Changes in plasma bile acid profiles after partial internal biliary diversion in PFIC2 patients

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Background: We ask if plasma bile acid profiles can be used to monitor the effectiveness of partial internal biliary diversion (PIBD) for treating uncontrolled cholestasis in progressive familial intrahepatic cholestasis type 2 (PFIC2) patients.

Methods: Plasma bile acids were profiled in 3 cases of ATP-binding cassette, sub-family B member 11 (*ABCB11*)-mutated PFIC2 children before and after PIBD compared to healthy controls and 8 PFIC2 patients. The quantitation of bile acids was performed by reversed-phase ultrahigh-performance liquid chromatography/multiple-reaction monitoring-mass spectrometry (UPLC/MRM-MS) with negative ion detection.

Results: Before PIBD, all three patients presented with >50-fold higher levels of total plasma bile acids, 2–7 folds higher ratios of taurine: glycine conjugated primary bile acids, and unchanged secondary bile acids levels compared to healthy controls. After PIBD, only one of the three patients (P3) showed relief of cholestasis. The bile acid profiles of the two nonresponding patients showed little change while that of the responding patient showed a 5-fold reduction in total plasma primary bile acids, a reduced taurine: glycine conjugate ratio, and an unexpected 26- and 12-fold increase in secondary bile acids DCA and LCA respectively. One year later, the responder suffered a recurrence of cholestasis, and the bile acid profile shifted back to a more pre-PIBD-like profile.

Conclusions: Plasma bile acid profiles may potentially be useful as sensitive biomarkers for monitoring the clinical course of PIBD patients. Relief of cholestasis after PIBD appears to be associated with significantly increased circulating toxic secondary bile acids and this may limit the utility of PIBD in PFIC2 patients in the long run.

Keywords: Biliary diversion; bile salt export pump (BSEP); jaundice; liquid chromatography-mass spectrometry

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Introduction

The accumulation of cytotoxic hydrophobic bile acids in the liver is considered to be the root cause of hepatobiliary injury in progressive intrahepatic cholestasis (PFICs) (1). The primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA) in humans, are synthesized from cholesterol in hepatocytes, conjugated, secreted into the bile, and then released into the duodenum. Ninety-five percent of biliary bile acids are reabsorbed into blood circulation through ileum enterocytes (1), and the rest enter the colon. Some of the bile acids in the colon are converted by the gut microbiome into the secondary bile acids, mainly deoxycholic acid (DCA) and lithocholic acid (LCA) (2-4), which are either reabsorbed into circulation or eliminated through feces.

Progressive familial intrahepatic cholestasis type 2 (PFIC2) is a fatal childhood disease caused by mutations in the ATP-binding cassette, sub-family B member 11 (*ABCB11*) gene, which encodes the Bile Salt Export Pump (BSEP) protein. PFIC2 is often associated with pruritus, jaundice, growth retardation, cirrhosis, liver failure, and death (5-9). Defects in BSEP result in the accumulation of toxic bile acids in hepatocytes with complex changes in bile acid metabolism (10-14). The recent development of a reversed-phase ultrahigh-performance liquid chromatography/multiple-reaction monitoring-mass spectrometry (UPLC/MRM-MS) method with negative ion detection allows quantitation of a wide range of modified bile acids with high sensitivity (15). Using this technique, novel polyhydroxylated bile acids have been detected in mice with a genetically impaired bile salt export pump (16-18). In a similar manner increased levels of tetrahydroxylated bile acids (THBAs) have also been detected in children with cholestatic diseases (14,19).

Biliary diversion (BD) is a first-line, non-liver transplant clinical intervention, for low gamma-glutamyl transferase (GGT) PFIC (20,21). Variants of BD (*Figure 1*) include partial external biliary diversion (PEBD) (20,24), partial internal biliary diversion (PIBD) (21,25), and total internal BD (22). Briefly, the PEBD procedure partially diverts the flow of bile from the gall bladder to an external stoma to reduce bile acids in the intestine for recirculation. For the PIBD procedure used in the current study, a conduit is performed between the terminolateral side of the gall bladder and distal colon using a segment of jejunum, to divert the biliary flow from the enterohepatic cycle without an external stoma (21). The procedure of total internal BD uses a jejunum conduit between the graft bile duct and the transverse colon (22).

PEBD is most commonly practiced and it has been shown to lessen the clinical symptoms of PFIC including pruritus, reduce plasma bile acid levels, improve the plasma lipoprotein profile, slow disease progression, and improve liver histopathology (23,26-32). Responses do vary amongst patients and the underlying mechanism for the effectiveness of BD is not well-understood. For example, how BD affects bile acid metabolism, including the distribution and composition of bile acids, has not been well investigated yet (20). It is also not understood how the different genotypes associated with PFIC respond to BD (20,29), and detailed comparisons of bile acids pre- and post-operation have usually not been undertaken (32-35). PIBD appears an effective alternative approach to PEBD for cholestatic diseases in PFIC, although only a few cases have been reported (21,23,36-39).

In the present study, we performed detailed analyses of plasma bile acids before and after PIBD in three genetically-defined PFIC2 patients. To our knowledge, no bile acid profiling of PFIC2 patients before and after BD has been reported (20,29). We have shown previously, that the profiling of plasma bile acids has provided valuable insights into cholestasis alleviation and tracked genetic and clinical status in *ABCB11* mutated patients (19). Thus, the current goal is to determine if bile acid profiling may also be useful for gaining insights into the treatment efficacy of PIBD in *ABCB11* mutated PFIC2 patients.

