

ORIGINAL ARTICLE

Clinical phenotype of adult-onset liver disease in patients with variants in *ABCB4*, *ABCB11*, and *ATP8B1*

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Abstract

Variants in *ATP8B1*, *ABCB11*, and *ABCB4* underlie the most prevalent forms of progressive familial intrahepatic cholestasis. We aim to describe variants in these genes in a cohort of patients with adult-onset liver disease, and explore a genotype–phenotype correlation. Patients with onset of liver disease aged above 18 who underwent sequencing of cholestasis genes for clinical purposes over a 5-year period were identified. Bioinformatic analysis of variants was performed. Liver histology was evaluated in patients with variants. Of the 356 patients tested, at least one variant was identified in 101 (28.4%): 46 *ABCB4*, 35 *ABCB11*, and 28 *ATP8B1*. Patients with *ABCB4* variants had chronic liver disease (71.7%) and pregnancy-associated liver dysfunction (75%), with a younger age of onset in more severe genotypes ($p = 0.046$). *ABCB11* variants presented with pregnancy-associated liver dysfunction (82.4%) and acute/episodic cholestasis (40%), with no association between age of onset and genotype severity. *ATP8B1* variants were associated with chronic liver disease (75%); however, they were commonly seen in patients with an alternate etiology of liver disease and variants were of low predicted pathogenicity. In adults with suspected genetic cholestasis, variants in cholestasis genes were frequently identified and were likely to contribute to the development of liver disease, particularly *ABCB4* and *ABCB11*. Variants were often in heterozygous state, and they should no longer be considered recessive Mendelian traits. Sequencing cholestasis genes in selected patients with adult-onset disease should be considered, with interpretation in close collaboration with histopathologists and geneticists.

Deepak Joshi and Richard J. Thompson share joint senior authorship.

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INTRODUCTION

Cholestatic liver disease includes a spectrum of pathophysiological processes and clinical phenotypes, ranging from a pure intrahepatic cholestasis to downstream biliary obstruction. Some patients are diagnosed with a recognized cholestatic syndrome, such as primary sclerosing cholangitis (PSC) or primary biliary cholangitis (PBC), whereas in many a clear diagnosis cannot be made. Progressive familial intrahepatic cholestasis (PFIC) is a group of early-onset cholestatic liver diseases of genetic origin related to variants in genes vital to the transport of biliary constituents and the stability of the canalicular membrane. The most commonly associated genes in PFIC are *ABCB4*, *ABCB11*, and *ATP8B1*. It is now evident that variants in these genes also contribute to later-onset cholestatic phenotypes.^[1]

ABCB4 and *ABCB11* encode proteins in the ABC transporter superfamily. Most of the family members have two highly conserved nucleotide binding sites and two membrane spanning domains. *ABCB4* encodes multidrug resistance protein 3 (MDR3), which “flops” phosphatidylcholine from the inner leaflet to the outer leaflet, where it is used to form mixed micelles with bile acids.^[2] Reduced MDR3 function results in a low bile phospholipid content, a reduction in mixed micelle formation, and consequently an increased free bile acid concentration. In turn, this leads to damage to the biliary epithelium and cholangiopathy, in addition to cholesterol crystal formation.^[3,4] *ABCB11* encodes bile salt export pump (BSEP), which transports bile acids from the cytoplasm of the hepatocyte into the canaliculus. Variants in *ABCB11* result in hepatocellular damage secondary to bile acid accumulation.^[5] *ATP8B1* encodes the familial intrahepatic cholestasis 1 (FIC1) protein, which is an aminophospholipid-transporting P-type ATPase.^[6] FIC1 is expressed on the lipid bilayer of the hepatocyte and “flips” phosphatidylserine from the outer leaflet to the inner leaflet. This contributes to the asymmetry of the lipid bilayer, which is essential for the function of other transporter proteins.^[7] FIC1 is expressed extensively, and variants in *ATP8B1* can result in liver disease as part of a multisystem disorder.^[8]

With an improved understanding of the genes and proteins involved in biliary homeostasis and the expanding access to genetic testing, more patients with adult-onset cholestasis are being tested for the cholestasis genes implicated in PFIC. From the limited data of genetic testing in adult-onset cholestasis, variants in *ABCB4*, *ABCB11*, and *ATP8B1* have been identified, although they usually have less loss of function and in many cases are only heterozygotes.^[1,9–12] The spectrum of phenotypes in adults with variants ranges from episodic cholestasis to advanced chronic liver disease and primary liver cancer.^[10,13] Multiple factors are likely to influence this wide range of clinical presentations, including the amount of residual protein function

and the interaction with environmental exposures such as hormones and drugs.

