Original Article

Urinary coproporphyrins as a diagnostic biomarker of Dubin-Johnson syndrome in neonates: A diagnostic pathway is proposed

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Abstract

Background: Dubin-Johnson syndrome (DJS) presents during the neonatal period with a phenotype that overlaps with a broad list of causes of neonatal cholestasis (NC), which makes the identification of DJS challenging for clinicians. We conducted a case-controlled study to investigate the utility of urinary coproporphyrins (UCP) I% as a potential diagnostic biomarker.

Methods: We reviewed our database of 533 cases of NC and identified 28 neonates with disease-causing variants in ATP-binding cassette-subfamily C member 2 (ABCC2) gene "Cases" (Study period 2008–2019). Another 20 neonates with cholestasis due to non-DJS diagnoses were included as "controls." Both groups underwent UCP analysis to measure CP isomer I percentage (%).

Results: Serum alanine aminotransferase (ALT) levels were within the normal range in 26 patients (92%) and mildly elevated in 2 patients. ALT levels were significantly lower in neonates with DJS than in NC from other causes ($P < 0.001$). The use of normal serum ALT levels to predict DJS among neonates with cholestasis had a sensitivity of 93%, specificity 90%, positive predictive value (PPV) 34%, and negative predictive value (NPV) 99.5%. The median UCPI% was significantly higher in DJS patients [88%, interquartile range (IQR) 1–IQR3, 84.2%–92.7%] than in NC from other causes [67%, (IQR1–IQR3, 61%–71.5%; Confidence interval 0.18–0.28; $P < 0.001$)]. The use of UCPI% >80% to predict DJS had a sensitivity, specificity, PPV, and NPV of 100%.

Conclusion: Based on the results from our study, we propose sequencing of the *ABCC2* gene in neonates with normal ALT, presence of cholestasis and UCP1% >80%.

Keywords: *ABCC2* gene, Dubin-Johnson syndrome, neonatal cholestasis, Saudi Arabia, urinary coproporphyrins

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INTRODUCTION

Dubin-Johnson syndrome (DJS) results from pathologic mutations in the ATP-binding cassette-subfamily C member 2 (*ABCC2*) gene that leads to absent, reduced expression or impaired function of a transporter protein known as

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multidrug resistance-associated protein 2 (MRP2).[1] The MRP2 protein is located on the canalicular membrane of hepatocytes and is important in the excretion of conjugated bilirubin from hepatocytes into bile.[2] In addition, coproporphyrins (CP) in plasma, the intermediates of heme-biosynthesis, are excreted predominantly in bile via MRP2.[3] Coproporphyrins exist in two isomeric forms [isomer I (CP I) and isomer III (CP III)] with MRP2 having a higher affinity for isomer I than for isomer III.[4] Animal studies showed that under physiologic conditions, CP I level in bile is 3 times CP III level resulting in a biliary CP I/(I + III) ratio >70%; the remaining CPs in plasma (mostly CP III) are excreted in urine via MRP2 in kidneys.[3] Hence, the resulting urinary CP I/(I + III) ratio in normal individuals is <30%. However, when MRP2 is absent or non-functional, this leads to a switch in excretion pattern, from excretion in bile to excretion in urine resulting in a urinary CP I/(I + III) ratio >80%.[3]

DJS has rarely been reported to present with neonatal cholestasis (NC).[5,6] The differential diagnosis of NC is very broad and identification of etiology is challenging to clinicians because the list includes many entities with overlapping clinical, biochemical, and histological features. Furthermore, the diagnostic workup must be completed in a short time, since some treatable conditions need prompt medical or surgical intervention. Because of these challenges, clinicians need helpful clinical and biochemical clues that assist in the approach of these patients. The pattern of CPs excretion and the measured CP I/(I + III) ratio in urine was reported to be a potential biomarker that correlates with MRP2 activity.[7] The usefulness of the urinary CP I/(I + III) ratio to help diagnose neonatal-onset DJS has been investigated in a single study that included four cases of DJS.[6] In our case-control study, we investigated the utility of the urinary CP I/(I + III) ratio as a potential diagnostic biomarker in 28 cases of neonatal-onset DJS in comparison to 20 cases of NC due to other etiologies. Based on the findings from our study, we have proposed a diagnostic pathway to facilitate the molecular diagnosis of DJS.

PATIENTS AND METHODS

Study settings and design

This was a retrospective case-control study in Riyadh city, the capital of Saudi Arabia, at King Fahad medical city, one of the largest tertiary referral centers for children with liver disorders in Saudi Arabia. We retrospectively reviewed our database of 533 cases of NC [defined clinically as the presence of jaundice with conjugated bilirubin of >17 μmol/l] that were presented to our center during the period from 2008 until 2019. We identified neonates with a disease-causing mutation in the *ABCC2* gene.

