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ORIGINAL ARTICLE

Poly-hydroxylated bile acids and their prognostic roles in Alagille syndrome

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Abstract

Background The liver manifestations of Alagille syndrome (ALGS) are highly variable, and factors affecting its prognosis are poorly understood. We asked whether the composition of bile acids in ALGS patients with good clinical outcomes differs from that in patients with poor outcomes and whether bile acids could be used as prognostic biomarkers.

Methods Blood for bile acid profiling was collected from genetically confirmed *JAG1*-associated ALGS patients before one year of age. A good prognosis was defined as survival with native liver and total bilirubin (TB) < 85.5 μmol/L, while a poor prognosis was defined as either liver transplantation, death from liver failure, or TB ≥ 85.5 μmol/L at the last follow-up.

Results We found that the concentrations of two poly-hydroxylated bile acids, tauro-2β,3α,7α,12α-tetrahydroxylated bile acid (THBA) and glyco-hyocholic acid (GHCA), were significantly increased in patients with good prognosis compared to those with poor prognosis [area under curve (AUC) = 0.836 and 0.782, respectively] in the discovery cohort. The same trend was also observed in the molar ratios of GHCA to glyco- chenodeoxycholic acid (GCDCA) and tetrahydroxylated bile acid (THCA) to tauro-chenodeoxycholic acid (TCDCA) (both AUC = 0.836). A validation cohort confirmed these findings. Notably, tauro-2β,3α,7α,12α-THBA achieved the highest prediction accuracy of 88.00% (92.31% sensitivity and 83.33% specificity); GHCA at > 607.69 nmol/L was associated with native liver survival [hazard ratio: 13.03, 95% confidence interval (CI): (2.662–63.753), $P = 0.002$].

Conclusions We identified two poly-hydroxylated bile acids as liver prognostic biomarkers of ALGS patients. Enhanced hydroxylation of bile acids may result in better clinical outcomes.

Keywords Alagille syndrome · Bile acid profile · Poly-hydroxylated bile acids · Prognostic biomarkers

Introduction

Alagille syndrome (ALGS, OMIM 118,450) is a multisystem autosomal dominant developmental disorder that is caused by pathogenic variants in either *Jagged 1* (*JAG1*) or *NOTCH2*, with *JAG1* variants accounting for approximately 95% of diagnosed cases [1–5]. It potentially leads to end-stage liver diseases requiring liver transplantation [6]. The poor prognosis of ALGS poses a substantial burden on clinical management, as well as on families and society economically and emotionally.

With highly variable clinical manifestations and outcomes and the lack of obvious correlation between genotype and phenotype [2], potential biomarkers indicating the liver prognosis of ALGS are urgently needed [7]. Previously, Kamath et al. reported that in some cases of ALGS, total bilirubin levels fell rapidly between 12 and 24 months of age,

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and the decrease may be associated with better outcomes [8]. However, to our knowledge, earlier (before one-year-old age) prognostic biomarkers that are important for the precision management of ALGS have not been reported to date.

Bile acids are amphipathic molecules essential for multiple physiological functions, such as lipid and energy homeostasis [9, 10]. However, hydrophobic bile acids at high concentrations are inherently cytotoxic and can induce inflammatory stress in the liver or intestines [11–13]. Hydroxylation and conjugation through amidation with glycine and taurine or esterification with sulfuric acids and glucuronic acids increase their hydrophilicity, representing two efficient mechanisms of bile acid detoxification [14, 15]. The compositions and relative hydrophobicity of different bile acids in biological materials can be analyzed by ultrahigh-performance liquid chromatography coupled to multiple-reaction monitoring–mass spectrometry (UPLC-MRM-MS) [16].

Hydroxylation can increase hydrophilicity and decrease the toxicity of bile acids. Poly-hydroxylated bile acids, such as trihydrocylated, including muricholic acids (MCA) and hyocholic acid (HCA), or tetrahydroxy bile acid (THBA), are highly hydrophilic bile acids compared to human primary bile acid, chenodeoxycholic acid. Glyco-hyocholic bile acid (GHCA), glycine-conjugated HCA, is decreased in nonalcoholic fatty liver disease and associated with disease severity [17]. In biliary stenosis, GHCA was decreased in malignant stenosis compared to benign stenosis and controls [18]. No report on GHCA in cholestatic disorders has yet been published.

THBAs are usually not detectable or are only present in trace amounts in healthy humans, but they are often detected in the serum and urine of cholestatic patients and mouse models. It has been speculated that THBA may act as a hepatoprotective agent in alleviating cholestatic stress [19–23]. In 2001, Wang et al. reported the presence of a large amount of THBAs in *Bsep*$^{-/-}$ mice, which displayed only very mild cholestasis in contrast to human BSEP (ABCB11) deficiency that results in fatal childhood disease, i.e., progressive familial intrahepatic cholestasis type 2 (PFIC 2). THBAs are proposed to protect mutant mice from severe cholestatic damage [24]. A follow-up study of *Mdr* 2$^{-/-}$ and *Bsep*$^{-/-}$ double knockout mice showed that increased hydrophilic bile acids, such as MCA, THBAs, and reduced cholic acid, prevented liver damage caused by the *Mdr*2$^{-/-}$ genotype, which typically presented with progressive liver damage due to severe cholangitis [25]. In a preliminary study in infants with intrahepatic cholestasis of mixed etiologies, a high level of THBAs in urine was observed to be associated with good clinical outcomes [21]. In a more focused study of cholestasis patients, in comparison with 35 healthy controls, some THBAs and tauro-THBAs were found to be elevated along with significantly reduced hydrophobicity of the bile acid pools in plasma of PFIC 2 and genetically undiagnosed cholestasis patients [20]. In a follow-up study of a subset of PFIC 2 patients who underwent partial internal biliary diversion, Liu et al. observed that changes in the level of THBAs were well correlated with disease relief and recurrence, which implies a potential use for poly-hydroxylated bile acids as prognostic indicators [26].

Since bile acids play a key role in cholestatic diseases, we aimed to investigate in the present study whether poly-hydroxylated bile acids could be potential liver prognostic biomarkers of ALGS outcomes.

