Sequencing of FIC1, BSEP and MDR3 in a large cohort of patients with cholestasis revealed a high number of different genetic variants

Graphical abstract

Highlights

- 149 patients with at least one disease-causing mutation in FIC1, BSEP or MDR3 were identified.
- 154 different genetic variants detected, of which 25 have not been described yet.
- All 13 novel missense mutations were predicted to be disease-causing.
- Risk alleles of common BSEP SNPs were overrepresented in the patient cohort, underscoring a contribution to cholestasis.

Authors

Carola Dröge, Michele Bonus, Ulrich Baumann, ..., Ralf Kubitz, Dieter Häussinger, Verena Keitel

Correspondence

haeussin@uni-duesseldorf.de (D. Häussinger)
verena.keitel@med.uni-duesseldorf.de (V. Keitel)

Lay summary

FIC1, BSEP, and MDR3 represent hepatobiliary transport proteins essential for bile formation. Genetic variants in these transporters underlie a broad spectrum of cholestatic liver diseases. To confirm a genetic contribution to the patients’ phenotypes, gene sequencing of these three major cholestasis-related genes was performed in 427 patients and revealed 154 different variants of which 25 have not been previously reported in a database. In patients without a disease-causing mutation, common genetic variants were detected in a high number of cases, indicating that these common variants may contribute to cholestasis development.
Sequencing of FIC1, BSEP and MDR3 in a large cohort of patients with cholestasis revealed a high number of different genetic variants

Carola Dröge1, Michele Bonus2, Ulrich Baumann3, Caroline Klinadt1, Elke Lainka4, Simone Kathemann4, Florian Brinkert6, Enke Grabhorn5, Eva-Doreen Pfister3, Daniel Wenning5, Alexander Fichtner4, Daniel N. Gotthardt7, Karl Heinz Weiss7, Patrick McKiernan8, Ratna Dua Puri4, I.C. Verma4, Stefanie Kluge4, Holger Gohlke2, Lutz Schmitt10, Ralf Kubitz1, Dieter Häussinger1, Verena Keitel1

1Department of Gastroenterology, Hepatology and Infectious Diseases, University Hospital Düsseldorf, Heinrich Heine University Düsseldorf, Germany; 2Institute for Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, Germany; 3Pediatric Gastroenterology and Hepatology, Department for Pediatric Kidney, Liver and Metabolic Diseases, Hannover Medical School, Germany 4Department of Pediatric Nephrology, Gastroenterology, Endocrinology and Transplant Medicine, Clinic for Pediatrics II, University Children’s Hospital Essen, University Duisburg-Essen, Germany; 5Pediatric Gastroenterology and Hepatology, University Children’s Hospital, University Medical Center Hamburg-Eppendorf, Germany; 6Department of General Pediatrics, Heidelberg University Hospital, Germany; 7Department of Internal Medicine IV, University Hospital Heidelberg, Germany; 8Pittsburgh Liver Research Center, University of Pittsburgh and Children’s Hospital of Pittsburgh of UPMC, Pittsburgh, USA; 9Institute of Medical Genetics & Genomics, Sir Ganga Ram Hospital, New Delhi, India; 10Institute of Biochemistry, Heinrich Heine University Düsseldorf, Germany

Abstract

In 427 patients with suspected inherited cholestasis, 149 mutations were identified, of which 25 have not been previously reported. All 13 novel missense mutations were disease-causing according to bioinformatic tools and 3D protein modeling. Eighty-two percent of patients carried at least one disease-causing mutation in FIC1, BSEP, or MDR3, respectively. Overall, 154 different mutations were detected in FIC1, BSEP, and MDR3, 25 of which were novel. All 13 novel missense mutations were disease-causing according to bioinformatic tools and homology modeling. Eighty-two percent of patients were sequenced in cholestatic patients with assumed genetic cause. The effects of new variants were evaluated by bioinformatic tools and 3D protein modeling.

Results: In 427 patients with suspected inherited cholestasis, 149 patients carried at least one disease-causing mutation in FIC1, BSEP or MDR3, respectively. Over all, 154 different mutations were identified, of which 25 were novel. All 13 novel missense mutations were disease-causing according to bioinformatic analyses and homology modeling. Eighty-two percent of patients with at least one disease-causing mutation in either of the three genes were children. One or more common polymorphism(s) were found in FIC1 in 35.3%, BSEP in 64.3% and MDR3 in 72.6% of patients without disease-causing mutations in the respective gene. Minor allele frequencies of common polymorphisms in BSEP and MDR3 varied in our cohort compared to the general population, as described by gnomAD. However, differences in ethnic background may contribute to this effect.

Conclusions: In a large cohort of patients, 154 different variants were detected in FIC1, BSEP, and MDR3, 25 of which were novel. In our cohort, frequencies for risk alleles of BSEP (p.V444A) and MDR3 (p.I237I) polymorphisms were significantly overrepresented in patients without disease-causing mutation in the respective gene, indicating that these common variants can contribute to a cholestatic phenotype.

Introduction

The three transport proteins familial intrahepatic cholestasis 1 (FIC1, gene symbol: ATP8B1), bile salt export pump (BSEP, ABCB11) and multidrug resistance P-glycoprotein 3 (MDR3, ABCB4) are located in the canalicular membrane of hepatocytes and mediate bile formation. The FIC1 protein is encoded by the ATP8B1 gene.

Keywords: FIC1; BSEP; MDR3; ATP8B1; ABCB11; ABCB4; ICP; LPAC; BRIC; PFIC; SNP.
Research Article

