DR. LEON ANTON ADAMS (Orcid ID : 0000-0002-3968-7909)

DR. ZHENGYI    WANG (Orcid ID : 0000-0002-1738-2207)

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# Bile acids associate with specific gut microbiota, low level alcohol consumption and liver fibrosis in patients with non-alcoholic fatty liver disease

Leon A Adams *MBBS FRACP PhD*[1,2], Zhengyi Wang *MD PhD*[1,2], Chris Liddle BSc *MBBS PhD FRACP FAASLD*[3], Phillip E. Melton *BA MA PhD*[4,5], Amir Ariff *PhD*[4], Harsha Chandraratna *MBBS FRACS*[6], Jeremy Tan *MBBS FRACS*[7], Helena Ching *BSc*[1], Sally Coulter *PhD*[3], Bastiaan de Boer *MBBS MD FRACPath*[8], Claus T Christophersen *MSc PhD*[9,10], Therese A. O'Sullivan *BSc PhD*[9], Mark Morrison *BSc MSc PhD*[11], Gary P Jeffrey *MBBS FRACP FRCP MD*[1,2]

1. Medical School, Faculty of Medical and Health Sciences, The University of Western Australia, Perth, Australia.
2. Department of Hepatology, Sir Charles Gairdner Hospital, Perth, Australia.
3. Storr Liver Centre, The Westmead Institute, Sydney, Australia.
4. Curtin/UWA Centre for Genetic Origins of Health and Disease, Curtin University and University of Western Australia, Perth, Australia.
5. School of Pharmacy and Biomedical Sciences, Curtin University, Bentley, Australia.
6. Obesity Surgery WA, Murdoch Hospital, Murdoch, Australia.
7. Department of Upper GI and Bariatric Surgery, Singapore General Hospital, Singapore.
8. Department of Anatomy, Pathwest, Nedlands, Australia.
9. School of Medical and Health Sciences, Edith Cowan University, Australia.

10. School of Molecular and Life Sciences, Curtin University, Bentley, Australia.

11. The University of Queensland Diamantina Institute, Faculty of Medicine Translational Research Institute, Australia.

**Corresponding author:**

Associate Professor Leon A Adams,

Level 2, Harry Perkins Institute of Medical Research,

School of Medicine, The University of Western Australia,

Nedlands, Western Australia, 6009

Tel: +6161510835          Email: leon.adams@uwa.edu.au

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**Abbreviations:** non-alcoholic fatty liver disease (NAFLD), bile acids (BA's), non-alcoholic steatohepatitis (NASH), farnesoid X receptor (FXR), fibroblast growth factor-19 (FGF19), deoxycholic acid (DCA), Body mass index (BMI), glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), lithocholic acid (LC).

**Conflicts of Interest:** None

## Abstract

**Background**: Bile acids (BAs) are synthesized by the liver and modified by gut bacteria, and may play an intermediary role between the gut microbiome and liver in promoting fibrosis in non-alcoholic fatty liver disease (NAFLD). We investigated the associations between serum and faecal BAs, gut microbiome and fibrosis in patients with and without NAFLD and examined the impact of diet and alcohol consumption on these relationships.

**Methods**: Adult patients (n=122) underwent liver biopsy and BAs characterization by high-performance liquid chromatography/mass spectrometry. Gut microbiome composition was analysed using next-generation 16S rRNA sequencing. Diet and alcohol intake were determined by 3-day food diary.

**Results**: Serum and faecal BA concentrations increased progressively between non-NAFLD controls (n=55), NAFLD patients with no/mild fibrosis (F0-2, n=58), and NAFLD with advanced fibrosis (F3/4, n=9). Progressive increases in serum BAs were driven by primary conjugated BA's including glycocholic acid [GCA] and secondary conjugated BA's. In contrast, faecal BA increase was driven by secondary unconjugated BA's (predominately deoxycholic acid [DCA]). Serum GCA levels and faecal DCA levels correlated with the abundance of *Bacteroidaceae* and *Lachnospiraceae,* and stool secondary BAs with an unclassifiable family of the order *Bacteroidales* (*Bacteroidales;other*). These bacterial taxa were also associated with advanced fibrosis. Modest alcohol consumption was positively correlated with faecal DCA levels and relative abundance of *Lachnospiracaea* and *Bacteroidales;other*.

**Conclusions:** Higher serum and faecal BA levels are associated with advanced fibrosis in NAFLD. Specific gut bacteria link alterations in BA profiles and advanced fibrosis, and may be influenced by low level alcohol consumption.

**Keywords**: Non-alcoholic steatohepatitis, fibrosis, microbiome, deoxycholic acid, diet

**Lay Summary:**

- Specific gut bacteria link alterations in BA profiles and advanced fibrosis, and may be influenced by low level alcohol consumption.

- Faecal levels of toxic deoxycholic acid [DCA] and its serum metabolite (glycodeoxycholic acid) are increased in patients with NAFLD and advanced (F3/4) fibrosis.
- Specific gut microbial taxa (*Lachnospiraceae, Bacteroidaceae, Bacteroidales;other*) associated with advanced fibrosis and with serum and faecal BA levels.
- Low-level alcohol consumption correlates with the relative abundance of gut *Lachnospiraceae*, *Bacteroidales;other* and faecal DCA levels.

## Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined by the presence of hepatic lipid in the absence of excess alcohol consumption and is typically associated with obesity and insulin resistance.(1) The prognosis of patients with NAFLD is determined principally by their degree of liver fibrosis, and patients with advanced liver fibrosis are at highest risk of developing cirrhosis and hepatocellular carcinoma.(2) The gut microbiome and the entero-hepatic circulation of bile acids (BA), have been increasingly implicated in the genesis of liver injury and fibrosis in patients with NAFLD.(3)

Collectively, BA's are steroid molecules originating from the metabolism of cholesterol and may be classified as primary (from the liver) or secondary (derived from gut microbial metabolism of primary BA's). BA's are important signalling molecules involved in glucose, lipid and energy metabolism.(4) In humans, serum levels of BA's correlate with levels of insulin resistance, whereas BA sequestrants alter lipid levels and improve serum glucose.(5,6) BA's may act on farnesoid X receptors (FXR) in the gut or liver to influence hepatic lipid metabolism and injury, or may be directly hepatotoxic in the case of deoxycholic acid (DCA).(7-9) Among patients with non-alcoholic steatohepatitis (NASH), the synthetic BA derivative obeticholic acid significantly improves liver fibrosis.(10) Therefore understanding the relationship between BA's within the gut and the systemic circulation, may provide insight into developing effective therapeutic strategies for NAFLD.