The American College of Medical Genetics and Genomics and the Association for Molecular Pathology guidelines are used widely by clinical genetics laboratories to classify the pathogenicity of variants.^[14] Classification into the five categories incorporates population frequency, and functional and segregation data. These guidelines have been helpful; however, this approach is conservative in ascribing pathogenicity to a variant and results in many variants being classified as being of uncertain significance. A further challenge in adopting this classification for *ABCB4*, *ABCB11*, and *ATP8B1* in adult-onset liver disease is that the terminology is designed for use in fully penetrant Mendelian disorders. Genetic variation in adult-onset liver disease is likely to contribute to the clinical phenotype, although it may not be the entire cause.

We hypothesize that a proportion of patients with adult-onset liver disease have variants in genes encoding proteins involved in bile homeostasis that are contributory factors for the development of liver disease, and the severity of the genotype is linked to the phenotype.

PATIENTS AND METHODS

Using a prospectively assembled database, patients who underwent sequencing of cholestasis genes over 5 years via the clinical service were identified. Patients were excluded if they had onset of liver disease below 18 years, or were asymptomatic patients tested as part of family screening. Patients with other possible etiologies of liver disease were, however, included, as the genetics were requested due to clinical suspicion of a genetic contribution to liver disease. This was conducted as a service evaluation of genetic testing in adults in accordance with institutional policy. It was not considered research as per the National Health Service's Health Research Authority decision tool and was exempt from institutional review board approval.

Data including demographics, clinical phenotype, liver histopathology, and clinical outcomes were collected from the electronic patient records where available. Information on the clinical diagnosis of chronic liver disease etiology before genetic testing was recorded.

Patient samples and identification of genetic variants

As part of the clinical service, genomic DNA was isolated from whole blood using the Promega Maxwell CSC Instrument. Next Generation Sequencing (NGS) of targeted genes was carried out using a custom-designed Illumina TruSeq custom application kit or

Agilent SureSelect-QXT capture library, and the Illumina MiSeq System. Reads were mapped to the reference human genome. Variants were called using Qiagen CLC Genomics workbench software. After automatic filtering of common variants, a variant call file was generated. Genetic variants were described according to the Human Genome Variation Society guidelines.

Bioinformatic analyses

Variants were matched with literature and databases from the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/clinvar>), Ensembl (<http://ensembl.org>), and the Genome Aggregation Database (gnomAD v2.1.1; <https://gnomad.broadinstitute.org/>). Information on the minor allele frequency (MAF) was retrieved from gnomAD, including the population MAF and the most common subpopulation MAF.

The predicted pathogenicity of identified variants was calculated using a combination of *in silico* computational tools, the effect on the amino acid substitution, and protein structure. A range of algorithms were used to predict potential pathogenicity of a variant. Combined Annotation Dependent Depletion score (<https://cadd.gs.washington.edu/snv>) provides a genome-wide ranking, in which higher scores are more likely to be deleterious. PolyPhen-2 provides a score, which is the probability that the substitution is damaging, and a qualitative prediction (*probably damaging*, *possibly damaging*, *benign*, or *unknown*). SIFT (Sorting Intolerant From Tolerant) provides a score that is a normalized probability that the substitution is tolerated, and a qualitative prediction (*tolerated* or *deleterious*). The impact of altered splice sites was analyzed using tools from Spliceman (<http://fairbrother.biomed.brown.edu/spliceman/>).

The severity of an individual's genotype for a particular gene was classified based on the predicted amount of functional protein produced and classified into *mild* (heterozygous for missense; heterozygous for mild splice site), *moderate* (heterozygous for loss of function; heterozygous for severe splice site; two missense; missense and mild splice site), and *severe* (loss of function and missense; loss of function and splice site; severe splice site and missense).

A variant was considered novel if it could not be identified on gnomAD or in the literature of patients with liver disease.

Liver histology

A liver histopathology database search was performed in patients with a genetic variant, and material available was retrieved. For patients with more than one

specimen, only the first available sample was included. Slides were re-analyzed by a single liver histopathologist, who was aware of a genetic variant in each patient but blinded to the individual genotype.

The median biopsy length for the liver biopsies was 18 mm (range 9–34 mm). Portal tracts were counted in 40 liver biopsies (excluded one biopsy with cirrhosis and a single regenerative nodule), and median number of portal tracts was 11 (range 4–34). Two biopsies had a suboptimal representation of portal tracts but were considered sufficient for qualitative assessment and were included in the study, given the descriptive nature of this work.

There was an *a priori* assumption of variable histological findings in this cohort, and consequently the main histopathology patterns of liver injury considered to classify the samples were minor histological abnormalities, acute lobular injury (including acute hepatitis and cholestatic hepatitis), fatty liver disease, chronic hepatitis, biliary disease (including early cholangiopathy, chronic biliary disease, and bile duct loss), vascular disease, or mixed patterns. Fibrosis stage is presented as a description, with advanced fibrosis including bridging fibrosis and cirrhosis.

Microscopic assessment of the injury pattern was performed using hematoxylin and eosin stain. Fibrosis was assessed with picosirius red in most cases or with the available stain for collagen fibers (unstained sections or block were not always available for referral biopsies), and the stain available depended on the choice from the referral laboratories, including Masson's trichrome, Elastic Van Gieson, and reticulin. Orcein stain for periportal copper-associated protein deposition and K7 immunohistochemistry for periportal hepatobiliary phenotype of the hepatocytes were used to support the diagnosis of chronic cholestasis, when available.

