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Original Article

Comprehensive Bile Acid Profiling of ABCB4-mutated Patients and the Prognostic Role of Taurine-conjugated 3α,6α,7α,12α-Tetrahydroxylated Bile Acid in Cholestasis

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Abstract

Background and Aims: We asked if comprehensive bile acid profiling could provide insights into the physiopathology of *ABCB4*-mutated patients and evaluated the prognostic value of taurine-conjugated tetrahydroxylated bile acid (tauro-THBA) in cholestasis. ***Methods:*** Serum bile acid profiles were evaluated in 13 *ABCB4*-mutated patients with 65 healthy controls by ultra-high-performance liquid chromatography/multiple-reaction monitoring-mass spectrometry (UPLC/MRM-MS). The concentration of tauro-THBA was compared between *ABCB4*-mutated patients with different prognoses. The areas under the curve (AUCs) of tauro-THBA were compared between *ABCB11*-mutated patients with native liver survival and those who died or underwent liver transplantation before 3 years of age by receiver operating characteristic curve (ROC), with another patient cohort for further verification. ***Results:*** The overall hydrophobicity indices of bile acids in *ABCB4*-mutated patients (12.99±3.25 m) were significantly lower than those of healthy controls (14.02±1.74 m, $p<0.000$). That was due to markedly increased bile acid modifications including conjugation, sulfation, and ketonization. Differences in the tauro-THBA concentration in *ABCB4*-mutated patients with different prognoses were not significant. ROC analysis indicated that levels of tauro-THBA of <60 nM yielded an AUC of 0.900 with a sensitivity of 80% and specificity of 87.5% for *ABCB11*-mutated patients with different prognoses ($p=0.0192$). Of the 15 patients with good prognosis, 14 were classified correctly and four of the five patients with a poor prognosis were classified correctly (14:15 vs. 1:5, $p=0.005$) with tauro-THBA as a classifier. ***Conclusions:*** Tauro-THBA concentration may be a biomarker for predicting the clinical outcome in low gamma-glutamyl transferase intrahepatic cholestasis patients.

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Introduction

Bile acids are synthesized in the liver from cholesterol. They are inherently cytotoxic and can function as detergents to aid digestion and as signaling molecules to regulate gene expression and metabolism. Disruption of bile acid homeostasis that results in the accumulation of hydrophobic bile acids within the hepatocyte or canaliculus can damage hepatocyte membranes because their detergent-like properties,[1,2] or by causing a mitochondrial membrane permeability transition that leads to necrosis and apoptosis, and promoting the production of reactive oxygen species and oxidative stress.[3–6] This is considered to be the common pathogenesis of cholestatic diseases characterized by jaundice, progressive inflammation, liver fibrosis, cirrhosis, and death.[7]

More than 20 transporters are involved in the enterohepatic circulation of bile acids.[8,9] The bile salt export pump (BSEP) protein, encoded by *ABCB11*,[10] plays a key role in the secretion of bile acids across the canalicular membrane of hepatocytes into bile to provide the osmotic driver for bile flow.[11,12] The absence, deficiency, or dysfunction of BSEP, or progressive familial intrahepatic cholestasis, type 2, (PFIC2)

Keywords: Bile acids; MDR3; BSEP; PFIC; Liquid chromatography-mass spectrometry.

Abbreviations: AUC, area under the curve; BSEP, bile salt export pump; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; GC-MS, gas-liquid chromatography-mass spectrometry; GGT, gamma-glutamyl transferase; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; MCA, muricholic acid; MDR3, multidrug resistance protein 3; MRP2, multidrug resistance-associated protein 2; PFIC, progressive familial intrahepatic cholestasis; ROC, receiver operating characteristic; RT, retention time; TBA, total bile acids; THBA, tetrahydroxylated bile acid; UDCA, ursodeoxycholic acid; UPLC/MRM-MS, ultra-high-performance liquid chromatography/multiple-reaction monitoring-mass spectrometry.

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is caused primarily by mutations in *ABCB11* or, secondarily, by mutations in *ATP8B1*, which encodes familial intrahepatic cholestasis 1 (FIC1),[13,14] *MYO5B* (encoding myosin VB),[15,16] or *NR1H4* (encoding farnesoid X receptor, FXR),[17] or can be due to drug toxicity, leading to the accumulation of bile acids in hepatocytes (cholestasis). Multidrug resistance protein 3 (MDR3), encoded by *ABCB4*, is expressed on the canalicular membrane of hepatocytes and is responsible for flippase activity in transporting phospholipids from hepatocytes into bile. Mutations in *ABCB4* can cause the absence or dysfunction of MDR3 leading to MDR3 deficiency or PFIC3. In PFIC3, the impaired flippase of phosphatidylcholine into bile leads to an overload of bile acids within the canaliculus as biliary bile acids are no longer being sequestered in mixed micelles with phospholipid and cholesterol leading to bile duct injury.[18]

The toxicity of bile acids depends primarily on their hydrophobicity.[19,20] Compensatory modifications of bile acids, such as the formation of hydrophilic taurine-conjugated bile acids, hydroxylated bile acids, sulfated bile acids, and alternative renal excretion routes for bile acids, reduce the toxic detergent effect of hydrophobic bile acids and are considered important compensation mechanisms that affect the disease phenotype.[21,22] How dysfunction of MDR3 in human beings affects bile acid metabolism, including the distribution and composition of bile acids, has not been well investigated.

Tetrahydroxylated bile acids (THBAs) are the most hydrophilic and the least cytotoxic bile acids identified in humans to date.[23–25] The bile acids found in humans are typically mono-, di-, and tri- hydroxylated. THBAs are often undetectable in healthy humans or are present only in trace amounts. They can be detected in the serum and urine of cholestatic patients and in mouse models of human liver diseases.[22] It has been proposed that THBAs are hepatoprotective agents in alleviating cholestatic stress.[22] Tetrahydroxylation of bile acids to increase solubility might be an alternative pathway for elimination of bile acids through urine,[26] for alleviation of cholestasis.[27]

In our previous study, the profiling of bile acids revealed increased levels of taurine-conjugated 3α,6α,7α, 12α-THBA (tauro-THBA) in PFIC2 patients, compared with healthy controls.[28] In infants with intrahepatic cholestasis of diverse etiologies, a high urine THBA level was associated with better outcomes.[29] In a follow-up study of a subset of PFIC2 patients who underwent partial internal biliary diversion, we observed that changes in the level of THBAs were correlated with disease relief and recurrence, which implies a potential use for THBAs as prognostic indicators.[30] Our recent study revealed several THBA isoforms that might have potential as prognostic biomarkers in Alagille syndrome patients.[25] The correlation of the concentration of tauro-THBA with the prognosis of PFIC2 patients was not evaluated in the previous study owing to lack of sufficient follow-up information. The same was true for PFIC3 patients. We were very interested to know whether the concentration of tauro-THBA may have the potential to be a prognostic biomarker in patients with either a BSEP deficiency or PFIC3. The study goal was to determine the bile acid profile in *ABCB4*-mutated patients, and to explore the potential use of tauro-THBA as a prognostic indicator in both *ABCB4*-mutated patients and patients with BSEP deficiency.

