



OPEN Conjugated bile acids in serum reflect disease severity and predict survival in chronic liver disease of humans and rats

Akiko Eguchi^{1,2✉}, Motoshi Iwasa^{1,3}, Teruo Miyazaki⁴, Mina Tempaku¹, Masako Ichishi⁵, Yasuyuki Tamai¹, Ryuta Shigefuku¹, Yoshinao Kobayashi¹, Akira Honda⁴, Masatoshi Watanabe^{2,5}, Tadashi Ikegami⁶ & Hayato Nakagawa¹

Chronic liver diseases (CLD) are closely linked to an increased load of conjugated bile acids (BAs), which contribute to liver damage and inflammation, although many aspects remain unclear. This study investigated the relationships between serum conjugated BAs, BA-related gene expression in the liver and ileum, and survival outcomes in both human and rat models of CLD. Serum BA levels, including tauro- and glyco-conjugated BAs such as taurochenodeoxycholic acid (TCDCA), were measured in 73 CLD patients and in carbon tetrachloride (CCl₄)-induced CLD rat models using LC-MS/MS. A higher conjugated-to-free BA ratio, elevated TCDCA levels, and a lower glycine-to-aurine ratio were significantly associated with impaired liver function, increased fibrosis markers, and worse survival rates in patients. Hepatic expression levels of BA-related genes were also associated with liver damage and survival rates in CLD rat models. The rat study suggested that increased serum conjugated BAs result from reduced intestinal excretion and enhanced bloodstream flow. These findings suggest that elevated serum conjugated BAs reflect disease severity and could serve as potential biomarkers for prognosis in CLD.

Keywords Conjugated bile acid, Taurochenodeoxycholic acid, Survival, *Slc10a2* gene, *Baat* gene, Liver cirrhosis, Fibrogenesis

Altered bile acid (BA) homeostasis has been implicated in various chronic liver diseases (CLD)¹. Exposure to elevated BA levels in diseased livers increases the risk of hepatotoxicity by activating inflammatory and necrotic cell death pathways². Higher serum BA levels are associated with worse clinical outcomes; for example, serum BAs are correlated with portal hypertension and the Model for End-Stage Liver Disease (MELD) score, and they can predict decompensation and transplant-free survival³.

BA conjugation processes produce less toxic and more water-soluble BA species, providing protection against damage caused by more toxic, hydrophobic BAs⁴. It is estimated that 98% of hepatic BAs are conjugated to either taurine or glycine by the enzymes such as including BA-CoA:amino acid N-acyltransferase (BAAT), which catalyzes the final step in conjugated synthesis⁵. However, recent studies have highlighted the involvement of tauro- and glyco-conjugated BAs in the pathogenesis of metabolic syndrome, dementia and cancer cachexia^{6,7}. In addition, elevated concentrations of conjugated BAs have been shown to significantly contribute to liver fibrosis and portal hypertension^{8,9}. However, in CLD, the associations between BA conjugation, including changes in *Baat* activity, and patient prognosis remain unclear. Furthermore, the mechanism underlying the marked increase in conjugated BA levels in CLD has yet to be clarified.

This study aimed to elucidate the associations between serum conjugated BA levels, the ratio of conjugated BA to free BA, the glycine-to-aurine (G/T) ratio, hepatic BA-related gene expression levels (including *Baat*), and survival rates in patients with CLD and in a rat model of CLD. To further investigate the mechanism underlying

¹Department of Gastroenterology and Hepatology, Mie University Graduate School of Medicine, Tsu, Japan.

²Biobank Center, Mie University Hospital, Tsu, Japan. ³Department of Gastroenterology, Murase Hospital, Suzuka, Japan. ⁴Joint Research Center, Tokyo Medical University Ibaraki Medical Center, Ibaraki, Japan.

⁵Department of Oncologic Pathology, Mie University Graduate School of Medicine, Tsu, Japan. ⁶Department of Gastroenterology and Hepatology, Tokyo Medical University Ibaraki Medical Center, Ibaraki, Japan. ✉email: akieguchi@med.mie-u.ac.jp

the increase in conjugated BAs, we examined the expression levels of genes involved in BA reabsorption in the ileum.

Results

Patient characterization

A total of 73 CLD patients, with average age 71.0 years, including 58 men and 15 women, were enrolled in the study. Diabetes was observed in 34 individuals (46.5%), hypertension in 42 (57.5%), dyslipidemia in 5 (6.8%), and hyperuricemia in 12 (16.4%). Atrial fibrillation, ischemic heart disease, emphysema, and cerebral infarction were reported in 4, 6, 4, and 2 individuals, respectively. Due to malignant tumors, three patients had a history of gastrectomy, and two had a history of colectomy. The cohort comprised patients with various underlying etiologies: 13 with hepatitis B virus (HBV), 21 with hepatitis C virus (HCV), 21 with metabolic-associated steatotic liver disease (MASLD), 16 with alcohol-related liver disease (ALD), and 2 with other causes. Patients infected with HBV or HCV were treated with direct-acting antivirals for HCV or nucleos(t)ide analogs for HBV as needed, and viral control was achieved. According to the Barcelona Clinic Liver Cancer (BCLC) staging system, 11 patients were classified as Stage 0, 29 as Stage A, 13 as Stage B, 19 as Stage C, and 1 as Stage D.

Serum BA levels, including conjugated BAs, showed no association with different CLD etiologies (Supplementary Fig. 1), except for GCDCA, which was significantly higher in CLD patients with ALD patients compared to those with HBV.

Correlation between BAs and laboratory variables

Glyco- and tauro-chenodeoxycholic acid (CDCA) were positively correlated with transaminases such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (γ -GT) and alkaline phosphatase (ALP) ($p < 0.05$ – 0.0001); hepatic functional reserve, including albumin (Alb), albumin-bilirubin (ALBI) score and prothrombin time (PT) ($p < 0.001$ – 0.0001); and platelet count (Plt) ($p < 0.01$), ammonia (NH_3) ($p < 0.05$ – 0.01), sodium (Na) ($p < 0.05$) and fibrosis index based on 4 factors (FIB-4) index ($p < 0.0001$) (Fig. 1a, b and Supplementary Fig. 2). The relationships between glyco- and tauro-cholic acid (CA), and taurochenodeoxycholic acid (TDCA) with transaminases, hepatic functional reserve, NH_3 , Na, and the FIB-4 index were similar to those observed between GCDCA, TCDCA, and these test values (Fig. 1a, b and Supplementary Fig. 2). The serum conjugated BA levels were not significantly correlated with the renal function test results. In addition, the serum conjugated BA level was not significantly correlated with tumor-node-metastasis (TNM) stage or maximum tumor size (Fig. 1c).

In the relationship between primary BAs and laboratory variables, several correlations were observed: Alb ($r_s = -0.457$, $p < 0.0001$), AST ($r_s = 0.284$, $p < 0.05$), ALP ($r_s = 0.294$, $p < 0.05$), Plt ($r_s = -0.234$, $p < 0.05$), PT ($r_s = -0.410$, $p < 0.001$), and ALBI score ($r_s = 0.414$, $p < 0.001$).