Clinical phenotypes

The following definitions of phenotypes were used: *family history of liver disease* (reported presence of liver disease in a first or second degree relative), *pregnancy-associated liver dysfunction* (new/worsening liver enzyme abnormalities during pregnancy or postpartum, or new/worsening pruritus during pregnancy or postpartum), *acute or episodic cholestasis* (one or more episodes of acute onset liver enzyme abnormalities), and *chronic liver disease* (persistent abnormality in liver enzymes, or a clinical diagnosis of chronic liver disease).

Statistics

Continuous variables are expressed as median (interquartile range [IQR]); categorical variables are represented as number (percentage), or where data

are missing number/number available (percentage). Subgroup differences were analyzed by chi-squared or Fisher's exact tests for categorical parameters, and Mann–Whitney U test or Kruskal Wallis test for continuous variables. Statistical analysis of the data was performed using SPSS software (version 26; SPSS Inc.).

RESULTS

A total of 356 individuals were identified for analysis: 219 (61.5%) female; median age at presentation with liver disease, 36.2 years (IQR, 28.2–48.0); and age at genetic testing, 39.4 years (IQR, 30.8–53.2). A variant in *ABCB4*, *ABCB11*, or *ATP8B1* was identified in 101 (28.4%) patients, including a subgroup of 9 patients with variants in more than one gene (four *ATP8B1* and *ABCB11*, four *ATP8B1* and *ABCB4*, and one *ABCB11* and *ABCB4*). Details of their phenotype and genotype are outlined in Table 1, and additional information is available in Tables S1 and S2. Novel variants from our cohort are found in Table S3.

ABCB4

One or more variants in *ABCB4* were identified in 46 (12.9%) patients. Loss of function (12, 37.5%) and biallelic (16, 34.8%) variants were seen more commonly in *ABCB4* than in *ABCB11* or *ATP8B1*, resulting in a classification of moderate and severe genotypes in 29 of 46 (63.0%).

There was a significant difference in the median age of presentation between the severities of genotype (mild 37.9 years [30.4–48.4], moderate 31.8 years [27.5–37.0], and severe 27.5 years [21.5–27.9]; $p = 0.046$). Subgroup analysis of the moderate group identified no difference in age of presentation for those with biallelic variants (30.6 years [26.1–33.6]) or those heterozygous for a loss of function variant (36.2 years [28.7–41.2]; $p = 0.17$).

Pregnancy-associated liver dysfunction was reported in 5 of 9 (55.6%) with mild, 10 of 11 (90.9%) with moderate, and no pregnant patients were reported with a severe genotype. Liver transplantation has been undertaken in 4 patients: no patients with mild, 1 of 25 (4%) with moderate, and 3 of 4 (75%) with severe genotype. Age at liver transplant was 28–36 years; all had cirrhosis with portal hypertension, and 2 of 4 had hepatocellular carcinoma (HCC). One patient with HCC was treated with bridging transarterial chemotherapy before transplant; the other was diagnosed with HCC on analysis of the explant; neither had elevated alpha-fetoprotein, vascular invasion, or extra-hepatic disease. One transplanted patient had a moderate genotype (homozygous for novel missense variant), whereas 3 had

a severe genotype (compound heterozygous for missense variant and loss of function variant; Table S1). One of the patients also had a novel missense variant in *ATP8B1*.

ABCB11

One or more variants in *ABCB11* were identified in 35 (9.8%) patients, and most had monoallelic variants (31 of 35, 88.6%). There was no difference in age at presentation between the mild and the combined moderate and severe groups (33.6 years [27.9–46.4] vs. 34.9 years [26.7–47.2]; $p = 0.77$).

The patient who underwent liver transplant had a mild genotype (heterozygote for missense variant) and no other cause for liver disease identified. Another patient with a moderate genotype (compound heterozygous for missense variant and variant with a mild effect on splicing) who had a history of episodic cholestasis and chronic liver disease was put forward for liver transplantation but declined for personal reasons.

ATP8B1

One or more variants in *ATP8B1* were identified in 28 (7.9%) patients, who were primarily a milder genotype with monoallelic variants in 24 of 28 (85.7%) and only 1 patient with a loss of function variant. Subgroups of genotype severity were not compared due to small numbers in moderate and severe groups. A co-existing diagnosis of chronic liver disease was present in 9 of 21 (42.9%).

Three patients with *ATP8B1* variants underwent liver transplantation, all with mild genotypes (heterozygous missense; Table S1). One patient also had a severe genotype of *ABCB4*, which was likely to be the main driver of their liver disease, and another had an existing diagnosis of PSC. The other had no other cause for liver disease identified.