Methods

Subjects

Study subjects were Chinese children who were admitted to the Children's Hospital of Fudan University, with consent, under a protocol approved by the Children's Hospital of Fudan University (2015-178 and 2020-402) and in accord with the ethical guidelines of the 1975 Declaration of Helsinki. Subject screening criteria for *ABCB4*-mutated patients included: (1) Clinical manifestations of cholestasis. (2) Exclusion of patients with infectious, other metabolic, and obstructive causes of conjugated jaundice by appropriate investigation. (3) Homozygous or compound heterozygous (likely) pathogenic variants in *ABCB4* based on a screening panel of 61 cholestasis-related genes or by whole exome sequencing.[31] (4) With available fasting serum samples. Sixty-five healthy subjects with fasting samples available and no indications of infectious, endocrinological, or metabolic diseases who were included in a previous study,[28,30] were used as controls for comprehensive bile acid profiling. The tauro-THBA concentrations in *ABCB4*-mutated patients with no jaundice (good prognosis) and those with decompensated cirrhosis or who died/underwent liver transplantation (bad prognosis) during follow-up were compared.

The tauro-THBA concentration in *ABCB4*-mutated patients were also compared with 17 *ABCB11*-mutated patients, with primary BSEP deficiency who participated in a previous study.[28] The areas under the curve (AUCs) of tauro-THBA in *ABCB11*-mutated patients with native liver survival (good prognosis) and those who died or underwent liver transplantation (bad prognosis) before 3 years of age were compared by receiver operating characteristic curve (ROC) analysis. The cutoff value derived from the *ABCB11*-mutated patients (primary BSEP dysfunction) was evaluated in another patient cohort, also previously reported, with secondary BSEP dysfunction, including five patients with *ATP8B1*, eight with *MYO5B* deficiency, and 12 with undiagnosed low gamma-glutamyl transferase (GGT) level cholestasis (GGT<100 IU/L)[28] to check whether tauro-THBA has the potential to be used as a prognostic biomarker.

Sample preparation and analysis of bile acids

The methods of sample preparation and analysis of bile acids were the same as previous reports.[28,30] Briefly, serum was separated from blood by routine centrifugation (1,000 × *g* for 5 m at 4°C), aliquoted (50 μL), freeze-dried, and stored at −80 °C until bile acid analysis was performed by reverse-phase ultra-high-performance liquid chromatography/multiple-reaction monitoring-mass spectrometry (UPLC/MRM-MS) with negative ion detection at the University of Victoria-Genome British Columbia Proteomics Center.[24] The quantitation of bile acids utilized standards of 60 bile acids, and 14 deuterium-labeled analogs of bile acids, as internal standards. The 60 targets, were previously described[16,24] and included all the major bile acids and some minor species, e.g., ursodeoxycholic acid (UDCA), hyocholic acid (HCA), hyodeoxycholic acid (HDCA), muricholic acids (MCAs), two THBAs (3α,6α,7α,12α-THBA, and tauro-THBA, which were custom-synthesized and provided by Victor Ling ([2]British Columbia Cancer Agency, Vancouver, BC, Canada). Linear-regression calibration curves of individual bile acids were constructed with internal calibration (Tables 1 and 2).

Assessment of the hydrophobicity of bile acids

The hydrophobicity of bile acids was calculated by the weighted retention time (RT) of bile acids in LC as:

$$\frac{\sum[\mathrm{C}(nM)\times\mathrm{RT}(min)]}{\mathrm{C}_{total}}$$

in which C is the concentration of individual bile acids and

Table 1. Serum concentrations of main primary and secondary bile acids in patients with *ABCB4* mutations, and healthy controls

RT in m		Healthy controls (*n*=65)	*ABCB4*-muated patients (*n*=13)	*p*-value
		M (Q1, Q3), nM	M (Q1, Q3), nM	
14.8	CA	45.6 (29.2, 89.9)	38.8 (30, 50.3)	0.246
9.6	tauro-CA	141.4 (67, 329.1)	4,528.8 (1,503, 8,650.1)	0.000
11.9	glyco-CA	690.7 (321.3, 1,230.8)	9,888.2 (2,083, 14,818.7)	0.000
18.6	CDCA	169.3 (96.7, 298.3)	156.4 (39.9, 364.5)	0.625
13	tauro-CDCA	683.1 (243, 1,522.2)	9,377.5 (6,978.9, 26,071.5)	0.000
15.2	glyco-CDCA	3,697.4 (2,031.3, 6,058.9)	32,616.8 (15,638.9, 70,515.3)	0.000
19.1	DCA	193.2 (83.2, 380.4)	53.7 (32.6, 195.9)	0.012
13.7	tauro-DCA	31.1 (5.8, 139.7)	25.4 (11.5, 214.3)	0.673
15.8	glyco-DCA	150.4 (8.8, 530)	84.9 (31.3, 960.5)	0.743
23.7	LCA	6.3 (1.7, 13.5)	1.8 (1.2, 22.1)	0.524
17	tauro-LCA	1 (0.2, 3.1)	2.8 (1.5, 5.7)	0.020
19.6	glyco-LCA	4.7 (1.8, 12.9)	19.6 (5.9, 83.1)	0.003

Primary bile acids: CA, tauro-CA, glyco-CA, CDCA, tauro-CDCA, glyco-CDCA. Secondary bile acids: DCA, tauro-DCA, glyco-DCA, LCA, tauro-LCA, glyco-LCA. RT, retention time (the elution time of compounds); m, minute.

C_{total} is the total bile acid concentration.[28] A lower hydrophobicity index indicates relatively lower hydrophobicity or higher hydrophilicity.

Statistical analysis

The statistical analysis was performed with SPSS 19.0 (IBM Corp., Armonk, NY, USA) to determine the significance of differences between the concentrations of tauro-THBA in the patient groups and healthy controls. Student's *t*-test was used when the data had a normal distribution. A nonparametric test, the Mann-Whitney *U* test, was used when the data did not have a normal distribution. Data were reported as means±standard deviations or medians and interquartile ranges (IQRs). ROC curve analysis was used to calculate AUC. Fisher's exact test was used in the R × C table. A *p*-value <0.05 was considered significant.

