



**Assessing the clinical value of faecal bile acid profiling to predict recurrence in primary *Clostridioides difficile* infection**

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**Title: Assessing the clinical value of faecal bile acid profiling to predict recurrence in primary *Clostridioides difficile* infection**

**Short running title: Bile profiles predict CDI recurrence**

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Authorship:

JRA conceptualized the study; ELM oversaw study administration,  
sample collection and metadata documentation; JRA and JRM  
supervised all aspects of work; BHM, EC, GDSC, MRL ,VH-VDL, JAKM  
and AP performed the metabolomic assays and initial bile acid analysis;

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LM-G undertook the bile acid modelling; JRFW performed further bile acid analysis; BHM performed all BSH assays; LAR helped with design of figures and the visual abstract; BHM and LM-G wrote the initial draft of the manuscript; all authors reviewed and approved the final version of the manuscript. Dr Benjamin H Mullish is the guarantor of the article.

**Statement of interests:** BHM has received consultancy fees from Finch Therapeutics Group, Ferring Pharmaceuticals, and Summit Therapeutics, outside of the submitted work. JRFW has received consultancy fees from GE Healthcare, Intercept Pharmaceuticals, and Novome Biotechnologies outside of the the submitted work. JRM has received consultancy fees from EnteroBiotix Ltd., outside of the submitted work. JRA has consulted for Finch Therapeutics Group, Seres, Pfizer, Janssen, Bristol Myer Squibb, Artugen, Iterative Scopes, Lilly, Merck and Abbvie, and has research support from Merck and Pfizer. All other authors declared no relevant statements of interest.

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1. Abstract:

**Background:** Factors influencing recurrence risk in primary *Clostridioides difficile* infection (CDI) are poorly-understood, and tools predicting recurrence are lacking. Perturbations in bile acids (BAs) contribute to CDI pathogenesis and may be relevant to primary disease prognosis.

**Aims:** To define stool BA dynamics in primary CDI patients, and explore signatures predicting recurrence.

**Methods:** Weekly stool samples were collected from primary CDI patients from the last day of anti-CDI therapy until recurrence, or through eight weeks post-completion otherwise. Ultra-high performance liquid chromatography-mass spectrometry was used to profile BAs; stool bile salt hydrolase (BSH) activity was measured to determine primary BA bacterial deconjugation capacity. Multivariate and univariate models were used to define differential BA trajectories in patients with recurrence *versus* those without, and assess faecal BAs as predictive markers for recurrence.

**Results:** Twenty (36%) out of 56 patients (median age: 57, 64% male) recurred; 80% of recurrence occurred within the first nine days post-antibiotic treatment. Principal component analysis of stool BA profiles demonstrated clustering by recurrence status and post-treatment timepoint. Longitudinal faecal BA trajectories showed recovery of secondary BAs and their derivatives in patients without recurrence only. BSH activity increased over time amongst only non-relapsing patients ( $\beta=0.056$ ; likelihood ratio test  $p=0.018$ ). A joint longitudinal-survival model identified five stool BAs with AUROC > 0.73 for predicting recurrence within nine days post-CDI treatment.

**Conclusions:** Gut bile acid metabolism dynamics differ in primary CDI patients between those developing recurrence versus those who do not. Individual BAs show promise as potential novel biomarkers to predict CDI recurrence.

## 2. Introduction:

*Clostridioides difficile* infection (CDI) continues to present a considerable global disease burden, with an estimated annual incidence of 462,100 cases in the USA on latest assessment, and this trend expected to increase in line with predicted worldwide growth of antimicrobial resistance [1]. While a growing proportion of cases appear to be community-acquired [2], CDI remains the major cause of nosocomial gastrointestinal infection [3], leading to increased hospitalisation time [4], and a higher burden of clinical complications and mortality [5]. Furthermore, CDI is associated with considerable healthcare expenditure, equating to \$1.5 billion annually within the USA [6].

Recurrent CDI remains a major clinical challenge. A key clinical dilemma in the management of CDI patients is prediction of the risk of recurrence caused by either re-exposure to *C. difficile* or reactivation of dormant spores within vulnerable patients. The rate of recurrence within eight weeks following treatment for a primary episode of CDI is 15-25%, and rises as high as 40-60% for patients experiencing further recurrences [7,8]. Updated CDI clinical guidelines recognise that the risk of recurrence in primary CDI patients may influence the preferred management approach and the need for preventative strategies, such as the potential benefit for bezlotoxumab in primary CDI patients with higher recurrence risk compared to those with lower risk [9,10]. However, at present, limited tools exist for the prediction of CDI recurrence [11], with risk of recurrence estimation based upon the use of broad clinical criteria (including age, immunocompromise, and severity of CDI at diagnosis [9,12]). The dynamic assessment of biomarkers could be important not only to stratify low- and high-risk patients, but also to further understand the mechanisms underlying recurrence.

One such biological area of interest relates to the contribution of gut microbiota-bile acid interactions to the pathogenesis of CDI [13]. Prior antibiotic exposure is well-established as the major risk factor for CDI [14] and entails a loss of bacterial microbiome community members possessing bile-metabolising enzymes (including bile salt hydrolases (BSHs) and 7- $\alpha$ -dehydroxylase) [15–18]. Bile salt hydrolases (BSH) are widely-distributed amongst bacteria resident in the gut, and their action (in removing the taurine or glycine groups of primary bile acids secreted into the gut) is the key rate-limiting step for microbiota-mediated 7 $\alpha$ / $\beta$ -dehydroxylation within the gastrointestinal tract [19]. Predicted bile salt hydrolase gene

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3 148 abundance has previously been demonstrated by our group to be reduced in stool in patients  
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5 149 with recurrent CDI compared to those with primary CDI or control patients [15], but no  
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7 150 comparison of BSH activity dynamics between primary CDI patients experiencing recurrence  
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9 151 vs no recurrence after initial treatment has been described previously. Regarding specific bile  
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11 152 acids and CDI risk, the antibiotic-exposed gut develops enrichment in primary bile acids  
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13 153 (including taurocholic acid (TCA), a major pro-germinant trigger to *C. difficile* [20]), and loss of  
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15 154 secondary bile acids, which have established roles in restricting the growth of *C. difficile* and  
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17 155 its toxin activity [21–23] and in modulating regulatory T cell activity [24]. In addition, CDI is also  
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19 156 characterised by reduced activity in the farnesoid X receptor (FXR)-fibroblast growth factor  
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21 157 (FGF) axis, important for bile acid homeostasis [25]. Faecal bile acid profiles have shown  
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23 158 promise for differentiating non-CDI diarrhoea from CDI-related diarrhoea [26].

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27 160 Our recent analysis of clinical factors in a cohort of patients experiencing a first episode of  
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29 161 uncomplicated CDI demonstrated that primary diagnosis of CDI via toxin enzyme  
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31 162 immunoassay (EIA) and treatment with metronidazole were both factors increasing the risk  
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33 163 of recurrence [27]. Extending upon this work, we here present a longitudinal analysis of faecal  
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35 164 bile acid profiles after anti-CDI therapy cessation until recurrence or until eight weeks post-  
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37 165 therapy of patients within this cohort, with the joint aims of better delineating differences in  
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39 166 bile acid dynamics in patients with primary CDI who recur or not, and in identifying potential  
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41 167 biomarkers that may predict future CDI recurrence.

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44 169 **3. Material and Methods:**  
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46 170 **3.1. Patient cohort:**  
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48 171 Clinical parameters of this cohort has been previously-described [27]. In brief, all participating  
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50 172 patients were recruited from the inpatient service at Brigham and Women’s Hospital (BWH;  
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52 173 Boston, USA) as well as two surrounding community hospitals (Brigham and Women’s  
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54 174 Faulkner Hospital and Newton Wellsley Hospital). Potentially eligible patients were identified  
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56 175 by daily reports of patients with positive stool tests for *C. difficile* provided by the BWH Clinical  
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58 176 Microbiology Laboratory. *C. difficile* infection was defined as the presence of diarrhoea,  
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60 177 positive laboratory tests (i.e. glutamate dehydrogenase (GDH) and enzyme immunoassay  
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179 (EIA) toxin or polymerase chain reaction for toxin B, depending upon the testing methods  
used at the associated hospital laboratory), and clinician decision to treat the patient for CDI.

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Primary CDI was defined as no prior episodes of CDI within the prior six months. Patients who were excluded were: patients with inflammatory bowel disease; patients with inherited or acquired immunodeficiencies; severe or fulminant CDI, as diagnosed by IDSA and/or ACG guidelines [9,10]; or the need for ongoing non-CDI antibiotic use that continued past the CDI antibiotic course. Initial powering of the study was derived focused upon the expected clinical outcomes [27], and suggested 20 patients with recurrence would be required to reach these primary outcomes; however, an interim analysis of bile acid profiles from samples from this study demonstrated that at least 10 patients experiencing recurrence would be sufficient for intended bile acid-related analyses [28].

Participating patients were recruited at the time of CDI diagnosis and samples collected through up to eight weeks post-completion of their anti-CDI therapy to assess for recurrence, with clinical assessments regarding presence or absence of diarrhoea occurring at each time point. Active stool collection began one day after completing anti-CDI antibiotics; stool was collected up to twice a week for the first two weeks post-therapy completion, and weekly through to week eight (in the case of no recurrence), or until the point of recurrence. Recurrence was suspected if participants developed diarrhoea (Bristol stool scale 6-7) and at least three bowel movements daily for three days; if these criteria were met, stool was assayed for *C. difficile* via GDH and EIA, and patients were considered to have had a recurrence if both laboratory assays were positive. If the patient developed recurrence, the final sample from that participant included in analysis was the last study protocol sample collected prior to the recurrence; no post-recurrence stool samples were collected. While 75 patients were part of the original clinical cohort [27], only 56 provided post-treatment serial stool samples, and hence the present study has an effective size of  $n=56$ .

Regarding the collection of stool samples, these were either collected on site from patients, or couriered from patients at home within one hour of production, and were all flash frozen on arrival at the storage facility. All samples were maintained at  $-80^{\circ}\text{C}$  until downstream processing.

The Institutional Review Board of BWH gave ethical approval for this work. In addition, a UK National Research Ethics Centre (13/LO/1867) also gave ethical approval for this study.

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**3.2. Bile acid profiling:**

Faecal samples were analysed using ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS). The protocols used for faecal extract preparation [29] and spectra acquisition [18,30] were as previously-described.

Faecal extracts were prepared by extracting from lyophilized dried faecal samples using a 2:1:1 (vol) mixture of water, acetonitrile and 2-propanol to 10mg/mL, via use of a Biospec bead beater with 1.0 mm Zirconia beads followed by centrifugation (16,000×g, 20 min) and filtering the supernatant through 0.45 µm microcentrifuge filters (Costar, Corning). Pooled study reference samples, used as quality control of the profiling data and to monitor assay performance, were prepared using equal parts of the faecal filtrates. In addition, for assessment of linearity of analyte response [31], a series of study reference sample dilutions was created by diluting with ice-cold LC-MS grade methanol to the concentrations of 100%, 80%, 60%, 40%, 20%, 10%, 1% and analyzed at the start and end of each set of sample analyses.