Methods

Study design

Serum or plasma samples for bile acid profiling were clinical specimens collected from genetically confirmed *JAG1*-associated ALGS patients before one year of age. These specimens were the leftover samples from clinical tests, which were deposited in biobanks in Jinshan Hospital (Shanghai, China) and Children's Hospital of Fudan University (Shanghai, China) according to ethics approvals (No. 2014-13-01 in Jinshan Hospital and No. 2015-178 in Children's Hospital of Fudan University). The study was approved by the Ethics Committee of Children's Hospital of Fudan University (Shanghai, China) (No. 2017-99) following the ethical standards of the Helsinki Declaration of 1964, as revised in 2000. Informed consent to participate in the study was obtained from participants or their parent or legal guardian in the case of children under 16 years old. The selection of patients and samples is outlined in Fig. 1. If one patient had more than one specimen collected before the age of one year, the earliest specimen was used. Samples collected from 01 January 2015 to 30 December 2017 were enrolled as the discovery cohort, and those collected from 01 January 2018 to 31 October 2020 were enrolled as the validation cohort.

Subjects and grouping

The clinical diagnosis of ALGS was made by standard clinical criteria [3]: the presence of bile duct paucity and at least three major clinical features or at least four of six major clinical features (cholestasis, cardiac murmur, skeletal abnormalities, ocular abnormalities, a characteristic face, and renal abnormalities) in the absence of paucity of bile ducts. Only patients with confirmed *JAG1* pathogenic/likely pathogenic variants were enrolled in this study. The exclusion criteria were as follows: (1) received the Kasai procedure; (2) patients who were alive with their native liver and less than one year old in the last follow-up or lost to follow-up before the age of one year, and (3) samples that were

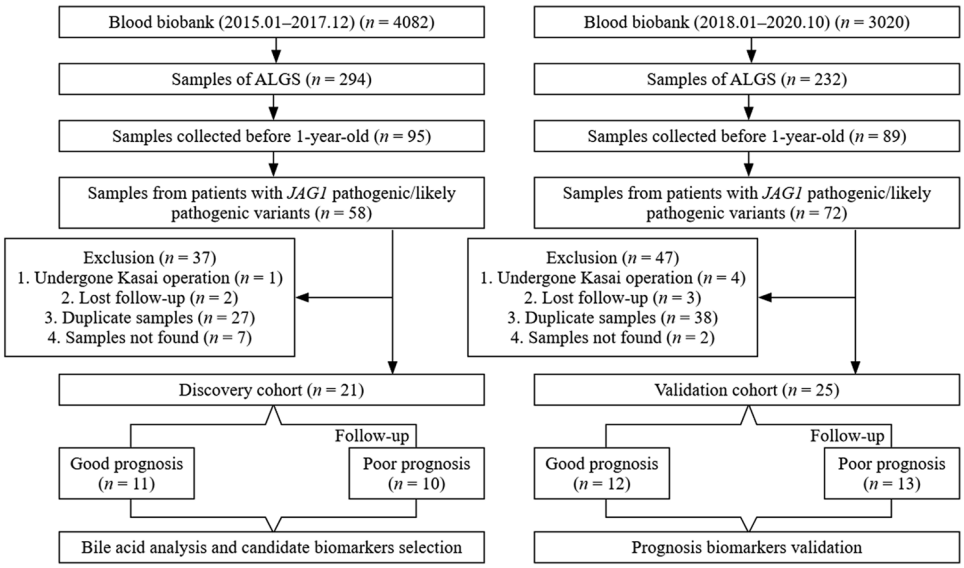

Fig. 1 Flow chart of samples and subjects enrolled in the discovery and validation cohorts (left and right side, respectively)

not available in the biobank. All patients received standard medical care, including ursodeoxycholic acid, supplementation with fat-soluble vitamins, and cholestyramine if needed.

The patient's prognosis was assessed according to both clinical data and liver function tests at the last follow-up, which were collected from the medical electronic record system or the parents of the patients. A good prognosis was defined as patients satisfying both of the following criteria: (1) survival with their native liver, and (2) total bilirubin (TB) < 85.5 μmol/L. Poor prognosis was defined if either of the following events occurred: (1) received liver transplantation or died of liver failure [27], and (2) TB ≥ 85.5 μmol/L.

According to the above criteria, 21 ALGS patients with *JAG1* mutations were enrolled in the discovery cohort. Among them, 11 were grouped as having a good prognosis, including six patients without jaundice and five with mild jaundice at the last follow-up. They were sampled at a median age of 7.8 months with an interquartile of 6.1 months to 10.7 months. The last follow-up was at a median age of three years four months (interquartile one year 10 months to five years five months). The other 10 were grouped as poor prognosis, including two who died before one-year-old, five received liver transplantation at ages of one year five months to two years seven months, and three lived with severe jaundice with the last follow-up at ages four years five months, three years 10 months, and six years two months, respectively. They were sampled at a median age of 7.9 months (interquartile 5.8 months to 11.25 months).

Totally 25 ALGS patients were enrolled in the validation cohort. Among them, 12 were grouped as having a good prognosis with a median sampling age of 4.5 months (interquartile 3.8 months to 4.6 months). Among them, nine patients lived without jaundice, and three lived with mild jaundice at the last follow-up, with a median age of two years seven months and an interquartile range of two years three months to four years. Thirteen were grouped as poor prognosis with a median sampling age at 6.4 months (interquartile 5.2 months to 9.5 months), among which 10 lived with severe jaundice after follow-up to a median age of three years and interquartile two years to four years nine months, one received liver transplantation at six months of age, and two died at the ages of nine months and two years 10 months. The demographic data and follow-up details of these patients are presented in Supplementary Table 1.

Sample collection and specimen preparation

Plasma from ALGS patients in the discovery cohort was separated from EDTA-treated peripheral blood by centrifugation, and serum was collected. The samples were aliquoted, lyophilized, and stored at −80 °C until bile acid analysis. The samples of the validation cohort were from frozen plasma or serum deposited in the biobanks.