on chromosome 18q21 and represents a P-type ATPase, which flips aminophospholipids from the outer to the inner membrane leaflet thereby maintaining membrane asymmetry, which is essential for the function of other canalicular transport proteins.1-4 BSEP (ABCB11) and MDR3 (ABCB4) are members of the subfamily B (MDR/TAP) of adenosine triphosphate (ATP)-binding cassette (ABC) transporters. BSEP excretes bile salts from the hepatocyte into the bile canalicus, which represents the major driving force of bile salt-dependent bile flow.5-7 While the human ABCB11 gene is located on chromosome 2q24,8 the ABC4 gene is positioned on chromosome 7q21.9 MDR3 acts as a floppase and transports lipids of the phosphatidylcholine family from the inner to the outer leaflet of the canalicular membrane.10-12 A reduction or even absence of functionally active FIC1, BSEP or MDR3 from the canalicular membrane of hepatocytes is the molecular mechanism underlying a wide spectrum of cholestatic disorders comprising both acquired forms due to infection or drugs as well as inherited cholestatic disorders. Mutations in the genes encoding FIC1 or BSEP may trigger cholestasis that is typically associated with normal values of gamma-glutamyltranspeptidase (low γGT cholestasis), since bile salt levels in bile and thus toxicity of bile salts on the biliary epithelium is reduced.13 In contrast, MDR3-related cholestasis is characterized by elevated γGT levels (high γGT cholestasis), which may be explained by reduced phospholipid concentrations in bile that impair the formation of mixed micelles, resulting in higher relative concentrations of bile salts in bile with subsequent toxic effects on bile ducts.14,15 Progressive familial intrahepatic cholestasis (PFIC) type 1, 2 or 3 represent severe forms of cholestasis with an early onset phenotype linked to homozygous or compound heterozygous mutations in either the FIC1, BSEP or MDR3 gene often necessitating liver transplantation in early childhood. In 10–15% of cases, neonatal cholestasis can be attributed to PFIC types 1–3.16,17 Heterozygous variants in these three genes have been linked to milder cholestatic phenotypes like intrahepatic cholestasis of pregnancy (ICP),18-21 contraceptive-induced cholestasis (CIC),19,22 drug-induced cholestasis,23 benign recurrent intrahepatic cholestasis (BRIC),3,4,24,25 or low phospholipid-associated cholestathiasis (LPAC) for MDR3 variants.26 Furthermore, late-onset phenotypes have been described to have mutations in all three genes.26-28 Specifically, MDR3 mutations can lead to a presentation in adulthood with biliary fibrosis or liver cirrhosis even without clinically overt cholestasis.21-29,31 These findings in individual patients or families have recently been confirmed by a whole genome sequencing of the Icelandic population.32,33 This study showed a highly significant association of MDR3 mutations and the common single nucleotide polymorphism (SNP) p.I237I with an elevation of liver enzymes and γGT, the development of gallstones and ICP but also with liver cirrhosis (odds ratio [OR] up to 5.5-fold) and hepatobiliary malignancies (OR up to 4.8-fold). Besides rare, disease-causing mutations, common variants with a minor allele frequency (MAF) of greater than 1%, denoted as SNPs,34 may underlie or contribute to the cholestatic phenotypes. This is well-established in the BSEP SNP p.V444A, which predisposes a person to both ICP and drug-induced liver injury (DILI), as well as for the synonymous MDR3 variant p.I237I, which has been associated with ICP and gallstones.18,19,32,35

To determine the contribution of mutations and common variants in the FIC1, BSEP and MDR3 genes to cholestatic disorders of differing disease onset and severity, we performed DNA sequencing on patients with a cholestatic phenotype consistent to the mentioned disorders at the Laboratory for Cholestasis Diagnostics in our clinic and assessed the potential impact of the identified variants on mRNA splicing or protein function by comparing with published data as well as by bioinformatics tools. Furthermore, the potential impact on protein structure or function of novel missense variants was characterized by 3D homology modeling.

Patients and methods

Patients' samples and identification of genetic variants

The study was performed according to the guidelines of the Declaration of Helsinki, approved by the local ethics committee (study numbers 2875 and 3115) and informed written consent was obtained from each patient or the patients' parents. First degree relatives of index patients were not included in the cohort of 427, but used for determination whether mutation status was compounded heterozygous or one-allelic. Genomic DNA was isolated from whole blood using MagNA Pure LC-2.0-DNA Isolation Kit (Roche, Mannheim, Germany) or QIAamp DNA Blood Kit (Qiagen, Hilden, Germany). Coding exons with flanking intron regions were amplified by PCR and sequenced.36-38 Reference sequences: AF339007 (Gene ID: 5205, BSEP), U1343520, FIC1 (Gene ID: 8647, ABCB11; UniProt: O95342, BSEP) and NM_003742.2 (Gene ID: 5244, ABCB4; UniProt: P21439, MDR3). Genetic variants were termed according to the Human Genome Variation Society.39 First coding exons are counted as exon 1, adename of ATG is denoted as c.1. It has to be noted, that all three genes encode one non-coding exon as well as 27 coding exons.

Databases

Variants were matched with literature and databases from NCBI (http://www.ncbi.nlm.nih.gov/projects/SNP/index.html) and the Genome Aggregation Database (gnomAD), Cambridge, MA (http://gnomad.broadinstitute.org/);40,41) (allele frequencies accessed June 2017). Our cohort represents a mixed population, however, numerous patients are of European descent therefore detected allele frequencies (AFs) were compared to AFs of either the worldwide population or the European population (non-Finnish) of gnomAD.

Bioinformatics analyses

Potential functional effects of detected missense mutations were predicted by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml);42,43) The impact of altered splice-sites was analysed using tools from Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/splice.html44) and NetGene2 Server (http://www.cbs.dtu.dk/services/NetGene2/45). Positioning of genetic variants in 2D protein models

TOP2O tool (http://www.sacs.ucsf.edu/cgi-bin/open-topo2.py) was used to create 2D protein models. Information concerning amino acid (AA) sequence and predicted transmembrane regions was taken from UniProt (http://www.uniprot.org/uniprot).

Homology modeling of human BSEP and MDR3

For AA sequences of human BSEP and MDR3 (UniProtKB IDs O95342-1, P21439-1), mammalian sequences with an E-Value of <0 and a sequence identity >15% were identified in the UniProtKB/Swiss-Prot database using DELTA-BLAST.46 A multiple sequence alignment (MSA) was constructed using MAFFT,47 incorporating the structural information available for P-glycoprotein (MDR1). The MSA was optimized by removing sequences with a high gap content, a distance tree was generated with neighbour joining, and the cluster of sequences that contained the respective query sequence was kept. After repeating the alignment procedure with the remaining sequences, homology models of human BSEP and MDR3 were generated using Schrödinger BioLuminate.48 The corrected PDB structure of murine Mdr1 (PDB ID: 4M1M49) served as a template. The sequence identity (similarity) between target and template is 48.4% (65.9%) for BSEP and 73.0% (82.2%) for MDR3.