Hospital protocol in investigating NC

In our protocol, we adopted a stepwise approach with high priority to promptly diagnose and treat treatable disorders. Our initial assessment of neonates with cholestasis included a detailed history, physical examination, baseline laboratory, serological, and extensive biochemical investigations to diagnose underlying infectious, endocrine, and metabolic causes, and ultrasound abdomen (± magnetic resonance cholangiography) to diagnose biliary anomalies. When the diagnosis remained undetermined after the initial extensive investigations, infants underwent molecular analysis using targeted gene sequencing, if the patient's phenotype was consistent with a specific genetic disease, or cholestasis panel. Earlier studies on DJS showed that serum alanine transaminase (ALT) levels were typically normal.[8-13] This important biochemical finding has shifted our diagnostic approach to request urine coproporphyrins analysis (UCPA) in babies with NC and normal ALT [International units/liter (IU)] to support the diagnosis of DJS.

Study subjects

Twenty-eight neonates with gene-confirmed DJS were diagnosed during the study period (study group). Another 20 neonates with cholestasis due to different etiologies, in whom UCPA was available, were included as a "control group," as illustrated in Figure 1. Twelve of the 20 cases in the control group underwent UCPA because they were initially suspected to have DJS because of normal ALT-cholestasis; the remaining eight neonates had elevated ALT-cholestasis [Figure 1].

Study procedures

a. Data collection
   Medical records were reviewed to collect clinical characteristics, total and direct bilirubin (TSB/DB) [the normal reference is 3.4–17.1 μmol/l and 0–6.8 μmol/l, respectively], liver transaminases, international normalized ratio (INR), gamma-glutamyl transferase (GGT), serum total bile acids, imaging findings, and urinary CP I/CP (I + III) ratio. Our laboratory used different ALT and aspartate transaminase (AST) tests during the study period with different normal cut-off reference ranges [Table 1], therefore in addition to reporting the absolute values of the transaminases, we calculated the proportion of ALT and AST to the upper limit of normal (ULN).
b. UCPA:
   Twenty-four-hour urine samples were obtained during admission for NC workup by urine bags and collected

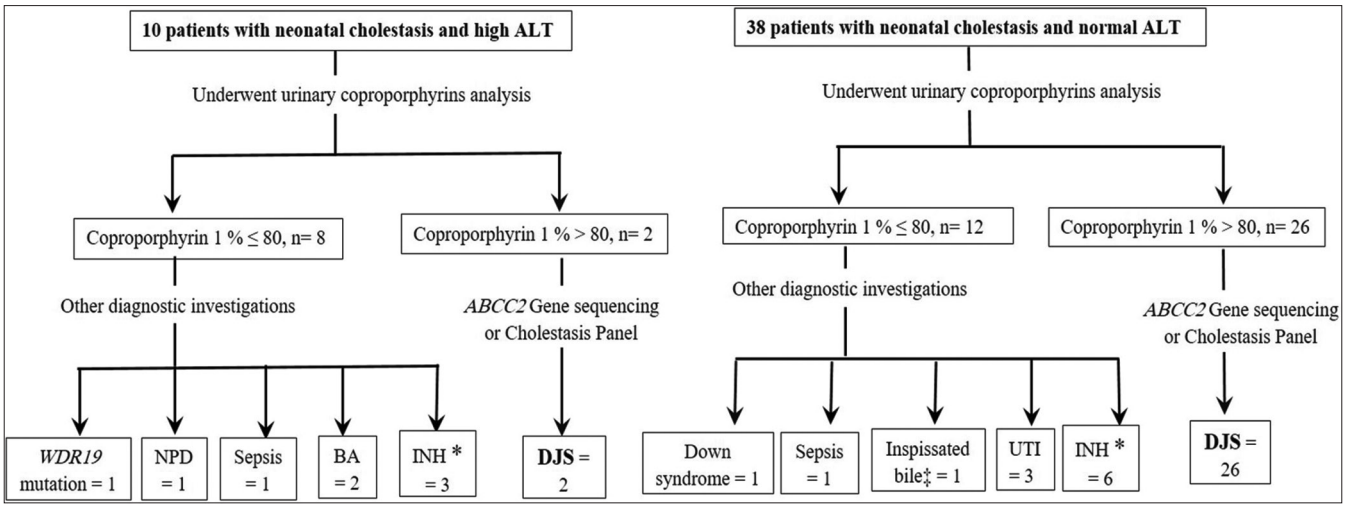

Figure 1: Diagram for the outcome of the 48 samples of urinary coproporphyrins collected over the study period (2008–2019). ALT = Alanine aminotransferase; BA = biliary atresia; INH = idiopathic neonatal hepatitis; NPD = Neiman-Pick disease; UTI = urinary tract infection; * = INH patients underwent cholestasis panel or whole exome sequence. ‡ Inspissated bile due to hemolysis secondary to ABO incompatibility

