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The identification and characterization of genetic variants in human hepatobiliary transporters

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ABSTRACT

The human liver is the main metabolic organ with a wide range of important functions such as bile secretion, metabolism, glycogen storage, detoxification, and endocrine function. A number of membrane proteins including members of the family of adenosine triphosphate binding cassette (ABC) transporters possess an outstanding importance in the secretory function of the liver. ABC transporters like the bile salt export pump (BSEP) and multidrug resistance protein 3 (MDR3) and the P-type ATPase familial intrahepatic cholestasis 1 (FIC1) are located in the canalicular membrane of hepatocytes and are essential for bile formation. Genetic variants in FIC1, BSEP, and MDR3 can result in an impaired bile salt homeostasis and therefore lead to cholestatic liver diseases of varying severity. The spectrum of these cholestatic diseases comprises severe forms affecting mostly children in the first years of life, the group of progressive familial intrahepatic cholestasis (PFIC) type 1 to 3. The benign recurrent intrahepatic cholestasis (BRIC) type 1 and 2, the intrahepatic cholestasis of pregnancy (ICP) as well as the low phospholipid-associated cholelithiasis (LPAC) represent less severe forms of cholestatic liver diseases with a later onset compared to PFIC. Not only rare disease causing mutations but also common single nucleotide polymorphisms (SNPs) as well as synonymous variants not affecting the amino acid sequence can predispose to a cholestatic phenotype and affect the clinical course of liver diseases. More than 600 genetic variants in FIC1, BSEP, and MDR3 are already known, nevertheless, only a few are characterized on a molecular level. A detailed knowledge of the genetic variants is important for a better assessment of disease process and especially for treatment decisions. Nowadays, a number of bioinformatics tools exist to predict the impact of genetic variants. Another method is the analysis of specific variants via *in vitro* models like cell-culture-based assays. Furthermore, the evaluation of the patient's liver tissue gives detailed insights concerning the effects of the genetic variants.

This work gives an overview of hepatic bile formation and the disease spectrum of cholestatic liver diseases, which can result from dysfunction in hepatobiliary transport systems. Genetic variants were identified from patients suffering from cholestasis and characterized on a molecular level using not only *in vitro* systems but also investigating the patient's liver tissue.

ZUSAMMENFASSUNG

Die humane Leber ist das Hauptstoffwechselorgan mit zahlreichen wichtigen Aufgaben wie Gallesekretion, Stoffwechsel, Glykogenspeicher, Entgiftung sowie endokriner Funktion. Eine Vielzahl von Membranproteinen, einschließlich Mitglieder der ATP-bindenden Kassette Transporter (ABC Transporter), besitzen eine herausragende Bedeutung für die sekretorische Funktion der Leber. ABC Transporter wie die Gallensalzexportpumpe BSEP und der Multidrogentransporter MDR3 oder auch die ATPase FIC1 sind in der kanalikulären Membran der Hepatozyten lokalisiert und unerlässlich für die Gallebildung. Genetische Varianten in FIC1, BSEP und MDR3 können zu einer gestörten Gallensalzhomöostase und somit zu verschiedenen cholestatischen Lebererkrankungen führen. Das Spektrum der cholestatischen Erkrankungen umfasst eine schwerwiegende Form wie die progressive familiäre intrahepatische Cholestase (PFIC) Typ 1 bis 3. Betroffen sind weitestgehend Kinder in den ersten Lebensjahren. Verglichen dazu sind die benigne rekurrente intrahepatische Cholestase (BRIC) Typ 1 und 2, die intrahepatische Schwangerschaftscholestase (ICP), sowie die mit niedrigen Phospholipid-Galle-Spiegeln assoziierte Cholelithiasis (LPAC) spätmanifeste Erkrankungen mit einem meist milderen Verlauf. Nicht nur seltene krankheitsassoziierte Mutationen sondern auch häufige Nukleotidpolymorphismen (SNPs) oder synonyme Varianten ohne direkten Einfluss auf die Aminosäuresequenz können für eine Cholestase prädisponieren und den klinischen Verlauf von Lebererkrankungen beeinflussen. Bisher sind über 600 genetische Varianten in FIC1, BSEP und MDR3 bekannt, nur wenige sind jedoch auf molekularer Ebene charakterisiert. Detaillierte Kenntnis der Varianten ist hilfreich, um den Krankheitsverlauf und auch die Behandlungsmöglichkeiten besser einschätzen zu können. Es existiert eine Vielzahl bioinformatischer Instrumente, um den Einfluss genetischer Varianten vorherzusagen. Eine weitere Möglichkeit ist die Analyse spezifischer Varianten mit Hilfe von *in vitro* Systemen wie zellkulturbasierten Assays. Des Weiteren gibt die Analyse des Lebergewebes der Patienten Aufschluss über die Auswirkungen der genetischen Varianten.

Die vorliegende Arbeit gibt einen Überblick über die hepatische Gallebildung und das Spektrum der cholestatischen Lebererkrankungen, die auf Funktionsstörungen in hepatobiliären Transportsystemen basieren. Bei Patienten mit cholestatischen Phänotypen wurden genetische Varianten identifiziert und auf molekularer Ebene charakterisiert. Dabei wurden nicht nur *in vitro* Systeme genutzt sondern auch das Lebergewebe betroffener Patienten analysiert.

1 INTRODUCTION

1.1 Liver anatomy and function

1.1.1 Topology and macroscopic liver anatomy

The liver (*hepar*) of mammals represents the main metabolic organ with characteristics of an exocrine gland and a wide range of important functions such as bile secretion, metabolism, glycogen storage, detoxification, synthesis of blood plasma components, immunoglobulin A (IgA) secretion, and endocrine function. The human liver is the heaviest internal organ, situated in the upper right quadrant of the abdominal cavity ending with the last costal arch. It weighs about 1 - 1.8 kg in adults depending on body weight. The organ is coated by the thin fibrous Glisson's capsule (*Capsula fibrosa*). The upper convex surface (*Facies diaphragmatica*) is smooth and adjacent to the diaphragm whereas the lower more complex shaped surface (*Facies visceralis*) covers the gallbladder (*Vesica biliaris*) and rests above the bowels. The liver is divided into a smaller left hepatic lobe (*Lobus sinister*) and a larger right hepatic lobe (*Lobus dexter*). The borders of these lobes are defined by the *Ligamentum falciforme hepatis* as well as the fissure of *Ligamentum teres* and *Ligamentum venosum*. From the visceral side, the *Porta hepatis* subdivides the medial part of the right hepatic lobe into two smaller lobes: *Lobus quadratus* (ventral) and *Lobus caudatus* (dorsal). The *Porta hepatis* represents the entry of the two main blood vessels: the hepatic artery (*Arteria hepatica*) and the portal vein (*Vena portae*). Furthermore, the common hepatic bile duct (*Ductus hepaticus communis*) exits the liver. Branches of the hepatic artery, the portal vein, and the bile ducts allow a lateral segmentation of the hepatic lobes. The vessels of the hepatic vein (*Vena hepatica dextra/intermedia/sinistra*) result in an additional vertical division of each lobe. Eight liver segments (I-VIII) are distinguished, numbered clockwise. 70 - 80 % of blood streaming into the liver derives from the unpaired internal organs and transports nutrients through the portal vein. 20 - 30 % of blood reaches the liver via the hepatic artery and is oxygen-rich. After passing the liver lobules, venous blood flows into the hepatic veins leading into the inferior vena cava (*Vena cava*). The liver is drained by the subperitoneal and intraparenchymatous lymph vessels and innervated by sympathetic as well as parasympathetic fibers (1, 2).

1.1.2 Microscopic liver anatomy and histology

The liver parenchyma is structured in small hexagonal lobules surrounding the endings of the central vein. These anatomic structures are therefore named central vein liver lobules (*Lobuli hepatici*) (Figure 1A). While the blood flow is directed from the lobule periphery to the center, the bile flows in the opposite direction. Each liver lobule is drained by multiple interlobular branches of the portal vein and the hepatic artery. Likewise, the bile of one lobule also flows into several bile ducts. Structures including branches of the hepatic portal vein, the proper hepatic artery, and the common bile duct are denoted as the portal triad (*Trias hepatica*) (Figure 1). A portal liver lobule describes a functional unit of the liver parenchyma which is provided by one periportal field (portal triad) in the center while the central veins are located at the corners of this triangle (Figure 1B).

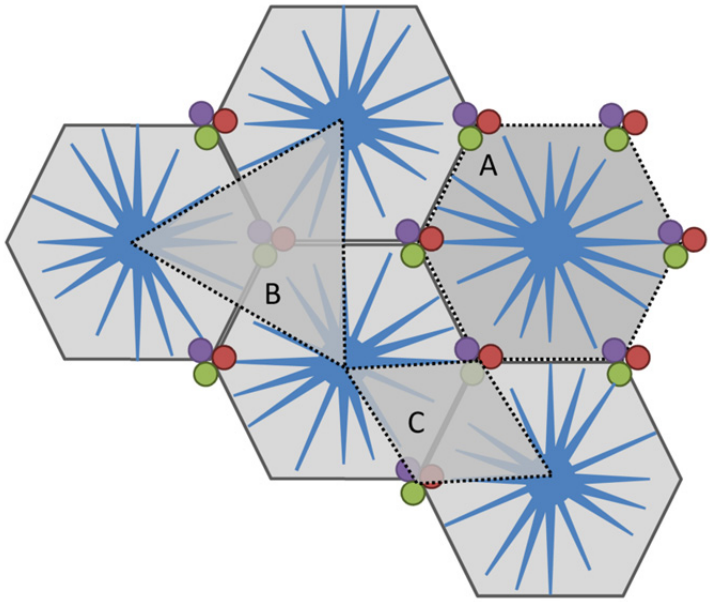

Figure 1. Schematic overview of the parenchyma liver lobules

The central vein drains the polygonal anatomic structure, the central vein lobules (A). The portal triad composed of branches of the hepatic portal vein (purple), the proper hepatic artery (red), and the common bile duct (green) encircles the central vein lobule and represents the center of the functional unit of the portal triad (B). The liver acinus depicts the functionally heterogeneous zoning of the liver parenchyma cells (C), while the periportal zone is in the liver lobule periphery and the pericentral zone is situated in the area of the central vein.

The character of the liver as an exocrine gland is expressed best by this classification with the interlobular bile ducts as glands in each center of the portal lobule. A common functional classification is the liver acinus, which reflects the hepatic oxygen supply and represents the metabolic zoning of the liver parenchyma (Figure 1C). Zone 1 is the most oxygenated area in the liver lobule periphery and thus the center of the acinus. Together with the adjacent zone 2, it forms the periportal zone. Zone 3 is located in the area of the central vein and builds the pericentral zone. The liver parenchyma cells in these zones are functionally heterogeneous due to a gradient of metabolites, oxygen, and hormones.

About 70 % of the liver volume is made up of hepatocytes, which are polarized parenchyma cells with a sinusoidal (basolateral) and a canalicular (apical) membrane (3). The sinusoidal membrane is adjacent to blood vessels and nutrients and xenobiotics are taken up across this membrane. The canalicular domains of adjacent hepatocytes are bound together by tight junctions and form the smallest bile ducts, the bile canaliculi (*Canaliculi biliares*), as schematically depicted in Figure 3 as well as in Chapters 3.2 (Figure 1), and 3.8 (Figure 1). The bile canaliculi empty into bile ducts via the canal of Hering. There is evidence for a hepatic stem cell niche in the canals of Hering, which are lined by hepatocytes and another group of epithelial cells, the cholangiocytes, which encompass the bile ducts (4). Moreover, the liver contains a number of non-epithelial cells. The fenestrated sinusoidal endothelial cells cover the sinusoidal surface to enclose the extravascular space of Disse. Within this space of Disse, the hepatic stellate cells (or Ito cells) are located. They can be activated by cytokines and transform into myofibroblast-like cells. Cytokines are released by the Kupffer cells, the resident macrophages of the liver, phagocytosing old erythrocytes and other particles deriving from the sinusoids.

The given overview of macroscopic and microscopic liver anatomy and liver function is derived from the corresponding book chapters of (1, 2, 5, 6).

1.2 Bile formation, secretion and physiological relevance

Bile formation is essential for the elimination of cholesterol and excretion of lipophilic components after biotransformation like conjugation, sulfation, and hydroxylation (7, 8). Within bile, mixed micelles composed of bile salts, phospholipids, and cholesterol enable the

elimination of the latter one. Three ABC transport proteins are involved in the excretion of the components of these mixed micelles. Bile salts are transported from the hepatocytes across the canalicular membrane by the bile salt export pump (BSEP) while the multidrug resistance protein 3 (MDR3) flops phosphatidylcholine from the inner to the outer membrane leaflet. The heterodimeric transporter ABCG5/ABCG8 secretes cholesterol from the hepatocytes into the bile canaliculi. Within bile, bile salts and phospholipids form mixed micelles together with cholesterol (9).To maintain membrane asymmetry required for proper transporter function, the P-type ATPase familial intrahepatic cholestasis 1 (FIC1) flips aminophospholipids from the outer to the inner membrane leaflet. The two primary bile acids cholic acid and chenodeoxycholic acid are synthesized in the hepatocytes via the oxidation of cholesterol, controlled by the rate-limiting-enzyme 7α-hydroxylase (CYP7A1) (10, 11). Bile acids are conjugated with either glycine or taurine resulting in negatively charged bile salts (12). Bile salts get from the canaliculi, via the larger bile ducts into the intestine, where they are partly deconjugated and dehydroxylated by bacteria, yielding in secondary bile acids/salts. While only a small amount of bile salts (0.2 - 0.6 g of 3 - 4 g) is excreted by the feces, about 90 % are reabsorbed in the ileum and recycled back to the liver via the portal vein several times a day (5, 13). A minor portion of bile salts circumvents the ileum passage and is already reabsorbed by the cholangiocytes and directly circled back to the liver via the periductular capillary plexus, which is referred to as cholehepatic shunting (12). The cholehepatic and enterohepatic circulation involves active transport steps in hepatocytes, cholangiocytes, and enterocytes (13). The major hepatic transporters to maintain bile salt homeostasis are the Na^+-taurocholate co-transporting polypeptide (NTCP; gene symbol *SL10A1*) as the main uptake transporter for bile salts at the sinusoidal membrane (14) and BSEP (*ABCB11*) at the canalicular membrane of hepatocytes. Other important transporters of cholangiocytes and enterocytes are the apical sodium-dependent bile acid transporter (ASBT; *SLC10A2*) and the organic solute transporters OSTα/OSTβ (*SLC51A/SLC51B*). Bile salts have several effects in hepatocytes, cholangiocytes, and enterocytes. At high concentrations, bile salts are toxic (15), can cause apoptosis (16, 17), and are involved in biological processes like liver regeneration (18), glucose homeostasis (19) or carcinogenesis (20). Therefore, the tolerable level of bile salts in blood, hepatocytes, bile, and intestine is limited. The enterohepatic circulation is schematically depicted in Figure 2. The mechanism of the enterohepatic and cholehepatic circulation including the involved transport mechanisms are further described in Chapter 3.2.

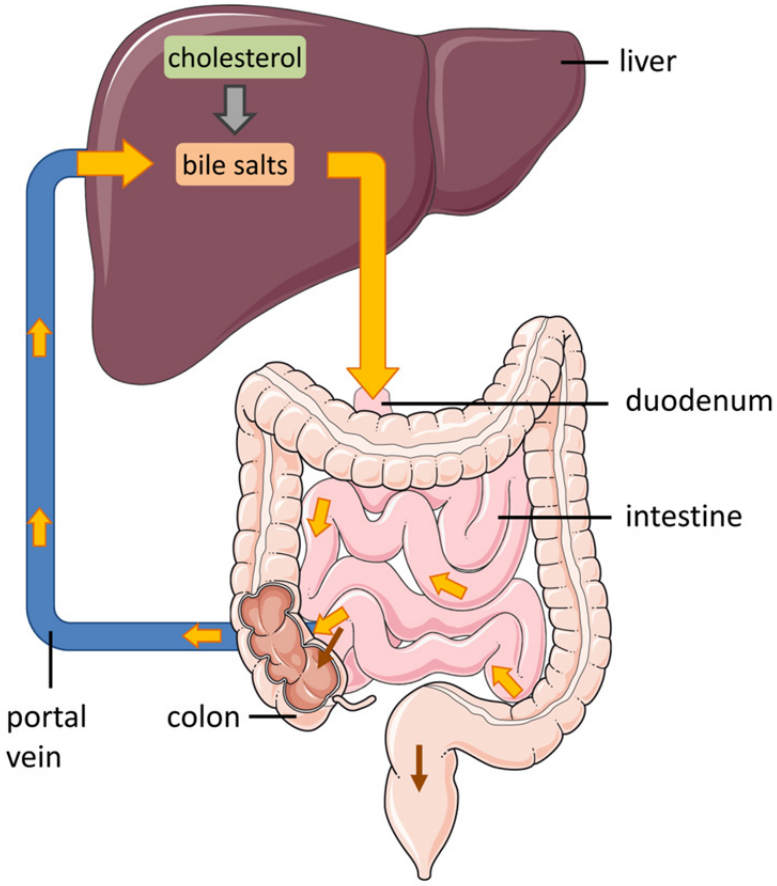

Figure 2. The enterohepatic circulation of bile salts

Bile salts are synthesized in the liver from cholesterol and secreted to the duodenum and used for digestion. Bile circulation is shown in yellow. About 90% of bile salts are reabsorbed in the terminal ileum and cycled back to the liver via the hepatic portal vein. Only a small proportion of bile salts reaches the colon and get lost with the feces (brown arrow). For involved transport proteins see Chapter 3.2, Figure 1. This figure includes elements taken from Servier Medical Art (www.servier.com).

1.3 Hepatobiliary transport mechanisms

Bile formation represents an osmotic process powered by the transport of solutes from blood into bile via sinusoidal and canalicular transport mechanisms. The asymmetric distribution of transporter proteins along the sinusoidal and the canalicular membrane of hepatocytes provides the basis for the vectorial transport of bile components from blood to bile (3). For an overview of hepatocellular transporters and their main substrates see Figure 3. Dysfunction

of the hepatobiliary transport proteins involved in bile flow is associated with liver disease of varying severity, which will be discussed in section 1.4.

1.3.1 Sinusoidal and canalicular transport systems

Several transport proteins are situated in the sinusoidal hepatocyte membrane and mediate not only the uptake of endogenous substrates and xenobiotics but also the export of substrates from the hepatocytes back to the bloodstream (21). NTCP is of great importance as it represents the main uptake system preferentially for conjugated bile salts from bloodstream into hepatocytes in a Na^+-dependent manner (22, 23). Additionally, the group of organic anion transporting polypeptides (OATPs) mediates the Na^+-independent bile salt uptake and import of bilirubin and other amphipathic compounds like steroids, thyroid hormones, peptides, and many drugs (21, 24, 25). In the human hepatocytes, OATP1A2, OATP1B1, OATP1B3, and OATP2B1 are expressed (26-30). Furthermore, the organic anion transporters (OATs), e.g. OAT2 and OAT5, facilitate the uptake of substances like methotrexate in a Na^+-independent manner (31, 32). Organic cations are imported by the sinusoidal organic cation transporters (OCT), especially by the liver-specific transport protein OCT1 (33). Besides these uptake mechanisms, the sinusoidal membrane also includes proteins with export function. The heterodimeric organic solute transporters OSTα/OSTβ as well as some multidrug resistance-associated proteins (MRP1, MRP3, MRP4) can mediate back-transport of bile salts into blood under cholestatic conditions and therefore may protect the hepatocyte from critical intracellular bile salt concentrations to some degree (3, 34-37). Additionally, MRP5 and MRP6 transport cyclic nucleotides and glutathione conjugates, respectively (3, 37). The canalicular membrane contains several ABC transport proteins, which transport different substrates from hepatocyte into bile against a concentration gradient enabled by ATP hydrolysis (For detailed function of ABC transporters see section 1.5). Similar to the breast cancer resistance protein (BCRP), the multidrug resistance protein 1 (MDR1) excretes cationic substances, drugs, and steroid hormones into bile (38), while MDR3 flops phospholipids from the inner to the outer leaflet of the canalicular membrane (39, 40). The bile salt export pump BSEP predominantly transports conjugated bile acids but also unconjugated bile salts (41). This transport protein can increase bile salt concentration in bile up to 1000-fold higher than in portal blood (12, 42). BSEP is the major force in bile salt-dependent bile flow. In contrast to the other MRPs, MRP2 is situated in the canalicular

membrane and transports a wide spectrum of conjugated substrates such as bilirubin conjugates (43-45). The heterodimeric transporter ABCG5/G8 mediates biliary cholesterol secretion, which is important for an equilibrated bile composition (46-48). Different from the canalicular ABC transporters, the P-type ATPase FIC1 functions as an aminophospholipidflippase to maintain membrane asymmetry (49). Disturbed function of FIC1, BSEP, and MDR3 is clearly associated with the manifestation of cholestatic liver diseases and will be discussed in detail below.