Bile acid analysis

Bile acid analysis was performed by ultrahigh-performance liquid chromatography coupled to multiple-monitoring reaction-mass spectrometry (UPLC/MRM-MS) according to the procedures as well as LC and MS operating parameters as previously described [16]. Briefly, bile acids in plasma or serum were extracted with a mixture of methanol/acetonitrile (1:1, v/v), followed by cleanup and enrichment by reversed-phase solid-phase extraction with the use of polymeric Strata-X cartridges (33 μm, 60 mg/1 mL, Phenomenx Inc. CA). Reversed-phase (C18) UPLC/MRM-MS with negative ion detection was used to separate and quantitate bile acids in the samples of the discovery cohort, which was

carried out at the University of Victoria-Genome British Columbia Proteomics Centre. Quantitation of bile acids in the samples of the validation cohort was performed at the Institutes of Biomedical Sciences of Fudan University. In total, 83 bile acids, including primary BAs [cholic acid, (CA); chenodeoxycholic acid (CDCA)] and secondary BAs [deoxycholic acid (DCA); lithocholic acid (LCA); ursodeoxycholic acid (UDCA)], and their metabolites in the classes of unconjugated, glycine-conjugated (glyco-), taurine-conjugated (tauro-), sulfated and glucuronidated BAs, together with a few keto-/diketo-BAs, unconjugated and taurine-conjugated THBAs, were quantified, with the use of their standard substances for the preparation of linearly regressed, internal standard calibration curves. Totally 14 deuterium-labeled bile acids were used as internal standards for accurate quantitation. For the BAs for which none of their isotope-labeling internal standards were available, glyco-CDCA-d4 was used as a common internal standard.

Total bile acids (TBA) are the sum of all detected bile acids. The total sulfated, and glyco- and tauro-bile acids were calculated by adding the concentrations of the corresponding conjugated forms in each category. For example, concentrations of CA 3-sulfate, DCA 3-sulfate, CDCA 3-sulfate, and LCA 3-sulfate were summed as the total of bile acid sulfates. The concentrations of unconjugated and conjugated CA and CDCA and those of unconjugated and conjugated DCA and LCA were summed as the total of primary and secondary bile acids, respectively. The percentages of individual BAs among the total of its category, the molar ratio of secondary BAs to primary BAs, and the molar ratio of total tauro-BAs to total glyco-BAs were also calculated. The molar ratios of conjugated to corresponding unconjugated BAs were used to reflect the specific metabolism processes, including hydroxylation [(GHCA:glyco- chenodeoxycholic acid (GCDCA) and tauro-hyocholic acid (THCA):tauro-chenodeoxycholic acid (TCDCA)], glyco-conjugation [glyco-cholic acid (GCA):CA, GCDCA:CDCA, glycol-ursodeoxycholic acid (GUDCA):UDCA, etc.], tauro-conjugation [tauro-cholic acid (TCA):CA, TCDCA: CDCA, tauro-ursodeoxycholic acid (TUDCA):UDCA, etc.], sulfonation [cholic acid-sulfate (CAS):CA, chenodeoxycholic acid-sulfate (CDCAS):CDCA, lithocholic acid-sulfate (LCAS):LCA, etc.], glucuronidation (CDCA-glu:CDCA), carbon shortening from C24 to C23 BAs (nor-CA:CA, nor-UDCA:UDCA, nor-THBA:THBA) and oxide reduction (7-keto-LCA:LCA) [20].

Statistical analysis

Median and interquartile range (IQR) for quantitative indexes are presented. The Mann-Whitney *U* test was used to determine the difference in the indexes between the two groups. Prognostic biomarkers were selected via receiver operating characteristic (ROC) curve analysis. Youden's index was used to define the optimal cut-off value. Fisher's exact test was used to test the predicted efficacy of biomarkers in the validation cohort. Multivariate Cox regression analysis was conducted to determine whether bile acids were independently associated with native liver survival. Kaplan-Meier curves were used to display survival curves. Graphs were generated using GraphPad Prism (version 8.0, GraphPad Software Inc.). Statistical significance was considered at $P < 0.05$ bilaterally.

Results

Bile acid profiles in the discovery cohort

The concentrations of individual bile acids in the good prognosis versus poor prognosis patients in the discovery cohort are presented in Table 1. Totally 83 bile acids were analyzed for each patient. GHCA in the good prognosis group showed higher concentrations than that in the poor prognosis group (median and IQR: 1168.03 nmol/L, 692.83–1863.72 nmol/L vs. 557.90 nmol/L, 339.18–1002.53 nmol/L, $P = 0.036$), and a similar trend was found in tauro-2β,3α,7α,12α-THBA (91.73 nmol/L, IQR: 52.07–298.87 nmol/L vs. 51.60 nmol/L, IQR: 34.72–69.77 nmol/L, $P = 0.013$). Other polyhydroxylated bile acids, such as THCA ($P = 0.099$), tauro-3α,6α,7α,12α-THBA ($P = 0.061$), and tauro-3α,6β,7α,12α-THBA ($P = 0.099$), also showed trends similar to those of GHCA and tauro-2β,3α,7α,12α-THBA, with borderline significance. No significant difference was observed in the concentrations of other bile acids except 3-oxo-CA.

To explore the overall metabolic process of bile acids, the concentrations of individual bile acids were summed according to their different categories (Supplementary Table 2). The concentration of total tauro-THBAs in the good prognosis group (3607.11 nmol/L, 1851.66–4506.49 nmol/L) was significantly higher than that in the poor prognosis group (1022.25 nmol/L, 749.59–1629.08 nmol/L; $P = 0.001$). No significant differences were observed in other categories, as well as the molar ratios of tauro-BAs to glyco-BAs and the secondary BAs to the primary BAs between these two patient groups.

To analyze in more detail the role of bile acid modification in these two groups of patients, the respective molar ratios of individual bile acids, conjugated versus unconjugated bile acids, and some atypical modifications versus unmodified forms were examined (Table 2). The process of hydroxylation (GHCA:GCDCA, THCA:TCDCA) was significantly enhanced in patients with a good prognosis compared to patients with a poor prognosis ($P = 0.013$ and 0.010, respectively). No significant differences were observed in

Table 1 Blood concentrations of individual bile acids in the discovery cohort of ALGS patients with different prognosis