in a container to which sodium carbonate was added to convert all coproporphyrins to their oxidized forms. Urinary coproporphyrin levels were determined by high-performance liquid chromatography in Mayo Clinic Laboratory. The CP I% was obtained by dividing the peak height (mV) of isomer I by the sum of the peak heights of isomers I and III and multiplying by 100 to obtain a percentage.

c. Molecular Genetic Investigations of *ABCC2*
Throughout the study period, blood DNA samples were examined by targeted sequencing, next-generation sequencing (Jaundice chip or cholestasis panel), or whole-exome sequencing (WES). Missense variations in the *ABCC2* gene that are predicted by PolyPhen-2, Sorting Intolerant From Tolerant (SIFT), or Mutation Taster tools to have a deleterious effect on the biological function of the MPR2 protein were considered pathologic, and confirmed the genetic diagnosis. Variants in the *ABCC2* gene were classified according to the classification guidelines of the American College of Medical Genetics and Genomics. Where available, parental blood DNA samples were analyzed for the mutation variant by Sanger sequencing. All procedures were conducted with informed consent.

Ethical consideration

The local review board has approved the study (number 14-012).

Statistical analysis

Numerical data are given as mean ± standard deviation or median [range, interquartile range (IQR) 1 and 3] as appropriate. To evaluate the utility of normal serum ALT level during the initial laboratory assessment of neonates with cholestasis in predicting the diagnosis of DJS, we identified all patients with normal-ALT neonatal-onset cholestasis that presented to our center during the study period and their final diagnoses, and calculated the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of normal serum ALT level. The diagnostic performance of UCPA and urinary CP I% in diagnosing neonatal-onset DJS was determined by calculating the sensitivity, specificity, PPV, and NPV, and 95% confidence intervals (95% CI). A Mann–Whitney test was performed to compare urinary coproporphyrins (UCP) I% between cases and controls.

RESULTS

The outcome of the biochemical analysis

A total of 78 of 533 cases with NC (14.6%) had normal-ALT during the 12-year study period; the causes of normal-ALT cholestasis are listed in Supplementary Table 1. The diagnoses of all hereditary diseases were confirmed by molecular analysis. All infants with inborn errors of metabolism and characteristic pattern of metabolites on metabolic screening tests were confirmed by molecular genetic diagnosis. Thirty-eight of the 78 patients and 10 more patients with elevated-ALT neonatal onset cholestasis underwent UCPA; the results of UCPA were stratified into two groups: 1) coproporphyrin I% >80% and 2) coproporphyrin I% <80%.

Figure 1 summarizes the outcome of the 48 urine samples submitted for CPA and the diagnoses of the 48 patients. Among the 48 patients with normal-ALT cholestasis, 26 were diagnosed with DJS. Another two patients with neonatal-onset DJS and mildly elevated ALT (1.6 and 2 X ULN) were

Table 1: Clinical and laboratory characteristics and outcome of the 28 neonates with Dubin-Johnson syndrome

