Research Article
Paediatric Hepatology

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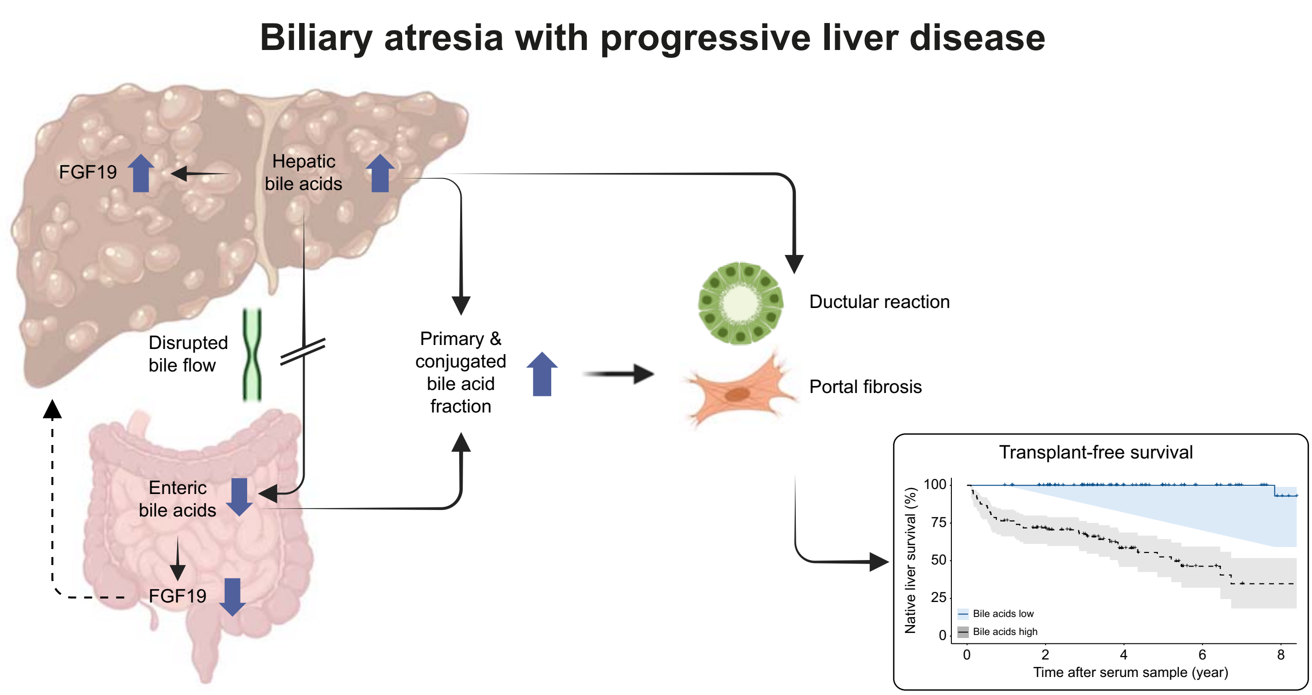
Graphical abstract



Highlights

- Biliary atresia is the most common indication for pediatric liver transplantation.
- Accumulation of altered serum bile acids predicted progression of liver disease.
- Low bile acid synthesis and aberrant liver FGF19 production were associated with poor outcomes.

Impact and implications

Biliary atresia, a fibro-obliterating biliary disease of infants, remains the most common indication for pediatric liver transplantation because of the rapid progression of liver injury. To identify predictive biomarkers of disease progression and to elucidate the pathophysiology of liver injury in biliary atresia, we profiled serum bile acids and studied their liver metabolism after Kasai portoenterostomy. Accumulation and altered composition of circulating bile acids predicted progression of liver disease and need for liver transplantation. Patients with poor prognosis showed low bile acid synthesis and abnormal liver expression of fibroblast growth factor 19.

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Accumulation of altered serum bile acids predicts liver injury after portoenterostomy in biliary atresia

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Background & Aims: Little is known about the mechanism of liver injury mediated by bile acids following Kasai portoenterostomy (KPE) for biliary atresia (BA). We sought to quantify individual serum bile acids, 7-alpha-hydroxy-4-cholesten-3-one (C4), and fibroblast growth factor 19 (FGF19) after KPE and to evaluate their prognostic utility and transcriptomic regulation.

Methods: Serum (n = 244) and liver specimens (n = 105) prospectively obtained from patients with BA (n = 54) after KPE were included. Bile acids were analyzed using mass spectrometry, gene expression with quantitative PCR, and histopathology using a neural network model.

Results: Following KPE, serum bile acids correlated positively with biochemical liver injury, pediatric end-stage liver disease score, liver stiffness, histological ductular reaction, and liver fibrosis. Bile acids were higher among patients who developed portal hypertension (79.6 *vs.* 11.9 μmol/L, p <0.0001), esophageal varices (91.6 *vs.* 16.2 μmol/L, p <0.0001), or required liver transplantation (LT, 115.3 *vs.* 22.0 μmol/L, p <0.0001) during follow-up; bile acids predicted these outcomes in time-dependent regression models. Accumulation of conjugated bile acids, cholic acid, and taurine conjugates predicted LT risk while associating with histological liver injury. Serum C4 (0.04 *vs.* 0.00 μmol/L, p = 0.04) and liver *CYP7A1* were higher in native liver survivors than in LT recipients (fold-change 16.9 *vs.* 7.0, p = 0.02). Primary bile acids correlated negatively with C4 (R = -0.38, p <0.001) and *CYP7A1* (R = -0.49, p = 0.01). Unlike in native liver survivors (R = -0.19, p = 0.66), serum FGF19 correlated with liver *FGF19* (R = 0.59, p = 0.04) without an inverse association with serum primary bile acids in LT recipients (R = 0.26, p = 0.08).

Conclusions: Accumulation and altered composition of serum bile acids predicted progressive liver disease and poorer transplant-free survival following KPE. Poor prognosis was associated with low bile acid synthesis and aberrantly increased liver FGF19.

Introduction

Biliary atresia (BA) is a progressive cholestatic disease of infancy leading to liver failure in the first years of life if untreated.[1] The first-line surgical treatment, Kasai portoenterostomy (KPE), aims to restore bile flow by replacing fibro-obliterated extrahepatic biliary remnants with a jejunal loop. Although serum bilirubin normalizes postoperatively in most patients, complications of progressive cholangiopathy and fibrosis necessitate liver transplant(ation) (LT) in 60-75% of individuals with BA before adulthood. While the underlying mechanism of the post-KPE cholangiopathy remains unclear, BA is the most common indication for LT in children globally.[1,2]

Bile acids facilitate absorption of lipids and act as important signaling molecules between the gut and liver while modulating intestinal microbiota.[3] After being synthesized from cholesterol by hepatocytes, primary bile acids (PBAs), cholic acid (CA), and chenodeoxycholic acid (CDCA) are conjugated to glycine or taurine.[3] PBAs are secreted via bile to the intestine where they undergo deconjugation and conversion to secondary bile acids (SBAs), deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) by intestinal bacteria. The enterohepatic bile acid pool is maintained by intrinsic synthesis, replacing the small amounts of bile acids escaping their efficient intestinal reabsorption. Synthesis is governed by cholesterol 7α-hydroxylase (CYP7A1), which is under tight negative feedback regulation of enterohepatic circulation. Bile acids downregulate their own synthesis by repressing CYP7A1 through activation of farnesoid X receptor (FXR), which induces SHP (small heterodimer partner) transactivation in the liver and FGF19 secretion in the ileum.[3]

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Due to obstructed bile drainage, bile acids accumulate to pathologically high levels in the liver and serum.[4–7] While bile acids have direct cytotoxic properties,[8] their accumulation drives parenchymal and biliary damage, ductular reaction (DR), and progressive fibrosis in experimental and clinical cholestatic conditions, including BA.[9] Elevated serum total bile acids (TBAs), their altered composition, and extreme suppression of the bile acid synthesis marker 7-alpha-hydroxy-4-cholesten-3-one (C4) are associated with poorer prognosis in cholestatic adults and children.[10–13] Recently, we showed that serum FGF19, but not serum bile acids, predicted outcomes of BA at the time of KPE, whereas, after KPE, increased serum bile acids coupled with the degree of DR which predicted native liver NL survival.[4,14] Accordingly, in patients who normalized their bilirubin, elevated serum TBA concentrations at 6 months after KPE were associated with complications of the liver disease and the need for LT.[6] Adult BA NL survivors showed slightly elevated, glycine-enriched serum bile acids in a recent report.[15] However, serum bile acid levels and composition, enterohepatic regulation of bile acids, and their relation to liver injury and disease progression after KPE have not been investigated.