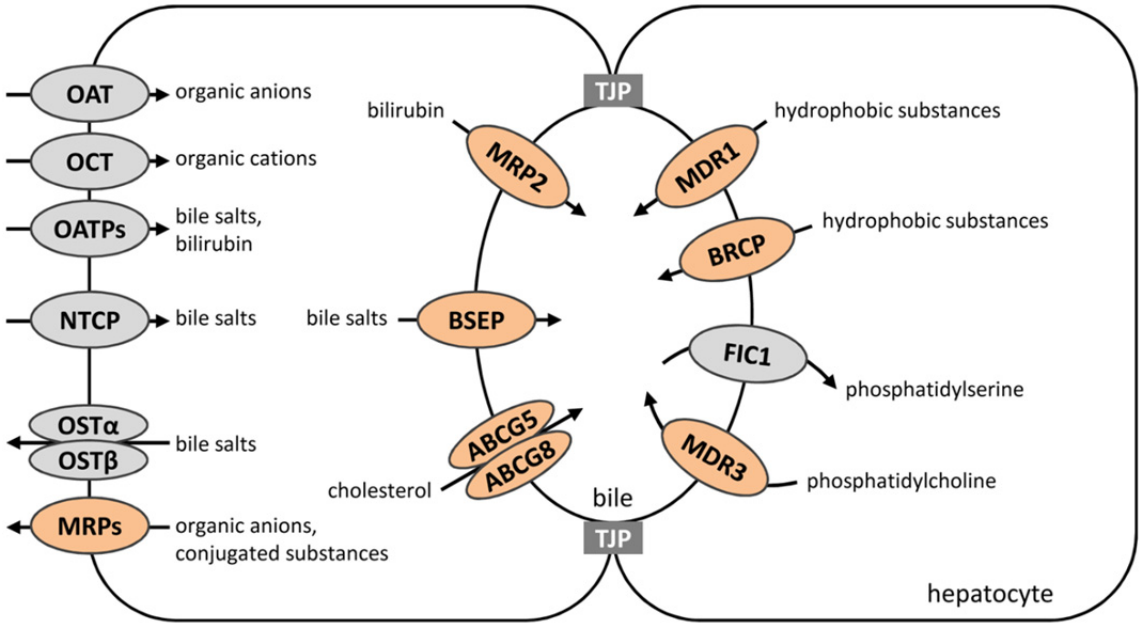

Figure 3. Hepatocellular transport systems

Transport proteins are differentially distributed in the plasma membrane of hepatocytes. The transporters at the sinusoidal (basolateral) membrane mediate the uptake and export of various substrates from and back into the blood stream. NTCP is the main hepatic bile salt uptake transporter. Proteins at the canalicular (apical) membrane mainly export substances from the hepatocyte into bile. The P-type ATPase FIC1 flips phosphatidylserine from the outer to the inner leaflet of the canalicular membrane to maintain membrane asymmetry. Bile salts and cholesterol, exported by BSEP and ABCG5/ABCG8, respectively, form mixed micelles in bile together with phosphatidylcholine, which is flopped from the inner to the outer leaflet by MDR3. The heterodimeric transporter OSTα/OSTβ and the sinusoidal MRPs represent an overflow mechanism when intracellular bile salt levels increase. ABC transporters are highlighted in orange. The canalicular membrane is separated from the basolateral membrane by tight junction proteins (TJPs).

1.3.2 BSEP expression, localization, and function

The bile salt export pump BSEP (gene symbol: *ABCB11*) is a member of the MDR/TAP subfamily of ABC transporters together with P-glycoprotein (P-gp/MDR1; *ABCB1*) and multidrug resistance protein 3 (MDR3; *ABCB4*). The human *ABCB11* gene is located on chromosome 2q24 and consists of an untranslated exon followed by 27 exons encoding the BSEP protein of 1321 amino acids (AA) with a molecular mass of about 160 kDa (50). The protein encompasses twelve transmembrane helices (TMHs) and two large intracellular nucleotide binding domains (NBDs).

BSEP is exclusively expressed at the canalicular membrane of hepatocytes and is responsible for bile-salt dependent bile flow. The main substrates are monovalent bile acids including the glycine and taurine conjugates of the primary bile acids cholic acid and chenodeoxycholic acid and the secondary bile acid deoxycholic acid (51). Furthermore, it can secrete ursodeoxycholic acid (UDCA) and related conjugates into bile. The calculated intrinsic clearance values (Vmax/Km) are ranked as it follows: taurochenodeoxycholate > glycochenodeoxycholate > taurocholate > glycocholate (52). It is likely that BSEP has a higher affinity to eliminate chenodeoxycholic acid and its conjugated forms because they are shown to be more toxic for hepatocytes *in vivo* as well as *in vitro* (17, 53). Targeting of BSEP from the Golgi apparatus to the canalicular membrane is stimulated by the p38MAP kinase and protein kinase C (PKC) (54). The HCLS1-associated protein X-1 (HAX1) regulates the internalization of BSEP from the canalicular membrane. A depletion of HAX1 as well as a dominant negative form the HAX1 interacting partner cortactin leads to an increased amount of BSEP protein at the apical membrane in polarized Madin-Darby canine kidney (MDCK) cells (55). Cortactin phosphorylation is a result of cell shrinkage resulting in endocytosis of rat Bsep and Mrp2 and subsequent cholestasis (56). A tyrosine-based motif at BSEP's C-terminus was shown to be essential for its dynamin- and clathrin-dependent endocytosis (57). It is known that cortactin interacts with dynamin (58) which possibly represents a link between external stimuli and endocytosis of transport proteins.

The plasma membrane asymmetry maintained by proper function of FIC1 is a prerequisite for correct BSEP function in the canalicular membrane of hepatocytes as well as to protect the plasma membrane towards the detergent effects of high bile acid concentrations (59, 60). An additional mechanism how FIC1 and BSEP may interact comprises the farnesoid X receptor (FXR) and the atypical PKCζ. The inhibition or downregulation of PKCζ reduced the nuclear

localization of FXR. FXR is a known transcription factor of BSEP. Reduced nuclear FXR localization may therefore reduce BSEP expression. Due to the interactions between FIC1 and BSEP, patients with defects in FIC1 or BSEP show similar phenotypes (low gamma-glutamyltransferase (γGT) cholestasis). Nevertheless, patients with FIC1-malfunction also suffer from extrahepatic manifestations like hearing-loss, diarrhea explained by the extrahepatic expression of FIC1.

1.4 Disorders of hepatobiliary transport

Malfunction of hepatobiliary transport mechanisms results in an impaired bile flow and excretion of bile components, which is defined as cholestasis (3, 61, 62). Main clinical symptoms are jaundice and pruritus. Laboratory findings include increased bilirubin levels as well as elevated serum bile salts. It is distinguished between extrahepatic and intrahepatic cholestasis. An obstruction of the draining bile duct system outside the liver due to gallstones, tumors, cysts, or bile duct stenosis is assigned as extrahepatic or obstetric cholestasis. In contrast, forms of intrahepatic cholestasis are predominantly caused by a markedly reduced or impaired capacity of hepatocytes to secrete bile (3). This work focuses on the spectrum of intrahepatic cholestasis as described in the following.

1.4.1 Three canalicular proteins related to intrahepatic cholestasis

Three transport proteins are of great importance for bile formation: the familial intrahepatic cholestasis 1 (FIC1; *ATP8B1*), the bile salt export pump (BSEP; *ABCB11*) and the multidrug resistance protein 3 (MDR3; *ABCB4*). They are located in the canalicular membrane of hepatocytes. In detail, FIC1 translocates aminophospholipids from the outer to the inner membrane leaflet (49). This process maintains membrane asymmetry, which is essential for the accurate function of other membrane transporters, especially for BSEP functioning (43, 63). The FIC1 protein of 1251 AA is encoded by the *ATP8B1* gene located on chromosome 18q21 containing one non-coding and 27 coding exons (64, 65). Two members of the subfamily B (MDR/TAP) of adenosine triphosphate (ATP)-binding cassette (ABC) transporters are essential for bile formation: BSEP (*ABCB11*) and MDR3 (*ABCB4*). The functioning of ABC

transporters is elucidated later in section 1.5. As mentioned before, BSEP is the major driving force of bile salt-dependent bile flow (3, 62, 66). The human *ABCB11* gene on chromosome 2q24 consists of 27 coding exons resulting in the BSEP protein of 1321 AA (50). *ABCB4* is localized on chromosome 7q21 with one non-coding followed by 27 coding exons resulting in 1286 AA of the MDR3 protein (67). MDR3 transports phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane (40, 68, 69). Genetic variants in these three transport proteins are the basis of cholestatic liver diseases of diverging severity. Intrahepatic cholestatic diseases due to impaired function of FIC1 or BSEP are predominantly associated with normal γGT values (low γGT), whereas MDR3-related cholestasis is characterized by elevated γGT levels. Patients' phenotypes range from milder forms like drug-induced liver injury (DILI) (70), intrahepatic cholestasis of pregnancy (ICP) (71-74), benign recurrent intrahepatic cholestasis (BRIC) (64, 65, 75, 76) or low phospholipid-associated cholelithiasis (LPAC) (74) to a severe, early-onset phenotype: progressive familial intrahepatic cholestasis (PFIC) type 1, 2 or 3 (50, 64, 65, 77), often necessitating liver transplantation in early childhood. The different forms of intrahepatic cholestasis and other hepatobiliary diseases are addressed in depth hereafter and summarized in Table 1. The genetic background based on FIC1, BSEP, and MDR3 was analyzed in more than 400 patients as presented in Chapter 3.3.

1.4.2 Progressive familial intrahepatic cholestasis (PFIC)

The first PFIC cases were identified in several siblings in an Amish family called Byler in 1969 and therefore this type of cholestasis was first termed Byler's disease (78). PFIC affects children in the first months or years of life suffering from symptoms as jaundice, pruritus, growth retardation, or coagulopathy. This progressive form of cholestasis can lead to fibrosis, cirrhosis and end stage liver disease and therefore, in most cases, liver transplantation is the only chance of recovery. Furthermore, PFIC-2 patients are known to have an increased risk for hepatocellular or cholangiocellular carcinoma (79-82). PFIC-1 and PFIC-2, due to impaired function of FIC1 or BSEP, are mainly associated with normal γGT levels, since bile salt levels in bile and therefore toxicity on cholangiocytes is reduced (83). In contrast, PFIC-3 is characterized by elevated γGT levels, due to reduced phospholipid concentrations in bile caused by disturbed MDR3 function. The impaired formation of mixed micelles results in higher relative concentrations of bile salts and thus toxicity on bile ducts (84, 85).

Homozygous mutations in FIC1, BSEP or MDR3 are related to the autosomal recessive disorders PFIC-1, -2, -3, respectively (50, 64, 65, 77). Compound heterozygosity with two heterozygous mutations on different alleles can have the same effect as one homozygous mutation. Parents of affected children carrying only one heterozygous mutation are usually asymptomatic, nevertheless, even one heterozygous variant can predispose for less severe forms of cholestasis like BRIC, ICP or LPAC as elucidated in the following paragraphs. Additional to mutations in FIC1 or BSEP, mutations in the tight junction protein 2 (TJP2) and in the myosin VB gene (MYO5B) were identified to cause forms of early onset low γGT cholestasis (86-88).

1.4.3 Benign recurrent intrahepatic cholestasis (BRIC)

In contrast to the early onset forms of PFIC, the benign recurrent intrahepatic cholestasis (BRIC) is less severe with later onset and temporary episodes of recovery. Genetic variants in FIC1 are related to BRIC-1, while BSEP variants are assigned to BRIC-2 (64, 65, 75, 76). Both forms of BRIC go along with normal γGT levels. BRIC-patients are homozygous or compound heterozygous for mutations in FIC1 or BSEP. BRIC-associated mutations often have a residual protein expression resulting in a partly preserved function. In some cases, one heterozygous mutation with detrimental effects is sufficient to cause a milder cholestatic phenotype (89). Furthermore, there is sometimes a transition between PFIC-2 and BRIC-2 phenotypes, illustrating that the forms of intrahepatic cholestasis are more a disease continuum (90-92). The clinical presentations of BRIC-1 and BRIC-2 are very similar, although there is a higher incidence of gallstones in BRIC-2 patients (76). The less severe form of cholestasis based on MDR3 variants is often denoted as low phospholipid-associated cholelithiasis (LPAC) and will be further described in paragraph 1.4.5 in the context of gallstone formation. Late onset of cholestasis also based on genetic variants in FIC1, BSEP, and MDR3 induced by pregnancy will be discussed in the following.

1.4.4 Intrahepatic cholestasis of pregnancy (ICP)

Hormones may contribute to the manifestation of a cholestatic phenotype. Intrahepatic cholestasis of pregnancy (ICP) is the most frequent liver disease in pregnant women affecting about 0.2 - 2.0 % of pregnancies in Europe (93-95). ICP usually occurs in late pregnancy, but

symptoms resolve rapidly after delivery (95-97). However, in subsequent pregnancies, symptoms reappear in 45 - 90 % (95, 96, 98). ICP is mainly characterized by pruritus and elevated serum bile acid as well as transaminase levels, whereas icterus and increased bilirubin levels are less common (95, 96). This form of cholestasis does not only affect the pregnant women, but is also related to a higher incidence of adverse fetal outcomes, e.g. premature delivery, respiratory distress, or even stillbirth (99-101). Although the ICP-associated symptoms resolve after delivery, these women have a higher incidence of some liver, biliary, pancreatic, metabolic, and immune-mediated disorders such as cholelithiasis, cholecystitis, cholangitis, gallstone-associated pancreatitis, nonalcoholic liver cirrhosis, or also diabetes, cardiovascular diseases, and thyroid disease (94, 102, 103). ICP-affected women even have an increased risk to develop hepatocellular or biliary carcinoma (94). Key factors for ICP development are not only the increased gestational hormones, e.g. estrogen and progesterone, but also genetic variants (96, 104). Genetic variants in the canalicular proteins FIC1, BSEP, and MDR3 as well as in the nuclear receptor FXR are clearly related to the manifestation of ICP (71, 73, 105-109). Similar to ICP, oral contraceptives can also trigger a cholestatic phenotype denoted as contraceptive-induced cholestasis (CIC), which is also based on genetic variants in BSEP and MDR3 (72, 95, 110-112). A women affected by a severe form of ICP is presented in detail in Chapter 3.11.

1.4.5 Low phospholipid-associated cholelithiasis (LPAC)

Gallstone disease is one of the most common diseases in Europe as well as in North and South America. Women are affected twice as often as men (5, 113). Gallstones are subdivided depending on their composition into cholesterol stones which make out about 90 % (5) and pigment/bilirubin stones (114, 115). Gallbladder stones can trigger cholecystitis whereas gallstones in the efferent bile ducts can cause obstructive cholestasis. In 25 - 30 % of symptomatic gallstones, genetic variants contribute to the phenotype (116-119). Additionally, environmental factors such as fasting, diabetes or insulin resistance as well as oral contraceptives and pregnancy are known to have an effect on the formation and progression of cholesterol gallstones (118, 120, 121). Thus, patients suffering from ICP are also predisposed for gallstone formation. Cholesterol gallstones develop due to an imbalanced bile composition of cholesterol, bile salts and phospholipids. Therefore, BSEP mutations associated with an altered bile salt excretion can lead to cholesterol gallstone formation as

well as MDR3 mutations cause the low phospholipid-associated cholelithiasis (LPAC) syndrome (74, 122, 123). An alteration in bile composition can also result from disturbed hepatocellular bile salt sensing due to mutations in CYP7A1 or FXR (124-126). The development of the less prevalent pigment gallstones is based on an increased hepatic excretion of unconjugated bilirubin (5). Specific genetic variants contributing to gallstone formation are listed in Chapter 3.1 (Table 2).

	gene/protein	**serum γGT**	**serum bile acids**	**clinical features**
PFIC-1	*ATP8B1*/FIC1	normal	elevated	severe cholestasis in early childhood
PFIC-2	*ABCB11*/BSEP	normal	elevated	severe cholestasis in early childhood
PFIC-3	*ABCB4*/MDR3	elevated	elevated	severe cholestasis in early childhood, decreased phospholipids in bile
BRIC-1	*ATP8B1*/FIC1	normal	elevated during episodes	recurrent episodes of cholestasis at any age
BRIC-2	*ABCB11*/BSEP	normal	elevated during episodes	recurrent episodes of cholestasis at any age
LPAC	*ABCB4*/MDR3	can be elevated	can be elevated	gallstones, recurrence after cholecystectomy
ICP-1	*ATP8B1*/FIC1	normal	elevated	symptomatic cholestasis with mainly pruritus in the third trimester, symptom relief after delivery
ICP-2	*ABCB11*/BSEP	normal	elevated	
ICP-3	*ABCB4*/MDR3	can be mildly elevated	elevated	

Table 1. Characteristics of intrahepatic cholestatic disorders of varying severity

1.4.6 Drug-induced liver injury (DILI)

Therapeutic drugs can cause hepatotoxicity, e.g. drug-induced liver injury (DILI). Not only genetic variants but also age, gender, nutrition, intake of alcohol or other drugs, and pre-

existing liver diseases can contribute to DILI (119, 127). The extent of hepatic manifestations can range from unspecific injury to hepatitis, cholestasis, vascular lesions or even tumors (127). Obviously, drugs are more likely to cause DILI when they are significantly metabolized in the liver. While DILI-associated genetic variants in BSEP or MDR3 are clearly related to hepatic manifestations, extrahepatic manifestations can also appear, e.g. when MRP2 variants are present (127-134). Genetic testing might be useful to identify non-responders for specific drugs or to avoid severe adverse reactions in some patients. Furthermore, drugs can also directly inhibit BSEP function and therefore induce DILI (135-137).

1.4.7 Antibody-induced BSEP deficiency (AIBD)

After liver transplantation, disease recurrence is a frequent problem for certain patient groups. Symptoms can reappear in patients transplanted for cholestatic diseases like primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) or for hepatitis B or C or hepatocellular carcinoma (HCC) (138, 139). For children, disease recurrence of autoimmune sclerosing cholangitis happens with additive effects of inflammatory bowel disease (140, 141). Furthermore, occurrence of autoimmune hepatitis is a common challenge in liver transplanted children necessitating immunosuppressants additional to the common antirejection therapy (142, 143). Since 2009, it is known that disease recurrence after liver transplantation due to PFIC-2 is mediated by de novo antibodies targeted against BSEP (144, 145). The first case was a girl transplanted for PFIC-2 with three genetically proven severe BSEP mutations (144). After successful liver transplantation, the PFIC-2 phenotype reappeared within one year. The two major findings were on the one hand canalicular antibody deposits in a biopsy of the transplanted liver and on the other hand BSEP-reactive antibodies in the post-transplant serum. In the following, similar cases of PFIC-2 recurrence were published by other groups (145-148). The affected patients published so far are summarized in Chapter 3.8. This review also gives an overview on the spectrum of PFIC diseases and the formation of PFIC-2 phenotype recurrence. Since the proof of these anti-BSEP antibodies, an algorithm was suggested for the diagnosis of BSEP antibodies after liver transplantation as outlined in Chapter 3.8, Figure 3. Before liver transplantation, the genetic background and BSEP protein expression should be elucidated because severe BSEP mutations with absent BSEP protein predispose for the development of AIBD. Post-liver transplantation, on the one hand, canalicular antibody deposits are demonstrable in the liver

transplant. On the other hand, the patient's serum contains BSEP-reactive antibodies as shown by transfected cells as well as visualized canalicular structures in varying dilutions on normal human liver tissue. Recently, a compilation of seven additional AIBD cases was published (149), which is included as Chapter 3.9. The patients' genetic and clinical parameters were carried together to elucidate the triggers for anti-BSEP antibody formation after liver transplantation. Major findings were that all patients had severe BSEP mutations causing the initial PFIC-2 phenotype. Furthermore, acute transplant rejection, virus infections and changes in immunosuppression often appeared in these patients (Chapter 3.9, Table 1).