Pt	Sex	Family (no.)	Family history of jaundice	Age at onset of jaundice (weeks)	Age at presentation (weeks)	Labs at presentation				UCP 1%	TSB/DB at last FU	Recurrence of jaundice (yes/no)	Last Follow up age (year)
						TSB/DB (μmol/l)	ALT/AST IU/l Proportion to ULN‡	GGT IU/l*	s.bile acid μmol/l				
1	F	A1	Yes	1	5	110/94	31/39 0.56/1.1	145	90	88%	44/29	Yes	8.5
2	F	A2	Yes	1	4	185/159	22/28 0.4/0.8	195	130	92%	23/12	No	5
3	F	B1	No	1	2	131/125	33/43 1/1.3	33	155	94%	NA	NA	7 Lost to FU
4	F	C1	NA	1	6	140/120	39/44 0.6/1.2	90	177	85%	11/9	No	10
5	M	D1	Yes	1	4	216/173	35/62 0.5/1.6	304	158	88%	25/20	No	11.5
6	M	E1	NA	1	4	118/102	65/72 1.6/1.7	55	70	84%	19/16	No	7
7	M	F1	Yes	1	8	174/162	25/32 0.6/0.8	130	48	89%	17/14	No	8.5
8	M	G1	No	1	16	42/31	22/34 0.4/1	22	36	82%	12/9	Yes	3
9	F	H1	No	1	3	55/46	15/26 0.3/0.8	69	77	92%	15/11	No	4
10	F	I1	Yes	1	3	173/140	64/73 1/2	183	117	95%	21/15	No	13
11	F	J1	Yes	1	5	137/111	23/18 0.35/0.5	131	156	86%	40/25	Yes	13
12	M	J2	Yes	1	7	89/78	33/48 0.8/1.2	149	57	82%	82/60	Yes	14
13	M	K1	Yes	1	2	355/270	36/34 0.9/0.9	207	177	94%	NA	No	5.5 Lost to FU
14	F	L1	No	1	2	707/470	14/33 0.2/0.9	148	28	86%	24/16	No	10
15	M	M1	NA	1	5	173/169	47/66 1.1/1.7	68	180	89%	15/13	No	6
16	F	N1	Yes	1	6	150/139	28/44 0.5/1.3	190	110	82%	56/39	Yes	13
17	M	N2	Yes	1	4	240/218	41/46 0.74/1.3	220	174	88%	52/35	Yes	15
18	M	O1	No	1	3	446/314	14/35 0.3/1	295	106	90%	19/15	Yes	2.5
19	M	P1	Yes	1	12	93/77	32/38 0.5/1	113	ND	82%	22/17	NA	13.5
20	F	P2	Yes	1	7	145/108	22/42 0.3/1.1	159	157	84%	20/18	NA	10
21	F	Q1	Yes	1	5	181/172	27/37 0.85/1.1	117	88	96%	30/21	Yes	6
22	M	R1	No	1	18	22/20	34/20 0.5/0.54	20	ND	97%	18/13	Yes	10.5
23	F	S1	Yes	1	2	186/167	18/25 0.6/0.8	87	37	88%	23/19	NA	8
24	M	T1	Yes	1	6	358/292	34/77 0.5/2	282	105	91%	14/11	No	11
25	F	T2	Yes	1	11	257/232	81/88 2/2.7	292	ND	85%	21/17	No	5
26	M	U1	Yes	1	4	212/97	26/46 0.4/1.2	100	72	95%	25/21	Yes	10
27	F	U2	Yes	1	8	160/153	20/38 0.64/1.2	179	49	93%	14/10	Yes	6.5
28	F	V1	No	1	5	178/158	38/44 0.7/1.3	220	ND	84%	52/40	Yes	11.5

TSB/DB=total serum bilirubin/direct bilirubin; ALT=alanine aminotransaminase; F/U=follow-up; GGT=Gamma Glutamyl transferase; M=male; F=female, ND=not done; NA=not available; *Normal values of GGT are as follows <1 month old, GGT <200 IUl; 1-2 month old, GGT <150 IU/l; 2-3 month old, GGT <100 IU/l; >6 months, GGT <60 IU/l[14,15]; ‡Our laboratory used different ALT and AST tests during the study period with different normal cut-off reference ranges, therefore, in addition to reporting the absolute values of the transaminases, we calculated the proportion of ALT and AST to the upper limit of normal (ULN)

diagnosed during the study period, resulting in a total study group of 28 patients *versus* 20 non-DJS patients in the control group. After tabulation of the results of serum ALT levels in the 533 cases with NC on a 2X2 table [Supplementary Table 2], the use of normal serum ALT level to predict for DJS among neonates with cholestasis had a sensitivity of 93%, specificity = 90%, PPV = 34%, and NPV = 99.5%.

Clinical and laboratory characteristics of the cases and controls

Table 1 summarizes the clinical and laboratory characteristics and outcomes of the 28 cases with DJS (from 22 Saudi unrelated families). Consanguinity was noted in all of the 22 families with a female: male ratio of 1:1. Twelve of the families (55%) reported a history of recurrent jaundice in at least one close relative. All patients were products of full-term pregnancy with a median birth weight of 2.7 kg (range, 2.3–4.5 kg) with the onset of jaundice within the first week of life. They presented to our center at a median age of 5 weeks (range, 2–18 weeks). All of the 28 neonates were well-looking at presentation, without hepatosplenomegaly, and had a biochemical profile characterized by normal liver synthetic function. ALT level was within the normal range in 26 patients (92%) and mildly elevated in 2 patients (1.6 times the ULN in patient E1 and 2 times ULN in patient T2). Jaundice resolved in all patients within the first 3–6 months of life. Ursodeoxycholic acid was prescribed in all patients for 3 to 4 months. All of the 28 patients are alive and well after a median follow-up period of 9.25 years (range 2.5–14 years). During the follow-up period, all patients had a persistent mild elevation of TSB/DB on repeated liver function tests, but 12 developed recurrent clinically visible jaundice (43%). Table 2 summarizes the clinical and laboratory characteristics of the 20 non-DJS patients.