Statistics

Statistical analyses were performed using chi-square test to determine variations of AFs detected in the present cohort compared with AFs described in the database (Table 2).
Results

ATP8B1, ABCB11 or ABCB4 genes were sequenced in 427 patients, who presented with a cholestatic phenotype of varying degree. Samples were collected between 2006 and 2016. Since variants in FIC1 and BSEP may underlie low γGT cholestasis, variants in BSEP and MDR3 may predispose to gallstone disease and variants in all three genes are linked to ICP. Two or all three genes were sequenced in 43% of patients resulting in a total of 651 gene analyses in our 427 patients (Fig. 1). Additional sequencing for detecting, potentially disease-causing variants was performed in first degree relatives of the 427 patients when available. In this cohort, 154 different genetic variants including 55 novel mutations were identified, 25 of which have not been described in the literature nor databases yet.

FIC1 sequencing results

FIC1 sequencing identified 18 patients with potentially disease-causing variants at least on one allele (Fig. 1, Table 1; Table S1). The median age at disease onset for these 18 patients was 0.75 years. While 10 patients presented before the first year of life, four patients presented with symptoms in adulthood (Table 1; Table S1). Overall, 28 different genetic variants were identified in the 184 patients analysed for FIC1 (Fig. 2; Table S1 and S4), including two common SNPs. These common FIC1 SNPs p.R952Q and c.3531+8G>T were found in 35 families and 41 families, respectively (Table S4). 35.3% of patients, in whom no disease-causing mutation in the FIC1 gene was identified, carried at least one of these two common FIC1 SNPs (Fig. 1, Tables 1 and 2). Five variants identified by FIC1 sequencing were neither found in the literature nor databases (Fig. 2; Table S4). In our cohort, 20 potentially disease-causing FIC1 mutations were detected. Nucleotide exchanges resulted in 14 missense mutations and 2 nonsense mutations (p.R930X; p.R1164X; Fig. 2; Table S1; FIC1 patients 4 and 10, Table S4). A premature stop codon was introduced by an adenine insertion (c.614_615insA) or a duplication (c.3422dup) each resulting in a frameshift (p.N205KfsX2; p.Q1142Tfs35X; Fig. 2, Table S1; FIC1 patients 8 and 1, Table S4). Two phenylalanine deletions (p.F529del; p.F971del; Fig. 2, Table S1; FIC1 patients 2, 10 and 6, Table S4) were caused by the deletion of one codon each.

The synonymous variant p.T82T was previously described at low frequencies in patients with ICP (AF: 0.7%) and chronic pancreatitis (AF: 1.6%). Nevertheless, it was not found in the respective control groups. We detected p.T82T in two unrelated patients. FIC1 sequencing in one of these patients revealed only the common variant c.3531+8G>T together with p.T82T, which seems insufficient to explain the PFIC-like phenotype. BSEP sequencing for low γGT PFIC showed three heterozygous
Common SNPs R952Q, 3531+8G

Patients carrying no disease-causing mutation but one/more common SNPs

Median age at DO (yr) pediatric cohort (Table 1): 0.75 yr, 1.0 yr, 4.7 yr

Age at disease onset

≤ 1 yr
> 1–10 yr
> 10–18 yr
> 18–40 yr
> 40 yr

Median age at DO (yr)

0.75 yr
0.3 yr
28 yr, n = 4
1.0 yr
23 yr, n = 9
31.5 yr, n = 13

Patients carrying no disease-causing mutation but one/more common SNPs

65/184
162/252
156/215

SNPs BSEP Y269F, BSEP V444A, BSEP A1028A, however, no disease-causing mutation. The second patient with p.T82T had a BRIC-phenotype and additionally carried the missense mutation p.N457E and the SNP p.R952Q (Table 5; Table 1: FIC1 patient 18). In this case, one heterozygous disease-causing mutation together with a heterozygous SNP and a synonymous variant may explain the cholestatic phenotype. Additionally, BSEP Y269F, BSEP A1028A, MDR3 N168N, and MDR3 R1893+6T were found in the absence of a disease-causing BSEP or MDR3 variant in this patient. Similarly, the new variant p.P553P was found in one patient with no other exonic FIC1 variant but in the presence of FIC1 3531+8G and BSEP V444A in the absence of other disease-associated BSEP variants. This patient presented with a cholestatic phenotype during the first months after birth suggesting that the synonymous p.P553P variant, c.3531+8G>T or an undetected variant within the UTR, introns or other genes besides FIC1 and BSEP may underlie the PFIC-like phenotype. Because of the distance of c.3531+8G>T from the splice-site, mRNA splicing is most likely not affected, which is in line with predictions from splice-site analysis tools. FIC1 variants with an influence on the protein sequence mostly affect the large intracellular protein domains as depicted in the 2D model (Fig. 2). For FIC1, no template structure was available from which a reliable 3D protein model including mutation sites could be constructed.

BSEP sequencing results

BSEP sequencing was performed in 252 unrelated patients and identified 88 patients with potentially disease-causing mutations on one or both alleles (Fig. 1, Table 1; Table S2). The median age of disease onset was 1 year in this cohort and while 44 patients presented before the first year of life, 9 patients first experienced symptoms in adulthood. Twenty-one patients (24%) with a disease-associated BSEP mutation at least on one allele underwent liver transplantation (LT) and three further patients are currently listed/planned for LT (Fig. 1; Table S2). Of the patients with LT for BSEP deficiency, 7 (33%) experienced recurrence of disease due to BSEP inhibitory antibodies.50–52

Overall, 83 different variants were identified in BSEP (Fig. 3; Table S5), of which 35 were described in our lab, 10 of which have never been reported to date. Missense mutations form the majority of detected variants (45/83) in this cohort. Based on the 2D protein model, nine of these are located in BSEP’s transmembrane helices (TMHs), while other types of mutations are exclusively situated in the extra- or intracellular loops and the nucleotide binding domain (NBD, Fig. 3A). The most common PFIC2-associated mutations p.E297G, p.D482G, and p.N591S were observed in 10, 11, and 6 families, respectively (Tables S2 and S5).53–56

The disease relevance of p.R698H, also detected in seven families, is unclear. Eleven nonsense mutations were identified, six of which were first described in our lab.51,56 Insertion or deletion of nucleotides resulted in four frameshift mutations, two deletions of one single AA, and a deletion of seven AA (Table S2). Only two of these mutations were described before (Fig. 3; Table S5). Seven splice-site mutations were detected in the direct vicinity to exon-intron boundaries. Six affect the core nucleotides of the splice-sites.57 Additionally, 11 synonymous variants were identified including the polymorphism p.A1028A, which was found in 197 of 252 families. Another well-known SNP is p.V444A, which appeared in 204 of 252 families of our cohort (Table S5). In the absence of disease-causing BSEP variants, either or both common SNPs were identified in 64% (162/252) of patients (Tables 1–3). Interestingly in the pediatric patients (<18 years of age), we identified only two patients who carried neither a disease-causing BSEP mutation nor one of these two common polymorphisms (Table 3).