In contrast, no significant relationships were found between secondary BAs and laboratory variables.

Correlation between the ratio of conjugated to unconjugated BAs and laboratory variables

The correlation between the ratio of conjugated to unconjugated BAs and laboratory variables was similar to the correlation mentioned above for conjugated BA values. In brief, this ratio (GCDCA/CDCA, TCDCA/CDCA, GCA/CA, TCA/CA, GDCA/CA, and TDCA/DCA) was correlated with transaminases such as AST, ALT, γ -GT and ALP; hepatic functional reserve, including Alb, ALBI score and PT; and the Plt, NH_3 , Na and FIB-4 index (Supplementary Fig. 3a, b). The ratio of conjugated to unconjugated BAs was not significantly correlated with the maximum tumor size or TNM stage (Supplementary Fig. 3c).

Correlation between the G/T ratio of conjugated BAs and laboratory variables

The G/T ratios of CDCA, CA, and DCA were negatively correlated with transaminases such as AST, ALT, γ -GT and ALP ($p < 0.05$ – 0.0001); hepatic functional reserve, including Alb, ALBI score and PT ($p < 0.05$ – 0.0001); and NH_3 ($p < 0.05$), Na ($p < 0.05$ – 0.01) and the FIB-4 index ($p < 0.01$ – 0.001) (Fig. 2a and Supplementary Fig. 4). The G/T ratio of conjugated BAs was not significantly correlated with TNM stage or maximum tumor size (Fig. 2b).

Correlation between BAs and laboratory variables in the non-cholestatic group

When defining the non-cholestatic group as cases meeting the criteria of total BA $< 10 \mu\text{mol/L}$ and T-Bil $< 1.2 \text{ mg/dL}$, 19 cases (26% of all cases) were identified. In this group, an analysis of the relationships between various BAs and laboratory variables revealed significant correlations between GCDCA and γ -GT ($r_s = 0.477$, $p < 0.05$), TCDCA and γ -GT ($r_s = 0.576$, $p < 0.01$) as well as ALBI ($r_s = 0.461$, $p < 0.05$). GCA showed significant correlations with AST ($r_s = 0.555$, $p < 0.05$), ALT ($r_s = 0.614$, $p < 0.01$), γ -GT ($r_s = 0.740$, $p < 0.001$), and ALP ($r_s = 0.563$, $p < 0.05$). Additionally, TCDCA/CDCA was correlated with γ -GT ($r_s = 0.459$, $p < 0.05$). GCA/CA and TCA/CA were correlated with γ -GT ($r_s = 0.556$, $p < 0.05$; $r_s = 0.394$, $p < 0.1$, respectively) and with ALP ($r_s = 0.463$, $p < 0.05$; $r_s = 0.432$, $p < 0.1$, respectively). The G/T ratios of CDCA were negatively correlated with γ -GT ($r_s = -0.402$, $p < 0.1$) and ALBI score ($r_s = -0.484$, $p < 0.05$). In contrast, no associations were observed between CDCA or CA and laboratory variables. These findings suggest that even in non-cholestatic CLD, conjugated BAs undergo alterations.

Serum conjugated BAs, the ratio of BAs to their free forms and the G/T ratio are prognostic factors for survival

The significant correlation between serum conjugated BAs and liver function led us to further investigate whether conjugated BA levels are associated with survival. Among the 73 patients, 23 (31.5%) died during the average follow-up period of 1005 ± 471 days after the study period. All causes of death were considered liver related, except for one case of pancreatic cancer and one case of pneumonia. Patients with high TCAs or low

