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Genetic contribution of *ABCC2* to Dubin-Johnson syndrome and inherited cholestatic disorders

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Abstract

Background and Aims: The *ABCC2* gene is implicated in Dubin-Johnson syndrome (DJS), a rare autosomal recessive liver disorder. The primary aim of this study was to determine the diagnostic value of *ABCC2* genetic testing in the largest cohort of DJS reported to date. The high number of patients with cholestatic manifestations in this series prompted us to evaluate the genetic contribution of rare, potentially pathogenic *ABCC2* variants to other inherited cholestatic disorders.

Methods: The cohort study included 32 patients with clinical DJS diagnosis, and 372 patients referred for the following disorders: low phospholipid-associated cholelithiasis (LPAC) syndrome, intrahepatic cholestasis of pregnancy (ICP) and benign recurrent intrahepatic cholestasis (BRIC). *ABCC2* was screened by next-generation sequencing.

Results: Most patients with clinical DJS had positive genetic diagnosis (n = 30; 94%), with a great diversity of point mutations and copy number variations in *ABCC2*. Strikingly, eight (27%) of these patients showed transient cholestatic features at presentation: four neonatal cholestasis, two ICP, one contraceptive-induced cholestasis and one sporadic cholestasis. Conversely, the frequency of rare, heterozygous, potentially pathogenic *ABCC2* variants in patients with LPAC, ICP or BRIC did not differ significantly from that of the general population.

Conclusions: This large series reveals that DJS is a highly homogeneous Mendelian disorder involving a large spectrum of *ABCC2* variants. Genetic testing is crucial to establish early DJS diagnosis in patients with atypical presentations, such as neonatal cholestasis. This study also provides no evidence for the contribution of rare, potentially pathogenic *ABCC2* variants to other inherited cholestatic disorders.

KEYWORDS

ABCC2, canalicular transporter, Dubin-Johnson syndrome, genetics, neonatal cholestasis

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BRIC, benign recurrent intrahepatic cholestasis; c.DNA, coding DNA; CNV, copy number variations; DJS, Dubin-Johnson syndrome; GGT, gamma glutamyl transpeptidase; ICP, intrahepatic cholestasis of pregnancy; LPAC, low phospholipid-associated cholelithiasis; NGS, next-generation sequencing; PFIC, progressive familial intrahepatic cholestasis; UDCA, ursodeoxycholic acid.

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1 | INTRODUCTION

Dubin-Johnson syndrome (DJS) is a rare autosomal recessive liver disorder characterized by chronic jaundice and predominantly conjugated hyperbilirubinaemia, without other features of hepatobiliary disease.[1] Patients also display an increase in the urinary excretion of coproporphyrin isomer I, a metabolic by-product of haem synthesis. There is no ultrasound liver anomaly and the liver function is normal. Finally, when liver biopsy is performed, histology reveals black-brown granular pigments in the cytosol of hepatocytes. The *ABCC2* gene was found to be implicated in DJS in 1997, following a candidate gene approach.[2,3]

This gene comprises 32 exons,[4] and is mainly expressed in the liver. It encodes a 190-kD glycoprotein called ABCC2 or MRP2, mainly localized in the canalicular or apical membrane of hepatocytes,[5] and belonging to the ATP-binding cassette transporter subfamily C.[6,7] ABCC2 mediates the ATP-dependent transport of a broad range of endogenous compounds including anionic conjugates, such as bilirubin glucuronides and xenobiotics, from hepatocytes into bile.[8] ABCC2 is also involved in the resistance of cancer cells to multiple chemotherapeutic drugs.[9,10]

Dubin-Johnson syndrome affects individuals of all ethnic origins. A study of 17 families from Iranian and Moroccan Jewish descent revealed that DJS is quite common in these populations (prevalence of 1/1300 individuals),[11] due to two distinct variants with a founder effect.[12] Most studies reporting *ABCC2* variants in DJS consist in case reports. As a corollary, the number of disease-causing variants reported to date in *ABCC2* is limited and cohort studies of DJS are still lacking.

Notably, variants in genes encoding several other hepatocyte canalicular transporters are responsible for paediatric liver disorders with a true Mendelian inheritance mode. In this regard, *ABCB4*, *ABCB11* and *ATP8B1* are implicated in different autosomal recessive forms of progressive familial intrahepatic cholestasis (PFIC). Variants in these genes have also been shown to constitute strong susceptibility factors to polygenic adult-onset liver diseases.[13] Along these lines, *ABCB4* has been involved primarily in the low phospholipid-associated cholelithiasis (LPAC) syndrome, when *ABCB11* and *ATP8B1* have been implicated in benign recurrent intrahepatic cholestasis (BRIC). Mutations in each of these transporters can also be associated with intrahepatic cholestasis of pregnancy (ICP).[14] As for *ABCC2*, if its causative role in the autosomal recessive DJS is well-established, its implication in ICP is debated,[15-17] and its contribution to other adult-onset cholestatic or cholelithiasic disorders has not been investigated. Nevertheless, a number of studies suggested that ABCC2 could play a significant role in cholestatic processes. First, ABCC2 transports divalent glucuronidated and sulphated bile acid conjugates.[18] *ABCC2* expression is decreased in patients with obstructive cholestasis.[19] In addition, rodent models of cholestasis display a decrease in *ABCC2* expression,[20,21] and an altered subcellular localization of this transporter.[22-25] More recently, several studies reported cholestasis in patients with neonatal DJS.[26-28]