The generation of secondary BA's occurs via bacterial metabolism of primary BA's within the intestine. Thus the composition of the gut microbiome has a significant influence on composition of the BA pool. Consequently, alteration of the gut microbiome by antibiotics or diet leads to change in BA profiles, and reductions in NAFLD and liver injury in animal models.(11,12) As yet, it is unknown whether diet plays a similar role in altering the gut microbiome and BA composition in NAFLD patients.

To date, it remains unresolved whether altered BA composition associated with gut dysbiosis is involved in the genesis of liver fibrosis in NAFLD patients. Studies examining serum BA levels have been conflicting whether secondary BA levels are increased or decreased in NASH.(13-15) Moreover, others have suggested altered BA levels are related to insulin resistance rather than NASH.(5) Furthermore, studies examining BA levels in NAFLD have focused primarily on their association with NASH and not fibrosis, and have examined just one compartment of BA metabolism (gut or serum). In addition, whether gut dysbiosis is associated with altered BA profiles and dietary composition in NAFLD patients is not well characterized.

We sought to describe the association between gut and serum BA profiles, NAFLD and fibrosis, in a large cohort of patients with a spectrum of liver histology. We hypothesized that BA profiles would differ according to the presence of NAFLD and advanced fibrosis, supporting a role of BA in the pathogenesis of NAFLD and fibrosis. Secondly, we aimed to investigate the relationship between BA profiles and the gut microbiome. We hypothesized that gut bacteria involved in BA metabolism would associate with faecal BA levels and that these relationships would be altered according to the presence of fibrosis. Lastly, we sought to assess the influence of diet and alcohol on gut microbiome and BA profiles in relation to liver fibrosis, hypothesizing that they may shape a microbiome and BA pool which promotes liver damage.

## Methods

**Subjects:** Adults undergoing liver biopsy as part of clinical care or during bariatric surgery were recruited from three centres located in Perth, Western Australia: the Hepatology Department and Department of Surgery (Sir Charles Gairdner Hospital) and Obesity Surgery WA (Murdoch Hospital). Exclusion criteria included age <20 or >65 years, weekly alcohol consumption >140 grams for females, and >210 grams for males, current use of antibiotics or immunosuppressants, major gastro-intestinal tract surgery, active gastrointestinal disease, secondary causes of NAFLD, concomitant liver disease, poorly controlled diabetes (HbA1c>8.5%) and contra-indication to liver biopsy.

Subjects were grouped into 3 categories based upon their liver histology: 1) controls with normal liver histology with <5% hepatic steatosis, 2) NAFLD with no/minimal fibrosis (F0-2) and (3) NAFLD with advanced fibrosis (F3,4). Controls were patients undergoing bariatric surgery and no evidence of liver disease on liver biopsy.

The study was approved by the human research ethics committees of Sir Charles Gairdner and Group and Murdoch Hospitals with all subjects providing written informed consent. A total of 122 patients were analysed of which 122 had faecal BAs and gut microbiome profiles and 120 with serum BA profiles.

**Clinical Assessment:** All subjects underwent a standardized assessment including a questionnaire regarding alcohol intake(16), medications and past medical history. Anthropometric measures were taken using standardized methods. Dietary data including quantification of alcohol consumption was available in 87 participants and was assessed by completion of a 3-day food record and analysed using FoodWorks®7 Pro (Xyris Software, Brisbane, Australia).

**Biospecimens:** Fasting serum was collected on the day of liver biopsy and was analysed for glucose, insulin, liver function, and lipids using standardized assays at the state hospital reference

laboratory (PathWest, Nedlands, WA). Liver biopsies were stained with hematoxylin-eosin and trichrome stains. Faecal specimens were collected within two weeks of the liver biopsy according to the Human Microbiome Project sampling protocol[17] before being stored at minus 80°C.

**Liver Injury Assessment:** Biopsies were scored according to the NASH CRN scoring system[18] by an expert liver pathologist (BdB).

**Serum and Faecal Bile Acid Characterization:** Sample extraction and preparation was adapted from Humbert *et al[19]*, and is outlined in the Supplementary Methods. Bile acid quantification was performed using Nextera Ultraperformance Liquid Chromotography (SHIMADZU, Kyoto, Japan) system used in combination with a Q-TRAP 5500 Mass Spectrometer (AB SCIEX, Toronto, Canada) with Analyst Software 1.6.2.

**Microbiome Characterisation:** Faecal bacterial DNA was extracted and sequenced using the MiSeq Illumina platform (see supplementary methods). Following demultiplexing and initial sequence quality control assessments, samples were analyzed using the QIIME pipeline.[20] Initially reads were grouped into operational taxonomic units at different distance thresholds using the Uclust algorithm. A representative sequence from each OTU was aligned against the Greengenes reference alignment as implemented in QIIME for phylogenetic identification.

**Statistical Analysis:** Clinical and BA profiles are described using means with standard deviations (±SD) or medians with interquartile ranges, according to their distribution. Bile acids representing >5% of the total BA pool are presented.[21] Differences between groups (non-NAFLD, NAFLD with F0-2 and NAFLD with F3/4) in clinical parameters or individual BA's were tested using Chi-squared test for categorical data and Mann-Whitney U test for continuous data. Differences in BA composition between groups was assessed using Jonckheere-Terpstra test with post-hoc pairwise comparisons with Bonferroni correction. The association between clinical and histological factors and total BA levels was performed using linear regression analysis. The association between

individual BA's and advanced fibrosis was examined using multivariate logistic regression following adjustment with clinical factors found to be associated with advanced fibrosis on univariate logistic regression. Correlation between BA profiles and individual microbial taxa was assessed using spearman correlation with Bonferroni adjustment for multiple testing. Analysis was performed using SPSS v24 (IBM, Armonk New York).

## Results

### Patient Characteristics

Overall the cohort (n=122) was middle aged and predominately female (81%). The majority were obese with 28% having type 2 diabetes and 37% having a history of hypertension.    Over half of the cohort had NAFLD (55%), with 13% of these having advanced (F3/4) hepatic fibrosis. The percentage of NAFLD patients with F0, F1, F2, F3 and F4 fibrosis was 46%, 34%, 6%, 9% and 4.5% respectively. Age, liver enzymes and metabolic features increased progressively across groups (Table 1). Body mass index (BMI) was evenly distributed between groups.