In this study, we quantified individual serum bile acids, C4, and FGF19 in children with BA during post-KPE follow-up. We next correlated these with histological liver injury and evaluated their prognostic utility for portal hypertension (PH), esophageal varices, and NL survival. Additionally, we analyzed liver transcriptomes of key genes regulating synthesis and transport of bile acids and localized hepatic FGF19.

Patients and methods

Patients and specimens

Fifty-four patients with BA – in whom prospectively collected archived serum (n = 224) and/or liver mRNA (n = 52) specimens were obtained at clinical follow-up visits after KPE in Helsinki University Hospital (from 2009–2020) – were enrolled (Table 1 and Table S1, Fig. 1). Among enrolled patients, a total of 105 post-KPE liver biopsies were available. Serum samples obtained at KPE in 19 patients were also included to address longitudinal changes. Clearance of jaundice (COJ) was defined as a decrease of serum bilirubin to <20 μmol/L.

Clinical records, routine serum liver biochemistry, PELD (pediatric end-stage liver disease) score, and liver stiffness measurements were collected. According to the national protocol, all patients with BA in Finland undergo endoscopic variceal surveillance and ultrasound-guided core needle liver biopsies at 1 year and at 5-year intervals after KPE.[16,17] Clinically significant PH was defined as endoscopic detection of esophageal or gastric varices or thrombocytopenia ($<150\times10^9$/L) with splenomegaly. Further details are provided in the Supplementary Methods.

Controls

Serum (n = 80) from healthy children (males 63%, median age 5.0 [range, 3.3-6.1] years) undergoing minor day-surgery operations were included as controls. Control liver specimens were pediatric donor liver biopsies (n = 10, males 50% median age 2.0 [1.0-9.5] years) and commercial liver tissue homogenates (n = 6, Sekisui XenoTech, Kansas City, MO) (Table S1).

Serum bile acids and biomarkers

Quantification of taurine and glycine conjugates, unconjugated CA, CDCA, UDCA, LCA, DCA, and C4 was performed using high-performance liquid chromatography–tandem mass spectrometry as previously described with minor modifications.[18] FGF19 concentrations were measured with ELISA. Detailed methods are provided in the Supplementary Methods.

Liver histopathology

The overall proportional area of DR (DR%), consisting of cholangiocytes and cytokeratin-7-positive parenchymal intermediate hepatocytes (PIHs), was quantified with an image-processing neural network model developed for BA as previously described.[14] PIHs were also scored manually by two pediatric pathologists. Liver fibrosis was assessed by Metavir staging and by measuring the proportional Sirius red-positive area as described previously.[4] See Supplementary Methods for details.

Liver mRNA expression

mRNA expression was assessed using qPCR and is presented as fold-changes. Details are provided in the Supplementary Methods.

Localization of liver FGF19 expression

FGF19 protein expression was localized with immunohistochemistry. *FGF19* mRNA co-localization with *KRT19* (keratin-19), *HPX* (hematopexin), and *ACTA2* (actin alpha 2) was assessed using *in situ* hybridization. See the Supplementary Methods for details.

Statistical analysis

RStudio (R version 4.3.1) was used for data analysis. Continuous variables are expressed as medians with IQRs and were compared using Mann-Whitney *U* test and Spearman rank correlations. Median concentrations or fractions for each patient with multiple follow-up serum samples were included in comparisons between groups. Temporal changes were analyzed with Wilcoxon signed-rank test. Principal components analysis (PCA) was performed using concentrations of the individual bile acids. As repeated follow-up serum samples were analyzed, survival analyses were performed with time-dependent univariable Cox proportional hazards regression models using continuous variables. Survival start time was defined as the date of serum withdrawal and stop time as the date of the next serum, event, or latest follow-up. The primary outcome event was LT or death. Secondary events were detection of PH or varices. Wald and log-rank tests choosing the least significant method were used for comparisons of the survival models. Optimal cut-offs for the primary event were selected with receiver-operating characteristic (ROC) analysis. Survival models and ROC analysis were validated internally with bootstrapping by randomly resampling the cohorts with replacement 1,000 times. Comparisons of mRNA expression analyses were corrected using the Benjamini-Hochberg procedure. *p* values <0.05 were considered significant.

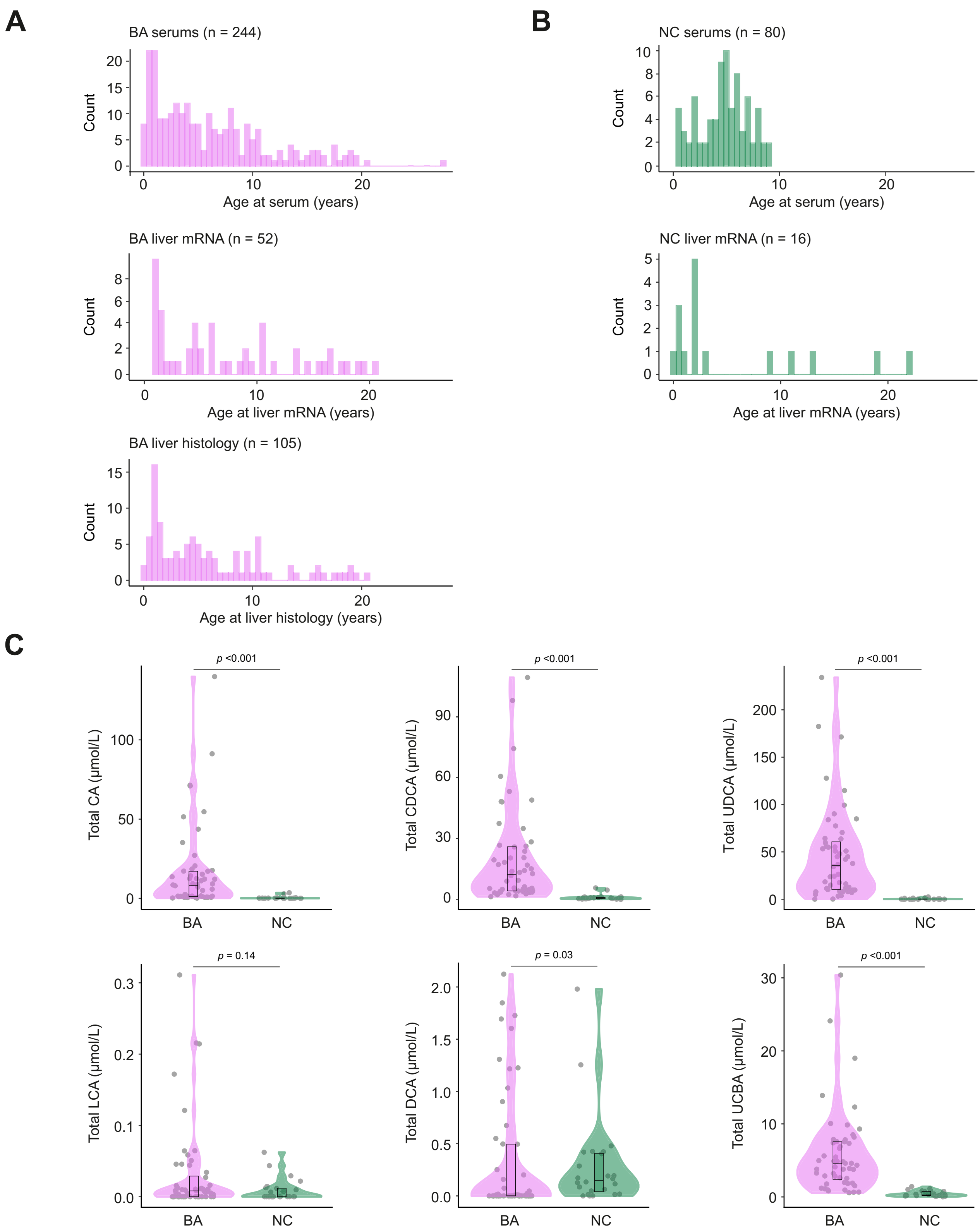

Fig. 1. Sample distributions and serum bile acid concentrations in patients and controls. Number of serum, mRNA, and histology specimens of (A) patients with BA and (B) controls according to age at sampling. (C) Concentrations of bile acid species and UCBAs in patients and controls (Mann-Whitney *U* tests). Median concentration of multiple measurements for each patient is included. Violin plots indicate median, IQR, and individual data points. BA, biliary atresia; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; NC, normal control; UCBA, unconjugated bile acid; UDCA, ursodeoxycholic acid.