Key points

- Dubin-Johnson syndrome (DJS) is a rare genetic liver disorder due to variants in the *ABCC2* gene.
- We report the largest cohort of DJS and show that, contrary to the initial disease description, DJS can present with transient cholestatic manifestations, notably in newborns.
- However, rare *ABCC2* pathogenic variants do not confer susceptibility to several other inherited cholestatic disorders.
- This has implications for diagnosis and genetic counselling.

Here, we describe the largest series reported so far of DJS patients, who were referred to the main French reference laboratory for genetic disorders of hepatobiliary transporters. We paid particular attention to patients with cholestatic manifestations, which are not classically considered as part of the clinical spectrum of the disease. We also evaluated the contribution of rare, heterozygous, potentially pathogenic variants of *ABCC2* in three cholestatic and/or cholelithiasic disorders with a strong genetic component, including ICP, LPAC and BRIC.

2 | METHODS

2.1 | Patients

All patients were referred to the French reference laboratory for genetic disorders of biliary transporters (Saint-Antoine Hospital, Paris). We investigated patients with the following clinical presentations: DJS (n = 32), LPAC (n = 192), ICP (n = 97), BRIC (n = 83). They were unrelated, except for patients 13 and 14, who were siblings. The clinical diagnosis of DJS was based on the following criteria: prolonged jaundice with predominantly conjugated hyperbilirubinaemia, no or very few alterations in liver enzyme activities, normal liver imaging and absence of any other cause of jaundice. Diagnosis of LPAC was retained in the presence of at least two of the following criteria: age at first symptoms (ie biliary pain) <40 years, recurrence of symptoms after cholecystectomy and evidence of intrahepatic hyperechoic foci or sludge or microlithiasis along the biliary tree on imaging. The diagnosis of BRIC was based on the following criteria: intermittent episodes of cholestatic jaundice with severe pruritus, normal or low

(<100 U/L) levels of gamma glutamyl transpeptidase (GGT) activity, and normal imaging of the liver and biliary tract. The diagnosis of ICP was established based on the following signs: pruritus starting during pregnancy, elevated serum levels of aminotransferases and total bile acids, normal imaging of the liver and absence of any other cause of hepatic cytolysis or pruritus.

Informed written consent was obtained from each individual. This study was approved by the Comité de Protection des Personnes Ile-de-France 5 (Paris, France).

2.2 | *ABCC2* screening

Genomic DNA was extracted from peripheral blood leucocytes using standard procedures. *ABCC2* was screened by next-generation sequencing (NGS) together with a gene panel. Exons and flanking intronic sequences of *ABCC2* were captured from fragmented DNA with the SeqCapEZ enrichment protocol (Roche NimbleGen). Paired-end massively parallel sequencing was achieved on a MiSeq platform (Illumina). Bioinformatic analysis was performed using the Sophia Genetics DDM pipeline®. Variants were confirmed either by Sanger sequencing (Big Dye Terminator sequencing kit; SeqScape v2.7 software - Applied Biosystems) or by quantitative PCR (SYBR green; ViiA™7 real-time PCR system - Applied Biosystems).

2.3 | Interpretation of variants

A variant was retained as potentially pathogenic according to several criteria: (a) alteration in *ABCC2* coding sequence (deletion, insertion, nonsense, missense, frameshift or essential splice site variants); (b) minor-allele frequency (MAF) <0.01 in control populations from public databases (ExAC, Exome Aggregation Consortium, http://exac.broadinstitute.org/); (c) for missense variants, prediction of pathogenicity by two independent softwares: SIFT v4.0.3 (http://sift.jcvi.org/) and Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/). The reference sequences used to report variants were NM_000392.4 for the *ABCC2* coding DNA (c.DNA) and NP_000383.1 for the protein sequence.

2.4 | Statistical analyses

Frequencies of variants observed in patients and in the general population were compared using the chi-squared test with Yates' correction or the Fisher's exact test when appropriate. A $P \leq .05$ was considered significant.