### Associations Between Serum Bile Acids and Advanced NAFLD

A progressive increase in total serum BA's was seen from controls, F0-2 NAFLD to F3/4 NAFLD (Figure 1, Supplementary Table 1). The largest contribution was related to increased concentrations of primary conjugated BA's and secondary conjugated BA's (Figure 1A). The proportion of primary conjugated BAs within the total primary BA pool also increased significantly ($p < 0.05$) between groups (Figure 2).    Specifically, the proportion of glycocholic acid (GCA) and glycodeoxycholic acid (GDCA) also increased as a proportion of total primary and secondary BAs respectively ($p < 0.05$ for all comparison).

Total serum BA levels were associated with serum ALT and GGT and histological features of steatosis, inflammation, ballooning and fibrosis (Supplementary Table 2) but not age, sex or metabolic factors including insulin resistance (HOMA-IR). Serum primary conjugated, secondary unconjugated and secondary conjugated BA levels were predictive for advanced (F3/4) fibrosis in the

cohort (Table 2), however following adjustment for clinical factors also associated with advanced fibrosis (outlined in supplementary table 3), only primary (including GCA and glycochenodeoxycholic acid [GCDCA]) and secondary conjugated BAs (including GDCA) remained significant. The association with total primary conjugated BA's and GCDCA remained significant after additional adjustment for gender and HOMA-IR (data not shown).

**Associations Between Faecal Bile Acids and Advanced NAFLD**

A progressive increase in secondary unconjugated BAs were noted between groups (Figure 1B, Supplementary Table 4) with specific increases in deoxycholic acid (DCA) and lithocholic acid (LC). Similarly, levels of secondary unconjugated BAs, DCA and LC were predictive of advanced fibrosis (Table 3). The association between faecal BA's and advanced fibrosis remained significant following additional adjustment for gender and insulin resistance (HOMA-IR, data not shown). There was no difference in the proportion of unconjugated BAs, DCA or LC within the total secondary BA pool between groups (data not shown).

**Association Between Gut Microbiome and Advanced Liver Fibrosis**

Alpha diversity was significantly lower in the F3/4 group (Chao1 Index 275.0 ± 72.4) compared to controls and NAFLD F0-2 subjects (347.9 ± 72.5, p=0.012 and 349.6 ± 65.1, p=0.003 respectively). Between group differences in diversity were also noted [Analysis of similiarities (ANOISM) p=0.007 (unweighted); p=0.049 (weighted) and ADONIS (PERMANOVA) p=0.002 (unweighted); p=0.01 (weighted)].

The relative abundance of microbial taxa differed significantly between groups, with a greater proportion of Firmicutes, Proteobacteria and Actinobacteria but fewer Bacteroidetes in NAFLD F3/4 patients (Supplementary Figure 1).    At the family level, (Figure 3) a greater abundance of *Actinomycetaceae* and *Lachnospiraceae* observed in F3/4 patients, but lower abundance of *Bacteroidaceae* and an unclassifiable family of the order *Bacteroidales* (*Bacteroidales;other*) (all

p<0.05, Bonferroni corrected). No significant associations were noted at the genus level following Bonferroni correction.

**Association Between Gut Microbiome and Serum and Faecal Bile Acids**

Significant correlations were observed between the abundance of gut microbial families and faecal BA concentrations (Figure 4). Specifically, faecal secondary unconjugated BA's and total secondary BA's correlated with taxa that were also associated with F3/4 fibrosis, namely *Bacteroidaceae, Bacteroidales;other* and *Lachnospiraceae.* Faecal DCA levels also correlated negatively with *Bacteroidaceae* and positively with *Lachnospiraceae.*

Significant correlations were also observed between the abundance of gut microbial taxa and serum BA concentrations, which were similar to the associations seen with faecal BA levels (Figure 4). Notably, serum GCA was positively correlated with *Lachnospiraceae* and negatively with *Bacteroidaceae* and *Rickenellaceae.*

**Associations Between Diet and Alcohol Intake and Gut Microbiome**

A significant positive correlation was seen between average daily alcohol consumption and the relative abundance of *Bacteroidales;other* whereas a negative correlation was observed with *Lachnospiraceae* (Table 4). In addition, alcohol consumers (n=40) compared with non-alcohol consumers (n=49) had a higher mean (standard deviation) abundance of *Bacteroidales;other* [1.6 (2.3) versus 0.6 (0.8), p<0.05 Bonferonni corrected]. *Lachnospiraceae* was lower in alcohol consumers [22.5 (10.4) versus 28.6 (14.4), p-0.045], however this lost statistical significance following Bonferonni correction.

The family *Barnesiellaceae* was correlated with total fat intake (spearman rho 0.22, p=0.04), percentage of energy from fat (spearman rho 0.24, p=0.02) and saturated fat (spearman rho 0.21, p=0.04) and inversely with percentage of energy from carbohydrates (spearman rho -0.29, p=0.006).

No additional associations between taxa and dietary intake of starch, fibre or protein were observed (data not shown).

**Associations Between Diet and Alcohol Intake and Bile Acids**

A significant inverse association was noted with total faecal BA (spearman rho -0.23, p=0.03) and faecal DCA levels (Spearman rho -0.20, p=0.05).    No association with alcohol consumption and serum BA's were noted. In addition, no significant association between diet and serum or faecal BA levels were noted (supplementary tables 5 and 6).

## Discussion

Our findings support the role of the gut-liver axis as an important pathway in the pathogenesis of liver fibrosis in humans with NAFLD. In this large series of patients with liver histology, we found levels of primary and secondary conjugated serum BAs and secondary conjugated faecal BAs were higher among patients with advanced fibrosis, and were also associated with microbial taxa which in turn also associated with advanced liver fibrosis. Despite the relatively small number of patients with F3/4 fibrosis, significant differences in BA and microbiome profiles were observed between groups, highlighting the magnitude of differentiation that occurs with different fibrosis levels in NAFLD. Specifically, serum GCA and GCDA increased as a proportion of the total primary and secondary BA pools respectively, and were independently associated with advanced liver fibrosis. Within the gut, faecal DCA was the dominant BA that increased in the presence of NAFLD and was also predictive of advanced fibrosis. Specific gut microbial taxa (*Lachnospiraceae; Bacteroidales;other; Bacteroidaceae*) were correlated with faecal BA levels (including DCA) and also had altered abundance in patients with advanced fibrosis. Lastly, we found that alcohol ingestion at modest amounts, was inversely associated with *Lachnospiraceae* and *Bacteroidales;other* and faecal DCA levels. This suggests that a complex and potentially pathogenic interaction between BA metabolism and gut microbiome occurs in the setting of NAFLD and advanced liver fibrosis, and that it may be modified by low level alcohol consumption.