MDR3 sequencing results

MDR3 was sequenced in 215 unrelated patients and 43 patients were identified as carrying at least one disease-causing mutation (Fig. 1; Table S3). The median age of disease onset was 4.7 years and was 1.8 years in the pediatric cohort. Thirteen patients first experienced symptoms in adulthood (Table 1; Table S3). Of the 43 patients, 10 (23%) underwent LT. Overall, 43 different genetic variants were identified, including 15 previously unknown vari-
Twenty-one missense mutations were detected, of which p.S320F is the only mutation in the loop region according to the 2D protein model (Fig. 4A). The missense mutation p.T175A was detected in eight families and was associated with cholestatic phenotypes of varying degree (Tables S3 and S6). Four of five frameshift variants and three of five splice-site mutations were not described previously. Furthermore, eleven synonymous variants were detected including the common SNPs p.L59L and p.I237I, found in 53, and 60 families, and p.N168N present in 164 families (Table S6). In 19% of patients with no disease-causing mutation, a combination of these three variants was identified. Besides p.L59L, p.N168N, and p.I237I, one PFIC-3 patient was compound heterozygous for p.F357Cfs12X and p.Y1086S (Table S3: MDR3 patient 20). In two other cases, an additional synonymous variant or SNP was found, either p.V712V or p.S498C. The synonymous variants p.T438T and p.N168N were found in two patients without severe MDR3 mutations, who presented with PFIC-like symptoms in the first months after birth or with an LPAC-phenotype at the age of 37, respectively. The MAF of p.T438T ranges between 0.5–4% (gnomAD,23). Overall, 172 patients analysed for MDR3 did not carry a disease-associated mutation, however, in 73%, at least one of the four common MDR3 SNPs p.L59L, p.N168N, p.I237I or p.R652G was identified (Table 1).

Potential impact of amino acid substitutions

Eighty of the 154 variants (51.9%) identified in our cohort in FIC1, BSEP, and MDR3 were missense mutations. The impact of missense mutations depends on their position as well as the kind of substitution. PolyPhen-2, SIFT, and Mutation Taster, were used to predict the influence of missense mutations on protein function (Tables S4–S7). Most algorithms used to classify AA substitutions do not consider structural information available for homologues of the target protein, although homology modeling is possible for BSEP and MDR3. To assess the impact of missense mutations on local structural stability and transporter function, all mutation sites in the homology models of BSEP and MDR3 were inspected for: i) their location in typical ABC transporter domains; ii) their occurrence in functionally important sequence motifs; and iii) a potential formation or breaking of interactions with the surrounding (≤4 Å) residue environment after mutation (Figs. 3 and 4, Table 4; Table S7). Thus, our structure-based interpretation complements the used prediction tools and their algorithms by exploiting structure-function relationships in terms of assessing the influence of a mutation on its local environment, in the context of the overall protein function. Since the homology model of BSEP did not include the N-terminal and linker regions, the structural and functional consequences of the missense variants were studied (Fig. 4B).
## Table 2. Allele frequencies of common SNPs.

<table>
<thead>
<tr>
<th>Gene (p.)</th>
<th>Gene (c.)</th>
<th>SNP ID</th>
<th>AF all patients</th>
<th>AF patients w/o mutation</th>
<th>AF1 (gnomAD)</th>
<th>AF2 European (gnomAD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIC1 (p.)</td>
<td>ATP8B1 (c.)</td>
<td>AF1 (gnomAD)</td>
<td>AF2 European (gnomAD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R952Q</td>
<td>2855G&gt;A</td>
<td>rs12968116</td>
<td>10.3% (p1 = 0.152; p2 = 0.315)</td>
<td>9.6% (p1 = 0.365; p2 = 0.193)</td>
<td>8.3%</td>
<td>12.0%</td>
</tr>
<tr>
<td>-</td>
<td>3531+8G&gt;T</td>
<td>rs34027711</td>
<td>13.0% (p1 = 0.686; p2 = 0.036)</td>
<td>14.2% (p1 = 0.838; p2 = 0.147)</td>
<td>13.8%</td>
<td>17.2%</td>
</tr>
<tr>
<td>BSEP (p.)</td>
<td>ABCB11 (c.)</td>
<td>AF1 (gnomAD)</td>
<td>AF2 European (gnomAD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V444A</td>
<td>1331T&gt;C</td>
<td>rs2287622</td>
<td>61.0% (p1 = 0.060; p2 = 0.560)</td>
<td>65.9% (p1 = 0.001; p2 = 0.024)</td>
<td>56.9%</td>
<td>59.7%</td>
</tr>
<tr>
<td>A1028A</td>
<td>3084A&gt;G</td>
<td>rs497692</td>
<td>56.0% (p1 = 0.494; p2 = 0.279)</td>
<td>59.8% (p1 = 0.055; p2 = 0.025)</td>
<td>54.5%</td>
<td>53.6%</td>
</tr>
<tr>
<td>MDR3 (p.)</td>
<td>ABCB4 (c.)</td>
<td>AF1 (gnomAD)</td>
<td>AF2 European (gnomAD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L59L</td>
<td>175C&gt;T</td>
<td>rs2302387</td>
<td>13.8% (p1 = 0.039; p2 = 0.928)</td>
<td>14.0% (p1 = 0.077; p2 = 0.993)</td>
<td>17.6%</td>
<td>13.9%</td>
</tr>
<tr>
<td>N168N</td>
<td>504C&gt;T</td>
<td>rs1202283</td>
<td>53.3% (p1 = 0.019; p2 = 0.282)</td>
<td>53.8% (p1 = 0.022; p2 = 0.438)</td>
<td>47.6%</td>
<td>55.9%</td>
</tr>
<tr>
<td>I237I</td>
<td>711A&gt;T</td>
<td>rs2109505</td>
<td>15.4% (p1 = 0.005; p2 = 0.210)</td>
<td>16.0% (p1 = 0.026; p2 = 0.396)</td>
<td>20.9%</td>
<td>17.7%</td>
</tr>
<tr>
<td>R652G</td>
<td>1954A&gt;G</td>
<td>rs2230028</td>
<td>6.3% (p1 = 0.004; p2 = 0.340)</td>
<td>6.4% (p1 = 0.012; p2 = 0.427)</td>
<td>10.5%</td>
<td>7.5%</td>
</tr>
</tbody>
</table>

The allele frequencies (AF) of common single nucleotide polymorphisms (SNPs) in FIC1, BSEP, and MDR3 in either the total cohort or patients without (w/o) a disease-causing mutation in the respective gene were compared to AFs available from the Genome Aggregation Database either of the worldwide population (AF1) or the European population (AF2, non-Finnish) (gnomAD; http://gnomad.broadinstitute.org/). p values were calculated using the chi-square test.