3 | RESULTS

3.1 | Genetic testing of *ABCC2* in patients with manifestations of DJS

As part of the French reference centre for inflammatory biliary diseases and autoimmune hepatitis, our laboratory has been the only genetic department performing *ABCC2* analysis in France until 2015. We screened this gene in 32 patients with a presentation of DJS. All patients were unrelated, except for patients 13 and 14, who were siblings. Twenty-two of these patients (69%) were referred by hepatology departments, six by genetic centres (19%) and four (12%) by other departments. Six queries for genetic testing (19%) were sent by clinicians from foreign countries. The diagnosis of DJS was confirmed by the identification of two *ABCC2* mutated alleles in 30 subjects (94%) (patients 1-30) (Table 1). Patient 31 was diagnosed with Rotor syndrome based on the identification of a homozygous deletion comprising the whole *SLCO1B1* gene and part of the *SLCO1B3* gene (exons 4-16). No molecular explanation was identified in patient 32.

3.2 | *ABCC2* mutational spectrum

Among the 30 patients with genetically confirmed DJS diagnosis, 17 patients (57%) were homozygotes and 13 (43%) compound heterozygotes for *ABCC2* variants. We identified 29 different molecular defects, including 18 new pathogenic variants and 11 variants previously reported in the literature. There was a great diversity in the nature of *ABCC2* pathogenic variants: seven missense variants (24%), eight nonsense variants (28%), one splice site variation (3%), 11 small deletions or insertions (<10 pb) (38%) and two large deletions involving more than one exon (7%). Among the 29 molecular defects identified, four were found in several individuals: p.Gly758Val (n = 2), p.Arg768Trp (n = 7), p.Arg1066* (n = 6) and p.Ile1173Phe (n = 2). As shown in Figure 1, variants were spread throughout the *ABCC2* gene.

Since we used the same gene panel to screen all patients referred for a suspicion of inherited liver disorder, we looked if any patient had a genetic diagnosis of DJS (ie carrier of biallelic variants in *ABCC2*) and another clinical presentation. Four patients presented such a genotype and all of them carried the same duplication of *ABCC2* exons 24 and 25 (c.3259_3614dup). A 24-year old lady carried this duplication in the homozygous state. She came to the hospital for gallstone-related acute pancreatitis. At that time, she had altered liver function tests (GGT: 700 U/L, alkaline phosphatase (ALP): 184 U/L, alanine aminotransferase (ALT): 1000 U/L), which subsequently normalized. The levels of total (23 µmol/L) and direct (15 µmol/L) bilirubin were moderately elevated, and were not associated with jaundice. In three additional patients with cholestatic and/or cholelithiasic disorders, we identified the same duplication of exons 24 and 25 in the heterozygous state in association with a second potentially pathogenic *ABCC2* variant. None of these patients had clinical manifestations evocative of DJS, suggesting that this copy number variation (CNV) is not a molecular defect causing DJS. No other patient with a genetic diagnosis of DJS was identified.

3.3 | Characteristics of patients with DJS and *ABCC2* positive genetic testing

Among the 30 patients with positive genetic diagnosis, the male to female ratio was 15/15, consistent with the autosomal recessive transmission of the disease. The mean age at onset of symptoms was 11 ± 3 years, whereas the mean age at diagnosis was 24 ± 3 years (Table 2). The mean concentrations of total and direct serum bilirubin

TABLE 1 Genotype at the *ABCC2* locus and characteristics of variants in patients with DJS

Patients with a homozygous genotype							
Patient number	**Sex/ age (years)**	**Origin**	***ABCC2* variant name (cDNA)/ (protein)**	**Location**	**ExAC frequency**	**Conservation throughout evolution**	**Previous report in DJS**
1	F/ 31	France	c.226_228del p.Leu76del	Exon 3	2.10^{-5}	Conserved	No
2	F/ 9	Germany	c.415_416del p.Ser139Aspfs*19	Exon 4	0	Na	No
3	M/ 6	Maroc	c.1901_2094del p.Asp634Glyfs*28	Exons 15-16	0	Na	No
4	F/ 3	Sri Lanka	c.2125T>C p.Trp709Arg	Exon 17	8.10^{-6}	Highly conserved	[27,31,43-45]
5	F/ 0.3	France	c.2260del p.Ile754Leufs*6	Exon 17	0	Na	No
6	F/ 10	Germany	c.2273G>T p.Gly758Val	Exon 18	0	Highly conserved	[26]
7	F/ 1	Germany	c.2273G>T p.Gly758Val	Exon 18	0	Highly conserved	[26]
8	M/ 10	Caucasus	c.2302C>T p.Arg768Trp	Exon 18	7.10^{-5}	Highly conserved	[3,4,27-32]
9	F/ 16	North Africa	c.2302C>T p.Arg768Trp	Exon 18	7.10^{-5}	Highly conserved	[3,4,27-32]
10	M/ 64	France	c.2325del p.Tyr776Thrfs*4	Exon 18	0	Na	No
11	M/ 26	India	c.2997G>A p.Trp999*	Exon 22	8.10^{-6}	Na	No
12	M/ 54	France	c.3196C>T p.Arg1066*	Exon 23	4.10^{-4}	Na	[2,32,46,47]
13	M/ 1	Maroc	c.3216dup p.Asp1073*	Exon 23	0	Na	No
14	F	Maroc	c.3216dup p.Asp1073*	Exon 23	0	Na	No
15	M/ 43	France	c.3517A>T p.Ile1173Phe	Exon 25	2.10^{-5}	Highly conserved	[12]
16	F/ 41	Algeria	c.3599G>A p.Trp1200*	Exon 25	0	Na	No
17	M/ 15	North Africa	c.3615_4146del p.Trp1206Thrfs*9	Exons 26-29	0	Na	No