Bile acids	RT	Median (IQR) nM[a]		AUC (95% CI:	P^b
	(min)	Good prognosis ($n=11$)	Poor prognosis ($n=10$)	lower limit–upper limit)	
CA	14.80	51.48 (22.52, 75.09)	34.92 (29.30, 46.09)	0.550 (0.237–0.863)	0.705
TCA	9.60	21,566 (10,197, 47,081)	48,097 (10,549, 65,579)	0.627 (0.433–0.825)	0.349
GCA	11.90	40,940 (13,059, 53,168)	34,798 (24,146, 72,742)	0.582 (0.263–0.901)	0.557
CAS	7.4	35.06 (12.75, 59.32)	19.29 (10.38, 32.92)	0.659 (0.369–0.945)	0.223
GCAS	4.5	505.6 (72.67, 700.4)	274.5 (69.96, 564.8)	0.591 (0.400–0.791)	0.512
AlloCAS	10.8	3.15 (0.88, 8.94)	2.16 (1.58, 3.55)	0.582 (0.291–0.873)	0.557
GalloCAS	4.4	12.37 (7.67, 52.34)	13.53 (5.20, 23.44)	0.600 (0.319–0.881)	0.468
THCA	5.4	1307 (635.6, 4639)	743.6 (556.9, 1372)	0.718 (0.495–0.941)	0.099
GHCA	6.3	1168 (692.8, 1864)	557.9 (339.2, 1003)	0.773 (0.561–0.985)	0.036
Nor-CA	9.0	95.04 (42.65, 144.2)	61.02 (38.30, 92.43)	0.536 (0.331–0.740)	1.000
3-oxo-CA	10.7	5.52 (0.73, 9.22)	19.34 (12.58, 28.68)	0.836 (0.636–0.972)	0.008
CDCA	18.60	64.54 (49.54, 78.69)	72.13 (52.54, 118.7)	0.577 (0.379–0.775)	0.557
TCDCA	13.00	30,312 (8026, 45,238)	52,417 (22,812, 68,302)	0.655 (0.367–0.946)	0.251
GCDCA	10.3	65,325 (21,265, 120,317)	121,154 (42,077, 137,028)	0.636 (0.352–0.910)	0.314
CDCAG	8.8	286.4 (210.8, 338.8)	114.4 (41.34, 275.5)	0.745 (0.492–0.994)	0.061
CDCAS	9.5	26.88 (18.78, 52.21)	39.69 (19.31, 80.54)	0.568 (0.369–0.767)	0.605
TCDCAS	4.9	10,148 (4402, 16,185)	13,089 (6698, 18,386)	0.618 (0.326–0.911)	0.387
GCDCAS	6.3	8406 (5013, 12,897)	11,668 (6477, 17,445)	0.627 (0.430–0.821)	0.349
DCA	19.10	102.9 (95.62, 114. 8)	92.63 (85.94, 108.2)	0.723 (0.549–0.897)	0.085
GDCAS	6.6	25.16 (18.50, 58.33)	36.06 (2.29, 61.25)	0.509 (0.300–0.711)	1.000
LCA	23.70	0.21 (0.15, 0.41)	0.40 (0.15, 0.79)	0.618 (0.326–0.909)	0.387
GLCA	19.60	6.98 (2.04, 19.15)	10.10 (3.88, 15.59)	0.532 (0.210–0.854)	0.809
LCAS	11.9	3.77 (0.61, 7.33)	3.10 (0.61, 10.69)	0.518 (0.223–0.815)	0.918
GLCAS	8.5	36.21 (25.07, 67.16)	2.68 (2.05, 105.40)	0.618 (0.326–0.910)	0.387
TLCAS	6.4	102.3 (58.05, 244.4)	131.0 (67.31, 294.8)	0.582 (0.261–0.902)	0.557
7-keto-LCA	12.6	4.91 (2.82, 7.75)	4.39 (3.90, 9.26)	0.523 (0.201–0.842)	0.863
UDCA	11.3	1584 (199.8, 5875)	538.9 (109.4, 4090)	0.627 (0.321–0.923)	0.349
TUDCA	6.2	16,330 (6877, 41,259)	24,644 (2282, 51,708)	0.536 (0.331–0.740)	0.809
GUDCA	7.2	34,069 (13,804, 52,817)	55,195 (6483, 81,022)	0.564 (0.364–0.768)	0.654
GUDCAS	4.1	112,603 (71,089, 137,659)	79,968 (26,029, 148,981)	0.582 (0.261–0.902)	0.557
Nor-UDCA	9.1	17.22 (0.15, 33.60)	26.70 (0.15, 31.90)	0.500 (0.297–0.703)	1.000
α-MCA	8.8	0.44 (0.30, 7.68)	0.28 (0.18, 0.95)	0.618 (0.328–0.909)	0.387
λ-MCA	10.1	10.19 (0.15, 12.35)	0.15 (0.15, 12.96)	0.605 (0.401–0.803)	0.426
ω-MCA	8.5	17.74 (6.74, 43.66)	7.10 (2.13, 34.69)	0.700 (0.519–0.879)	0.132
3α,4α,7β,12α-THBA	4.9	9.67 (2.14, 51.63)	5.40 (2.43, 23.71)	0.555 (0.245–0.851)	0.705
Nor-THBA	3.8	44.06 (36.95, 57.24)	37.36 (32.03, 62.58)	0.568 (0.255–0.881)	0.605
Tauro-3α,6α,7α,12α-THBA	3.6	262.6 (31.70, 453.3)	44.09 (19.93, 102.16)	0.745 (0.520–0.971)	0.061
Tauro-3α,6β,7α,12α-THBA	2.8	306.4 (48.47, 651.2)	95.85 (44.24, 180.5)	0.718 (0.485–0.951)	0.099
Tauro-2β,3α,7α,12α-THBA	4.0	91.73 (52.07, 298.9)	53.60 (33.94, 73.46)	0.818 (0.634–1.000)	0.013

RT retention time, *IQR* interquartile range, *AUC* area under the curve, *CI* confidence interval, *CA* cholic acid, *TCA* tauro-cholic acid, *GCA* glyco-cholic acid, *CAS* cholic acid-sulfate, *GCAS* glyco-cholic acid-sulfate, *AlloCAS* allo-cholic acid-sulfate, *GalloCAS* glyco-allocholic acid-sulfate, *THCA* tauro-hyocholic acid, *GHCA* glyco-hyocholic acid, *CDCA* chenodeoxycholic acid, *TCDCA* tauro-chenodeoxycholic acid, *GCDCA* glyco-chenodeoxycholic acid, *CDCAG* chenodeoxycholic acid-glucuronidation, *CDCAS* chenodeoxycholic acid-sulfate, *TCDCAS* tauro-chenodeoxycholic acid-sulfate, *GCDCAS* glyco-chenodeoxycholic acid-sulfate, *DCA* deoxycholic acid, *GDCAS* glyco-deoxycholic acid-sulfate, *LCA* lithocholic acid, *GLCA* glyco-lithocholic acid, *LCAS* lithocholic acid-sulfate, *GLCAS* glyco-lithocholic acid-sulfate, *TLCAS* tauro-lithocholic acid-sulfate, *7-keto-LCA* 7-ketonized lithocholic acid, *UDCA* ursodeoxycholic acid, *TUDCA* tauro-ursodeoxycholic acid, *GUDCA* glyco-ursodeoxycholic acid, *GUDCAS* glyco-ursodeoxycholic acid-sulfate, *Nor-UDCA* 23C-ursodeoxycholic acid, *MCA* muricholic acid, *THBA* tetrahydroxylated bile acid, *Nor-THBA* 23C-tetrahydroxylated bile acid