Study and pooled study reference samples were prepared for the UHPLC-MS profiling by aliquoting 75 µL of filtered faecal extracts onto a 96-well plate and adding 75 µL of LCMS grade water and 75 µL of internal standard (IS) solution, followed by the addition of 75 µL of water to each well for study samples, and 75 µL of ice-cold LC-MS grade methanol to each well for study samples. Samples were mixed for two minutes on a plate mixer (1400 rpm at 2-8 °C), incubated at -20°C for 4 hours, then mix briefly and centrifuged at 3486 x g for 10 minutes at 4°C. Aliquots of 125 µL of clear supernatants of each sample were carefully transferred to an analytical 96-well plate that was heat sealed and placed into the autosampler maintained at 4°C for the analysis. Blank samples were prepared in the same way but with an empty tube (i.e. processing neat extraction solution through all steps, including the filter, allowing tracing back of contamination peaks if present).

ACQUITY UHPLC-MS (Waters Ltd., Elstree, UK) coupled to a Xevo G2-S Q-ToF mass spectrometer with an electrospray ionization source operating in negative ion mode (ESI-) (Waters, Manchester, UK) was used for bile acids profiling. LC separation was conducted on a

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ACQUITY BEH C8 column (1.7  $\mu\text{m}$ , 100 mm  $\times$  2.1 mm) maintained at 60°C. A gradient was applied consisting of 10:1 water:acetonitrile, 1 mM ammonium acetate, pH 4.15 (A) and 1:1 isopropanol: acetonitrile (B). Details of the linear gradient method 90% A to 65% are described elsewhere [18]. Injection volume was of 5  $\mu\text{L}$ .

Mass spectrometry parameters were as follows: capillary voltage was set at 1.5 kV, cone voltage at 60 V, source temperature at 150 °C, desolvation temperature at 600 °C, desolvation gas flow at 1000 L/h, and cone gas flow at 150 L/h. Masslynx software (Waters, Manchester, U.K.) was used for data acquisition and visual inspection.

A total of 81 bile acid authentic chemical standards were used to help annotation of endogenous bile acids in faecal samples. The standards were split into eight mixtures prepared in 1:3 water: methanol mixture and analyzed in the beginning and the end of the run. The standards were also spiked into pooled study reference sample analyzed across the run. This helped monitor any potential retention time shifts for each bile acid species and to determine retention time windows (regions of interest) used as an input together with  $m/z$  values for the targeted extraction and integration of annotated bile acids using peakPantherR package [27].

Waters .RAW LC-MS data files were converted to .mzML format using Proteowizard msconvert [32], with removal of signals with less than 100 ion counts. A total of 81 bile acid species were annotated using authentic reference standards to determine their retention times and their relative abundance integrated with the R package, peakPantherR [33]. To adjust for spectra intensity decay along the run, each feature was divided by a LOWESS curve fitted on the study reference samples' intensity [34]. Features with a coefficient of variation greater than 30% in normalised study reference samples, with a Pearson correlation with dilution factor below 0.7 (estimated using a dilution series of pooled SR samples), or below LOD in more than 20% of study samples were discarded. Drift-correction and feature filtering were performed with the nPYc-Toolbox [35]. Features were log-transformed and zeros imputed using impute.QRILC from the *imputeLCMD* R package. For statistical analyses, features were also mean-centered.

### 3.3. Bile salt hydrolase activity assays:

A spectrophotometry-based assay was used to assess the activity of bacterial bile salt hydrolase (BSH) in a subset of stool samples from the cohort, as previously-described [29].

### 3.4. Data analysis and statistics:

Statistical analyses were performed in R. Principal component analysis (PCA) of the processed UHPLC-MS bile acid profiling data was performed using the *ropIs* R package [36]. Bile acid longitudinal trajectories were fitted using mixed effects models with the R package *nlme*, stratifying samples by recurrence, with time (transformed as square root of days for ease of modelling and visualisation; **Supplementary Figure 1**) as a co-variate and subject as random intercept and time slope effect. For each feature, log-likelihood ratio tests of nested models were used to determine the addition of non-linear (squared and cubic) time terms. Area under the curve (AUC) of subject-specific fitted bile acid trajectories up to the maximum timepoint of recurrence was calculated with *pracma* function *trapz* and compared across recurring and non-recurring patients using a Mann-Whitney test, with Benjamini-Hochberg false discovery rate correction (FDR) for multiple testing applied. FDR-adjusted P-values ( $P_{adj}$ )  $< 0.1$ , corresponding to a 10 % false discovery rate, were considered significant.

To assess if bile acids contributed to recurrence, a Bayesian method was used to jointly model bile acid longitudinal changes with the hazard risk of recurrence using *jointModelBayes* function in the *JMbayes* package [37] with default parameters. All joint survival models controlled for gender and factors that could associate with risk of recurrence, namely age, previous antibiotics (yes/no), and our two previously identified risk factors: anti-*C. difficile* antibiotic type (vancomycin/metronidazole) and diagnostic test (EIA/ PCR) [27].

Mean area under the receiver operating characteristic curve (AUROC) was calculated for each feature joint model with the *rocJM* method from *JMbayes* package, using 1,000 Monte Carlo permutations of training sets randomly built with 80% of samples from recurring patients and 80% of samples from non-recurring patients, and the remaining 20% of samples as test set. Three sets of ROC curves were built to predict recurrence within nine days post-treatment, using only one, two or three first timepoints of longitudinal bile acid measurements, while controlling for all the other covariates. Predictive performance for a multivariate joint model

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including the top five predictive features was assessed using the same train-test permutation settings with functions `jm` and `tvROC` from `JMbayes2` package. Only the first timepoint measurement for each feature was used to predict recurrence within nine days post-treatment, while controlling for all the other covariates. Other relevant metrics (including sensitivity, specificity, positive predictive value and negative predictive value) were also recorded. Plots were generated using `tidyverse`, `ggpubr` and `GGally` R packages.

#### 4. Results:

##### 4.1. Participant details:

Key clinical details of the 56 included patients are presented in **Table 1**. No patients had received treatment with ursodeoxycholic acid, and two patients were treated with bile acid sequestrants (one with colestipol, one with cholestyramine). Out of the 56 patients, 20 (36%) developed recurrence, with 80% of recurrence occurring within the first nine days post-antibiotic treatment. Despite a higher proportion of the patients in the non-recurrence group of this cohort having used systemic antibiotics prior to CDI diagnosis than in the recurrence group, our earlier clinical analysis identified no statistically significant difference in use of any major antibiotic group between patients with or without recurrence [27].

##### 4.2. Timepoint since diagnosis and recurrence status influence faecal bile acid profile in primary CDI:

In total, 71 bile acids were detected and relatively quantified in the peakPanther dataset (see **Supplementary Table 1** for full list of bile acids).

We first sought to explore the overall impact of recurrence status and post-treatment sampling time upon faecal bile acid profiles; as such, all sample profiles were first visualised using multivariate statistical analysis. On PCA of the annotated bile acid profiles of all included samples and time points ( $n=273$ ), samples clustered mostly according to timepoint of sampling and by recurrence status of the patient (**Figure 1A**). Individual trajectories showed that patients who did not recur had more pronounced dynamic changes in their faecal bile acid composition than those who recurred (**Figure 1B**). This higher temporal variability in non-recurring patients was reflected in the comparison of mean Euclidean distance of within-individual longitudinal samples collected the first nine days post-treatment, which showed a

higher - but not statistically significant - dissimilarity in bile acid composition across time in non-recurring patients (Wilcoxon  $p = 0.13$ ; **Figure 1C**).

#### **4.3. Recovery of bacterial bile acid metabolism and restoration of the pre-morbid bile acid milieu occurs in primary CDI patients without recurrence:**

To explore the temporal changes in bile acid metabolism in patients recovering from primary CDI – and how recurrence may impact upon this – we first compared the longitudinal trajectories (from day 1 post-treatment up to 27 days, the last recorded recurrence event) of faecal bile acids for the 20 patients experiencing recurrence *versus* those 36 with no recurrence (**Figure 2A, B and C**).

Of the identified bile acids, 24 were found to have marked differences in longitudinal trajectories, assessed via comparison of area under curve (AUC) of patient-specific trajectories among recurring vs non-recurring patients (**Figure 2C and Supplementary Table 1**). Two of the faecal bile acids with the most marked difference in AUC between recurring and non-recurring patients were the secondary bile acids DCA and LCA, with AUCs for both significantly higher in non-recurring patients (DCA:  $P_{adj} = 2.3 \times 10^{-5}$ ; LCA:  $P_{adj} = 4.1 \times 10^{-3}$ , Mann-Whitney with Benjamini-Hochberg FDR; **Figure 2A**). Other faecal bile acid AUCs that strongly differentiated between patients who recurred and those who did not (with larger AUC in non-recurring patients) were notable for also being secondary bile acids, and particularly derivatives of DCA and LCA. This included glycine-conjugated forms (glycoDCA and glycoLCA;  $P_{adj} = 2.4 \times 10^{-5}$  and  $6.6 \times 10^{-3}$  respectively, Mann-Whitney with Benjamini-Hochberg FDR), as well as forms presumably derived from bacterial hydroxysteroid dehydrogenase (HSDH) enzyme activity. Specifically, this latter group included: isoDCA (5- $\beta$ -cholanolic acid-3- $\beta$ , 12- $\alpha$ -diol;  $P_{adj} = 2.3 \times 10^{-5}$ ), the 3 $\beta$ -HSDH-derived epimer of DCA; 3-oxoDCA (5- $\beta$ -cholanolic acid 12- $\alpha$ -ol-3-one;  $P_{adj} = 7.3 \times 10^{-5}$ ), the 3 $\alpha$ -HSDH-mediated dehydrogenated derivative of DCA; and 12-ketoDCA (3- $\alpha$ -hydroxy-12-ketolithocholic acid;  $P_{adj} = 7.3 \times 10^{-5}$ ), the 12 $\alpha$ -HSDH-mediated dehydrogenated derivative of DCA. In contrast, participants experiencing recurrence (relative to those with non-recurrence) had higher AUC levels of primary bile acid derivatives, sulfated and/or amide-conjugated ursodeoxycholic acid (UDCA), or those similar to the 5 $\beta$ -cholanolic acid (ursocholanolic acid) ‘skeleton’ from which all bile acids are derived. In particular, this

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group included glycoursocholic acid ( $P_{adj}= 2.5 \times 10^{-5}$ ), chenodeoxycholic acid-3-sulfate ( $P_{adj}= 0.027$ ), as well as a number of oxo-derivatives of cholic acid, i.e. 3,7,12-dehydrocholic acid, 3- $\alpha$ -hydroxy-7,12-diketocholeic acid, and 12-dehydrocholic acid|7-dehydrocholic acid ( $P_{adj}= 6.7 \times 10^{-4}$ ,  $2 \times 10^{-3}$  and 0.012 respectively).