Methods

Subjects

Study subjects were Chinese children, with informed consent, under a protocol approved by the Children's Hospital of Fudan University, in accordance with ethical guidelines.

Subjects screening criteria include (I) carrying confirmed *ABCB11* mutations based on the screening of a panel of 61 cholestasis-related genes (40); (II) undergone biliary diversion because of sub-optimal response to ursodeoxycholic acid (UDCA) treatment; and (III) with fasting serum samples pre- and post-operation. Immunostaining for BSEP expression was performed as previously described (13,19). The patients' plasma bile acid profiles were determined before and after PIBD. Data for 40 age-matched healthy subjects, age from 3 months to

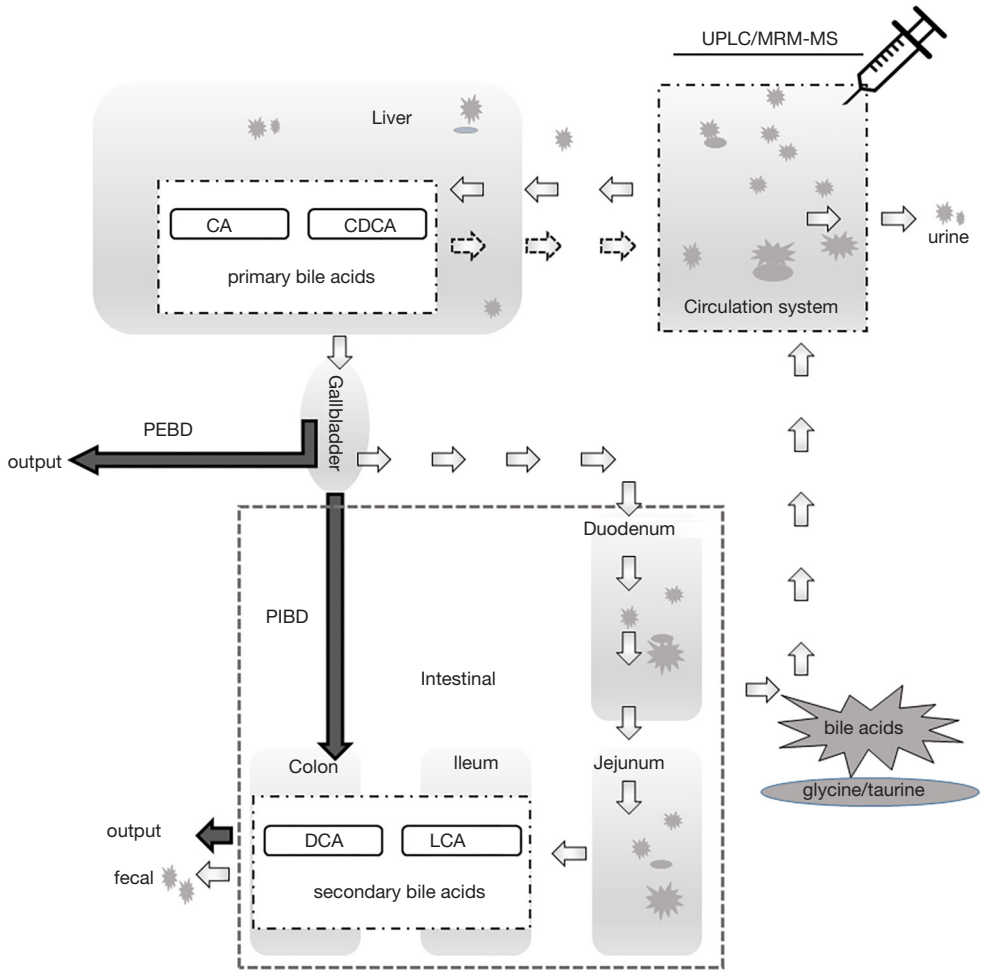

Figure 1 Enterohepatic circulation of bile salts after biliary diversion, and under normal conditions. This figure illustrates the physiology and the enterohepatic circulation of bile salts after biliary diversion (dark arrows) and under normal conditions (light arrows). Briefly, CA and CDCA (primary bile acids) are synthesized and conjugated with taurine or glycine in the liver before been secreted and concentrated in the gallbladder. They are secreted into the duodenum with bile to facilitate digestion during meals. Most of the bile acids are deconjugated in the intestines, while some are dehydroxylated by the flora in the ileum and the colon to generate secondary bile acids (DCA and LCA). Some of the bile acids are secreted into the circulation system through the sinusoidal membrane during cholestasis (1). The procedure of partial internal biliary diversion (PIBD) (22), partially diverts the biliary flow into the colon to diminish the absorption of the bile acids from the upstream intestines (22), while the procedure of partial external biliary diversion (PEBD) delivers gallbladder bile to the exterior of the body to diminish the secretion of bile acids into the intestines (23).

18 years with fasting samples and no infectious or endocrine genetic metabolic diseases, collected for another study (19), were used as healthy controls. Blood samples from eight PFIC2 patients, whom were receiving UDCA administration were collected as PFIC2 controls to differentiate the UDCA therapy effects [of whom 6 were collected for another study (19)] (*Table S1*).