Clinical phenotypes tested

Pregnancy-associated liver dysfunction

Pregnancy-associated liver dysfunction was noted in 59 females who underwent testing. In 31 (52.5%) there was an additional liver phenotype: 22 with chronic liver disease (13 chronic cholestasis, 5 autoimmune liver disease, 3 chronic hepatitis B, 1 nonalcoholic fatty liver disease [NAFLD]), and 9 acute or episodic cholestasis. A variant was identified in 28 of 59 (47.5%) patients: 15 *ABCB4* and 14 *ABCB11* (including a patient with variants in *ABCB4* and *ABCB11*).

TABLE 1 Demographics, clinical phenotypes, and genotype information of subgroups with variants in *ATP8B1*, *ABCB11*, and *ABCB4*

	<i>ABCB4</i>	<i>ABCB11</i>	<i>ATP8B1</i>
Number of patients (% of all tested)	46 (12.9%)	35 (9.8%)	28 (7.9%)
Female (%)	31 (67.4%)	27 (77.1%)	14 (50%)
Median age at presentation, years (IQR)	33.5 (27.1–39.2)	34.3 (27.0–45.9)	31.8 (25.7–50.7)
Median age at testing, years (IQR)	36.1 (31.3–49.4)	36.9 (30.9–48.7)	42.3 (31.1–55.5)
Median interval between presentation and testing, months (IQR)	25.6 (7.9–107.9)	9.3 (3.8–34.5)	56.6 (12.6–127.2)
Clinical phenotype			
Family history of liver disease (%)	14 (30.4%)	4 (11.4%)	2 (7.1%)
Acute or episodic cholestasis (%)	5 (10.9%)	14 (40%)	7 (25%)
Pregnancy-associated liver dysfunction (% reported pregnancies)	15/20 (75%)	14/17 (82.4%)	0/3
Chronic liver disease (%)	33 (71.7%)	14 (40%)	21 (75%)
<i>NAFLD</i>	4	2	5
<i>Autoimmune liver disease</i>	4	2	4
<i>Chronic hepatitis B</i>	1	1	0
No co-existent diagnosis	24	11	12
HCC (%)	2 (4.3%)	0	1 (3.6%)
Liver transplant (%)	4 (8.7%)	1 (2.9%)	3 (10.7%)
Genotype			
Number of different variants	32	29	17
Previously reported in liver disease (%)	11 (34.4%)	12 (41.4%)	5 (29.4%)
Novel variants (%)	14 (43.4%)	9 (31%)	4 (23.5%)
Loss-of-function variants (%)	12 (37.5%)	3 (10.3%)	1 (5.9%)
Number of patients with loss-of-function variants	17 (37%)	5 (14.3%)	1 (3.6%)
Missense variants (%)	17	24	14
Predicted pathogenicity of missense variants			
SIFT <i>deleterious</i> (%)	15 (88.2%)	13 (54.2%)	4 (28.6%)
PolyPhen-2 <i>probably damaging</i> (%)	12 (70.6%)	12 (50%)	2 (14.3%)
CADD score > 20 (%)	17 (100%)	20 (83.3%)	7 (50%)
Number of alleles affected in patients			
1	30 (65.2%)	31 (88.6%)	24 (85.7%)
2 (compound heterozygous/ homozygous)	16 (9/7) (34.8%)	4 (3/1) (11.4%)	4 (3/1) (14.3%)
Severity of genotype			
Mild	17 (37.0%)	27 (77.1%)	24 (85.7%)
Moderate	25 (54.3%)	6 (17.1%)	4 (14.3%)
Severe	4 (8.7%)	2 (5.7%)	0

Abbreviations: CADD, Combined Annotation Dependent Depletion; HCC, hepatocellular carcinoma; IQR, interquartile range; NAFLD, nonalcoholic fatty liver disease; SIFT, Sorting Intolerant From Tolerant.

Drug-induced liver injury

A history of drug-induced liver injury was reported in 49 (13.8%) patients from the cohort. In addition to drug-induced liver injury, 11 of 49 (22.4%) had chronic liver disease and 5 of 29 (17.2%) females had pregnancy-associated liver dysfunction. A variant was identified in 11 of 49 (22.4%) patients: 1 with *ABCB4*, 8 with *ABCB11*, and 3 with *ATP8B1* (including a patient with variants in *ABCB11* and *ATP8B1*). The most common

potentially causative medications in patients with variants were 4 on antibiotics, 3 on oral contraceptive pill, and 2 on nonsteroidal anti-inflammatory medications.

Autoimmune liver disease

Genetic testing was performed in 48 patients with a diagnosis of autoimmune liver disease: 29 with PSC (15 large duct, 12 small duct, 2 large duct with

cholangiocarcinoma), 11 with PBC (9 anti-mitochondrial antibody positive, 2 anti-mitochondrial antibody negative), 6 with autoimmune hepatitis (AIH), and 2 with overlap syndrome (1 with PSC/AIH, 1 with PBC/AIH). A variant was identified in 10 of 48 (20.8%) patients (5 with PSC, 3 with PBC, 2 with AIH), and all were heterozygotes: 4 with *ABCB4* (large-duct PSC, small-duct PSC, PBC with pregnancy-associated liver dysfunction, AIH with cholestasis); 4 with *ATP8B1* (small-duct PSC, large-duct PSC requiring liver transplantation, PBC, AIH with recurrent jaundice and pruritus); 2 with *ABCB11* (PBC with pregnancy-associated liver dysfunction, large-duct PSC with pregnancy-associated liver dysfunction). The existing diagnosis of autoimmune liver disease was not changed for any of the patients with variants.