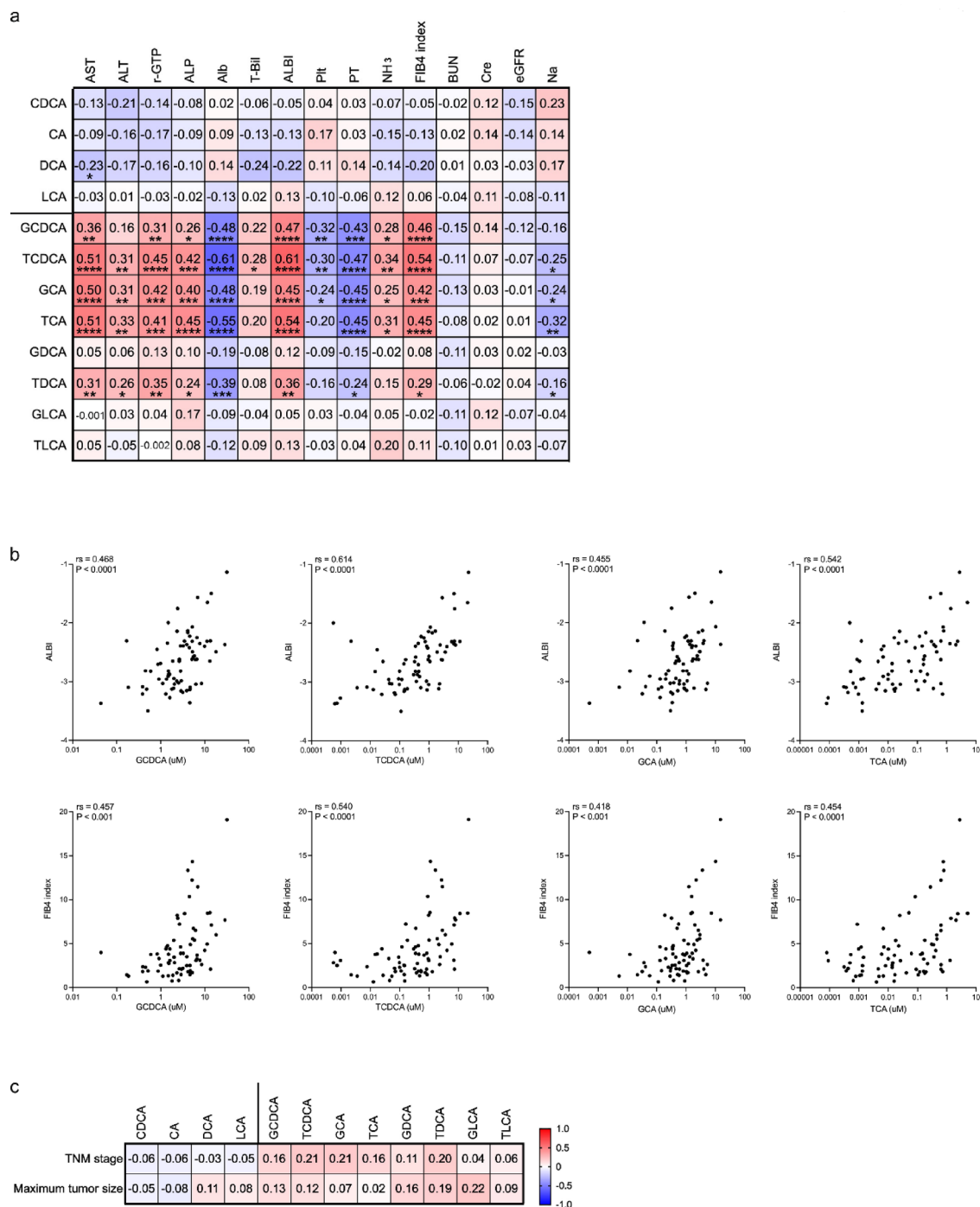


Fig. 1. (a) Correlations between BAs and laboratory variables; (b) Scatter plot between conjugated BAs and the ALBI or FIB4 index; (c) Correlations between BAs and the TNM stage or maximum tumor size. Positive correlations are shown with varying shades of red, whereas negative correlations are represented with varying shades of blue, according to their strength. The values in the figure represent the correlation coefficients, and * indicates statistical significance. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. BA, bile acid; CDCA, chenodeoxycholic acid; CA, cholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; G, glyco; T, tauro; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ -GT, gamma-glutamyltransferase; ALP, alkaline phosphatase; Alb, albumin; T-Bil, total bilirubin; ALBI, albumin-bilirubin; Plt, platelet count; PT, prothrombin time; NH₃, ammonia; FIB-4, fibrosis index based on 4 factors; BUN, blood urea nitrogen; Cre, creatinine; eGFR, estimated glomerular filtration rate; Na, sodium; TNM, tumor node metastasis.

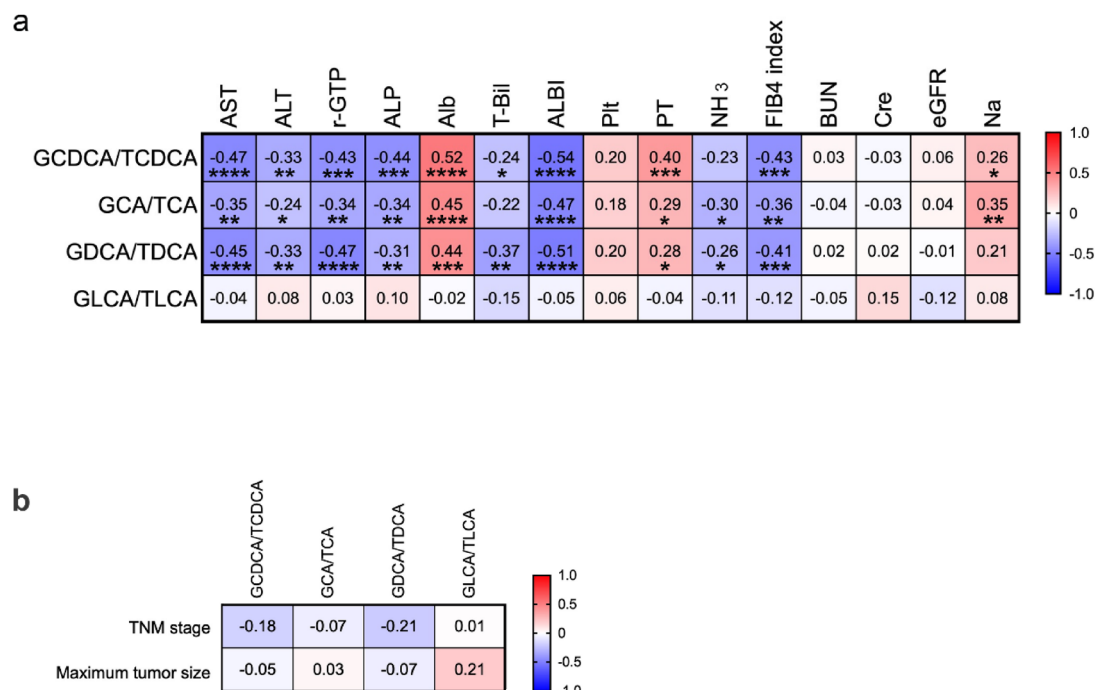


Fig. 2. (a) Correlation between the G/T ratio of conjugated BAs and laboratory variables; (b) Correlation between the G/T ratio of conjugated BAs and TNM stage or maximum tumor size. Positive correlations are shown with varying shades of red, whereas negative correlations are represented with varying shades of blue, according to their strength. The values in the figure represent the correlation coefficients, and * indicates statistical significance. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. G/T, glycine-to-aurine; BA, bile acid; G, glyco; T, tauro; CDCA, chenodeoxycholic acid; CA, cholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ -GT, gamma-glutamyltransferase; ALP, alkaline phosphatase; Alb, albumin; T-Bil, total bilirubin; ALBI, albumin-bilirubin; Plt, platelet count; PT, prothrombin time; NH₃, ammonia; FIB-4, fibrosis index based on 4 factors; BUN, blood urea nitrogen; Cre, creatinine; eGFR, estimated glomerular filtration rate; Na, sodium; TNM, tumor node metastasis.

GCA/TCA ratios (≥ 0.20 $\mu\text{mol/L}$, < 6.1) had significantly worse overall survival than those with lower or higher levels ($p < 0.05$, $p < 0.01$) (Fig. 3a). Patients with high TCDCA, GCDCA, and the ratio of TCDCA to its free form (TCDCA/CDCA) or low GCDCA/TCDCA levels (≥ 0.15 , ≥ 1.33 $\mu\text{mol/L}$, ≥ 0.25 , < 11.3 , respectively) had significantly worse overall survival than those with lower or higher levels ($p < 0.01$, $p < 0.01$, $p < 0.05$, $p < 0.05$, respectively) (Fig. 3b). Patients with high TDCA/DCA or low GDCA/TDCA levels (≥ 0.045 , < 8.2) had significantly worse overall survival than those with lower or higher levels ($p < 0.05$) (Fig. 3c).

The contribution of BA, ALBI score, and FIB-4 index to overall survival was evaluated by univariate analysis using a Cox proportional hazards model. Significant predictors of overall survival included TCDCA ($p < 0.001$) and TCA ($p < 0.001$), as well as ALBI score ($p = 0.001$) and FIB-4 index ($p < 0.001$) (Table 1). However, no factors were identified as significant in the multivariate analysis.

Correlations between BAs and liver function parameters, as well as fibrotic and inflammatory genes, in CLD rats (animal sample 1)

To explore the association between conjugated BAs and liver function, we measured BAs in CLD rats treated with CCl₄. The levels of serum total BAs and liver BAs were increased compared to wild-type (WT); however, the differences were not statistically significant (Supplementary Fig. 5a). Similarly, serum primary BAs, secondary BAs, and the primary/secondary BA ratio were elevated compared to WT, but these differences were also not statistically significant (Supplementary Fig. 5b). Muricholic acid (MCA) (β) or MCA (ω) was correlated with AST and ALT ($p < 0.05$ – 0.001). Glyco- and tauro-CDCA, CA, DCA, and tauro-MCA (TMCA, $\alpha + \beta$) were correlated with transaminases such as AST and ALT ($p < 0.05$ – 0.001) and hepatic functional reserve, including ALB and total bilirubin (T-Bil) ($p < 0.05$ – 0.001) (Fig. 4a and Supplementary Fig. 6). Notably, Glyco- and tauro-CDCA, CA, DCA, and TMCA were positively correlated with the expression of genes involved in liver fibrosis and inflammation, such as alpha-smooth muscle actin (α -Sma) ($p < 0.05$ or 0.01), collagen type I alpha 1 chain (*Col1a1*) ($p < 0.001$ or 0.0001), connective tissue growth factor (*Ctgf*) ($p < 0.05$ – 0.001), metalloproteinase inhibitor 1 (*Timp1*) ($p < 0.01$ – 0.0001), nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (*Nlrp3*) ($p < 0.01$ – 0.0001), and cysteine aspartate-specific protease 1 (*Casp1*) ($p < 0.01$ – 0.0001).