Sample preparation and analysis of bile acids

Plasma was separated from whole blood by centrifugation (2,000 × g) for 10 min at 4 ℃, with sodium heparin as an anticoagulant, and was aliquoted into 1.5-Eppemdorf tubes in 50 μL for freeze-drying before storage at −80 ℃. For determination of bile acids, 50 μL of water was used

to reconstitute an aliquot of each sample and 50 µL of an internal standard (IS) solution containing 14 deuterium-labeled bile acids (15) was added to each tube. After vortex-mixing and 3-s spin-down, 250 µL of methanol-acetonitrile (1:1, v/v) was added to each tube followed by 30-s vortex-mixing and 3-min sonication in an ice-water bath. Protein was pelleted by centrifugation at 21,000 ×g and 10 ℃ for 10 min and the clear supernatant was transferred and completely mixed with 270 µL of water inside a polymeric Strata-X reversed-phase solid-phase extraction cartridge (200 mg/3 mL, Phenomenex Inc. CA, USA), which was activated with 3 mL of methanol and reconditioned with 3 mL of water before use. Under a 3-psi positive pressure, the flow-through fraction was discarded and bile acids retained on the resin were eluted with 3 mL of methanol-acetonitrile (1:1). The collected eluant in a 5-mL glass test tube was dried down under a gentle nitrogen gas flow at 30 ℃ and the residue was dissolved in 200 µL fo 50% aqueous methanol. 20 µL was injected to run reversed-phase UPLC/MRM-MS with negative ion detection, on an Agilent 1290 UPLC system coupled to a Sciex QTRAP 4000 mass spectrometer, using the same procedure as described before (15). Along with preparation of the sample solutions, a mixture of 60 bile acids as reference standards as described before (13,15) was used to make serially diluted, working calibration solutions containing the same deuterium-labeled internal standards , in 50% aqueous methanol. The 60 bile acids included all of the major bile acids and some minor species, e.g., UDCA, hyocholic acid (HCA), hyodeoxycholic acid (HDCA), muricholic acids (MCAs), two THBAs [3α,6α,7α,12α-THBA, and taurine-3α,6α,7α,12α-THBA (tauro-THBA)] which were custom-synthesized (*Table S2*). Linear-regression calibration curves of individual bile acids were constructed with internal calibration. For those bile acids without their isotope-labeled analogues, deuteriated glyco-CDCA was used as a common internal standard. Concentrations of bile acids in plasma were calculated by interpolating the corresponding calibration curves with the analyte-to-internal standard peak area ratios measured from each sample solution.

Results

Clinical and ABCB11 mutation status of study subjects

Briefly, the patients in the current study were identified from a cohort of 77 patients with confirmed *ABCB11* deficiency by genome sequencing accrued by our Center in the last 16 years. Of the ten patients from this cohort who underwent PIBD procedure, three died, one underwent liver transplantation, four suffered recurrent jaundice, and two patients were lost to follow-up. Three PFIC patients, who have undergone PIBD because of uncontrolled cholestasis (progressive jaundice and/or intractable pruritus with unsatisfied response to drug therapy) were recruited. Patient 1 carried two missense mutations (c.3457C>T, p.R1153C/c.3623 A>G, p.Y1208C). Patient 2 carried a splicing mutation and a missense mutation (c.2815-8A>G/c.3458G>A, p.R1153H), and Patient 3 carried a missense mutation and a nonsense mutation (c.1460G>A, p.R487H/c.3169C>T, p.R1057X) (*Table 1*). All three patients were treated with UDCA before and after PIBD. Patient P1 was a boy with no BSEP expression by immunohistochemistry, who underwent PIBD at 5 months but showed little postoperative improvement, with no alleviation in jaundice and decrease of liver enzymes (*Table 1*). The patient died at 1.5 y because of infection with severe cholestasis. The liver biopsy specimen acquired during PIBD indicated severe liver fibrosis (developing cirrhosis) at the time of the operation.

Patient P2 was a girl presenting with jaundice and abnormal liver indicators at admission as a 2.6-year-old. The liver biopsy specimen during PIBD procedure indicates fibrosis without obvious cirrhosis. BSEP immunostaining was not performed. No obvious improvement in jaundice and liver indicators were observed after PIBD at age of about 3.5 years old, or during later follow-ups at 4.0 and 4.3 years of age.

Patient P3 was a girl, who presented with persistent jaundice two days after birth, pruritus from 6 months of age onward, and persistent abnormal liver function (*Table 1*). The liver biopsy at age of 2 months suggested cholestasis, with no-detectable BSEP expression by immunohistochemistry, and no severe fibrosis/cirrhosis by histology. She underwent PIBD at age 8 because of increasing levels of abnormal liver enzymes and pruritus. Cholestasis was relieved after the PIBD, with resolved jaundice and pruritus. Liver enzymes, including TB, DB, ALT and AST levels were within the normal range except for increased ALP (*Table 1*). P3 suffered a relapse a year later with mild jaundice, pruritus, and elevated liver enzymes. The patient's plasma total bilirubin rose sharply to 183 µmol/L at age 10 (up from 13.2 µmol/L at age 9.5 year, and close to the pre-PIBD level of 190.9 µmol/L) (*Table 1*).

Both P2 and P3 have survived with native livers as of this submission.