Although there are some treatment options (e.g. adjustment of immunosuppression, plasmapheresis, immunoadsorption, B cell depletion, re-transplantation) (139, 150), there is no universal therapy concept yet and the selection of treatment is dependent on the severity of symptoms. A patient suffering from disease recurrence after liver transplantation due to PFIC-2 was treated successfully with immunoadsorption and B-cell depletion. Anti-BSEP antibodies were demonstrably diminished in the patient's serum by immunoadsorption therapy whereas the B-cells were depleted by the administration of rituximab. This treatment was successful to ameliorate the clinical condition of this patient. The patient's clinical background as well as the treatment details are depicted in Chapter 3.10.

1.4.8 Dubin-Johnson syndrome (DJS)

Bilirubin is a metabolic product of heme catabolism. Genetic variants can lead to syndromes of hyperbilirubinemia, subdivided into unconjugated and conjugated hyperbilirubinemia (151). The Dubin-Johnson syndrome (DJS) was first described in 1954 and represents a form of conjugated hyperbilirubinemia based on mutations in the multidrug resistance-associated protein 2 (MRP2; *ABCC2*) (152-154). In the canalicular hepatocyte membrane, MRP2 mediates biliary excretion of organic anions including bilirubin glucuronides (Figure 3) (155). More than 200 MRP2 variants are known, but the functional relevance is not evident for all variants (154). The manifestation of the Dubin-Johnson syndrome is based on mutations leading to a non-functional or even absent MRP2 protein (153, 156-158). Nevertheless, despite an impaired bilirubin glucuronide excretion, the affected patients do not suffer from progressive liver disease because alternative elimination mechanisms, such as MRP3 at the sinusoidal membrane, are activated (154, 159). Specific genetic variants associated with hyperbilirubinemia syndromes are listed in Chapter 3.1, Table 1 (160).

1.4.9 Genetic variants associated with cholestatic diseases

Varying phenotypes of the mentioned cholestatic liver diseases are associated with different types of genetic variants. The majority of the variants are single nucleotide exchanges leading to the substitution of one AA, so called missense mutations. The impact of missense mutations depends on their position as well as the kind of substitution. A variety of bioinformatics tools is available to predict the potential impact of the AA exchange such as PolyPhen-2 (161, 162), SIFT (163), MutationTaster (164), and MutPred (165), which are mainly based on sequence, phylogenetic and structural information, and the degree of AA conservation. Furthermore, 3D protein modeling can be useful to simulate the effect of the AA substitution on protein structure and dynamics (166-168).

Common AA exchanges and other types of variants with a minor allele frequency (MAF) of more than 1 %, depending on the geographical or ethnical background, are denoted as single nucleotide polymorphisms (SNPs) (169). Although SNPs are also found in control cohorts compared to patients, these common variants may also contribute to cholestatic phenotypes, e.g. the most common BSEP SNP c.1331C>T (p.V444A) has an allele frequency in the general population of about 55 % (1000 Genomes, (73, 170, 171)). Nevertheless, this variant is clearly associated with ICP (71-73, 172) and DILI (70).

Genetic variants affecting the canonical splice-sites make up about 15 % of point mutations associated with human genetic diseases (173). The disruption of splice-sites can cause exon-skipping or activation of cryptic splice-sites and thereby introduce intronic regions. Alternative splicing is the basis for different transcripts as known for the three MDR3 isoforms (174). An alternative splice-site also results in varying BSEP transcripts, which is depicted in Chapter 3.2, Figure 2 (175).

The impact of synonymous variants, e.g. nucleotide exchanges not resulting in an altered AA sequence, is often indeterminate. However, they can modulate mRNA processing and have detrimental effects on splicing when situated in close vicinity to the exon-intron border (173). Exonic as well as intronic nucleotide exchanges far away from the splice-sites can also affect splicing when located in exonic or intronic splicing enhancer or silencer sequences (176).

Furthermore, nonsense, frameshift or also splice-site mutations can result in a premature termination codon, which may entail nonsense-mediated mRNA decay (NMD), a degradation of deficient mRNA transcripts (177-179). These severe mutations implicate a missing protein expression and therefore result in a progressive phenotype.

1.4.10 Therapeutic options for cholestatic liver diseases

Ursodeoxycholic acid (UDCA) is a naturally occurring bile acid in Ursidae (180). It is applied as standard treatment in a wealth of cholestatic liver diseases where mechanisms of action are probably the replacement of hydrophobic bile acids and other toxic bile compounds by the hydrophilic UDCA as well as an increased choleresis (181-184). UDCA is a standard treatment for PFIC patients and is known to be effective in PFIC-1 (185), PFIC-2 (186-188), and PFIC-3 (74, 85, 189). Nevertheless, in many cases, UDCA treatment fails requiring other therapeutic approaches (190-196). UDCA is probably more useful in patients with residual protein function (85, 197). Initially, treatment of all types of intrahepatic cholestasis should be started with UDCA (189, 197, 198). For ICP-affected women, amelioration under UDCA therapy is also known (199-202). Another therapeutic option mainly to reduce pruritus is rifampicin, which can lower intrahepatocytic bile salt concentrations by a lower bile salt uptake with NTCP and an increased bile salt export via BSEP and MRP4 (203-205). Additionally, 4-phenylbutyrate is a chaperone, which is known to improve FIC1 and BSEP expression and increases correct BSEP localization at the canalicular membrane (206-210). We also described two patients with PFIC-2, where steroid treatment was successful and therefore, liver transplantation was avoided (Chapter 3.7) (211). In these cases, a residual BSEP expression and function was present.

Apart from the mentioned medical treatment options, the partial external biliary diversion (PEBD) represents a surgical therapeutic approach. In some patients suffering from PFIC-1/-2, PEBD can relieve pruritus and delay disease progression (193, 195, 212-214). PEBD bypasses the terminal ileum and thus removes bile acids from the enterohepatic circulation. Clinical symptoms, growth, histology and liver function tests can be improved (195).

When other therapy options fail, in most cases for progressive intrahepatic cholestasis, especially for those patients with severe mutations resulting in a massive loss of protein expression or function, orthotopic liver transplantation (OLT) represents the only alternative (196, 215). PFIC patients make out about 10 % of pediatric OLTs (215). Long-term outcome after pediatric OLT in general is excellent with one-year graft survival rates of about 90 % (216, 217). Survival of patients who were liver transplanted for PFIC is not different to other OLT indications (196). Therapeutic options for cholestatic liver diseases are summarized by Stapelbroek *et al*. (218).

There is no definite therapy for AIBD yet but there are some experimental therapeutic approaches presented in detail in Chapters 3.8 (139), 3.9 (149), and 3.10 (150).

1.5 Detailed description of ABC transport proteins

Molecules like glucose are ingested with food and metabolized. Subsequently, the energy is stored in the triphosphate group of ATP molecules (219). ATP hydrolization releases the stored energy, which is needed for endergonic cellular processes. Binding of ligands such as ATP represents a crucial step and is strictly dependent on the three-dimensional structure of the protein's ligand-binding site and the ligand itself. Specific binding motifs have evolved to bind nucleotides like ATP accurately (220). The phosphate-binding loop, or P-loop , is highly conserved in amino acid sequence and structure and represents the most abundant nucleoside triphosphate-binding motif (220). In 1982, the P-loop was identified by Sir John Walker and colleagues and thus is also named the "Walker A" motif (221). This highly conserved sequence is a glycine-rich loop, which accurately positions the triphosphate. Besides the Walker A motif, another highly conserved motif is the "Walker B" motif for binding a water-bridged magnesium ion.

P-loop motifs are present in membrane proteins involved in the transport of substances across membranes (220). Biological membranes are required to form intracellular compartments as well as the cell itself to build a selective-permeable barrier between inside and outside (222). Membranes consist up to 80 % of membrane proteins. The main function of transmembrane (TM) proteins is to facilitate the transport of large, polar molecules and ions across membranes. Membrane transport proteins can be subdivided into five classes (223). ABC transporters such as BSEP and MDR3 belong to class III of membrane transport proteins comprising primary active transporters. BSEP as well as MDR3 use binding and hydrolysis of ATP to transport bile salts and phospholipids, respectively, across the canalicular membrane of hepatocytes. In general, ABC transporter consist of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) comprising the P-loop. The first ABC transporter identified in the mid-1980s was the human P-glycoprotein (P-gp, MDR1, *ABCB1*) as a transporter responsible for drug-resistance in cancer cells (224-226). Like MDR1, BSEP, and MDR3 all human ABC transporters belong to the class of exporters as no importers

were identified in eukaryotes. Human ABC (transport) proteins are divided into seven families (A-G) (227).

Twelve ABC transporters with a variety of substrates are localized in the liver. With respect to cholestatic liver diseases, this work focused mainly on the bile salt export pump (BSEP, *ABCB11*) and on the multidrug resistance protein 3 (MDR3, *ABCB4*).

1.5.1 Functional domains of ABC transporters

The transmembrane domains (TMDs) of ABC exporters are made up of helical bundles crossing the membrane, while each helical bundle consists of six transmembrane helices (TMHs) as first observed for the bacterial ABC transporter Sav1866 (228). The confirmation of the TMDs are assumed to determine substrate specificity by providing a binding pocket with a high substrate affinity (229). The TMDs not only cross the membrane but also have intracytosolic loops, called coupling helices (CHs), which form the interface between the TMDs and NBDs and facilitate the translation of ATP binding and hydrolysis (230).

Compared to TMDs, the NBDs are more preserved concerning AA sequence and structure. The NBDs contain the highly conserved P-loop and form the cytosolic part of the transport proteins. By ATP binding and hydrolysis, these domains drive the transport of substances across the membrane (231, 232). Each NBD consists of a catalytic subdomain (CSD) and a helical subdomain (HSD) hinged by the Q- and the Pro-loop. The P-loop or Walker A motif is included in the CSD. Additional conserved NBD structures are the A-, C-, D-, and H-loop (233). ATP-binding requires the dimerization of two opposite NBD monomers in a head to tail arrangement.

The communication between the TMDs and NBDs is mediated by the intracytosolic loops (ICLs), also termed coupling helices (CHs). ABC exporter have two helices (CH1, CH2) per TMD, while each NBD is in contact with two CHs, one from each TMD (228).

The ATP-switch model is well-recognized for the transport mechanism of ABC transporters (234, 235). The model is divided into four steps. Step I: substrate binding to the TMDs induces conformational changes via the CHs to the NBDs initiating transport by ATP binding and bringing the NBDs together. Step II: the closed NBD dimer leads to an inverted TMD configuration from inward-facing to outward-facing (in case of exporters). The ligand is

secreted. Step III: ATP hydrolysis and opening the NBD dimer. Step IV: ADP and Pi release from the NBDs and reset of the transporter to the initial confirmation.

ABC transporters like BSEP (*ABCB11*), MDR3 (*ABCB4*), MRP2 (*ABCC2*), or ABCG5/G8 are expressed in the liver. Genetic variants in these transporters are clearly related to the manifestation of liver diseases such as the spectrum of intrahepatic cholestasis, the Dubin-Johnson syndrome, or hypercholesterolemia and cholelithiasis (for an overview see Chapters 3.1, 3.2, and 3.8). Knowledge about sequence, structure and function of these transport proteins helps to understand and to predict the possible effects of genetic variants.

2 AIMS AND OBJECTIVES

The bile salt export pump (BSEP), the multidrug resistance protein 3 (MDR3) as well as the familial intrahepatic cholestasis 1 (FIC1) play central roles in hepatic bile formation. Genetic variants of these transporters are the basis for cholestatic liver diseases of diverging severity.

The aim of the present thesis was to identify and further characterize genetic variants of patients with cholestatic phenotypes. First, the underlying genetic variations were identified from human blood samples. Moreover, the patients' liver tissue samples were analyzed on the DNA, RNA, and protein level to evaluate mutational effects on mRNA processing as well as protein expression and localization using sequencing techniques, quantitative real time PCR and immunofluorescent staining.

Despite the investigation of the patients' blood and tissue samples, cell culture-based methods were applied to illustrate the impact of specific genetic variants, e.g. on mRNA splicing or protein expression and function.

The results are of great importance in the field of genetic-based cholestatic liver diseases in children and adults and represent the basis in understanding the underlying mechanisms of disease manifestation and progression. Furthermore, the detailed knowledge of mutational effects is important for the development of further therapeutic options.

3 PUBLICATIONS

3.1 Genetic variants in adult liver diseases

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Genetic variants in adult liver diseases

Genetische Varianten bei Lebererkrankungen im Erwachsenenalter

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Key Words
monogenetic, single nucleotide polymorphism, autosomal, genotype, phenotype, disease modifier

Schlüsselwörter
monogenetisch, Polymorphismus, autosomal, Genotyp, Phänotyp, Krankheitsmodifikator

Abstract
In the last decades, understanding of genetic variants contributing to liver disease development has considerably improved through novel genotyping techniques. Genetic variants of single genes are known to be decisive for the development of monogenetic liver diseases of varying severity. Identification of genetic variants is an important part of the diagnostic process, e.g. the majority of patients with high iron [Fe] (HFE)-associated hemochromatosis carry the homozygous mutation p.C282Y. Detection of mutations in genes encoding hepatobiliary transport proteins like familial intrahepatic cholestasis 1 (FIC1), bile salt export pump (BSEP), or multidrug resistance protein 3 (MDR3) is the basis to differentiate various forms of intrahepatic cholestasis. Moreover, genetic variants in a variety of genes are known to act as disease modifiers and represent risk factors for disease progression and the development of cirrhosis or even hepatocellular carcinoma. Success of drug treatment or appearance of severe side effects can also be influenced by specific genetic variants. All these aspects underscore the increasing importance of genetic variants, which in the future may help to identify patients at risk for disease progression or help to guide treatment decisions. In the present overview, specific frequent genetic variants are summarized that play roles in monogenetic liver diseases, forms of intrahepatic cholestasis, gallstone development, fatty liver disease, drug-induced liver injury, and liver disease progression as well as hepatocellular carcinoma development.

Zusammenfassung
In den letzten Jahrzehnten hat sich das Verständnis von Lebererkrankungen mit Hilfe neuer Techniken erheblich verbessert. Genetische Varianten einzelner Gene sind für die Entwicklung monogenetischer Lebererkrankungen unterschiedlichen Schweregrads entscheidend. Die Identifizierung genetischer Varianten ist ein wichtiger Teil des Diagnostikprozesses, z.B. trägt sie Mehrheit der Patienten mit *high iron [Fe]* (HFE)-assoziierter Hämochromatose die homozygote Mutation p.C282Y. Der Nachweis von Mutationen in Genen, die hepatobiliäre Transportproteine wie FIC1 (*familial intrahepatic cholestasis 1*), BSEP (*bile salt export pump*) oder MDR3 (*multidrug resistance protein 3*) kodieren, ist die Grundlage für die Diagnose verschiedener Formen intrahepatischer Cholestasen. Des Weiteren beeinflussen genetische Varianten in einer Vielzahl von Genen den Krankheitsverlauf unterschiedlicher Lebererkrankungen und stellen Risikofaktoren für die Entwicklung einer Leberzirrhose oder des hepatozellulären Karzinoms dar. Der Behandlungserfolg oder das Auftreten schwerer Nebenwirkungen kann ebenfalls durch spezifische genetische Varianten beeinflusst werden. All diese Aspekte verdeutlichen, dass die Einbeziehung genetischer Informationen im Bereich Diagnostik, Risikostratifizierung und Therapieentscheidung bei Patienten mit Lebererkrankungen in Zukunft weiter an Bedeutung gewinnen wird. In der vorliegenden Arbeit sind spezifische genetische Varianten zusammengefasst, die bei monogenetischen Lebererkrankungen, intrahepatischen Cholestasen, Gallensteinentwicklung, Fettleber, medikamentös-toxischer Leberschädigung, Leberzirrhose und hepatozellulärem Karzinom eine Rolle spielen.

Introduction

Genetic variants provide the basis for a broad range of disorders affecting the liver. In this review we summarize liver diseases with clinical manifestation in adulthood in which genetic variants contribute to disease development or progression. Monogenetic disorders like HFE-associated hemochromatosis or Alpha1-antitrypsin deficiency are based on specific variants in one gene, respectively. In contrast, genetic variants in different genes can contribute to development of more frequent liver disorders such as non-alcoholic steatohepatitis, cholestatic liver disease or gallstone disease. A wide spectrum of cholestatic syndromes ranging from progressive to benign recurrent forms can be caused by different mutations within the same gene, underscoring that the severity of the mutations as well as other host factors and environmental aspects contribute to the clinical phenotypes. Genetic variants not only affect disease development but can also modify disease course or response to treatment.

Monogenetic variants as cause of liver disease with clinical manifestation in adulthood

Inborn errors of metabolism can not only manifest in the neonatal period or early childhood but also in adulthood often necessitating liver transplantation due to end-stage liver disease. The following paragraph will summarize monogenetic diseases which affect liver function like HFE-associated hemochromatosis, Wilson disease, Alpha1-antitrypsin deficiency, and Citrin deficiency, with an emphasis on hepatic disease and the underlying genetic aspects.

HFE-associated hemochromatosis represents an inborn error in iron metabolism with massive iron overload leading to tissue and organ damage often resulting in liver cirrhosis. Since 1995, when mutations in the high iron [Fe] (*HFE*) gene were first identified to cause hemochromatosis [1], other genes involved in iron metabolism were uncovered to be related to this disease like transferrin receptor (*TFR*) 2, ferroportin (*FPN*), hepcidin antimicrobial peptide (*HAMP*), and hemojuvelin (*HJV*). The HFE protein plays a critical role in iron sensing and forms a complex with TFR1 at the hepatocyte membrane [2]. This complex regulates hepcidin expression encoded by the *HAMP* gene [3]. Hepcidin in turn is involved in the post-translational modification of FPN, thereby regulating FPN internalization and degradation thus decreasing iron absorption in the enterocytes [2,4,5]. When HFE-associated hemochromatosis is suspected because of elevated serum ferritin levels and elevated serum transferrin saturation, genetic testing especially for the HFE gene mutation c.845G>A (p.C282Y) is the method of choice for diagnosis confirmation [6]. In Caucasians of Northern European descent, 80-90% of patients with hemochromatosis carry this homozygous HFE mutation p.C282Y (c.845G>A, rs1800562; Table 1) with a minor allele frequency (MAF) of 1.3% (all MAF sources: 1000 Genomes,) [6,7]. Nevertheless, due to a low penetrance and additional host, environmental and lifestyle factors, there are homozygous carriers of this variant without clinical manifestations of HFE-associated hemochromatosis including progressive liver disease [2,7]. It is estimated, that about 75% of men and 50% of women homozygous for p.C282Y have elevated serum transferrin-iron saturation (TS) and serum ferritin (SF) levels [8]. In a recent study, patient populations of Northern European descent with manifestations of HFE-associated hemochromatosis and homozygous for p.C282Y were screened for other risk loci of iron metabolism previously selected via genome-wide association studies (GWAS) [9]. The C allele of the intronic variant c.1156-1135C>G (rs236918) of the protease PCSK7 was identified to be related to progressive liver fibrosis or cirrhosis in these patients that points to a gene-gene interaction [9]. Another frequent HFE mutation is p.H36D (c.187C>G, rs1799945; Table 1) with a MAF of 7.3%, however, homozygous carriers of this variant show no progressive clinical disease [2]. Although both missense mutations are quite frequent in HFE-associated hemochromatosis, compound heterozygosity of p.H36D/p.C282Y is found in about 5% of patients with hemochromatosis [2,6]. While therapeutic phlebotomy represents an effective treatment to lower blood iron concentrations especially addressing patients in the precirrhotic stage, liver transplantation is the only option for patients with end stage liver disease [2,6,10]. Moreover, cirrhotic patients have a high risk for hepatocellular carcinoma (HCC) development [6,10].