(Continues)

TABLE 1 (Continued)

Patients with a compound heterozygous genotype							
Patient number	**Sex/ age (years)**	**Origin**	***ABCC2* variant name (cDNA)/ (protein)**	**Location**	**ExAC frequency**	**Conservation throughout evolution**	**Previous report in DJS**
18	F/ 29	France	c.334-2_337del p.Leu112Glnfs*37	Exon 4	0	Na	[46]
			c.2736_2737del p.Leu913*	Exon 20	0	Na	[46]
19	F/ 19	France	c.537_542del p.Ile180_Phe181del	Exon 5	0	Na	[46]
			c.3196C>T p.Arg1066*	Exon 23	4.10^{-4}	Na	[2,32,46,47]
20	F/ 29	France	c.697dup p.Met233Asnfs*11	Exon 7	0	Na	No
			c.2302C>T p.Arg768Trp	Exon 18	7.10^{-5}	Highly conserved	[3,4,27-32]
21	M/ 54	Scotland	c.821_822del p.Pro274Argfs*19	Exon 7	0	Na	[48]
			c.1135C>A p.Gln379Lys	Exon 9	0	Moderately conserved	[48]
22	F/ 40	France	c.974C>G p.Ser325*	Exon 8	2.10^{-5}	Na	[49]
			c.2302C>T p.Arg768Trp	Exon 18	7.10^{-5}	Highly conserved	[3,4,27-32]
23	M/ 16	France	c.1505T>G p.Met502Arg	Exon 11	0	Moderately conserved	No
			c.1882C>T p.Arg628*	Exon 14	8.10^{-6}	Na	No
24	M/ 38	France	c.1695T>G p.Tyr565*	Exon 13	0	Na	No
			c.3196C>T p.Arg1066*	Exon 23	4.10^{-4}	Na	[2,32,46,47]
25	M/ 20	France	c.1963C>T p.Arg655*	Exon 15	2.10^{-5}	Na	No
			c.2302C>T p.Arg768Trp	Exon 18	7.10^{-5}	Highly conserved	[3,4,27-32]
26	M/ 16	Turkey	c.2077G>C p.Gly693Arg	Exon 16	8.10^{-6}	Highly conserved	No

(Continues)

TABLE 1 (Continued)

Patients with a compound heterozygous genotype							
Patient number	**Sex/ age (years)**	**Origin**	***ABCC2* variant name (cDNA)/ (protein)**	**Location**	**ExAC frequency**	**Conservation throughout evolution**	**Previous report in DJS**
			c.3517A>T p.Ile1173Phe	Exon 25	2.10^{-5}	Highly conserved	[12]
27	F/ 15	France	c.2302C>T p.Arg768Trp	Exon 18	7.10^{-5}	Highly conserved	[3,4,27-32]
			c.3196C>T p.Arg1066*	Exon 23	4.10^{-4}	Na	[2,32,46,47]
28	F/ 47	Russia	c.2302C>T p.Arg768Trp	Exon 18	7.10^{-5}	Highly conserved	[3,4,27-32]
			c.3196C>T p.Arg1066*	Exon 23	4.10^{-4}	Na	[2,32,46,47]
29	M/ 24	France	c.2391del p.Lys797Asnfs*14	Exon 18	0	Na	No
			c.3741+1G>T p.?	Intron 26	5.10^{-5}	Na	No
30	M/ 20	France	c.3196C>T p.Arg1066*	Exon 23	4.10^{-4}	Na	[2,32,46,47]
			c.4536del p.Ile1513*	Exon 32	0	Na	No

Note: Reference sequences used are NM_000392.4 for *ABCC2* cDNA and NP_000383.1 for the corresponding protein.
Abbreviations: ExAC, Exome Aggregation Consortium; DJS, Dubin-Johnson syndrome; Na, not applicable.

were 58 ± 7 μmol/L and 41 ± 6 μmol/L respectively. The mean direct to total bilirubin ratio was 66 ± 5%. Liver biopsy was performed in only two patients prior to genetic testing and confirmed the diagnosis. The characteristic urinary coproporphyrin excretion pattern (ie elevated proportion of coproporphyrin I over 80% of total coproporphyrin with a normal level of total coproporphyrin) was reported in five patients and was not evaluated in others. Consistent with the typical presentation of DJS, the majority of patients (73%) had no clinical or biochemical features of cholestasis, with neither pruritus nor abnormal levels of ALP, GGT, ALT or aspartate aminotransferase (AST). Nevertheless, eight patients had a history of cholestatic manifestations.