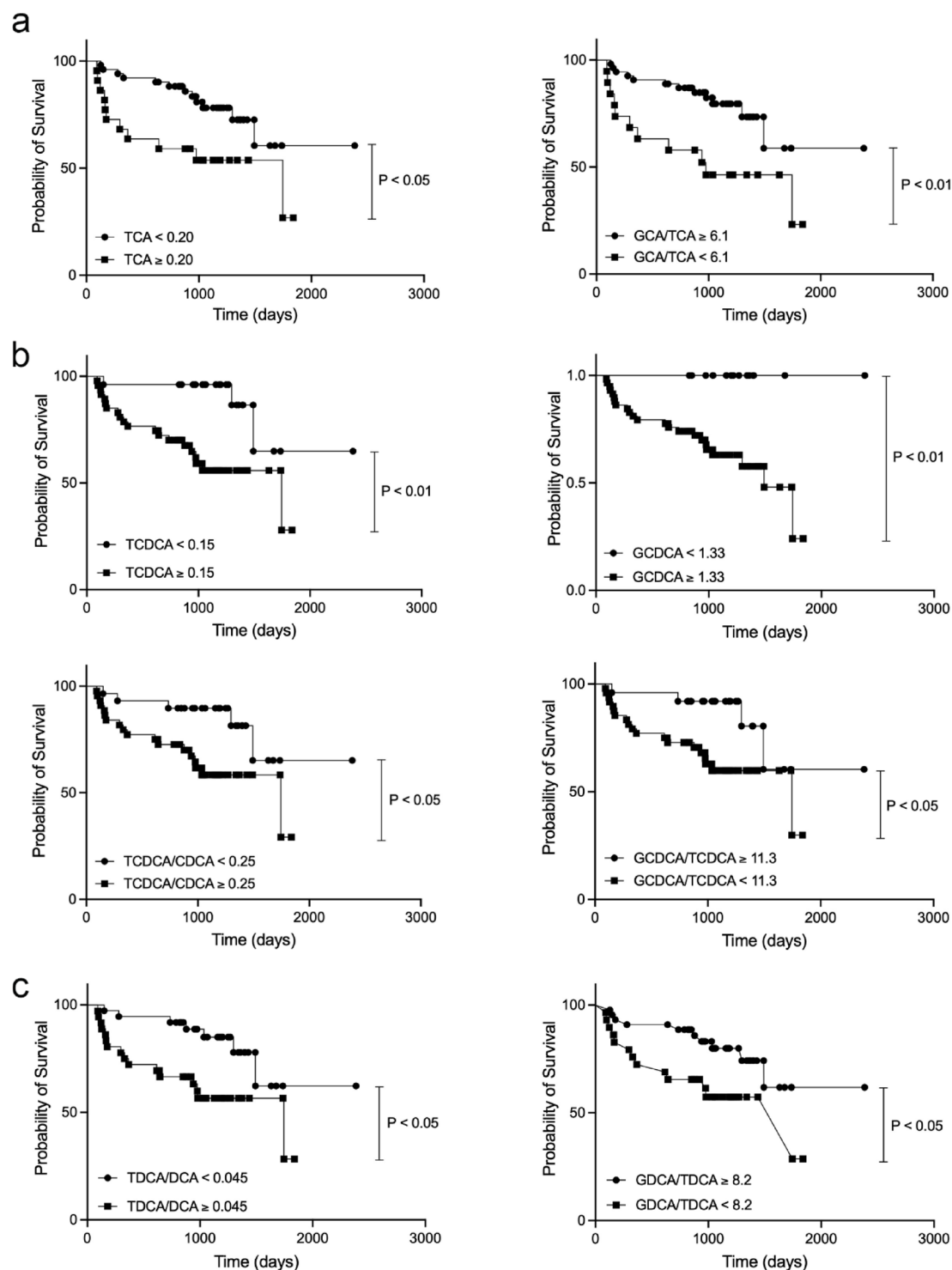


Fig. 3. (a–c) Serum conjugated BAs, the ratio of BAs to their free forms and the G/T ratio are prognostic factors for survival. BA, bile acid; G/T, glycine-to-aurine; GCDCA, glycochenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; GCA, glycocholic acid; TCA, taurocholic acid; TDCA, taurodeoxycholic acid.

MCA (β) exhibited a correlation with *Timp1* ($p < 0.01$) (Fig. 4b and Supplementary Fig. 7). The levels of glyco- and tauro-CDCA, CA, and DCA were significantly greater (TCA and TDCA; $p < 0.05$ and $p < 0.01$, respectively) or tended to be greater (GCDCA, GDCA, and TMCA; $p < 0.1$, $r = 0.51$, 0.56 , and 0.51 , respectively) in the CLD rats than in the WT rats (Fig. 4c).

Variables	Univariate	
	Hazard ratio (95%CI)	P value
GCDCA	1.041 (0.966–1.101)	0.213
TCDCA	1.167 (1.056–1.271)	0.0008
GCA	1.125 (0.995–1.229)	0.024
TCA	2.354 (1.430–3.646)	0.0002
GDCA	0.830 (0.490–1.099)	0.371
TDCA	0.872 (0.218–1.879)	0.794
GLCA	1.532 (7.4e–6–1632)	0.800
TLCA	1.46e–88 (0–79,823)	0.404
ALBI score	3.820 (1.675–8.442)	0.001
FIB-4 index	1.194 (1.070–1.320)	0.0008

Table 1. Univariate cox regression analysis for predictors of survival. CI, confidence interval; GCDCA, glycochenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; GCA, glycocholic acid; TCA, taurocholic acid; GDCA, glycodeoxycholic acid; TDCA, taurodeoxycholic acid; GLCA, glycolithocholic acid; TLCA, tauroolithocholic acid; ALBI, albumin-bilirubin; FIB-4, fibrosis-4.

Expression levels of genes involved in BA reabsorption in the ileum (animal sample 1)

Furthermore, we examined the expression of genes involved in BA reabsorption, including solute carrier family 51 alpha/beta (*Slc51a/Slc51b*) and the solute carrier family 10 member 2 (*Slc10a2*), in the rat ileum. Corresponding to serum conjugated BA levels, the expression of *Slc51a*, *Slc51b* and *Slc10a2* genes was significantly enhanced compared to WT rats ($p < 0.01$) (Fig. 4d), suggesting that BA reabsorption in the ileum is elevated in CLD. However, there was little correlation between the expression levels of these genes and the serum BA levels or liver function test results (data not shown).