Results

Clinical status and genetic variations, study subjects

There were 13 *ABCB4*-mutated patients (10 females and 3 males). The age of sampling ranged from 1.1 to 20.5 years with a median age of 6.7 years. Seven of the patients were receiving UDCA therapy, two had not received UDCA treatment when screened, and four did not have clear medical records. In five patients, cholestasis resolved during follow-up (good prognosis). Three had decompensated cirrhosis, two underwent liver transplantation later, and one child died at 6.5 years of age because of liver failure (bad prognosis). One child was lost to follow-up at age of 3 years of age, and another, with moderate cholestasis at 19 years of age, was excluded from the prognostic analysis (Table 3).

The baseline information, including age, the clinical status of *ABCB11*-mutated patients and patients with undiagnosed low GGT level cholestasis, are shown in Supplementary Tables 1 and 2. Among the 17 *ABCB11*-mutated patients, 12 were screened before 2 years of age. One patient was lost to follow-up and eleven were followed up to ages of 1–9 years, with a median age of 3 years. Five patients had a bad prognosis (died or underwent liver transplantation before 3 years of ag, and eight had a good prognosis (survived with native liver until 3 years of age).

Bile acid profiles of ABCB4-mutated patients

Increased conjugation of primary bile acids in the *ABCB4*-mutated patients: We compared the concentrations of primary bile acids, including cholic acid (CA), chenodeoxycholic acid (CDCA), and their tauryl or glycyl conjugates in healthy controls and *ABCB4*-mutated patient groups. The concentration differences of unconjugated CA and CDCA, were not significant, but the concentrations of their conjugates [tauro-CA (32-fold), glyco-CA (14-fold), tauro-CDCA (13.7-fold), and glyco-CDCA (8.8-fold)] were significantly higher, in the *ABCB4*-mutated patient group than in the healthy controls (Table 1). Secondary bile acids, including deoxycholic acid (DCA), lithocholic acid (LCA), and their conjugates, represent biliary bile acids released into the intestine and reabsorbed into the blood for recirculation. We observed no significant differences in tauro-DCA, glyco-DCA, LCA, values between healthy controls and *ABCB4*-mutated patient groups. The concentrations of tauro-LCA (2.8-fold) and glyco-LCA (4.2-fold) values were significantly higher in the *ABCB4*-mutated patient group compared with the healthy controls (Table 1).

Increased concentrations of glycine/taurine-conjugated bile acids, sulfated bile acids, THBAs, and some atypical bile acid species in the *ABCB4*-mutated patient group: Bile acids can be modified *in vivo* in many ways, e.g. taurine/glycine conjugation, sulfation, norabieta/demethylation (nor-, 23 carbon bile acid analog with a four, instead of five, carbon side chain), ketonization/oxidoreduction (keto-), hydroxylation, dehydroxylation (DH-), and isomerism (iso-/apo-).[32] Representative atypical bile acids detected in this study are listed in Table 2.

The total bile acid (TBA) concentration and the concentrations of UDCAs, including UDCA, tauro-UDCA, tauro-UDCA-3-sulfate, and glyco-UDCA-3-sulfate and some atypical bile acid species including 12-keto-CDCA, 7,12-keto-LCA, alloisoLCA, DH-LCA, apoCA, and tauro-HDCA were significantly increased in the *ABCB4*-mutated patient group compared

Table 2. Serum concentrations of tetrahydroxylated bile acids (THBAs) and some atypical hydrophilic bile acid species in patients with *ABCB4* mutations, and healthy controls

RT in m		Healthy controls (*n*=65)	*ABCB4*-mutated patients (*n*=13)	*p*-value
		M (Q1, Q3), nM	M (Q1, Q3), nM	
3.3	glyco-UDCA-3-sulfate	101.6 (50.4, 157.3)	1,406.2 (507.5, 19,234.2)	0.000
3.3	tauro-THBA	0.9 (0.5, 1.5)	14.9 (2.7, 25.7)	0.000
3.9	tauro-UDCA-3-sulfate	8.7 (0.3, 19.5)	1,155.1 (266.9, 5,020.8)	0.000
4.3	glyco-CA-3-sulfate	1.6 (0.6, 2.9)	1.2 (0.5, 3.1)	0.893
4.4	tauro-DH-CA	0.2 (0.2, 0.6)	0.2 (0.2, 0.3)	0.946
5	tauro-ω-MCA	2.8 (0.2, 8.9)	38.2 (10.4, 86.3)	0.001
5.4	tauro-α-MCA	11.7 (3.4, 52.6)	44.1 (22.4, 304.6)	0.007
5.7	tauro-β-MCA	0.7 (0.2, 2.3)	32.3 (16.5, 309.9)	0.000
6.2	glyco-DH-CA	0 (0, 2.2)	0 (0, 0)	0.665
6.8	3α, 6α, 7α12α-THBA	0.3 (0.1, 0.5)	0.3 (0.3, 1.1)	0.498
7.3	glyco-DCA-3-sulfate	70.9 (7.2, 203.6)	0 (0, 5.1)	0.000
7.3	tauro-CDCA-3-sulfate	75.7 (14, 144)	1,084.1 (246.7, 1,570)	0.001
7.4	tauro-HCA	13.5 (6.4, 33)	266 (42.5, 675.1)	0.000
7.9	CA-3-sulfate	0.9 (0.4, 1.7)	3 (1.4, 6.6)	0.007
7.9	tauro-DCA-3-sulfate	7.5 (0.1, 53.6)	0 (0, 13.6)	0.144
9	tauro-HDCA	3.4 (0, 18.9)	4,390.3 (593.2, 17,573.7)	0.000
9	tauro-UDCA	25.4 (11.7, 70.1)	11,745.8 (1,915, 29,718.6)	0.000
9.9	glyco-LCA-3-sulfate	50.7 (6.4, 194.8)	38.9 (16.3, 195.8)	0.380
9.9	UCA	3.6 (2, 7.3)	2.2 (1.7, 3.2)	0.024
10.2	7, 12-keto-LCA	0.8 (0.3, 6.9)	69.4 (35.7, 170.7)	0.000
10.3	glyco-HCA	73.7 (35.2, 106.3)	345.2 (138.5, 693.3)	0.000
10.6	tauro-LCA-3-sulfate	1 (0.1, 28.9)	1.4 (0, 3.2)	0.803
10.7	DH-CA	3.7 (0, 11.7)	7.3 (2.6, 13.5)	0.170
11.6	ω-MCA	5.6 (1.3, 16.7)	7.7 (4.3, 8.8)	0.856
12.3	Nor-UDCA	8.3 (3.3, 20.2)	16.8 (8.9, 18.4)	0.161
12.5	7-keto-DCA	3.6 (1.6, 9.1)	1.4 (1.1, 1.8)	0.004
12.6	DCA-3-sulfate	0.1 (0, 0.7)	0.6 (0.1, 3.9)	0.047
12.6	β-MCA	1 (0.5, 4.5)	0.8 (0.1, 2)	0.151
13.4	12-keto-CDCA	1.6 (0, 17.3)	275 (38.3, 488.3)	0.000
13.8	HCA	16.7 (8, 39.5)	15.1 (11.7, 19.7)	0.835
14	MCA	1.4 (0.4, 6.3)	0.4 (0.3, 0.5)	0.036
14.4	3-keto-CA	1 (0.2, 2.3)	1.2 (0.7, 1.7)	0.456
14.6	alloCA	18.8 (6.9, 47.3)	1 (0.8, 1.6)	0.000
15.1	UDCA	99.2 (37.9, 194)	628.5 (281.1, 1,444.7)	0.001
15.3	HDCA	0 (0, 1.4)	0.2 (0, 3.3)	0.358
16.6	7-keto-LCA	9.8 (3.1, 23.1)	17.4 (7.3, 89.9)	0.054
16.6	6, 7-keto-LCA	3.3 (0.3, 6.8)	0.5 (0.3, 2.1)	0.155
17	Nor-DCA	2.6 (0.4, 8.8)	1.3 (1, 2.8)	0.648
17.1	12-keto-LCA	2.2 (0.1, 8.5)	0.5 (0.1, 0.8)	0.200
17.2	apoCA	0 (0, 0.1)	0.1 (0.1, 0.3)	0.025
21.5	alloisoLCA	0.5 (0, 1.2)	1.2 (0.9, 1.7)	0.007
21.9	isoLCA	5.3 (0.1, 11)	0.9 (0, 3.3)	0.228
22.2	isoDCA	0.1 (0, 0.3)	0.1 (0.1, 0.1)	0.378
24.4	DH-LCA	0 (0, 0.7)	0.7 (0.2, 1.6)	0.031
	TBA	8,374.3 (5,267.8, 15,325.6)	133,961.9 (79,907.4, 226,171.6)	0.000