3.4 | History of cholestatic manifestations in patients with DJS

Unexpectedly eight patients with DJS manifestations and positive genetic testing (27%) had a history of cholestatic manifestations (patients 3, 5, 13, 14, 16, 18, 19 and 23). Among them, four patients (patients 3, 5, 13 and 14) presented with transient intrahepatic neonatal cholestasis, as described below. All of them carried *ABCC2* pathogenic variants: patient 3 carried a homozygous deletion of exons 15 and 16 (c.1901_2094del; p.Asp634Glyfs*28); patient 5 carried a homozygous 1-bp deletion in exon 17 leading to a frameshift (c.2260del; p.Ile754Leufs*6); patients 13 and 14 carried a 1-bp homozygous insertion in exon 23, leading to a premature stop codon (c.3216dup; p.Asp1073*). Two patients (patients 16 and 18) were diagnosed in a clinical context of ICP. Patient 16 was referred for jaundice, a slight elevation of total serum bile acid levels (13.1 μmol/L), elevated levels of total (41-86 μmol/L) and direct (31-65 μmol/L) bilirubin, and normal liver enzyme activities. This woman carried an *ABCC2* homozygous nonsense variant (c.3599G>A; p.Trp1200*). Patient 18 displayed persistent jaundice and pruritus during pregnancy, with normal liver imaging and normal liver enzyme activities but a transient increase in total bile acid levels in sera (40.0 μmol/L). She carried two heterozygous deletions in *ABCC2* (c.334-2_337del; c.2736_2737del) leading to a frameshift and a premature stop codon (p.Leu112Glnfs*37; p.Leu913*) respectively. Patient 19 was a 19-year old woman, who presented with jaundice and transient parallel elevation in total serum bile acids (26 μmol/L) following oral contraceptive introduction. She carried a nonsense (c.3196C>T; p.Arg1066*) and an inframe deletion (c.537_542del; p.Ile180_Phe181del) in *ABCC2*. Finally, patient 23 was a 16-year old boy, who started his disease at the age of 12 by an unexplained episode of acute mixed hepatitis (ALT: 357 U/L, ALP: 289 U/L, GGT: 256 U/L). He then recovered normal liver tests, except for isolated hyperbilirubinaemia with elevated serum concentrations of total (71 μmol/L) and direct (35 μmol/L) bilirubin. *ABCC2* screening revealed two pathogenic variants: p.Met502Arg (c.1505T>G) and p.Arg628* (c.1882C>T). Overall, all eight patients, who initially presented with various manifestations of cholestasis, ultimately developed a typical picture of DJS. None of them carried any disease-causing variant in the other genes known to be involved in genetic cholestatic disorders (ie, *ABCB4*, *ABCB11*, *ATP8B1* and *NR1H4*).

3.5 | Neonatal cholestasis in DJS

Since the DJS manifested itself by neonatal cholestasis in patients 3, 5, 13 and 14 (13% of cases), we investigated the disease characteristics in more detail in these patients (Table 3). All four patients presented with jaundice and had elevated levels of direct bilirubin. Acholic stools were observed in patients 5, 13 and 14. In addition, patients 3, 5 and 14 displayed elevated levels of total serum bile acids: 15 μmol/L, 214 μmol/L and 85 μmol/L respectively. In patient 5, cholestasis was also evidenced by histological features of hepatocellular cholestasis. In patients 3 and 5, there was a transient increase in GGT and AST levels. To treat these cholestatic signs, all four patients received ursodeoxycholic acid (UDCA) for a period of 14 months, 1 year, 3 months and 6 years respectively. In all cases, usual causes of neonatal cholestasis (eg infections, biliary atresia, alpha-1 antitrypsin deficiency, progressive familial intrahepatic cholestasis, Alagille syndrome and metabolic diseases) were excluded by appropriate investigations, including screening of other genes responsible for neonatal cholestasis, and liver biopsy when deemed necessary. The cholestatic features resolved in all of them within 6 to 12 months, whereas isolated conjugated hyperbilirubinaemia remained present.