Fig. 3. 2D and 3D illustration of identified BSEP variants. (A) BSEP TOP2 model with affected AA depicted as coloured squares or stars. The first affected position is marked for frameshift variants. Red: missense, green: nonsense, blue: deletion/frameshift, cyan: splice-site, purple: synonymous, grey: polymorphism, stars highlight new variants. (B) Schematic view of ABC transporter function forced by ATP (adenosine triphosphate). TMD, transmembrane domain; CH, coupling helix; NBD, nucleotide binding domain. (C) Specific missense mutations in a 3D BSEP model based on P-gp structure (PDB-Code 4M1M). TMH, transmembrane helix.
functional consequences of the mutations p.V43I, p.R698C, and p.Y721C were evaluated by molecular dynamic simulations assisted by disorder predictions (see Supplementary data and Figs. S1–S4). Although we did not detect significant differences between wild-type BSEP and the p.V43I mutant regarding secondary structure or order/disorder propensity, calculations of the conformational effective energy revealed that the substitution of valine by isoleucine may have occurred at a structural weak spot in the wild-type. While in the p.R698C variant, we detected a significant shift towards structural order with respect to the wild-type, the p.Y721C variant was revealed to introduce more structural disorder around the mutation site.

**Discussion**

Genetic analysis of FIC1, BSEP, and MDR3 was performed for 427 patients with a cholestatic phenotype. We identified 149 patients with at least one disease-causing mutation in one of these three genes, which represents 35% of our study cohort. 82.6% of

<table>
<thead>
<tr>
<th>Table 3. Appearance of variants in pediatric and adult patients.</th>
</tr>
</thead>
<tbody>
<tr>
<td>184 patients sequenced for FIC1</td>
</tr>
<tr>
<td>Pediatric patients (&lt;18 yr)</td>
</tr>
<tr>
<td>Median age of pediatric patients at genetic analysis</td>
</tr>
<tr>
<td>Adult patients (≥18 yr)</td>
</tr>
<tr>
<td>Median age of adult patients at genetic analysis</td>
</tr>
</tbody>
</table>

18 patients with FIC1 mutations 88 patients with BSEP mutations 43 patients with MDR3 mutations

| Pediatric patients with mutations | 12/18 (66.7%) | 72/88 (81.8%) | 28/43 (65.1%) |
| Median age at genetic analysis of pediatric patients with mutations | 1.4 yr | 2.3 yr | 4.0 yr |
| Adult patients with mutations | 6/18 (33.3%) | 16/88 (18.2%) | 15/43 (34.9%) |
| Median age at genetic analysis of adult patients with mutations | 33.3 yr | 23.4 yr | 43.6 yr |

65 patients with FIC1 SNPs only 162 patients with BSEP SNPs only 156 patients with MDR3 SNPs only

| Pediatric patients with SNPs only | 52/65 (80.0%) | 111/162 (68.5%) | 65/156 (41.7%) |
| Median age at genetic analysis of pediatric patients with SNPs only | 1.8 yr | 1.4 yr | 3.8 yr |
| Adult patients with SNPs only | 13/65 (20.0%) | 51/162 (31.5%) | 91/156 (58.3%) |
| Median age at genetic analysis of adult patients with SNPs only | 32.1 yr | 36.1 yr | 39.8 yr |

Values are given for the patients sequenced, patients with disease-causing mutations, and patients without mutations but SNPs. Total amounts and percentages concern: i) the analysed genes FIC1, BSEP, MDR3; and ii) pediatric and adult patients, respectively. Classification of pediatric (<18 yrs) and adult patients (≥18 yrs) as well as the calculation of median ages at genetic analysis given in years (yrs). SNP, single nucleotide polymorphism. For details see Tables S1–S3.
Table 4. Possible effects of yet uncharacterized BSEP and MDR3 AA substitutions.