HC, healthy control; RT, retention time (time before compound elution); TBA, total bile acids (62 bile acids listed in Table 1 and Table 2); THBA, tetrahydroxylated bile acid; m, minute.

with the healthy controls. It is interesting to note that some atypical taurine/glycine-conjugated trihydroxy and tetrahydroxy bile acids and their conjugates were significantly increased in the *ABCB4*-mutated patient group, including glyco-HCA, tauro-HCA, tauro-α-MCA, tauro-β-MCA, tauro-ω-MCA, tauro-THBA. The concentrations of sulfated bile acids, including CA-3-sulfate, DCA-3-sulfate, and tauro-CDCA-3-sulfate, were significantly increased in the *ABCB4*-mutated patient group compared with the healthy controls. Overall, levels of hydrophilic bile acids modified by hydroxylation, conjugation, sulfation, and ketonization, were significantly increased in the *ABCB4*-mutated patients, as confirmed by their RTs (Tables 1 and 2).

Concentrations and proportions of calculated bile acid species in the serum of *ABCB4*-mutated patients and healthy controls: Concentrations of calculated bile acid species including CA, CDCA, DCA, LCA, MCA, THBA and their modified conjugates or isomers were calculated (Fig. 1A–F). The total concentrations of CAs (11.6-fold), CDCAs (8.8-fold), LCAs (2.6-fold), and MCAs (4.9-fold) were significantly increased in the *ABCB4*-mutated patient group compared with healthy controls. The proportions of calculated bile acid species were determined relative to TBA levels. UDCA concentration was excluded from the TBA to reduce interference, as most *ABCB4*-mutated patients were treated with UDCA during sampling (Table 3). The proportions of total CAs, and total CDCAs, were not significantly different, but the proportions of total DCAs (1.33±1.72% vs. 12.26±10.64%, p=0.000), total LCAs (0.76±0.74% vs. 3.71±4.27%, p=0.001), total MCAs (1.96±2.25% vs. 2.85±2.07%, p=0.042) and total THBAs (0.07±0.27% vs. 0.21±0.25%, p=0.002) were reduced in *ABCB4*-mutated patients compared with healthy controls (Fig. 1G–L).

Molar ratios of modified and native bile acids in *ABCB4*-mutated patients: Molar ratios of MCA:CDCA, HCA:CDCA, α-MCA:CDCA, β-MCA:CDCA, and ω-MCA:CDCA in the *ABCB4*-mutated patient group and healthy controls were not significantly different. The molar ratios of tauro-CA:CA, tauro-DCA:DCA, tauro-CDCA:CDCA, tauro-LCA:LCA, tauro-UDCA:UDCA, tauro-HCA:HCA, tauro-α-MCA:α-MCA, tauro-β-MCA:β-MCA, tauro-ω-MCA:ω-MCA, and tauro-THBA:THBA were significantly increased in *ABCB4*-mutated patients compared with healthy controls (p<0.05). Those of glyco-CA:CA, glyco-DCA:DCA, glyco-CDCA:CDCA, glyco-LCA:LCA, glyco-UDCA:UDCA, glyco-HCA:HCA, 3-keto-CA:CA, 12-keto-CDCA:CDCA, 7-keto-LCA:LCA, and 7,12-keto-LCA:LCA were also increased (Table 4). Another noticeable change in the *ABCB4*-mutated patient group was a consistent increase in levels of taurine-conjugated, rather than glycine-conjugated, bile acids. Increases in molar ratios were found for tauro-CDCA: glyco-CDCA, tauro-UDCA: glyco-UDCA, tauro-UDCA-3-sulfate: glyco-UDCA-3-sulfate, tauro-HCA: glyco-HCA, and tauro-DH-CA: glyco-DH-CA (Supplementary Table 3).

Increased hydrophilicity of total bile acids in *ABCB4*-mutated patients: The hydrophobicity index of total bile acids was significantly lower in *ABCB4*-mutated patient group (12.99±3.25 m) than in healthy controls (14.02±1.74 m, p=0.000) (Fig. 2A). UDCA and its conjugates were not calculated to exclude interference from UDCA therapy.

Prognostic role of tauro-THBA for cholestasis patients

In patients with MDR3 deficiency [14.9 (2.7, 25.7) nM], tauro-THBA concentration was significantly elevated (19.5-fold), compared with healthy controls (p<0.001), but were relatively lower than those of patients with BSEP [40.4 (18.3, 226.9) nM] (p=0.035) (Fig. 2B).

Tauro-THBA concentration in ABCB4-mutated/MDR3 deficient patients with different prognoses

No significant difference of the concentration of tauro-THBA was observed between *ABCB4*-mutated patients with no jaundice (good prognosis) and those suffering with decompensated cirrhosis or died/underwent liver transplantation (bad prognosis) (data not shown).