NAFLD

There was a diagnosis of presumed NAFLD before genetic testing in 25 patients. A variant was identified in 8 of 25 (32%) patients with presumed NAFLD: 3 with *ABCB4* (2 heterozygotes for loss of function variant, 1 compound heterozygous for missense and splicing variant); 1 with *ABCB4* and *ATP8B1* (heterozygous for *ABCB4* splicing variant, homozygote for *ATP8B1* missense variant); 3 with *ATP8B1* (heterozygotes for missense variants); and 1 with *ATP8B1* and *ABCB11* (heterozygote for missense variants in *ATP8B1* and *ABCB11*). The 4 patients with *ABCB4* variants had moderate or severe genotypes, and in the 2 who underwent biopsy, they had biliary disease without fatty liver disease on histology; therefore, the original diagnosis of NAFLD was reconsidered.

Histopathology

Liver histology specimens were available from 43 patients (41 liver biopsies, 2 liver resections). All specimens included were considered satisfactory for diagnostic interpretation.

The most frequent patterns of injury (Table 2) were biliary disease in 27 (63%) biopsies and acute lobular injury in 11 (26%), with co-existent chronic biliary disease and acute lobular injury in 3 patients. Features of fatty liver disease were present in 5 (12%) samples. In addition, there were 3 (7%) cases with advanced cirrhosis and suspected fatty liver disease. Absence of fibrosis was observed in 9 (21%) samples. There was early fibrosis in 17 (40%), and 17 (40%) showed advanced fibrosis (including 7 with established cirrhosis).

Representative clinical examples with typical histology for each gene are outlined in Table 3. Most of the patients with *ABCB4* variants had biliary disease (80%), and 45% had advanced fibrosis. Acute lobular injury (58%) and biliary disease (33%) were most commonly seen with *ABCB11*, and only a minority (8%) had advanced fibrosis. A range of patterns was present in *ATP8B1* variants, including fatty liver disease (25%) and nonspecific cirrhosis (19%). All 3 patients with variants in *ABCB4* and *ATP8B1* had biliary disease, and 1 patient with *ABCB11* and *ATP8B1* had acute lobular injury, whereas the other had fatty liver disease.

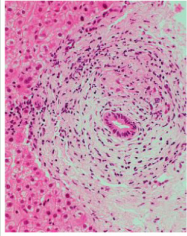
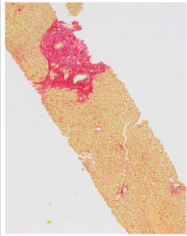
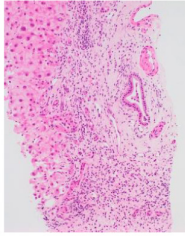

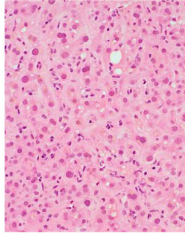
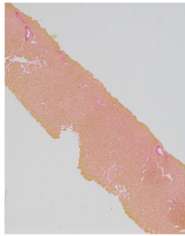
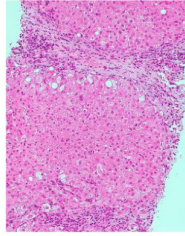
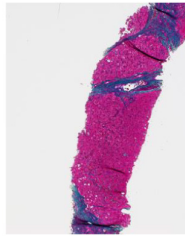


DISCUSSION

In this study we report the results of testing a large cohort of patients with a suspected genetic contribution to the etiology adult-onset liver disease at a specialist liver center, and describe the subgroup of patients with variants in genes involved in bile homeostasis (*ABCB4*, *ABCB11*, and *ATP8B1*), including clinical phenotype data. From our cohort of 356 patients, at least one potentially disease-causing variant was identified in 101 patients (28.4%), with a spectrum of liver injury, ranging from benign to end-stage liver disease. We identified different phenotypes of liver disease related to each gene, and support this with patterns of histological injury. To explore a genotype–phenotype association, we classified genotype severity by a combination of number of alleles affected (monoallelic vs. biallelic) and type of variant (missense vs. loss of function), and demonstrated an association for *ABCB4* variants.