<table>
<thead>
<tr>
<th>BSEP</th>
<th>Domain</th>
<th>Substructure</th>
<th>Possible effects/interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>V43I</td>
<td>CL0</td>
<td>CL0</td>
<td>Remote residue; likely alters secondary structure propensity of N-terminus → could impair membrane targeting (see Supplementary Information)</td>
</tr>
<tr>
<td>M217R</td>
<td>TMD</td>
<td>TMH3</td>
<td>Near inner membrane leaflet; likely involved in formation of a hydrophobic pocket for initial substrate recognition → likely impairs ligand translocation (corresponds to G191R in MDR1, which reduces MDR towards vinca alkaloids, paclitaxel and etoposide;30 corresponding region in second pseudohalf in MDR1 binds QZ-Val30)</td>
</tr>
<tr>
<td>W330R</td>
<td>TMD</td>
<td>TMH5</td>
<td>Resides in putative translocation cavity, neighboring R1033; induces local electrostatic repulsion → could widen the translocation cavity or locally destabilize the structure and thus perturb substrate translocation</td>
</tr>
<tr>
<td>W342G</td>
<td>TMD</td>
<td>TMH5</td>
<td>Near membrane interface; unspecific effect (W can act as membrane anchor for helices and modulate responses to hydrophobic mismatch;44 W315R in MDR1 is a suppressor mutant44)</td>
</tr>
<tr>
<td>G374S</td>
<td>TMD</td>
<td>TMH6</td>
<td>Precedes a kink in TMH6; serine alters helix bending in TM regions → conformations may be altered drastically</td>
</tr>
<tr>
<td>A382G</td>
<td>TMD</td>
<td>TMH6</td>
<td>At membrane interface, preceded by gating residues, followed by NBD; A → G mutations impair helix stability44 → could impair substrate entry and TMD-NBD communication</td>
</tr>
<tr>
<td>D440E</td>
<td>NBD</td>
<td>RecA-like</td>
<td>Surface-exposed residue; perturbs interaction network with N424, N439 and N442 → local structural destabilization</td>
</tr>
<tr>
<td>S462R</td>
<td>NBD</td>
<td>Walker A</td>
<td>Conserved residue in Walker A motif; essential for nucleotide binding → abolishes nucleotide binding</td>
</tr>
<tr>
<td>D473V</td>
<td>NBD</td>
<td>RecA-like</td>
<td>Surface-exposed residue; perturbs interaction with R470, R487 and R495 → local destabilization</td>
</tr>
<tr>
<td>D496V</td>
<td>NBD</td>
<td>RecA-like</td>
<td>Surface-exposed residue; perturbs interaction with R575 → could uncouple correlated motions between RecA-like subdomain and helical subdomain</td>
</tr>
<tr>
<td>A570V</td>
<td>NBD</td>
<td>helical</td>
<td>Resides in hydrophobic core, directly follows ABC signature motif; stabilizes the overall NBD fold, influences helicity of preceding ABC signature motif → unspecific functional impairment (A570T variant is associated with BRIC-2)</td>
</tr>
<tr>
<td>G648V</td>
<td>NBD</td>
<td>RecA-like</td>
<td>Connecting loop between terminal NBD-helices; increases helix propensity, promotes helix joining → likely to affect structure and function of the linker region</td>
</tr>
<tr>
<td>R698C</td>
<td>LNK</td>
<td>-</td>
<td>Terminal ends of linker region; function highly debated, contains several phosphorylation sites43 and influences ligand specificity as well as conformational coupling.40 R698C promotes an disorder-to-order transition, whereas Y721 promotes an order-to-disorder transition (see Supplementary Information)</td>
</tr>
<tr>
<td>Y721C</td>
<td>LNK</td>
<td>-</td>
<td>Terminal ends of linker region; function highly debated, contains several phosphorylation sites43 and influences ligand specificity as well as conformational coupling.40 R698C promotes an disorder-to-order transition, whereas Y721 promotes an order-to-disorder transition (see Supplementary Information)</td>
</tr>
<tr>
<td>I879R</td>
<td>TMD</td>
<td>TMH9</td>
<td>Transmembrane region; insertion of charged residue in hydrophobic environment causes local structural instability → impaired membrane insertion or loss of function</td>
</tr>
<tr>
<td>A1028E</td>
<td>TMD</td>
<td>TMH12</td>
<td>Near putative substrate entry gate formed by TMH9 and TMH10; obstructs substrate entry gate → likely impairs substrate translocation</td>
</tr>
<tr>
<td>G1032R</td>
<td>TMD</td>
<td>TMH12</td>
<td>Resides in putative translocation cavity or locally destabilize the structure and thus perturb substrate translocation</td>
</tr>
<tr>
<td>D1098E</td>
<td>TMD</td>
<td>TMH12</td>
<td>Hydrophilic cluster near X-loop and signature motif; breaks hydrophobic cluster with F1200, V1201 and V1219, leading to local destabilization → impaired nucleotide binding</td>
</tr>
<tr>
<td>A1283V</td>
<td>NBD</td>
<td>RecA-like</td>
<td>Near central β-sheet; might destabilize the local structure due to its bulkiness → impaired nucleotide binding</td>
</tr>
<tr>
<td>S1120N</td>
<td>NBD</td>
<td>Walker A</td>
<td>Conserved residue in Walker A motif; essential for nucleotide binding → abolishes nucleotide binding</td>
</tr>
<tr>
<td>S1173D</td>
<td>NBD</td>
<td>helical</td>
<td>Surface-exposed, near CH; overstabilizes the overall NBD fold by interacting with R1231 or disrupts local hydrophobic interactions → various outcomes</td>
</tr>
<tr>
<td>L1204P</td>
<td>NBD</td>
<td>helical</td>
<td>Hydrophobic cluster near X-loop and signature motif; breaks hydrophobic cluster with F1200, V1201 and V1219, leading to local destabilization → impaired nucleotide binding</td>
</tr>
<tr>
<td>A1283V</td>
<td>NBD</td>
<td>RecA-like</td>
<td>Near central β-sheet; might destabilize the local structure due to its bulkiness → impaired nucleotide binding</td>
</tr>
<tr>
<td>S1321N</td>
<td>NBD</td>
<td>C-terminus</td>
<td>C-terminal residue; no evident influence on local stability, could likely serve as a kinase target</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MDR3</th>
<th>Domain</th>
<th>Substructure</th>
<th>Possible effects/interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>V428D</td>
<td>NBD</td>
<td>RecA-like</td>
<td>Directly precedes Walker A motif; influences Walker A dynamics → impairs nucleotide binding</td>
</tr>
<tr>
<td>A794R</td>
<td>TMD</td>
<td>TMH5</td>
<td>Close to CH3; induces a kink and breaks the helical structure → impaired TMD-NBD communication</td>
</tr>
<tr>
<td>R816G</td>
<td>TMD</td>
<td>TMH9</td>
<td>N-terminal portion of TMH9; abolishes interaction with E689; lifts conformational constraints on linker, thus further increases linker flexibility → impaired linker phosphorylation</td>
</tr>
<tr>
<td>N902D</td>
<td>TMD</td>
<td>TMH10/CH4</td>
<td>Boundary residue between TMH10 and CH4; alters dynamics of X-loop by introducing a negative charge in the vicinity of E528 → impaired cross-talk of TMD and NBD88</td>
</tr>
<tr>
<td>S951P</td>
<td>TMD</td>
<td>TMH11</td>
<td>Transmembrane region; induces a kink and breaks the helical structure → decreased TMD stability</td>
</tr>
<tr>
<td>A984T</td>
<td>TMD</td>
<td>TMH12</td>
<td>Near putative substrate entry gate formed by TMH9 and TMH10; obstructs substrate entry gate → likely impairs substrate translocation</td>
</tr>
<tr>
<td>S1076N</td>
<td>NBD</td>
<td>Walker A</td>
<td>Corresponds to S1120N in BSEP</td>
</tr>
<tr>
<td>Y1086S</td>
<td>NBD</td>
<td>RecA-like</td>
<td>Residue at the interface to CH4; loss of hydrogen bond to D804 → impaired TMD-NBD communication</td>
</tr>
<tr>
<td>E1106G</td>
<td>NBD</td>
<td>RecA-like</td>
<td>Interaction cluster of charged residues in NBD; destabilization of local interactions → impaired nucleotide binding</td>
</tr>
<tr>
<td>Q1181E</td>
<td>NBD</td>
<td>RecA-like</td>
<td>In-between X-loop and signature motif; causes electrostatic repulsion with X-loop residue 1177D → impaired cross-talk between TMD and NBD</td>
</tr>
<tr>
<td>H1238Y</td>
<td>NBD</td>
<td>H-loop</td>
<td>In highly conserved H-loop histidine → obliterates catalytic activity</td>
</tr>
</tbody>
</table>