**Significance of Altered BA metabolism in Advanced NAFLD**

It is likely that the increased gut BA levels seen in NAFLD F3/4 subjects altered the gut microbial composition, with high levels favouring proliferation of gram positive bacteria such as *Lachnospiraceae* but reducing Bacteroidetes (gram negative) which includes the families *Bacteroidales, Rikenellaceae, Bacteroideaceae and Barnesiellaceae.*(22-24)    Notably in decompensated cirrhosis where faecal BA levels fall, gut gram positives including *Lachnospiraceae* are lower and the abundance of gram negatives (including *Enterobacteriaceae*) are higher.(25) The gut microbiome may in turn, influence secondary BA levels via bacterial enzymes including 7α-dehydroxylase which converts CA to DCA and is present in the *Clostridium XIVa* cluster, a member of the *Lachnospiraceae* family.(22,26,27)    In our cohort, faecal DCA was associated with the abundance of *Lachnospiraceae* and advanced liver fibrosis. Serum G-DCA levels, which represent the hepatic conjugated product of gut DCA, were also increased and associated with advanced fibrosis, consistent with increased liver exposure to gut-derived DCA. In animal models, DCA is cytotoxic and increases gut permeability potentially increasing the exposure of the liver to gut derived toxins (eg bacterial lipopolysaccharide), which promotes liver injury in animal models of NAFLD.(28) Gut derived DCA also promotes a senescence-associated secretory phenotype in hepatic stellate cells in obesity related fatty liver in mice, which in turn secretes inflammatory and tumour-promoting factors, facilitating NASH and HCC development.(29) Thus, our data supports the hypothesis that an interplay between increased BAs and gut microbiome composition favours the production of increased hepato-toxic DCA among NAFLD patients with advanced liver fibrosis.

Our study extends earlier reports that serum BAs increase among patients with NASH (13,14) to demonstrate that levels increase significantly among patients with advanced liver fibrosis and that this relationship was independent of insulin resistance. Similar to our findings, Caussy and colleagues recently demonstrated serum BA levels as well as the proportion of conjugated primary BAs, also increase with increasing fibrosis in patients with NAFLD.(30) We also demonstrate that serum levels (as opposed to proportions) of BA species change significantly with fibrosis in NAFLD.    In addition, we demonstrate that faecal BAs, which are representative of 80% of the total BA pool (in contrast to serum which is <1%), also increase in concentration in the presence of NAFLD compared to controls

and further again in those with advanced hepatic fibrosis.   Thus the combination of increased faecal BA loss in combination with increased serum BA is strongly suggestive of increased hepatic BA synthesis. This supports previous findings in children where increased hepatic gene expression of CYP7A1 (the rate limiting enzyme in BA synthesis) was observed in obese patients with NASH compared with lean healthy controls.(13)

## Influence of Diet and Alcohol

Animal studies suggest that a high fat diet may drive increased BA synthesis with subsequent alterations in the gut microbiome composition favoring increased production of secondary BAs such as DCA.(31) Human studies have been conflicting as to the impact of dietary fat on BA profiles.(32,33) We did not find an association between dietary fat intake and BA profiles though found a modest correlation with *Barnsiellaceae* which correlated with total faecal secondary BA levels. However, we found that low level alcohol consumption, at levels of less than three standard drinks a day, was associated with favorable microbial taxa and had an inverse association with unfavourable microbial taxa and faecal BA species. This raises the possibility that low-level alcohol impacts on gut microbiome-BA interactions to produce an environment that is not injurious to the liver. The association between alcohol and liver injury in NAFLD is controversial, with some but not all studies demonstrating low-level alcohol consumption being associated with reduced fibrosis levels(16).

The reduced faecal BA levels seen with alcohol consumption may reflect reduced faecal excretion due to increased intestinal BA uptake or reduced production of secondary BA (DCA) due to alcohol related reduction in dehydroxylating bacteria such as *Lachnospiraceae(34,35)*. Notably other studies have demonstrated increased faecal DCA levels in alcoholic cirrhosis, however this is presumably related to the higher doses of alcohol ingested and the advanced state of liver disease(36).

## Context of Microbiome Findings with the Literature

We found specific associations between gut microbial composition and NAFLD and advanced fibrosis with increased Proteobacteria, Firmicutes and Actinobacteria, but reduced Bacteroidetes noted at the phylum level. At the family level, reduced *Bacteroidaceae*, an unclassifiable family of the order *Bacteroidales* (*Bacteroidales;other*) but increased *Actinomycetaceae* and *Lachnospiraceae* were observed in F3/4 patients.    Similar to our findings, a consistent association between increased Proteobacteria and NAFLD has been reported previously(37-39), whereas associations between other phylum and NAFLD and fibrosis have been discordant.(39) For example, both increased and decreased Bacteroides have been reported to be associated with advanced fibrosis/cirrhosis using metagenomic methods.(38,40) Reduced *Bacteroidaceae* has previously been found to be associated with liver cirrhosis, whereas in contrast to our findings, reduced *Lachnospiraceae* has been documented to be associated with cirrhosis although this may be related to falling faecal BA levels in the setting of decompensation.(35,39) The associations between *Actinomycetaceae* have not been previously reported to our knowledge and need to be confirmed in larger cohorts. Different methods of microbiome characterization, bio-informatics and analysis as well as the influence of other environmental (eg diet) and patient factors (eg obesity) are all likley contribute to the conflicting findings in the literature.

**Study Limitations**

Whilst the size of the cohort compares favourably with other studies examining BA and gut microbiome profiles, there were only nine subjects in the group with advanced fibrosis limiting our ability to detect associations at the genus level. Despite statistically significant differences being found at other taxonomic levels, we acknowledge that the generalizability may be limited and replication in a different cohort is required. This is particularly relevant given the multiple comparisons performed in our study. Lastly, longitudinal studies characterizing the change in gut microbiome, BA levels and liver histology in response to diet are required before the clinical significance of these findings can be fully understood.

In this prospective study of adults matched for BMI, we demonstrate that BA metabolism is increasingly altered in the presence of NAFLD and subsequent advanced fibrosis. Serum and faecal BA levels progressively increase and become enriched in injurious BAs. In parallel, the gut microbiome profile is enriched with bacteria capable of increasing secondary BA levels, in particular DCA which could promote hepatic inflammation. Lastly, low level alcohol consumption was inversely associated with deleterious BA and gut microbiome profiles. This suggests a significant role of the gut environment in the development of NAFLD and liver fibrosis and supports the concept of modification of the gut microbiome and BA pool as therapeutic strategy.