Correlations between hepatic BAs, BA-related genes, and prognosis, as well as fibrotic and inflammatory genes, in CLD rats (animal sample 2)

To further explore the associations between hepatic BAs, BA-related genes, and prognosis, we analyzed the changes in hepatic BAs and BA-related genes in CLD rats. In the livers of CLD rats, the gene expression of solute carrier family 10 member 1 (*Slc10a1*), which is involved in BA uptake, sterol 12 α -hydroxylase (*Cyp8b1*), which is involved in BA synthesis, *Baat*, ATP-binding cassette subfamily B member 11 (*Abcb11*), multidrug resistance-associated protein (*Mrp*) 2, which are involved in BA excretion, and farnesoid X receptor (*Fxr*), which regulates BA composition, was downregulated, whereas oxysterol 7 α -hydroxylase (*Cyp7b1*) was upregulated compared with that in WT rats ($p < 0.05$). There were no significant changes in the gene expression of small heterodimer partner (*Shp*), cholesterol 7 α -hydroxylase (*Cyp7a1*), sterol 27-hydroxylase (*Cyp27a1*), and *Mrp4* (Fig. 5a). There were no changes in liver BA levels compared to WT (Fig. 5b). The liver gene expression of *Slc10a1*, *Baat*, *Abcb11*, *Mrp2*, and *Mrp4* was decreased in the deceased group, whereas the expression of *Cyp8b1* and sterol *Cyp27a1*, which is involved in BA synthesis, was elevated compared with that in the survival group ($p < 0.05$ – 0.01). The liver gene expression of *Shp* and *Cyp7b1* tended to decrease in the deceased group ($p < 0.1$, $r = 0.47$ and 0.42 , respectively). On the other hand, there was no correlation between the gene expression of *Cyp7a1* and *Fxr* and subsequent survival or death (Fig. 5c). Moreover, liver BAs were significantly decreased in the deceased rats ($p < 0.001$) (Fig. 5d). The hepatic gene expression of *Baat* was strongly negatively correlated with the expression of genes involved in liver fibrosis and inflammation, such as *Ctgf* ($p < 0.0001$), *Timp1* ($p < 0.0001$), hyaluronan synthase (*Has*) 3 ($p < 0.0001$), interleukin-6 (*Il-6*) ($p < 0.0001$), and toll-like receptor 4 (*Tlr4*) ($p < 0.0001$). Additionally, hepatic *Baat* gene expression showed a strong negative correlation with serum AST ($p < 0.01$) and T-Bil ($p < 0.01$).

Discussion

Detailed serum BA analysis has reinforced previous findings of elevated serum conjugated BA levels and an increased ratio of conjugated BAs to unconjugated BAs in patients with CLD, with these changes being more pronounced in advanced stages of the disease^{10,11}. Some of these relationships were observed even in patients without cholestasis, and changes in conjugated BAs occurred even in the early stages of CLD. Furthermore, we are the first to find that an increase in these conjugated BAs is significantly associated with an increased mortality rate in patients with CLD. In a CLD rat model, serum conjugated BAs correlated with liver function test values and liver fibrotic or inflammatory gene expression. *Baat* mRNA levels also correlated with liver fibrotic and inflammatory genes and were linked to survival rates. In rodents such as mice and rats, most of the CDCA is converted into MCAs by the enzyme *Cyp2c70*. As a result, MCAs along with CA are the predominant BAs found in the liver and bile of these species, while CDCA levels remain low. Therefore, it is appropriate to consider that MCAs in rodents is comparable to CDCA in human¹². These findings have not been previously reported; however, further validation is needed to confirm them. In this study, we found that in rats with end-stage liver failure, the intrahepatic BA concentration was decreased, associated with a reduction in BA synthesis. Additionally, in rats, the expression of *Abcb11* and *Mrp2* in the liver was significantly decreased, while *Mrp4* expression was preserved. This suggests a reduction in BA excretion into the intestines and an increased flow

into the bloodstream. The elevated BAs in the bloodstream are reabsorbed by the kidneys and return to the damaged liver. These processes are considered to contribute to the increased serum levels of conjugated BAs in CLD patients and rats. Although multiple studies have suggested an association between conjugated BAs and hepatocellular carcinoma (HCC)^{13–15}, we observed a limited correlation with liver cancer in this cohort. Instead, elevated conjugated BAs appear to result primarily from the progression of underlying liver disease.

We found that *Slc51a*, *Slc51b* and *Slc10a2* expression was upregulated in the ileum of CLD rats. This is consistent with the findings of a previous study reporting increased expression of these genes¹⁶ and is presumed to be a compensatory response to the reduced BA levels in the intestinal lumen, as described above. Interestingly, SLC10A2 inhibitors have been reported to improve experimental nonalcoholic steatohepatitis or cholestatic disease by targeting SLC10A2 function in the intestine and liver, resulting in the clearance of circulating BAs and protection of the liver^{17,18}. Conversely, our study observed a decrease in hepatic *Slc10a1* expression, which encodes Ntcp. This reduction in hepatic BA uptake leads to increased plasma conjugated BA levels. This elevation may contribute to the exacerbation of liver injury and inflammation. Consequently, the potential therapeutic application of *Slc10a1* inhibition in CLD is currently under investigation¹⁹. These reports also indirectly support the findings of the present study.

In addition, a shift toward taurine conjugation rather than glycine conjugation in patients with CLD, which becomes more pronounced in advanced stages of the disease, is consistent with previous findings^{10,11,20}. In the present study, the G/T ratios of conjugated BAs, such as GCA/TCA, GCDCA/TCDCa, and GDCA/TDCA, were determined to be associated with patient survival. BAAT-mediated amino acid conjugation with either glycine or taurine is the final step in BA synthesis²¹. *Baat*^{-/-} mice are almost completely devoid of taurine-conjugated BAs in the liver, suggesting that BAAT is the primary taurine-coupled enzyme²². In the present study, *Baat* expression in the liver decreased in CLD model rats; however, the ratio of taurine conjugation increased in parallel with the progression of liver disease in CLD patients. Thus, it can be inferred that its function in taurine conjugation may be maintained until the terminal stage of liver failure. Furthermore, given that BAAT has a lower affinity for glycine than taurine does²¹, the reduced influx of unconjugated BAs from the blood into the liver may lead to a lower demand for conjugation and thus a relatively greater reduction in glyco-conjugated BAs. However, the precise mechanism underlying the preferential suppression of glyco-conjugated BAs in CLD is not clear and requires further investigation.