Information on possible effects is given as follows: “Location in detail; possible effect(s) on the structural level → functional consequences”. AAS, amino acid substitution; CH, coupling helix; CL, cytoplasmic loop; NBD, nucleotide binding domain; PL, phospholipid; TMD, transmembrane domain; TMH, transmembrane helix.

patients with at least one disease-causing mutation were below the age of 18 years at symptom onset, while 22.2%, 10.2% and 30.2% of patients with FIC1, BSEP and MDR3 mutations, respectively, first experienced symptoms or were first diagnosed with inherited cholestasis in adulthood. Our data are in line with findings from other studies, demonstrating that especially patients...
with disease-associated MDR3 mutations can first present in adulthood with LPAC, biliary fibrosis or even cirrhosis. Women carrying disease-associated MDR3 mutations often experience symptoms after intake of oral contraceptives or during pregnancy and therefore tend to present earlier (median age at symptom onset in our study 29.1 years, n = 6) than men (median age 47.6 years, n = 7). In total, 85 patients were homozygous or compound heterozygous and about half of these patients presented with symptoms before the first year of life, which is a typical feature of PFIC. While patients with PFIC-1 and PFIC-2 usually present within the first months of life, which was also observed in our cohort, the median age of symptom onset was later for patients with PFIC-3 (1.8 years in our study). Interestingly, 25 patients, who experienced cholestatic symptoms within the first year of life only carried one heterozygous, disease-causing variant in either of these genes, indicating that mutations outside the sequenced areas (e.g. the promoter, the untranslated regions or intronic regions), mutations in other genes, epigenetic changes or environmental factors may contribute to the severity of the phenotype. In line with this, mutations in the tight junction protein 2 (TJP2), the farnesoid X receptor (FXR) and Myosin 5B (MYO5B) have recently been identified in patients with low GT cholestasis, while variants in Claudin-1 and doublecortin domain containing protein 2 (DCDC2) can confer a sclerosing cholangitis-like phenotype with high GT. Of the 21 patients who received a LT due to BSEP deficiency, 7 (33%) experienced recurrent disease with development of anti-BSEP inhibitory antibodies. This is the first study calculating the risk of recurrent BSEP disease after LT and the percentage in our cohort is higher than the previously estimated 8%.

We identified 154 different genetic variants in our cohort of 427 patients. The majority (51.5%) of the genetic variants detected by sequencing of FIC1, BSEP, or MDR3, resulted in a rare AA substitution. The impact of the 41 yet uncharacterized missense mutations on protein function was evaluated using prediction tools, which are based on sequence, phylogenetic and structural information (PolyPhen-2) as well as the degree of AA conservation (SIFT), 75%, 95%, and 100% of these FIC1, BSEP, and MDR3 missense mutations, respectively, were considered to be detrimental by at least one of the three prediction tools. However, differing consequences for the same mutation were found for 62.5% (5/8) of FIC1, 31.8% (7/22) of BSEP and 27.3% (3/11) of MDR3 missense mutations (Table S7). Thirteen of these missense mutations were considered novel, since they were not found in databases nor in the current literature. All 13 novel missense mutations were considered detrimental by at least one prediction tool (Table S7). Additionally, 3D protein modeling was used to assess the impact of the yet uncharacterized BSEP and MDR3 missense mutations and to possibly reconcile contradictory predictions with regard to functional consequences as suggested by PolyPhen-2 and SIFT (Figs. 3 and 4, Table 4; Tables S4–S7). 3D protein modeling revealed a potential effect of the BSEP missense mutations p.V444A, p.D440E and p.Y721C, which were classified as benign variants by at least two predictions tools (Table S7). The AA substitutions detected in FIC1 were not assessed by 3D protein modeling because of the lack of an appropriate template structure, preventing the calculation of a reliable FIC1 model.

Variants affecting the canonical splice-site sequences account for about 15% of point mutations involved in human genetic diseases. In our cohort, 12 splice-site mutations were observed in BSEP and MDR3. For FIC1, only the common SNP c.3531+8G>T was detected in proximity to a splice-site. Disruption of splice-sites can lead to exon-skipping or activation of cryptic splice-sites and thereby introduces intronic regions in the mutated protein. Alternative splicing results in three MDR3 isoforms. Our reference sequence for MDR3 (NM_018849.2, isoform B) includes 21 intronic nucleotides upstream of coding exon 25, resulting in 7 additional AA. Interestingly, there is one reported variant in this region. While the nucleotide exchange would be an intronic splice-site variant (c.3280-3C>G) in NM_000443.3 (MDR3-A), it would result in an AA substitution (p.G1100D) in NM_018849.2 (MDR3-B). However, we did not detect any variation in these seven AA in our cohort.

The impact of synonymous variants is difficult to predict, since they do not result in an altered AA sequence. However, they may potentially modulate mRNA processing and affect splicing due to their location in close vicinity to the exon-intron border with detrimental effects on splicing as described for FIC1 (A93A (c.279G>A, rs761575295), which in a homozygous state can underlie PFIC-1. For the BSEP variants p.R33R (c.97A>C, rs191280849) and p.Q1100Q (c.477A>C, rs191040213) bioinformatics tools predict no impact on splicing. The disease association of these two variants is uncertain, since they are only listed in databases and not described in cholestatic patients to date. The synonymous BSEP G1100G variant was present in a patient with a BRIC-like phenotype together with p.V444A, and p.A1028A. According to prediction tools, the altered acceptor splice-site is either not properly recognized or remains unaltered. For MDR3, two known synonymous variants are located near the exon-intron border. The frequent variant p.I237I (c.711A>T) at the third position of coding exon 7 is not predicted to affect the splice-site, while p.L688L (c.2064T>C, rs375846939), only mentioned in databases so far, affects the last nucleotide of coding exon 15. This exchange is expected to result either in a slight improvement or no alteration of the splice donor.

The contribution of common SNPs to a cholestatic phenotype varies. The AFs for the common FIC1 SNPs p.R952Q (c.2855G>A) and c.3531+8G>T were 10.3% and 13.0% in our cohort, respectively, which corresponds to the AFs in the general population (Table 2; taken from gnomAD) and previous studies that found a similar incidence of either p.R952Q or c.3531+8G>T in patients with ICP and chronic pancreatitis or in healthy controls. In the European population, the mentioned FIC1 SNPs were more frequent, as in our cohort (Table 2).