Ethics

The study protocol was approved by the Research ethics committee (345/13/03/03/2008) and review board of Helsinki University Hospital (§70HUS/284/2019) and conforms to the Declaration of Helsinki and Istanbul. Informed consent for the use of samples in research was obtained from the patients' legal guardians.

Results

Patient characteristics

Baseline patient characteristics and serum biochemistry results are presented in Table 1. Eight (15%) patients received LT by 2 years, 11 (20%) by 5 years, and 19 (35%) during the entire median follow-up of 10.8 (5.2-14.3) years (Fig. S1).

Longitudinal changes from KPE to follow-up

Among the patients with both KPE and (first) follow-up serum (n = 19) acquired at median 1.2 (0.3-8.2) months postoperatively, PBAs decreased (88.9 [40.1-118.6] *vs.* 34.9 [18.8-46.6] μmol/L, p = 0.03) and SBAs (consisting mainly of supplemented UDCA) increased (0.1 [0.1-0.4] *vs.* 66.9 [26.8-83.0] μmol/L, p = 0.001) following KPE. Overall, the composition shifted to increased enrichment with UDCA, unconjugated bile acids (UCBAs), and glycine-conjugated bile acids after KPE. In NL survivors, concentrations of TBAs and PBAs decreased more and SBAs increased less after KPE than in patients requiring LT (Fig. S2).

Comparison of follow-up samples to controls

In serum samples obtained during post-KPE follow-up, median TBA, PBA, and UDCA concentrations were 31-, 17- and 343-fold higher than in controls, respectively (Fig. 1, Table S1). Although the UDCA fraction was markedly higher, fractions of other bile acids (especially CDCA and DCA) decreased (Table S1). Although the absolute concentration of UCBAs was increased, patients with BA had a lower UCBA fraction than controls (Fig. 1). Serum FGF19 increased, while C4 was comparable to controls (Table S1).

Effect of UDCA treatment

The effect of UDCA supplementation on bile acid concentration and profile was assessed, since patients with BA are frequently treated with UDCA following KPE.[19] Based on clinical records, 50 (96%) patients were prescribed UDCA after KPE. However, some patients had disproportionate UDCA levels compared with the retrospectively acquired UDCA treatment status. Therefore, serum samples obtained after KPE were divided into UDCA-supplemented and non-supplemented subgroups based on the highest total UDCA concentration (2.45 μmol/L) and fraction (22%) in controls. Altogether, only six patients with BA had 12 measurements below these cut-offs. TBAs (44.4 [16.7-113.1] *vs.* 2.8 [1.7-4.4] μmol/L, p <0.001), PBAs (17.8 [5.4-41.8] *vs.* 1.6 [1.0-3.3] μmol/L, p <0.001), and SBAs (17.8 [10.9-65.4] *vs.* 1.0 [0.8-1.6] μmol/L, p <0.001) were higher and the PBA fraction (36.4 [30.1-47.6] *vs.* 66.5 [47.9-73.6] %, p <0.001) was lower in the UDCA-supplemented subgroup compared with the non-supplemented subgroup.

Correlations with markers of liver injury

Following KPE, TBA concentration correlated positively with total and conjugated bilirubin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyltransferase, and APRI (aspartate aminotransferase-to-platelet ratio index), and negatively with liver synthetic function (Fig. 2, Table S2). Liver stiffness positively correlated with TBAs (Fig. 2) and CA/CDCA ratio (R = 0.45, p <0.001), and negatively correlated with UCBA fraction (R = -0.49, p <0.001) and glycine/taurine (G/T) ratio (R = -0.48, p <0.001). PELD score was similarly correlated with TBAs (Fig. 2), UCBA fraction (R = -0.23, p = 0.005) and G/T ratio (R = -0.17, p = 0.04).

Associations with histological liver injury

Neural network quantified DR correlated positively with absolute TBA, SBA, and PBA concentrations (Fig. 2, Table S3). CA/CDCA ratio and CA and taurine-CBA fraction were also positively correlated with DR. A negative correlation was found with UCBA, LCA and DCA fractions, G/T ratio, and concentrations of FGF19 and C4. The correlations were even stronger for PIHs (Table S3).

No correlations between TBA, PBA, or SBA concentrations with proportional Sirius red liver fibrosis were observed (Table S4). However, portal fibrosis as assessed by Metavir staging was positively associated with TBA, PBA, and SBA concentrations and CA/CDCA ratio. UCBA and DCA fractions and G/T ratio were negatively correlated, and taurine-CBAs were positively correlated with fibrosis assessments.

Liver disease complications and transplant-free survival

Patients with persistently elevated bilirubin after KPE showed higher absolute TBA concentrations post-KPE compared to patients achieving COJ (Fig. 3). Increased TBAs were also found in patients who developed PH or varices and in patients requiring subsequent LT (Table S5). Additionally, LT recipients presented with lower UCBA, LCA, and DCA fractions and G/T ratio and markedly increased CA and taurine-CBA fractions and CA/CDCA ratio than NL survivors (Table S5). In absolute terms, LT recipients showed increased CA, CDCA, and UDCA concentrations and decreased DCA concentrations (Fig. 3). Overall, the bile acid composition after KPE in NL survivors more closely resembled that in controls, whereas in patients with PH or requiring subsequent LT it showed similarities with that of treatment-naïve patients at KPE-naive patients (Fig. 4).

Prognostic utility

Among patients requiring LT, TBA concentration was consistently higher during the entire post-KPE follow-up compared with NL survivors who had steadily lower and close to normal TBA levels (Fig. 5, first samples: [113.8 (89.6-183.1) *vs.* 22.3 (11.8-55.4) μmol/L, p <0.001], last samples: [107.2 (62.8-235.9) *vs.* 24.0 (4.1-26.8) μmol/L, p <0.001]). In univariable regression models, higher absolute TBA, PBA, and SBA concentrations predicted development of PH and varices and shorter NL survival (Tables S6–8). In ROC analysis, TBA concentrations exceeding 31.9 μmol/L predicted LT with 83% accuracy, 95% sensitivity, and 62% specificity (Fig. 5, Table S9). Importantly, the prognostic effects of TBAs, PBAs, and SBAs were maintained when only patients with COJ after KPE or

Table 1. Patient characteristics and baseline biochemistry.