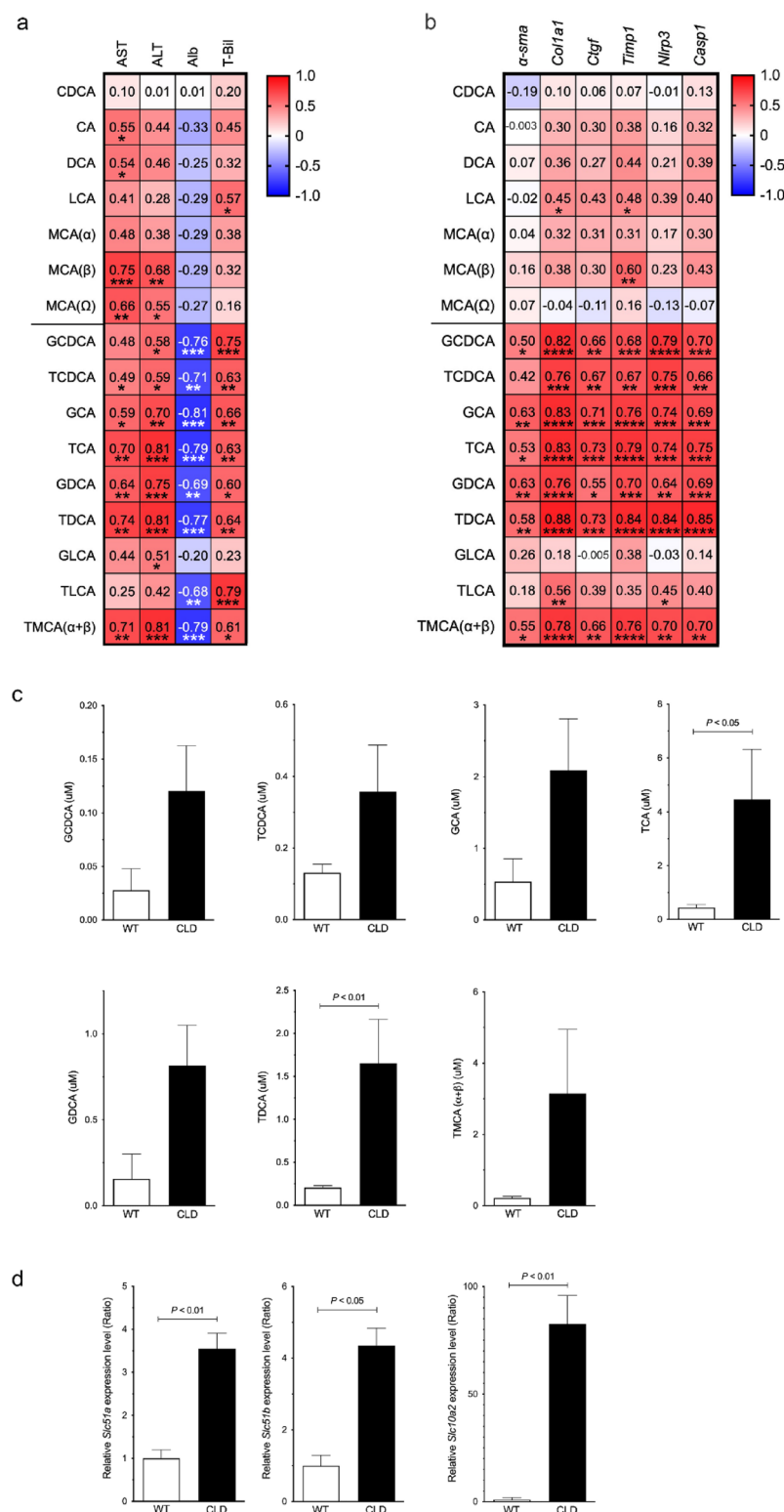
In this study, TCDCA, GCDCA and TCA were identified as BAs that determine the prognosis of patients with CLD. TCDCA has been reported to activate a phosphatidylinositol 3-kinase (PI3-K)-mediated survival pathway, which induces liver damage and hepatocyte apoptosis²³. Another report suggested that a progressive increase in TCDCA, GCDCA and TCA in parallel with disease severity in patients with alcoholic cirrhosis²⁴. Furthermore, a strong association between serum TCDCA levels and the hepatic venous pressure gradient has also been reported²⁵. Multiple reports indicate that TCDCA, GCDCA and TCA are deeply involved in the pathogenesis of liver diseases and that their levels can serve as biomarkers for predicting the progression of these diseases. However, further elucidation of the mechanisms by which these two conjugated BAs affect the liver is needed.

Studies have reported that BAs induce hepatic fibrosis and inflammation. For example, Feng et al. reported that GCDCA induces hepatic fibrosis via the NLRP3 inflammasome pathway, and FXR inhibits NLRP3 activity by restraining its phosphorylation²⁶. Furthermore, Ali et al. reported that taurine-conjugated BAs, especially TCDCA and TCA, are the most biologically active BAs in HCV and may contribute to liver disease pathogenesis through hepatic spingosine-1-phosphate receptor 2 (S1PR2)-mediated hepatic inflammation²⁷. Indeed, serum conjugated BAs, the ratio of conjugated BAs to free BAs, and the G/T ratio were correlated with transaminases and the FIB-4 index in patients with CLD in our study. Additionally, TCDCA, GCDCA, and TCA, which have been implicated in the development of hepatic fibrosis, were identified in our study as well. The observed decrease in hepatic *Fxr* expression revealed in this study may also contribute to hepatic fibrosis. Conjugated BAs can promote cell survival and antiapoptotic pathways, which are thought to mitigate their inherent toxicity²⁸. Therefore, the accumulation of taurine-conjugated BAs observed in CLDs is conventionally interpreted as an adaptive compensatory response to increase the elimination and detoxification of BAs. However, as mentioned above, conjugated BAs themselves may contribute to the progression of hepatic fibrosis. These findings suggest that the profibrotic effects of conjugated BAs may negatively impact patient outcomes.

Another interesting observation was that, as liver fibrosis progressed, while the gene expression of *Slc10a1*, *Cyp8b1*, *Baat*, *Abcb11*, *Mrp2*, and *Fxr* decreased, whereas *Cyp7b1* expression increased, and *Cyp7a1* expression showed a tendency to increase, which was associated with the reduction in *Fxr* expression. The extent of the expression of these genes was strongly associated with the survival rate of the rats. In CLD, BA synthesis is maintained while BA conjugation and enterohepatic circulation appear to be impaired, with decreased mRNA levels of *Slc10a1*, *Baat*, *Abcb11*, *Mrp2*, and *Mrp4*, which is consistent with the findings of a recent study²⁷.

Despite our important findings, the present study has several limitations. First, this retrospective study at a single institution involving human subjects does not exclude the potential influence of HCC on BAs. Prospective studies of CLD patients without HCC are anticipated in the future. Second, we were unable to obtain human liver tissues or fecal samples (gut microbiota), so the relationships among these factors, BA metabolism, and prognosis could not be examined. Additionally, the relationship between the serum BA concentration and survival rate could not be evaluated in the rat study. The limitations of this study include the limited inclusion of advanced CLD with cholestasis and the inability to demonstrate a direct causal relationship between conjugated BAs and CLD progression.

In conclusion, our study demonstrated that the ratio of serum conjugated BAs to free BAs increases, whereas the G/T ratio decreases in parallel with liver damage, fibrosis and inflammation. Furthermore, the increase in conjugated BAs is associated with subsequent survival rates in both humans and rat models of CLD.