TABLE 2 Histological pattern and staging in patients with variants (patients with variants in more than one gene and represented in each group [3 patients with variants in *ABCB4* and *ATP8B1*, **2 patients with variants in *ABCB11* and *ATP8B1*])

	<i>ABCB4</i>	<i>ABCB11</i>	<i>ATP8B1</i>
Number of samples	20	12	16
Biliary disease, <i>n</i> (%)	16 (80%)*	4 (33%)	7 (44%)*
Acute lobular injury (acute or cholestatic hepatitis), <i>n</i> (%)	3 (15%)	7 (58%)**	4 (25%)**
Minor histological changes, <i>n</i> (%)	1 (5%)	1 (8%)	1 (6%)
Fatty liver disease, <i>n</i> (%)	1 (5%)	1 (8%)**	4 (25%)**
Nonspecific cirrhosis, <i>n</i> (%)	1 (5%)	0	3 (19%)
Advanced fibrosis, <i>n</i> (%)	9 (45%)	1 (8%)	10 (63%)

TABLE 3 Clinical examples of patients with variants in each gene and representative histology (stains and magnification provided for each image)

Clinical features	Genotype and severity	Histological findings	H&E	Collagen stains
<i>ABCB4</i>				
Male who presented with chronic elevations in ALP and GGT aged 57; family history of autoimmune hepatitis	Heterozygous c.1769G>A	Focal (one portal tract) cholangitic damage with periductal edema and loose granuloma formation. Additional findings were focal copper-associated protein deposition and periportal biliary metaplasia of the hepatocytes with K7 staining (H&E, ×200)		
Autoimmune and viral screen negative; no cholangiopathy on MRCP	p.Arg590Gln Mild genotype severity	Fibrosis stage (picrosirius red, ×40): focal portal fibrosis		
Male who presented with decompensated cirrhosis, aged 33 with elevated ALP and GGT; sister died of cryptogenic cirrhosis in early adulthood	Homozygous	Chronic biliary disease, with mild to moderate portal inflammation, cholangitic injury, focal bile duct loss, and ductular reaction; additional findings were abundant periportal copper-associated granules and hepatocellular biliary metaplasia with K7 staining (H&E, ×100)		
Autoimmune and viral screen negative	c.716C>T p.Ser239Leu Moderate genotype severity	Fibrosis stage (picrosirius red, ×20): cirrhosis		
<i>ABCB11</i>				
Male who presented with recurrent episodes of jaundice and pruritus, aged 42 and likely drug-induced liver injury; liver blood tests were normal in between episodes	Heterozygous	Cholestatic hepatitis with moderate lobular disarray, mild scattered inflammation and ceroid-laden macrophages, and bilirubinostasis; no significant portal damage (H&E, ×200)		
Autoimmune and viral screen negative; no cholangiopathy on MRCP	c.2036C>T p.Ala679Val Mild genotype severity	Fibrosis stage (Elastic Van Gieson, ×40): no fibrosis		
<i>ATP8B1</i>				
Male who presented with decompensated cirrhosis aged 53, with elevated ALP and GGT; history of obesity and diabetes mellitus	Heterozygous c.134A>C	Advanced liver disease with features in keeping with fatty liver disease progression; mild residual steatosis; chronic cholestasis with periportal cholate stasis and copper-associated protein deposition, likely related to the advanced stage; no significant inflammation or bile duct injury (H&E, ×100)		
Autoimmune and viral screen negative	p.Asn45Thr Mild genotype severity	Fibrosis stage (Masson's trichrome, ×20): cirrhosis		

Abbreviations: ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; H&E, hematoxylin and eosin; MRCP, magnetic resonance cholangiopancreatography.

In patients with early-onset pediatric MDR3 deficiency, they primarily have biallelic variants in *ABCB4*, whereas milder phenotypes are seen in children with missense variants, which is thought to be related to the residual transport activity of MDR3.^[15] In our cohort, patients were primarily heterozygotes for variants in *ABCB4*, similar to other reports of adults with *ABCB4* variants.^[1,9,11] Patients with low phospholipid-associated cholelithiasis with truncating variants in *ABCB4* were found to have an earlier onset of symptoms compared to missense^[16]; and in our cohort we found a similar genotype–phenotype correlation with an earlier age in more severe *ABCB4* genotypes, as well as an increased requirement for liver transplantation. With less residual MDR3 function there is likely to be increased free bile acids in turn damaging the biliary epithelium with their detergent activity, and a more rapid progression of the consequent cholangiopathy. We hypothesize that there is a relatively linear relationship between MDR3 protein function and progression of the clinical phenotype, and MDR3 function is sufficiently reduced in *ABCB4* heterozygotes to present with cholangiopathy and chronic liver disease as adults.^[13] The predominant histopathological pattern in our MDR3-associated cohort was biliary disease, which was present in all genotype severities, and supports the pathogenic role of *ABCB4* variants even in heterozygotes with milder genotypes. Comparison of degree of fibrosis by genotype is challenging, as age at biopsy specimen differs significantly and is a confounder.

