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The value of blood and urine metabolomics in differential diagnosis of cholestasis in infants

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Abstract

Background Early detection of biliary atresia (BA) is a great challenge providing the main useful way to improve its clinical consequence. Promising metabolomics provides an effective method for determining innovative biomarkers and biochemical ways for improving early diagnosis. This study aimed to determine the benefit of serum and urinary potential bile acid metabolites in the differentiation of BA from non-biliary atresia (non-BA) cases using tandem mass spectrometry (MS/MS). Fourteen bile acids metabolites were measured quantitively by MS/MS in serum and urine samples from 102 cholestatic infants and 102 control infants, in addition to the assay of the total serum bile acid enzymatically.

Results After the diagnostic clinical and laboratory workflow, cholestatic infants were divided into BA (37 infants) and non-BA (65 infants) subgroups. Remarkably on analysis of serum individual bile acid concentrations, there were significant differences between cholestatic BA and non-BA regarding serum (glycocenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA), taurocholic acid (TCA), and GCDCA/chenodeoxycholic acid (CDCA) ratio) ($p < 0.001$, for all), while there was no significant difference between the two groups regarding serum level of (cholic acid (CA), glycocholic (GCA), or TCDCA/CDCA ratio). There were no significant differences in either the urinary individual bile acids or urinary primary bile acids (conjugated or unconjugated) between BA and non-BA. Further principal component analysis (PCA) analysis was done and receiver operating characteristic (ROC) analysis was performed using score plots of the positive factors in the first two principal components PC1 (CA, GCA, GCDCA, TCA, TCDCA) and PC2 (CA, CDCA, lithocholic (LCA), ursodeoxycholic acid (UDCA)) for establishing the differences between the two diseased groups and revealed that the area under the curve (AUC) for PC1 was (0.770) higher than AUC for PC2 (0.583) indicating that the positive components of PC1 may be potential biomarkers for differentiation between the two cholestatic groups.

Conclusions Metabolomics of serum bile acid levels using tandem mass spectrometry might change the paradigm differentiating BA from non-BA saving patients from unnecessary invasive procedures.

Keywords Biliary atresia, Liquid chromatography-mass spectrometry, Tandem mass spectrometry, Bile acid profile, Metabolites

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Introduction

Neonatal cholestasis (NC) is a common neonatal hepatobiliary disorder occurring within the first three months of life [1]. Biliary atresia (BA) is considered the major reason of NC occurring in (35–41%) of the cases followed by progressive familial intrahepatic cholestasis (PFIC) (10%), preterm neonates (10%), metabolic and endocrinological conditions (9–17%), Alagille syndrome (AGS) (2–6%), infectious diseases (1–9%), mitochondriopathy (2%), biliary sludge (2%), and, lastly, idiopathic causes (13–30%) [2].

BA is a life-threatening disease that is characterized by a rapidly developing fibro-obliteration of the bile ducts resulting in liver failure [3]. It is considered the commonest cause of end-stage liver disease in infancy [4] and the main indication for liver transplantation during childhood [5]. It is a great challenge to differentiate it from other causes of cholestasis and obstructive cholangiopathy due to shared clinical, biochemical, imaging, and histopathological features [6].

Despite being the golden standard of the diagnosis of BA, intraoperative cholangiogram (IOC) is a procedure that can clearly visualize the biliary tree, confirming the diagnosis of BA, but it is an invasive and undesirable procedure and could considerably increase morbidity. Furthermore, the diagnosis should be done as early as possible to facilitate timely Kasai portoenterostomy (KPE) before 3 months of life to improve native liver survival and decrease the need for liver transplantation during childhood [7]. So, we are in urgent need to identify a less invasive and non-operative technique to rule out BA without delay and avoid an unnecessary operation or liver biopsy [8].

Bile acids are the end-products of cholesterol catabolism in the liver [9, 10]. Conjugated primary bile acids are synthesized and secreted into the bile where deconjugation and conversion into secondary bile acids occur by intestinal bacteria [11], then they undergo enterohepatic circulation as most bile acids are reabsorbed with a trace amount lost in feces or spilled out into the systemic circulation and urine [12].

The importance of bile acids involved in physiological functions, their role in pathological processes [13], and their potential pharmacological uses have sparked an increased interest in deciphering their patterns in various biological matrices [14]. Changes in bile acid profiles can be exploited as disease biomarkers [15, 16], hence complete bile acid profiling, particularly those detected at relatively low concentrations in pathological settings, necessitates precise, and sensitive approaches [17]. With advances such as mass spectrometer-dependent metabolomics, it is possible to identify and quantify in vivo metabolites with molecular mass <1.5 KDa and so develop a profile of biomarkers for certain diseases or diseases [18]. Compared with traditional diagnostic methods, even small changes in metabolites can help to detect early pathologic changes more sensitively [19].

The metabolic profiling of bile acids varies in humans of different ages and different disease states [20], both gastrointestinal and non-gastrointestinal diseases [21, 22]. As bile acids are the major constituents of bile, their excretion is disrupted in BA with a decrease in luminal bile acid concentration and less generation of deconjugated and secondary bile acids that results in alterations in the enterohepatic circulation and disruption in total bile acid pool [8].

Thus, the aim of this study was to evaluate the potential value of the intermediates involved in bile acid metabolism (bile acid profiles) in the differentiation between BA and non-BA cholestasis in infants.

Methods

The institutional research board of the National Liver Institute, Menoufia University, approved the study (00134/2018 INTM), and written informed consent was obtained from participants' parents or guardians. A case-control study was conducted on (204) infants, distributed as (102) cholestatic infants, aged from 1 to 365 days, and (102) matched control infants aged from 5 to 365 days. The cholestatic infants were selected from the inpatient wards, pediatric hepatology, gastroenterology, and nutrition department, National Liver Institute Hospital, Menoufia University, in the period from March 2019 to March 2020, using a nonprobability convenience sampling method. The control infants were recruited from Menoufia University Hospitals, complaining of other diseases such as chest infection or gastroenteritis. Cholestasis in children was defined as a conjugated bilirubin level of more than 1 mg/dL with a total bilirubin level of less than 5 mg/dL or when the conjugated bilirubin is more than 20% of the total bilirubin that is more than 5 mg/dL [23].