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**Tables**

**Table 1:** Cohort clinical and metabolic characteristics.

| | Control N=55 | NAFLD F0-2 N=58 | NAFLD F3/4 N=9 | P value |
|---|---|---|---|---|
| Age (years) | 45.6 ± 10.0 | 49.5 ± 10.2 | 51.6 ± 11.2 | 0.075 |
| Male, n (%) | 5 (9.1) | 18 (31.0) | 3 (33.3) | 0.008 |
| BMI, kg/m$^2$ | 42.7 ± 8.9 | 42.2 ± 8.9 | 39.9 ± 8.8 | 0.694 |
| Waist circumference, cm | 118 ± 18 | 124 ± 17 | 125 ± 20 | 0.170 |
| Mean alcohol (gm/week) | 3.7 (9.5) | 3.6 (6.2) | 3.0 (9.2) | 0.997 |
| Obese, n (%) | 52 (94%) | 53 (95%) | 8 (100%) | 0.978 |
| Diabetes, n (%) | 5 (9.1) | 21 (36.8) | 7 (87.5) | < 0.001 |
| Hypertension, n (%) | 16 (29.1) | 25 (45.5) | 4 (50.0) | 0.146 |
| SBP, mmHg | 129 ± 17 | 132 ± 15 | 133 ± 14 | 0.525 |
| DBP, mmHg | 81 ± 11 | 78 ± 8 | 75 ± 13 | 0.178 |
| Bilirubin (mmol/l) | 8.9 ± 4.1 | 9.2 ± 5.9 | 9.2 ± 7.9 | 0.955 |
| ALT (IU/l) | 30 ± 15 | 48 ± 44 | 61 ± 84 | **0.016** |
| AST (IU/l) | 25 ± 29 | 28 ± 15 | 41 ± 16 | 0.247 |
| ALP (IU/l) | 86 ± 26 | 87 ± 26 | 123 ± 48 | 0.393 |
| Albumin (mg/dl) | 40 ± 3 | 40 ± 3 | 38 ± 5 | 0.284 |
| Platelet count (x10$^9$/l) | 277 ± 66 | 285 ± 74 | 205 ± 60 | 0.011 |
| Fasting glucose (mmol/l) | 5.3 ± 0.9 | 6.6 ± 2.4 | 8.9 ± 3.2 | **< 0.001** |
| Fasting insulin    (mmol/l) | 17.2 ± 11.2 | 27.0 ± 20.8 | 34.7 ± 19.0 | **0.003** |
| HOMA-IR[*] | 4.2 ± 3.0 | 8.4 ± 11.1 | 15.8 ± 13.5 | **0.002** |
| Triglycerides[*] (mmol/l) | 1.5 ± 0.7 | 1.9 ± 1.1 | 1.4 ± 0.6 | 0.082 |
| Total cholesterol (mmol/l) | 5.2 ± 1.1 | 4.8 ± 1.2 | 4.3 ± 1.4 | 0.055 |
| LDL-cholesterol (mmol/l) | 3.2 ± 1.0 | 2.7 ± 1.0 | 2.4 ± 0.8 | **0.014** |
| HDL-cholesterol (mmol/l) | 1.3 ± 0.3 | 1.2 ± 0.3 | 1.0 ± 0.1 | **<0.001** |

**Table 2:** Association between serum bile acids and advanced (F3/4) fibrosis in NAFLD (n=120).

| Serum Bile Acid | OR | 95% CI | P value | OR | 95% CI | P value |
|---|---|---|---|---|---|---|
| | | Univariate Analysis | | | Multivariate Analysis* | |
| **Total Bile Acid Level** | 2.81 | 1.35-5.87 | 0.006 | 3.49 | 1.22-9.99 | 0.02 |
| Total primary unconjugated BAs | 1.09 | 0.67-1.76 | 0.72 | | | |
| **CA** | 0.80 | 0.47-1.32 | 0.40 | | | |
| **CDCA** | 1.01 | 0.62-1.65 | 0.97 | | | |
| Total primary conjugated Bas | 2.76 | 1.40-5.45 | 0.003 | 3.99 | 1.37-11.57 | 0.01 |
| **GCA** | 2.09 | 1.24-3.52 | 0.006 | 2.41 | 1.15-5.05 | 0.02 |
| **GCDCA** | 2.92 | 1.39-6.13 | 0.005 | 4.68 | 1.37-16.02 | 0.01 |
| Total Secondary unconjugated BAs | 2.30 | 1.01-5.23 | 0.048 | 1.70 | 0.70-4.12 | 0.24 |
| **DCA** | 2.58 | 1.04-6.18 | 0.03 | 1.72 | 0.67-4.39 | 0.26 |
| Total Secondary conjugated BAs | 3.22 | 1.41-7.37 | 0.006 | 4.08 | 1.35-12.33 | 0.01 |
| **GDCA** | 3.08 | 1.45-6.53 | 0.003 | 3.34 | 1.31-8.54 | 0.01 |

Using logistic regression with bile acids log-transformed. *Adjusted for age, diabetes and serum total cholesterol.

**Table 3**: Association between stool bile acids and advanced (F3/4) fibrosis in NAFLD (n=122).

| Faecal Bile Acids | OR | 95% CI | P value | OR | 95% CI | P value |
|---|---|---|---|---|---|---|
| | | **Univariate Analysis** | | | **Adjusted Analysis** | |
| **Total BAs** | 3.86 | 1.46-10.19 | 0.006 | 3.19 | 1.04-9.78 | 0.04 |
| **Total primary BAs** | 1.40 | 0.99-1.96 | 0.05 | 1.32 | 0.87-2.00 | 0.2 |
| Total primary unconjugated BAs | 1.33 | 0.98-1.80 | 0.06 | - | - | - |
| **CA** | 1.31 | 0.99-1.73 | 0.06 | - | - | - |
| **Total Secondary BAs** | 4.66 | 1.52-14.1 | 0.006 | 3.38 | 1.08-10.57 | 0.04 |
| Total Secondary unconjugated BAs | 4.68 | 1.54-14.22 | 0.006 | 3.38 | 1.08-10.54 | 0.04 |
| **DCA** | 4.18 | 1.42-12.30 | 0.01 | 3.06 | 1.04-9.00 | 0.04 |
| **LC** | 2.89 | 1.19-7.04 | 0.02 | 2.00 | 0.89-4.50 | 0.09 |

Footnote: Using logistic regression with bile acids log-transformed. *Adjusted for age, diabetes and total cholesterol. CA=cholic acid, DCA=deoxycholic acid, LC=lithocholic acid.