HFE for hemochromatosis and *ATP7B* for **Wilson disease** were the first genes mapped and cloned that are related to monogenetic diseases with predominant liver phenotypes [11]. Wilson disease is a hereditary disorder with defective copper metabolism leading to high accumulation of inorganic copper mostly in liver and brain resulting in tissue injury [12]. The *ATP7B* gene encodes a protein mainly expressed in hepatocytes that acts as a chaperone to facilitate intracellular copper transport as well as excretion. Mutations in *ATP7B* are related to Wilson disease [13]. There are over 500 ATP7B variants associated with a Wilson disease phenotype and most patients are compound heterozygous, indicating that direct genetic diagnosis of this disease is difficult [11,14]. Two missense mutations appear in a large number

of Wilson disease patients depending on their ethnicity. About 40% of patients with Northern European descent carry the variant p.H1069Q (c.3207C>T/A) while in the Asian population p.A778L is frequent (30% of patients; Table 1). Nevertheless, there is no clear genotype-phenotype correlation between genetic variants and clinical disease [2], underscoring that clinical and biochemical parameters are essential for diagnosis of Wilson disease [14]. Gene sequencing to identify mutations in the *ATP7B* gene is generally applied to confirm the diagnosis and also to allow testing of relatives before onset of symptoms [14]. The development of steatosis in patients with Wilson disease may also be triggered or aggravated by a common single nucleotide polymorphism (SNP) in the *PNPLA3* (p.I148M, rs738409) gene that is generally known to contribute to the progression of non-alcoholic fatty liver disease (NAFLD), which will be discussed later on [15]. Treatment of Wilson disease aims to induce copper excretion, however, in patients with end stage liver disease or fulminant hepatic failure liver transplantation is indicated.

One of the most frequent genetic-based liver diseases is **Alpha1-antitrypsin deficiency** (AATD) affecting either the liver and/or the lung (for reviews see [16,17]). The *SERPINA1* gene encodes the alpha1 antitrypsin glycoprotein (α1-AT), which inhibits the proteolytic effect of neutrophil proteases, elastase, cathepsin G and proteinase 3 [2]. Mutations in *SERPINA1* are known to affect α1-AT protein structure and are classified according to their migration rate in a gel electrophoresis either as M, Z or S. A medium migration rate is named α1-ATM (=M) and this represents the most common allele with an allele frequency of 95% [18], where the phenotype is named PiMM when homozygous for α1-ATM. The missense mutation p.Q342K (c.1096G>A, rs28929474; Table 1) results in the mutant α1-ATZ molecule that forms abnormal polymers accumulating in the ER [19-22]. People homozygous for the α1-ATZ allele (PiZZ) account for 96% of AATD patients [23]. However, appearance of α1-ATZ or α1-ATS allele due to the missense mutation p.Q264 (c.863A>T, rs17580; Table 1) varies according to ethnic origin while α1-ATZ (p.Q342K) is the most prevalent one in Northern European Countries [2,23]. The manifestation of liver-affecting AATD is supposed to be based on toxic gain-of-function mutations as known for PiZZ which leads to accumulation of misfolded α1-AT molecules in endoplasmic reticulum of hepatocytes. Diagnosis is verified by gel electrophoresis. At present, treatment comprises only supportive care whereas liver transplantation is intended for patients with acute hepatic failure or end stage liver disease. AATD represents a risk factor for liver cirrhosis whereas the data concerning HCC development are controversial [24-26]. In a mouse model, it was shown that the PiZ genotype leads to increased hepatocellular proliferation especially in male animals [27]. There are no established treatments for AATD-associated liver disease, however, carbamazepine, a well-established drug, was investigated in PiZZ mice as well as recently in induced pluripotent stem cells (iPSCs) obtained from patients with a PiZZ phenotype. A decrease of α1-ATZ aggregation was demonstrated, mediated by carbamazepine-induced autophagy [28,29]. Furthermore, 4-phenylbutyrate that functions as a chaperone was identified to have positive effects in the mouse model of the PiZZ phenotype [30]. This agent is also known to increase membrane expression of some BSEP mutants [31]. Substitution therapy with α1-AT is only indicated for AATD affecting the lung but not the liver [17].

Citrin deficiency or Citrullinemia comprises two subgroups of patients with neonatal cholestasis caused by citrin deficiency (NICCD) or adult onset citrullinemia type 2 (CTLN2) mainly found in Japan, the latter affecting 1:230,000 up to 1:100,000 Japanese (Table 1) [32,33]. In 1999, mutations in the *SLC25A13* gene encoding the mitochondrial aspartate-glutamate carrier 2 (AGC2, citrin) were identified to cause this disease [34]. Frequency of citrin deficiency-associated variants depends on ethnic background. In the Asian population, c.851delGTAT (rs80338720) and IVS11+1G>A (rs80338722) are the most common mutations, which are found in about 80% of citrin deficient patients (Table 1) [35]. In the French-Canadian population, two nonsense mutations (p.R43X, p.R355X) are causative for this disease in most known cases [36]. In contrast, for most Caucasians and other populations, the majority of mutations are rare and occur only in one or two independent families [2]. Patients with CTLN2 probably have increased lipid synthesis resulting in the development of steatohepatitis and subsequent fibrosis, cirrhosis and HCC [37]. Diagnosis of citrin deficiency is based on molecular testing to identify mutations in the *SLC25A13* gene. Treatment options are limited to liver transplantation with good outcome.

Bilirubin is a metabolic product of heme catabolism. In humans, there are different types of hyperbilirubinemia syndromes (for review see [38]). Inborn defects of bilirubin conjugation are mainly based on genetic variants of the bilirubin conjugating enzyme UDP glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1). They are characterized by unconjugated hyperbilirubinemia and encompass liver diseases of differing severity. Severe *UGT1A1* mutations result in a non-

functional protein leading to Crigler-Najjar type 1 with lethal effects while mutations with residual UGT1A1 activity are associated with the less severe form Crigler-Najjar type 2. Variants in the first exon or in the TATAA element of the promoter region of *UGT1A1* are mostly related to a benign form of unconjugated hyperbilirubinemia named **Gilbert-Meulengracht syndrome** with no hepatic disease manifestation and not requiring any treatment [39,40]. Due to the fact that the first exon is variable in *UGT1A*, mutations in this exon only have milder phenotypes because they affect only one of the *UGT1A* isoforms [39]. Up to now, more than 110 *UGT1A1* variants are known [38]. The insertion of TA (A(TA)7TAA, rs8175347; Table 1) in the promoter is a very common variant in the Caucasian population where homozygous carriers are frequent [40,41]. Other variants causing Gilbert-Meulengracht syndrome are known to affect people of Asian, Indian, or African descent [38]. Although patients with this syndrome do not require any medication and some variants are even supposed to have a protective function, genetic variants can also represent potential risk factors for specific drug side effects. UGT1A1 is not only responsible for bilirubin glucuronidation but also for conjugation of other substrates comprising therapeutic drugs. Several UGT1A1 variants are associated with unwanted drug side effects including toxic reactions after administration of irinotecan, atazanavir, and indinavir (summarized in [38]). Variants of UGT1A1 are also under suspicion to increase the risk of gallstone development, which will be discussed later.

Dubin-Johnson syndrome represents a form of conjugated hyperbilirubinemia caused by mutations in the *ABCC2* gene encoding the multidrug resistance-associated protein 2 (MRP2; Table 1), which facilitates biliary excretion of organic anions including bilirubin glucuronides [42-45]. MRP2 is located in the apical membrane of polarized cells, including hepatocytes. Despite impaired bilirubin glucuronide excretion, patients with Dubin-Johnson syndrome do not suffer from progressive liver disease due to the fact that alternative elimination pathways e.g. across the basolateral hepatocyte membrane via MRP3 are activated [44,46]. Over 200 variants in the *ABCC2* gene are described but not all of them with known functional relevance [44]. Mutations that entail a non-functional or even absent protein in the canalicular membrane of hepatocytes are the basis for Dubin-Johnson syndrome [42,47,48]. Although there is no overall common disease-causing mutation as mentioned for HFE-associated hemochromatosis, some mutations become more frequent in families with Jewish origin as shown for p.I1173F (c.3517A>T) and p.R1150H (c.3449G>A) [49]. Moreover, since MPR2 is an efflux pump for multiple drugs *ABCC2* mutations are not only related to Dubin-Johnson syndrome but also form risk factors for drug toxicity as shown for methotrexate [50,51]. The variant p.V417I (c.1249G>A, rs2273697) with a MAF of 18.7% is associated not only with tenofovir-induced renal proximal tubulopathy [52] but also with neurological adverse drug reactions of patients under carbamazepine therapy [53]. Another study identified p.V1188E (c.3563T>A, rs17222723) and p.C1515Y (c.4544G>A, rs8187710) with MAFs of 3.8% and 6.8%, respectively, to be related to anthracycline-induced cardiotoxicity whereas these SNPs seemed to have a protective effect for proximal tubulopathy [52,54]. Thus, although genetic analysis is not part of the regular diagnostic process of Dubin-Johnson syndrome, it could definitely contribute to the understanding of drug-induced toxicities.

Sitosterolemia is based on impaired function of ABCG5 or ABCG8 that form a transporter complex expressed in the liver and intestine. Patients suffer from hypercholesterolemia, phytosterolemia, and premature coronary heart disease [55-57]. Since phytosterols represent the preferred substrate of ABCG5/G8, patients with Sitosterolemia show a markedly reduced biliary sterol excretion [58-60]. Therapy options comprise low phytosterol and low-cholesterol diet, resin therapy, and ileal bypass (summarized in [61]). Restored ABCG5/G8 activity in the liver after liver transplantation for end-stage disease leads to the assumption that hepatic ABCG5/G8 transporter activity is required to maintain non-cholesterol sterol balance [62]. One variant associated with sitosterolemia is the ABCG8 variant p.G575R (c.1720G>A, rs137852988; Table 1) with an allele frequency of 0.02% (NHLBI Exome Sequencing Project (ESP)) [61,63].

Some of these monogenetic diseases are caused by a limited number of hot spot mutations, which makes direct genetic diagnosis feasible. In contrast, in diseases associated with a wide variety of underlying mutations, results from gene sequencing are more difficult to interpret since the impact of newly detected variants on the patient's phenotype will have to be established by bioinformatics, introduction of the mutations into cell culture models or recombinant proteins. Altogether, the occurrence of common variants in the affected genes of the mentioned diseases often depends on the ethnic origin due to a founder effect. Mainly, there are differences in the Asian, Caucasian, or North American populations. While isolated populations show a small number of highly recurrent genetic variants as described in detail for Greenland Familial Cholestasis [64] or for

the Hutterites in the United States [65], most populations show no such common variants . Moreover, the clinical phenotype of the presented genetic-based liver diseases in adulthood are also influenced by genetic and environmental factors like alcohol intake (ASH) or hormones (ICP) that can influence manifestation, age of onset and severity.

Disease	Inheritance	Incidence	Phenotype MIM no.	*Gene* Protein	Frequent disease-associated variants
Hemochromatosis (Type 1)	autosomal recessive	not available	#235200	*HFE* HFE	p.H63D (rs1799945) p.C282Y (rs1800562)
Wilson disease	autosomal recessive	1:30,000 - 1:100,000 [2]	#277900	*ATP7B* ATP7B	p.A778L p.H1069Q (rs76151636)
Alpha1-antitrypsin deficiency	autosomal co-dominant	1:2,000 - 1:3,000 [66]	#613490 (liver affecting)	*SERPINA1* A1AT (liver affecting)	p.Q264V (PiS, rs17580) p.Q342K (PiZ, rs28929474) (liver affecting)
Citrin deficiency (citrullinemia type 2, CTLN2)	autosomal recessive	1:100,000 - 1:230,000 [33]	#603471	*SLC25A13* AGC2	c.851delGTAT (rs80338720) IVS11+1G>A (rs80338722) p.R43X p.R355X
Dubin-Johnson syndrome	autosomal recessive	1:3,000 - 1:300,000 [44]	#237500	*ABCC2* MRP2	no specific variants
Gilbert-Meulengracht syndrome	autosomal recessive	1:10 (in Caucasians) [38]	#143500	*UGT1A1* UDPGT	A(TA)7TAA (rs8175347) [40]
BRIC-1	autosomal recessive	not available	#243300	*ATP8B1* FIC1	p.I661T (rs121909100) [67,68]
BRIC-2	autosomal recessive	not available	#605479	*ABCB11* BSEP	p.A570T [69,70] p.R1050C [69,70]
LPAC/ Gallbladder disease 1		not available	#600803	*ABCB4* MDR3	no specific variants
ICP-1			#147480	*ATP8B1* FIC1	no specific variants
ICP-2	autosomal dominant	1:5 - 1:2,000 (varying with geographic location and ethnicity) [71]	not available	*ABCB11* BSEP	p.V444A (rs2287622) [72] p.N591S (rs11568367) [70,73]
ICP-3			#614972	*ABCB4* MDR3	p.S320F (rs72552778) [74]
ICP			not available	*NR1H4* FXR	p.M173T (rs61755050) [71,75]
Sitosterolemia	autosomal recessive	<1:1,000,000 [ORPHA2882]	#210250	*ABCG5/G8* ABCG5/G8	p.G574R (ABCG8; rs137852988) [61]

Table 1. Overview of genetic-based liver diseases with clinical manifestation in adulthood

Genetic basis of benign recurrent intrahepatic cholestasis (BRIC) and intrahepatic cholestasis of pregnancy (ICP)

Bile salt secretion is needed for removal of cholesterol which is insoluble in water. In bile, cholesterol forms mixed micelles together with bile salts and phospholipids [76]. Bile salts are secreted from hepatocytes into bile via the bile salt export pump (BSEP) encoded by the *ABCB11* gene [77]. *ABCB4* encodes the multidrug resistance protein 3 (MDR3) excreting phosphatidylcholine into bile [78,79]. The aminophospholipidflippase familial intrahepatic cholestasis 1 (FIC1, gene: *ATP8B1*) facilitates membrane asymmetry that is required for proper function of hepatobiliary transport proteins like BSEP and MRP2, therefore genetic variants leading to impaired FIC1 function indirectly hamper BSEP and MRP2 transport activity by disturbed membrane asymmetry [80-82]. In general, mutations leading to dysfunction of FIC1, BSEP, and MDR3 are related to different types of cholestatic diseases of varying degree ranging from progressive intrahepatic cholestasis type 1 to 3 (PFIC-1/-2/-3) to less severe forms like benign recurrent intrahepatic cholestasis type 1 or 2 (BRIC-1/-2), low phospholipid-associated cholelithiasis (LPAC) or intrahepatic cholestasis of pregnancy (ICP) (Table1; for reviews see [83-88]).

PFIC patients present with clinical manifestation in early childhood and therefore will not be further discussed here. There is clear evidence that milder homozygous or compound heterozygous mutations in FIC1, BSEP or MDR3 are the genetic basis to develop intrahepatic cholestasis with adult onset and that even heterozygous mutations have an influence, e.g. predispose to develop ICP (Table 1) [72,89-97]. Milder mutations indicate that there is a residual activity to fulfill function of keeping membrane asymmetry or bile salt and phospholipid secretion. Two frequent BSEP mutations associated with BRIC-2 are p.A570T (c.1708G>A) and p.R1050C (c.3148C>T) (Table 1) but other BSEP missense mutations can also underlie a BRIC-2 phenotype [70,98]. It is difficult to assign mutations to PFIC, BRIC or ICP because there is no distinct genotype-phenotype correlation, thus these cholestatic syndromes represent a continuum of varying severity [99,100]. However, nonsense and frameshift mutations resulting in a premature stop codon or splice site variants entailing exon skipping are more likely to cause a severe phenotype based on complete loss of functional BSEP. Missense mutations with residual BSEP function are more common in milder forms of intrahepatic cholestasis [68,88,95,101-103]. Estrogen and other pregnancy hormones contribute to ICP development which most frequently occurs in the last trimester of pregnancy. Cholestasis in genetically predisposed subjects can also be promoted through administration of oral contraceptives or hormonal replacement therapy [104-109]. In ICP and also BRIC, patients with elevated gamma-GT values, the *ABCB4* gene encoding MDR3 should be screened for mutations since it is postulated that about 15% of ICP cases are based on MDR3 mutations [70,91,96,110]. Besides the homozygous variant p.S320F (c.959C>T, rs72552778; Table 1) identified in ICP patients, a variety of other MDR3 mutations have been found in ICP patients [70,110-112]. For BSEP, the common polymorphism p.V444A (c.1331T>C, rs2287622; Table 1) with a MAF of 41.1% represents a predisposing factor for ICP [72,93]. Another frequent BSEP mutation found in ICP is p.N591S (c.1772A>G) [70]. Nevertheless, mutations in FIC1, BSEP, and MDR3 explain only part of inherited forms of ICP [105,113]. One MRP2 mutation (rs3740066) was designated to be associated with ICP [114]. In a recent study, the occurrence of common variants in *ABCB4*, *ABCB11*, *ABCC2*, *ATP8B1*, *NR1H4*, and *FGF19* was investigated in ICP patients [93]. A significant association was shown for six variants in or surrounding the gene loci for *ABCB11* and *ABCB4.* The highly significantly associated variants comprised one non-coding variant located upstream from *ABCB11* (rs7577650) and one synonymous variant in *ABCB4* (p.I237I, rs2109505) [93]. Additionally, genetic variants of the farnesoid X receptor (FXR), a key transcription factor with an important role in BSEP and MDR3 expression [115,116], are also known to predispose for ICP as described for c.518T>C/p.M173T (Table 1) [71,75].

Genetic variants for gallstone formation

Gallstone disease is common in Europe as well as North and South America [117]. Gallstones can be classified according to composition into two major subtypes either cholesterol gallstones or pigment/bilirubin gallstones [118,119]. Most patients with gallbladder stones do not have any symptoms but there is a risk of complications. Family studies of affected patients with gallstones or high prevalence rates in ethnically distinct populations pointed out genetic variants in different genes predisposing for gallstone formation. Multiple genetic factors assign for 25-30% of symptomatic gallstones [120-123]. Alterations in bile composition due to impaired biliary secretion of cholesterol by ABCG5/G8, bile salts by BSEP or phospholipids by MDR3 influence cholesterol gallstone formation. To determine specific genetic variants related to cholelithiasis, the first GWAS for hepatobiliary disorders was performed. Via this analysis, the p.D19H variant (c.55G>C, rs11887534) in ABCG8 as a part of the cholesterol transporter complex ABCG5/G8 was identified to be strongly associated with gallstone formation (Table 2) [11,124]. The nucleotide exchange p.E604Q in the other hemitransporter ABCG5 has also been linked to cholelithiasis (Table 2) [125]. Nevertheless, additional environmental factors play a critical role in formation and progression of cholesterol gallstones, e.g. pregnancy, fasting, diabetes and insulin resistance [122]. Women are known to have an increased frequency of gallstones due to oral contraceptives and pregnancy [126,127]. Although ICP patients seem to have a predisposition for gallstones, common ICP-associated variants in BSEP or MDR3 do not appear more often in patients with cholelithiasis [128]. However, BRIC-2 patients with mutations in BSEP develop cholesterol gallstones based on altered bile composition also known for patients with MDR3 mutations, the latter is called low phospholipid-associated cholelithiasis (LPAC). Interestingly, BRIC-1 patients carrying FIC1 mutations do not have an increased risk for gallstones. Disturbed hepatic bile salt sensing displays another probable mechanism for altered bile salt excretion resulting in gallstone formation. This effect can result from mutations in the cholesterol 7α-hydroxylase (CYP7A1), the rate limiting enzyme of hepatic bile acid synthesis, or

the *NR1H4* gene encoding the nuclear transcription factor farnesoid X receptor (FXR) resulting in lower BSEP activity [129-131]. Three FXR variants were described in patients with cholelithiasis with males being more affected than women [130]. In some populations, genetic variants of the apical sodium-dependent bile acid transporter (ASBT) are also supposed to display a risk factor for gallstones due to impaired enterohepatic circulation of bile salts as described for the intronic variant c.378-105A>G (rs9514089; Table 2) [132,133]. Formation of pigment gallstones can result from an increased bilirubin in the enterohepatic circulation by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), encoded by the *ABCC7* gene (Table 2) [129]. The prevalence of gallstones in patients with CFTR mutations is probably modified by genetic variants in other genes like *UGT1A1* that is associated with Gilbert-Meulengracht syndrome. Impaired function of hepatobiliary transporters involved in gallstone formation is not only directly based on genetic variants in the corresponding genes or indirectly on regulating factors like FXR but also on pharmaceutical agents. Certain drugs like cyclosporin and tacrolimus can predispose for cholesterol gallstone formation by inhibiting BSEP function as well as inducing retrieval from the plasma membrane, thus reducing the amount of functional transport protein in the canalicular hepatocyte membrane [134,135].