	At follow-up	At KPE
Patients, n	54	19
Serum samples, n	244	19
Bile acids, n[†]	174	16
FGF19, n	241	19
Liver biopsies, n		
Histology	105	19
mRNA expression[‡]	57	0
Age at serum samples, years	4.81 (1.83–9.22)	0.15 (0.07–0.21)
Male, n (%)	27 (50)	9 (47)
Type of BA, n (%)		
I or II	5 (9)	0 (0)
III	49 (91)	19 (100)
Cystic disease, n (%)	7 (13)	3 (16)
Splenic malformation, n (%)	8 (15)	0 (0)
Isolated disease, n (%)	34 (63)	12 (63)
Age at KPE, days	61 (39–79)	54 (27–75)
COJ, n (%)	47 (87)	14 (74)
Outcome, n (%)		
NL	35 (65)	12 (63)
LT	19 (35)	7 (37)
NL survival, years	7.14 (3.81–13.11)	3.68 (1.29–6.10)
2-year, n (%)	46 (85)	13 (68)
5-year, n (%)	43 (80)	12 (63)
Age at LT, years	2.52 (0.99–9.29)	0.98 (0.82–1.29)
Liver biochemistry		
Bilirubin, μmol/L	13 (7–31)	124 (100–162)
Conjugated bilirubin, μmol/L	6 (3–23)	98 (66–127)
ALT, U/L	56 (28–98)	116 (59–226)
AST, U/L	71 (46–121)	149 (90–263)
GGT, U/L	68 (28–253)	201 (102–507)
ALP, U/L	361 (261–490)	379 (312–689)
APRI	0.71 (0.39–1.57)	0.69 (0.52–1.22)
Prealbumin, mg/L	152 (121–181)	99 (86–110)
Albumin, g/L	35 (32–39)	29 (24–36)
Triglycerides, mmol/L	0.84 (0.56–1.23)	1.29 (1.12–1.62)
Total cholesterol, mmol/L	3.9 (3.4–5.0)	4.2 (3.4–5.0)
LDL-cholesterol, mmol/L	1.9 (1.7–2.4)	2.0 (1.5–2.9)
HDL-cholesterol, mmol/L	1.3 (1.0–1.8)	0.5 (0.3–1.0)
Creatinine, μmol/L	23 (19–34)	19 (18–23)

All patients with serum samples at KPE also had follow-up serum obtained. Median value of multiple measurements for each patient is included. Data are presented as medians with IQRs or frequencies.

ALP, alkaline phosphatase; ALT, alanine aminotransferase; APRI, AST-to-platelet ratio index; AST, aspartate aminotransferase; BA, biliary atresia; COJ, clearance of jaundice; FGF19, fibroblast growth factor 19; GGT, gamma-glutamyltransferase; KPE, Kasai portoenterostomy; LT, liver transplantation; NL, native liver; PBAs, primary bile acids; PH, portal hypertension; SBAs, secondary bile acids; TBAs, total bile acids; UCBAs, unconjugated bile acids; UDCA, ursodeoxycholic acid.

[†]Serum bile acid quantification was available for 52 patients.

[‡]Liver mRNA expression measurement was available for 34 patients.

UDCA-treated patients were included in subgroup analysis (Tables S10–11). When the predictive ability of TBAs was compared to other previously recognized prognosticators of NL survival after KPE,[20] only TBAs remained significant in a multivariable model including TBAs, bilirubin, gamma-glutamy ltransferase, APRI, and liver stiffness (Table S12).

When addressing the prognostic ability of bile acid profiles, higher PBA and taurine-CBA fractions and lower UCBA fraction and G/T ratio predicted inferior transplant-free survival (Table S6). In subgroup analyses including patients with COJ after KPE or UDCA-supplemented patients, the prognostic ability of UCBA fraction, taurine-CBA fraction, and G/T ratio remained significant (Tables S10–11). Additionally, higher UDCA fraction in the UDCA-supplemented subgroup was associated with improved transplant-free survival (Table S11).

Regulation of bile acid synthesis and transport

We next compared the underlying regulation of bile acid synthesis and transport after KPE between NL survivors and patients undergoing LT (Fig. 6). Liver *CYP7A1* expression was higher in NL survivors than in LT recipients (p = 0.02, Mann-Whitney U test) or controls. Among feedback repressors of bile acid synthesis, liver expression of FXR (*NR1H4*) was reduced, whereas *FGF19* expression was increased in LT recipients (p = 0.02, Mann-Whitney U test) compared to NL survivors. Expression of both basolateral and canalicular bile acid transporters and *CYP27A1* were similarly increased in both NL survivors and LT recipients compared to controls.

Consistent with increased *CYP7A1* expression, serum C4 was higher among NL survivors than LT recipients (Fig. 7). PBA concentration was inversely associated with *CYP7A1*, C4, and

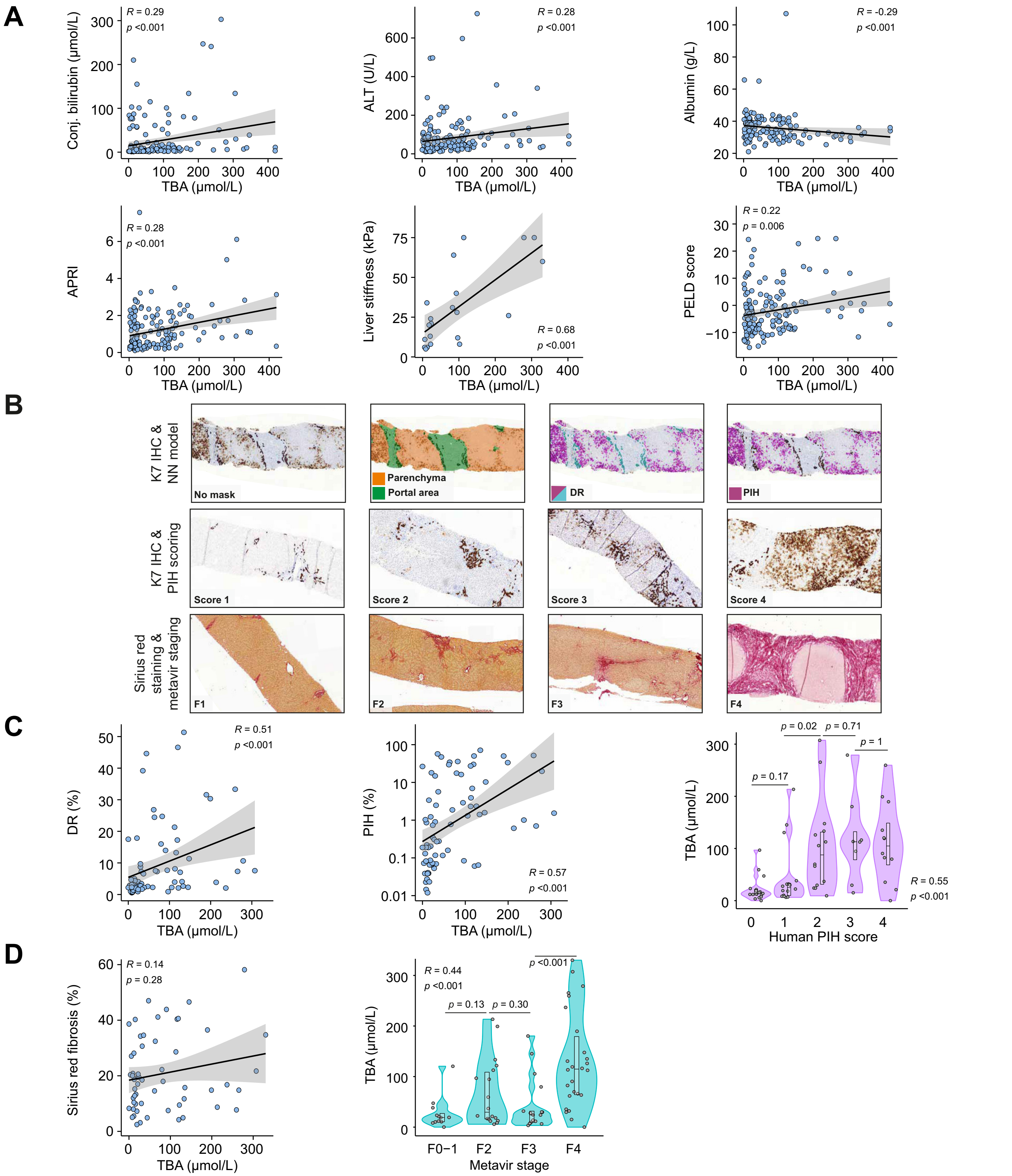

Fig. 2. Association with biochemical and histopathological liver injury. (A) Spearman rank correlation of TBAs with conjugated bilirubin, ALT, albumin, APRI, liver stiffness, and PELD score. (B) Representative images of neural network analysis, K7 immunohistochemistry, and Metavir stages of Sirius red-stained sections. (C) Associations of TBAs with DR%, PIH% (Spearman rank correlations), and PIH scores (Mann-Whitney *U* tests) and (D) with Sirius red-quantified fibrosis (Spearman rank correlation) and Metavir staging (Mann-Whitney *U* tests). Violin plots indicate median, IQR, and individual data points. ALT, alanine aminotransferase; APRI, aspartate aminotransferase-to-platelet ratio index; DR, ductular reaction; K7, cytokeratin 7; PELD, pediatric end-stage liver disease score; PIH, parenchymal intermediate hepatocytes; TBAs, total bile acids.