Serum tauro-THBA level as a potential biomarker of the prognosis of patients with BSEP dysfunction and intrahepatic cholestasis of unknown cause (ROC curve analyses)

Levels of serum tauro-THBA were compared between *ABCB11*-mutated patients (primary BSEP deficiency) with different prognoses. Tauro-THBA concentration was significantly higher in patients with good prognosis than in those with bad prognosis (Fig. 2C). ROC curve analysis indicated that tauro-THBA had an AUC of 0.900 (95% CI: 0.702, 1.000), (p=0.0192; Fig. 2D). A concentration of tauro-THBA <60 nM was chosen as a marker for bad prognosis with a sensitivity of 80% and specificity of 87.5%. We then asked whether the bile acid classifier identified in *ABCB11*-mutated patients also applied to cholestatic patients in whom disease was ascribed to secondarily impaired BSEP function, as with *ATP8B1*-mutations,[13,14] *MYO5B*-mutations,[15,16] or with undiagnosed cholestasis. Of the 15 patients (7 with MYO5B, 3 with ATP8B1, and 5 with undiagnosed cholestasis) with good prognosis, 14 were classified correctly 4 of 5 were classified correctly (two ATP8B1 and three undiagnosed cholestasis patients) with severe prognosis using tauro-THBA of <60 nM the cutoff (15:16 vs. 1:5, p=0.005, with a sensitivity of 93.75% and specificity of 80%) (Table 5).

Discussion

In this study, we performed an analyses of serum bile acids in genetically-defined *ABCB4*-mutated patients. To our knowledge, no bile acid profiling in *ABCB4*-mutated patients has been reported. The principal difference observed was a marked increase in conjugated primary bile acids and hydrophilic bile acids modified by conjugation, sulfation, or ketonization, were significantly elevated. There were also significant increases in atypical hydrophilic trihydroxy, MCAs, and tauro-THBA in *ABCB4*-mutated patients (Tables 1, 2 and 4). All these modifications resulted in an increase in the hydrophilicity of the bile acid profile, as confirmed by the lower hydrophobicity indices of total serum bile acids in the *ABCB4*-mutated patient group compared with healthy controls (Fig. 2A). These findings support the hypothesis that abnormal metabolism of bile acids in *ABCB4*-mutated patients contributed to the activation of multipathway compensatory mechanisms to increase bile acid hydrophilicity, thereby alleviating bile acid toxicity in such patients. The factors that confer or lessen this ability await further definition. Of note, the concentrations and proportions of the main secondary bile acids were not increased compared with the main primary bile acids (Fig. 1 and Table 1), suggesting that the secretion of primary bile acids into the intestine was not increased, as not so many additional secondary bile acids were generated. Quantitation of secondary bile acids in the feces might help to confirm this.

A separate study arm assessed the relationship between tauro-THBA production and the disruption of bile acid homeostasis. We included patients in whom the pathogenesis of cholestasis was clear, i.e. caused by defects of transporters that led to bile acid overload, including the main canalicular bile acid transporter, BSEP, as well as PFIC3, another typi-