To link these bile acid profile changes with microbiome functionality, we went on to explore BSH activity dynamics between primary CDI patients experiencing recurrence vs those who did not after initial treatment. For these experiments, we undertook BSH activity assays on a subset of primary CDI patients who experienced CDI recurrence ( $n=9$ ) and those with non-recurrence ( $n=18$ ). BSH activity increased over time amongst patients who did not relapse ( $\beta= 0.056$ ; likelihood ratio test  $p= 0.018$ ; **Figure 3A**), while not enough data points were available to test changes in BSH activity over time in patients who relapsed. Comparison of BSH activity in patients experiencing recurrence vs no recurrence showed no significant difference in activity one day after antibiotic treatment cessation, and a trend towards a lower BSH activity was observed in patients who recurred at the latest timepoints measured, corresponding to a mean  $\pm$  SD of  $8 \pm 7$  days for recurring patients, and  $35 \pm 5$  days for non-recurring patients (Wilcoxon,  $p= 0.078$ ; **Figure 3B**).

#### 4.4. Faecal bile acid profiles immediately following antibiotic treatment for primary CDI are related to risk of recurrence, and may be a predictive tool for recurrence:

Comparison of AUC under the longitudinal trajectories allowed us to evaluate the overall recovery of bile acid species over a time interval, but it does not provide information on the imminent effect bile acids have upon the risk of recurrence at a discrete timepoint. More specifically, it would be of clear clinical interest if it was feasible to use a 'snapshot' faecal bile acid profile obtained soon after the end of antibiotic treatment for primary CDI and use this to gain insight into future recurrence risk. As such, we sought to further investigate the relationship of specific bile acids to recurrence risk, and whether they had potential utility in use as a predictive marker of recurrence.

To explore the contribution of faecal bile acids to the risk of recurrence, we undertook a Bayesian joint longitudinal and survival modelling approach [37] (**Figure 4A** and

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**Supplementary Table 2).** Given that 80% of recurrences within this cohort occurred within nine days post-completion of anti-CDI therapy, this time period was the area of focus for prediction of recurrence. Recognising the potential impact of key clinical factors of this cohort upon both clinical outcome and bile acid profiles (including antibiotic exposure prior to CDI diagnosis, use of either metronidazole or vancomycin as initial anti-CDI antibiotic, and diagnostic test used for CDI), these factors were all incorporated as co-variables within the model, as described in **Section 3.4**. We found 16 bile acids associated with increased risk of recurrence, while 15 were associated with reduced risk. Particularly noteworthy was the marked increased risk of recurrence associated with faecal ursocholanic acid ( $p=0.006$ ), together with its conjugated derivatives, glycoursocholanic acid ( $p<0.001$ ) and tauroursocholanic acid ( $p=0.034$ ). In addition, UDCA and its sulfated and tauro-conjugated forms were also positively associated with recurrence. Conversely, it was noteworthy that those bile acids most strongly associated with a reduced risk of recurrence were predominantly glycine-conjugated bile acids, with many of them also sulfated (**Figure 4A**).

We next aimed to assess the model's performance in predicting recurrence in patients with primary CDI using faecal bile acid profiles. Given the time frame at which recurrences occur both within this study and in clinical practice more generally, we modelled prediction of recurrence within the first nine days post-treatment, focusing on using only the measurements of post-treatment day 1, as it would be more translatable to clinical practice than conducting daily faecal samplings and BA measurements (**Figure 4D**). Area under the receiver operating characteristic curve (AUROC) showed good prediction of recurring patients within nine days post-treatment by 5- $\alpha$ -cholanic acid-3, 6-dione (AUROC=0.81; **Figure 4D**). Four other faecal bile acids were also identified to have AUROCs > 0.73 for prediction of recurrence (**Figure 4D**). When using two or three consecutive measurements, 5- $\alpha$ -cholanic acid-3, 6-dione continued to be the best predictor of recurrence, but AUROC was slightly smaller (0.79 and 0.78, respectively) (**Figure 4B, 4C**); notably, this bile acid was also associated with sensitivity > 80% and negative predictive value > 90% in all three models, further supporting its clinical utility (**Table 2**). Similarly, prediction performance did not improve when adding all top five performing bile acids in the model, with an AUROC of 0.6; this was due to the collinearity among the features (**Supplementary Figure 2**). Altogether, measuring

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5- $\alpha$ -cholanic acid-3,6-dione on the first day post-treatment might be sufficient to predict the risk of recurrence.

## 5. Discussion:

The body of evidence supporting a close interaction between perturbed bile acid composition and risk of CDI has been growing over the past decade [13]. In particular, enriched gut TCA and loss of gut secondary bile acids (particularly DCA and LCA) that characterise the CDI gut upon the ability of *C. difficile* to undergo germination, vegetative growth, and toxin activity. However, limited data have been described on the degree to which this axis is perturbed in primary CDI specifically, and – if so – whether it might be exploitable as a biomarker to predict recurrence within this condition. Our large, intensively-sampled primary CDI cohort allowed us an opportunity to explore this question with greater granularity than has been possible previously.

We chose to focus only on faecal bile acid profiling for several reasons. Firstly, metabolites are the final endpoint of host-microbial metabolic reactions, as opposed to metagenomics, which represent microbial functional potential but not its true activity. Therefore, our findings provide valuable mechanistic insights that could later on be further investigated with other omics. Secondly, there remains significant gaps in knowledge related to metagenomic annotation of bile metabolising enzyme genes [38], with current state-of-the-art metabolomics techniques being able to detect a far more diverse milieu of bile acid species than currently known enzymes. Thirdly, the high-throughput, highly-sensitive nature of metabolomic assays make them an attractive approach to develop potential novel clinical biomarkers.

Previous work from our laboratories described enrichment in gut primary bile acids and loss of secondary bile acids in patients with both primary and recurrent CDI, together with reduced predicted stool *bsh* gene abundance in recurrent CDI compared to primary CDI or controls; however, this was assessed only cross-sectionally rather than longitudinally [15]. One confounder in interpreting cross-sectional datasets in CDI (particularly primary CDI) is that vancomycin (the major anti-CDI therapy that most participants are initially treated with) has itself been associated with an altered gut microbiota profile, as well as by loss of faecal

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secondary bile acids and enrichment in primary unconjugated bile acids [39,40]; in contrast, metronidazole has less marked effects upon microbial bile acid metabolism [41]. While these collateral effects of vancomycin upon gut microbial bile acid metabolism may be expected to facilitate *C. difficile* growth, such effects need to be balanced against the efficacy of the drug in its effect against *C. difficile* itself, with vancomycin having clear superiority in this regard compared to metronidazole [27]; a focus for antibiotic discovery within this area has been on molecules with narrow-spectrum activity against *C. difficile* but which cause minimal perturbation to microbially-derived bile acids, with ridinilazole being a promising agent with this regard [42]. More broadly, these data support and extend upon our previous results by suggesting an increase of BSH functionality in non-recurring patients over time, as does the associated recovery of transition of primary to secondary bile acids and their derivatives (a further nuance in this system relates to the recent demonstration that different BSHs, due to their different substrate specificity, have different degrees of protective ability against CDI [43]).

The conventional clinical interpretation of recurrence after an episode of primary CDI is to view this as a pathogen-derived event (e.g. related to germination of residual spores/ growth of residual *C. difficile* vegetative cells, or re-exposure of a vulnerable host to *C. difficile*); this factor likely relates at least partly to efficacy of the initial therapy used, and the strategy to overcome this is escalated or alternative anti-*C. difficile*-focused therapies. However, our data suggest that recurrence may alternatively be viewed as a failure of sufficient recovery of enterohepatic bile-metabolising functionality, and suggest by extension that targeted microbiome-focused therapies or bile acid co-treatments may have a potential role in mitigating the risk of recurrence. Supportive of this view (albeit from the context of recurrent rather than primary CDI) is the finding that a key mechanism underlying the efficacy of faecal microbiota transplant (FMT) in recurrent CDI is through restoration of bacterial BSH functionality, and the associated restoration of a pre-morbid gut bile acid profile [18,23,44,45]. Similarly, both a spore-based 'microbiome therapeutic' derived from alcohol-shocked healthy donor stool and a live biotherapeutic product consisting of eight *Clostridia* strains were both shown to rapidly and sustainably restore stool secondary bile acids [46,47].

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Much research to date on gut microbiome-bile acid interactions in the context of CDI has focused on the bacterial enzymes BSH and 7- $\alpha$ -dehydroxylase [13], and it was therefore unsurprising to see two bile acids that are direct products of this pathway – DCA and LCA – as having longitudinal trajectories within the gut that differed between those patients with and without recurrence. However, our data also give novel insight into disruption to complex additional interactions within this domain. For example, ursocholic acid (also known as 5- $\beta$ -cholic acid, the ‘skeleton’ from which other bile acids are derived, lacking the characteristic bile acid hydroxyl groups at positions 3, 7 and 12) and its simple derivatives had higher AUC in recurring patients compared to those who did not recur, and were associated with recurrence risk in the joint longitudinal-survival model. As another example, bile acids with oxo-/keto- groups appeared to associate with risk of recurrence across both models; this particularly applied to 3-oxo groups (likely to represent gut bacterial 3 $\alpha$ -hydroxysteroid dehydrogenase (HSDH) activity), with such bile acids more predictive of increased recurrence risk in the joint longitudinal-survival model. Conversely, one particularly interesting finding relates to isoDCA (5- $\beta$ -cholic acid-3- $\beta$ , 12- $\alpha$ -diol), which was observed in both models to be associated with non-recurrence; this bile acid is the 3- $\beta$ -hydroxy epimer of DCA, with its presence in stool likely resulting from metabolism of DCA first via bacterial 3 $\alpha$ -HSDHs to 3-oxoDCA, before bacterial 3 $\beta$ -HSDH epimerization. As such, this suggests a role for gut bacterial 3 $\beta$ -HSDH activity as being overall associated with protection from recurrence. This finding extends upon previous work *in vitro* demonstrating that a broad range of gut bacterial derived secondary bile acids (including iso- forms) inhibit different aspects of the life cycle of *C. difficile* and its toxin activity [21]. The range of gut bacteria with 3 $\alpha$ -HSDH functionality is focused around (but not based purely within) the class of *Clostridia*, and includes a number of bacteria with other well-characterised bile-metabolising functionality, e.g. *Clostridium scindens*, the archetypal bacterium recognised to possess 7- $\alpha$ -dehydroxylation functionality [38]; bacteria with 3 $\beta$ -HSDH functionality include *Ruminococcus gnavus* [48]. This observation leads us to conclude that the presence/ recovery of even very particular microbial bile-metabolising functions within the gut microbiota may be sufficient to profoundly affect clinical outcome. In particular, our data allow us to propose that primary CDI patients may typically retain gut bacterial 3- $\alpha$ -HSDH functionality after anti-CDI therapy, but only the additional presence of 3- $\beta$ -HSDH activity results in reduced recurrence risk. Of note, HSDH

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activity depends also on the gut pH and availability of NAD(H) or NADP(H) cofactors [49], leading us to hypothesise that patients who recur may have a more 'hostile' pro-oxidative gut environment. The relevance of 3 $\beta$ -HSDH-mediated biotransformations of secondary bile acids to human health has been of recently-growing interest; one pertinent example of this is the recent demonstration that isoallothocholic acid produced from this enzyme system by *Odoribacteriaceae* strains had potent effects against the growth of *C. difficile* and other pathobionts [50]. Furthermore, isoLCA has recently been described to suppress pro-inflammatory T<sub>H</sub>17 cell differentiation, and to be at reduced levels in patients with colitis (at least colitis caused by inflammatory bowel disease) [51].