[a]Only integer parts were displayed in values > 1000, values from 100 to 1000 were rounded to 1 decimal place and 2 decimal places in values < 100. [b]Mann-Whitney *U* test

Table 2 Molar ratios of conjugated vs. unconjugated bile acids, and atypical modifications versus unmodified forms in the discovery cohort

	Molar ratio	Median (IQR)[a]		AUC (95% CI)	P^b
		Good prognosis ($n=11$)	Poor prognosis ($n=10$)		
A	GHCA:GCDCA	0.0236 (0.0092, 0.0405)	0.0065 (0.0034, 0.0183)	0.836 (0.697–0.980)	0.013
	THCA:TCDCA	0.0792 (0.0382, 0.1295)	0.0213 (0.0113, 0.0420)	0.836 (0.696–0.983)	0.010
B	CAS:CA	0.5500 (0.4000, 1.030)	0.5213 (0.3204, 0.9313)	0.573 (0.371–0.773)	0.605
	CDCAS:CDCA	0.5009 (0.3712, 0.8506)	0.5812 (0.4509, 0.7205)	0.518 (0.223–0.816)	0.918
	LCAS:LCA	5.728 (4.001, 26.33)	8.591 (3.253, 21.34)	0.536 (0.331–0.739)	0.809
	GCAS:GCA	0.0092 (0.0047, 0.0249)	0.0059 (0.0042, 0.0083)	0.718 (0.469–0.970)	0.099
	GalloCAS:GalloCA	0.0190 (0.0145, 0.1456)	0.0451 (0.0223, 0.0621)	0.590 (0.400–0.791)	0.512
	GCDCAS:GCDCA	0.1137 (0.0894, 0.2357)	0.1031 (0.0683, 0.1701)	0.609 (0.410–0.809)	0.426
	GLCAS:GLCA	6.252 (1.804, 17.58)	2.249 (0.1657, 17.32)	0.636 (0.359–0.918)	0.314
	GUDCAS:GUDCA	2.522 (1.726, 6.064)	1.073 (0.7374, 2.593)	0.709 (0.530–0.881)	0.114
	TCDCAS:TCDCA	0.3377 (0.2496, 0.5186)	0.2921 (0.1826, 0.4012)	0.627 (0.430–0.821)	0.349
C	TCA:CA	622.9 (268.5, 1411)	1329 (274.7, 1635)	0.618 (0.326–0.910)	0.387
	TCDCA:CDCA	595.5 (175.6, 637.0)	612.3 (271.0, 1017.0)	0.581 (0.260–0.901)	0.557
	TUDCA:UDCA	17.68 (3.790, 52.20)	20.47 (10.06, 139.5)	0.645 (0.369–0.919)	0.251
	TCDCAS:CDCAS	242.3 (110.03, 408.4)	292.1 (115.4, 411.6)	0.518 (0.223–0.816)	0.918
	TLCAS:LCAS	30.19 (16.37, 95.11)	27.92 (13.07, 99.63)	0.509 (0.217–0.799)	1.000
D	GCA:CA	781.8 (540.5, 989.4)	1036 (604.5, 1932)	0.663 (0.481–0.839)	0.223
	GCDCA:CDCA	1012 (503.1, 1175)	1528 (840.3, 1919)	0.645 (0.369–0.919)	0.282
	GalloCA:alloCA	88.26 (57.76, 1506)	103.5 (81.82, 192.5)	0.509 (0.216–0.801)	0.973
	GLCA:LCA	14.96 (9.347, 64.35)	18.77 (10.66, 52.42)	0.500 (0.297–0.793)	1.000
	GUDCA:UDCA	38.29 (3.156, 183.5)	65.29 (28.25, 199.6)	0.645 (0.369–0.919)	0.282
	GCAS:CAS	10.84 (5.863, 21.87)	8.371 (6.454, 20.46)	0.509 (0.213–0.800)	0.973
	GCDCAS:CDCAS	259.9 (156.4, 342.4)	220.5 (133.1, 420.5)	0.509 (0.216–0.801)	1.000
	GLCAS:LCAS	10.47 (1.434, 41.08)	4.210 (0.2201, 51.67)	0.590 (0.311–0.879)	0.512
E	CDCAG:CDCA	5.035 (1.428, 8.654)	1.7607 (0.5234, 3.2535)	0.745 (0.501–0.985)	0.061
F	Nor-CA:CA	1.772 (1.205, 3.580)	2.000 (1.004, 2.4403)	0.555 (0.237–0.863)	0.705
	Nor-UDCA:UDCA	0.0059 (0.0006, 0.0561)	0.0460 (0.0041, 0.3794)	0.727 (0.551–0.902)	0.143
	Nor-THBA:THBA	3.543 (0.7225, 16.60)	3.400 (0.9112, 7.3637)	0.536 (0.331–0.740)	0.809
G	7-keto-LCA:LCA	23.52 (8.275, 43.36)	15.20 (4.910, 37.54)	0.573 (0.371–0.772)	0.605