Gene **Protein**	**Frequent disease-associated variant/syndrome**	**Mechanism of action**
ABCB4 MDR3	LPAC [136]	impaired excretion of phospholipids into bile
ABCB11 BSEP	BRIC-2	impaired excretion of bile salts into bile
ABCC7 CFTR	Cystic fibrosis	induced enterohepatic circulation of bilirubin, hypersecretion of bilirubin conjugates into bile, reduced pH in bile
ABCG5/G8 ABCG5/G8	p.E604Q (ABCG5) p.D19H (ABCG8, rs11887534)	increased secretion of cholesterol into bile
ADRB3 ADRB3	p.W64R (rs35724) [121]	decreased gall bladder motility
APOB APOB	Familial hypobetalipoproteinemia [137]	cholesterol hypersecretion into bile
CYP7A1 CYP7A1	c.-267C>A (rs3808607) [131,138]	affected regulation of hepatic bile acid synthesis
NR1H4 FXR	rs56163822 [130] rs7304328 [130] rs35724 [130] depending on gender and weight	modified synthesis and excretion of bile salts
SL10A2 ASBT	c.378-105A>G (rs9514089) contrasting studies [132,133]	impaired enterohepatic bile salt circulation
UGT1A1 UGT1A1	A(TA)7TAA (rs8175347) modifier combined with CFTR variants rs6742078 [139]	increased excretion of monoglucuronidated bilirubin, bilirubin-core formation in cholesterol-based gallstones increased plasma bilirubin levels, increased symptomatic gallstone disease

Table 2. Genetic risk factors for gallstone formation (based on [129])

Genetic factors in drug-induced liver injury (DILI)

Therapeutic agents can cause hepatotoxicity, e.g. drug-induced liver injury (DILI). It is estimated that DILI occurs in fewer than 1:100,000 persons under drug treatment while multiple factors like genetic variants, age, gender, nutrition, intake of other drugs or alcohol, or preexisting liver diseases influence frequency [140]. Hepatic manifestations of DILI range from unspecific injury to hepatitis, cholestasis, vascular lesions and tumors [140]. More precisely, DILI causes about 10% of hepatitis cases in adult patients, and over 40% of hepatitis events in adults older than 50 years [71,141-143]. Genetic variants can contribute to DILI development [123,140]. In this section we focus on non-HLA variants contributing to DILI. For MDR3, two variants were identified in a patient population with DILI: p.I764L and p.L1082Q [70,144]. MDR3-associated DILI can be based on transport by MDR3 or MDR3 inhibition by certain

drugs (e.g. digoxin, paclitaxel, vinblastine, verapamil, cyclosporine) [70,145]. For BSEP, the ICP-associated very common variant p.V444A is also related to DILI with a higher allele frequency in DILI patients (76%) compared to controls (59%), leading to an odds ratio (OR) of 3 for DILI development [70,144]. Hepatotoxicity of various drugs correlates with their pharmacological interaction with BSEP [134,146,147]. Furthermore, variants in UGT2B7 (UDP glucuronosyltransferase 2 family, polypeptide B7), CYP2C8 (cytochrome P450, family 2, subfamily C, polypeptide 8), and MRP2 were shown to be associated with diclofenac-induced hepatic injury [148]. The polymorphism rs1495741 and others in N-acetyltransferase 2 (NAT2) are related to isoniazid-induced DILI in patients under tuberculosis therapy [149-151]. In contrast to DILI-associated genetic variants in MDR3 or BSEP, MRP2 variants also cause extrahepatic manifestations of drug toxicity as described above [50-53]. It is evident that drugs are more likely to cause DILI by undergoing significant hepatic metabolism [140]. Enzymes of the CYP superfamily are responsible for metabolizing over 90% of current therapeutic drugs [152]. Therefore, genetic variants of the variety of *CYP* genes, especially with reduced or lacking enzyme activity, are likely to be associated with DILI [152]. In the future, timely performed genetic testing to identify slow and fast metabolizers for a broad spectrum of drugs might be useful to exclude severe adverse reactions and to identify patients who would not benefit from a particular drug [152].

Genetic variants predispose for development of fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) comprises a wide spectrum of liver diseases characterized by increased fat incorporation in hepatocytes [153]. This disease ranges from hepatic steatosis or non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH) to liver fibrosis, cirrhosis, and HCC [154]. Worldwide prevalence of NAFLD has been estimated between 9-37% [155]. In the United States, prevalence ranges around 30%, whereby men are more often affected (30-40%) than women (15-20%) [155,156]. It is believed that in 2030, NAFLD will be the most frequent indication for liver transplantation in western industrial nations [156]. According to this, the frequency of NAFLD-/NASH-associated HCC rose significantly in the United States [157]. NASH development is a multifactorial-process. Besides factors like obesity, reduced physical activity, insulin resistance and type 2 diabetes, genetic variants in the patatin-like phospholipase domain-containing protein 3 (PNPLA3) or transmembrane 6 superfamily member 2 (TM6SF2) represent disease modifiers. Additionally, the composition of the intestinal microbiome, modulated by diet as well as the composition and size of the bile acid pool, influences NAFLD as well as HCC progression [154,158]. Up to now, there is no NAFLD-/NASH-specific drug therapy. One reason for this might be the lack of an appropriate mouse model [158]. Since hepatic steatosis, NASH as well as cirrhosis accumulate in some families and ethnicities, inheritance is estimated to account or contribute to almost 40% of this disease [159]. In 2008, the amino acid exchange p.I148M (c.444C>G, rs738409) in PNPLA3 was identified to be associated with NAFLD (Table 3) [160]. Moreover, this variant represents an important genetic factor that exacerbates the progression of alcoholic-based liver disease [161,162]. The MAF of this SNP in PNPLA3 is 26.2% and compared to controls, the risk to develop liver disease or NASH is about 3-fold higher [163]. The adjusted OR (for gender, age, diabetes, obesity, cirrhosis) for HCC development in NAFLD patients carrying the GG allele (e.g. homozygous for p.I148M) was 5.1 as compared to patients with a CC allele [164]. Since this PNPLA3 variant is that important, it was suggested to summarize fatty liver diseases associated with p.I148M 'PASH' for PNPLA3-associated steatohepatitis [165]. Another NAFLD-contributing genetic variant besides p.I148M for PNPLA3 is p.E167K (c.499G>A, rs58542926) in the *TM6SF2* gene which is also supposed to play a protective role for the risk of cardiovascular disease [166-168]. Besides *PNPLA3* and *TM6SF2*, other genes have been identified to contribute to NAFLD development (see Table 3). To date, besides the p.I148M variant in PNPLA3 mutations in the Adiponectin gene (*ADIPOQ*) are not only associated with NAFLD susceptibility but also contribute to disease progression [158,160,169].

Gene **Protein**	**NAFLD-associated variant**	**MAF***	**Odds ratio with NAFLD**	
ADIPOQ Adiponectin	c.214+62G>T (rs1501299)	3.0%	5.04	1.73
	c.214+135A>G (rs2241767)	15.0%	7.47	3.83
	c.215-414A>G (rs3774261)	49.7%	3.55 susceptibility	1.89 [169] progression
APOC3 Apolipoprotein C3	c.-501C>T (rs2854116)	45.2%	nd	
	c.-528T>C (rs2854117) [170]	49.9%		
GCKR Glucokinase regulatory protein	p.P446L (rs1260326)	29.3%	1.45 [158]	
	c.1423-418T>C (rs780094) SNPs in linkage disequilibrium [171]	30.2%		
LYPLAL1 Lysophospholipase-like protein 1	rs12137855	16.4%	1.37 [158]	
NCAN Neurocan core protein	p.P92S (c.274C>T, rs2228603)	4.4%	1.65 [158]	
PNPLA3 Patatin-like phospholipase domain-containing protein 3	p.I148M (c.444C>G, rs738409)	26.2%	3.26 [158]	
PPP1R3B Protein phosphatase 1 regulatory subunit 3B	rs4240624	11.4%	0.93 [158]	
TM6SF2 Transmembrane 6 superfamily member 2	p.E167K (c.499G>A, rs58542926) [167]	6.7%	nd	

Table 3. Genetic variants associated with NAFLD
*Minor allele frequency from '1000 Genomes' (http://www.ncbi.nlm.nih.gov/SNP/); Genes with high Odds ratio for NAFLD susceptibility as well as progression are highlighted in grey

Genetic variants contributing to HCC development in patients with chronic hepatitis C, alcoholic liver disease or NAFLD

Patients with liver cirrhosis are at an increased risk to develop hepatocellular carcinoma (HCC) [172]. SNPs have been identified in the promoter region of the myeloperoxidase gene G(-463)A-(MPO). The -463G allele creates a stronger SP1 binding site, leading to increased transcription of the gene and elevated MPO mRNA levels and most likely enhanced generation of reactive oxygen species [173,174]. Patients, which were homozygous for the G variant, had a higher risk to develop HCC than patients carrying at least one A-MPO allele (HR 3.8, p=0.002) [175]. The GG-MPO genotype also contributed to HCC development in patients with alcoholic liver cirrhosis [176]. In this population the high activity associated G-MPO allele was also a risk factor for death or complications of hepatic failure without HCC [176]. Another SNP identified in Japanese patients with chronic HCV infection is located in the 5' flanking region of the MHC class I polypeptide-related sequence A (*MICA*) gene (rs2596542) [177,178]. The risk allele of this SNP was associated with lower levels of the MICA protein and was significantly correlated with progression from chronic HCV infection to HCC (OR 1.4). Additionally, the PNPLA3 variant p.I148M (rs738409, G allele) represents a risk factor for liver disease progression and HCC development not only in NAFLD but also in alcoholic liver disease (ALD) (OR 2.2 for HCC development in ALD) [154,158,163,179].

Genetic contribution to cryptogenic liver cirrhosis

Despite a wide spectrum of modern diagnostic tools, cryptogenic liver cirrhosis, e.g. cirrhosis of unknown etiology represents a clinical challenge. NASH is considered to be the major cause of formerly cryptogenic liver cirrhosis [180]. Some patients with NAFLD present for the first time with cirrhosis since NASH remains asymptomatic for a long time in most patients [165]. Non-invasive diagnostic investigations like liver elastography and Controlled Attenuation Parameter (CAP) are needed for better understanding of NASH and to test therapeutic approaches to arrest or reverse disease progression [181]. To the present, liver biopsy is the method of choice to determine hepatic inflammation and fibrosis [158,165]. Homozygosity of the above mentioned PNPLA3 variant p.I148M (rs738409) can represent a cause of cryptogenic liver cirrhosis [165]. Furthermore, MDR3 variants, AATD, HFE-associated hemochromatosis, are associated with a broad spectrum of liver disease also with late-onset of symptoms and therefore should be considered in cases presenting with "cryptogenic" cirrhosis [182,183].

Conclusion

With progress in genotyping techniques genetic contribution to liver disease, which manifest in adulthood has become more evident. In this review, we focused on the genetic contribution to liver disease, which present in adulthood. In the last years certain common polymorphisms contributing to liver disease development and progression have been identified and may in the future allow for individualized genetically based risk stratification, implementation of surveillance programs (e.g. for HCC) and therapy.

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3.2 Genetic variations of bile salt transporters

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DRUG DISCOVERY TODAY TECHNOLOGIES

Transporter assays

Genetic variations of bile salt transporters

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Bile salt transporters directly or indirectly influence biological processes through physicochemical or signalling properties of bile salts. The coordinated action of uptake and efflux transporters in polarized epithelial cells of the liver, biliary tree, small intestine and kidney determine bile salt concentrations in different compartments of the body. Genetic variations of bile salt transporters lead to clinical relevant phenotypes of varying severity ranging from a predisposition for drug-induced liver injury to rapidly progressing end-stage liver disease. This review focuses on the impact of genetic variations of bile salt transporters including BSEP, NTCP, ASBT and OSTα/β and discusses approaches for transporter analysis.

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Bile formation and tissue distribution of bile salt transporters

Bile formation is necessary for the elimination of cholesterol and for the excretion of lipophilic compounds after their biotransformation (e.g. hydroxylation, sulfation, conjugation). Solubilization of cholesterol within bile involves the formation of mixed micelles, which contain large amounts of bile salts (BS) and phospholipids. The primary bile acids cholic acid and chenodeoxycholic acid are synthesized in hepatocytes involving oxidation of cholesterol by the rate limiting 7α-hydroxylase (CYP7A1). Conjugation with glycine or taurine introduces a fully ionized carboxyl or sulfonate group into bile acids, resulting in negatively charged bile salts (BS). BS reach the intestine *via* canaliculi and bile ducts. Within the large intestine BS are partly dehydroxylated and deconjugated by bacteria, resulting in secondary bile salts/acids. To provide the required amounts of bile salts they are not only synthesized in the liver but also reabsorbed in the biliary tree and the small bowel and cycled back to the liver several times per day. This cholehepatic and enterohepatic circulation involves transport steps in hepatocytes, cholangiocytes and enterocytes. Bile salts have numerous effects within these cells. At high concentrations bile salts are toxic [89], can induce apoptosis [44,92], and interfere with some biological processes such as liver regeneration [36], glucose homeostasis [114] or carcinogenesis [3]. These properties limit the tolerable level of bile salts in blood, hepatocytes, bile and intestine.

To maintain BS homeostasis several transport systems have evolved. The major transporters are the Na^+-taurocholate cotransporting polypeptide (abbreviated protein name NTCP; common gene symbol *SLC10A1*), the bile salt export pump (BSEP; *ABCB11*), the apical sodium-dependent bile acid transporter (ASBT; *SLC10A2*), and the organic solute transporters OSTα/OSTβ (*SLC51A/SLC51B*) (Fig. 1).

NTCP (NTCP/Ntcp for the human and animal orthologs, respectively) is the major uptake system for bile salts at the sinusoidal membrane of hepatocytes [106], and utilizes the sodium gradient generated by the Na^+/K^+-ATPase. Members of the organic anion-transporting polypeptide (OATP; *SLCO*)

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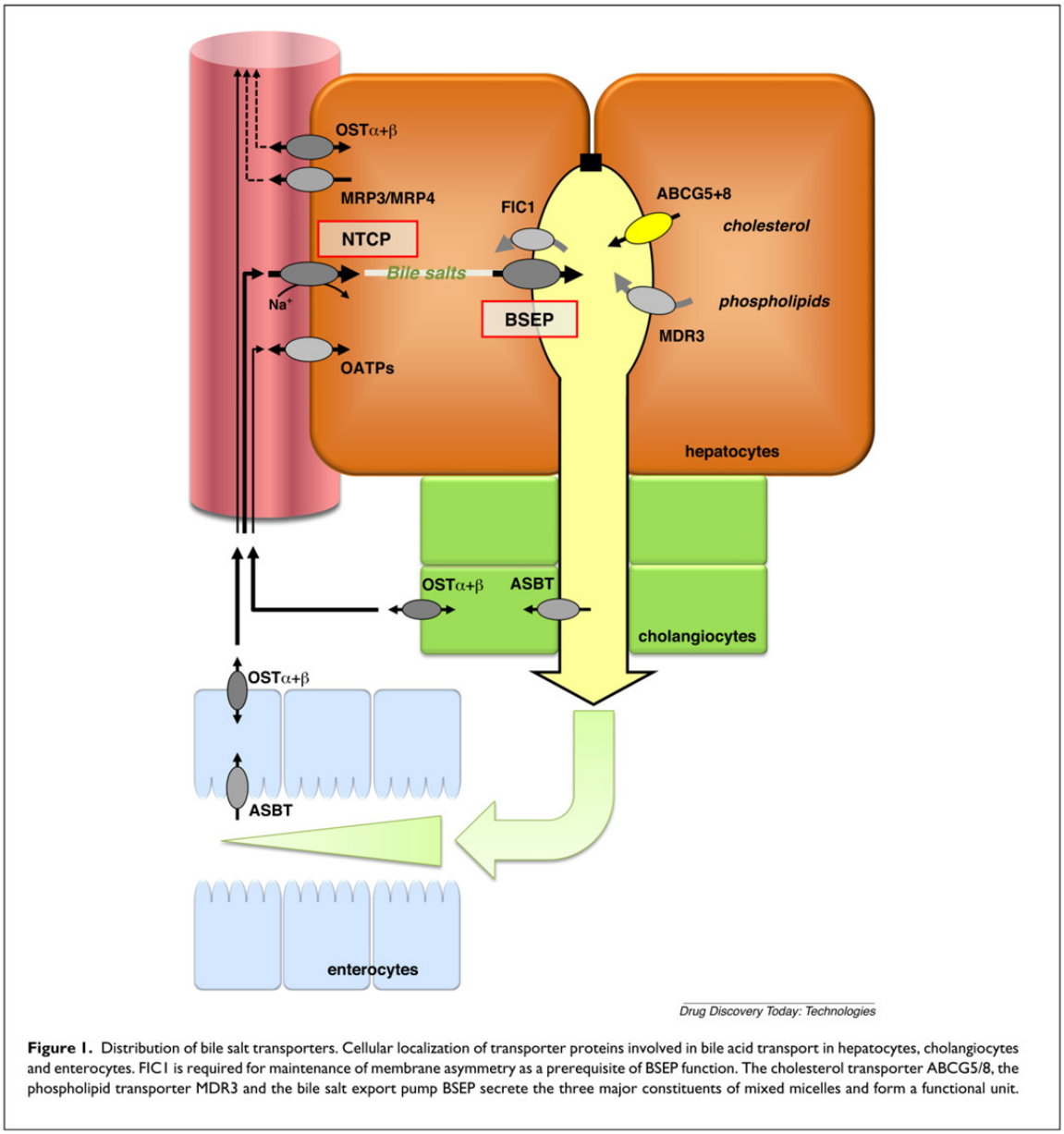

Figure 1. Distribution of bile salt transporters. Cellular localization of transporter proteins involved in bile acid transport in hepatocytes, cholangiocytes and enterocytes. FIC1 is required for maintenance of membrane asymmetry as a prerequisite of BSEP function. The cholesterol transporter ABCG5/8, the phospholipid transporter MDR3 and the bile salt export pump BSEP secrete the three major constituents of mixed micelles and form a functional unit.

family transport bile salts in addition to a wide range of organic anions. They act as anion exchangers, for example, transporting taurocholate in exchange to HCO_3^- [26]. In addition, the multidrug resistance-associated proteins 3 and 4 (MRP3 and 4) are able to transport (sulfated) bile salts [94,95,132] back into blood. In cholestasis with increased intracellular BS concentrations, this may represent an important overflow mechanism. In line with this an almost tenfold up-regulation of MRP4 was demonstrated in livers from paediatric patients with inherited cholestasis [47]. Likewise, MRP4 is induced by ursodeoxycholic acid (UDCA) in humans, which in part explains the beneficial effect of UDCA in cholestasis [68]. Apart from MRP3 and 4, the organic solute transporter OSTα/OSTβ is expressed at the sinusoidal membrane of hepatocytes and mediates the efflux of BS back into blood to a not well-defined degree [2].