First, history taking and physical examination of all patients were conducted carefully, and their data were collected retrospectively from electronic records including etiologic work-up of cholestasis hepatitis B surface antigen, hepatitis C virus antibody, TORCH screening (Toxoplasma, rubella, herpes simplex virus (HSV) type 1 HSV type 2), and cytomegalovirus (CMV) immunoglobulin M and immunoglobulin) G) and abdominal ultrasound in all diseased cases for differential diagnosis. Some cases underwent specifically selected investigations according to their provisional diagnoses, such as CMV polymerase chain reaction, succinylacetone in urine, galactose and galactose-1-phosphate in the blood, magnetic resonance cholangiopancreatography (MRCP), and

hepatobiliary scintigraphy (HBS). Liver tissue biopsies were taken from suspected cases of cholestasis as part of the diagnostic work except for cases with infection, Down syndrome, cases with endocrinal causes, Arthrogryposis–renal dysfunction–cholestasis (ARC) syndrome, acute liver failure, and hepatic focal lesion. From all, 69 cases underwent liver biopsy. Biliary obstruction was confirmed in 39 cholestatic infants. Then, they underwent IOC and 37 cases were diagnosed as biliary atresia and a Kassi operation was done. Two cases of them had a patent biliary tract that exclude the diagnosis of biliary atresia. Exclusion criteria: (i) cholestatic children with other comorbidities like diabetes mellitus or viral hepatitis and (ii) cases that underwent treatment.

Sample collection

Blood and urine samples were collected from all subjects included in this study at the same time in the early morning after fasting for 3 h. Six milliliters of the venous blood was divided as follows: 3ml was added to the plain vacationer tube, was left 15 min for coagulation, and centrifuged at 3000 rpm for 5 min, and then, the sera were aliquoted into 2 vials; one was used for measurement of liver and kidney function tests using RX Daytona plus, clinical chemistry analyzer (Randox Laboratories Limited, UK), and total bile acids using auto chemistry analyzer CDT240 (Diasystem Scandinavia AB, Sweden). The other vial was stored at −80 until analysis of bile acid metabolites by MS/MS (ACQUITY UPLC/MS/MS. Waters Corporation, Milford MA, USA). Two milliliters was collected into an EDTA tube for a complete blood count using Coulter Counter T660 (Coulter Electronics, Hialeah, Fl, USA). The remaining 1 ml was aliquoted into a sodium citrate tube for measurement of prothrombin time. Urine samples were collected from patients in special sterile plastic tubes and then evacuated in a special container without the addition of any preservatives and stored immediately at −80°C until analysis.

Chemicals and reagents

Bile acid standards

Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycoursodeoxycholic acid (GUDCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), tauroursodeoxycholic acid (TUDCA), and taurolithocholic acid (TLCA) were purchased from Sigma Chemical Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Methanol, acetonitrile, and formic acid were of HPLC grade and purchased from Fisher Scientific (Loughborough, UK). HPLC grade water was obtained from Millipore pure water purification system (Bransteasd Diamond RO model D12671) (USA).

Sample preparation and assay method

Both blood and urine samples were prepared according to Sugita et al. [24] method with some modifications. First, 100 µL of serum/urine was added to 400-µL ice-cold methanol to precipitate proteins and vortexed,

Table 1 Laboratory and diagnostic workflow for differential diagnosis between biliary atresia and non-biliary atresia patients

Work-up test	Biliary atresia		Non-biliary atresia		*P* Value
Acholic stool (%)	36/37	97.3%	32/65	35.4%	**<0.001***
Total bilirubin (mg/dL)	10.59 ± 5.64		11.27 ± 6.33		0.549
Direct bilirubin (mg/dL)	7.79 ± 4.72		7.85 ± 4.54		0.661
GGT (IU/L)	938.14± 635.60		426.26 ± 603.97		**<0.001***
Serum total bile acids (µMol/L)	132.08 ± 75.26		109.32 ± 70.69		0.158
CMV IgM					
Positive	15	40.5%	16	24.6%	0.093
Negative	22	59.5%	49	75.4%	
CMV IgG					
Positive	30	81.1%	52	80%	0.895
Negative	7	18.9%	13	20%	
Ultrasound (%)					
Small, contracted, or non-visualized gallbladder (%)	36/37	97.3%	22/65	31.9%	**<0.001***
Liver biopsy (%)	37/37	100%	32/65	49.23 %	
Intraoperative cholangiogram (%)	37/37	100%	2/65	3.076%	**<0.001***

GGT Gamma glutamyl transaminase, *CMV IgM* cytomegalovirus immunoglobulin M, *CMV IgG* cytomegalovirus immunoglobulin G

Table 2 Comparison between the pathological findings in the two cholestatic groups (biliary atresia and non-biliary)

	Biliary atresia (n= 37)		Non-biliary atresia (n = 32)		χ^2	p
	No.	%	No.	%		
Visible bile plug						
Absent	1	(2.7)	14	(43.8)	29.507*	**<0.001***
Bile duct	0	(0.0)	3	(9.4)		
Canalicular	10	(27.0)	10	(31.3)		
Bile duct and canalicular	26	(70.3)	5	(15.6)		
Portal tract edema						
Absent	4	(10.8)	25	(78.1)	31.912*	**<0.001***
Present	33	(89.2)	7	(21.9)		
Portal tract fibrosis						
Absent or fibrous expansion of some portal area	3	(8.1)	19	(59.4)	34.232*	**<0.001***
Fibrous expansion of most portal area	7	(18.9)	11	(34.4)		
Focal portal to portal bridging	16	(43.2)	2	(6.3)		
Marked bridging	11	(29.7)	0	(0.0)		
Portal tract inflammation						
Absent	0	(0.0)	6	(18.8)	10.247*	**^{MC}p=0.004***
Mild	15	(40.5)	16	(50.0)		
Moderate	22	(59.5)	10	(31.3)		
Periductular neutrophils						
Absent	3	(8.1)	19	(59.4)	24.709*	**<0.001***
Mild	22	(59.5)	12	(37.5)		
Marked	12	(32.4)	1	(3.1)		
Ductular proliferation						
None	1	(2.7)	12	(37.5)	41.999*	**<0.001***
Focal	5	(13.3)	18	(56.3)		
Generalized	31	(83.8)	2	(6.3)		
Mononuclear inflammatory cells in duct						
Absent	3	(8.1)	21	(65.6)	25.868*	**<0.001***
Mild	12	(59.5)	9	(28.1)		
Multiple	222	(32.4)	2	(6.3)		
Hepatocytes swelling						
Absent	17	(45.9)	11	(34.4)	9.422*	**0.009***
Less than 50%	18	(48.6)	11	(34.4)		
More than 50%	2	(5.4)	10	(31.3)		
Pseudo-rosette formation						
Absent	2	(5.4)	11	(34.4)	14.930*	**^{MC}p=0.001***
Present	34	(91.9)	16	(50.0)		
Prominent	1	(2.7)	5	(15.6)		
Hepatocyte multinucleated giant cells						
Absent	22	(59.5)	9	(28.1)	12.464*	**0.002***
Present	13	(35.1)	11	(34.4)		
Prominent	2	(5.4)	12	(37.5)		
Extramedullary hematopoiesis						
Absent	31	(83.8)	19	(59.4)	6.811*	**^{MC}p=0.018***
Present	6	(16.2)	4	(12.5)		
Extensive	0	(0.0)	9	(28.1)		
Steatosis						
Absent	36	(97.3)	28	(87.5)	2.451	^{FE}p=0.175
Present	1	(2.7)	4	(12.5)		
Lobular necroinflammation						
Absent	330	(81.1)	18	(56.3)	5.082	**^{MC}p=0.077**
Few	5	(13.5)	11	(34.4)		
Many	2	(5.4)	3	(9.4)		