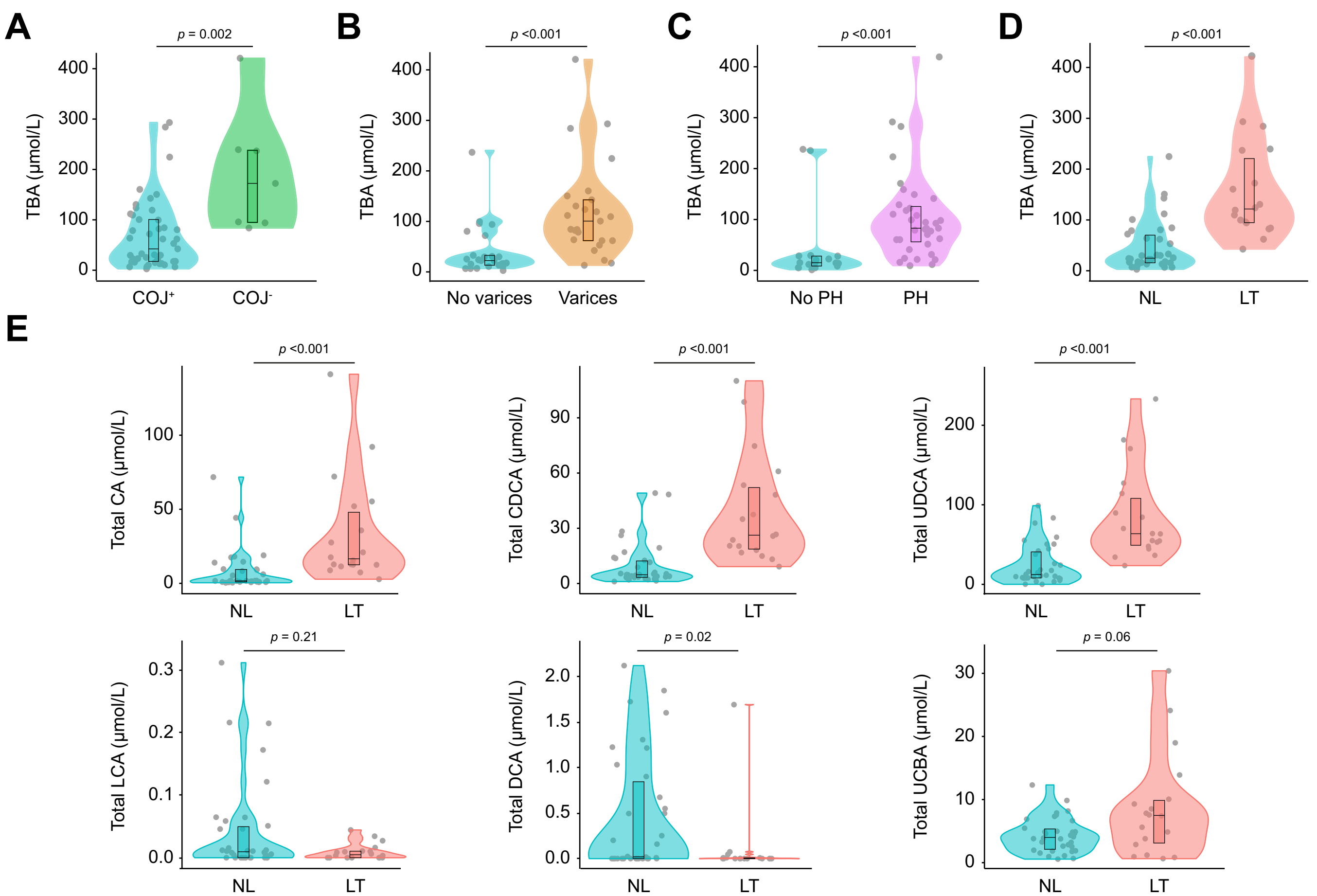

Fig. 3. Association with liver disease complications and outcome. TBAs according to (A) COJ, (B) detection of varices (C) and PH, and (D) outcome (Mann-Whitney *U* tests). (E) Concentrations of bile acid species and UCBAs according to outcome (Mann-Whitney *U* tests). Median concentration of multiple measurements for each patient is included. Violin plots indicate median, IQR, and individual data points. CA, cholic acid; CDCA, chenodeoxycholic acid; COJ; clearance of jaundice; DCA, deoxycholic acid; LCA, lithocholic acid; LT, liver transplantation; NL, native liver; PH, portal hypertension; TBA, total bile acids; UCBA, unconjugated bile acid; UDCA, ursodeoxycholic acid.

FXR (*NR1H4*) (Fig. S4). Serum FGF19 levels were similarly increased both in NL survivors (218 [160-290] pg/ml) and LT recipients (267 [184-561] pg/ml) compared with controls (119 [76-174] pg/ml, p <0.001 for both). Although serum FGF19 was not significantly correlated with C4 in NL survivors (R = -0.16, p = 0.07) or LT recipients (R = 0.14, p = 0.37), liver *FGF19* correlated negatively with *CYP7A1* expression (Fig. S4). Liver *FGF19* expression correlated positively with bilirubin levels and tended to correlate positively with PBAs, while serum FGF19 correlated negatively with PBAs only in NL survivors but not in LT recipients (Fig. 7 and Fig. S4). Serum FGF19 levels correlated positively with liver *FGF19* expression in LT recipients but not in NL survivors (Fig. S4).

Liver FGF19 immunostaining was found mainly in hepatocytes and weakly in cholangiocytes in LT recipients (Fig. S5). *In situ* hybridization showed *FGF19* mRNA co-localization with *HPX* without *KRT19* or *ACTA2* co-expression.

Discussion

We observed that an increased concentration and altered profile of serum bile acids was closely associated with histopathological progression of liver injury, severity of liver disease, and poor prognosis after KPE. In addition, poor prognosis was associated with low bile acid synthesis and aberrantly increased liver expression of FGF19.

Consistent with the present findings, moderately elevated TBA (>40 µmol/L) measured at 6 months after successful KPE is associated with complications of liver disease and poor NL survival in BA.[6] Although no comparative studies are currently available, collectively these findings suggest postoperative bilirubin may not reflect progression of the underlying disease or cholestasis as accurately as serum bile acids. In support of these findings, only TBAs predicted NL survival in a multivariable model including bilirubin among other established non-invasive predictors of transplant-free survival. In addition to the well-established connection between TBAs and cirrhosis, a recent study including a limited number of patients with BA listed for LT identified a link between TBA accumulation and peri-transplant adverse events, broadening the relationship between elevated TBAs and poor prognosis.[21,22] Our optimal cut-off concentration of 31.9 µmol/L for transplant-free survival was remarkably similar to the 40 µmol/L used in a previous study of patients with successful KPE, both with and without UDCA treatment.[6,21] Although bile acids peaked in the early postoperative period, their concentration also remained