3.6 | Involvement of *ABCC2* heterozygous variants in inherited cholestatic and/or cholelithiasic disorders

Considering the putative role of *ABCC2* in cholestatic processes, we evaluated the contribution of this gene in patients referred for molecular diagnosis of different adult-onset cholestatic and/or cholelithiasic disorders, which are known to be oligogenic. Our cohort included 372 patients with the following clinical presentations: LPAC syndrome (n = 192), ICP (n = 97) and BRIC (n = 83). We focused on rare *ABCC2* variants displaying the characteristics of true disease-causing mutations, as described in the Methods section. Six patients with LPAC were found to carry a potentially pathogenic *ABCC2* variant in the heterozygous state (patients 33-38, Table S1), so that the rate of presumed pathogenic variants in this group was 1.6% (6/384 alleles). To compare this mutation rate with that of the general population, we used the sequencing data set from the ExAC database comprising 60 706 unrelated individuals. Among the 1410 different variants of *ABCC2* identified in the general population, 341 found in 1508 alleles were potentially pathogenic, with a global frequency of 1.2% (1508/121 412). Therefore, there was no enrichment of pathogenic variants in the present LPAC cohort as compared to the general population ($P = .57$). Notably, patient 38 also carried a disease-causing variant in *ABCB4*, thereby providing a first susceptibility factor to the disease. An *ABCC2* potentially pathogenic variant was also identified in one patient with ICP and one patient with BRIC (patients 39 and 40, respectively, Table S1). The comparison of the mutation rate between the ICP (0.5%; 1/194 alleles) and BRIC (0.6%; 1/166 alleles) cohort with the general population did not either reveal any significant difference ($P = .53$; $P = .73$ respectively). Taken together, these data show that there is no overrepresentation of *ABCC2* pathogenic variants in LPAC, BRIC and ICP.