FE Fisher's exact, *MC* Monte Carlo

then centrifuged at 13500 rpm for 15 min. The resulting supernatant was separated in clean Eppendorf and centrifuged again at 13,500 rpm for another 15 min. Finally, 50 µL of the supernatant was mixed with 100 µL water/formic acid solution and injected into (ACQUITY UPLC H-Class. Waters corporation, Milford MA, USA) using ACQUITY UPLC BEH C18, 1.7 µm, 2.1×50mm, column (Waters), at 50°C and flow rate of 0.28 mL/min. Mobile phase A was water/formic acid (1000: 1, v/v), and mobile phase B was 100% acetonitrile. The samples were eluted with 80% mobile phase A and 20% mobile phase B for an initial 2.1 min after injection, then with a linear gradient of mobile phase B of 20 to 30% over 5.2 min, followed by mobile phase B at 80% over 8 min, which was held for 0.5 min. The column was equilibrated with 80% mobile phase A for 2 min before the injection of the next sample. The injection volume of the sample was 5 µL, and the mass spectrometer was operated on electrospray negative mode using the multiple reaction monitoring (MRM). Data analysis was performed using Target Lynx applications manager software ver. 4.1 (waters) to obtain the calibration equations and the quantitative concentration of each bile acid in the samples. The validation range was from 0 to 40 µmol/L.

Pathological assessment

The liver specimens were fixed in 10% buffered formaldehyde paraffin-embedded and stained with the routine hematoxylin and eosin stain. Ancillary special stains, Masson's trichrome stain, and Perls' stains were done to assess fibrosis and iron deposition, respectively. Liver biopsies were assessed by an independent hepatopathologist for adequacy of biopsy. Each liver tissue was assessed according to Russo et al. [25] scoring system for the presence of the followings: visible bile plugs, portal tract edema, portal tract inflammation, bile duct proliferation, and periductal neutrophilic infiltration. Lobular necroinflammation, giant cell transformation, hepatocyte swelling, extramedullary hematopoiesis, canalicular cholestasis, and fatty infiltration were also assessed.

Statistical analysis

Data analysis was performed using SPSS software for Windows version 20.0 (Armonk, NY: IBM Corp). Differences between patients and controls were evaluated using Student's unpaired *t* test (for continuous variables) and the chi-square (χ^2) test (for categorical variables). The Mann–Whitney *U* test is applied for statistical comparison between two sets of data if one or both have a skewed distribution. Comparison between more than 2

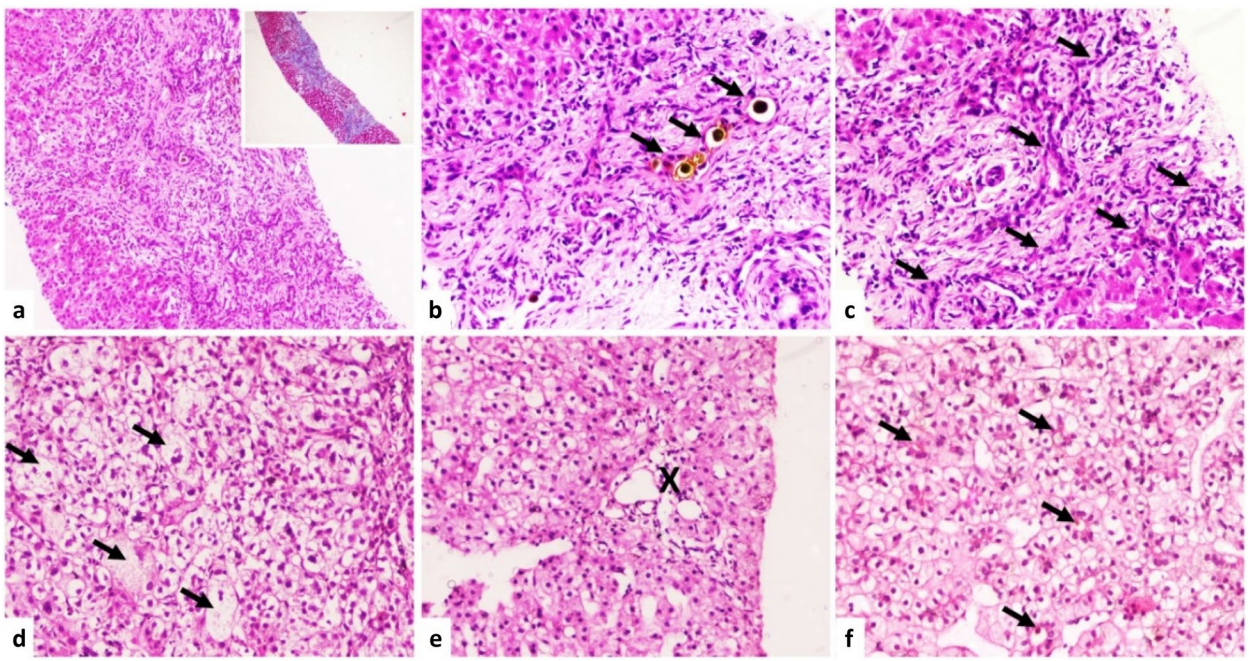

Fig. 1 Histopathological characteristics of biliary atresia versus non-biliary atresia cholestatic diseases. **a** A case of biliary atresia showing marked portal tract expansion by edema (H&E 100×) and fibrosis (incite, Masson trichrome 40×). **b** A case of biliary atresia showing pathognomic intraluminal bile plugs (arrows) (H&E 200×). **c** A case of biliary atresia showing bile ductular proliferation (arrows) (H&E 200×). **d** A case of giant cell hepatitis showing numerous giant cell transformations (arrows) (H&E 200×). **e** A case of Alagille syndrome showing lacks of the main bile duct branch, not accompanying the artery (X mark) (H&E 200×). **f** A case of progressive familial intrahepatic cholestasis showing prominent hepatic rosetting with cholestatic rosettes formation (arrows) (H&E 200×)

patient groups for non-parametric data is done using the Kruskall-Wallis test. Fisher's exact and Monte Carlo are correct for chi-square when more than 20% of the cells have an expected count of less than 5. PCA was used as a multivariate technique for analysis in metabolomics to detect the metabolites that augment the potential of the metabolic biomarkers analyzed in the biological fluids. ROC curves were constructed based on the data of PCA for differentiation between BA and non-BA infants.