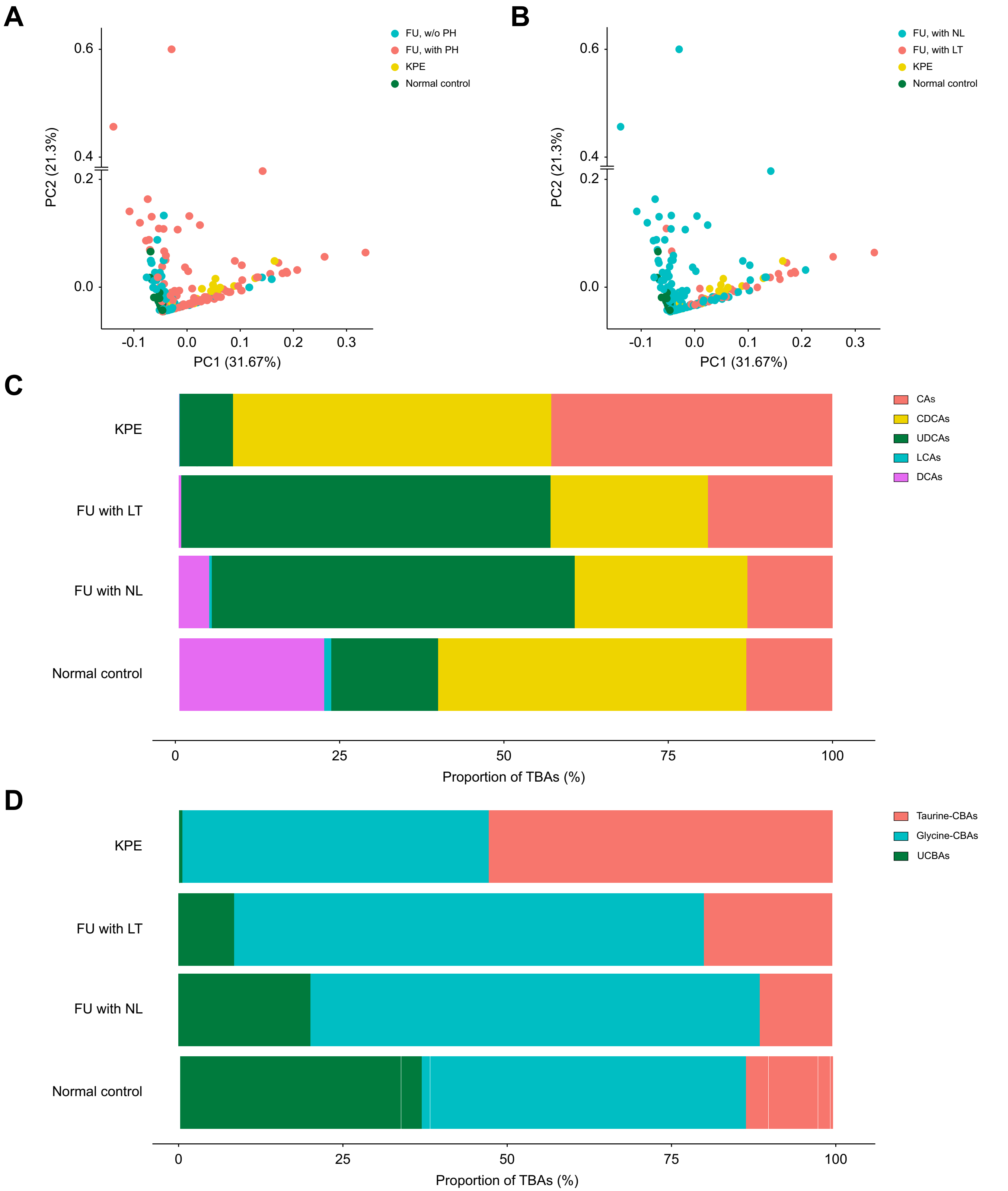

Fig. 4. Bile acid profile in patients according to prognosis. PCA of individual bile acids in controls and in BA at KPE and follow-up (A) according to PH or (B) outcome. (C) Fractions of bile acid species and (D) bile acid conjugation status in controls and in patients with BA at KPE and follow-up according to outcome. BA, biliary atresia; CA, cholic acid; CBA, conjugated bile acids; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; FU, follow-up; KPE, Kasai portoenterostomy; LCA, lithocholic acid; LT, liver transplantation; NL, native liver; PCA, principal components analysis; UCBAs, unconjugated bile acids, UDCA, ursodeoxycholic acid.

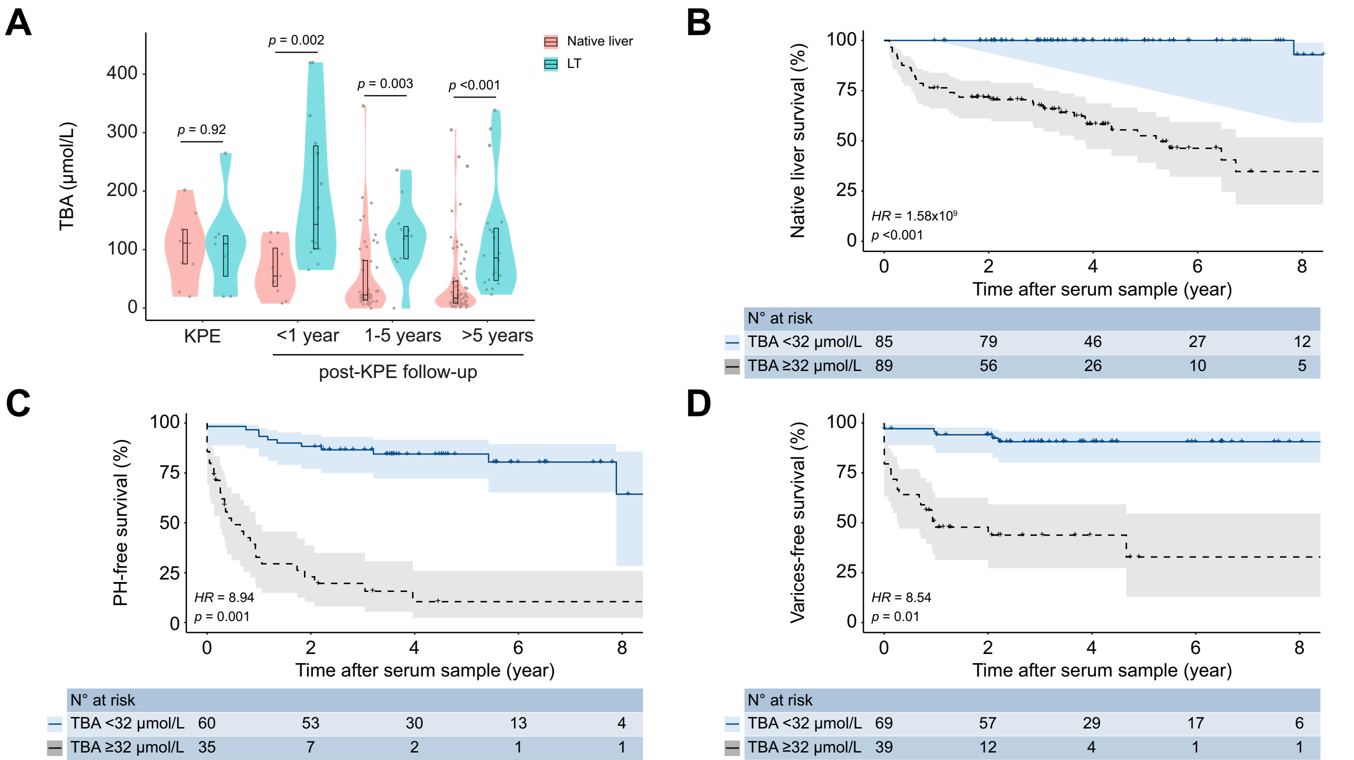

Fig. 5. Prediction of progressive liver disease and LT after KPE. (A) Serum TBAs during follow-up according to age and subsequent outcome (Mann-Whitney *U* tests). Kaplan-Meier curves for (B) NL survival, (C) detection of PH, or (D) varices according to optimal cut-off for serum TBAs. All follow-up serum bile acid measurements are included in the Kaplan-Meier analyses. Violin plots indicate median, IQR, and individual data points. The shaded areas of the curves represent 95% CIs. Two patients were not assessed endoscopically before LT and were excluded from survival analyses for varices. KPE, Kasai portoenterostomy; LT, liver transplantation; PH, portal hypertension; TBAs, total bile acids.

longitudinally elevated in patients who developed PH or required LT during long-term follow-up after successful KPE, suggesting postoperative timing of the sampling is not critical for predictive ability. Consistent with this, TBAs also predicted NL survival in patients with COJ, while most of the included children survived with their NL beyond 2 years.