Comparison of the cases and controls

A comparison of the 28 patients with neonatal DJS and the 20 patients with other causes of NC revealed no differences in sex ratio, age at presentation, serum bilirubin levels, serum GGT, and serum bile acids [Table 3]. Levels of AST and ALT were significantly lower in infants with DJS ($P = 0.001$ and $P < 0.001$, respectively), while the median of UCPI% was significantly higher in DJS patients [88%, (IQR1–IQR3, 84.2%–92.7%)] than in infants with cholestasis from other causes [67%, (IQR1–IQR3, 61%–71.5%; Confidence interval, 0.18–0.28; $P < 0.001$)] [Figure 2]. After tabulation of the results of UCPA on a 2X2 table, the use of UCPI% >80% to predict DJS among neonates with cholestasis had a sensitivity, specificity, PPV, and NPV of 100% [Supplementary Table 3]. Two of the 20 non-DJS patients (number 12 and 20) had a repeat of UCPA after the neonatal period; in patient 12, the UCPI% dropped to 21% at age 3 months, and in patient 20, the UCPI% dropped to 30% at age 6 months and to 21% at age 21 months.

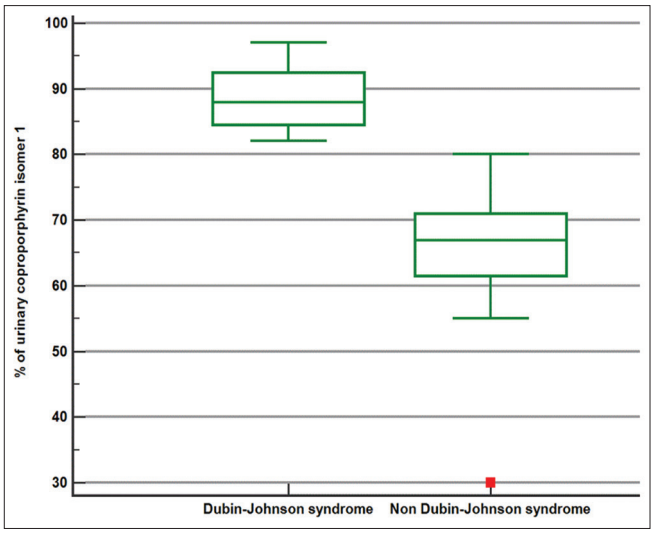

Figure 2: Comparison of urinary coproporphyrin I percentage among Dubin-Johnson syndrome patients and control cases

Molecular analysis

The molecular genetic features of the 28 patients with DJS are summarized in Table 4. We identified four homozygous mutations in the *ABCC2* gene, one is splicing (c.3258+1G>A; p.?) and three are missense mutations; one of the four mutations (in two siblings T1 and 2) is a novel missense mutation [c.1594G>A(p.Glu532Lys)]. All of the four mutations are considered pathologic or likely pathologic according to the American College of Medical Genetics and Genomics, or by using in-silico predictions.

DISCUSSION

This is the largest case series reported to date that describes the clinical, biochemical, and molecular features of 28 neonatal-onset DJS patients and compares them to 20 neonates with cholestasis due to other etiologies. Our study highlights several important observations. First, the results of our study provide further evidence of the very high accuracy of UCPA in diagnosing neonatal-onset DJS and differentiating DJS from other causes of NC; the UCPI fraction of >80% was 100% specific and sensitive for DJS. Second, the high sensitivity, specificity, and NPV of normal serum ALT level makes this test an important biochemical marker in the initial diagnostic evaluation of NC; normal serum ALT level is useful to direct the investigations toward DJS, while ALT level >2X ULN makes the DJS diagnosis unlikely. However, mildly elevated ALT (up to 2X ULN) should not exclude DJS diagnosis. Another interesting

Table 2: Clinical and laboratory characteristics of 20 non-Dubin-Johnson syndrome neonates