Results

The patient's data were retrospectively evaluated for their ability to distinguish BA from other causes of cholestasis and the laboratory and diagnostic workflow were performed (Table 1), which revealed that there was no significant difference between the cholestatic BA infants and the cholestatic non-BA, regarding the serum total bile acids and total bilirubin while, there was a highly significant difference regarding GGT (p <0.001). On ultrasound examination, 36 cases in the BA group showed small, contracted, or nonvisualized gallbladder versus 32 cases of cholestatic non-BA (p <0.001). Bile duct and canalicular plugs, periductal neutrophilic infiltrate, ductular proliferation, and mononuclear infiltrate in the duct were significantly observed in BA cases compared to non-BA atresia cases (p <0.001, for all). The presence of significant portal tract edema, marked fibrosis, and inflammation was significantly found in BA cases compared to non-BA atresia cases ($p < 0.001$, $p < 0.001$, and $p = 0.004$, respectively). On the other hand, hepatocyte swelling, prominent pseudo-rosette formation, hepatocyte multinucleated giant cells, and extramedullary hematopoiesis

Table 3 Comparison between the two cholestatic groups (biliary atresia and non-biliary) and control regarding primary and secondary bile acids in conjugated and unconjugated forms in serum and urine

		Biliary atresia ($n = 37$)	Non-biliary atresia ($n = 65$)	Control ($n = 102$)	*H*	*p*
Serum	**Primary unconjugated**					
	Min.–Max.	0.01–0.39	0.0–2.21	0.0–1.20	6.671*	**<0.036***
	Mean ± SD.	0.09 ± 0.15	0.28 ± 0.41	0.28[a] ± 0.21		
	Median (IQR)	0.16 (0.01–0.13)	0.13 (0.02–0.34)	0.25 (0.15–0.30)		
	Primary conjugated					
	Min.–Max.	31.60–418.70	0.35–495.0	0.33–10.43	135.435*	**<0.001***
	Mean ± SD.	182.02 ± 103.32	127.93 ± 109.95	3.03[ab] ± 2.29		
	Median (IQR)	146.0 (97.56–141.85)	97.90 (51.40–160.7)	2.85 (1.09–4.0)		
	Secondary unconjugated					
	Min. – Max.	0.0–0.51	0.0–1.80	0.0–0.60	0.732	0.694
	Mean ± SD.	0.19 ± 0.20	0.20 ± 0.32	0.18 ± 0.13		
	Median (IQR)	0.12 (0.01–0.30)	0. 2 (0.01–0.35)	0.14 (0.09–0.24)		
	Secondary conjugated					
	Min.–Max.	0. 33–6.14	0.05–16.23	0.19–3.03	94.794*	**<0.001***
	Mean ± SD.	2.69 ± 1.7	4.13[a] ± 3.26	0.80[ab] ± 0.66		
	Median (IQR)	2.16 (0.4–2.98)	3. 13 (0.05–4.43)	0.49 (0.19–0.55)		
Urinary	**Primary unconjugated**					
	Min.–Max.	0.0–0.21	0.0–0.81	0.0–1.03	0.976	0.614
	Mean ± SD.	0.04 ± 0.06	0.07 ± 0.17	0.07 ± 0.17		
	Median (IQR)	0.0 (0.0–0.04)	0.0 (0.0–0.06)	0.01 (0.0–0.04)		
	Primary conjugated					
	Min.–Max.	1.81–188.0	0.02–137.0	0.0–6.90	123.127*	**<0.001***
	Mean ± SD.	51.44 ± 49.69	29.27 ± 29.92	1.20[ab] ± 1.54		
	Median (IQR)	37.40 (12.70–76.80)	20.02 (6.26–43.0)	0.53 (0.10–2.20)		
	Secondary unconjugated					
	Min.–Max.	0.0–0.19	0.0–0.34	0.0–0.36	0.289	0.865
	Mean ± SD.	0.03 ± 0.06	0.06 ± 0.09	0.04 ± 0.08		
	Median (IQR)	0.0 (0.0–0.04)	0.0 (0.0–0.10)	0.01 (0.0–0.03)		
	Secondary conjugated					
	Min.–Max.	0.0–2.35	0.0–8.77	0.0–1.02	55.367*	**<0.001***
	Mean ± SD.	0.63 ± 0.58	1.14 ± 1.44 [a]	0.14[ab] ± 0.21		
	Median (IQR)	0.4 (0. 0–0.95)	0.65 (0.01–1.39)	0.02 (0.0–0.24)		

were significantly observed in non-BA compared to BA cases ($p = 0.009$, $p < 0.001$, p 0.002, and $p = 0.018$, respectively) (Table 2) (Fig. 1).

Remarkably, two non-BA infants underwent intraoperative cholangiogram despite their preoperative workup. Together, these data highlighted the challenge of differentiating BA from non-BA without operative intervention.

Bile acid profiling in cholestatic BA, cholestatic non-BA infants, and healthy control

The serum and urinary primary conjugated bile acids were significantly elevated ($p < 0.001$) in both BA and non-BA groups compared with control samples (182.25 ± 103.4 and 127.93 ± 109.95 versus 3.30 ± 2.36 µmol/L and 51.44 ± 49.69 and 29.27 ± 29.92 versus1.20 ± 1.54 µmol/L). There was a significant difference between the cholestatic biliary atresia subgroup and control regarding serum primary unconjugated bile acids ($p < 0.036$). Furthermore, there were significant differences between the cholestatic BA subgroup and the cholestatic non-BA subgroup regarding serum and urinary secondary conjugated bile acids ($p < 0.001$ for both) with no difference in serum or urinary levels of primary (unconjugated and conjugated) and secondary unconjugated bile acids (Table 3).

On analysis of individual bile acid concentrations, there were significant differences between cholestatic BA and non-BA regarding serum levels of (GCDCA, TCDCA, TCA, and GCDCA/CDCA ratio), while there was no significant difference regarding serum level of CA, CDCA, GCA, CDCA TCDCA/CDCA ratio or urinary CA, CDCA, GCDCA, TCDCA, TCA (Tables 4 and 5). Moreover, there was no statistically significant difference regarding serum or urinary levels of both conjugated and unconjugated secondary individual bile acids between the two patients' groups (Tables 6 and 7).