IQR interquartile range, *AUC* area under the curve, *CI* confidence interval, *GHCA* glyco-hyocholic acid, *GCDCA* glyco-chenodeoxycholic acid, *THCA* tauro-hyocholic acid, *TCDCA* tauro-chenodeoxycholic acid, *CAS* cholic acid-sulfate, *CA* cholic acid, *CDCDS* chenodeoxycholic acid-sulfate, *CDCA* chenodeoxycholic acid, *LCAS* lithocholic acid-sulfate, *LCA* lithocholic acid, *GCAS* glyco-cholic acid-sulfate, *GCA* glyco-cholic acid, *GalloCAS* glyco-allocholic acid-sulfate, *GalloCA* glyco-allocholic acid, *GCDCAS* glyco-chenodeoxycholic acid-sulfate, *GCDCA* glyco-chenodeoxycholic acid, *GLCAS* glyco-lithocholic acid-sulfate, *GLCA* glyco-lithocholic acid, *GUDCAS* glyco-ursodeoxycholic acid-sulfate, *GUDCA* glyco-ursodeoxycholic acid, *TCDCAS* tauro-chenodeoxycholic acid-sulfate, *TCDCA* tauro-chenodeoxycholic acid, *TCA* tauro-cholic acid, *TUDCA* tauro-ursodeoxycholic acid, *UDCA* ursodeoxycholic acid, *TCDCAS* tauro-chenodeoxycholic acid-sulfate, *TLCAS* tauro-lithocholic acid-sulfate, *CDCAG* chenodeoxycholic acid-glucuronidation, *Nor-CA* 23C-cholic acid, *Nor-UDCA* 23C-ursodeoxycholic acid, *Nor-THBA* 23C-tetrahydroxylated bile acid, *THBA* tetrahydroxylated bile acid, *7-keto-LCA* 7-ketonized lithocholic acid. A, hydroxylation; B, sulfation; C, taurine conjugation; D, glycine conjugation; E, glucuronidation; F, 23C bile acid; G, oxidoreduction. [a]Values displayed to 4 significant figures rounded up to 4 decimal places; [b]Mann-Whitney *U* test

the processes of sulfonation, taurine or glycine conjugation, glucuronidation, or oxide reduction between the two groups.

Bile acid profiles in the validation cohort

To determine whether the results observed in the discovery cohort could be reproduced, the same set of bile acids was profiled in a validation cohort of another 25 *JAG1*-variant confirmed patients. The difference in polyhydroxylated bile acids, including GHCA, THCA, and three tauro-THBAs (tauro-3α,6α,7α,12α-THBA, tauro-3α,6β,7α,12α-THBA and tauro-2β,3α,7α,12α-THBA), between the two different prognostic groups was confirmed and even more pronounced in the validation cohort (Table 3). Additionally, the molar ratios of GHCA to GCDCA and THCA to TCDCA as indicators of the bile acid metabolism process via hydroxylation were significantly higher in the good prognosis group than in the poor prognosis group. However, no significant

Table 3 The results of blood concentrations and molar ratios of selected bile acids in the validation cohort

Bile acids	Median (IQR)[a]		AUC	P^b
	Good prognosis ($n=12$)	Poor prognosis ($n=13$)	(95% CI)	
GHCA (nmol/L)	2517.13 (1256.22, 3553.13)	710.42 (355.14, 1719.05)	0.814 (0.603–0.949)	0.008
THCA (nmol/L)	3366.61 (2552.91, 4689.99)	1227.12 (687.99, 1496.65)	0.885 (0.718–0.987)	0.001
3-oxo-CA	0.86 (0.32, 4.38)	1.36 (0.33, 3.48)	0.506 (0.300–0.712)	1.000
Tauro-3α,6α,7α,12α-THBA (nmol/L)	118.14 (67.94, 437.30)	28.23 (0.09, 48.70)	0.904 (0.769–1.000)	< 0.001
Tauro-3α,6β,7α,12α-THBA (nmol/L)	424.33 (184.88, 1227.66)	69.00 (42.12, 104.11)	0.942 (0.827–1.000)	< 0.001
Tauro-2β,3α,7α,12α-THBA (nmol/L)	267.87 (132.75, 303.96)	0.09 (0.09, 36.69)	0.907 (0.752–1.000)	< 0.001
GHCA:GCDCA	0.0177 (0.0129, 0.0261)	0.0036 (0.0020, 0.0110)	0.904 (0.731–1.000)	< 0.001
THCA:TCDCA	0.0843 (0.0512, 0.1145)	0.0194 (0.0112, 0.0594)	0.923 (0.788–0.994)	< 0.001

CA cholic acid, *GHCA* glyco-hyocholic acid, *THCA* tauro-hyocholic acid, *THBA* tetrahydroxylated bile acid, *GCDCA* glyco-chenodeoxycholic acid, *THCA* tauro-hyocholic acid, *TCDCA* tauro-chenodeoxycholic acid, *IQR* interquartile range, *AUC* area under the curve, *CI* confidence interval. [a]Results are rounded to two decimal points. [b]Mann-Whitney *U* test

difference was observed for 3-oxo-CA between the two prognosis groups in the validation cohort.

Selection and validation of Alagille syndrome prognostic biomarkers

We then focused on poly-hydroxylated bile acids to determine if they could be used as biomarkers to predict the outcomes of young (one-year-old or less) ALGS patients. The variables with P value < 0.05 and area under the curve (AUC) > 0.7, both in the discovery and validation cohorts, were included as the candidates (Table 4). Poly-hydroxylated bile acids, GHCA and tauro-2β,3α,7α,12α-THBA, and the molar ratios of GHCA to GCDCA and THCA to TCDCA were initially enrolled.

Optimal cutoffs (GHCA: 607.69 nmol/L, tauro-2β,3α,7α,12α-THBA: 79.88 nmol/L, GHCA:GCDCA: 0.0220, and THCA:TCDCA: 0.0762) were determined using the Youden index in the discovery cohort (Table 4), and these values were applied to predict prognostic outcomes in the validation cohort. The results are shown in Table 5, where tauro-2β,3α,7α,12α-THBA achieved an accuracy of 88.00% (92.31% sensitivity and 83.33% specificity), and the molar ratio of THCA to TCDCA achieved a prediction accuracy of 84.00% (100% sensitivity and 67.67% specificity) in the validation cohort.