In addition to pure excess of TBAs, a taurine-conjugated, UCBA-depleted, and cholic acid-enriched bile acid profile was predictive for LT following KPE and was associated with DR and portal fibrosis. The bile acid composition among children with BA showed substantial similarity with those reported for adults with primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC), suggesting that post-KPE liver disease shares similarities with these cholangiopathies.[11,23] Consistent with our findings, a correspondingly altered profile predicted hepatic decompensation in PSC and was associated with clinical severity of PBC.[11,23] Although TBAs were slightly elevated, a glycine-enriched profile was also found in long-term adult NL survivors who had undergone successful KPE for BA in infancy.[15] Pointing to possible theoretical mechanisms underlying these clinical observations, taurocholate has been reported to promote DR, hepatic stellate cell activation, and fibrosis in human cultured hepatocytes.[24,25] Furthermore, bile acids enriched with CA and taurine conjugates were closely associated with DR, PIHs, and fibrosis in the portal regions of the liver exposed to the highest bile acid load.[26] Previously, a strong connection between NL survival and DR was found in patients with BA.[14] Here, NL survivors showed higher fractions of UCBAs, LCA, and DCA produced by deconjugation and dehydroxylation by enteric bacteria.[3] The increased fraction of bacterially modified bile acids indicates restored bile flow into the intestine after successful KPE in NL survivors, explaining their positive prognostic features and reverse associations with histopathological liver injury.

Although our transcriptional analysis suggested comparably increased hepatic uptake and canalicular and basolateral export of bile acids in NL survivors and LT recipients after KPE, higher serum C4 and liver *CYP7A1* expression in NL survivors indicates more active bile acid synthesis in patients with favorable outcome. This may have been contributed by nearly uniform UDCA supplementation, as UDCA antagonizes FXR leading to CYP7A1 activation while decreasing circulating FGF19 in morbid obesity and PBC.[23,27] However, in contrast to NL survivors, liver FXR and *CYP7A1* expression and C4 were reduced in LT recipients with more advanced liver disease despite even higher absolute UDCA levels. Extreme suppression of bile acid synthesis is associated with poor outcomes in PSC, indicating that synthesis has a limited compensatory ability to overcome overwhelming bile acid accumulation when bile drainage is severely impaired.[10] Similar to the present study, previous research in BA has demonstrated upregulated expression of basolateral bile acid exporters at the time of LT,[28] representing an adaptive mechanism to high intracellular bile acid load and contributing to their increased serum levels. Unexpectedly, NTCP (responsible for portal bile acid uptake) was also upregulated. It remains unclear if the increased expression of NTCP, a negatively regulated FXR target gene, was affected by UDCA-exerted FXR antagonism. Consistent

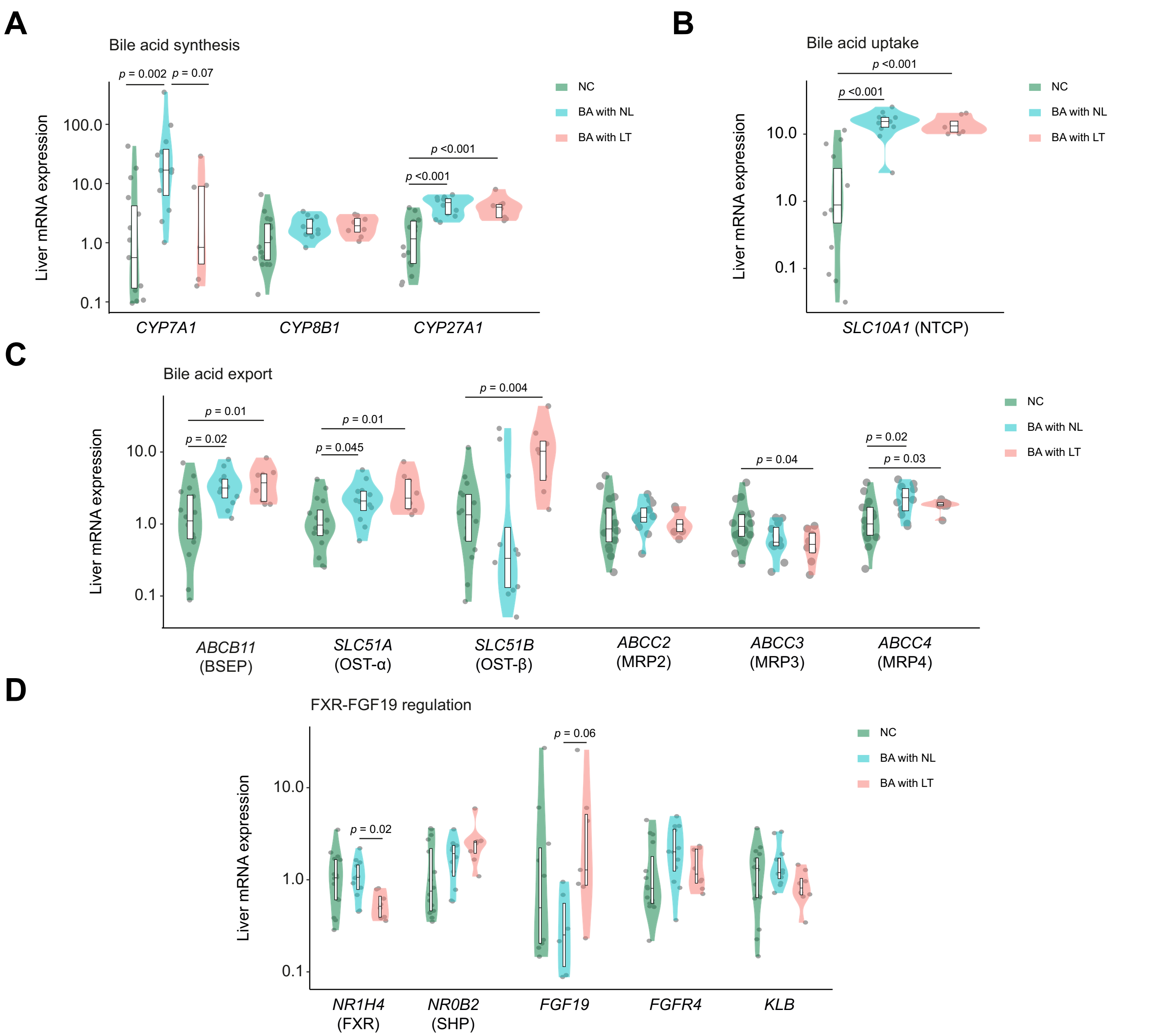

Fig. 6. Liver mRNA expression of genes regulating synthesis and transport of bile acids. Expression of (A) *CYP7A1, CYP8B1, CYP27A1*, (B) *SLC10A1* (NTCP), (C) *ABCB11* (BSEP), *SLC51A* (OST-α), and *SLC51B* (OST-β), *ABCC2* (MRP2), *ABCC3* (MRP3), *ABCC4* (MRP4), and (D) *NR1H4* (FXR), *NR0B2* (SHP), *FGF19, FGFR4*, and *KLB* in patients with BA with NL (n = 23) or after LT (n = 11) during follow-up and in controls (n = 16) (Mann-Whitney *U* tests with Benjamini-Hochberg procedure). The most recent liver specimen for each patient was included. Violin plots indicate median, IQR, and individual data points. BA, biliary atresia; LT, liver transplantation; NC, normal control; NL, native liver.

with our findings, UDCA supplementation upregulates the main canalicular bile acid transporter BSEP (bile salt export pump) in mice while increasing bile acid secretion in humans.[29,30]

Elevated serum levels and aberrantly increased liver expression of FGF19 have been previously reported in BA both at KPE and LT as in other cholestatic conditions.[4,5,31–33] In microdissected hepatocytes obtained from patients with BA at LT, FGF19-mediated suppression of bile acid synthesis was dysfunctional, whereas the FXR/SHP pathway appeared to be normal.[28] Here, serum FGF19 was moderately increased above control levels during post-KPE follow-up. Serum FGF19 correlated positively with liver *FGF19* expression in LT recipients, who also showed higher liver *FGF19* expression than NL survivors and lacked the inverse association between serum FGF19 and PBAs. These findings indicate elevated serum FGF19 originated from the liver instead of the ileum[4,5,33] without downregulating bile acid synthesis in patients who required LT. In these patients, cholestasis and bile acid accumulation may have triggered aberrant hepatocyte expression of FGF19 to downregulate bile acid synthesis. In contrast, FGF19-mediated feedback regulation appeared to function in NL survivors who showed a negative correlation between serum FGF19 and PBAs. Finally, the inverse association of PBAs with C4 and *CYP7A1* indicate that direct negative feedback regulation by bile acids was maintained in patients with BA following KPE.