The most common BSEP variants are p.V444A and p.A1028A, with AFs for the variant alleles in the general population of 56.9% and 54.5%, respectively (Table 2). While BSEP is clearly associated with ICP and DILI there was no higher frequency of either BSEP or BSEP in primary biliary cholangitis (PBC) or primary sclerosing cholangitis (PSC). In our cohort, BSEP (c.1331T>C) showed a significantly higher AF in patients with no disease-causing BSEP mutation compared to the worldwide population (65.9%, p = 0.001; Table 2). BSEP (c.3084A>G) appeared at a slightly increased AF in patients without relevant BSEP mutations (59.8%, p = 0.055; Table 2). In comparison with AFs of the European population, the AFs of BSEP and BSEP were significantly more frequent in our cohort of patients with no disease-causing BSEP mutation (V444A: p2 = 0.024; A1028A: p2 = 0.025; Table 2).

In pediatric patients without disease-causing BSEP mutation, only two patients carried neither BSEP nor BSEP , sug-
Research Article

gestig a contribution of these common SNPs to a cholestatic phenotype. We and others have previously shown, that the presence of BSEP[V444A] can aggravate the ICP phenotype in women with MDR3 mutations, leading to early onset of symptoms and severe elevation of bile acid levels.55,71,72 While BSEP[V444A] has been linked to reduced protein amounts, BSEP[A1028A] was shown to reduce splice activity in vitro.73,74 further supporting the hypothesis that the combination of these two common variants can predispose to cholestasis development. For MDR3, three common synonymous variants were detected in a large number of patients with AFs of 13.8% (MDR3[59L]), 53.3% (MDR3[N168N]), and 15.4% (MDR3[237L]). These differ from the reported AFs of gnomAD in the worldwide population. Detected AFs of MDR3[237L] were significantly lower in our patient cohort irrespective of present MDR3 mutations (all patients: p1 = 0.005; patients without mutation: p2 = 0.026; Table 2). This is in line with the finding that the more common allele of MDR3[237L] (c.711A>T) confers the risk for MDR3-associated cholestatic diseases, as shown in a whole genome-wide sequencing analysis of the Icelandic population. Here, this common MDR3 variant was associated with elevated liver enzymes, higher risk for early onset gallstone disease as well as ICP.72,73,76 Nevertheless, the detected AFs of common synonymous MDR3 variants in our cohort correspond to AFs in the European population (17.7%, Table 2). The impact of the common MDR3 SNP p.R652G is unclear. In controls, the heterozygous variant is roughly observed at the same frequency as in cholestatic patients or patients with DILI or gallstones.77,78 Nevertheless, it may be associated with decreased biliary phospholipids or ICP.5,74 In our cohort, this was the only non-synonymous MDR3 SNP present in 25 patients with a suspected MDR3-deficient phenotype. In total, the AF of p.R652G in our cohort was 6.3% and therefore significantly below the AF of gnomAD in the worldwide population (10.5%, p1 = 0.04; Table 2). However, the detected AF in our cohort matched the AF in the European population (7.5%, Table 2). Of patients without disease-causing MDR3 mutations, 72.6% (156/215) carried at least one of these four common SNPs.

The impact of SNPs and synonymous variants on disease phenotype is often unclear, especially when variants have AFs of about 50% or greater as described for BSEP[V444A], BSEP[A1028A], or MDR3[N168N]. Variants appearing at these frequencies challenge the selection of the reference sequence. For our analyses, AF038007 was used as FIC1 reference sequence. Compared to AF038007, NM_005603.4 differs at two nucleotide positions (c.696T>C, p.D232D, rs319438; c.811A>C, p.R271R, rs319443). In a recent study, the C allele of rs319443 was present in 100% of controls and 99.6% of pancreatitis patients.49 The AFs according to gnomAD are 0.2% and 0.3% for c.696T and c.811A, respectively, thus, AF038007 was used.

In summary, we analysed FIC1, BSEP, and MDR3 in a cohort of 427 patients with suspected genetic disposition to cholestasis. We identified 154 variants of which 55 were first described by our lab and 25 have never been reported to date. A total of 149 patients carried at least one disease-associated variant in the genes analysed. The risk alleles of the common SNPs BSEP[V444A], BSEP[A1028A], MDR3[59L], MDR3[N168N], MDR3[237L], and MDR3[R652G] were frequently different in our cohort as compared to database entries for the general worldwide population, supporting a role for common SNPs as well as synonymous variants to disease susceptibility. Nevertheless, our cohort represents a mixed population and AFs vary in subpopulations depending on the ethnic background. The composition of the subpopulations is not obvious, which makes it more difficult to compare the data to an adequate control population. The number of patients carrying neither a disease-associated mutation nor one of the common BSEP and MDR3 SNPs was surprisingly low with 0.8% (2/252) and 7.4% (16/215) for BSEP and MDR3, respectively, underscoring the important role of these two transport proteins in the pathogenesis of cholestasis. Additional studies using next generation sequencing techniques will help to identify additional genes contributing to the broad spectrum of genetically-determined cholestasis.

Financial support

This work was funded by the German Research Foundation (DFG) through the Clinical Research Group KFO217 and the Collaborative Research Centre SFB974.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors’ contributions

C.D., S.K., R.K., D.H., and V.K. designed the study and analysed patients’ and sequencing data, U.B., C.K., E.L., S.K., F.B., E.G., E.D., P., D.W., A.F., D.N.G., K.H.W., P.M., R.D.P., and IC.V. collected and analysed the patients’ data. C.D. performed 2D protein modeling and applied prediction tools, M.B., H.G., and L.S. performed mutational effect prediction and 3D protein modeling. C.D. and V.K. wrote the manuscript; all authors critically revised the manuscript.

Acknowledgements

The authors thank the physicians for sending the patients’ samples for evaluation to Düsseldorf. Furthermore, expert technical assistance by Nathalie Walter, Lisa Knopp, Paulina Philippaki and Annette Tries is gratefully acknowledged. We are grateful to the “Zentrum für Informations- und Medientechnologie (ZIM) at the Heinrich Heine University Düsseldorf for providing computational support and to the Jülich Supercomputing Center for granting computing time on the supercomputer JURECA (NIC project ID HDU19) to HG.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2017.07.004.
References

Author names in bold designate shared co-first authorship


JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Cholestasis and Autoimmune Diseases
Research Article