A further interesting observation relates to the presence of UDCA and its derivatives as associating with increased risk of recurrence in the joint longitudinal model; this observation seems initially counterintuitive, given the apparent benefits associated with the exogenous use of UDCA in a rodent CDI model (albeit potentially more through its impact upon host bile acid receptor signalling systems rather than via direct effects on the life cycle of *C. difficile* [52]). However, we found that only bile acids without 7- $\alpha$ -hydroxyl groups had higher AUCs in patients who did not experience recurrence. From these data, we conclude that recovery of 7- $\alpha$ -dehydroxylation enzymatic activity over time drives bile acid biotransformation in a way that reduces recurrence risk. By possessing a 7 $\beta$  group, UDCA is not readily acted upon by 7- $\alpha$ -dehydroxylase-producing bacteria, and may undergo downstream metabolism via more unconventional routes [53] (such as isomerisation back to 7 $\alpha$  followed by 7 $\alpha$ -dehydroxylation, or direct 7 $\beta$ -dehydroxylation to LCA) that have less impact than immediate 7- $\alpha$ -dehydroxylation of primary bile acids upon direct bile acid-*C. difficile* interactions.

Due to the current lack of clinical biomarkers to prognosticate recurrence in primary CDI – we further explored whether stool bile acid signatures may have utility as a predictive tool for recurrence of CDI. Work to date within this field is relatively limited, although we previously demonstrated that stool bile acid profiles could differentiate primary from recurrent CDI with 84.2% accuracy [15], and faecal LCA (combined with urinary p-cresol sulfate) has been demonstrated to predict response to FMT for recurrent CDI with 100% accuracy [54]. Our data here identifies at least five stool bile acids whose day 1 post-treatment measurement was

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558 enough to predict recurrence within 9 days with AUROC ranging from 0.81 to 0.73. Regarding  
559 the actual bile acids that predicted recurrence, it was of particular interest that an unusual  
560 'allo' bile acid - 5- $\alpha$ -cholanic acid-3, 6-dione - was the most robust predictor of recurrence  
561 (in terms of AUROC, as well as high sensitivity and negative predictive value), especially since  
562 formation of allo bile acids is recognised to be a function of the gut microbiome of particular  
563 mammals (including rabbits and rats) but not of humans [55]; further work is needed to  
564 establish the potential biological significance of this bile acid to this disease state.

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566 The level of accuracy of our bile acid model in recurrence prediction is similar to a previous  
567 study that used 16S rRNA gene sequencing of a single stool sample from each of 88 primary  
568 CDI patients to derive a bacterial signature 'risk index' to predict recurrence, where an AUROC  
569 of 0.78 for prediction of recurrence was reported [56] (unfortunately, no DNA was available  
570 from our current study to explore where taxa with recognised bile metabolising functionality  
571 and/or specific relevant genes from within the microbiome (i.e. *bsh*) may be contributory to  
572 our analyses). Collectively, data such as these allow us to suggest that microbiome and  
573 metabolome profiling in primary CDI may hold promise as accurate biologically-rationale  
574 biomarkers to predict recurrence. However, such studies would need replication in larger  
575 cohorts - including a broader range of patients - before they could be considered for use  
576 within clinical practice. While we used internal validation of the model we developed here,  
577 prospective external validation would also clearly be desirable. It is also of note that while  
578 our model included the key clinical features of this particular study population as co-variables  
579 (including antibiotic use), these may differ between different CDI patient groups, and may  
580 even evolve over time, which may require models to be recalibrated. For instance, recognition  
581 of metronidazole use being associated with higher risk of recurrence from the earlier clinical  
582 analysis of this cohort - coupled with recognition of their relatively poor efficacy in treatment  
583 of hypervirulent *C. difficile* strains - means that use of this drug in primary CDI therapy is likely  
584 to decline further in Western settings, although factors including medication availability and  
585 healthcare resource may mean that it still used for this role in other settings [57]. A further  
586 limitation of our model is that we may not have considered or controlled for factors that may  
587 differ between groups and influence recurrence; for example, while our clinical assessment  
588 of this cohort demonstrated that prior antibiotic use and overall dietary type were not factors  
589 that influenced CDI recurrence risk [27], we did not perform a detailed analysis of whether

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non-CDI medications or specifically dietary types/ components differed between the groups with and without recurrence, when such factors impact upon microbiome functions including bile acid metabolism [58]. For our model and analyses to have greater applicability, it would be interesting to explore the dynamics of stool bile acid changes after a second course of treatment following recurrence of CDI, but we did not have access to such patients or samples in this study. Pragmatic aspects regarding use of stool bile acid assays (i.e. potentially time consuming multi-step aspect of sample preparation and batch effects between samples runs in mass spectrometry analysis) would also require careful consideration prior to use clinically. As such, while a stool bile acid-based assay is not ready for clinical adaptation at present, this study may be a launchpad to exploring the development of targeted bile acid point-of-care tests that may identify patients at risk of CDI recurrence earlier in their clinical course.

In conclusion, our data demonstrate distinctive differential gut bile acid trajectories that differentiate recurrence from non-recurrence in patients with primary CDI, and define the degree to which different gut bile acids associate with recurrence risk. These data extend upon existing knowledge regarding the influence of microbiome-mediated bile acid metabolism pathways upon clinical outcomes in CDI. By extension, these data suggest a clinical case for focus upon therapies targeted at recovery of microbiome-bile acid interactions to potentially reduce the risk of recurrence after primary CDI, and a feasible role for stool bile acids as novel, rationale biomarkers to predict CDI recurrence.

**Data availability:** The bile acid profiling data generated for this project is available on the MetaboLights Repository, study number: MTBLS657. All data produced in the present study are available upon reasonable request to the authors.

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Tables:

**Table 1: Patient Characteristics.** Abbreviations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mean index; CDI: *Clostridioides difficile* infection; EIA: enzyme immunoassay; Hct: haematocrit; INR: international normalised ratio; PCR: polymerase chain reaction; Plts: platelet count; PPI: proton pump inhibitor; PT: prothrombin time; SD: standard deviation; WBC: white blood cell count.

| Variable Name                               | Patients with recurrence (n = 20) | Patients without recurrence (n = 36) |
|---------------------------------------------|-----------------------------------|--------------------------------------|
| Age (Mean ± SD)                             | 58.2 ± 18.5                       | 57.5 ± 15.2                          |
| Male N,%                                    | 13 (65.0%)                        | 23 (63.8%)                           |
| Race N,%                                    |                                   |                                      |
| Black                                       | 1 (5%)                            | 8 (22.2%)                            |
| White                                       | 19 (95%)                          | 28 (77.7%)                           |
| BMI (Mean ± SD)                             | 28.1 ± 6.5                        | 28 ± 6.6                             |
| Received Antibiotics prior to Diagnosis N,% | 13 (65.0%)                        | 27 (75%)                             |
| Prior PPI use N,%                           | 9 (45.0%)                         | 18 (50%)                             |
| History of Cirrhosis N,%                    | 1 (5.0%)                          | 2 (5.5%)                             |
| Dietary restrictions N,%                    |                                   |                                      |
| No                                          | 18 (90.0%)                        | 34 (94.4%)                           |
| Vegan                                       | 1 (5%)                            | 0 (0%)                               |
| Vegetarian                                  | 1 (5%)                            | 1 (2.7%)                             |
| Gluten Free                                 | 0 (0%)                            | 1 (2.7%)                             |
| Lactose Free                                | 0 (0%)                            | 0 (0%)                               |
| Smoking status                              |                                   |                                      |
| Never                                       | 14 (70%)                          | 9 (25%)                              |
| Former                                      | 5 (25%)                           | 16 (44.4%)                           |
| Current                                     | 1 (5%)                            | 1 (2.7%)                             |
| Diagnosis of Irritable Bowel Syndrome N,%   | 0 (0%)                            | 6 (16.6%)                            |
| Baseline diarrhoea or constipation N,%      |                                   |                                      |
| No                                          | 16 (80%)                          | 26 (72.2%)                           |
| Diarrhoea                                   | 1 (5%)                            | 5 (13.8%)                            |
| Constipation                                | 2 (10%)                           | 4 (11.1%)                            |
| Both                                        | 1 (5%)                            | 1 (2.7%)                             |
| Baseline Bristol Score (Mean ± SD)          | 5 ± 1.3                           | 2.8 ± .98                            |
| Ursodiol Use N,%                            | 0 (0.0%)                          | 0 (0.0%)                             |
| Cholestyramine Use N,%                      | 1 (5%)                            | 0 (0.0%)                             |
| Cholestid Use N,%                           | 0 (0.0%)                          | 1 (2.7%)                             |
| CDI Treatment Regimen                       |                                   |                                      |
| Metronidazole                               | 6 (30%)                           | 7 (19.5%)                            |
| Vancomycin                                  | 14 (60%)                          | 29 (80.5%)                           |
| Other current antibiotics (not for C diff)  | 0 (0%)                            | 0 (0%)                               |
| Test Used for Diagnosis N,%                 |                                   |                                      |
| PCR                                         | 3 (15%)                           | 19 (52.7%)                           |
| EIA Toxin                                   | 17 (85%)                          | 17 (47.3%)                           |
| Baseline Lab Values (Mean ± SD)             |                                   |                                      |
| WBC                                         | 8.2 ± 3.5                         | 10.8 ± 5.9                           |
| Hct                                         | 35.5.1 ± 6.0                      | 36.5.1 ± 5.6                         |
| Plts                                        | 199.7 ± 73.1                      | 256.8 ± 114.5                        |
| ALT                                         | 20.2± 14.0                        | 61.7± 117.4                          |
| AST                                         | 23 ± 14.8                         | 53.7 ± 114.5                         |
| Alkaline Phosphatase                        | 74.2 ± 28.1                       | 87.2 ± 46.3                          |
| Total bilirubin                             | 0.6 ± 0.36                        | 0.6 ± 0.5                            |
| Blood urea nitrogen                         | 20.8 ± 26.2                       | 20.1 ± 22.1                          |
| Creatinine                                  | 1.1 ± .59                         | 1.2 ± .15                            |

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|-----|----------------|-----------------|
| PT  | $15.6 \pm 1.2$ | $25.5 \pm 34.3$ |
| INR | $1.2 \pm 0.09$ | $1.3 \pm 0.37$  |

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**Table 2: Longitudinal-survival model performance metrics.** Longitudinal-survival model was used to predict probability of ‘survival’ [37] (in this context, survival defined as CDI recurrence) within nine days post-CDI treatment, using up to three, two or one timepoints of bile acid measurements. Tables show best sensitivity and specificity (as determined by the Youden index), and predictive values of the ROC curves shown in Figure 4B-D. PPV: positive predictive value; NPV: negative predictive value.