Principle component analysis (PCA)

PCA of the metabolic profiling of serum individual bile acids of the two cholestatic groups (BA and non-BA) showed that out of all the extracted PCs, the first two PCs (PC1, PC2) had eigenvalues larger than one (eigenvalue >1) with a value 2.803 and 1.652, respectively (Table 8). The serum bile acids possessing positive loading factors in the first two extracted PCs with contribution percent to the total variance (20.02% and 11.81%, respectively) were CA, GCA, GCDCA, TCA, and TCDCA for PC1 and CA, CDCA, LCA, and UDCA for PC2.

- Then, the ROC analysis was performed using score plots of the first two principal components for visualization of the data and establishing the differences between the two diseased groups; the area under the curve (AUC) for PC1 was 0.770 and for PC2 was .583 (Figs. 2 and 3).

The non-BA cholestatic infants were followed up by the pediatrician and were diagnosed furthermore as following infection (n=20), PFIC (n=16), inspissated bile syndrome (n=9), patients with metabolic causes (n=7), patients with Down syndrome (n=3), with endocrinal causes (n=2), AGS syndrome (n=3), one patient for

Table 4 Comparison between the two cholestatic groups (BA & non-BA) and control regarding primary unconjugated bile acids in serum and urine (µmol/L)

Primary unconjugated bile acids (µmol/L)		Biliary atresia ($n = 37$)	Non-biliary atresia ($n = 65$)	Control ($n = 102$)	*H*	*p*
Serum	Cholic acid (CA)					
	Min. – Max.	0.0 – 0.23	0.0 – 1.20	0.0 – 0.50	12.19*	0.002*
	Median (IQR)	0.09 (0.0 – 0.11)	0.05 (0.01 – 0.190)	0.13[a] (0.07 – 0.21		
	Chenodeoxycholic acid (CDCA)					
	Min. – Max.	0.0–0.24	0.0 – 0.9	0.0 – 0.90	16.1961*	<0.001*
	Median (IQR)	0.05 (0.01 – 0.09)	0.01 (0.0 – 0.12)	0.0 [a] (0.0 – 0.03)		
Urinary	Cholic acid (CA)					
	Min. – Max.	0.0 – 0.20	0.0 – 0.80	0.0 – 0.30	0.244	0.885
	Median (IQR)	0.0 (0.0 – 0.04)	0.0 (0.0 – 0.04)	0.0 (0.0 – 0.03)		
	Chenodeoxycholic acid (CDCA)					
	Min. – Max.	0.0–0.13	0.0–0.40	0.0–0.90	2.012	0.366
	Median (IQR)	0.0 (0.0–0.01)	0.0 (0.0–0.01)	0.0 (0.0–0.03)		

H H for Kruskal-Wallis test, pairwise comparison bet. 2 groups was done using post hoc test (Dunn's for multiple comparisons test)

IQR Interquartile range, *SD* Standard deviation

p p value for comparing the studied groups

*Statistically significant at $p \leq 0.05$

[a] Statistically significant with the biliary atresia group

[b] Statistically significant with the non-biliary atresia group

each of ARC syndrome, giant cell hepatitis, acute liver failure, hepatic focal lesion, and choledochal cyst.

Discussion

Since the Kasai hepatoportoenterostomy had evolved over time, a growing number of studies have shown that the younger the age at Kasai, the better the native liver survival and overall BA survival [19, 26]. However, distinguishing BA from other causes of cholestatic jaundice remains difficult due to the limitations of traditional methods, and numerous noninvasive tests that have been developed to aid in the diagnosis. Growing evidence implies that metabolite profiling may be beneficial in identifying diagnostic biomarkers in BA [27]. The changes in the serum and urinary bile acid levels in the cholestatic patients compared to the control suggest different disturbances in metabolism [28].

This study assessed the serum total bile acid (enzymatically) and the metabolic profile of 14 bile acids in cholestasis infants for differential diagnosis of the BA group from the non-BA group. There was no statistically significant difference regarding serum total bile

Table 5 Comparison between the two cholestatic groups (BA & non-BA) and control regarding primary conjugated bile acids in serum and urine (μmol/L)

Primary conjugated bile acids (μmol/L)		Biliary atresia ($n = 37$)	Non-biliary atresia ($n = 65$)	Control ($n = 102$)	*H*	*p*
Serum	**Glycholic acid (GCA)**					
	Min. – Max.	0.80 – 144.0	0.0 – 281.0	0.0 – 3.0	126.824*	**<0.001***
	Median (IQR)	22.9(14.60 – 44.0)	18.0 (6.60 – 40.30)	0.10[ab] (0.02-0.70)		
	Glychochenodeoxycholic acid (GCDCA)					
	Min. – Max.	6.80 – 163.0	0.02 – 167.0	0.0 – 2.50	130.766*	**<0.001***
	Median (IQR)	42 (30.20 – 66.30)	22.80[a] (12.2 -52.0)	0.08[ab] (0.04 -0.60)		
	Taurochlolic acid (TCA)					
	Min. – Max.	0.12 – 155.70	0.0 – 65.90	0.0 – 0.86	113.598*	**<0.001***
	Median (IQR)	24.7 (12.80 -39.8)	17.8[a] (4.2 -32.9)	0.06[ab] (0.01 –0.21)		
	Taurochenodeoxycholic acid (TCDCA)					
	Min. – Max.	0.13 – 183.0	0.0 – 208.0	0.0 – 1.30	113.738*	**<0.001***
	Median (IQR)	45.0 (36.90 –68.60)	20.5[a] (9.0 – 45.50)	0.03[ab] (0.0 – 0.50)		
	TCDCA/CDCD ratio					
	Min. – Max.	13.0 – 11920.0	0.0 – 20800.0	0.40 – 100.0	116.004*	**<0.001***
	Median (IQR)	686.0(260.0– 2740)	683.33(91.4 -2420)	9.0[ab] (3.58 – 20.0)		
	GCDCA/CDCA ratio					
	Min. – Max.	230.0 – 16300.0	0.25 – 16700.0	0.50 – 600.0	124.972*	**<0.001***
	Median (IQR)	3860.0(2240 -6300)	1720.0[a] (404.2-3640)	22.50[ab] (8.0 –85.0)		
Urinary	**Glycholic acid (GCA)**					
	Min. – Max.	0.0 – 68.0	0.0 – 64.50	0.0 – 3.0	101.57*	**<0.001***
	Median (IQR)	6.60 (3.10 – 18.0)	6.30(1.40– 12.50)	0.10[ab] (0.02 –0.70)		
	Glychochenodeoxycholic acid (GCDCA)					
	Min. – Max.	0.0 – 67.0	0.0 – 52.0	0.0 – 2.50	36.142*	**<0.001***
	Median (IQR)	8.60 (0.30 – 17.0)	0.60 (0.15 – 7.40)	0.08[ab] (0.04 – 0.60)		
	Taurochlolic acid (TCA)					
	Min. – Max.	0.0 – 83.80	0.0 – 33.0	0.0 – 0.86	103.752*	**<0.001***
	Median (IQR)	5.60 (2.40– 17.40)	2.60 (0.44 – 9.50)	0.06[ab] (0.01 –0.21)		
	Taurochenodeoxycholic acid (TCDCA)					
	Min. – Max.	0.0 – 56.70	0.0 – 65.60	0.0 – 1.30	55.941*	**<0.001***
	Median (IQR)	1.20 (0.20 – 17.0)	0.60 (0.17 – 6.30)	0.03[ab] (0.0 – 0.50)		