Table 4 The receiver operating characteristic curve analysis and the optimal cut-off values of selected candidates prognostic biomarkers in the discovery cohort

Bile acids	Cut-off value	AUC	95% CI		Sensitivity	Specificity	+LR	–LR	P^a
			Lower limit	Upper limit					
GHCA (nmol/L)	607.69	0.773	0.561	0.985	0.909	0.600	2.27	0.15	0.036
THCA (nmol/L)	1033.96	0.718	0.495	0.941	0.727	0.700	2.42	0.39	0.099
Tauro-3α,6α,7α,12α-THBA (nmol/L)	102.84	0.745	0.520	0.971	0.727	0.800	3.64	0.34	0.061
Tauro-3α,6β,7α,12α-THBA (nmol/L)	187.80	0.718	0.485	0.951	0.727	0.800	3.64	0.34	0.099
Tauro2β,3α,7α,12α-THBA (nmol/L)	79.88	0.818	0.634	1.000	0.727	0.900	7.27	0.30	0.013
GHCA:GCDCA	0.0220	0.818	0.638	0.999	0.545	1.000	0.54	0.00	0.013
THCA:TCDCA	0.0762	0.827	0.651	1.000	0.545	1.000	0.54	0.00	0.010

GHCA glyco-hyocholic acid, *THCA* tauro-hyocholic acid, *THBA* tetrahydroxylated bile acid, *GCDCA* glyco-chenodeoxycholic acid, *TCDCA* tauro-chenodeoxycholic acid, *AUC* area under the curve, *CI* confidence interval, *+LR* positive likelihood ratio, *–LR* negative likelihood ratio. [a]Mann-Whitney U test

Univariable and multivariate Cox proportional hazard models

Next, statistical tests of the poly-hydroxylated bile acids (GHCA, THCA, and three tauro-THBAs) and the derivative indexes (GHCA:GCDCA and THCA:TCDCA) by Cox proportional hazard model analysis were used to assess their associations with native liver survivability in the two cohorts using the optimal cutoff values determined above (Table 4). Univariable analysis showed that the concentrations of GHCA, THCA, tauro-3α,6β,7α,12α-THBA, tauro-2β,3α,7α,12α-THBA, and the ratio of THCA to TCDCA affected native liver survival (Table 6 and Fig. 2), while multivariable analysis indicated that the

Table 5 The prognostic efficiency of the candidate indices in the validation cohort

Criterion	Good prognosis ($n=12$)	Poor prognosis ($n=13$)	P^a	$\chi 2$	Sensitivity (%)	Specificity (%)	Correct prediction rate (%)	+LR	–LR
GHCA < 607.69 (nmol/L)	0/12	6/7	0.015	7.29	46.15%	100%	72%	0.00	0.54
Tauro-2β,3α,7α,12α-THBA < 79.88 (nmol/L)	2/10	12/1	< 0.001	14.49	92.31%	83.33%	88%	5.54	0.09
GHCA:GCDCA < 0.0220	8/4	13/0	0.039	5.16	100%	33.33%	68%	1.49	0.00
THCA:TCDCA < 0.0762	4/8	13/0	< 0.001	12.75	100%	67.67%	84%	3.03	0.00

GHCA glyco-hyocholic acid, *THBA* tetrahydroxylated bile acid, *GCDCA* glyco-chenodeoxycholic acid, *THCA* tauro-hyocholic acid, *TCDCA* tauro-chenodeoxycholic acid, *+LR* positive likelihood ratio, *–LR* negative likelihood ratio. [a]Fisher's exact test

Table 6 Univariable and multivariable Cox proportional analysis of the hydroxylated bile acids associated with a reduced native liver survival rate

Bile acids criterion	Univariable analysis		Multivariable analysis	
	Hazard Ratio (95% CI)	P^a	Hazard Ratio (95% CI)	P^a
GHCA < 607.69 (nmol/L)	41.85 (7.91–221.6)	< 0.001	13.03 (2.662, 63.753)	0.002
THCA < 1033.96 (nmol/L)	4.66 (1.253–17.33)	0.042	–	0.699
Tauro-3α,6α,7α,12α-THBA < 102.84 (nmol/L)	3.51 (0.96–12.78)	0.089	–	–
Tauro-3α,6β,7α,12α-THBA < 187.80 (nmol/L)	4.72 (1.34–16.68)	0.028	–	0.331
Tauro-2β,3α,7α,12α-THBA < 79.88 (nmol/L)	7.04 (1.99–24.84)	0.0035	–	0.082
GHCA:GCDCA < 0.0220	3.948 (0.8396–18.56)	0.0821	–	–
THCA:TCDCA < 0.0762	4.6598 (1.13–19.21)	0.0332	–	0.228

GHCA glyco-hyocholic acid, *THCA* tauro-hyocholic acid, *GCDCA* glyco-chenodeoxycholic acid, *TCDCA* tauro-chenodeoxycholic acid, *THBA* tetrahydroxylated bile acids, *CI* confidience interval. *log-rank test, –not available

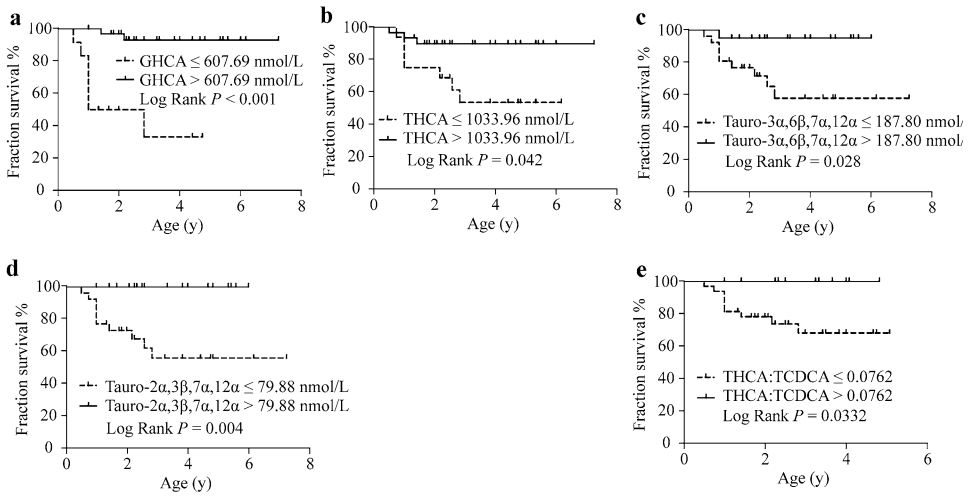

Fig. 2 Kaplan-Meier curves. Increased native survival of the ALGS patients is observed with a blood level of **a** GHCA > 607.69 nmol/L; **b** THCA > 1033.96 nmol/L; **c** tauro-3α,6β,7α,12α-THBA > 187.80 nmol/L; **d** tauro-2β,3α,7α,12α-THBA > 79.88 nmol/L. **e** THCA: TCDCA > 0.0762. *GHCA* glyco-hyocholic acid, *THCA* tetrahydroxylated bile acid, *THBA* tetrahydroxylated bile acids, *TCDCA* tauro-chenodeoxycholic acid

GHCA concentration was the single independent factor influencing native liver survival [hazard ratio: 13.03, 95% confidence interval (CI) 2.662–63.753, $P = 0.002$] (Table 6). The ALGS patients with blood GHCA concentrations lower than 607.69 nmol/L had a significantly higher death and/or transplantation rate (7/13 died or received liver transplantation at a median age of one year) than patients with GHCA concentrations higher than 607.69 nmol/L (2/33 received liver transplantation at one year five months old and two years two months old, respectively).