No.	Sex	Age at onset of symptoms (weeks)	Age at presentation (weeks)	Diagnosis	Labs at presentation				UCPI%
					TSB/D (μmol/l)	ALT/AST (IU/l) Proportion to ULN	GGT (IU/l)*	s.Bile acid μmol/l	
1	M	1	4	INH	187/165	55/58 0.84/1.5	20	110	73%
2	F	2	5	INH	254/207	50/85 1.6/2.6	243	98	70%
3	F	1	2	INH	218/172	56/47 0.86/1.3	55	119	63%
4	M	2	6	UTI	54/46	14/36 0.25/1	90	78	60%
5	F	1	3	UTI	161/93	31/33 0.5/0.9	329	145	60%
6	M	1	5	INH	155/92	52/53 0.95/1.5	139	93	55%
7	M	1	3	UTI	77/54	7/46 0.2 1.2	47	67	57%
8	M	2	11	Sepsis	84/82	45/58 1.1/1.5	230	56	68%
9	F	2	20	Sepsis	481/363	55 1.8	58	139	30%
10	F	1	2	INH	121/96	21/41 0.7/1.3	133	180	67%
11	F	1	2	Inspissated bile§	96/55	43/49 0.67/1.3	163	198	70%
12	M	1	2	Down syndrome	195/57	35/53 0.54/1.4	138	76	UCPI% # 1 (age 3 wks)=65% UCPI% # 2 (age 12 wks)=21%
13	F	1	4	Niemen-Pick disease	529/437	109/574 3.5/18	22	113	67%
14	F	1	8	Biliary atresia	151/123	579/494 18.6/14	163	157	67%
15	F	1	5	Biliary atresia	204/172	167/303 5.3/9.5	730	155	67%
16	M	1	4	INH	308/224	170/358 5.4/11	98	180	80%
17	M	2	6	INH	150/139	151/161 3.6/4.2	48	157	67%
18	M	1	9	WDR19 gene mutation	381/336	814/1189 20/31	65	144	75%
19	M	1	4	INH	137/107	36/48 0.55/1.3	ND	113	73%
20	M	1	3	INH	167/110	60/108 0.9/2.9	139	91	UCPI% # 1 (age 7 wks)=72% UCPI% # 2 (age 24 wks)=30% UCPI% # 3 (age 84 wks)=23%

INH=idiopathic neonatal hepatitis; ALT=alanine aminotransaminase; GGT=gmma glutamyl transferase; M=male; F=female, ND=not done; mo=month; TSB/DB=total serum bilirubin/direct bilirubin; UCP 1%=urinary Coproporphyrins 1 percentage; wk=weeks; *Normal values of GGT are as follows <1 month old, GGT <200 IUl; 1-2 month old, GGT <150 IU/l; 2-3 month old, GGT <100 IU/l; >6 months, GGT <60 IU/l.[14,15]; ‡Our laboratory used different ALT and AST tests during the study period with different normal cut-off reference ranges, therefore in addition to reporting the absolute values of the transaminases; we calculated the proportion of ALT and AST to the upper limit of normal (ULN); §=Inspissated bile due to hemolytic anemia secondary to ABO incompatibility

observation is that the measurements of the UCPI% in the 20 patients with NC, not due to DJS, demonstrated wide interindividual variability, with a median of 67% (range, 30–80%), which is intermediate between those of healthy adults (20 to 30%)[16] and patients with DJS (>80%).

In this study, we demonstrate the typical phenotype of patients with neonatal-onset DJS that is characterized by being well at presentation, normal-ALT direct hyperbilirubinemia since the first week of life which resolved within 3 to 6 months of age, followed by a benign course that was punctuated in some patients by recurrent episodes of jaundice on long term follow-up, and a direct bilirubin fraction that does not normalize between episodes. This phenotype during the neonatal period overlaps with a broad list of causes of NC, which makes the identification of DJS challenging for clinicians. Some neonates with DJS underwent invasive procedures (liver biopsy and intraoperative cholangiography) to exclude biliary atresia (BA), a scenario that happened frequently in some reported case series.[5,17,18] Hence, early consideration and prompt diagnosis of DJS is a very important step

Table 3: Comparison of 28 Dubin-Johnson syndrome neonates to 20 non-Dubin-Johnson syndrome neonates