**Table 4.**   Association between gut microbial taxa (family level) and alcohol intake (n=89).

| Microbial taxa | Alcohol (g/day) | | Alcohol intake / total energy intake | |
|---|---|---|---|---|
| | Spearman rho | P value | Spearman rho | P value |
| *Actinomycetaceae* | -0.09 | 0.4 | -0.9 | 0.4 |
| *Bacteroidaceae* | 0.07 | 0.5 | 0.07 | 0.5 |
| *Bacteroidales_other* | 0.36 | <0.001 | 0.37 | <0.001 |
| *Barnesiellaceae* | 0.14 | 0.2 | 0.07 | 0.5 |
| *Coriobacteriaceae* | -0.05 | 0.7 | -0.05 | 0.6 |
| *Lachnospiraceae* | -0.22 | 0.04 | -0.22 | 0.04 |
| *Odoribacteraceae* | 0.01 | 0.9 | 0.01 | 0.9 |
| *Rickenellaceae* | -0.06 | 0.6 | 0.17 | 0.1 |

Footnote: Taxa associated with altered faecal BA levels examined.

**Figure Legends**

**Figure 1.** The distribution of bile acids in control patients, patients with NAFLD and no/minimal fibrosis and patients with NAFLD and advanced fibrosis, in serum ( figure 1A, n=120) and stool (figure 1B, n=122).

Footnote: Median and interquartile range presented. * p<0.05 comparing with controls, # p<0.05 comparing with NAFLD F0-2 using Kruskall-Walis test with Bonferroni adjustment.    CA=cholic acid, CDCA= chenodeoxycholic acid, GCA=glycocholic acid, GCDCA=glycochenodeoxycholic acid, DCA=deoxycholic acid, GDCA=glycodeoxycholic acid, LC=lithocholic acid.

**Figure 2 (A-C).**    The proportion of serum primary conjugated bile acids, glycocholic acid and glycodeoxycholic acid increased significantly between controls, NAFLD with F0-2 and NAFLD with F3/4 (n=120).

Footnote: Percentage with standard deviation presented. Comparisons performed using one-way ANOVA with Bonferroni adjustment for post-hoc comparisons. * p<0.05 vs. control; # p<0.05 vs NAFLD F0-2.

**Figure 3.**    Abundance of gut microbial taxa (family level) among non-NAFLD controls, NAFLD with no/minimal fibrosis and NAFLD with advanced fibrosis (n=122).

Footnote**:**   Differences in gut taxa between patient groups analysed using Kruskall-Walis test with Bonferroni correction, *p<0.05.    Blue heat map demonstrates median relative abundance of taxa in each patient group. Bacteroidales_Other represents an unidentified family within the Bacteroidales order.
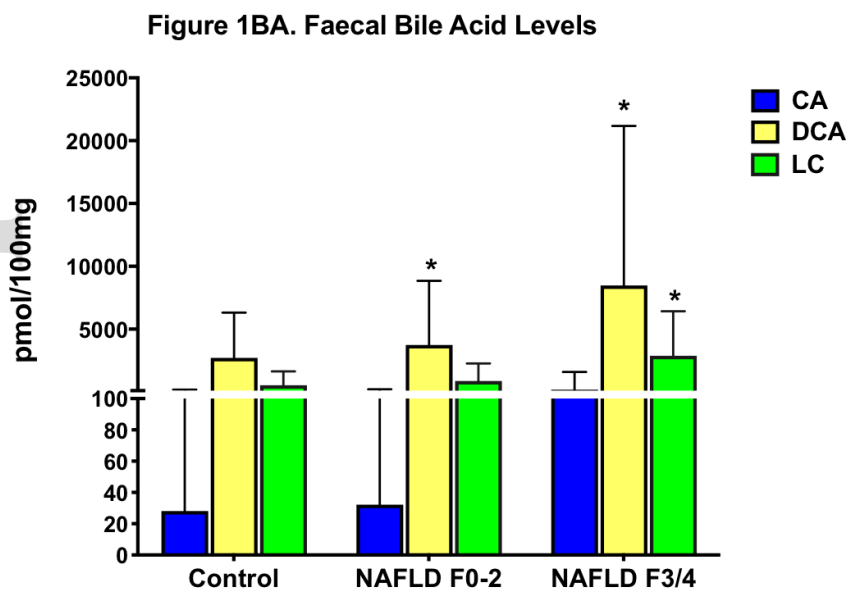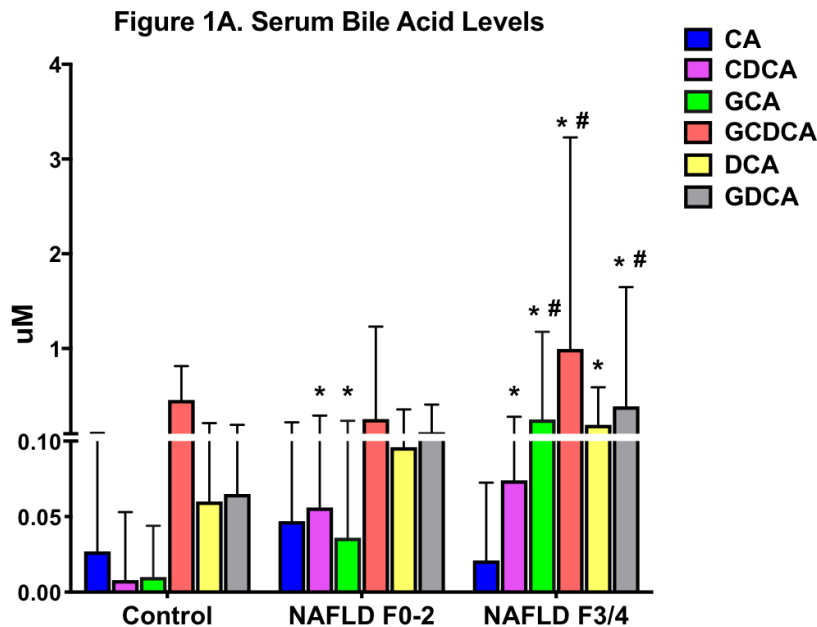
**Figure 4.**   Association between abundance of gut microbial taxa (family level) and serum (n=120) and faecal (n=122) bile acid concentrations.