Table 1 Background information, subjects with confirmed *ABCB11* mutations

P	G	Sampling age	UDCA (mg)	ALP	ALT	AST	GGT	TB	DB	ABCB11 mutations/BSEP consequences	(BSEP)	Status and age at last follow-up
ABCB11 (NM_003742)												
1	M	5 m	50 qd	504	248	385	34	151.5	112.8	c.3457C>T, p.R1153C/ c.3623 A>G, p.Y1208C	(–)	PIBD at age 5 m, died at 1.5 y
		7 m	50 qd	N/A	356	404	20	71.5	55.5			
2	F	2.6 y	80 bid	278	191	207	15	92.3	78.1	c.2815-8A>G/c.3458G> A, p.R1153H	N/A	PIBD at age 3.5 y, persistent cholestasis at 4.3 y
		4.0 y	125 qd	204	157	285	20	131.4	111.9			
		4.3 y	125 qd	204	141	320	29	193.9	157.6			
3	F	7.5 y	62.5 bid	286	39	75	27	190.9	139	c.1460G>A, p.R487H/ c.3169C>T, p.R1057X	(–)	PIBD at age 8 y, recurrent cholestasis at 10 y
		8.8 y	125 bid	656	26	28	11	20	10.3			
		9.5 y	125 bid	536	28	26	11	13.2	7.5			
		10 y	125 bid	405	36	61	14	183	115.6			

Samples for liver function test were used for bile acid profiling during the study except for P3 at 10 y. P, patient; G, gender; M/F, male/female; ALP, alkaline phosphatase (42–383 IU/L); ALT, alanine aminotransferase (0–40 IU/L); AST, aspartate aminotransferase (15–60 IU/L); GGT, gamma-glutamyl transferase (7–50 IU/L); TBIL, total bilirubin (5.1–17.1 μmol/L); DBIL, direct bilirubin (3.1–5.2 μmol/L); N/A, not available or lost to follow up; (BSEP), BSEP expression on immunostaining; PIBD, partial internal biliary diversion.

Plasma primary and secondary bile acids before and after PIBD

Before PIBD, all three patients exhibited bile acid profiles similar to PFIC2 controls, with much lower concentrations of unconjugated CA and CDCA and dramatically increased tauro-CA, glyco-CA, tauro-CDCA, and glyco-CDCA levels compared to healthy controls (*Table 2* and *Table S2*). The concentrations of DCA, LCA, and their conjugates in patients before PIBD were relatively lower than the controls.

After PIBD, no significant changes in total plasma bile acid concentrations were observed in P1 and P2; however, in P3, with relief of cholestasis, as expected, the total plasma bile acid concentrations were reduced by 5.5 fold (*Table 2* and *Table S2*) and the bile acid profile shifted toward that of the healthy controls, with increased levels of unconjugated CA, CDCA, DCA, and LCA and decreased levels of conjugated primary bile acids (tauro-CA, tauro-CDCA, and glycol-CDCA). What was not anticipated is that the level of secondary bile acids increased dramatically (25-fold) after PIBD in P3 (*Figure 2*). Total plasma DCA and LCA increased 26-fold and 12-fold respectively (*Table 2*). When cholestasis recurred in P3, the bile acid profile reverted toward that seen before PIBD and the total bile acids in the plasma increased by 3.5-fold compared to the level after PIBD, when cholestasis had been relieved (*Table 2* and *Table S2*). Notably, unconjugated CDCA was also increased after BD in P2.

In addition, taurine conjugation was the predominant conjugated form of the primary bile acids in the plasma of these patients prior to PIBD consistent with what is observed in other cholestatic PFIC2 patients (19,41,42). The molar ratios of taurine to glycine conjugates were 1.42/0.56/14.81/0.2 for tauro-CA:glyco-CA, and 1.20/0.33/0.44/0.14 for tauro-CDCA:glyco-CDCA in P1/P2/P3/healthy controls (*Table 2*). In patient P3, the ratios changed towards that of healthy controls with the relief of cholestasis, with glycine conjugation predominating and molar ratios of tauro-CA:glyco-CA equal to 0.13 and tauro-CDCA:glyco-CDCA equal to 0.11.

UDCAs, MCAs, THBAs and some atypical bile acids in patients before and after PIBD

Plasma UDCA and its conjugates were present at concentrations 100s of times higher in all three patients compared to healthy controls (*Table S2*). This was expected since the patients were undergoing UDCA treatment. It was noted that in P3 (but not the non-responders) the tauro-UDCA-3-sulfate level was greatly reduced after PIBD (from 11,961 to 7 nM), while the glycol-UDCA-3-sulfate level remained essentially unchanged at over 100 times

Table 2 Concentrations of main primary and secondary bile acids (nM) in patients with ABCB11 mutations before and after biliary diversion