Methods

Patient cohort

All methods were carried out in accordance with relevant guidelines and regulations. The study protocol was approved by the Clinical Research Ethics Review Committee of Mie University Hospital (H2019-063). This study was performed retrospectively on stored samples, and subjects were allowed to opt out of their data being used. Written informed consent was obtained from all subjects at the time of blood sampling. This cohort has been previously described in detail²⁹. Briefly, a total of 73 treatment-naïve patients with HCC with CLD were included in this retrospective study. BCLC and TNM stages were determined on the basis of the criteria for TNM staging

Fig. 4. (a) Correlation between BAs and liver function in rats treated with CCl_4 ; (b) Correlation between BAs and hepatic expression of fibrosis or inflammation markers in rats treated with CCl_4 . Positive correlations are shown with varying shades of red, whereas negative correlations are represented with varying shades of blue, according to their strength. The values in the figure represent the correlation coefficients, and * indicates statistical significance. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; (c) Comparison of BA between wild-type and CCl_4 -treated rats; (d) Comparison of ileal expression of BA reabsorption markers between wild-type and CCl_4 -treated rats. BA, bile acid; CDCA, chenodeoxycholic acid; CA, cholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; MCA, muricholic acid; G, glyco; T, tauro; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Alb, albumin; T-Bil, total bilirubin; α -Sma, alpha-smooth muscle actin; *Col1a1*, collagen type I alpha 1 chain; *Ctgf*, connective tissue growth factor; *Timp1*, metalloproteinase inhibitor 1; *Nlrp3*, nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3; *Casp1*, cysteine aspartate-specific protease 1; *Slc51a*, solute carrier family 51 member a; *Slc51b*, solute carrier family 51 member b; *Slc10a2*, solute carrier family 10 member 2;

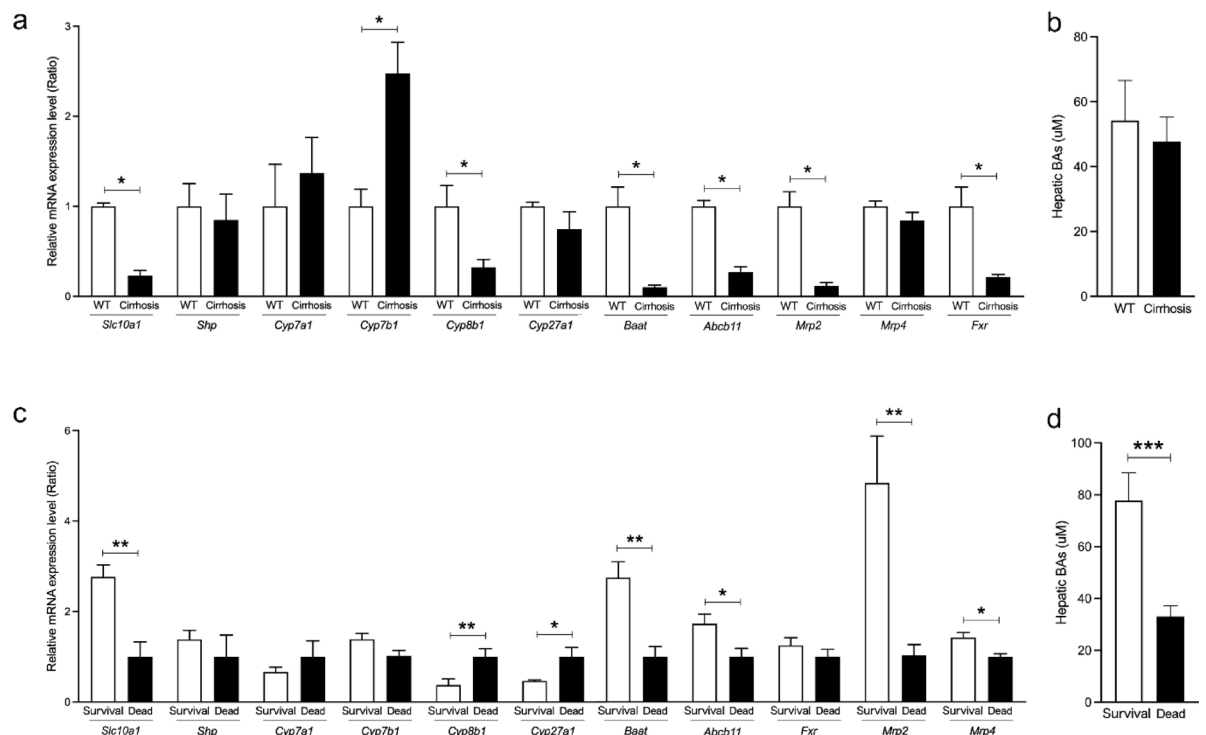


Fig. 5. (a, b) Comparison of hepatic BA-related gene expression (a) and BA concentrations (b) between wild-type and cirrhotic livers in rats. (c, d) Comparison of hepatic BA-related gene expression (c) and BA concentrations (d) between the survival and dead (deceased) groups of rats. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. BA, bile acid; *Slc10a1*, solute carrier family 10 member 1; *Shp*, small heterodimer partner; *Cyp7a1*, cholesterol 7 alpha-hydroxylase; *Cyp7b1*, oxysterol 7a-hydroxylase; *Cyp8b1*, sterol 12a-hydroxylase; *Cyp27a1*, sterol 27-hydroxylase; *Baat*, bile acid coenzyme A: amino acid N-acyltransferase; *Abcb11*, ATP-binding cassette subfamily b member 11; *Fxr*, farnesoid X receptor; *MRP*, multidrug resistance-associated protein.

of HCC presented by the Liver Cancer Study Group of Japan and were used to evaluate tumor progression. All the treatments were performed in accordance with the Japanese practical guidelines for HCC³⁰. Posttreatment follow-up for HCC patients included laboratory tests, such as measurements of tumor markers, at least every 3 months, and dynamic CT or MRI scans, at least every 6 months.

Blood preparation

The blood collection was performed in the early morning after an overnight fast. Clinical records, including AST, ALT, γ -GT, ALP, Alb, T-Bil, Plt, PT, NH_3 , blood urea nitrogen (BUN), creatinine (Cre), estimated glomerular filtration rate (eGFR) and Na, were retrospectively evaluated. The ALBI score³¹ and fibrosis index based on FIB-4³² were calculated. Serum was separated from blood at 1500 g for 10 min and stored at -80°C until the measurement of the BA composition.