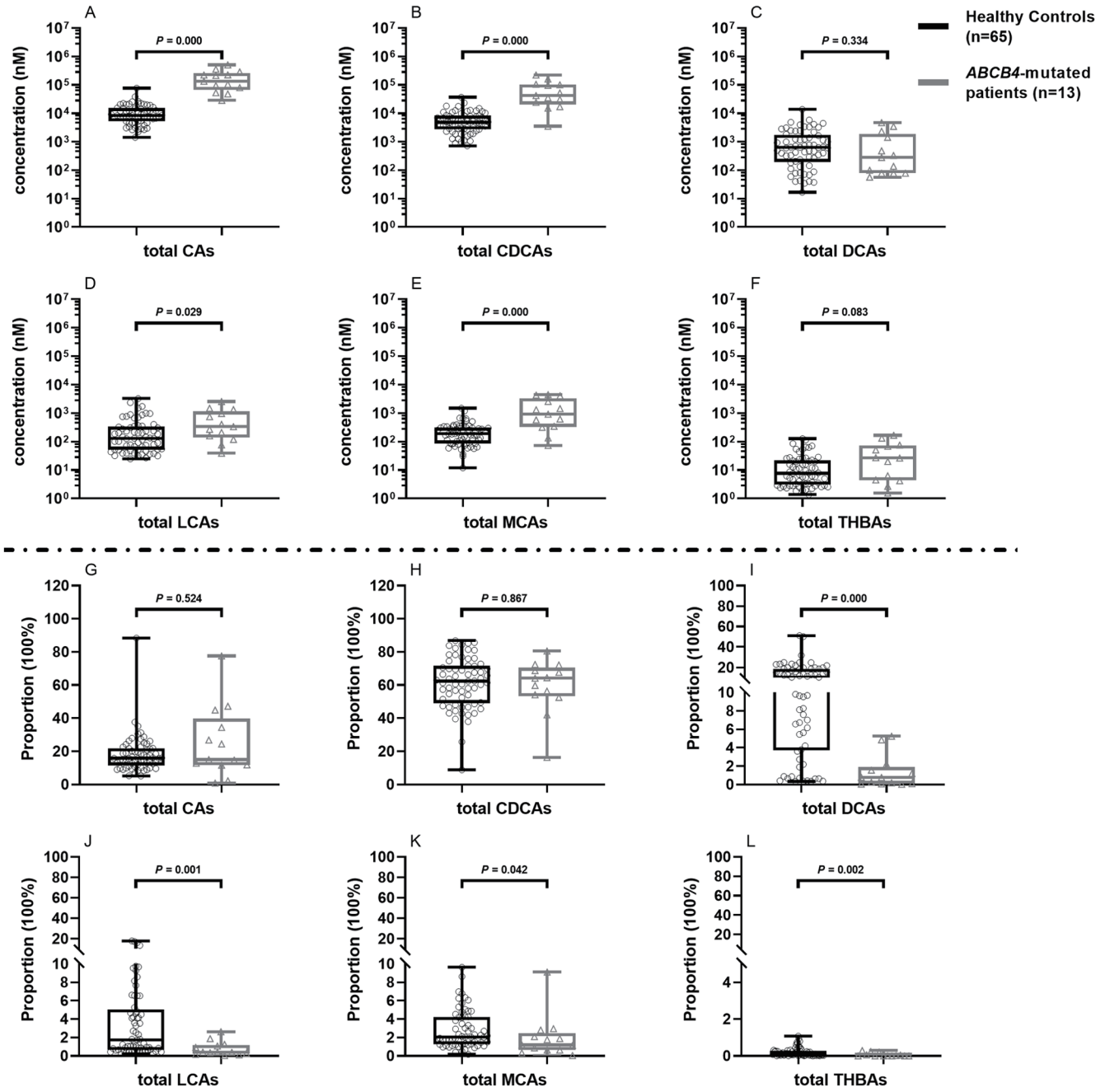

Fig. 1. Concentrations and proportions of calculated bile acids in the serum of *ABCB4*-mutated patients and healthy controls (concentrations in nM). The proportions of hydroxylated bile acids, including CAs, MCAs and THBAs were not significantly increased in *ABCB4*-mutated patient group compared to healthy controls. Total CAs includes CA, tauro-CA, glyco-CA, CA-3-sulfate, glyco-CA-3-sulfate, Nor-CA, 3-keto-CA, DH-CA, glyco-DH-CA, tauro-DH-CA, alloCA, apoCA and UCA; Total CDCAs includes CDCA, tauro-CDCA, glyco-CDCA, tauro-CDCA-3-sulfate and 12-keto-CDCA; Total DCAs includes DCA, tauro-DCA, glyco-DCA, DCA-3-sulfate, glyco-DCA-3-sulfate, tauro-DCA-3-sulfate, Nor-DCA, 7-keto-DCA and isoDCA; Total LCAs includes LCA, DH-LCA, tauro-LCA, glyco-LCA, tauro-LCA-3-sulfate, glyco-LCA-3-sulfate, 7-keto-LCA, 6,7-keto-LCA, 12-keto-LCA, 7,12-keto-LCA, isoLCA, and alloisoLCA; Total MCAs includes HCA, tauro-HCA, glyco-HCA, α-MCA, tauro-α-MCA, β-MCA, tauro-β-MCA, ω-MCA and tauro-ω-MCA; Total THBAs includes 6α,7α-THBA, tauro-6α,7α-THBA, 6β,7α-THBA and 2α,3α,7α,12α-THBA. Proportion %: proportion of calculated bile acids, e.g., proportion of total CA in calculated TBA (UDCA, Nor-UDCA, glyco-UDCA, tauro-UDCA, glyco-UDCA-3-sulfate, and tauro-UDCA-3-sulfate were excluded). CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; DH-, dehydroxylated-; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; MCA, muricholic acid; TBA, total bile acids; THBA, tetrahydroxylated bile acid; UDCA, ursodeoxycholic acid.

cal of cholestatic disease. In patients with BSEP deficiency, the secretion of bile acids from hepatocyte into canaliculus is impaired and bile acids are considered to accumulate within the hepatocytes. In patients with PFIC3, impaired flippase of phosphatidylcholine into the bile led to an overload of bile acids within the canaliculus as biliary bile acids were no longer being sequestered in mixed micelles with phospholipids and cholesterol.[18] High levels of THBAs have been observed in

Table 3. Background information in subjects with *ABCB4* variants

Patient	S	Sampling age in years	UDCA in mg	Nucleotide change/amino acid change	Status and age in years at last follow-up
ABCB4 (NM_000443.4)					
1	M	1.1	185 bid	c.2963G>A (p.Gly988Glu); c.1356+5G>C	jaundice free, 3.2
2	M	8.1	250 bid	c.2963G>A (p. Gly988Glu); c.1356+5G>C	jaundice free, 15
3	M	3	N/A	c.1965-1969delAAATG (p.Pro655ProfsTer8); c.1965-1969delAAATG (p.Pro655ProfsTer8)	N/A, 3
4	M	6.7	160 bid	IVS22-27C>T; c.2914G>A (p.Asp972Asn)	jaundice free, 20
5	M	20.5	N/A	c.924A>G (p.Ile308Met); c.3507+1G>A	jaundice free, 20.5
6	F	5.3	N/A	c.1376A>G (p.Asp459Gly); c.3825_3826delA (p.Met1276TrpfsTer1308)	Die, 6.5
7	F	2.2	stopped 2w	c.344+2_+3insT; c.2077_2078delC (p.Pro693HisfsTer698)	Decompensated cirrhosis, 16
8	M	19.1	500 bid	c.81-2A>G, c.1378A>T (p.Ile460Val); c.1954A>G (p.Arg652Gly)	Moderate jaundice, 19.1
9	M	4	N/A	c.139C>T (p.Arg47Ter); c.1745G>A (p.Arg582Gln)	Decompensated cirrhosis, 13
10	M	17	250 tid	c.1768C>T (p.Arg590Ter); c.3306C>T (p.Leu1102Leu)	jaundice free, 16
11	M	13.1	250 tid	c.1415T>C (p.Ile472Ter); c.1808T>C (p.Phe603Ser)	Decompensated cirrhosis, 13.1
12	M	8.2	no UDCA	c.469A>T (p.Ile157Phe); c.469A>T (p.Ile157Phe)	Liver transplantation, 15
13	F	4.8	250 qd	c. 469A>T (p.Ile157Phe); c.469A>T (p.Ile157Phe)	Liver transplantation, 13

M/F, male/female; N/A, data not available; S, sex; UDCA, ursodeoxycholic acid.

ABCB11$^{-/-}$ mice.[23,33] It has been speculated that the production of THBA was the major compensatory mechanism allowing for reduction of cholestatic stress in the mice. Significant elevation of THBA was not observed in *Mdr2*$^{-/-}$ mice (*ABCB4*),[18] which had a more severe phenotype of intrahepatic cholestasis. It was speculated that it is only when the bile duct injury eventually spills over onto the hepatocytes that the enzymes for producing THBA were stimulated, as cholestasis and bile acid accumulation eventually become more pronounced because of mechanical blockage of damaged bile ducts. Tauro-THBA concentration was significantly elevated in patients with BSEP deficiency compared with healthy controls and patients with MDR3 deficiency (Fig. 2B). That is in line with the hypothesis that THBA production is triggered by the accumulation of bile acid within hepatocytes, where P450 hydroxylases modify and detoxify bile acids by adding hydroxyl groups. The proportion of trihydroxy and tetrahydroxy bile acids including CAs, MCAs, and THBAs were not significantly elevated in *ABCB4*-mutated patients, compared with healthy controls (Fig. 1). In addition, differences of the molar ratios of MCA:CDCA, HCA:CDCA, α-MCA:CDCA, β-MCA:CDCA, and ω-MCA:CDCA in *ABCB4*-mutated patients and healthy controls were not significant (Table 4). The results suggest that, unlike patients with *ABCB11*-mutations and cholestasis for undiagnosed reasons,[28] hydroxylation was not the predominant form of bile acid modification in *ABCB4*-mutated patients. However, unlike in the mouse model, the concentration of tauro-THBA was significantly elevated in *ABCB4*-mutated patients, although to relatively low levels compared with BSEP-deficient patients. It is not known whether that was because of the secondary accumulation of bile acids within hepatocytes after bile duct injury or if there are other mechanisms for activating this compensatory mechanism. These observations warrant further follow-up studies.