	1	1*	2	2*	2*	3	3*	3*	HC (n=40), M (Q1, Q3)	PFIC2 control (n=8), M (Q1, Q3)
Sampling age	5 m	7 m	2.6 y	4.0 y	4.3 y	7.5 y	8.8 y	9.5 y		
CA	14.8	45.7	25.2	26.8	24.9	22.2	107.5	56.7	41.3 (26.6, 69.3)	32.2 (9.4, 43.6)
tauro-CA	41,969.0	38,944.9	39,335.6	18,553.2	12,074.6	35,946.1	1,015.7	10,438.4	66.6 (33.7, 151.3)	16,378 (12,118, 52,241.9)
glyco-CA	29,657.2	40,692.9	70,335.0	46,591.8	34,995.3	2,427.4	7,589.2	46,937.7	442.4 (187.9, 745.6)	40,021.6 (28,211.8, 65,710.1)
T:G (CA)	1.42	0.96	0.56	0.40	0.35	14.81	0.13	0.22	0.2 (0.13, 0.4)	0.41 (0.24, 0.72)
Total CA	71,641	79,684	109,696	65,172	47,095	38,396	8,712	57,433	583 (299, 978.9)	64,543.4 (43,812.9, 95,838)
Proportion (%)	31.60	34.11	47.26	19.79	14.48	24.06	17.77	46.26	13.6 (9.41, 20.87)	41.05 (32, 49.44)
CDCA	35.6	44.8	16.0	91.4	196.2	28.2	123.7	37.5	156.4 (68.2, 279.8)	51.1 (21.8, 99.9)
tauro-CDCA	84,437	62,412	30,480	54,346	57,107	37,054	2,309	7,650	243.6 (113, 706.3)	42,328.1 (31,922.6, 51,978.7)
glyco-CDCA	70,515	91,373	91,785	209,509	220,558	83,477	21,881	49,158	2,179 (1,218.6, 4,136.5)	60,232.8 (38,923.8, 117,941.9)
T:G (CDCA)	1.20	0.68	0.33	0.26	0.26	0.44	0.11	0.16	0.14 (0.08, 0.18)	0.53 (0.34, 0.76)
Total CDCA	154,988	153,829	122,281	263,947	277,861	120,560	24,314	56,846	2,859.6 (1,598.2, 5,048.1)	105,683.8 (82,402.4, 162,918.1)
Proportion (%)	68.36	65.84	52.68	80.16	85.43	75.54	49.58	45.79	70.88 (64.17, 80.33)	58.86 (50.54, 67.91)
DCA	45.2	71.2	55.4	44.8	184.7	37.9	627.2	181.2	135.3 (81.9, 296)	39.7 (27.4, 75.5)
tauro-DCA	9.9	13.1	15.6	19.0	16.0	167.6	2,271.1	1,710.3	23.3 (1, 40.7)	9.4 (4.8, 18.1)
glyco-DCA	18.5	21.9	38.9	42.7	46.6	364.5	12,317.0	7,542.8	93.4 (3.4, 260.6)	16.7 (11.6, 24.4)
T:G (DCA)	0.54	0.60	0.40	0.45	0.34	0.46	0.18	0.23	0.25 (0.11, 0.37)	0.56 (0.45, 0.9)
Total DCA	73.7	106.1	109.9	106.6	247.3	570.0	15,215.3	9,434.3	351.2 (99.8, 594.5)	60.2 (51, 100.1)
Proportion (%)	0.03	0.05	0.05	0.03	0.08	0.36	31.03	7.60	9.82 (2.15, 20.25)	0.04 (0.02, 0.08)
LCA	1.7	1.2	0.3	0.2	0.0	2.2	39.9	13.0	2.2 (0.9, 6.2)	2.1 (1.2, 5.5)
tauro-LCA	7.0	3.2	0.2	4.5	4.2	15.2	210.9	39.8	0.5 (0.1, 1.4)	2.8 (2, 3.9)
glyco-LCA	26.0	14.8	14.9	43.7	47.1	45.9	548.2	383.8	5.8 (1.8, 19.1)	14.2 (8.2, 22.8)
T: G (LCA)	0.27	0.22	0.02	0.10	0.09	0.33	0.38	0.10	0.05 (0.03, 0.15)	0.15 (0.11, 0.2)
Total LCA	34.8	19.2	15.4	48.5	51.4	63.3	798.9	436.6	10.9 (3.5, 22.5)	20.8 (14.5, 46.7)
Proportion (%)	0.02	0.01	0.01	0.01	0.02	0.04	1.63	0.35	0.36 (0.06, 0.71)	0.01 (0.01, 0.02)

Main primary bile acids: CA, tauro-CA, glyco-CA, CDCA, tauro-CDCA, glyco-CDCA. Main secondary bile acids: DCA, tauro-DCA, glyco-DCA, LCA, tauro-LCA, glyco-LCA. Proportion %: proportion of calculated bile acids, e.g., proportion of total CA (total CA, tauro-CA, glyco-CA) in calculated main primary (CA, tauro-CA, glyco-CA, CDCA, tauro-CDCA, glyco-CDCA) and secondary bile acids (DCA, tauro-DCA, glyco-DCA, LCA, tauro-LCA, glyco-LCA). HC, healthy controls. *Bile acid profiles after PIBD.

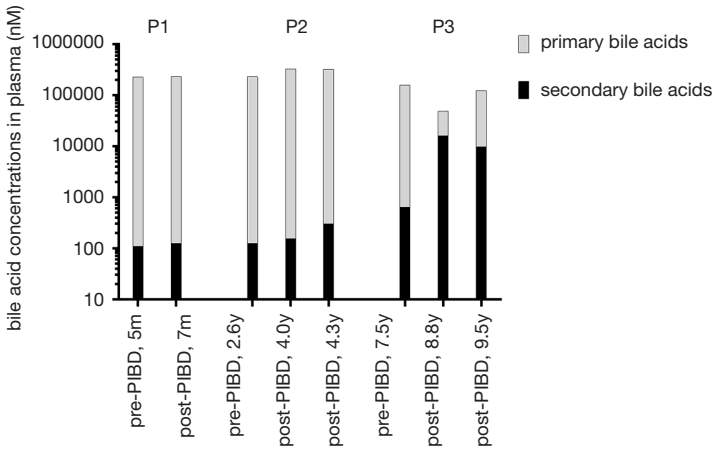

Figure 2 Concentrations of the main primary and secondary serum bile acids in patients pre- and post-PIBD procedure. Concentrations (nM) of main primary (grey columns) and secondary (black columns) serum bile acids in patients pre- and post-PIBD procedures are illustrated in this figure. No major changes were seen in P1 and P2 after PIBD. In P3, with relief of cholestasis, the concentrations of secondary bile acids increased over 25-fold after PIBD. See *Tables S1* and *S2* for details.