BA concentrations were determined blindly as described by Ando et al.³³ with minor modifications. A mixture of internal standards (ISs) consisting of 41.6 ng of [²H₄]CA, 57.5 ng of [²H₄]CDCA, 32.8 ng of [²H₄]DCA, 22.4 ng of [²H₄]LCA, 100 ng of [²H₃]TCA, and 20 µl of ethanol–water (9:1, v/v) was added to 20 µl of serum. After the addition of ISs and 2 mL of 0.5 M potassium phosphate buffer (pH 7.4), BAs were extracted via Bond Elut C18 cartridges (200 mg, Agilent Technologies, Santa Clara, CA). After washing the cartridge with 1.6 mL of water, BAs were eluted in 3 mL of ethanol–water (9:1, v/v). The eluate was evaporated to dryness at 100 °C under a nitrogen stream and redissolved in 20 mM ammonium acetate buffer (pH 7.5)-methanol (1:1, v/v). After centrifugation at 12,000×g for 1 min, an aliquot of the supernatant was injected into the liquid chromatography–mass spectrometry (LC-MS/MS) system for analysis. The LC-MS/MS system consisted of a TSQ Vantage triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an HESI-II probe and a Prominence ultra-fast liquid chromatography (UFLC) system (Shimadzu, Kyoto, Japan). Chromatographic separation was performed via a Hypersil GOLD column (150 × 2.1 mm, 3.0 µm; Thermo Fisher Scientific) at 40 °C. The mobile phase consisted of (i) 20 mM ammonium acetate buffer (pH 7.5)-acetonitrile-methanol (70:15:15, v/v/v) and (ii) 20 mM ammonium acetate buffer (pH 7.5)-acetonitrile-methanol (30:35:35, v/v/v). The following gradient program was used at a flow rate of 200 µL/min: 0–100% B for 20 min, 100% B for 10 min, and 100% A for 8 min. The linearity and reproducibility of BA analyses in human serum were shown in Supplemental Table 1.

Animal samples 1

All methods were carried out in accordance with relevant guidelines and regulations. Our animal protocol (HKD43046) was reviewed and approved by the Institutional Animal Care and Use Committee at Hokudo Co., Ltd. (Sapporo, Japan). This rat model of CLD has been previously described in detail³⁴. Briefly, Wistar male rats (SRF, CLEA Japan, Tokyo, Japan) aged 7 weeks were fed a solid normal diet, CE2 (CLEA Japan), under conventional conditions and were orally administered carbon tetrachloride (CCl₄) at 1.0 mL/kg twice a week for 4 weeks to induce advanced fibrosis or cirrhosis. The CLD state was maintained with two weekly administrations of 0.5 mL/kg CCl₄ for 6 weeks (10 weeks total). The analysis included 17 out of 20 rats that died from CCl₄ during the experimental period. Ten-week-old male Wistar rats were used as controls, along with three WT rats. All rats were sacrificed at the conclusion of our treatment protocol under anesthesia with isoflurane (DS-pharma, Osaka, Japan). The depth of anesthesia was confirmed by the absence of the pedal withdrawal reflex, followed by euthanasia via exsanguination. Liver and ileum tissues were collected and stored at –80 °C for the analysis of BA concentrations and BA-related genes. Serum was separated from blood at 1500 g for 10 min and stored at –80 °C. Serum was used for measurement of AST, ALT, ALB, and T-Bil at Hokudo Co., Ltd. Serum BAs were measured at CMIC Pharma Science Co., Ltd. and hepatic BAs were measured using commercially available Total Bile Acid Assay kits (Cell Biolabs, San Diego, CA, USA) following the manufacturer's instructions. The study is reported in accordance with ARRIVE guidelines.

Animal samples 2

All methods were carried out in accordance with relevant guidelines and regulations. Our animal protocol (HKD10-CAM-01) was reviewed and approved by the Institutional Animal Care and Use Committee at Hokudo Co., Ltd. (Sapporo, Japan). This rat model of CLD has been previously described in detail³⁵. Briefly, Wistar male rats (SRF, CLEA Japan, Tokyo, Japan) aged 6 weeks (n = 21) were orally administered 1.0 mL/kg CCl₄ twice a week for 5 weeks to establish a rat model of advanced fibrosis. To maintain the cirrhotic state, the rats received 0.5 mL/kg CCl₄ orally twice a week for 16 weeks (total of 21 weeks). We divided the rats into a survival group and a deceased group. The survival group included rats that survived for 21 weeks after CCl₄ administration, whereas the deceased group included rats that died within 21 weeks of CCl₄ administration. The control designation indicates advanced fibrotic rats 5 weeks after CCl₄ administration. For the rats that died, we checked their condition every day, sacrificed them under anesthesia with isoflurane just before death. All rats were sacrificed at the conclusion of our treatment protocol under anesthesia with isoflurane. The depth of anesthesia was confirmed by the absence of the pedal withdrawal reflex, followed by euthanasia via exsanguination. Serum was used for measurement of AST, ALT, ALB, and T-Bil at Hokudo Co., Ltd. Liver tissues were collected and stored at –80 °C for the analysis of BA concentrations and BA-related genes. Hepatic BAs were measured using commercially available Total Bile Acid Assay kits (Cell Biolabs, San Diego, CA, USA) following the manufacturer's instructions. The study is reported in accordance with ARRIVE guidelines.

Gene expression

Total RNA was isolated from rectus abdominis muscle using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. cDNA was synthesized from total RNA via a cDNA synthesis kit (Takara, Shiga, Japan). Real-time PCR quantification was performed using the SYBR Green PCR mixture (Thermo Fisher Scientific, Inc.) and Quantstudio1 (Thermo Fisher Scientific, Inc.). The PCR primers used to amplify each gene are listed in Supplementary Table 2. The mean values of the mRNAs were normalized to those of beta 2 microglobulin (β2 m).

Statistical analyses

Continuous variables are presented as the mean ± SEM or median (minimum–maximum), and categorical variables are presented as the number of patients. Continuous data were compared between two groups via the Mann–Whitney U test. The correlation between the serum BA levels and clinical data was examined via Spearman's rank correlation coefficient. The categorical data were compared via the chi-square test. Receiver operating characteristic (ROC) curves and the corresponding receiver operating characteristic curves (AUCs) were used to obtain cutoff values for the outcomes. The Youden index was applied to calculate the optimal

cutoff point. Overall survival was measured via the Kaplan–Meier method and compared via the log-rank test. Associations between predictor variables and overall survival were determined using Cox proportional hazards regression. All the statistical analyses were performed via SPSS 23.0 software (IBM, Armonk, NY, USA) or Prism 9 (GraphPad Software, Inc., CA, USA). Differences were considered to be significant at $p < 0.05$. Along with p values, effect sizes for the Mann–Whitney U test were calculated and expressed as r . Effect sizes were interpreted as small ($0.1 \leq r < 0.3$), medium ($0.3 \leq r < 0.5$), and large ($r \geq 0.5$).

Data availability

The data are not publicly available due to ethical restrictions. However, data are available from the corresponding authors upon reasonable request and with the permission of Ethics Committee of Mie University.

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Author contributions

Conception and design of study: AE and MI; Acquisition of data: TM, MT, MI, YT, RS, MW; Interpretation of data: YK, AH, MW, TI and HN; Drafting of the manuscript: AE and MI. All authors reviewed and approved the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

H2019-063.

Informed consent

Opt out.

Human and animal resource

HKD43046

Additional information

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Correspondence and requests for materials should be addressed to A.E.

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