Although children with early-onset BSEP deficiency generally have biallelic variants in *ABCB11* and present with severe early-onset liver disease,^[8,17] including HCC,^[18] our cohort with *ABCB11* variants had primarily acute or episodic cholestasis, without apparent chronic liver disease, and were heterozygous for missense variants. BSEP transports bile acids from the cytoplasm to the canaliculus, and in health there is more capacity than is required. In fact, the p.Val444Ala variant in *ABCB11* is more common than the wild type and is predicted to have approximately 50% of BSEP function. It has been speculated that both acute and chronic hepatocyte damage can develop below a clinically significant threshold of approximately 20%–25% of ideal BSEP function.^[13] Individuals with less than 20% function will generally have biallelic changes in *ABCB11* and are at risk of childhood disease, with those with loss of function variants at particular risk of worse prognosis.^[19,20] Above this threshold of 20%, including *ABCB11* heterozygotes, there is likely to be sufficient BSEP to cope with usual conditions, thereby avoiding the development of chronic liver damage. However, when exposed to challenges that either reduce functional capacity or increase bile acid demand, an accumulation of toxic bile acids in the hepatocyte can occur with resultant acute hepatocellular dysfunction.^[13] This

acute liver injury generally resolves once the insult is removed and equilibrium is restored, which is highlighted in our cohort who primarily have an acute lobular injury and no evidence of underlying chronic liver disease, with low rates of advanced fibrosis. There were no differences in age at presentation among the genotypic severities seen in adults. Although numbers are small, we believe that this probably reflects the need for environmental factors to initiate liver injury, in a genetically susceptible individual, and that the timing of these exposures is more influential than genotype, on the age of presentation.

Chronic liver disease was the predominant phenotype in individuals with variants in *ATP8B1*; however, nearly half had an alternative cause of liver disease before genetic testing, and a range of histological patterns of liver injury were present. Furthermore, patients had monoallelic variants of low predicted pathogenicity on computational models; therefore, it may be that *ATP8B1* variants are, at most, genetic contributors for the development of chronic liver disease in this cohort, as opposed to having primary *ATP8B1*-related liver disease. Functional work suggests that residual FIC1 function determines the clinical phenotype of patients with biallelic variants in *ATP8B1*, with most patients with early-onset FIC1 deficiency predicted to have a complete loss of function, and those with benign recurrent intrahepatic cholestasis 5%–20% function of a healthy subject.^[21] The correlation with higher levels of functional FIC1 and phenotype has not been explored, and most patients from our cohort are heterozygotes and therefore have much higher levels of FIC1, at least 50% of healthy subjects. The exact pathogenesis of FIC1 deficiency is not clear^[22]; however, it appears integral in the role of other proteins, including BSEP, farnesoid X receptor, and cystic fibrosis transmembrane conductance regulator.^[7,23,24] A mild disruption of signaling and transporter pathways in patients with *ATP8B1* variants could exacerbate underlying pathogenic liver disease processes, or put an individual at increased risk in the presence of existing risk factors.

Although specific treatment to address MDR3, BSEP, and FIC1 deficiencies are not currently available, there is a potential role for therapeutic intervention. Treatment with ursodeoxycholic acid has been demonstrated to be of benefit in children with PFIC^[8,25] and adults with *ABCB4* variants,^[9] and is suggested where tolerated for adult-onset liver disease.^[13] Apical sodium-dependent bile acid transporter inhibitors have been subjected to clinical trials in children with PFIC, and one is now licensed.^[26,27] They may be a future option for adults with variants. *In vitro* models have demonstrated the potential to rescue defective *ABCB4* and *ATP8B1* protein function using pharmacological chaperones^[28] and therapeutics developed for use in cystic fibrosis.^[29–31] With the growing role of personalized medicine, early identification of genotype will

TABLE 4 Most frequently identified variants in *ABCB4*, *ABCB11*, and *ATP8B1* in patients in our cohort (frequency in population databases and predicted pathogenicity of variants on computational tools [SIFT, PolyPhen-2, CADD])

DNA variant	Predicted protein consequence	Number of patients in cohort	MAF population (MAF highest subgroup)	Grantham distance	SIFT	PolyPhen-2	CADD	Comment
<i>ABCB4</i>								
c.523A>G	p.Thr175Ala	3	0.01145 (0.03845 Finnish)	58	0.05 Tolerated	0.734 Possibly damaging	24	Unclear pathogenicity; previously reported in liver disease
c.1769G>A	p.Arg590Gln	5	0.004542 (0.006745 European)	43	0.01 Deleterious	0.928 Probably damaging	31	Likely pathogenic; previously reported in liver disease
c.2363G>A	p.Arg788Gln	5	0.008163 (0.08311 African)	43	0 Deleterious	0.899 Possibly damaging	23.6	Likely pathogenic; previously reported in liver disease
c.2800G>A	p.Ala934Thr	9	0.001246 (0.01354 African)	58	0 Deleterious	0.973 Probably damaging	29.6	Likely pathogenic; previously reported in liver disease
c.3136C>T	p.Arg1046Ter	3	0.000003983 (0.00003266 South Asian)	–	NA	NA	36	Likely pathogenic (loss of function); not previously reported in liver disease
<i>ABCB11</i>								
c.1772A>G	p.Asn591Ser	4	0.01266 (0.1112 South Asian)	46	0.06 Tolerated	0.883 Possibly damaging	23.9	Unclear pathogenicity; previously reported in liver disease
c.2093G>A	p.Arg698His	5	0.003732 (0.01434 Ashkenazi Jewish)	29	0.06 Tolerated	0.01 Benign	24.4	Unclear pathogenicity; previously reported in liver disease
<i>ATP8B1</i>								
c.134A>C	p.Asn45Thr	6	0.004936 (0.007882 European)	65	0.11 Tolerated	0.112 Benign	15.69	Unclear pathogenicity; previously reported in liver disease
c.208G>A	p.Asp70Asn	4	0.003104 (0.009851 Ashkenazi Jewish)	23	0.23 Tolerated	0.926 Probably damaging	22.5	Unclear pathogenicity; previously reported in liver disease
c.1739G>A	p.Ser580Asn	6	0.004214 (0.04413 African)	46	0.54 Tolerated	0.493 Possibly damaging	20.3	Unclear pathogenicity; not previously reported in liver disease