higher than healthy controls. It was also noted that the concentration of tauro-THBA was high in all three patients prior to PIBD (*Table S2*), but after PBID, tauro-THBA remained unchanged in P1, was reduced by 12-fold with no obvious relief of cholestasis in P2 and was reduced to a non-detectable level with relief of cholestasis in P3. On the recurrence of cholestasis in P3, the patient's plasma tauro-THBA levels increased to 33 times higher than in healthy controls (*Table S2*).

Discussion

Biliary diversion (BD) is a first-line, non-liver transplantation, surgical intervention for low gamma-glutamyl transferase (GGT) PFIC (20,21). PEBD drains gallbladder bile to the exterior of the body to reduce enterohepatic recirculation of bile acids and thus the stress in the liver for processing and recirculation (23). PIBD involves the creation of an isolated jejunal conduit, anastomosed proximally to the gallbladder and distally to the distal colon (22) (*Figure 1*). The PIBD procedure redirects bile partially into the colon, which would likely change the metabolic dynamics of circulating bile acids although little is known (21,22). To our knowledge no direct comparison of these two surgical approaches as therapeutic interventions of PFIC has been performed (2,21,25,29,36-39,43). Moreover, few comparative analyses of bile acids pre- and post-operation of BD have been undertaken (32-35). Thus, the current study, albeit limited, is an attempt to determine if plasma bile acid profiling may have clinical utility in managing BD patients.

In this study, all three PFIC2 patients had uncontrolled cholestasis with 50-fold higher total plasma bile acids levels than that of healthy controls before PIBD treatment (*Table S2*). After PIBD, one patient (P3) showed clinically meaningful relief of jaundice and pruritus, and a decrease in liver enzymes to normal ranges. The other two patients, P1 and P2, failed to show significant symptom relief (*Table 1*).

Significant changes in the plasma bile acid were seen in P3 (the responder) after PIBD but not in either in P1 nor P2 (*Table 2*). The change in the postoperative plasma bile acid profile of P3, corresponding to relief of cholestasis, is noteworthy. P3 displayed an approximately 5-fold decrease in total plasma primary bile acids (CA and CDCA), and a 25-fold elevation in secondary bile acids (DCA and LCA). It is important to note that the level of total bile acids in the bile of a responding PEBD patient also decreased 5-fold after the operation, but that the levels of biliary LCA and DCA in that patient were reduced by 13.5-fold and 2.5-fold, respectively (29). The difference in DCA and LCA is likely due to the nature of the PIBD procedure, where much of the bile is bypassed into the colon. Thus, more bile acid becomes available to the gut microbiome (2) for conversion to secondary bile acids. These increases in the amounts of toxic secondary bile acids in circulation need to be taken into account, as they could cause additional damage post-PIBD. For example, it is known that the increased chronic exposure of secondary bile acids increases

the colon cancer risk (44). In a recent study by Alrabadi *et al.* (45), it was found that a PIBD procedure might be associated with the development of macrovesicular steatosis post liver-transplantation, which may have been reversed by PEBD in a patient with PFIC1. This observation appears to be consistent with the clinical course of P3 reported in the current study. It is not unreasonable to assume that what Alrabadi *et al.* observed was associated with changes in bile acid metabolism. It is certainly worth a follow-up study. Further investigations with more cases are needed to confirm these initial observations.

Along the same vein, we note that taurine-conjugated bile acids have been shown to be more hydrophilic than their glycine counterpart and they are often increased during cholestasis likely as part of an overall compensatory mechanism to relieve cholestatic stress (19,41,42). In the current study, we found that taurine-conjugated bile acids, especially tauro-CDCA, were greatly reduced in the plasma of P3 after PIBD (*Table 2*), along with the relief of cholestasis by liver enzymes (*Table 1*). An increased molar ratio of taurine:glycine-conjugated bile acids was also found in all three PFIC2 patients. Interestingly, the fluctuation of taurine:glycine conjugate ratio in P3 coincides with the course of disease: it decreased after PIBD along with the relief of cholestasis, and increased again upon recurrence (*Table 2*). Thus, the taurine:glycine conjugate ratios of plasma bile acids could be potentially useful for monitoring the treatment efficacy of BD in PFIC patients, since the concentration of total serum bile acid levels could fluctuate depending on feeding status.

All three patients in this study were treated with UDCA raising the question of whether or not such treatments might affect their bile acid profiles. In a previous study (19), we have compared the bile acid profiles of PFIC2 patients with or without UDCA treatment. We observed that UDCA treatment affected levels of only a few species of bile acids, including UDCAs and ω-MCA, tau-CDCA, tau-LCA, tau-β-MCA and tau-ω-MCA within PFIC2 patients.

