 = 0.4$, $p =$
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25 765 0.001). **(B)** Variable importance in projection (VIP) of PLS-DA showing the plasma
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27 766 bile acids that discriminate Cir_sAH from Cir_noAH patients (VIP score >1). The
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29 767 colored boxes on the right indicate the relative concentrations of the corresponding
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31 768 plasma bile acids in each group.
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35 769 **Figure 4: Faecal bile acids profiles in alcoholic liver disease. (A)** PCA ordination
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37 770 plot with 95% confidence ellipse for all faecal bile acids showing clustering of patients
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39 771 according to the liver complication. The first two components of the PCA explained
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41 772 57% of the total variance (component 1 = 39.3%; component 2 = 17.9%). **(B)** Total
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43 773 faecal bile acids, total unconjugated, secondary and secondary unconjugated levels
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45 774 of faecal bile acids. **(C)** and **(D)** faecal bile acids composition (% of total faecal bile
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47 775 acids). **(E)** Individual faecal bile acids levels. $*p < 0.05$, $**p < 0.01$. CA: cholic acid;
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781 tauroursodeoxycholic acid; UDCA: ursodeoxycholic acid; _3s: sulfated forms.

782 **Figure 5: Specificity of faecal bile acids profiles depending on alcoholic-**
783 **induced liver inflammation. (A)** PLS-DA score plot of faecal bile acids
784 concentrations of Cir_sAH vs. Cir_noAH patients with 95% confidence ellipse
785 showing a significant difference between the two groups ($R^2 = 0.8$, $Q^2 = 0.6$, $p =$
786 0.001). **(B)** Variable importance in projection (VIP) of PLS-DA showing the faecal bile
787 acids that discriminate Cir_sAH from Cir_noAH patients (VIP score >1). The colored
788 boxes on the right indicate the relative concentrations of the corresponding faecal
789 bile acids in each group.

790 **Figure 6:** Heatmap representation of the Spearman's r correlation coefficient
791 between bacterial taxa (phylum and genus level) and bile acids profiles in plasma of
792 Cir_noAH **(A)** and Cir_sAH **(B)** patients and feces of Cir_noAH **(C)** and Cir_sAH **(D)**.
793 Only the bacteria for which at least one significant correlation with bile acids was
794 found are displayed (p, phyla; g, genus). CA: cholic acid; CDCA: chenodeoxycholic
795 acid; DCA: deoxycholic acid; LCA: lithocholic acid; GCA: glycocholic acid; GCDCA:
796 glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic
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798 taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid; TLCA: tauroolithocholic
799 acid; TUDCA: tauroursodeoxycholic acid; UDCA: ursodeoxycholic acid; A:
800 Actinobacteria, B: Bacteroidetes, F: Fusobacteria; P: Proteobacteria. *Adjusted p
801 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 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; GDCDA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glyoursodeoxycholic acid; sAH: severe alcoholic hepatitis; TCA: taurocholic acid; TDCDA: taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid; TLCA: tauroolithocholic acid; TUDCA: tauroursodeoxycholic acid; UDCA: ursodeoxycholic acid; _3s: sulfated forms.

254x190mm (300 x 300 DPI)

A

Score Plot

Component 1 (28.4%)

Component 2 (16.4%)

100 yeast
100 human

B

TUGCA
TODCA
Prostate_Tumor
Falk_glioma
THH_DCCA
Targ_Primary
SGCCA
Pam_Ovpo
Test_Compact
AB_HL
Total_glycemia
DCA
CA
TCA
Tum_Cat

VFP scores

Q² (Low to High)

254x190mm (300 x 300 DPI)

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Figure 4

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: glyoursodeoxycholic acid; SAH: severe alcoholic hepatitis; TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid; TLCA: tauroolithocholic acid; TUDCA: taoursodeoxycholic acid; UDCA: ursodeoxycholic acid; _3S: sulfated forms.

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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: glycooursodeoxycholic acid; TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid; TLCA: tauroolithocholic acid; TUDCA: taurooursodeoxycholic 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 comparaisons).

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 comparaisons).

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).