Discussion

To our knowledge, no in-depth bile acid profiling in ALGS patients has been reported previously. The present study focused on analyzing the profiles of bile acids, especially poly-hydroxylated bile acids, in ALGS patients with different clinical outcomes to explore the potential liver prognostic indicators of the disease. We took advantage of a relatively large cohort of patients with follow-up data and a comprehensive panel of different bile acids, including multiple bile acids in the classes of unconjugated THBAs and tauro-THBAs, and used a well-developed bile acid profiling method [16]. We discovered that some poly-hydroxylated bile acids could serve as excellent prognostic biomarkers, and enhanced bile acid poly-hydroxylation may have the ability to predict a good prognosis of ALGS.

Increased hydroxylation makes the bile acid pool more hydrophilic, which is believed to be a common compensatory response observed in animals and patients experiencing cholestatic stress [19]. THBAs, bile acids with four hydroxyl groups, and MCA, bile acids with three hydroxyl groups, in their molecular structures, have been found to be greatly elevated in *Bsep*$^{-/-}$ mice, and the expression of high levels of such bile acids prevented the progressive liver pathology associated with the *Mdr2*$^{-/-}$ mutation [25]. Taurine conjugates are the major form of bile acid conjugation in mice [16, 28] and are often elevated in cholestatic human patients [20, 26, 29, 30]. We successfully quantified six new synthetic tauro-THBAs in this study. Consistent with the previous findings that the levels of tauro-THBAs, THCA, and GHCA were increased in patients with *ABCB11* deficiency and patients with undiagnosed cholestasis [20], we found that the levels of tauro- or glyco-conjugated polyhydroxylated bile acids in ALGS patients were also increased (compared to those in healthy controls determined by the same methodology in ref 20). The poly-hydroxylated bile acids in ALGS were mainly in tauro- or glyco- conjugated forms rather than unconjugated forms (Supplementary Table 2). More importantly, we observed and verified that higher blood levels of tauro-2β,3α,7α,12α-THBA and GHCA are associated with a better liver and overall prognosis of patients. Both conjugation and hydroxylation are common pathways of bile acid metabolism for increased hydrophilicity and cellular detoxification of hydrophobic bile acids [15]. However, in this study, we found no difference in the molar ratios of bile acid conjugation, such as glyco- to tauro-conjugation or sulfonation, in the ALGS patients associated with outcomes. This suggests that the processes of glyco-, tauro-conjugation, and sulfonation may not be involved in the differentiation of cholestatic responses in ALGS, as often seen in other forms of cholestasis in humans [20, 26, 29]. However, we observed and verified that the molar ratios GHCA to GCDCA and THCA to TCDCA were associated with different prognosis outcomes in this study, indicating that the enhanced bile acid poly-hydroxylation in ALGS patients may contribute to better clinical outcomes and survivability, which was consistent with the results in a cholestasis mouse model by Wang et al. [25]. It is therefore assumed that the high concentration of bile acids in ALGS patients due to cholestasis induces bile acid detoxification by producing poly-hydroxylated bile acids, which are more hydrophilic and less cytotoxic than the usual bile acids found in the control population [19].

One limitation of this study is that we were not able to compare the mRNA expression profiles of hydroxylases in the liver tissue of ALGS patients with different outcomes, as we did not have liver tissues from patients with a good prognosis. In *Mdr 2*$^{-/-}$ and *Bsep*$^{-/-}$ mice, it was observed that the process of hydroxylation is enhanced by the up-regulation of hydroxylases [25]. Meanwhile, more studies are warranted to explore whether poly-hydroxylated bile acids can be used as prognostic biomarkers for other forms of cholestasis or cholestatic liver diseases, especially the more prevalent entities, primary biliary cholangitis or primary sclerosing cholangitis, to extend the use of these biomarkers.

The current study has clinical relevance. The presentations and survivability of ALGS patients vary widely [1]. Some severely affected patients require liver transplantation, while others survive and even thrive with the native liver [6]. Unavoidably, some patients die while waiting for a suitable donor. An accurate prognostic biomarker(s) could help to triage patients to more appropriate treatments. The finding that lower levels of blood poly-hydroxylated bile acids indicated poor prognosis with high rates of mortality or liver transplantation implied that more aggressive treatment and more comprehensive management could be clinically applied. The current findings provide some leads for future medical therapeutic development of ALGS. They raise the possibility that enhancing poly-hydroxylation of bile acids might be an effective therapeutic target for patients with cholestasis.

In conclusion, the findings from this study indicate that the blood level of two poly-hydroxylated bile acids as liver prognostic biomarkers of ALGS patients: tauro-2β,3α,7α,12α-THBA in ALGS patients before one year of age could be an excellent prognostic biomarker and that GHCA can predict native liver survival of such patients. The increased polyhydroxylated bile acids and enhanced hydroxylation process associated with good clinical outcomes may point to a potential therapeutic target.

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Data availability statement All data generated or analyzed during this study are included in this published article and its supplementary information files. And the primary data would be available on request from the authors.

Declarations

Conflict of interest No financial or non-financial benefits have been received or will be received from any party related directly or indirectly to the subject of this article.

Ethical approval The study was approved by the Ethics Committee of Children's Hospital of Fudan University (Shanghai, China) (No. 2017-99) following the ethical standards of the institutional committee on human experimentation and with the Helsinki Declaration of 1964, as revised in 2000. Informed consent to participate in the study has been obtained from participants or their parent or legal guardian in the case of children under 16.

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