Conclusions

The findings from this study show that (I) the unexpectedly high levels of toxic secondary bile acids DCA and LCA in the plasma of the responding patient after PIBD, may limit the long-term effectiveness of PIBD. (II) Longer follow ups with more cases and bile acid profiling of patients' liver, bile, stool and urine are needed to confirm these initial findings. (III) Plasma bile acid profiles should be monitored during the clinical course of PIBD patients.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was under a protocol approved by the Children's Hospital of Fudan University, in accordance with ethical guidelines, with a signed consent from the parents was also achieved.

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Supplementary

Table S1 Background information, subjects with genetically-defined PFIC2 for PFIC2 control group

Patient	G	Sampling age	UDCA (mg)	ABCB11 mutations; BSEP consequences	BSEP expression on immunostaining	Status and age at last follow-up
N1	M	12.3 m	30 bid	c.G634A, p. A212T, c.A849C, p. E283D c.G1638T, p.Q546H	ND	Died, 1.5 y
N2	M	1.5 y	250 qd	c.612-(2_4) CTA>TT/-	(-)	Persistent cholestasis, 7.3 y
N3	F	3 m	50 bid	c.145C>T, p.Q49X c.1809G>C, p.K603N	(-)	Liver-transplantation, 4.5 y
N4	M	3.7 m	60 bid	c.409G>A, p.E137K c.2216delC, p.P740Qfs*6	(-)	Recurrent cholestasis, 6 y
N5	M	1.7 y	83.3 bid	c.1243C>T, p.R415X c.1493T>C:p.I498T	(-)	Persistent cholestasis, 2 y
N6	F	5.3 m	83.3 bid	c.1459C>T/p.R487C c.2594C>T/p.A865V	(+) in cytoplasma	Jaundice free, 3.1 y
N7		6.5 m	62.5 bid	c.1127C>A/p.A376D c.1637A>G/p.Q546R	ND	Jaundice free, 3.5 y
N8	M	2.9 m	62.5 bid	c.1460G>A/p.R487H c.2086C>T/p.R696W	(+) on canalicular membrane	Jaundice free, 3.1 y

N1 (P4), N2 (P5), N3 (P7), N4 (P10), N5 (P16) has been reported in previous study (19). M/F, male/female; ND, not done.

Table S2 Concentrations of UDCAs, THBAs and some atypical bile acids (nM) in patients with *ABCB11* mutations before and after biliary diversion