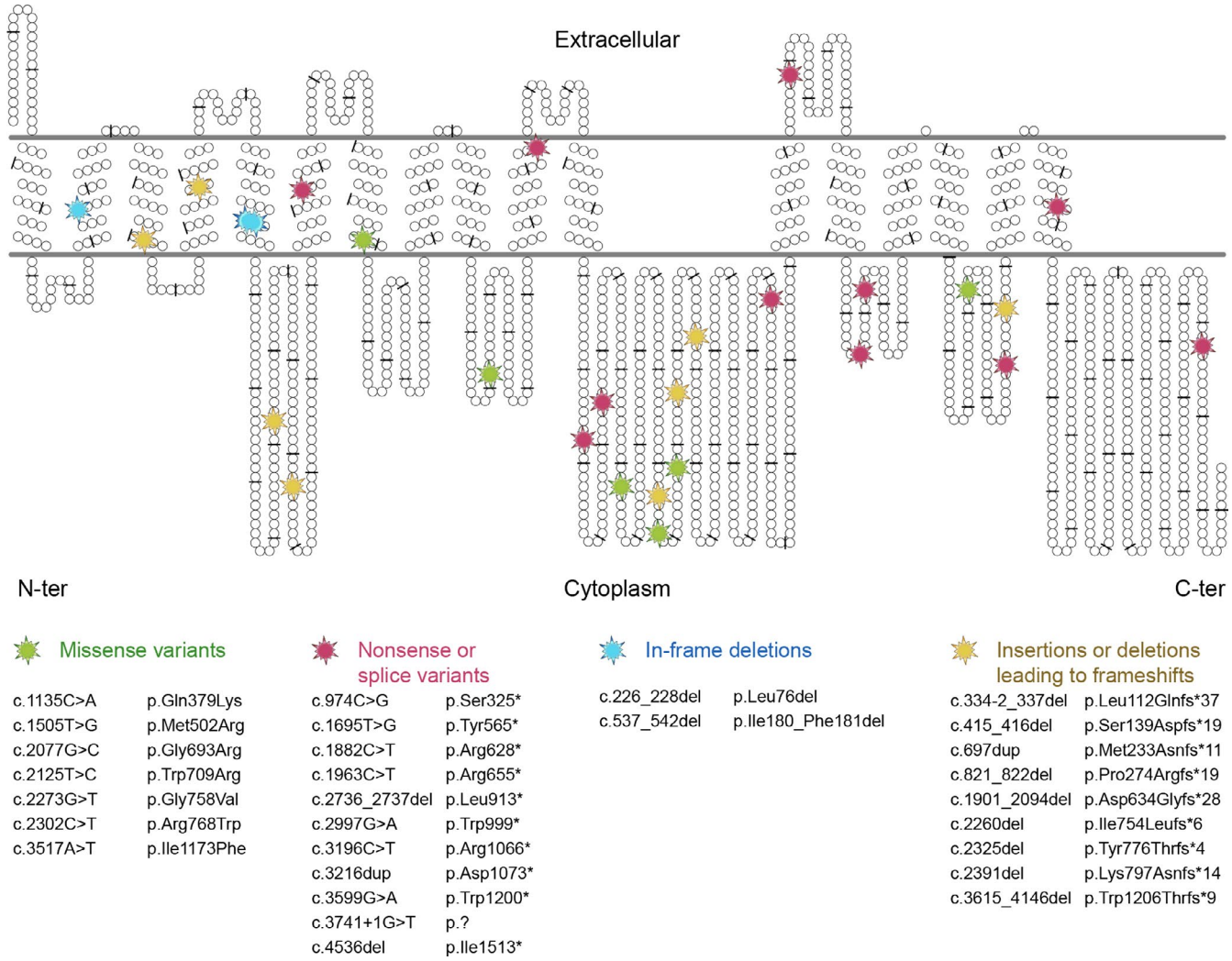

FIGURE 1 Localization in the ABCC2 canalicular transporter of pathogenic variants identified in patients with Dubin-Johnson syndrome (DJS). Coloured stars indicate the position of mutated amino acids identified in the 30 patients with genetically confirmed DJS diagnosis. For frameshift variants, yellow stars point to the localization of the first mutated residue. Black lines separate every ten residues

TABLE 2 Clinical and biological characteristics associated with DJS in patients with confirmed *ABCC2* genetic testing (n = 30)

Male to female ratio	15/15
Age at symptom onset (y)	11 ± 3
Age at diagnosis (y)	24 ± 3
Jaundice at presentation	100%
Total bilirubin level (μmol/L)	58 ± 7
Direct bilirubin level (μmol/L)	41 ± 6

Note: Age and bilirubin levels are expressed as means ± SD.

4 | DISCUSSION

In the present study, 30 independent patients with *ABCC2*-positive genetic testing were investigated, which represents the largest series of DJS reported to date. DJS remains poorly known and underdiagnosed, as attested here by the mean time interval of 13 years between the onset of symptoms and diagnosis. This might be explained by the low prevalence and the pauci-symptomatic nature of the disease.

In total, 94% of patients with a clinical DJS (30/32) carried two *ABCC2* mutated alleles, showing a high genetic homogeneity in this disorder. One patient referred for DJS was proven to have Rotor syndrome. Regarding the remaining patient with no genetic diagnosis, he might carry variants located in the promoter or intronic regions of *ABCC2*, *SLCO1B1* or *SLCO1B3*, that were not detected by the gene panels used. We can also not formally exclude that some clinical forms mimicking DJS are due to variants in other so far unidentified genes. The fact that numerous patients with DJS carry very rare variants in the homozygous state underlines that consanguinity plays a crucial role in this rare autosomal recessive disease. Our study identified 29 molecular defects responsible for DJS. The p.Arg768Trp (c.2302C>T) variant seems to correspond to a mutation hotspot since it was found in seven unrelated cases (23% of patients with positive genetic diagnosis) from different

TABLE 3 Characteristics of DJS patients with neonatal cholestasis

Patient number	Total bilirubin (µmol/L) [0-200:0-14 d] [5-50:14-1 mo] [0-17: >1 mo]	Direct bilirubin (µmol/L) [< 5]	Acholic stools	AST (UI/L) [30-110:0-3 d] [18-27:4 d-3 y]	ALT (UI/L) [3-30:0-3 d] [7-40:4 d-13 y]	GGT (UI/L) [10-270:0-1 m] [7-160:1-4 m] [5-45:4-7 m] [5-25:7 m-15 y]	Total serum bile acids (µmol/L) [<10:0-11 y]	Initiation of UDCA therapy
3	219 (8 d) 60 (3 mo)[a] 26 (4 y)	54 (8 d) 36 (3 mo)[a] 12 (4 y)	No	— 52 (3 mo)[a] 44 (4 y)	— N (3 mo)[a] N (4 y)	340 (3 d) N (3 mo)[a] N (4 y)	— 122 (3 mo)[a,b] 15 (3 y)	Yes
5	115 (1 mo) 26 (4.5 mo)[a] 26 (4 y)	81 (1 mo) — 21 (4 y)	Yes	N (1 mo) 40 (4.5 mo)[a] N (4 y)	N (1 mo) N (4.5 mo)[a] N (4 y)	142 (1 mo) 73 (4.5 mo)[a] N (4 y)	214 (1 mo) 125 (4.5 mo)[a,c] 24 (4 y)	Yes
13	197 (3 d) 74 (2 mo)	40 (3 d) 41 (2 mo)	Yes	— N (2 mo)	N (4 d) N (2 mo)	N (4 d) N (2 mo)	— 8 (2 mo)	Yes
14	88 (birth) 66 (1 mo) 17 (2 y)[a]	— 46 (1 mo) 9 (2 y)[a]	Yes	— N (1 mo) N (2 y)[a]	— N (1 mo) N (2 y)[a]	— N (1 mo) N (2 y)[a]	— 85 (1 mo)	Yes

Abbreviations: [ ], reference values according to the age of patients. The age of patients when observations where made is indicated in brackets; ALT, alanine aminotransferase; AST, aspartate aminotransferase; d, day; F, female; GGT, gamma glutamyl transpeptidase; M, male; mo, month; N, normal; UDCA, ursodeoxycholic acid; y, year.