Abbreviations: MAF, minor allele frequency; NA, not available.

be key to facilitating individualized pharmacotherapy, which may be based on the specific type of protein defect.^[32]

There are limitations to our retrospective study. Patients included in our cohort were seen in a specialist liver center and were referred for genetic testing by their caregiver due to clinical concerns for a genetic contribution to liver disease, as opposed to sequential analysis of consecutive patients. In the absence of clear guidelines on which patients should be tested, there is a risk of referral bias, as each clinician would have a different threshold for testing. The inclusion of clinical characteristics is key to interpreting the contribution of variants in cholestasis-related genes, which is a strength of this study; however, there are missing data, and we are unable to capture all environmental exposures that may have played a role in the development of cholestasis. Furthermore, the contribution of genes involved in rarer forms of PFIC (*TJP2*, *DCDC2*) and other childhood genetic liver diseases (*JAG1*, *NOTCH2*, *MYO5B*) was not assessed. Due to the large number of different variants in *ABCB4*, *ABCB11*, and *ATP8B1* (78 different variants in our cohort), it is not possible to perform functional analysis of all of them; therefore, *in silico* models have been used to evaluate the impact of missense variants. Although these tools have good accuracy,^[33] there is variable performance and a potential circularity, as overlapping data are used for training and validation of the algorithms.^[34] The impact has been mitigated by using multiple computational tools, in addition to other modes of assessment such as genotype–phenotype associations reported in the literature, and describing the location on the protein related to protein function.^[35] The analysis was not designed to detect whether the most commonly identified variants in our cohort (Table 4) were more enriched than in population databases; however, it is likely that clinical phenotype and histological analysis of groups of patients with the same genotype will be more useful in ascribing potential causality than frequency of individual variants. Our classification of genotype severity is simplistic, although more severe phenotypes in patients with loss-of-function variants have been previously described in patients with PFIC.^[15,20,36] Our genotype severity subgroups are likely to be heterogeneous in terms of the amount of residual function, particularly the moderate severity genotype where it could theoretically range from 0% to 100%, which prevents a more detailed genotype–phenotype correlation.

In conclusion, we describe a high frequency of rare variants in the cholestasis-related genes *ABCB4*, *ABCB11*, and *ATP8B1* using NGS technology, in patients with a spectrum of adult-onset liver disease. Despite the difficulties in predicting pathogenicity of the variants and reliance on *in silico* tools, it is apparent from our data that these genes should no longer be considered recessive Mendelian traits, at least in adult-onset disease, and that these are contributors to the development of liver disease.

We suggest that the phenotypes should be seen as a spectrum related to residual protein function, and thresholds vary by individual gene, particularly with *ABCB4* heterozygotes and chronic liver disease, and *ABCB11* heterozygotes and acute cholestasis in the presence of environmental factors. Current clinical guidelines for the description of variants are based on the assumption of fully penetrant Mendelian inheritance,^[14] and therefore clinical reports of variants defined as “benign,” “possibly benign,” and “uncertain significance” should not be discounted as genetic contributors to liver disease. Our data support testing for cholestasis genes in selected patients with adult-onset liver disease, and close collaboration with geneticists and histopathologists to aid interpretation of variants.

AUTHOR CONTRIBUTIONS

Study concept and design: Jeremy S. Nayagam, Deepak Joshi, and Richard J. Thompson. *Generation, collection, assembly, analysis, and/or interpretation of data:* All authors. *Manuscript draft:* Jeremy S. Nayagam and Richard J. Thompson. *Critical revision of the manuscript and approval of the final version of the manuscript, including authorship list:* All authors.

CONFLICT OF INTEREST

RJT is a consultant to Generation Bio, Rectify Pharma, Albireo Pharm, and Mirum, and holds shares/options in Generation Bio and Rectify Pharma.

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SUPPORTING INFORMATION

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

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