IQR Interquartile range, *SD* Standard deviation

H H for Kruskal-Wallis test, pairwise comparison bet. 2 groups was done using post hoc test (Dunn's for multiple comparisons test)

p p value for comparing the studied groups

*Statistically significant at $p \leq 0.05$

[a] Statistically significant with the biliary atresia group

[b] Statistically significant with the non-biliary atresia group

acid in BA and non-BA patients ($p = 0.549$ and 0.158), respectively as it was reported by Golden et al. [29] and Zhao et al. [8].

Using the breakthrough in the technology, particularly tandem mass spectrometry, Zhou et al. [30] and Mushtaq et al. [31] hypothesized that the individual bile acids were higher in BA when compared to other types of cholestatic hepatobiliary disorders or neonatal jaundice [30]. In this regard, the present study showed that there were significant differences regarding the conjugated bile acids (TCA, TCDCA, GCDCA, and GCDCA/CDCA) while no significant differences were reported regarding secondary bile acids between cholestatic BA and cholestatic non-BA. Similarly, Golden et al. [29] reported that there was a significant difference in serum primary BAs (TCDCA and GCDCA) between BA and cholestatic non-BA in infants. These results revealed that biliary obstruction causes a drastic buildup of primary individual bile acids [16]. Such increases were formerly reported in other cholestatic situations, such as intrahepatic cholestasis of pregnancy [32], supporting the possible role of primary bile acids as a biomarker for altered biliary circulation [16].

The enterohepatic circulation was significantly blocked in BA, and only a minimal amount of bile acids reached the colon, causing a great reduction of CA and CDCA in plasma. As the block of enterohepatic circulation, conjugated BAs (TCDCA, GCDCA, TCA, and GCA) in the liver were considerably elevated and eliminated via alternative export systems at the hepatic sinusoidal membrane which cause increased conjugated bile acid levels in plasma [33].

Furthermore, Zhao et al. [8] reported that levels of CA and CDCA were significantly lower in cholestatic infants than in control. While CDCA level was significantly lower in the BA group than the non-BA group because of the more severe fibrotic or cirrhotic changes. CDCA is hydrophilic bile acid and supposed to provide a hepatoprotective function which impaired due to cirrhosis [8].

Accordingly, Zhou et al. [34] studied plasma bile acid profiles in BA and neonatal hepatitis syndrome and reported that unconjugated bile acids (CA and CDCA) were significantly decreased in BA and neonatal hepatitis than in control.

In contrast to primary bile acids, secondary species are not at comparable levels in serum samples, and their relative abundance was insignificant between the two cholestatic subgroups. These findings were due to the absence of the interaction of the intestinal bacteria with primary bile acids in BA, which decrease the levels of deconjugated and secondary BAs [29].

Fortunately, the mean level of primary conjugated bile acids was higher than the primary unconjugated, as conjugation results in less toxic and more water-soluble bile acids, thus protecting against cellular damage from such toxic compounds that initiate oxidative stress and cell death signaling [35].

Table 6 Comparison between the two cholestatic groups (BA & non-BA) and control regarding secondary unconjugated bile acids in serum and urine (μmol/L)

Secondary unconjugated bile acids (μmol/L)		Biliary atresia ($n = 37$)	Non-biliary atresia ($n = 65$)	Control ($n = 102$)	*H*	*p*
Serum	**Ursodeoxycholic acid (UDCA)**					
	Min. – Max.	0.0 – 0.12	0.0 – 1.20	0.0 – 0.13	0.15	0.926
	Median (IQR)	0.01 (0.0 – 0.06)	0.01 (0.0 – 0.07)	0.01 (0.0 – 0.04)		
	Lithocholic acid (LCA)					
	Min. – Max.	0.0 – 0.60	0.0 – 0.5	0.0 – 0.48	3.169	0.205
	Median (IQR)	0.03 (0.0 – 0.10)	0.01 (0.0 – 0.10)	0.05 (0.0 – 0.09)		
	Deoxycholic acid (DCA)					
	Min. – Max.	0.0 – 0.56	0.0 – 1.40	0.0 – 0.40	15.08*	**<0.001***
	Median (IQR)	0.02 (0.0 – 0.09)	0.01 (0.0 – 0.10)	0.07[ab] (0.02 –0.11)		
Urinary	**Ursodeoxycholic (UDCA)**					
	Min. – Max.	0.0 – 0.15	0.0 – 1.40	0.0 – 0.04	18.129*	**<0.001***
	Median (IQR)	0.0 (0.0 – 0.01)	0.0 (0.0 – 0.02)	0.0[ab] (0.0 – 0.0)		
	Lithocholic acid (LCA)					
	Min. – Max.	0.0 – 0.49	0.0 – 0.36	0.0 – 0.10	0.014	0.993
	Median (IQR)	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.02)		
	Deoxycholic acid (DCA)					
	Min. – Max.	0.0 – 0.18	0.0 – 0.20	0.0 – 0.30	3.724	0.155
	Median (IQR)	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.01)		

Table 7 Comparison between the two cholestatic groups (BA & non-BA) and control regarding secondary conjugated bile acids in serum and urine (µmol/L)