[a]Corresponding values have been obtained in a patient taking UDCA.

[b]For this value of 122 µmol/L, a chromatographic study indicated that there was 109 µmol/L of UDCA and 13 µmol/L of other bile acid species.

[c]For this measurement, chromatography revealed that there was 107 µmol/L of UDCA and 18 µmol/L of other bile acid species.

origins (Caucasus, France, North Africa and Russia). Eight previous studies reported this variant in 18 patients with DJS, also originating from different countries (Japan and Turkey).[3,4,27-32] The fact that this variant was found in numerous patients from various origins supports the hypothesis of a recurrent mutational event. Consistently, this variant affects a CpG dinucleotide and methylcytosines of CpG are known to spontaneously deaminate to thymine leading to recurrent mutational events.[33] In addition, its frequency has been shown to vary between populations and the number of DJS cases related to this variant has been shown to be especially high in Japan.[27] The same reasoning could apply for the c.3196C>T (p.Arg1066*) variant, which also alters a CpG dinucleotide.

This study also benefited from the development of NGS, which allows the rapid and concomitant study of multiple genes and the identification of molecular defects missed by Sanger sequencing, such as CNV. In this regard, we identified three different CNV in *ABCC2* involving more than one exon: two deletions (exons 15-16, exons 26-29) and one duplication (exons 24-25). If the pathogenicity of exonic deletions in *ABCC2* has been previously reported,[34] the deleterious effect of duplication has not been proven. The herein identified duplication was previously described in the general population at a frequency of 3.10^{-3} (Database of Genomic Variants, reference esv3423829).[35] This CNV was also identified in a patient with ICP but its causality was not established.[36] In our study, it was present in four unrelated patients, who did not present a clinical picture of DJS, strongly arguing against its pathogenic effect.

In most patients with positive genetic diagnosis, the clinical presentation fits perfectly well with the historical description of DJS characterized by predominantly conjugated hyperbilirubinaemia in the absence of cholestasis. Nevertheless, our work allowed us to establish DJS diagnosis in a significant number of patients with transient cholestatic manifestations, especially in pregnant women and neonates. DJS is rarely diagnosed in the neonatal period and for a long time, such cases of neonatal DJS were considered as anecdotal observations.[37] Among clinical cases published prior to the identification of *ABCC2* as the disease-causing gene, a few clinical studies reported patients with neonatal cholestasis[38-41] There is now a growing number of studies reporting neonatal intrahepatic cholestasis in DJS.[26-28,42] In the present study, the diagnosis of neonatal cholestasis was retained by clinicians in four patients based on a combination of various signs: serum-conjugated hyperbilirubinaemia (n = 4), serum bile acid levels >10 µmol/L (n = 3), acholic stools (n = 3), elevated levels of GGT (n = 2) and histological features of cholestasis on liver biopsy (n = 1). This led clinicians to initiate UDCA therapy in all four patients. Our results are consistent with those recently published by Togawa et al, who also showed true neonatal cholestasis evidenced by increased levels of total serum bile acids and additional biological and clinical features in ten patients with a genetically confirmed DJS diagnosis.[27] The *ABCC2* pathogenic variants identified in our cohort and in previous studies of DJS-associated neonatal cholestasis are not the same, so that we have no evidence to indicate that some particular variants are specifically associated with such a neonatal-onset form of the disease. As discussed previously, the exact mechanisms by which a genetic defect in *ABCC2* might lead to transient cholestasis remain unknown, but a role of ABCC2 in cholestatic processes was underlined in several previous publications,[19,20,22-25] and it should be underlined that biliary excretion of specific bile salt species, including sulphated bile acids, is mediated by ABCC2.[18] Overall, these data demonstrate that cholestatic manifestations should not exclude DJS diagnosis, notably in newborns. This has important implications regarding the indication of *ABCC2* genetic testing for accurate diagnosis of neonatal DJS and differential diagnosis with other causes of neonatal cholestasis.

One new challenge for geneticists and clinicians is the interpretation of variants in oligogenic disorders with a complex mode of inheritance. In this regard, the finding of genetic defects found in rare monogenic disorders, such as DJS, might facilitate the study of more common polygenic diseases by identifying subsets of patients with rare highly penetrant variants in multiple genetic loci. Association studies have investigated the role of *ABCC2* common variants in ICP with inconsistent results.[15-17] In the current study, we did not observe any statistical difference in the frequency of rare, potentially pathogenic variants of *ABCC2* in LPAC, ICP or BRIC, as compared to the general population. Overall, these data suggest that *ABCC2* does not play a major role in the pathogenesis of these cholestatic and/or cholelithiasic genetic disorders. This does not formally exclude that some of the identified variants can take part in the pathophysiological process but the genetic contribution of *ABCC2*, if any, is too low to be evidenced.

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CONFLICT OF INTERESTS

All the authors declare to have no competing interest in link with this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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