Variables	Dubin-Johnson syndrome patients (*n*=28)	Non-Dubin-Johnson syndrome patients (*n*=20)	Confidence interval	*P*
Gender (M: F ratio)	13 (46.4%)/15 (53.6%)	11 (55.0%)/9 (45.0%)	-	0.385
Age at presentation (weeks) median (IQR 1-3)	5 [3.25-7]	4 [3-6]	[-1.92-2.98]	0.473
Total serum bilirubin (μmol/l) median (IQR 1-3)	173 [121.25-215]	164 [125-245]	[-89.68-66.75]	0.691
Direct bilirubin (μmol/l) median (IQR 1-3)	146.5 [98.25-172.75]	116.5 [84.5-198.25]	[-60.14-62.99]	0.645
ALT (u/l) median (IQR 1-3)	31.5 [22-37.5]	53.5 [35.25-140.5]	[-191.99–1.08]	0.001
AST (u/l) median (IQ1 I-3)	38.5 [33-44.75]	58 [48.25-267.5]	[-284.5–18.16]	<0.001
GGT (u/l) median (IQR 1-3)	146.5 [87.75-204]	133 [55-163]	[-85.94-79.84]	0.374
Serum bile acids (μmol/l) median (IQR 1-3)	105.5 [60.25-156.75]	116 [91.5-156.5]	[-44.91-10.85]	0.238
% of UCPI median (IQR 1-3)	88% [84.25-92.75%]	67% [60.75-71.5%]	[0.18-0.28]	<0.001

UCPI%=urinary coproporphyrins 1 percentage; IQR=interquartile range

in the workup of a neonate with cholestasis, to avoid subjecting a patient with a benign prognosis to unnecessary invasive and costly evaluation. In contrast to the vast majority of causes of NC, DJS is not associated with obvious liver injury, as evidenced by normal ALT, normal INR, and the absence of hepatosplenomegaly. There is a subgroup of patients with NC and normal ALT who manifest an overlapping phenotype with DJS (as shown in Supplementary Table 1) including urinary tract infection, sepsis, inspissated bile due to hemolysis, tyrosinemia, and parenteral nutrition-associated cholestasis. These diagnoses are readily identifiable by following a structured, stepwise diagnostic approach that incorporates clinical assessment and laboratory investigations (e.g., urine and blood cultures, complete blood count and reticulocytes, and metabolic workup). Therefore, based on our data, we propose a diagnostic pathway to improve the diagnosis of DJS [Figure 3] that entails screening of a well-looking neonate with normal ALT-cholestasis, not due to infection or hemolysis, for DJS. UCPA is the most reliable non-invasive screening test with a very high diagnostic performance; UCP1% >80 is consistent with DJS and should lead to direct sequencing of the *ABCC2* gene and further diagnostic procedures in neonates with cholestasis could be skipped. This approach could reduce the cost by making an earlier diagnosis of DJS and avoiding unnecessary costly investigations of other etiologies of NC, and guiding physicians to perform direct testing of the *ABCC2* gene instead of the more costly cholestasis panel or whole exome sequence.

The UCPI% has been used as a biomarker of *ABCC2* function in clinical studies because it provides quantitative information about the *in vivo* activity of MRP2.[7] The UCPI% showed trimodal distribution based on the function of MRP2 protein being high in DJS patients (>80%), normal in healthy children and adults (<30%), and intermediate (30–80%) in healthy infants and in individuals who carry a heterozygous mutation in the *ABCC2* gene.[7,16,19,20] Our data and data from other studies[6] provide proof that the UCPI% in young infants with cholestasis not due to DJS, follows similar excretion percentages to healthy non-cholestatic neonates,[20] but none showed values above the diagnostic cut-off for DJS (>80%). In the few cases when UCPA was repeated, the UCPI% dropped sharply after the first 3 months and approached the adult values (<30%) by 4 to 6 months of life. This observation could be due to the physiologically lower MRP2 activity during the first 3 months of life. One caveat clinicians need to be aware of is that UCPI% can be increased in hemolysis, anemia, congenital erythropoietic porphyria, or due to drugs (e.g., rifampicin).[21,22]

Table 4: The four pathologic variants in the *ABCC2* gene

Family ID	*ABCC2* variant	Zygocity	Type	Frequency in SHGP	Frequency in gnomAD	Classification (ACMG guidelines)	Prediction of pathogenicity			Variant Novelty
							Polyphen2	SIFT	Mutation Taster	
A1 and S1	NM_000392.5: c. 2273G>T; p.Gly758Val	Homozygous	Missense	15/2936	1/251268	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Reported (PMID: 31544333)
T1 and T2	NM_000392.5: c. 1594G>A; p.Glu532Lys	Homozygous	Missense	Absent	Absent	Likely pathogenic	Probably damaging	Deleterious	-	Novel
U1 and U2	NM_000392.5: c. 3258+1G>A; p.?	Homozygous	Splicing	1/2936	21/282862	Pathogenic	Pathogenic	-	-	Reported (PMID: 23429660)
V1	NM_000392.5: c. 2439G>C; p.Lys813Asn	Homozygous	Missense	Absent	Absent	Likely pathogenic	Probably damaging	Deleterious	Disease causing	Novel