Secondary conjugated bile acids (µmol/L)		Biliary atresia ($n = 37$)	Non-biliary atresia ($n = 65$)	Control ($n = 102$)	*H*	*p*
Serum	**Tauroursodeoxycholic acid (TUDCA)**					
	Min. – Max.	0.0 – 1.3	0.0 – 5.00	0.0 – 0.18	40.991*	**<0.001***
	Median (IQR)	0.24 (0.0 – 0.45)	0.33 (0.01 – 1.10)	0.06[b] (0.01 – 0.13)		
	Taurolithocholic acid (TLCA)					
	Min. – Max.	0.0 – 0.64	0.0 – 0.65	0.0 – 0.30	3.853	0.14
	Median (IQR)	0.13 (0.02 – 0.24)	0.10 (0.0 – 0.24)	0.07 (0.0 – 0.13)		
	Taurodeoxycholic acid (TDCA)					
	Min. – Max.	0.0 – 1.78	0.0 – 3.9	0.0 – 0.90	24.17*	**<0.001***
	Median (IQR)	0.60 (0.20 –.0.50)	0.35 (0.06 – 0.58)	0.14[ab] (0.03 –0.16)		
	Glycoursodeoxycholic acid (GUDCA)					
	Min. – Max.	0.0 – 4.34	0.0 – 7.3	0.0 – 1.5	30.520*	**<0.001***
	Median (IQR)	1.0 (0.13 – 2.26)	1.40 (0.15 – 1.20)	0.08[ab] (0.03 – 0.27)		
	Glychodeoxycholic acid (GDCA)					
	Min. – Max.	0.0 –0.80	0.0 – 0.90	0.0 – 1.40	1.065	0.587
	Median (IQR)	0.2 (0.01 – 0.30)	0.10 (0.01 – 0.31)	0.10 (0.04 – 0.16)		
Urinary	**Tauroursodeoxycholic acid (TUDCA)**					
	Min. – Max.	0.0 – 0.8	0.0 – 1.6	0.0 – 0.15	30.958*	**<0.001***
	Median (IQR)	0.0 (0.0 – 0.15)	0.10 (0.0 – 0.20)	0.0[ab] (0.0 – 0.01)		
	Taurolithocholic acid (TLCA)					
	Min. – Max.	0.0 – 0.19	0.0 – 0.26	0.0 – 1.0	0.397	0.820
	Median (IQR)	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.02)	0.0 (0.0 – 0.02)		
	Taurodeoxycholic acid (TDCA)					
	Min. – Max.	0.0 – 0.50	0.0 – 0.70	0.0 – 0.20	5.646	0.059
	Median (IQR)	0.03 (0.0 – 0.15)	0.0 (0.0 – 0.12)	0.0 (0.0 – 0.07)		
	Glycoursodeoxycholic acid (GUDCA)					
	Min. – Max.	0.0 – 0.9	0.0 – 7.2	0.0 – 0.20	54.651*	**<0.001***
	Median (IQR)	0.20 (0.0 – 0.55)	0.40 (0.01–1.35)	0.0[ab] (0.0 – 0.02)		
	Glychodeoxycholic acid (GDCA)					
	Min. – Max.	0.0 – 0.37	0.0 – 0.3	0.0 – 0.30	4.731	0.094
	Median (IQR)	0.0 (0.0 – 0.06)	0.0 (0.0 – 0.01)	0.0 (0.0 – 0.02)		

TCDCA and GCDCA are generated by taurine and glycine conjugation of CDCA in the liver. As the higher (TCDCA and GCDCA levels) and lower CDCA levels in BA, the ratio of TCDCA: CDCA and GCDCA: CDCA could be used to compare BA with other cholestatic diseases [8, 30]. In this regard, the present study reported that the ratio of GCDCA: CDCA was significantly higher in BA than in non-BA. However, there was no significant difference between them regarding TCDCA: CDCA ratio. Similarly, Zhao et al. [8] reported that GCDCA: CDCA ratio was significantly higher in BA infants than in non-BA infants. Accordingly, Zhou et al. [34] reported that TCDCA: CDCA ratio was significantly higher in BA infants than in neonatal hepatitis.

In addition to inhibition of *CYP7A1*, biliary obstruction also down-regulates *CYP8B1* expression in the liver [36]. This gene encodes sterol 12α-hydroxylase, a main pathway in the bile biosynthesis favoring synthesis of other primary bile acids instead of CDCA synthesis, and thus determines the TCDCA to CDCA and GCDCA to CDCA ratios [16].

Regarding urinary individual bile acids, the present study revealed that the most abundant bile acid species in cholestatic patients compared to control were TCA, TCDCA, GCA, and GCDCA but at a lower level than serum with no significant difference between BA and non-BA subgroups. These results were in line with Golden et al. [29] who reported that bile acids in the urine were predominantly

Table 8 Component matrix of PC1 and PC2

Component matrix[a]		
Component		
Serum individual bile acids	**PC1**	**PC2**
Cholic acid (CA)	*0.353*	*0.396*
Chenodeoxycholic acid (CDCA)	−0.286	*0.387*
Glycholic acid (GCA)	*0.338*	−0.146
Glychochenodeoxycholic acid (GCDCA)	*0.415*	−0.121
Tarochlolic acid (TCA)	*0.389*	−0.211
Taurochenodeoxycholic acid (TCDCA)	*0.404*	−0.083
Ursodeoxycholic (UDCA)	−0.086	*0.434*
Lithocholic acid (LCA)	−0.009	*0.389*
Eigenvalues	**2.803**	**1.652**
Variance %	**20.02**	**11.81**
Cumulative%	**20.02**	**31.83**

The individual component of PC1 and PC2 that had a positive loading factor was presented in italics

conjugated with no significant difference between cholestatic non-BA and BA.

Suzuki et al. [37] had previously investigated urinary bile acid metabolites as a possible screening tool without any conclusive results; however, in a study performed by Nanashima et al. [38], they reported that urinary sulfated bile acids increased in patients with cholestasis. Another study done by Trottier et al. [39] had revealed that biliary obstruction changes differentially the circulating and/or urinary levels of the various bile acids.

To display and explore analysis purposes for variables of BAs in the metabolome, modern chemometric tools were used such as principal component analysis (PCA), as simultaneous comparisons of a large number of complex objects were facilitated by reducing the dimensionality of the data set. The resulting data were displayed as score plots which are generally reflected by the first two principal components, representing the distribution of samples in multivariate space [40]. Accordingly, the present study revealed that the identified serum BAs possessing positive loading factors in the first two extracted PCs were CA, GCA, GCDCA, TCA, and TCDCA for PC1 and CA, CDCA, LCA, and UDCA for PC2.