Prediction using three timepoints (Figure 4B)

| Bile acid                                                   | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|-------------------------------------------------------------|-----------------|-----------------|---------|---------|
| 3,6-Diketocholelanc Acid   3,12-Diketocholelanc Acid (0.73) | 64              | 73              | 38      | 89      |
| 3,7-Diketocholelanc Acid (0.73)                             | 68              | 68              | 34      | 90      |
| 5-alpha-Cholelanc Acid-3, 6-dione (0.78)                    | 81              | 68              | 38      | 94      |
| 9(11), (5-beta)-Cholelanc Acid-3-alpha-ol-12-one (0.75)     | 70              | 68              | 34      | 90      |
| Chenodeoxycholic Acid-3-Sulfate (0.74)                      | 68              | 71              | 35      | 90      |

Prediction using two timepoints (Figure 4C)

| Bile acid                                                   | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|-------------------------------------------------------------|-----------------|-----------------|---------|---------|
| 3,6-Diketocholelanc Acid   3,12-Diketocholelanc Acid (0.74) | 63              | 74              | 43      | 87      |
| 3,7-Diketocholelanc Acid (0.75)                             | 68              | 68              | 39      | 87      |
| 5-alpha-Cholelanc Acid-3, 6-dione (0.79)                    | 80              | 69              | 44      | 92      |
| 9(11), (5-beta)-Cholelanc Acid-3-alpha-ol-12-one (0.75)     | 57              | 80              | 47      | 88      |
| Chenodeoxycholic Acid-3-Sulfate (0.73)                      | 59              | 82              | 51      | 87      |

Prediction using one timepoint (Figure 4D)

| Bile acid                                                   | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|-------------------------------------------------------------|-----------------|-----------------|---------|---------|
| 3,6-Diketocholelanc Acid   3,12-Diketocholelanc Acid (0.76) | 69              | 72              | 45      | 88      |
| 3,7-Diketocholelanc Acid (0.76)                             | 73              | 68              | 43      | 89      |
| 5-alpha-Cholelanc Acid-3, 6-dione (0.81)                    | 87              | 68              | 47      | 94      |
| 9(11), (5-beta)-Cholelanc Acid-3-alpha-ol-12-one (0.77)     | 61              | 81              | 52      | 87      |
| Chenodeoxycholic Acid-3-Sulfate (0.73)                      | 64              | 82              | 53      | 88      |

**Figure Legends:**

**Figure 1: Initial multivariate statistical analysis of faecal bile acid profiles obtained from patients with primary CDI.** Principal component analysis (PCA) of annotated bile acid relative intensities for all included samples ( $n=273$ ), as coloured by time point of collection (A) or recurrence status (B). C) Average within-individual Euclidean distance of bile acid composition across timepoints for participants with more than one sample collected up to the first 9 days post-treatment ( $n= 45$ ; 12 patients with recurrence and 33 patients without recurrence).  $p$  value was calculated using Wilcoxon test.

**Figure 2: Longitudinal trajectories of faecal bile acids of primary CDI patients, comparing those experiencing recurrence versus those with no recurrence.** As measured across days to weeks following the end of initial antibiotic therapy. Longitudinal trajectories are demonstrated for A) deoxycholic acid and B) lithocholic acid; thick lines correspond to the mean fit for patient with recurrence and patients without recurrence, while thinner lines are the fitted individual trajectories, from which area under curve (AUC) was calculated. Dots correspond to the  $\log_{10}$ -transformed and mean-centered BA relative quantification. C) Area under curve of subject-specific trajectories, ordered by increasing median in recurrers.  $P$  values were obtained using a Wilcoxon test and were adjusted ( $P_{adj}$ ) using the Benjamini-Hochberg method. Only significant features ( $P_{adj} < 0.1$ ) are shown.

**Figure 3: Comparative temporal dynamics of stool bile salt hydrolase (BSH) activity changes in patients with primary CDI experiencing recurrence versus those with no recurrence.** As assessed using plate-based precipitation assay, and measured in units of mmol of deoxycholic acid (DCA) produced per mg of faecal protein per minute. A) Time course of stool BSH activity. B) Comparison of stool BSH activity at one day post-treatment (left) and at the latest measured time point (right).  $P$  values were generated using Wilcoxon test (patients with recurrence:  $n=9$ ; patients without recurrence:  $n=18$ ).

**Figure 4: The contribution of faecal bile acids to risk of recurrence in patients with primary CDI, and their use as a predictive tool of recurrence.** As assessed at early time points after completion of antibiotics as therapy for primary CDI. A) Bayesian joint longitudinal and survival model; a positive regression coefficient means that an increase in this particular bile

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905 acid will increase risk of recurrence, while a negative regression coefficient means that an  
906 increase in this bile acid will lower the risk of recurrence. CI denotes credibility interval.  
907 Longitudinal-survival model fitted to each bile acid was used to predict recurrence within 9  
908 days post-CDI treatment using measurements at B) three timepoints; C) two time points; D)  
909 one time point only. Predictions were made using 80% of the data as training set and the  
910 other 20% as test set. Receiver operating characteristic plots show the mean of 1,000 Monte  
911 Carlo permutations for each bile acid, and the mean area under the receiver operating  
912 characteristic curve (AUROC) value is indicated in parenthesis in the figure legend.  
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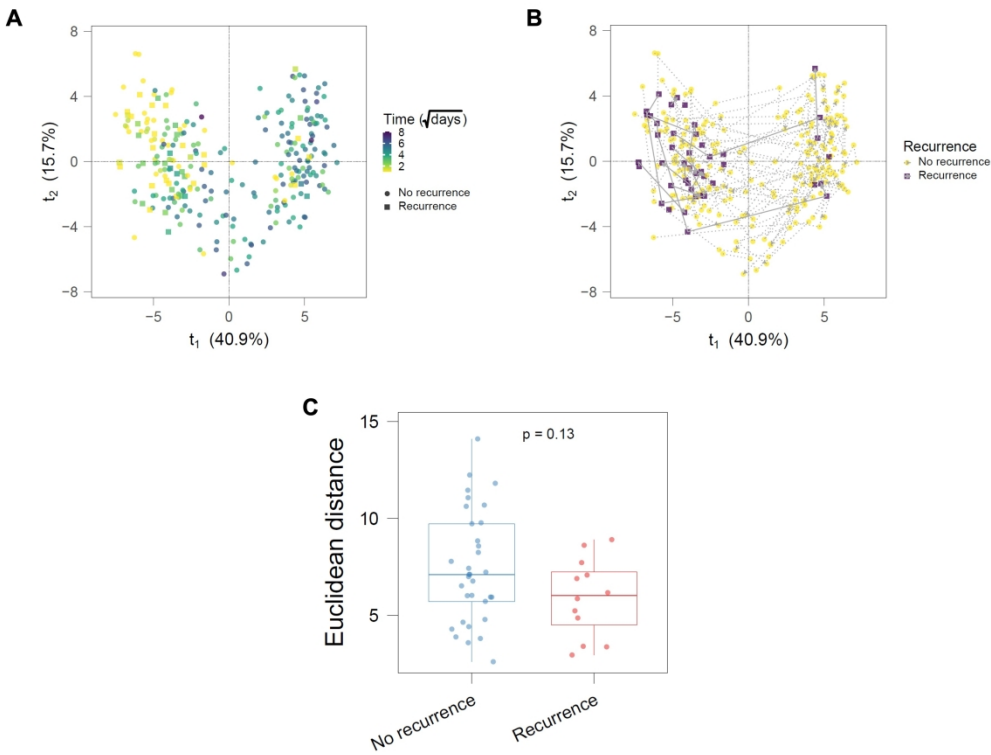


Figure 1

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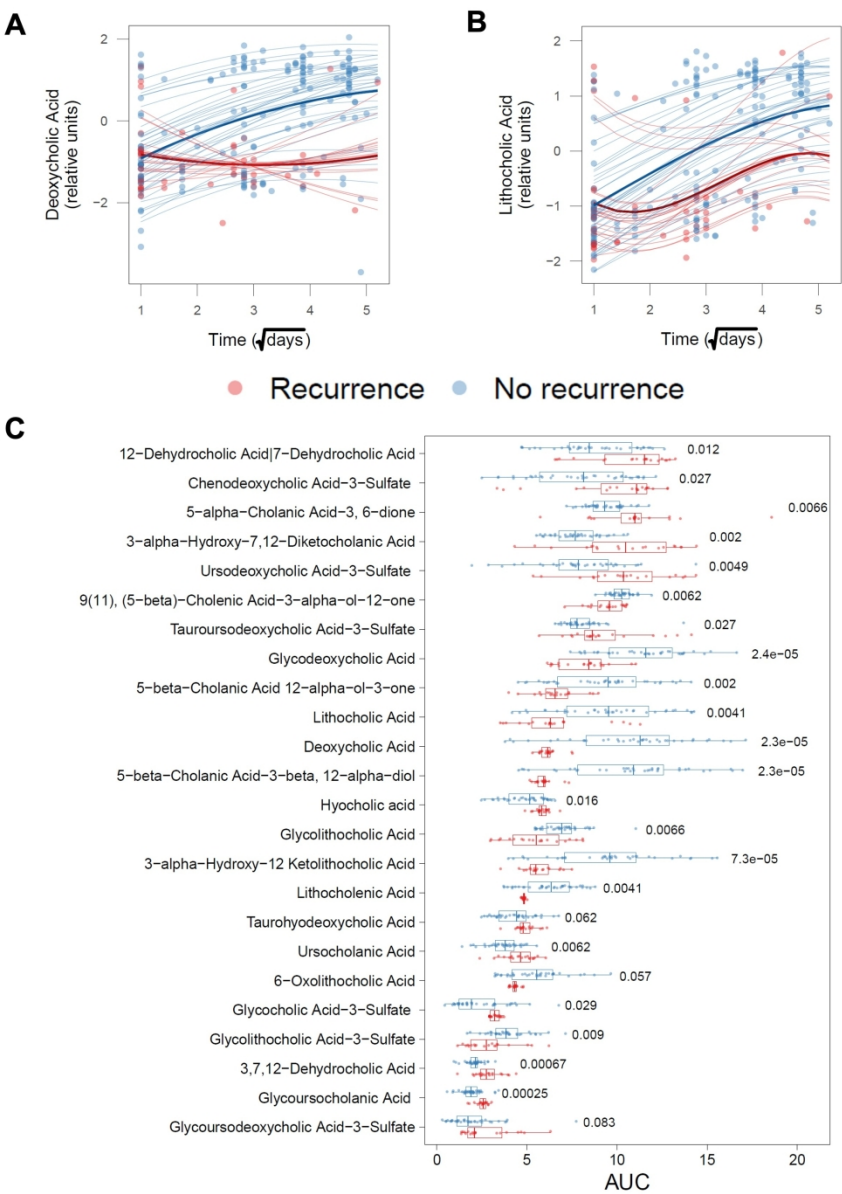