ACMG=American Academy of Medical Genetics; genomAD=Genome Aggregation Database; SHGP=Saudi Human Genome Project

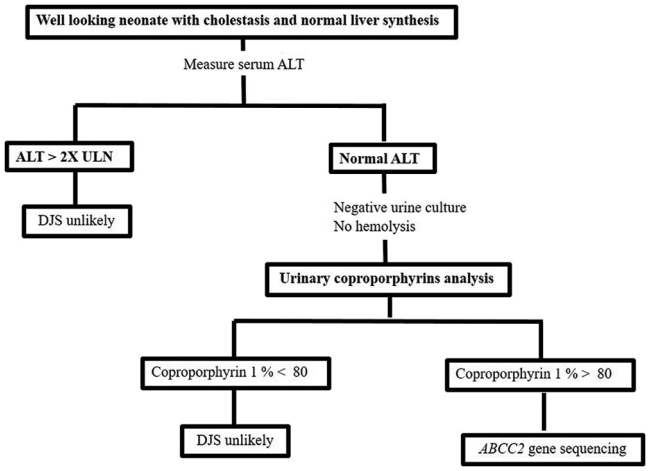

Figure 3: Simplified diagnostic pathway to diagnose Dubin-Johnson syndrome in a neonate with cholestasis. Urinary coproporphyrins 1% = coproporphyrins 1/total of coproporphyrins 1 + 3

The mutational spectrum of the *ABCC2* gene in DJS patients varies across different ethnic groups.[5,8,11,17] Our molecular analysis of the *ABCC2* gene showed that the p.Gly758Val variant is the most common variant in Saudi patients, affecting 19 of 22 families (86%) with DJS. This variant is unique to the Saudi population and found in several major tribes in the center of the Arabian peninsula, with a cumulative carrier frequency of 0.0051 (1 in 195), strongly suggesting that this variant is of common ancestral origin, which accounted for the observed cluster. For this reason, genetic testing in DJS in Saudi Arabia could be undertaken via targeted genotyping of the predominant variant (starting with p.Gly758Val) for rapid molecular analysis, particularly in patients originating from specific tribes.

In conclusion, a finding of UCPI% >80 in a neonate with normal ALT-cholestasis is suggestive of DJS and should lead to direct sequencing of the *ABCC2* gene.

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Conflicts of interest

There are no conflicts of interest.

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Supplementary Table 1: Causes of normal ALT-Neonatal cholestasis (*n*=78)

Diagnosis	Number of cases
Dubin-Johnson syndrome	26
Idiopathic neonatal hepatitis	15
Urinary tract infection	9
Tyrosinemia	6
Sepsis	3
Inspissated bile due to hemolysis	2
TPN-associated cholestasis	2
Caroli disease	2
Leukemia	2
USP53 Gene mutation (TJP mutation)	1
ARC syndrome	1
Edward Syndrome	1
Down syndrome	1
Joubert syndrome	1
Mitochondrial hepatopathy	1
Cystic fibrosis	1
Transaldolase deficiency	1
Gestational alloimmune liver disease	1
Gaucher disease	1
Cortisol deficiency	1
Total	78

Supplementary Table 2: Sensitivity and specificity of normal serum ALT in predicting the diagnosis of 28 cases of neonatal onset Dubin-Johnson Syndrome

	Diagnosis of Dubin-Johnson Syndrome		*P*
	Yes	No	
Normal ALT	(a)26	(c)52	78
Elevated ALT	(b) 2	(d)453	455
Total	28	505	533

$$\text{Sensitivity} = \frac{a}{a+b} \approx 26/28 = 93\%$$

$$\text{Specificity} = \frac{d}{c+d} \approx 453/505 = 89.7\%$$

$$\text{Positive predictive value} = \frac{a}{a+c} \approx 26/78 = 34\%$$

$$\text{Negative predictive value} = \frac{d}{b+d} \approx 453/455 = 99.5\%$$

Supplementary Table 3: Sensitivity and specificity of Urinary coproporphyrin >80% in predicting the diagnosis of DJS

	Diagnosis of Dubin-Johnson syndrome		
	Positive	Negative	
Urinary coproporphyrin >80%	(c)28	(c) 0	28
Urinary coproporphyrin <80%	(d)0	(e)20	20
Total	28	20	48

$$\text{Sensitivity} = \frac{a}{a+b} \approx 28/28 = 100\%$$

$$\text{Specificity} = \frac{d}{c+d} \approx 20/20 = 100\%$$

$$\text{PPV} = \frac{a}{a+c} \approx 28/28 = 100\%$$

$$\text{NPV} = \frac{d}{b+d} \approx 20/20 = 100\%$$