The AUC is a direct indication of the efficiency and clinical applicability of the diagnostic method [41]. According to the previous data, the current study showed that the ROC curves had varied diagnostic powers for the significant metabolites in the first two components between the two cholestatic groups as the AUC of PC1 was 0.770 while it was for PC2 0.583. These results indicated that the positive components of PC1 had the highest score and deemed as "potential marker metabolites" for differentiation between the two cholestatic groups

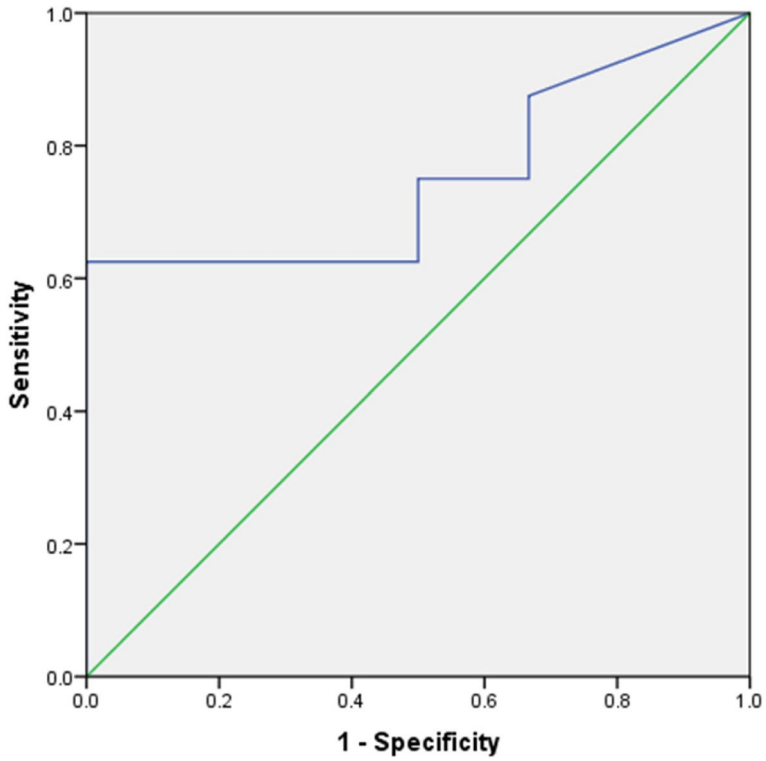

Fig. 2 Receiver operating characteristic (ROC) curve of PC1 model of serum individual bile acids for differentiating between BA and non-BA patients

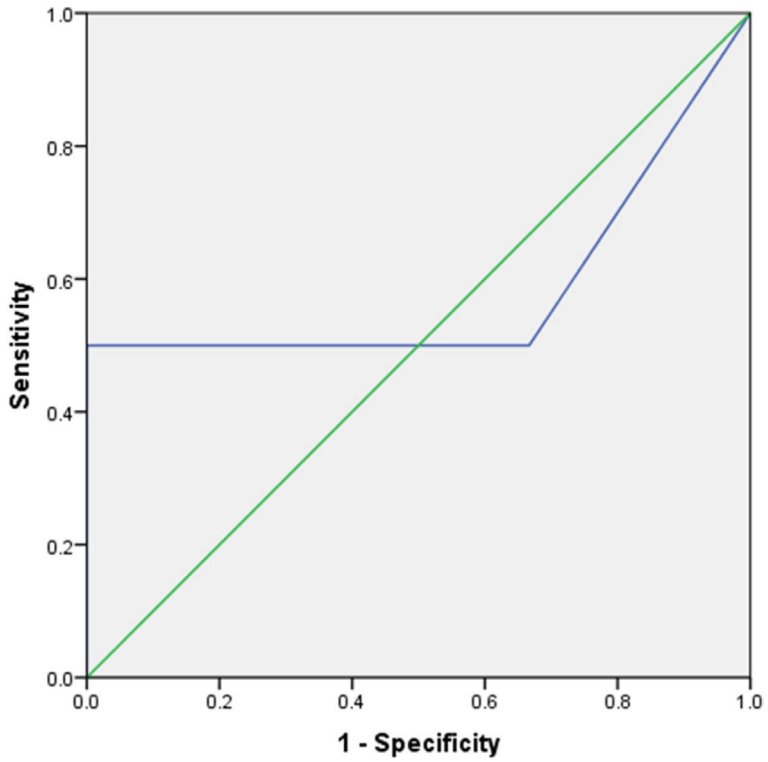

Fig. 3 Receiver operating characteristic (ROC) curve of PC2 model of serum individual bile acids for differentiating between BA and non-BA patients

This study had some limitations such as the variable pathologies of the cholestatic non-BA patients, which may clarify the wide variability in the concentrations of urine, and serum bile acids in this study in addition to, the difficult subgroups analysis due to the small sample size, although they may provide valuable information that can be potentially a useful diagnostic tool for understanding the mechanism of the disease.

Conclusion

Assessing the serum BA levels of CA, GCA, GCDCA, TCA, and TCDCA collectively using tandem mass spectrometry may be helpful in differentiating the cholestatic biliary atresia from cholestatic non-biliary atresia infants with avoidance of unnecessary invasive procedures. This might provide new insights into the biochemical changes in cholestasis.

Abbreviations

ARC	Arthogryposis—renal dysfunction cholestasis
AGS	Alagille syndrome
AUC	Area under the curve
BA	Biliary atresia
CA	Cholic acids
CDCA	Chenodeoxycholic acid
CMV	Cytomegalovirus
DCA	Deoxycholic acid
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GUDCA	Glycoursodeoxycholic acid
HSV	Herpes simplex virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IOC	Intraoperative cholangiogram
KPE	Kasi portoenterostomy
LC-MS	Liquid chromatography-mass spectroscopy
LCA	Lithocholic acid
MS/MS	Tandem mass spectrometry
NC	Neonatal cholestasis
Non-BA	Non-biliary atresia
PCA	Principal component analysis
PFIC	Progressive familial intrahepatic cholestasis
ROC curve	Receiver operating characteristic
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
TDCA	Taurodeoxycholic acid
TLCA	Taurolithocholic acid
UDCA	Ursodeoxycholic acid
GGT	Gamma glutamyl transaminase

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Authors' contributions

SAE, SA, and DS were the major contributor in performing the practical section of this work and the interpretation of the laboratory data. They also

contributed to the writing of the manuscript. SAA and SAE performed full clinical examination (general and hepatological), complete history taking regarding nutrition and growth charts with stress on birth date, sex, gestational age, antenatal, prenatal history, consanguinity, pedigree construction, recording of previous neonatal deaths, and similar affected cases within the family. DS had performed the pathological section of the study. SAA contributed to the interpretation of the clinical data and supervision of the final diagnosis of the cases. HE and AE contributed to the conception, design, and revision of the work. The authors have read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this article.

Declarations

Ethics approval and consent to participate
Approval was obtained from the Ethics Committee of the National Liver Institute, Menoufia University (00134/2018 INTM). Informed written consent was obtained from the guardians of participants before enrollment in the study.

Competing interests
The authors declare that they have no competing interests.

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