Figure 2

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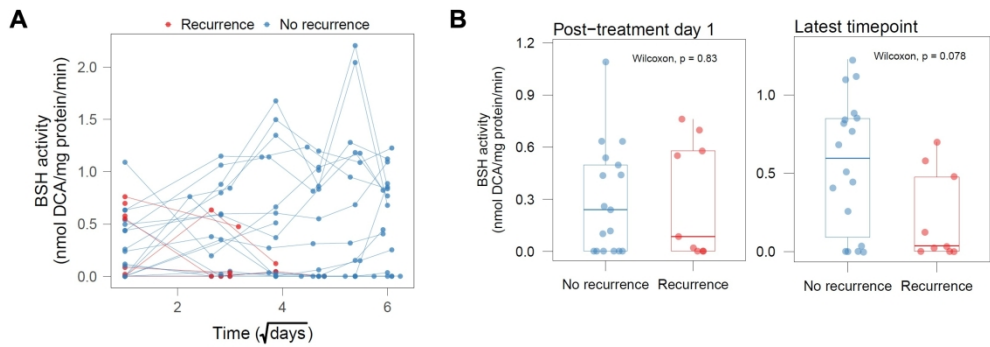


Figure 3

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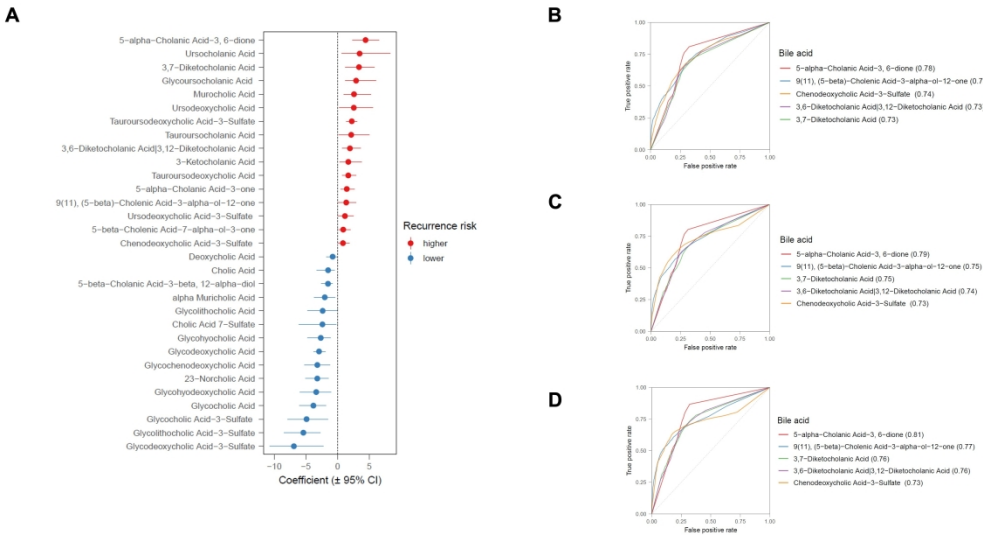


Figure 4

321x172mm (330 x 330 DPI)

## Supplementary Material

## Supplementary Material:

# Assessing the clinical value of faecal bile acid profiling to predict recurrence in primary *Clostridioides difficile* infection

## Supplementary Tables:

**Supplementary Table 1: Full results from longitudinal trajectory analysis.** AUC of predicted within-individual trajectories were compared between patients experiencing recurrence and those without recurrence. *p* values were obtained using a Mann-Whitney test and were adjusted using the Benjamini-Hochberg method. Mass: charge ratio (*m/z*) and retention time of these bile acids as previously-described [1]. Bile acids sorted by *Padj*.

| Name                                            | Mean (non-recurring patients) | Mean (recurring patients) | <i>P</i> | <i>Padj</i> |
|-------------------------------------------------|-------------------------------|---------------------------|----------|-------------|
| 5-beta-Cholanic Acid-3-beta, 12-alpha-diol      | 10.73                         | 5.92                      | 3.58E-07 | 2.30E-05    |
| Deoxycholic Acid                                | 10.74                         | 6.19                      | 6.47E-07 | 2.30E-05    |
| Glycodeoxycholic Acid                           | 11.43                         | 8.19                      | 1.02E-06 | 2.40E-05    |
| 3-alpha-Hydroxy-12 Ketolithocholic Acid         | 9.48                          | 5.67                      | 4.15E-06 | 7.30E-05    |
| Glycoursocolanic Acid                           | 1.94                          | 2.51                      | 1.80E-05 | 0.00025     |
| 3,7,12-Dehydrocholic Acid                       | 2.10                          | 2.83                      | 5.72E-05 | 0.00067     |
| 3-alpha-Hydroxy-7,12-Diketocholanic Acid        | 7.75                          | 10.32                     | 0.00023  | 0.00200     |
| 5-beta-Cholanic Acid 12-alpha-ol-3-one          | 9.26                          | 6.62                      | 0.00023  | 0.00200     |
| Lithocholonic Acid                              | 6.25                          | 4.83                      | 0.00059  | 0.00410     |
| Lithocholic Acid                                | 9.54                          | 6.66                      | 0.00059  | 0.00410     |
| Ursodeoxycholic Acid-3-Sulfate                  | 7.95                          | 10.39                     | 0.00077  | 0.00490     |
| 9(11), (5-beta)-Cholenic Acid-3-alpha-ol-12-one | 10.26                         | 9.44                      | 0.00116  | 0.00620     |
| Ursocholic Acid                                 | 3.68                          | 4.55                      | 0.00116  | 0.00620     |
| 5-alpha-Cholanic Acid-3, 6-dione                | 9.36                          | 10.97                     | 0.00141  | 0.00660     |
| Glycolithocholic Acid                           | 6.99                          | 5.49                      | 0.00141  | 0.00660     |
| Glycolithocholic Acid-3-Sulfate                 | 3.93                          | 2.92                      | 0.00207  | 0.00900     |
| 12-Dehydrocholic Acid   7-Dehydrocholic Acid    | 8.67                          | 10.68                     | 0.00281  | 0.012       |
| Hyocholic acid                                  | 4.91                          | 5.83                      | 0.00401  | 0.016       |
| Chenodeoxycholic Acid-3-Sulfate                 | 7.95                          | 9.82                      | 0.00785  | 0.027       |
| Tauroursodeoxycholic Acid-3-Sulfate             | 8.07                          | 9.28                      | 0.00744  | 0.027       |
| Glycocholic Acid-3-Sulfate                      | 2.43                          | 3.22                      | 0.00874  | 0.029       |
| 6-Oxolithocholic Acid                           | 5.47                          | 4.33                      | 0.018    | 0.057       |
| Taurohyodeoxycholic Acid                        | 4.31                          | 4.90                      | 0.021    | 0.062       |
| Glycoursodeoxycholic Acid-3-Sulfate             | 1.97                          | 2.72                      | 0.028    | 0.083       |
| Glycohyocholic Acid                             | 4.65                          | 5.37                      | 0.066    | 0.18        |
| 5-alpha-Cholanic Acid-3-one                     | 9.30                          | 8.59                      | 0.089    | 0.24        |

Supplementary Material

|                                                          |       |       |      |      |
|----------------------------------------------------------|-------|-------|------|------|
| Taurodeoxycholic Acid-3-Sulfate                          | 2.84  | 3.13  | 0.11 | 0.28 |
| Glycochenodeoxycholic Acid                               | 4.57  | 3.99  | 0.14 | 0.35 |
| Cholic Acid 7-Sulfate                                    | 2.20  | 2.05  | 0.19 | 0.45 |
| Glycocholic Acid                                         | 6.14  | 5.55  | 0.19 | 0.45 |
| Taurocholic Acid-3-Sulfate                               | 6.91  | 6.88  | 0.20 | 0.46 |
| Isolithocholic Acid                                      | 10.89 | 9.99  | 0.22 | 0.48 |
| 3-Dehydrocholic Acid                                     | 5.40  | 6.12  | 0.24 | 0.50 |
| Glycodeoxycholic Acid-3-Sulfate                          | 3.29  | 3.26  | 0.27 | 0.55 |
| 23-Norcholic Acid                                        | 3.25  | 2.90  | 0.34 | 0.60 |
| 5-beta-Cholanic Acid-3-alpha, 6-alpha-diol-7-one         | 12.91 | 12.67 | 0.31 | 0.60 |
| Cholic Acid-3-Sulfate                                    | 14.04 | 14.53 | 0.33 | 0.60 |
| Glycohyodeoxycholic Acid                                 | 3.68  | 3.89  | 0.31 | 0.60 |
| Taurochenodeoxycholic Acid-3-Sulfate                     | 5.31  | 5.82  | 0.34 | 0.60 |
| Tauroursocholanolic Acid                                 | 1.95  | 2.07  | 0.35 | 0.60 |
| 3-alpha-Hydroxy-7 Ketolithocholic Acid                   | 7.14  | 7.56  | 0.36 | 0.61 |
| 5-beta-Cholenic Acid-7-alpha-ol-3-one                    | 6.90  | 7.47  | 0.35 | 0.61 |
| 3,7,12-Taurodehydrocholic Acid                           | 2.62  | 2.49  | 0.43 | 0.65 |
| 5-alpha-Cholanic Acid-3-alpha-ol-6-one                   | 5.07  | 4.77  | 0.42 | 0.65 |
| Chenodeoxycholic Acid                                    | 13.68 | 12.87 | 0.43 | 0.65 |
| Taurocholic Acid                                         | 5.74  | 6.52  | 0.40 | 0.65 |
| Glycoursodeoxycholic Acid                                | 5.82  | 6.47  | 0.44 | 0.66 |
| Tauro omega-Muricholic Acid                              | 6.17  | 6.51  | 0.45 | 0.66 |
| Isoallolithocholic Acid                                  | 7.10  | 6.51  | 0.50 | 0.70 |
| Omega Muricholic Acid                                    | 6.26  | 6.16  | 0.50 | 0.70 |
| Taurolithocholic Acid                                    | 3.34  | 2.93  | 0.55 | 0.75 |
| Alpha Muricholic Acid                                    | 5.47  | 5.65  | 0.64 | 0.83 |
| Deoxycholic Acid-3-Sulfate                               | 6.25  | 5.99  | 0.65 | 0.83 |
| Lithocholic Acid 3-Sulfate                               | 13.71 | 13.29 | 0.63 | 0.83 |
| Taurohyocholic Acid                                      | 2.69  | 3.04  | 0.64 | 0.83 |
| Glycochenodeoxycholic Acid-3-Sulfate                     | 3.00  | 3.19  | 0.73 | 0.91 |
| 3-Ketocholanolic Acid                                    | 9.37  | 9.69  | 0.77 | 0.93 |
| 3,6-Diketocholanolic Acid   3,12-Diketocholanolic Acid   | 12.65 | 12.84 | 0.77 | 0.93 |
| 3,7-Diketocholanolic Acid                                | 9.89  | 10.36 | 0.79 | 0.94 |
| 5-Cholenic Acid-3-beta-ol                                | 8.17  | 8.19  | 0.85 | 0.98 |
| Beta Muricholic Acid                                     | 5.78  | 5.84  | 0.93 | 0.98 |
| Cholic Acid                                              | 21.63 | 20.81 | 0.93 | 0.98 |
| Murocholic Acid                                          | 6.30  | 5.99  | 0.97 | 0.98 |
| Tauro-beta Muricholic Acid   Tauro-alpha Muricholic Acid | 3.88  | 3.78  | 0.98 | 0.98 |
| Taurochenodeoxycholic Acid                               | 4.88  | 5.02  | 0.98 | 0.98 |
| Taurodeoxycholic Acid                                    | 4.03  | 3.73  | 0.94 | 0.98 |
| Taurolithocholic Acid 3-Sulfate                          | 3.02  | 2.99  | 0.98 | 0.98 |