Footnote**:**   Association between gut taxa and serum and stool bile acid concentrations analysed using spearman correlation with Bonferroni adjustment for multiple comparisons, *p<0.05. Red/green heat map demonstrates spearman correlation co-efficient between relative abundance of each taxa and concentration of bile acid.
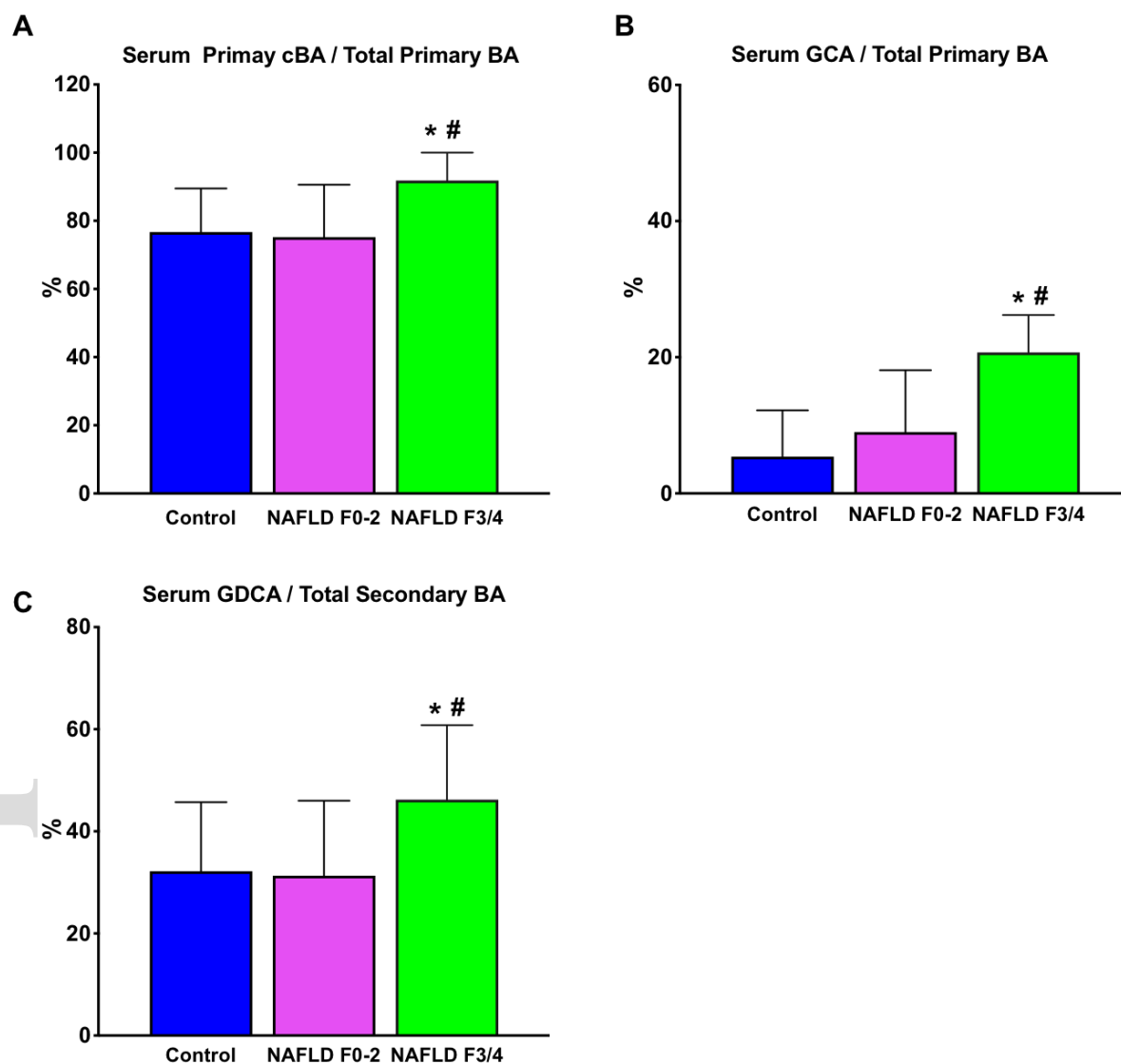
**Figures**

**Figure 1.** The distribution of bile acids in control patients, patients with NAFLD and no/minimal fibrosis and patients with NAFLD and advanced fibrosis, in serum (Figure 1A, n=120) and stool (Figure 1B, n=122).



Figure 1A. Serum Bile Acid Levels



Figure 1BA. Faecal Bile Acid Levels

Footnote: Median and interquartile range presented. * p<0.05 comparing with controls, # p<0.05 comparing with NAFLD F0-2 using Kruskall-Walis test with Bonferroni adjustment. CA=cholic acid, CDCA= chenodeoxycholic acid, GCA=glycocholic acid,
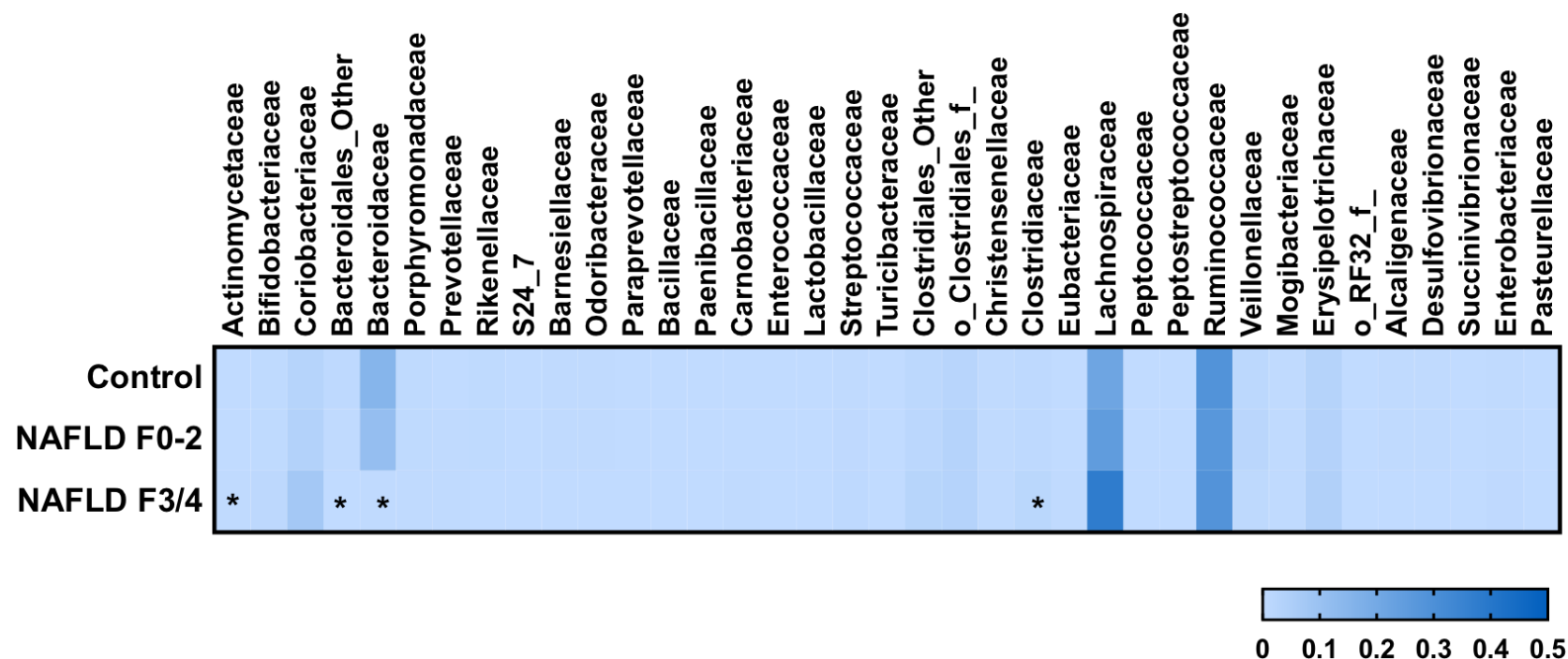
GCDCA=glycochenodeoxycholic acid, DCA=deoxycholic acid, GDCA=glycodeoxycholic acid, LC=lithocholic acid.