The major bile salt transporter at the canalicular membrane is BSEP, which belongs to the ATP binding cassette (ABC)

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transporter family. BSEP is able to concentrate bile salts more than 1000-fold from hepatocyte into bile. Canalicular secretion of bile salts is regarded as one of the rate-controlling steps [27,28,117], which in addition to BSEP involves other energy-dependent transporter proteins such as the transporter 'familial intrahepatic cholestasis' (FIC1; *ATP8B1*). FIC1 is a P-type ATPase [84,118] that maintains membrane asymmetry of the canalicular membrane, which is required for proper function of BSEP. Furthermore, the multidrug resistance-associated protein 2 (MRP2), the canalicular bilirubin transporter [50], mediates transport of certain BS such as 6α-glucuronosylhyodeoxycholate and 3α-sulfatolithocholyltaurine into bile [42], which, however, only represent small amounts of BS in normal bile.

Cholangiocytes express ASBT at the luminal (apical) membrane [1] together with the chloride channel cystic fibrosis transmembrane conductance regulator (CFTR; *ABCC7*) and the Cl^-/HCO_3^--exchanger AE2 (anion exchanger, member 2). In a process termed cholehepatic shunting, BS can be reabsorbed by ASBT at the cholangiocellular level and recycle *via* the peribiliary plexus back to hepatocytes for re-secretion into bile. This process has been suggested to serve intracellular signalling by BS and to help maintaining canalicular BS secretion even when bile drainage is impaired in obstructive cholestasis [129].

By contrast to other cells, cholangiocytes are more resistant towards high and otherwise cytotoxic BS concentrations. Cholangiocytes are protected by biliary HCO_3^- secretion. The combined action of CFTR and AE2 is required for net bicarbonate excretion into the biliary lumen to generate this protective 'bicarbonate umbrella' [4,35]. At the basolateral membrane of cholangiocytes the heterodimeric transporter OSTα/OSTβ secretes BS into the blood of the peribiliary plexus, eventually reaching the sinusoidal space and the hepatocytes.

Enterocytes in the terminal ileum and colon express ASBT (also termed ileal bile acid transporter IBAT or ileal sodium/bile acid co-transporter ISBT) as well as OSTα/OSTβ (for review see [15]; Fig. 1). In the ileum BS reabsorption is mediated by the sodium gradient-driven ASBT from the intestinal lumen and are secreted into the portal circulation by OSTα/OSTβ. Because of the large surface area, more than 90% of BS are reabsorbed by this mechanism and undergo the enterohepatic circulation. Coordination of bile salt transport in enterocytes and hepatocytes is tightly regulated. On the level of enterocytes high BS concentrations lead to an activation of the farnesoid X receptor (FXR), an intracellular BS sensor. As a response to FXR activation, the expression of fibroblast growth factor 19 (FGF19) is induced in enterocytes. FGF19 reaches the hepatocytes *via* the portal blood. In hepatocytes, FGF19 binds to FGF receptor 4 (FGFR4) and leads to repression of CYP7A1, the key enzyme of bile acid synthesis (for review see [91]). This negative feedback mechanism connects the intestine and liver and contributes to bile acid/salt homeostasis.

BSEP

Expression and function of BSEP

The bile salt export pump BSEP is a member of the MDR family (ABC subfamily B), which also includes the multidrug resistance protein 1 (also termed P-glycoprotein; MDR1; *ABCB1*) and 3 (MDR3; *ABCB4*). Human *ABCB11* is localized on chromosome 2 (2q24) and consists of one un-translated exon at the 5′-end and 27 coding exons, which are translated into 1.321 amino acids with a molecular mass of approx. 160 kDa [109]. As compared to the most common reference sequence NM_003742/NP_003733 [108] two other potential variants have been described in the context of 'The sequence of the human genome' by Venter *et al.* [122] (NCBI, DBSource accession number CH471058.2). On the cDNA level, isoform CRA_b (here termed BSEP-B) is characterized by an insertion of 42 base pairs of the intron sequence between the first and the second coding exon, whereas isoform CRA_c (BSEP-C) includes 27 base pairs of the intronic region between coding exon 15 and 16 (Fig. 2a). By contrast to BSEP-C, BSEP-B was detectable in human liver samples to a varying degree (Fig. 2b). The potential function and clinical relevance of these BSEP isoforms remain to be determined.

BSEP is localized at the canalicular membrane of hepatocytes. Low or even absent canalicular BSEP expression is a typical feature of many disease-related BSEP mutations [40,47,56,108] and may be caused by reduced or aberrant transcription, altered mRNA stability, impaired translation, increased degradation at the level of endoplasmic reticulum (ER)-associated degradation (ERAD) and lysosomes or disturbed intracellular trafficking. It is of note that in humans BSEP deficiency cannot be compensated by MDR1 or MRP2, because these ABC transporters have low affinities for human primary bile salts. For example, MRP2 transports divalent sulfated or glucuronidated, but no monovalent bile salts [50,57]. Targeting of BSEP to the canalicular membrane is influenced by four N-linked glycosylation sites of the first extracellular loop of rat Bsep [74], which are also present in human BSEP. It has been suggested that HCLS1-associated protein X-1 (Hax1) regulates the abundance of BSEP at the canalicular membrane [83]. Depletion of Hax1 or cortactin (an interacting partner of Hax1) increased BSEP expression in the apical membrane of Madin Darby canine kidney (MDCK) cells [83]. Interestingly, cell shrinkage leads to endocytosis of Bsep [97] and induces the interaction of cortactin with the Src kinase Fyn [9], suggesting that the actin binding protein cortactin is involved in the retrieval mechanism of Bsep. On the other hand, the taurine conjugate of UDCA, which is widely used to treat cholestatic liver diseases, stimulates insertion of Bsep into the canalicular membrane in a $p38^{MAP}$-kinase sensitive manner [58].

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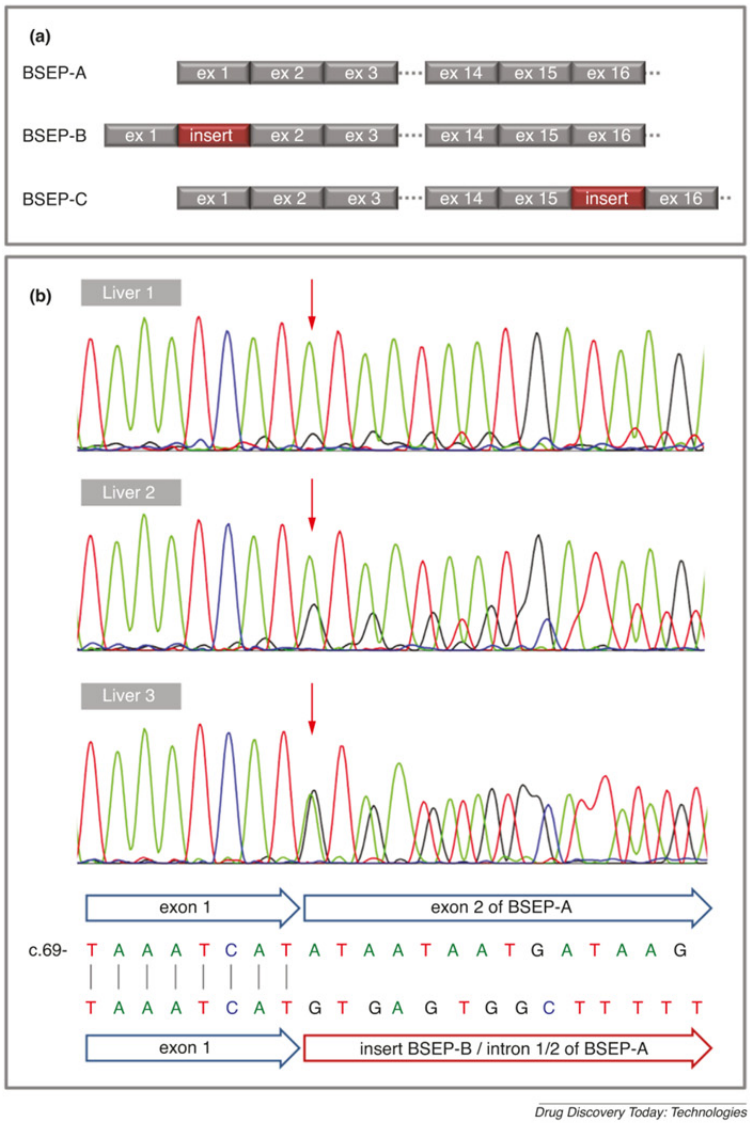

Figure 2. Splicing variants of human BSEP. **(a)** Three splice variants of human BSEP (*ABCB11*) have been described. Compared to the common variant BSEP-A (NM_003742.2), BSEP-B has an insertion of 42 nucleotides between coding exons 1 and 2 while BSEP-C includes 27 additional nucleotides of the intron sequence between coding exons 15 and 16. **(b)** By contrast to BSEP-C, BSEP-B mRNA was detectable in human liver samples in varying amounts. Following clear peaks of exon 1, there is an overlapping sequence (red arrow) of regular exon 2 and the sequence specific for cDNA of isoform BSEP-B.

BSEP is the major driving force of bile salt-dependent bile flow. Major substrates of human BSEP are taurine and glycine conjugates of cholic acid (CA) and chenodeoxycholic acid (CDCA), the two primary bile acids [103]. K_m-values were determined to be 6.2 μM for taurocholate (TC), 6.6 μM for taurochenodeoxycholate (TCDC), 7.5 μM for glycochenodeoxycholate (GCDC) and 21.7 μM for glycocholate (GC) [31]. However, 'clearance rates' (V_{max}/K_m) were higher for CDCA-derivatives as compared to TC-derivatives, with a rank order of TCDC > GCDC > TC > GC [31]. Given that CDCA and its conjugates are potentially more toxic for hepatocytes *in vivo* [101] and *in vitro* [92], a more efficient elimination of CDCA appears biologically favourable.

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Genetic variants of BSEP

Mutations of BSEP are associated with progressive familial intrahepatic cholestasis type 2 (PFIC-2) [6,109], or a milder phenotype called benign recurrent intrahepatic cholestasis type 2 (BRIC-2) [56,120]. In addition, intrahepatic cholestasis of pregnancy (ICP) has been linked to mutations and polymorphisms of BSEP [17,72,88].

PFIC-2 is a severe cholestatic liver disease typically leading to liver cirrhosis in childhood. The patients normally present within the first year of life with pruritus, growth failure or bleeding complications. In some patients, drainage of the remaining bile flow (e.g. by partial external biliary diversion (PEBD)) may ameliorate the progression towards liver cirrhosis [18,126]. It has been reported that PEBD is indicated in about 40–50% of PFIC patients and is successful in three quarters of these patients [18], whereas in the remaining cases liver transplantation is required. In comparison with PFIC-2, BRIC-2 is self-limiting, but recurrent and liver injury resolves between episodes of cholestasis. More recently, PFIC-2 and BRIC-2 have been recognized as parts of a disease continuum and this group of illnesses may be summarized as 'BSEP deficiency' [52] or 'BSEP disease'. Patients with BSEP disease (PFIC-2 or BRIC-2) have normal or low serum levels of gamma-glutamyltransferase (γGT), an enzyme localized in the canalicular membrane and the epithelium of cholangiocytes [86]. Low γGT-levels despite severe cholestasis are attributed to the low concentration of free bile salts within the biliary lumen in patients with BSEP deficiency [38]. Symptoms of BSEP disease like pruritus and jaundice can be ameliorated by UDCA or rifampicin therapy [12,39]. In most cases, BRIC-2 is responsive to UDCA, but deterioration over time is also possible [56,107].

More than 150 mutations of BSEP have been associated with cholestatic liver diseases [55]. Among these p.E297G, p.D482G and p.N591S belong to the more frequent mutations. While homozygosity of p.E297G or p.D482G is linked to PFIC-2, heterozygosity of these mutations was detected in about 1.4% of 491 patients with intrahepatic cholestasis of pregnancy (ICP) [17]. ICP is characterized by pruritus and elevated serum BS concentrations during pregnancy with spontaneous resolution after delivery. ICP can be treated with UDCA, which resolves pruritus to some extent and reduces the risk of preterm birth, which is the major challenge of ICP [19,20]. The clinical presentation of contraceptive-induced cholestasis (CIC) is similar to ICP. Trans-inhibition of BSEP by oestrogen and progesterone metabolites is a probable trigger of cholestatic episodes in CIC and ICP [105,119]. Patients with ICP or CIC have a higher prevalence of the BSEP polymorphism p.V444A/c.1331T>C (exchange of valine to alanine at amino acid position 444; 'p' stands for 'protein', 'c' stands for 'coding DNA') [17,72].

Individual genetic variants/mutations may be analyzed by functional studies. For this approach, BSEP expression in insect cell vesicles [80], yeast membrane vesicles [107] or single or double transfected cell lines [73] has been used. Several mutations do not alter function, but influence intracellular processing or targeting [45,123]. A characteristic finding in BSEP-related cholestasis is a reduced canalicular expression of BSEP *in vivo* [30,40,47,90,108,124] and an inverse correlation between plasma membrane expression of BSEP and severity of the clinical phenotype has been described [59]. For example, the two 'BRIC-2 mutations' p.A570T and p.R1050C had lower expression levels than the 'ICP mutation' p.N591S, but higher expression levels as compared to the PFIC-2 mutations p.D482G and p.E297G [59]. However, the mutation p.G374S is an exception of this relationship. This mutation is associated with a late onset PFIC-2 phenotype, but has an expression level comparable to wild-type BSEP *in vivo* and *in vitro* [107]. Interestingly, almost all missense mutations within the transmembrane helices are associated with severe phenotypes of BSEP disease (PFIC-2). It was suggested that the transmembrane helices are particularly sensitive to missense mutations due to their role in substrate recognition, binding and translocation of BS [107]. It is of note that only a small fraction of the 200 known missense mutations of BSEP [55] has been characterized at the molecular level. Therefore, a more in-depth genotype-phenotype characterization has to be addressed in the future: While premature stop-codon or frameshift mutations are evident causes of reduced protein expression, the role of missense mutations for deficient canalicular BSEP expression is less clear. On the mRNA level, splicing efficiency may be affected as shown by a minigene assay [7]. In this approach, exons with or without mutations and their adjacent introns are cloned into plasmids between exons and introns of a 'reporter gene' (e.g. the human fibronectin gene). After transfection of such minigene-constructs the effect on splicing efficiency of single mutations (as compared to the wild-type exon) can be analyzed by the amount and length of the resulting transcripts as determined by real-time PCR. For example reduced splicing and exon skipping was reported for the typical PFIC-2 mutation c.1445A>G (p.D482G), but also for the frequent synonymous variant c.3084A>G (p.A1028A) of BSEP [7] (Table 1).

Missense mutations may induce misfolding during protein translation resulting in an increased degradation by proteasomes (ERAD), which degrade up to 30% of normal proteins [99]. The missense mutations p.G238V, p.E297G, p.G982R, p.R1153C and p.R1268Q all led to a reduced expression at the apical membrane, when expressed in MDCK cells [124]. It was shown *in vitro* that the two BSEP mutations p.Y818F and p.G982R caused intracellular retention of BSEP. When in addition to these two mutations the common BSEP polymorphism p.V444A was present, complete degradation of triple-mutated BSEP *via* ERAD was observed [46], suggesting that p.V444A contributes to impaired stability of BSEP. A

Table 1. Consequences of genetic variations of BSEP/Bsep, NTCP and ASBT [8,30,32,33,46,51,59,80,85,104]. For BSEP, more than 300 variants/mutations are known [55,108], but only a few have been investigated on a molecular level.

	BSEP/Bsep		NTCP		ASBT	
Exon skipping	E186G	G1116R				
	G319G	R1128C				
	T463I	R1128H				
	A926P	E1186K				
	A1028A[a]	R1231W				
	A1110E					
Aberrant splicing	E297K	R1153H				
	R832C	S1154P				
	S1144R					
No splice product	T586I					
	R1231Q					
Reduced plasma membrane expression	E135K	A570T	I223T			
	E297G[b]	N591S[b]				
	V444A	R1050C				
Intracellular retention	Y818F					
	G982R					
Reduced or absent bile salt transport	A570T	R432T	A64T	K314E	V98I[c]	M264V
	I206V	Q558H	I223T		C144Y	P290S
	E297G[b]	N591S[b]	S267F		L243P	
	G374S	E1186K	I279T		T262M	

[a] A1028A induces significant exon skipping *in vitro* but probably not *in vivo* (unpublished data; Dröge, Häussinger, Kubitz).
[b] Some variants (such as E297G or N591S of BSEP) may affect transport handling on different levels.
[c] The variant V98I of ASBT showed reduced transport in one study [34], whereas another study reported unaffected transport by ASBT^{V98I} [66].

patient homozygous for p.V444A of BSEP and p.S320F of MDR3 (*ABCB4*), suffered from a severe form of ICP. BSEP expression (but not MDR3 expression) was massively reduced during cholestasis [49] underscoring the potential role of p.V444A for half-life of BSEP. Furthermore, transporter recycling at the canalicular membrane may influence the expression level. For the two common BSEP mutations p.E297G and p.D482G it has been reported that treatment with 4-phenylbutyrate prolongs half-life at the canalicular membrane and consecutively overall expression of BSEP [29].

Recently, a new type of cholestasis has been recognized, which is due to anti-BSEP antibodies and which occurred after liver transplantation due to PFIC-2 [41,46,67]. It is hypothesized that complete absence of BSEP in PFIC-2 livers results in a lack of auto-tolerance towards this transporter. Therefore, liver transplantation results in exposure towards BSEP as a new antigen and anti-BSEP antibodies develop. These antibodies bind BSEP from the extracellular space [46] and reach the canalicular space by a not yet identified pathway. Most importantly, in some cases these antibodies inhibit BSEP activity, thereby inducing cholestasis with a phenotype similar to PFIC-2.

Drug-induced liver injury (DILI) and BSEP

Drug-induced liver injury is an important hazard of drug treatment and often limits the clinical use of new drugs. Human hepatocytes have a lower capacity than rat hepatocytes to secrete BS back into blood. It was suggested that this reduced fractional elimination rate at the sinusoidal membrane contributes to a higher susceptibility towards increased intracellular BS concentrations and cholestatic drugs in human hepatocytes [43]. The low hepatocytes-to-blood transport in humans may explain, why a relatively strong correlation between inhibition of human BSEP and hepatotoxicity in humans could be observed [77]. Recent studies correlated the potential of different drugs to inhibit BSEP with the risk of DILI [16,77,82,115,121] (Table 1). Molecular size, hydrophilicity and non-polar surface area were factors associated with the inhibitory potential of compounds tested [16].

The frequent BSEP polymorphism p.V444A represents the mildest form of BSEP deficiency. An association between p.V444A and drug-induced liver injury [61] has been demonstrated. The allele frequency of c.1331T>C (alanine) was 76% in patients with DILI as compared to 59% in controls with an odds ratio of 4.0 [61]. The association of p.V444A with such disturbances may be explained by the decreased expression of BSEP in the presence of the polymorphism [71], leading to increased bile salt concentrations in hepatocytes. Furthermore, hepatitis C infected patients, who are homozygous for valine of p.V444A have less severe liver fibrosis [37] and some but not all HCV-cohorts show better treatment responses towards interferon/ribavirin treatment [54].

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NTCP

Expression and function of NTCP

The Na^+-taurocholate co-transporting polypeptide (NTCP; *SLC10A1*) belongs to the SLC10 family, which consists of seven members including the apical bile salt transporter ASBT (*SLC10A2*) and the sodium-dependent organic anion transporter (SOAT; *SLC10A6*). The human Na^+-taurocholate co-transporting polypeptide was cloned in 1994 [25]. It consists of 349 amino acids resulting in an apparent molecular mass of about 38 kDa. It transports taurocholate with a lower K_m (~6 μM) as compared to Ntcp from rat (~25 μM) [25]. Three amino acid residues (D115, E257 and C266) were identified to be essential for transport function of rat Ntcp [131]. These three amino acids are also detectable in human NTCP (NP_003040.1).