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|                           |       |       |      |      |
|---------------------------|-------|-------|------|------|
| Tauroursodeoxycholic Acid | 5.34  | 5.84  | 0.91 | 0.98 |
| Ursocholic Acid           | 21.63 | 20.81 | 0.93 | 0.98 |
| Ursodeoxycholic Acid      | 8.77  | 8.66  | 0.86 | 0.98 |

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**Supplementary Table 2: Full results from joint longitudinal and survival modelling approach.**

Bayesian joint longitudinal-survival model was fitted to each bile acid. Table shows the summary of the obtained survival regression coefficients ( $\theta$ ), with the 95% credibility interval (CI) and the P value, calculated as  $2 \times \min\{P(\theta > 0), P(\theta < 0)\}$ . Mass: charge ratio ( $m/z$ ) and retention time of these bile acids as previously-described [1]. Bile acids sorted by coefficient value.

| Name                                              | Value | 2.5% CI | 97.5% CI | P       | Significance |
|---------------------------------------------------|-------|---------|----------|---------|--------------|
| Glycodeoxycholic Acid-3-Sulfate                   | -6.89 | -10.71  | -2.25    | 0.0020  | *            |
| Glycolithocholic Acid-3-Sulfate                   | -5.40 | -8.47   | -2.70    | <0.0010 | *            |
| Glycocholic Acid-3-Sulfate                        | -4.89 | -7.85   | -1.53    | <0.0010 | *            |
| Glycocholic Acid                                  | -3.82 | -5.99   | -1.83    | <0.0010 | *            |
| Glycohydoxycholic Acid                            | -3.37 | -5.95   | -1.04    | 0.0010  | *            |
| 23-Norcholic Acid                                 | -3.20 | -5.02   | -1.43    | <0.0010 | *            |
| Glycochenodeoxycholic Acid                        | -3.19 | -5.23   | -1.17    | 0.001   | *            |
| Glycodeoxycholic Acid                             | -2.92 | -3.78   | -1.90    | <0.0010 | *            |
| Glycohyocholic Acid                               | -2.65 | -4.75   | -1.10    | <0.0010 | *            |
| Cholic Acid 7-Sulfate                             | -2.39 | -6.07   | -0.22    | 0.034   | *            |
| Glycolithocholic Acid                             | -2.35 | -4.71   | -0.02    | 0.048   | *            |
| Alpha Muricholic Acid                             | -2.02 | -3.73   | -0.41    | 0.011   | *            |
| 5-beta-Cholanic Acid-3-beta, 12-alpha-diol        | -1.51 | -2.53   | -0.75    | <0.0010 | *            |
| Cholic Acid                                       | -1.49 | -3.30   | -0.44    | 0.005   | *            |
| Deoxycholic Acid                                  | -0.77 | -1.75   | -0.073   | 0.033   | *            |
| Chenodeoxycholic Acid-3-Sulfate                   | 0.86  | 0.13    | 1.85     | 0.019   | *            |
| 5-beta-Cholenic Acid-7-alpha-ol-3-one             | 0.90  | 0.17    | 2.00     | 0.020   | *            |
| Ursodeoxycholic Acid-3-Sulfate                    | 1.17  | 0.14    | 2.49     | 0.026   | *            |
| 9(11), (5-beta)-Cholenic Acid-3-alpha-ol-12-one   | 1.37  | 0.07    | 2.91     | 0.038   | *            |
| 5-alpha-Cholanic Acid-3-one                       | 1.46  | 0.48    | 2.65     | 0.0020  | *            |
| Tauroursodeoxycholic Acid                         | 1.70  | 0.78    | 2.94     | <0.0010 | *            |
| 3-Ketocholanic Acid                               | 1.70  | 0.36    | 3.83     | 0.0060  | *            |
| 3,6-Diketocholanic Acid  3,12-Diketocholanic Acid | 1.96  | 0.72    | 3.63     | <0.0010 | *            |
| Tauroursocholanic Acid                            | 2.16  | 0.15    | 5.02     | 0.036   | *            |
| Tauroursodeoxycholic Acid-3-Sulfate               | 2.25  | 1.41    | 3.06     | <0.0010 | *            |
| Ursodeoxycholic Acid                              | 2.57  | 0.29    | 5.56     | 0.029   | *            |
| Murocholic Acid                                   | 2.60  | 0.96    | 5.28     | 0.0040  | *            |
| Glycoursocholanic Acid                            | 2.93  | 1.25    | 6.04     | <0.0010 | *            |
| 3,7-Diketocholanic Acid                           | 3.39  | 1.23    | 5.79     | <0.0010 | *            |
| Ursocholanic Acid                                 | 3.48  | 0.66    | 8.35     | 0.0060  | *            |
| 5-alpha-Cholanic Acid-3, 6-dione                  | 4.42  | 2.34    | 6.54     | <0.0010 | *            |
| Taurodeoxycholic Acid-3-Sulfate                   | -2.56 | -7.60   | 0.82     | 0.15    |              |

*Supplementary Material*

|                                                          |       |        |       |       |  |
|----------------------------------------------------------|-------|--------|-------|-------|--|
| Lithocholic Acid 3-Sulfate                               | -1.90 | -4.01  | 0.34  | 0.082 |  |
| Glycochenodeoxycholic Acid-3-Sulfate                     | -1.38 | -5.83  | 0.23  | 0.20  |  |
| 5-alpha-Cholanic Acid-3-alpha-ol-6-one                   | -1.01 | -2.63  | 0.37  | 0.16  |  |
| Lithocholic Acid                                         | -0.85 | -3.29  | 0.28  | 0.16  |  |
| Isolithocholic Acid                                      | -0.59 | -1.35  | 0.18  | 0.12  |  |
| Taurocholic Acid-3-Sulfate                               | -0.45 | -1.92  | 1.08  | 0.51  |  |
| 3-alpha-Hydroxy-12 Ketolithocholic Acid                  | -0.40 | -1.31  | 0.31  | 0.30  |  |
| Ursocholic Acid                                          | -0.38 | -1.26  | 0.61  | 0.41  |  |
| Taurochenodeoxycholic Acid-3-Sulfate                     | -0.28 | -0.84  | 0.30  | 0.28  |  |
| Taurodeoxycholic Acid                                    | -0.28 | -1.07  | 0.44  | 0.47  |  |
| Taurohyocholic Acid                                      | -0.27 | -1.02  | 0.52  | 0.44  |  |
| Chenodeoxycholic Acid                                    | -0.21 | -1.34  | 0.76  | 0.71  |  |
| Lithocholenic Acid                                       | -0.21 | -1.48  | 1.11  | 0.72  |  |
| 3-alpha-Hydroxy-7,12-Diketocholanic Acid                 | -0.18 | -0.78  | 0.46  | 0.51  |  |
| Taurochenodeoxycholic Acid                               | -0.17 | -0.84  | 0.46  | 0.58  |  |
| 5-beta-Cholanic Acid 12-alpha-ol-3-one                   | -0.11 | -1.62  | 0.76  | 0.96  |  |
| 5-Cholenic Acid-3-beta-ol                                | -0.09 | -0.81  | 0.64  | 0.80  |  |
| Taurocholic Acid                                         | -0.08 | -0.63  | 0.50  | 0.74  |  |
| Tauro-beta Muricholic Acid   Tauro-alpha Muricholic Acid | -0.02 | -0.93  | 0.98  | 0.95  |  |
| Tauro omega-Muricholic Acid                              | 0.04  | -0.55  | 0.73  | 0.97  |  |
| Taurolithocholic Acid                                    | 0.10  | -1.11  | 2.08  | 0.99  |  |
| Deoxycholic Acid-3-Sulfate                               | 0.20  | -0.80  | 2.23  | 0.78  |  |
| 3,7,12-Taurodehydrocholic Acid                           | 0.33  | -4.01  | 2.12  | 0.49  |  |
| 5-beta-Cholanic Acid-3-alpha, 6-alpha-diol-7-one         | 0.37  | -0.82  | 1.95  | 0.63  |  |
| Omega Muricholic Acid                                    | 0.51  | -0.97  | 1.96  | 0.51  |  |
| Glycoursodeoxycholic Acid-3-Sulfate                      | 0.67  | -0.46  | 1.82  | 0.19  |  |
| 3,7,12-Dehydrocholic Acid                                | 0.72  | -0.34  | 1.84  | 0.14  |  |
| 6-Oxolithocholic Acid                                    | 0.74  | -0.54  | 2.63  | 0.31  |  |
| Isoallolithocholic Acid                                  | 0.92  | -0.20  | 2.05  | 0.090 |  |
| 3-Dehydrocholic Acid                                     | 0.92  | -0.90  | 2.82  | 0.31  |  |
| Hyochoolic acid                                          | 1.09  | -0.24  | 4.77  | 0.12  |  |
| Beta Muricholic Acid                                     | 1.09  | -0.15  | 2.25  | 0.08  |  |
| Glycoursodeoxycholic Acid                                | 1.25  | -0.43  | 4.00  | 0.10  |  |
| Cholic Acid-3-Sulfate                                    | 1.34  | -0.46  | 3.60  | 0.17  |  |
| Taurolithocholic Acid 3-Sulfate                          | 1.56  | -10.16 | 10.54 | 0.75  |  |
| 12-Dehydrocholic Acid   7-Dehydrocholic Acid             | 1.66  | -0.27  | 4.05  | 0.16  |  |
| Taurohyodeoxycholic Acid                                 | 1.71  | -0.93  | 4.10  | 0.22  |  |
| 3-alpha-Hydroxy-7 Ketolithocholic Acid                   | 1.83  | -0.49  | 5.58  | 0.53  |  |