These findings suggest that bile acid accumulation and dysfunctional enterohepatic regulation contribute to post-KPE

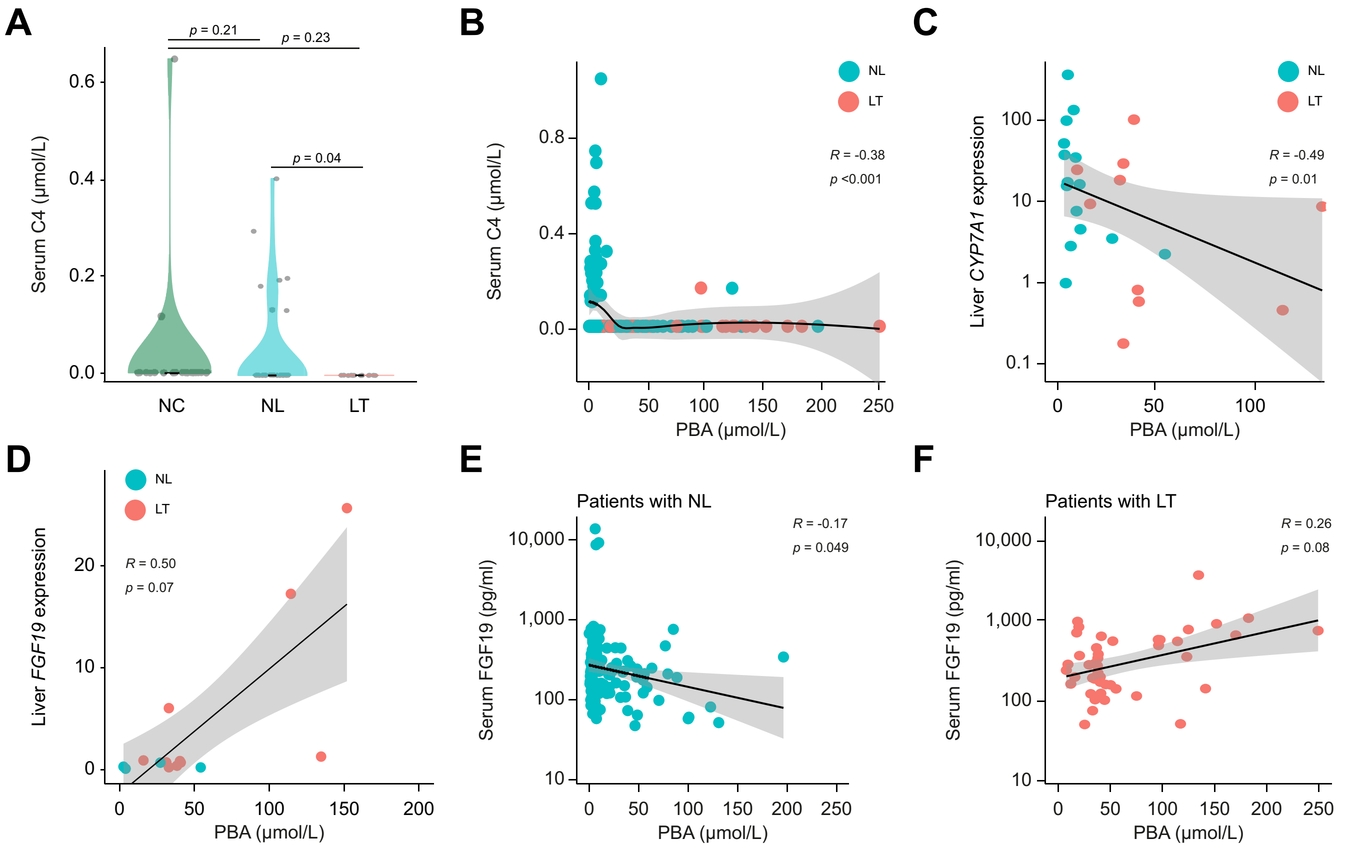

Fig. 7. Regulation of bile acid synthesis. (A) Serum C4 in NL survivors, LT recipients, and controls (Mann-Whitney *U* tests). (B) Spearman rank correlation between PBAs and serum C4, (C) between PBAs and *CYP7A1* expression, and (D) between PBAs and hepatic *FGF19* expression. (E) Spearman rank correlation between PBAs and serum FGF19 in NL survivors and (F) in LT recipients. Violin plots indicate median, IQR, and individual data points. BA, biliary atresia; C4, 7-alpha-hydroxy-4-cholesten-3-one; FGF19, fibroblast growth factor 19; LT, liver transplantation; NC, normal control; NL, native liver; PBAs, primary bile acids.

liver injury and clinical disease progression. Considering the bile acid-reducing potential of IBAT (ileal bile acid transporter) inhibitors and FXR agonists in pediatric and adult cholestatic diseases, future trials should examine whether pharmacological lowering of bile acids by modulation of the FXR-FGF19 axis can delay or prevent progression of histological liver injury and development of end-stage liver disease following KPE in BA.[34–36] However, the phase II trial (NCT04524390) of the IBAT inhibitor maralixibat in BA failed to meet its primary or secondary endpoints according to a press release.

This study had important limitations. The study was conducted in a single center with a limited number of patients, although the large amount of prospectively collected multifaceted research specimens was combined with comprehensive long-term follow-up data. Because of sample volume limitations, we were unable to quantify extremely low concentrations or unusual bile acid species. Lack of cholestatic controls is another limitation of the study, making it impossible to evaluate whether the findings are unique to BA. Despite providing information on temporal changes of bile acid metabolism during the disease course, the number and timing of sampling varied among patients. To address these confounders, only median values for each patient were included in subgroup analyses and time-dependent prognostic models were utilized. Finally, these clinical findings do not demonstrate causality between accumulated bile acids and liver injury despite the association.

In conclusion, accretion and altered composition of serum bile acids was associated with histopathological liver injury and predicted development of liver disease complications and transplant-free survival after KPE. Serum total and primary bile acids and fraction of UCBAs could be useful endpoints in future clinical trials on BA. Independent cohorts are needed to validate our findings and to establish optimal cut-offs.

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Abbreviations

BA, biliary atresia; C4, 7-alpha-hydroxy-4-cholesten-3-one; CA, cholic acid; CBA, conjugated bile acid; CDCA, chenodeoxycholic acid; COJ, clearance of jaundice; CYP7A1, cholesterol 7α-hydroxylase; DCA, deoxycholic acid; DR, ductular reaction; FGF19, fibroblast growth factor 19; FXR, farnesoid X receptor; G/T, glycine/taurine; KPE, Kasai portoenterostomy; LCA, lithocholic acid; LT, liver transplant(ation); NL, native liver; PBAs, primary bile acids; PCA, principal components analysis; PH, portal hypertension; SBAs, secondary bile acids; TBAs, total bile acids; UCBAs, unconjugated bile acids; UDCA, ursodeoxycholic acid.

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Conflict of interest

NS reports consulting income from Aiforia Technologies unrelated to the submitted work. The funding source had no role in the design, practice, or analysis of this study. All other authors declare no conflicts of interest for this article.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Supervision, study concept: MPP. Data analysis and interpretation, manuscript draft: IN, MPP. Bile acid metabolomics: MNe, MNi. Developing histopathological neural network model: NS, JA. Histopathological scorings: AiM, JL. Liver *in situ* hybridization: SH, OC. Data acquisition, critical revision, and final approval of the submitted article: all authors.

Data availability statement

Anonymized, aggregated data supporting the findings are available within the main article and supplementary data sheet. Study protocols and analytic methods are available from the corresponding author (IN) upon reasonable request.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2025.01.025.

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