Study of the *Mdr2*$^{-/-}$ mouse model has demonstrated that some THBA species stimulate bile flow after either intravenous injection or feeding, suggesting that THBAs have potential as therapeutic agents for cholestatic diseases.[34] Recent studies have also indicated that the ability to generate THBA as a compensatory mechanism for the alleviation of cholestasis affects the disease phenotype or prognosis.[22] In this lim-

Table 4. Molar ratios of modified hydrophilic bile acids to native bile acids in *ABCB4*-mutated patients compared with healthy controls

Modification	nM:nM	Healthy controls (*n*=65)	*ABCB4*-mutated patients (*n*=13)	*p*-value
A	MCA:CDCA	0.07±0.21	0.01±0.26	0.198
	HCA:CDCA	0.19±0.31	0.31±0.8	0.524
	α-MCA:CDCA	0.03±0.14	0.04±0.27	0.247
	β-MCA:CDCA	0.04±0.14	0.01±0.27	0.178
	ω-MCA:CDCA	0.07±0.18	0.09±0.27	0.490
B	CA-3-sulfate:CA	0.03±0.14	0.14±0.28	0.000
	DCA-3-sulfate:DCA	0±0.12	0.02±0.26	0.010
C	tauro-DH-CA:DH-CA	1.1±3.84	0.07±0.26	0.336
	tauro-CA:CA	9.93±35.71	156.11±156.27	0.000
	tauro-DCA:DCA	0.35±0.46	2.37±4.8	0.019
	tauro-CDCA:CDCA	7.94±14	163.36±195.88	0.000
	tauro-LCA:LCA	0.31±0.38	1.94±2.04	0.000
	tauro-UDCA:UDCA	0.85±1.11	18.72±20.53	0.000
	tauro-HCA:HCA	2.22±4.92	23.41±24.78	0.001
	tauro-α-MCA:α-MCA	31.16±61.18	171.04±250.35	0.034
	tauro-β-MCA:β-MCA	2.06±4.74	624.89±1,109.28	0.000
	tauro-ω-MCA:ω-MCA	0.94±2.43	11.65±15.65	0.000
	tauro-6α,7α-THBA:6α,7α-THBA	5.47±9.11	67±97.85	0.000
D	glyco-CA:CA	37.1±129.54	303.65±316.91	0.000
	glyco-DCA:DCA	1.25±1.58	5.34±10.01	0.025
	glyco-DH-CA:DH-CA	190.81±561.8	0.01±0.27	0.813
	glyco-CA-3-sulfate:CA-3-sulfate	3.64±7.78	10.56±18.48	0.135
	glyco-CDCA:CDCA	36.12±44.64	530.42±567.53	0.000
	glyco-LCA:LCA	2.16±3.11	12.35±20.39	0.000
	glyco-UDCA:UDCA	5.86±7.33	52±65.32	0.006
	glyco-HCA:HCA	11.14±33.97	38.99±74.72	0.023
E	3-keto-CA:CA	0.06±0.34	7.44±25.72	0.025
	7-keto-DCA:DCA	0.09±0.23	0.02±0.26	0.909
	12-keto-CDCA:CDCA	0.2±0.57	3.22±4.34	0.001
	7-keto-LCA:LCA	9.16±29.71	15.49±21.39	0.023
	6,7-keto-LCA:LCA	2.11±7.29	50.16±167.2	0.743
	12-keto-LCA:LCA	0.83±1.76	1.34±3.07	0.331
	7,12-keto-LCA:LCA	3.74±8.83	97.22±148.2	0.002
F	nor-CA:CA	1.38±4.29	1±1.02	0.941
	nor-DCA:DCA	0.05±0.16	0.03±0.26	0.102
	nor-UDCA:UDCA	0.28±0.67	0.04±0.27	0.001
G	DH-CA:CA	0.29±0.55	0.64±1.53	0.075
	DH-LCA:LCA	0.31±1.09	0.37±0.89	0.142

Data (nM) are means±standard deviations. A, hydroxylation; B, sulfation; C, taurine conjugation; D, glycine conjugation; E, oxidoreduction.

ited number of cases, the concentration of tauro-THBA indicated no significant difference between *ABCB4*-mutated patients with no jaundice (good prognosis) and those suffering with decompensated cirrhosis or who died/underwent liver transplantation (bad prognosis). It would be interesting to know whether, by enhancing the production of THBA or oral administration of THBAs, one might improve the prognosis of *ABCB4*-mutated patients.

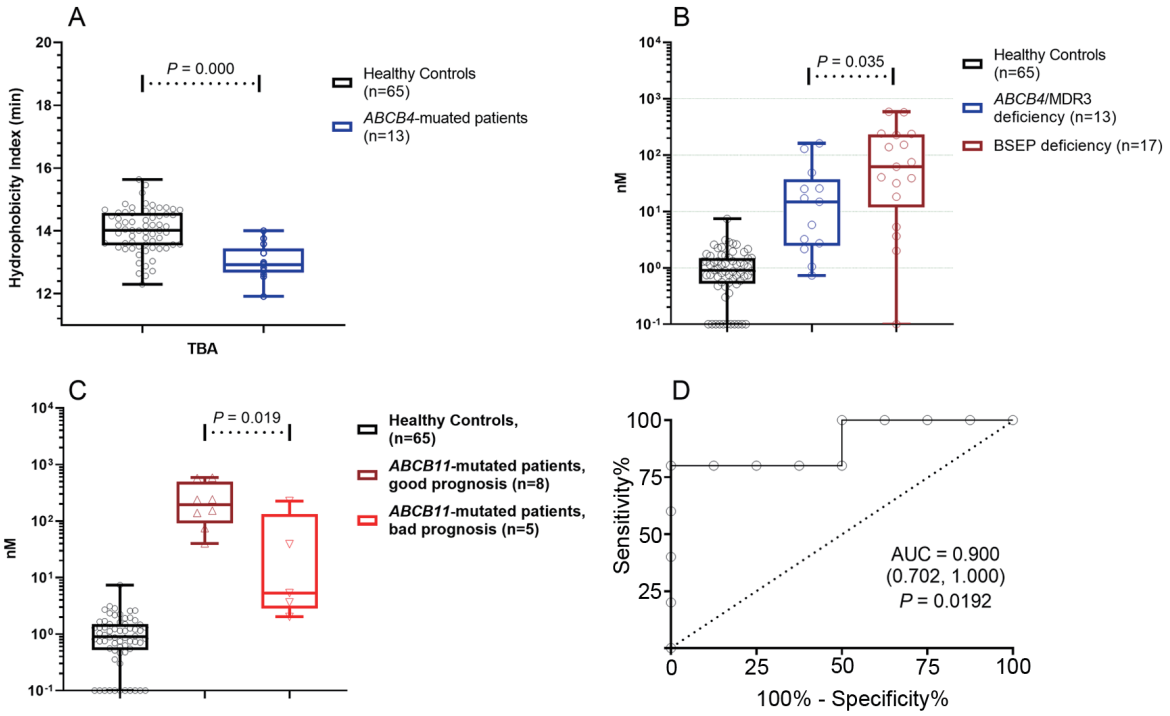

Fig. 2. Hydrophobicity indices, tauro-THBA concentrations and the ROC analyses. (A) Hydrophobicity indices of total bile acids (UDCA and its conjugates were not included) among *ABCB4*-mutated patients and healthy controls. (B) Levels of serum tauro-THBA concentration in *ABCB4*-mutated patient group and *ABCB11*-mutated patient group. (C) Levels of serum tauro-THBA concentration in *ABCB11*-mutated patients with different prognosis and (D) ROC curve analyses of serum tauro-THBA concentration for the prognosis of *ABCB11*-mutated patients. ROC, receiver operating characteristic; RT, retention time; THBA, tetrahydroxylated bile acid; UDCA, ursodeoxycholic acid.