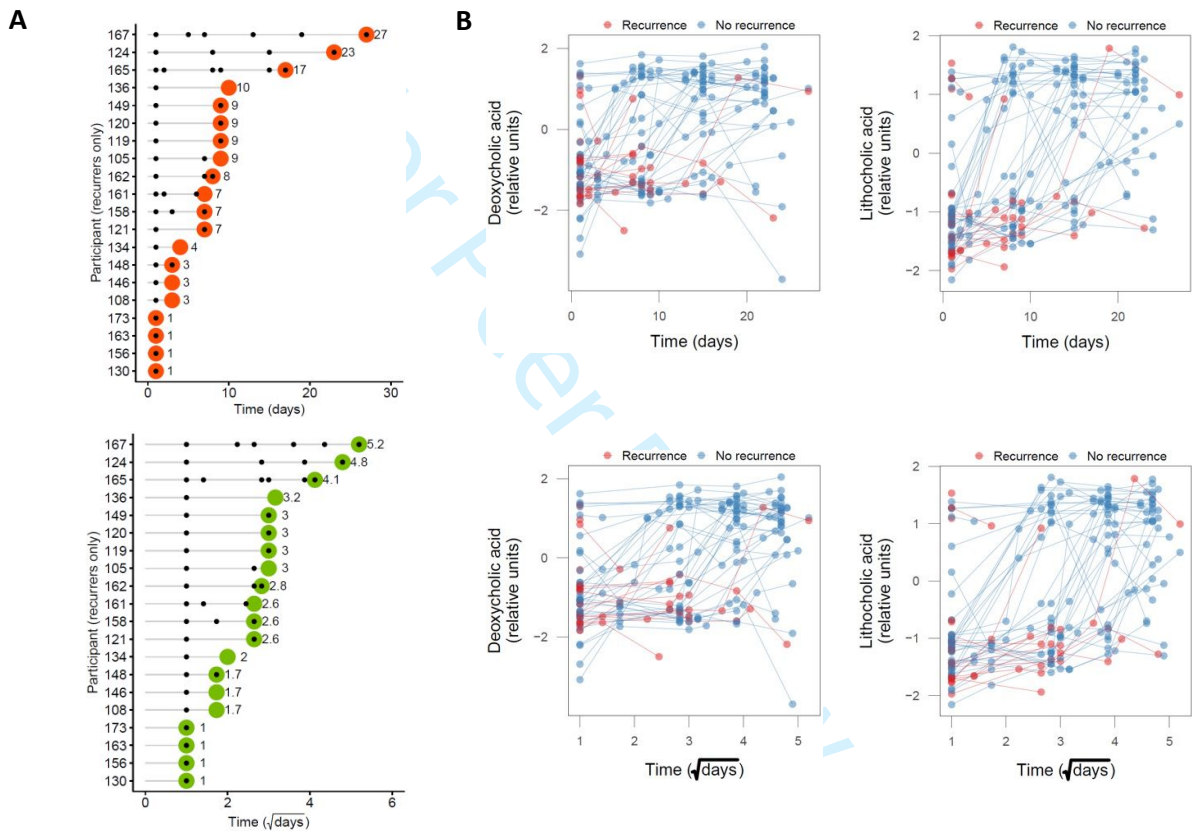
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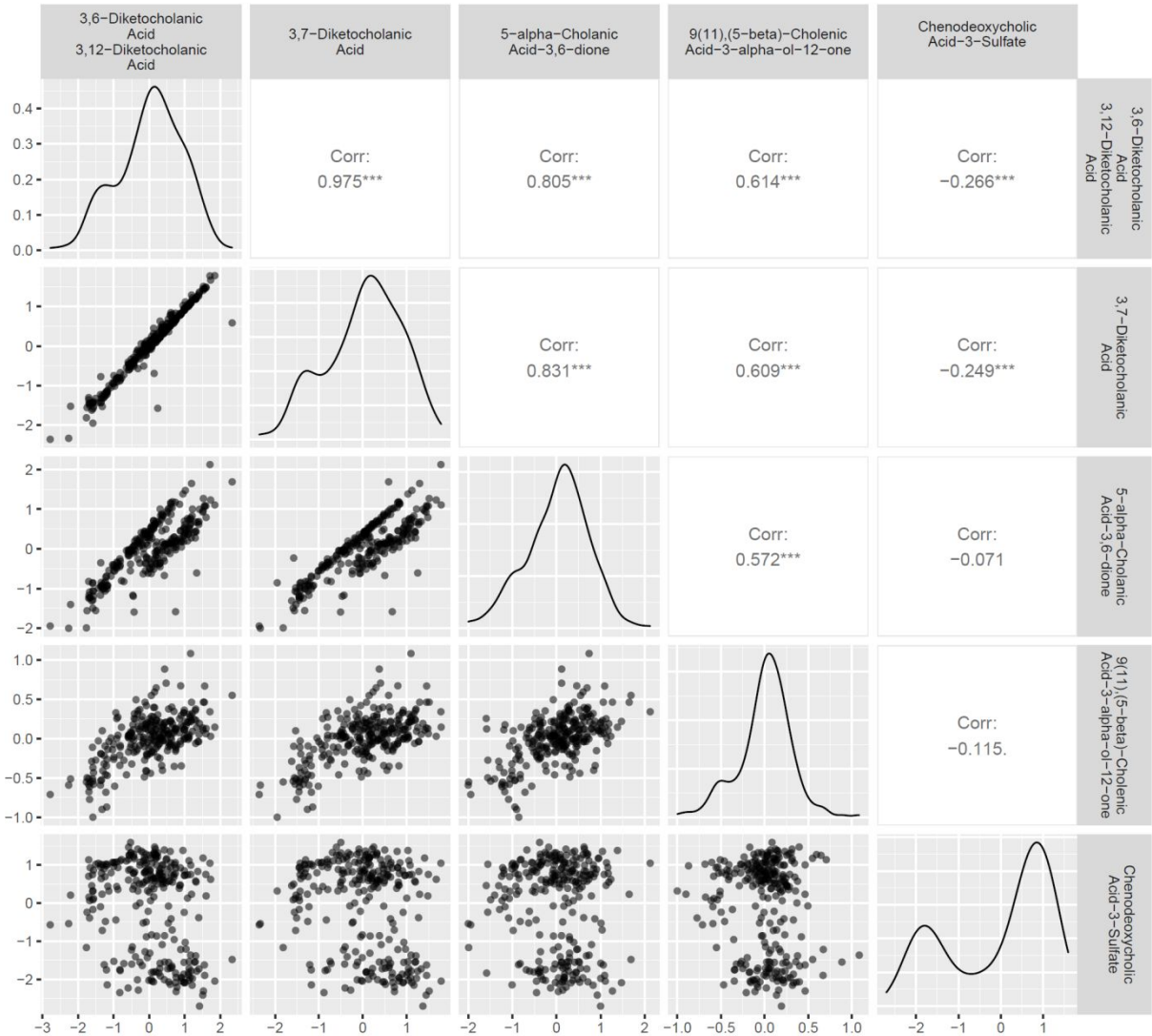
Supplementary Figures:

**Supplementary Figure 1. Dynamics of recurrence.** A) Recurrence event for each patient who experienced a recurrence ( $n=20$ ) with time as days (top) or as the square root of days (bottom). Red (top) or green (bottom) dots and labels indicate the time of recurrence while black dots indicate the times when a faecal sample was obtained. B) Deoxycholic acid (left) and lithocholic acid (right) individual trajectories across time in days (top) or in squared root of days (bottom). The y-axis shows log<sub>10</sub>-transformed and mean-centred relative intensities.



Supplementary Material

**Supplementary Figure 2: Pairwise correlations of the top five performing bile acids for prediction of recurrence risk in the joint survival longitudinal model.** Values at the upper triangle correspond to the Pearson correlation coefficient and significance (\*\*\*:  $p<0.001$ ; \*\*:  $p<0.01$ ; \*:  $p<0.05$ , .:  $p<0.10$ ). Bile acid relative intensities have been log-transformed and mean-centred, as indicated in methods.



Supplementary Material

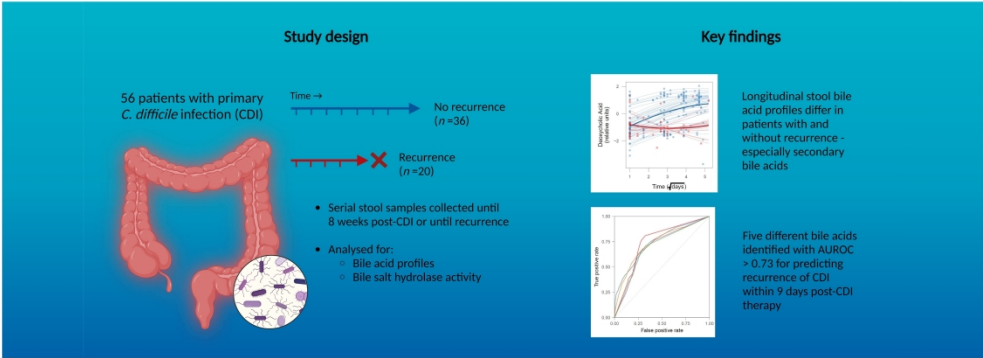
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3 77 **Supplementary References:**  
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5 78 [1] Sarafian MH, Lewis MR, Pechlivanis A, Ralphs S, McPhail MJW, Patel VC, et al. Bile Acid  
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ASSESSING THE CLINICAL VALUE OF FAECAL BILE ACID PROFILING TO PREDICT  
RECURRENCE IN PRIMARY *CLOSTRIDIoidES DIFFICILE* INFECTION



Mullish\*, Martinez-Gili\* et al. *Aliment. Pharmacol. Ther.*

AP&T

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