Human NTCP forms dimers early in the secretory pathway. Truncation of the 43 C-terminal amino acids of NTCP (p.Y307X) not only inhibits the exit of NTCP from the endoplasmic reticulum but also retains dimerized full length NTCP [5]. Interestingly, co-expression of wild-type NTCP and $NTCP^{E257N}$, an inactive variant, did not inhibit targeting to the plasma membrane or function of wild-type NTCP, indicating that single NTCP molecules are functionally active within the dimer [5]. Sorting to the sinusoidal (basolateral) membrane depends on the C-terminal, cytoplasmic tail of rat Ntcp [112], or more specifically, on tyrosine 321 of rat Ntcp [113]. NTCP not only forms dimers but is also organized in non-caveolar membrane microdomains. Interestingly, cholesterol depletion resulted in a reversible shift of NTCP into non-raft microdomains and was associated with increased transport activity [75]. Our own studies showed that a dileucine motif within the third putative intracellular loop (Leu222 and Leu223) is also important for targeting to the membrane and endocytosis of Ntcp [111].

Using a 3D-pharmacophore-model based on NTCP substrates, five interacting residues (two hydrogen bond acceptors and three hydrophobic sites) were predicted and a virtual compound library was screened for inhibitors of NTCP [22]. The most potent inhibitor was an anthraquinone derivative. Transport assays of bile acid analogues with different substitutions at carbon atoms 3 or 7 demonstrated that substitutions at C3 were better transported, whereas analogues with substitutions at C7 were not transported by NTCP [53]. Using HeLa cells transfected with human NTCP several drugs were identified, which inhibited taurocholate transport, including cyclosporin (IC_{50} = 1 μM), furosemide (IC_{50} = 15 μM) or ketoconazole (IC_{50} = 264 μM) [51]. Even UDCA, which is used for the treatment of cholestatic liver diseases, inhibits taurocholate uptake by NTCP with a low IC_{50} of about 3.6 μM [51]. The anti-retroviral drugs ritonavir (IC_{50} = 2.1 μM) or saquinavir (IC_{50} = 6.7 μM), but not efavirenz (IC_{50} = 43 μM), inhibited NTCP in a similar range [70].

Recently, NTCP has been identified as a receptor for the hepatitis B (HBV) and D (HDV) viruses [130]. It was shown that the pre-S1 protein of the large surface protein specifically interacts with NTCP. The amino acid residues 157–165 are essential for this interaction [130]. The selective expression of NTCP in the liver may explain the organotropism of HBV/HDV. Differences of NTCP-orthologs in the regions of amino acids 157–165 may be the reason for the species-specificity of HBV. It is currently not known, if transport activity of NTCP is required for infection of hepatocytes by the hepatitis B virus. In that case dysfunctional variants (such as p.A64T and p.S267F) could confer some resistance against HBV resulting in an evolutionary advantage. The exact mechanisms of viral entry are currently unknown. It was recently suggested that plasma membrane localization but not kinase activity of protein kinase C delta (PKCδ) [87] is required for cAMP-mediated exocytosis of NTCP [23,69,79]. On the opposite, endocytosis of rat Ntcp and subsequent lysosomal degradation are induced by PKC activation [110] and triggered by taurochenodeoxycholate (TCDC), a major substrate of Ntcp [78].

Cysteine and tyrosine residues may be important for the regulation of NTCP. NO-donors were shown to induce S-nitrosylation of cysteines [98] and nitration of tyrosines [21] of NTCP, resulting in decreased V_{max} of NTCP and therefore in a reduced uptake of bile salts. *In vivo* NO-production can be stimulated by bile salts *via* the membrane bound bile acid receptor TGR5 in sinusoidal endothelial cells (SEC) [48]. It may be speculative that SEC could protect hepatocytes from excessive bile acid uptake in a paracrine manner *via* this NO-mediated mechanism.

Genetic variants of NTCP

So far, no direct association of genetic variants of NTCP and diseases has been observed. Ho and co-workers detected four non-synonymous genetic variants including p.S267F (c.800C>T), p.I223T (c.668T>C), p.I279T (c.836T>C) and p.K314E (c.940A>G) in different American subpopulations. All four variants displayed decreased transport activity for taurocholate and taurochenodeoxycholate (Table 1). Interestingly, the p.S267F variant has an allele frequency of about 7.5% in Americans of Chinese descent [33]. It has a higher frequency in Chinese as compared to Korean persons [85] and was also detected in a Japanese study along with one synonymous (p.T75T) and 15 intronic variants [96]. The variant p.I223T had an allele frequency of 5.5% in African Americans and it was associated with a reduced surface expression as compared to wild-type NTCP and the other variants described in the study [33]. Genetic analysis in a Korean population identified six single nucleotide polymorphisms (SNPs) within the *SLC10A1* (NTCP) and 14 SNPs in the *SLC10A2* (ASBT) gene, including the two non-synonymous NTCP-variants p.A64T and p.S267F [85]. Both NTCP-variants are associated with decreased bile acid uptake (Table 1). An impact of genetic variants of NTCP on drug

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hepatotoxicity has been suggested but remains to be proven [64].

ASBT

Expression and function of ASBT

The apical sodium-dependent bile acid transporter (ASBT; *SLC10A2*) is expressed in the apical membrane of cholangiocytes [1,62], and epithelial cells from the terminal ileum, the colon and the renal proximal convoluted tubule [11,14]. The human *SLC10A2*-gene is localized on chromosome 13q33.1 and consists of six coding exons, spanning approx. 24 kb of DNA [128]. ASBT is composed of 348 amino acids with a molecular mass of 38–40 kDa in the glycosylated form. ASBT preferentially transports conjugated trihydroxy BS (tauro- and glycocholate) as compared to dihydroxy BS and unconjugated bile acids [24].

Genetic variants of ASBT

The genetic polymorphism p.P290S (proline to serine; rs56398830) was the first genetic variant identified in a sodium-dependent bile acid transporter in humans. It was initially found in a patient with Crohn's disease [127]. $ASBT^{P290S}$ had reduced transport activity [127], whereas expression and localization was not affected when transfected in COS-1 cells (Table 1). *In silico* modelling of ASBT suggested a functional defect of p.P290S due to a loss of binding of bile acids by this mutation [133]. This model of ASBT was based on a seven transmembrane topology of photoreceptor bacteriorhodopsin and identified four distinct binding sites for cholic acid, a major substrate for ASBT [133]. Subsequent analysis showed an allele frequency of p.P290S of about 5.2% in the control populations compared to 2.6% in gallstone carriers [93]. This study identified eight further non-synonymous SNPs (allele frequencies in brackets) with corresponding amino acid changes: p.W66X (0.4%), p.V98I (4.3%), p.V159I (4.3%), p.A171S (14.2%), p.L243P, p.T262M, p.F296L (0.4%) and p.F304L (0.8%). Interestingly, an intronic SNP c.378–105A>G (rs9514089, intron 1) with an allele frequency of 36.6% was more frequent in gallstone carriers (with odds ratios between 2.04 and 11.00 in two independent cohorts) [93], especially in male obese persons with gallstones. However, in a subsequent study covering three cohorts the association could not be confirmed [116].

In a family with hereditary or primary bile acid malabsorption (PBAM), characterized by diarrhoea, steatorrhea and reduced plasma cholesterol levels [81], Oelkers *et al.* identified four mutations including three missense and one splice site mutations. Two of these missense mutations (p.L243P and p.T262M) showed absent transport of conjugated and unconjugated bile acids in transfected COS cells, whereas the third missense mutation (p.A171S) had no effect on uptake (Table 1). A subsequent study investigated 13 adult patients with PBAM. Here, the two variants p.A171S (4 of 13 patients) and p.V98I (1 of 13) were found at the same frequencies as in control subjects [76], implying that these variants were not the reason for PBAM. The variant p.A171S was also found in a Korean population along with 13 SNPs in the non-coding region of *SLC10A2* [85]. Taken together, p.A171S and p.V98I most probably do not represent the cause of primary bile acid malabsorption.

Another study of 20 patients with familial hypertriglyceridemia (FHTG) detected three of the above mentioned variants (p.V98I, p.V159I and p.A171S). These missense variants displayed unaffected bile acid transport (in transfected COS cells) and showed the same frequencies in FHTG patients as compared to controls [66]. In a single patient, a new frameshift mutation (c.646insG), which abolished bile acid transport, was detected. However, a causative relation between genetic variants of ASBT and familial hypertriglyceridemia cannot be based on this observation.

Ho *et al.* identified three non-synonymous SNPs of human ASBT in healthy individuals of different ethnic background: c.292G>A (p.V98I), c.431G>A (p.C144Y) and c.790A>G (p.M264V) [34]. These variants were not associated with differences in mRNA- or protein-expression or in intracellular trafficking but exhibited different degrees of impaired transport capacity. The first two had a partially reduced taurocholate uptake while the latter one showed an almost complete loss of transport activity [34] (Table 1).

Taken together, there is no clear association between genetic variants of ASBT and clinical phenotypes (diseases) or functional consequences.

OSTα/OSTβ

Expression and function of OSTα/OSTβ

The two genes, Ostα/Ostβ (*Slc51A/Slc51B*) were first identified from a cDNA library of the little skate *Raja erinacea* [125]. The human OSTα and OSTβ genes are encoded on different chromosomes, positions 3q29 and 15q22, respectively. OSTα consists of nine exons coding for a protein of 340 amino acids (approx. 37 kDa) with seven potential transmembrane domains, OSTβ consists of four exons encoding 128 amino acids (approx. 19 kDa) with a predicted single-pass transmembrane domain [2,100,125]. The heteromeric transporter is expressed at the basolateral membrane of cholangiocytes and hepatocytes in the liver, as well as of enterocytes of the ileum. The 'mechanism' of transport by OSTα/OSTβ is facilitated diffusion; it is bi-directional and therefore follows chemical gradients. Under physiological conditions Ostα/Ostβ is responsible for the export of bile acids from enterocytes into the portal circulation. In cholestasis up-regulation of OSTα/OSTβ in hepatocytes mediates back-transport of BS from hepatocytes into the sinusoidal compartment, which may protect from liver injury [14]. Like other hepatobiliary transporters, the OSTα/OSTβ heterodimer is positively regulated by the farnesoid X receptor (FXR) [60,63]. It was shown by

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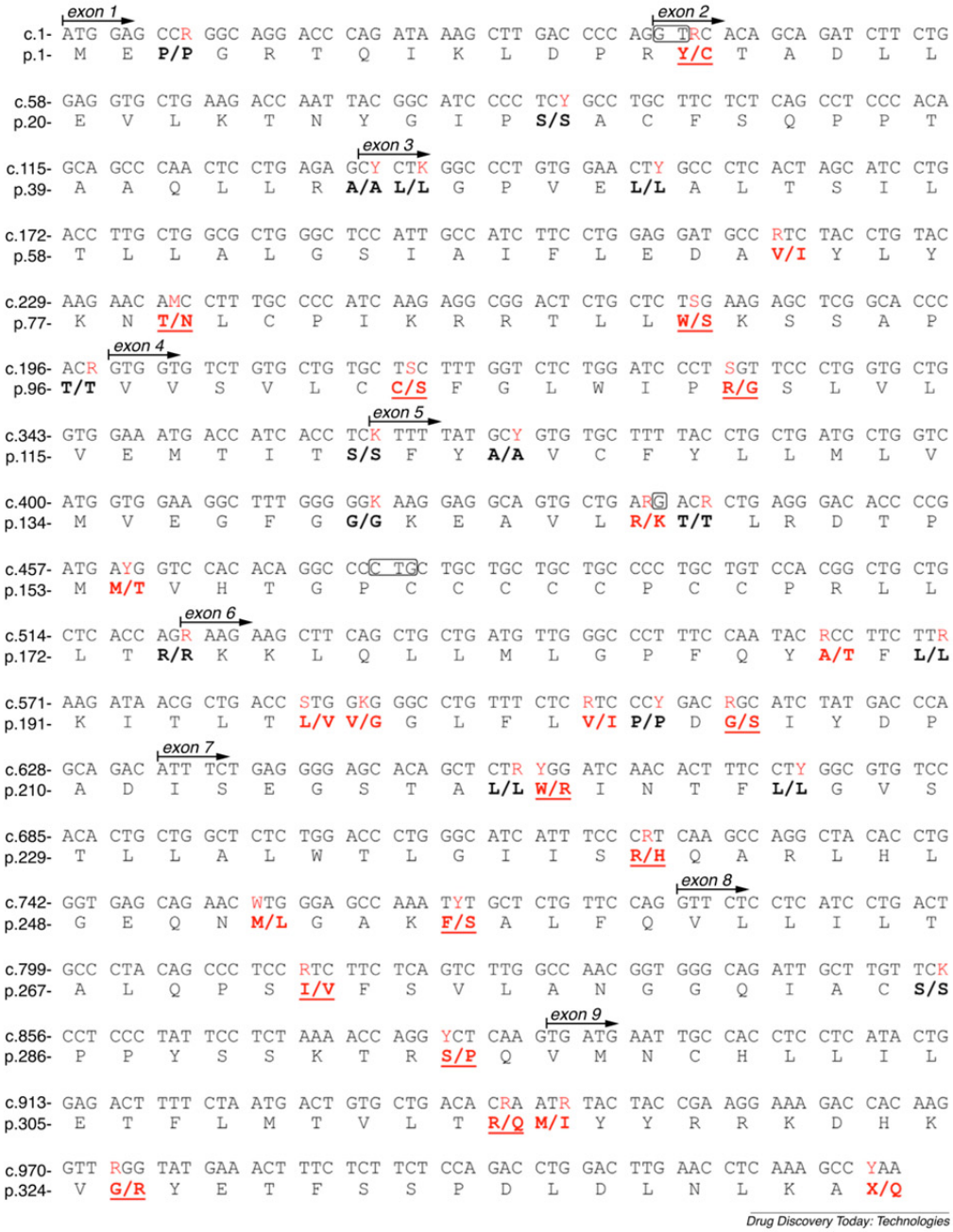

Figure 3. Nucleotide and amino acid sequences and genetic variants of OSTα. Coding sequence and corresponding amino acids of human OSTα including known genetic variants. 50 genetic variants are reported for human OSTα (*SLC51A*, NM_152672.2, http://www.ncbi.nlm.nih.gov/snp), but their clinical relevance is not known. Here, 23 missense mutations (red) and 16 synonymous variants (black) are depicted on the amino acid level. 13 of the missense mutations (red, underlined) are predicted by PolyPhen-2 to be probably damaging (http://genetics.bwh.harvard.edu/pph2/index.shtml). Five nucleotide changes in the coding sequence are located close to splice sites. Three deletions are described on cDNA level (signed by frames), two of them resulting in frameshifts. On cDNA level, letters are used according to the IUPAC nucleotide code: R = A or G; Y = C or T; K = G or T; M = A or C; S = G or C; W = A or T.

expression in MDCK cells that co-expression of mouse Ostα/Ostβ, but not the individual subunits, stimulated Na^+-independent bile acid uptake and the apical-to-basolateral transport of taurocholate. Co-expression was also required to convert the Ostα subunit to a mature glycosylated form [13]. Furthermore, co-expression is necessary for membrane delivery and stability of Ostα and Ostβ [65]. More specifically, the C-terminal end of Ostβ supports transport mechanisms, the transmembrane region interacts with Ostα and lysine/arginine residues are essential for proper extra- and intracellular topology of Ost β [10]. Deletion of the 50 N-terminal amino acids of human OSTα abolished transport activity, plasma membrane localization, and the ability to co-immunoprecipitate OSTβ [65]. Interestingly, human OSTα or OSTβ can be complemented by skate Ostβ or Ostα [100], although amino acid identities are only 32% (α) and 45% (β), respectively.

Genetic variants of OSTα/OSTβ

To date, there are no clinical data available on genetic variants of OSTα/OSTβ, that is, there is no known association between single variants and diseases. Nevertheless, several variants with single amino acid exchanges in the protein sequence of OSTα are available (Fig. 3). The impact of these variants on expression, function or intracellular trafficking of OSTα has not been analyzed in detail. However, *in silico* analyses suggest that some of these changes may result in serious dysfunction of the protein (Fig. 3). For OSTβ some variants, most of them in the intronic regions of the gene, were described (http://www.genecards.org) but their functional relevance was not investigated. Whether decreased activity of this heterodimeric transporter is of benefit in cholestasis remains open, although this has been suggested from a mouse model, where Ostα knock out protected from liver injury during oral bile acid feeding [102].

Conflict of interest

The authors have no conflict of interest to declare.

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3.3 Sequencing of FIC1, BSEP and MDR3 in 427 unrelated patients with cholestasis revealed 153 different genetic variants

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Own contribution: Compilation and analysis of sequencing data obtained from > 400 patients (analyzed in the "Lab of Cholestasis Diagnostics" in this department) including the identification of new genetic variants in comparison with the literature and databases

In silico prediction for specific genetic variants

2D protein modeling

Statistical analysis

Writing the main text

Preparing the tables and figures

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Sequencing of FIC1, BSEP and MDR3 in 427 unrelated patients with cholestasis revealed 153 different genetic variants

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Abstract

Background&Aims: The bile salt export pump (BSEP, *ABCB11*), multidrug resistance protein 3 (MDR3, *ABCB4*) and the ATPase familial intrahepatic cholestasis 1 (FIC1, *ATP8B1*) mediate bile formation. Mutations in these genes underlie cholestatic liver diseases ranging from intrahepatic cholestasis of pregnancy, benign recurrent intrahepatic cholestasis or low phospholipid-associated cholelithiasis to progressive familial intrahepatic cholestasis.

Methods: Coding exons with flanking intron regions of *ATP8B1*, *ABCB11,* and *ABCB4* were sequenced in cholestatic patients with assumed genetic cause. Effects of new variants were evaluated by bioinformatics tools and 3D protein modelling.

Results: In 248 patients with low gamma-glutamyltransferase (γGT) cholestasis, 28 FIC1 and 82 BSEP variants were detected including 39 novel ones. In 179 cases of high γGT cholestasis or gallstone disease, MDR3 analysis revealed 43 variants, 15 of them new. All novel missense mutations were disease-relevant according to bioinformatics analyses and homology modelling. 147 patients carried at least one rare, disease-relevant mutation in one of the three genes. 11 patients, who were sequenced for MDR3 only, showed no variants. In patients without severe mutations, common variants like FIC1$^{3531+8G>T}$ or FIC1^{R952Q} were found in 61.5% and 46.2%, respectively, BSEP$^{V444A+A1028A}$ in 71.3% and MDR3$^{L59L+N168N+I237I}$ in 18.6%.

Conclusions: In 427 unrelated patients, 153 different variants were detected in *FIC1*, *BSEP*, and *MDR3*. No disease-causing mutation was found in 90.2% *FIC1*, 65.1% *BSEP*, 80.9% *MDR3* sequences. Patients without disease-causing mutations carried common polymorphisms in 35.3% (*FIC1*), 65.1% (*BSEP*), 72.6% (*MDR3*) of cases. Mutations outside the sequenced areas, epigenomic and non-genomic factors may also contribute to cholestatic phenotypes.

3.4 The bile salt export pump (BSEP) in health and disease

Ralf Kubitz, Carola Dröge, Jan Stindt, Katrin Weissenberger, Dieter Häussinger

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Own contribution: Compilation and classification of all known genetic variants of BSEP based on literature, databases and sequencing data of the "Lab of Cholestasis Diagnostics" in this department

Summary of BSEP promoter regulation

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The bile salt export pump (BSEP) in health and disease

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Abstract

The bile salt export pump (BSEP) is the major transporter for the secretion of bile acids from hepatocytes into bile in humans. Mutations of BSEP are associated with cholestatic liver diseases of varying severity including progressive familial intrahepatic cholestasis type 2 (PFIC-2), benign recurrent intrahepatic cholestasis type 2 (BRIC-2) and genetic polymorphisms are linked to intrahepatic cholestasis of pregnancy (ICP) and drug-induced liver injury (DILI). Detailed analysis of these diseases has considerably increased our knowledge about physiology and pathophysiology of bile secretion in humans. This review focuses on expression, localization, and function, short- and long-term regulation of BSEP as well as diseases association and treatment options for BSEP-associated diseases.