Patient	1	1*	2	2*	2*	3	3*	3*	HC (n=40), M (Q1, Q3) nM	PFIC2 control (n=8), M (Q1, Q3)
Sampling age	5 m	7 m	2.6 y	4.0 y	4.3 y	7.5 y	8.8 y	9.5 y		
tauro-THBA	227	232	12	1	0	32	0	26	0.8 (0, 1.4)	156.5 (14.1, 273.6)
glyco-UDCA-3-sulfate	5,194	8,045	14,895	11,517	8,790	11,714	13,250	34,294	90 (48.6, 143.1)	14,391.8 (5,136.6, 83,232.4)
tauro-UDCA-3-sulfate	4,171	5,337	19	13	9	11,961	7	19	0.3 (0.1, 14.5)	8,690 (2,070.5, 27,430.1)
glyco-CA-3-sulfate	17	4	233	76	19	1	47	223	0.8 (0.3, 1.8)	183.3 (2.2, 319.6)
tauro-dehydro-CA	0.4	0.5	0	0	0	0.2	0	0	0.2 (0, 0.2)	0.3 (0.2, 1)
tauro-ω-MCA	68.5	0	0	0	0	37.4	2.6	42.7	0.2 (0, 2)	137.9 (22.8, 476.7)
tauro-α-MCA	931	506	100	62	54	43	1	47	6.3 (0.5, 20.1)	730.6 (84.5, 1,711)
tauro-β-MCA	1,096	590	191	32	39	6	20	283	1.5 (0.2, 2.4)	396.8 (50, 1,865.3)
glyco-dehydro-CA	0.1	0	0	6.3	1.7	0	0	0	0 (0, 1.2)	0 (0, 2)
THBA	0.4	0.4	6.3	3.5	4.4	0.2	0	0.1	0.4 (0.3, 2.5)	1.8 (0.3, 33.5)
glyco-DCA-3-sulfate	0	0	2.3	0	0	0	1,293	1,417.9	50.1 (4.3, 85.8)	0.8 (0, 1.8)
tauro-CDCA-3-sulfate	27,854	16,699	16	16	8	5,292	0	0	0.7 (0.1, 90.3)	10,196 (4,111.4, 29,233.7)
tauro-HCA	3,837	2,852	419	330	172	198	48	648	8.6 (0, 21.6)	1,713.5 (1,273.9, 2,818)
CA-3-sulfate	54.9	110.5	20.8	10.9	3.4	6.9	7.4	24.7	0.8 (0.3, 1.8)	27.5 (18.7, 41.4)
tauro-DCA-3-sulfate	0	0	0	0.4	0	252.6	1	2.7	0.1 (0, 3.4)	0.2 (0, 3.3)
tauro-HDCA	19387	4286.2	1.7	0.3	0.2	61,032	4.7	4.2	0 (0, 14.1)	8,690 (2,070.5, 27,430.1)
tauro-UDCA	20,149	4,739	14,691	13,979	39,234	61,662	1,271	11,856	16.2 (4.8, 26.6)	10,812.1 (1,719.8, 32,152.5)
glyco-LCA-3-sulfate	120.7	0	24.9	40.4	28.1	123.7	1,398.6	1,762.5	50 (7.7, 140.7)	54.2 (39.1, 68.7)
UCA	2.1	2.3	7.8	5.3	1.9	2.2	0	0	2.6 (1.3, 4.7)	4.1 (2.1, 16.8)
7,12-keto-LCA	379.2	576.7	2.8	0.1	2.3	594.8	0	0	3.4 (0, 9.9)	28.4 (2, 151.4)
glyco-HCA	1,322	1,647	406	400	167	141	348	1,276	42.9 (30.3, 96.8)	1,156.3 (338.2, 1,515.1)
tauro-LCA-3-sulfate	91.4	12.4	0.2	0.1	0.1	0.7	1.4	0	0.1 (0, 0.7)	20.5 (4.1, 88.1)
dehydro-CA	5	3.5	1.4	1.8	0	2	3.7	14.7	10.2 (6.7, 18.6)	2.3 (0.6, 10.3)
ω-MCA	0.3	0.3	3.5	9.9	18.2	0.5	8	22.2	2.9 (0.3, 15.9)	2.8 (1.5, 3.9)
glyco-UDCA	30,655	25,544	56,811	70,169	141,921	156,371	17,697	123,036	293.5 (87.8, 531.2)	53,559.2 (25,854.3, 99,666.6)
glyco-HDCA	0	0	19.1	28.5	27	0	19.3	0	2 (0, 4.5)	16.2 (0, 18,612.7)
α-MCA	0.5	0.7	30.8	29.1	1.8	0.9	1.9	4.1	4.9 (0.4, 15.1)	6.6 (2.9, 7.5)
nor-CA	32	28.2	50.6	60.3	27.4	28.8	29.5	38.5	21.9 (12.9, 33.7)	36 (14.7, 46.9)
nor-UDCA	30.4	3.5	2.6	4.7	1.5	14.4	63.4	17.9	4.4 (2.3, 10.5)	29.9 (19.2, 31.3)
7-keto-DCA	1.3	1.9	0.3	8.5	7.5	1.7	8.9	5.2	2.3 (1.2, 4.1)	0.9 (0.1, 4.8)
DCA-3-sulfate	0	1.2	0.1	0.1	0.1	0.6	12	26.1	0.7 (0.3, 1.4)	0.2 (0.1, 0.5)
β-MCA	0.3	2	0.5	0	0.4	0.4	3.5	0.9	1 (0.3, 4.1)	2.8 (1, 11)
12-keto-CDCA	877	1114	0	78	3	920	0	2	2.9 (0, 24.3)	154.9 (12.2, 644.9)
HCA	5.1	7.5	0	0	0	0.7	28.5	14.2	12.3 (8, 28)	4.3 (1.6, 19.3)
MCA	1	0.4	0	0	0	0.4	0	0	0.5 (0, 1.8)	0.5 (0.2, 2.4)
3-keto-CA	3.3	1.7	0.2	0	1.3	0.5	3.3	3.9	0.2 (0, 1)	1.7 (0.9, 4.4)
alloCA	1.1	0.9	0.6	0.8	0	0	5.9	6.5	10.9 (5.3, 45.1)	2.1 (1.3, 8.4)
UDCA	148	39	119	116	818	411	575	3,522	58.6 (22.7, 148.6)	1,064.1 (320.2, 6,419.4)
HDCA	1.5	0.2	0	0	0	1.2	0	0	0 (0, 1.1)	2.5 (0, 4.5)
7-keto-LCA	1	1.3	4	6	23.8	4.3	20.8	3.1	5.7 (0.9, 11.9)	5.7 (4, 8.3)
6,7-keto-LCA	0	0	1	12.4	16.8	0.8	6.8	9.3	0.5 (0.2, 3.6)	0.2 (0.1, 9.3)
nor-DCA	1.5	7.6	0	0	0.4	0	0	0	0.4 (0, 1.3)	2.2 (1.1, 5.9)
12-keto-LCA	0.3	0.2	3.2	0.8	1.9	0.1	32.1	4.1	0.2 (0, 4.1)	0.2 (0.1, 1.5)
apoCA	0.6	0.2	0	0	0	0.1	9.5	0	0 (0, 0.5)	0 (0, 0.1)
alloisoLCA	0.5	0.6	2.1	2.3	2.7	0.8	0.3	0.2	0.9 (0.6, 1.6)	0.8 (0.3, 3.9)
isoLCA	0.8	1	0	0	0	1	7.1	0	0 (0, 1.8)	0.2 (0, 1.1)
isoDCA	0.2	0.2	0	0	0	0.1	1.8	0	0 (0, 0.1)	0.3 (0.1, 3.3)
dehydro-LCA	1.8	0.9	0	0	0	2.3	0	0	0.2 (0, 1.1)	0.1 (0, 1.2)
TBA	343,407	306,040	320,255	426,327	516,707	470,453	85,289	302,824	6,128.4 (3,250, 8,364)	335,544 (216,087, 612,401)

HC, healthy controls. PFIC2 control: PFIC2 patients who were underwent UDCA therapy with no biliary diversion procedure. *, Bile acid profiles after PIBD. TBA, total bile acids (60 bile acids listed in *Table 2* and *Table S2*).