In a previous study, the urinary excretion of bile acids was investigated by gas-liquid chromatography-mass spectrometry (GC-MS) in seven infants with α1-antitrypsin deficiency. It was found that the proportion of THBAs increased but that the patients who developed cirrhosis during the observation period had a lower concentration of THBAs than those with a more favorable course.[27] A follow-up study by Lee *et al*.[29] that studied the proportion of THBAs in the urine of 40 infants with intrahepatic cholestasis by GC-MS found that a higher THBA proportion was associated with good outcomes and that urinary tauro-THBA proportion predicted good prognosis with high sensitivity (95.24%) and specificity (84.21%, $p<0.0001$). However, the case numbers in these studies were small, and most of the cases in Lee *et al*.[29] had an undefined etiology. In addition, the sensitivity of the GC-MS method was lower than that of the LC-MS method, and THBA is weakly detectable in serum samples using the GC-MS method,[22,24,27,29] detects unconjugated forms of bile acids. A deconjugation step is needed to convert conjugated bile acids, such as glycine-conjugated, taurine-conjugated, glucuronidated, and sulfated forms. Therefore, what was measured in those studies was the total unconjugated and variously modified forms of the bile acids for each compound.[27,29] Only the proportion of urinary THBA was evaluated in those studies. The THBA concentration was not quantitated or was only partially quantitated by the GC-MS method.

Table 5. Prognostic test of tauro conjugated tetrahydroxylated bile acid (tauro-THBA) in patients with variants in *ATP8B1* and *MYO5B* and undiagnosed cholestasis

Prognosis	Mutation	Positive	Negative	Total
Good	ATP8B1	3	0	3
	MYO5b	7	1	8
	Undiagnosed cholestasis	5	0	5
	Total	15	1	16
Bad	ATP8B1	1	1	2
	MYO5b	0	0	0
	Undiagnosed cholestasis	0	3	3
	Total	1	4	5
Total		16	5	21

Good prognosis indicates survival with native liver at last follow-up; Bad prognosis indicates death or having undergone liver transplantation during follow-up.

Therefore, it is not known whether any of the conjugated bile acids, including tauro-3α,6α,7α,12α-THBA, could be a potential prognostic biomarker for the disease, because GC-MS is not suitable for direct analysis of any of the very polar and conjugated bile acids. In our study, LC-MRM/MS was used[24] with custom-synthesized taurine-3α,6α,7α,12α-THBA as standard substance.[28] Compared with GC-MS, it provides higher sensitivity, allows direct analysis of tauro-3α, 6α, 7α, and 12α-THBA, and does not need time-consuming sample preparation before LC-MS runs. Therefore, the results for tauro-3α,6α, 7α,12α-THBA in the serum of patients with confirmed hereditary cholestasis, as seen in this study, is new and complements previously published results using urine.

Multiple THBA isomers have been identified in patients with liver diseases.[27,29] The prognostic value of taurine-3α,6α,7α,12α-THBA is explored in the current study, as it is one of the main forms of THBAs in patients with PFIC2[28] and with Alagille syndrome.[25] However, a study limitation as that only taurine-3α,6α,7α,12α-THBA was measured, as we only had access to custom-synthesized taurine-3α,6α,7α,12α-THBA when we performed the study. No authentic compounds of other tauro-THBAs are commercially available. In addition, tauro-3α,6α,7α,12α-THBA has been reported as a major THBA in animal models of PFIC2. That is why we hypothesized it might be a biomarker in patients of PFIC2. It will be very interesting to know whether there are other THBA isomers that have even higher specificity and sensitivity for prognostic purposes, and that warrants further study.

The distribution of bile acids in both serum and urine reflected the metabolism of bile acids during disease. The detection of urine samples is noninvasive, but serum samples were much easier to collect for liver function tests in children. Collection of urine samples was usually not convenient as the majority of our patients were infants who were being followed at outpatient clinics. For this study, only serum samples collected from the patients were available in our biobank and not enough urine samples were available for analysis. This study focused on the analysis of bile acids in serum only. In addition, serum bile acids are derived from two main sources, the intestine and the liver, which directly reflect the bile acid metabolism. Our study of serum samples complements previous reports in urine samples. Whether the concentration of taurine-3α,6α,7α,12α-THBA in urine is also associated with the severity of disease and has the potential to work as a prognostic biomarker warrants further study.

In the current study, we asked whether the THBA concentration was associated with the severity of disease and if it has the potential to work as a prognostic biomarker. We assessed the relationship between levels of tauro-THBA and prognosis in *ABCB11*-mutated patients and found that serum tauro-THBA concentrations of >60 nM were a sensitive biomarker for predicting clinical outcome in patients with hereditary cholestasis caused by both BSEP dysfunction and cases of undiagnosed low GGT cholestasis (Fig. 2C–D and Table 5).

Conclusions

(1) Profiling of serum bile acids revealed increased conjugation, sulfation, and ketonization of bile acids and that hydroxylation was not the predominant modification of bile acids in *ABCB4*-mutated patients. (2) Analysis of tauro-THBA in genetically-defined *ABCB4*- and *ABCC11*-mutated patients suggested that the generation of hydroxylated THBA was triggered by the accumulation of bile acids within hepatocytes. (3) The concentration of tauro-THBA may be a biomarker for predicting the clinical outcome of low GGT intrahepatic cholestasis patients.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceived and designed the experiments (RXW, VL, JSW), performed the experiments, drafted the manuscript (JH, TL), critically revised the manuscript (JSW, VL), collected the data and samples (ZDL, TL, XXX, LJZ), and proofed the language presentation in the final document (JAS).

Ethical statement

All procedures were performed in accordance with the ethical standards of the institution and the 1975 Helsinki Declaration. Study subjects were Chinese children who were admitted to the Children's Hospital of Fudan University, with consent, under a protocol approved by the Children's Hospital of Fudan University (2015-178 and 2020-402).

Data sharing statement

No additional data are available.

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