**Figure 2 (A-C).** The proportion of serum primary conjugated bile acids, glycocholic acid and glycodeoxycholic acid increased significantly between controls, NAFLD with F0-2 and NAFLD with F3/4 (n=120).
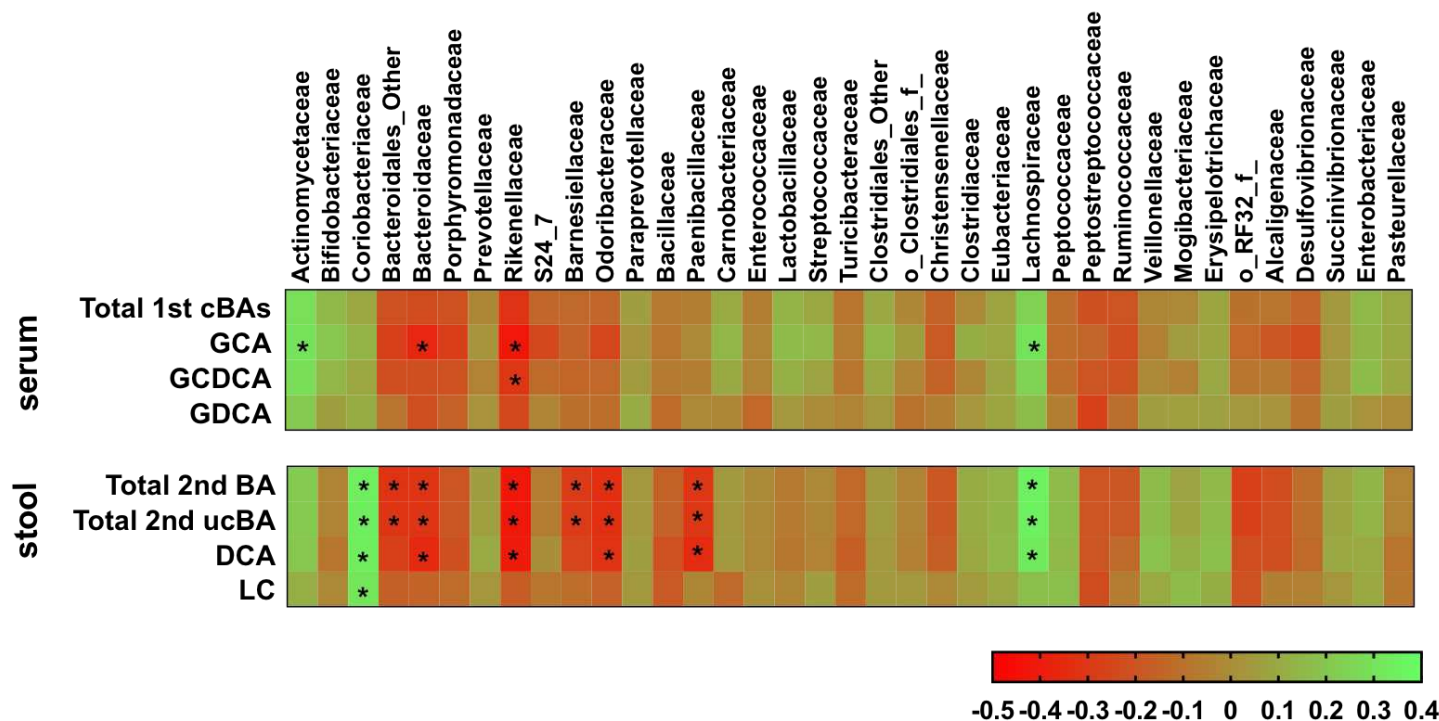


Footnote: Percentage with standard deviation presented. Comparisons performed using one-way ANOVA with Bonferroni adjustment for post-hoc comparisons. * $p<0.05$ vs. control; # $p<0.05$ vs NAFLD F0-2.

**Figure 3.** Abundance of gut microbial taxa (family level) among non-NAFLD controls, NAFLD with no/minimal fibrosis and NAFLD with advanced fibrosis (n=122).



**Footnote:** Differences in gut taxa between patient groups analysed using Kruskall-Walis test with Bonferroni correction, *p<0.05. Blue heat map demonstrates median relative abundance of taxa in each patient group. Bacteroidales_Other represents an unidentified family within the Bacteroidales order.

**Figure 4.** Association between abundance of gut microbial taxa (family level) and serum and faecal bile acid concentrations.



**Footnote:** Association between gut taxa and serum and stool bile acid concentrations analysed using spearman correlation with Bonferroni adjustment for multiple comparisons, *p<0.05. Red/green heat map demonstrates spearman correlation co-efficient between relative abundance of each taxa and concentration of bile acid.