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3.5 Exon-skipping and mRNA decay in human liver tissue: molecular consequences of pathogenic bile salt export pump mutations

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Experimental design

Cloning of minigene constructs

Transfection and cell culture

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Establishing of mRNA sequencing from FFPE and snap frozen human liver tissue including cDNA synthesis and PCR

Confocal laser microscopy

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Preparing the figures and table

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Exon-skipping and mRNA decay in human liver tissue: molecular consequences of pathogenic bile salt export pump mutations

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The bile salt export pump BSEP mediates bile formation. Over 150 BSEP mutations are associated with progressive familial intrahepatic cholestasis type 2 (PFIC-2), with few characterised specifically. We examined liver tissues from two PFIC-2 patients compound heterozygous for the splice-site mutation c.150 + 3A > C and either c.2783_2787dup5 resulting in a frameshift with a premature termination codon (child 1) or p.R832C (child 2). Splicing was analysed with a minigene system and mRNA sequencing from patients' livers. Protein expression was shown by immunofluorescence. Using the minigene, c.150 + 3A > C causes complete skipping of exon 3. In liver tissue of child 1, c.2783_2787dup5 was found on DNA but not on mRNA level, implying nonsense-mediated mRNA decay (NMD) when c.2783_2787dup5 is present. Still, BSEP protein as well as mRNA with and without exon 3 were detectable and can be assigned to the c.150 + 3A > C allele. Correctly spliced transcripts despite c.150 + 3A > C were also confirmed in liver of child 2. In conclusion, we provide evidence (1) for effective NMD due to a BSEP frameshift mutation and (2) partial exon-skipping due to c.150 + 3A > C. The results illustrate that the extent of exon-skipping depends on the genomic and cellular context and that regulation of splicing may have therapeutic potential.

The bile salt export pump BSEP (gene symbol: *ABCB11*) belongs to subfamily B (MDR/TAP) of adenosine triphosphate-binding cassette (ABC) transporters. BSEP is exclusively localised at the canalicular membrane of hepatocytes and mediates bile salt-dependent bile flow by transporting bile salts from the hepatocyte into the bile canaliculus, where bile salts form mixed micelles together with cholesterol and phospholipids[1]. Human BSEP is composed of 1321 amino acids encoded by the *ABCB11* gene, which is located on chromosome 2 (2q24)[2] and consists of a leading non-coding and 27 coding exons. BSEP mutations are the basis of cholestatic liver diseases of varying severity ranging from milder forms as intrahepatic cholestasis of pregnancy (ICP)[3–5] or benign recurrent intrahepatic cholestasis type 2 (BRIC-2)[6,7] to progressive familial intrahepatic cholestasis type 2 (PFIC-2) often resulting in end-stage liver disease in early childhood necessitating liver transplantation[2,8–11]. More than 290 genetic variants of BSEP are known and about 150 mutations were identified to be associated with PFIC-2 including missense mutations, deletions, insertions, frameshift and nonsense mutations with premature termination codons (PTCs) as well as splice-site mutations (for review see[12]). Donor and acceptor splice-sites specify exon boundaries, which are not only defined by the GT-AG rule[13], with GT at the 5′ end and AG at the 3′ end of an intron, but also by consensus sequences where the first six intronic positions of the donor splice-site are mostly GT(A/G)AGT[14]. Most influencing splice-site mutations concern +1/+2 (GT) or −1/−2 (AG) intron positions[15]. Nevertheless, more distal nucleotide exchanges within the consensus sequences are also known to be relevant. The putative splice-site mutation c.150 + 3A > C ("c." for "coding DNA") shown in two patients with a PFIC-2 phenotype leads to an exchange of adenine to cytosine at the third intron position downstream to *ABCB11* coding exon 3. In the present study, the impact of this more distally located *ABCB11* donor splice-site mutation was

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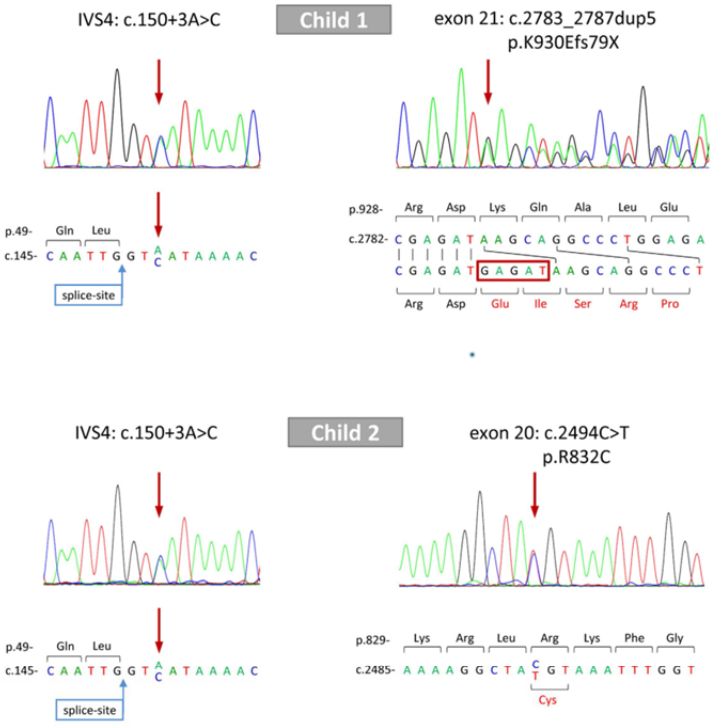

Figure 1. BSEP (*ABCB11*) mutations of two children with PFIC-2. Sequencing of all 27 coding exons with adjacent intron regions of *ABCB11* from gDNA revealed two relevant variants for child 1 (upper panel) and child 2 (lower panel) compared to reference sequence NM_003742.2 (Gene ID: 8647, first translated exon denoted as exon 1, adenine from ATG counted as c.1). Both children were compound heterozygous for the donor splice-site mutation c.150 + 3A > C as well as one exonic mutation. Child 1 had a duplication of GAGAT in exon 21 (c.2783_2787dup5) resulting in a frameshift with a premature termination codon (p.K930fs79X). Child 2 had a nucleotide exchange in exon 20 (c.2494C > T) leading to the missense mutation p.R832C. Corresponding sequences on nucleotide (c.) and protein (p.) level are shown in detail below. IVS4: intervening sequence, intron surrounded by coding exons 3 and 4.

proven not only in cultured cells by a minigene splicing assay but also by messenger RNA (mRNA) analyses from liver tissue of two patients with PFIC-2 phenotypes.

Results

Genetic analysis. Male child 1 was liver transplanted at the age of 3 due to progressive cholestatic liver disease with normal gamma-glutamyltransferase (gGT) levels. BSEP disease/PFIC-2 was considered, and sequencing of *ABCB11* revealed a heterozygous duplication of five nucleotides (GAGAT) in coding exon 21 (c.2783_2787dup5) (Fig. 1). This mutation, inherited by the father, causes a frameshift and a premature termination codon (PTC) after 78 altered codons (p.K930Efs79X; "p." for "protein sequence"). Furthermore, the heterozygous intronic mutation c.150 + 3A > C (Fig. 1; transmitted by the mother) concerning the donor splice-site of coding exon 3 was found in addition to two frequent polymorphisms (p.V444A and p.A1028A) and five more intronic variants further away from the splice-sites. All detected BSEP variants of both children are listed in Supplementary Table S1.

Child 2 (male) presented with pruritus, fatigue and elevated bile salt concentrations at 2.5 years of age. gGT levels were always within normal ranges, therefore low gGT-PFIC was assumed. *ABCB11* sequencing showed a heterozygous exchange of cytosine to thymine at position 2494 in coding exon 20 (c.2494C > T) leading to a missense mutation with arginine replaced by cysteine at amino acid position 832 (p.R832C; Fig. 1). This mutation was inherited by the mother whereas the splice-site mutation c.150 + 3A > C was inherited by the father (Fig. 1). Additionally, p.V444A and p.A1028A as well as four other intronic variants in a distance ≥15 nucleotides

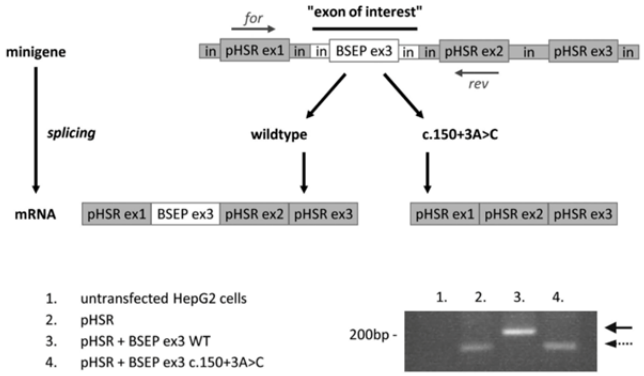

Figure 2. Analysis of c.150 + 3A > C by a minigene splicing assay. HepG2 cells were transiently transfected with a minigene plasmid (pHSR) and pHSR containing BSEP exon 3 with flanking intron regions, wildtype (WT) and mutant (c.150 + 3A > C), respectively. After transfection, RNA preparation, reverse transcription and PCR, agarose gel electrophoresis was performed. Lower bands (dashed arrow) include parts of the spliced pHSR exons 1 and 2 (165 bp) whereas the upper band (black arrow) additionally contains BSEP exon 3 (52 + 165 bp). The *ABCB11* donor splice-site mutation c.150 + 3A > C results in complete skipping of exon 3 *in vitro*. bp: base pairs, ex: exon, for: forward primer, in: intron, rev: reverse primer, WT: wildtype.

from the splice-sites were detected (Supplementary Table S1). Child 2 was listed for liver transplantation but was removed from the list two years later because cholestasis was effectively controlled by budesonide[16].

***In silico* analysis.** The intrinsic strength of the exon 3 splice donor and its mutation c.150 + 3A > C was calculated *in silico*. Wildtype sequence analysis by Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/splice.html[17]) resulted in 0.41 (possible values: 0 to 1) indicating a weak donor splice-site *per se*. It was no longer recognised in presence of c.150 + 3A > C, suggesting that this mutation leads to a disruption of this splice-site. In line with this, the cytosine at the third intron position was predicted to cause aberrant splicing with high probability according to HBond score algorithm (http://uni-duesseldorf.de/rna/index.php)[18,19].

Splicing analysis by a minigene assay. To analyse splicing outcome of c.150 + 3A > C, BSEP exon 3 and flanking intron regions with or without the nucleotide exchange (A > C) were cloned into a heterologous splicing reporter pHSR. After transient transfection into HepG2 or HEK293 cells, RNA extraction, reverse transcription and PCR were performed. Separation by gel electrophoresis (Fig. 2) resulted in a product of about 165 bp from cells transfected with the parental minigene pHSR. Insertion of BSEP exon 3 led to a shift of plus ~50 bp indicating that splice-sites were correctly recognised. In contrast, in presence of c.150 + 3A > C the shift was missing and the PCR product had the same size as seen for cells transfected with the empty minigene (Fig. 2). Sequencing revealed that both 165 bp products consist only of the amplified first two minigene exons whereas the larger product additionally contains 52 bp of inserted BSEP exon 3. In conclusion, the minigene assay indicates that BSEP c.150 + 3A > C leads to complete exon-skipping *in vitro*. The original uncropped gel is available as Supplementary Fig. S1.

RNA analysis in human liver tissue. For analysis of the patient's liver tissue, formalin-fixed paraffin-embedded (FFPE) tissue of the explanted liver was used in case of child 1 whereas for child 2, a snap frozen liver biopsy was available.

gDNA and RNA were isolated from liver tissue of child 1. The splice-site mutation as well as the duplication, located on different alleles, was verified on gDNA level (Fig. 3a). However, c.2783_2787dup5 was not detectable on mRNA level (Fig. 3b,c) strongly suggesting that mRNA transcripts from the affiliated allele are completely degraded. The duplication results in a premature termination codon (PTC) in exon 22, therefore, nonsense-mediated mRNA decay (NMD) is the most likely mechanism of mRNA degradation[20–22]. As a consequence, all proven BSEP mRNA transcripts must arise from the allele carrying the splice-site mutation, either excluding (Fig. 3b) or including (Fig. 3c) exon 3. The use of specific PCR forward primers made it possible to distinguish mRNA transcripts with or without exon 3.

In order to quantify the amounts of mRNA transcripts including or excluding exon 3, the relevant area was sequenced for the patients' liver tissues and 14 normal human liver samples. Reverse sequencing starting from exon 4 towards exon 1 (displayed as reverse complement sequence; Fig. 4) is depicted to avoid an additional overlap due to an insertion in isoform BSEP-B[23]. All control samples showed clear signals of exon 4, 3, and 2. In contrast, BSEP mRNA of child 1 and child 2 revealed an overlapping sequence composed of exon 2 and 3. The relative distribution of mRNA transcripts including and excluding exon 3 was calculated. Peak areas for each nucleotide of the overlap were matched and revealed for child 1 that skipping due to c.150 + 3A > C was observed in 63.1 ± 8.2%

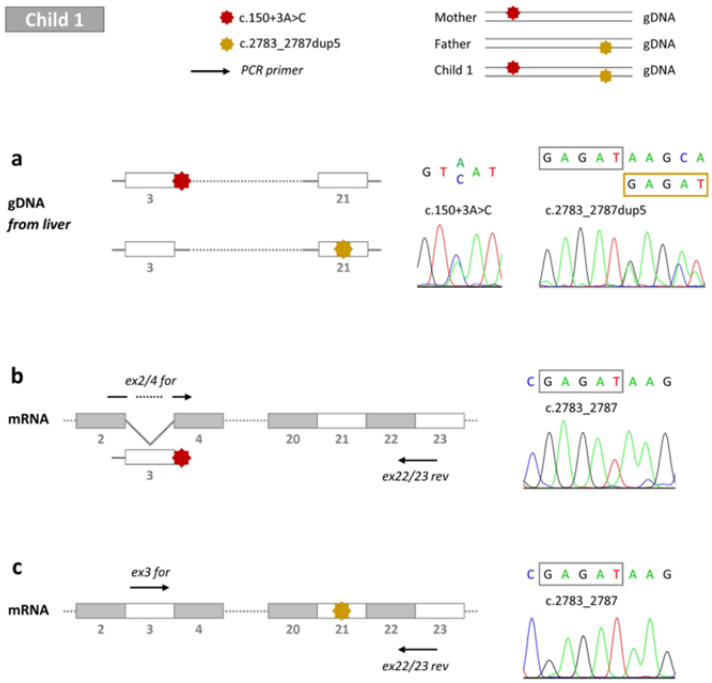

Figure 3. gDNA and mRNA sequencing from liver tissue of child 1. For child 1, the *ABCB11* splice-site mutation c.150 + 3A > C (red) is inherited by the mother whereas c.2783_2787dup5 (yellow) is transmitted by the father. (**a**) These mutations were detectable by sequencing of gDNA isolated from explanted liver of child 1. (**b**,**c**) For mRNA analysis, PCR forward primers *ex2/4_for* and *ex3_for* were used together with *ex22/23_rev*. Sequencing of BSEP exon 21 of both PCR products revealed the wildtype sequence. The GAGAT duplication (c.2783_2787dup5) was not detectable in mRNA transcripts with or without exon 3. ex: exon, for: forward primer, gDNA: genomic DNA, mRNA: messenger RNA, rev: reverse primer.

of transcripts (upper white bar, Fig. 4). Providing that detectable BSEP mRNA of child 1 entirely originates from the allele containing the splice-site mutation, it can be concluded that exon 3 is only partially skipped in the presence of c.150 + 3A > C in the patient's liver tissue. Furthermore, liver-specific mRNA sequencing of child 2 revealed that exon 3 is skipped in 37.1 ± 7.7% of transcripts (lower white bar, Fig. 4). In contrast to child 1, mRNA transcripts of both alleles of child 2 were included. The second mutation c.2494C > T (p.R832C) of child 2 does not affect mRNA processing.

Based on the missense mutation p.R832C, it was possible to distinguish the patient's alleles and to further prove exon-skipping in another approach (Fig. 5). Two different specific forward primers were combined with a reverse primer covering position c.2494 of the nucleotide exchange C > T in exon 20. Primer *ex2/4* is complementary to the exon transition of exon 2 and 4 in case of skipped exon 3 whereas primer *ex3* only binds when this exon is present. In the setting with a skipped exon 3, cytosine (C) from the wildtype sequence was almost exclusively found in exon 20 at position c.2494, whereas thymine (T) appears only to a very small amount (Fig. 5a). The ratio of peak areas (C/T) was 22/1, indicating that exon-skipping predominantly affects the allele containing the splice-site mutation. On the contrary, when exon 3 was present, the T peak was higher at c.2494 than the C peak (Fig. 5b) with a ratio of 2/1 (T/C). In summary, correctly spliced mRNA transcripts of child 2 arose not only from the maternal allele carrying c.2494C > T but also to some extent from the paternal allele despite the splice-site mutation (Fig. 4, lower panel, Fig. 5b).

BSEP protein expression. In normal human liver tissue BSEP and the bilirubin transporter MRP2 co-localise within the canalicular membrane and show a distinct immunoreactivity. Although to a lesser extent, BSEP was detectable in livers of both children co-localising with MRP2 (Fig. 6). In child 1, c.2783_2787dup5 excludes protein expression because BSEP mRNA transcripts from the affiliated allele were not observable (Fig. 3b,c). Therefore, detection of canalicular BSEP expression in child 1 confirms that mRNA splicing and processing from the allele with the splice-site mutation works properly to some extent.

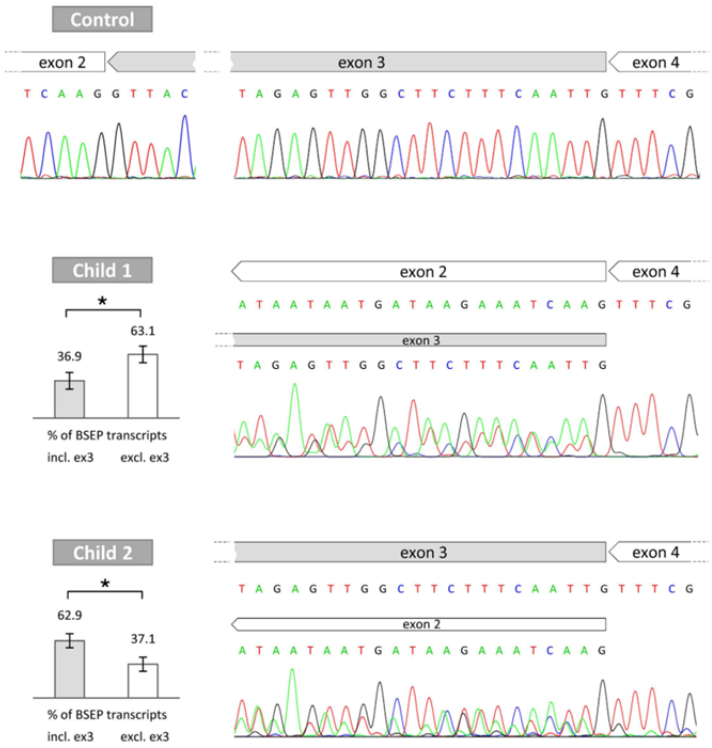

Figure 4. BSEP mRNA splicing effect due to c.150 + 3A > C in liver tissues of child 1 and 2. RNA from human liver tissue was used for reverse transcription and subsequent PCR. Reverse sequencing results are depicted in reverse complement. PCR product sequencing of control liver tissue displays clear peaks of BSEP exons 2, 3 and 4 (n = 14). In contrast, PCR product sequencing of child 1 and 2 shows overlapping peaks of BSEP exons 2 and 3, proving co-existence of mRNA transcripts with and without BSEP exon 3. Peak areas of each nucleotide within the overlap were measured and revealed amounts of 36.9 ± 8.2% for transcripts including exon 3 whereas in 63.1 ± 8.2% of transcripts exon 3 is missing in case of child 1. For child 2, higher amounts of transcripts with exon 3 (62.9 ± 7.7%) compared to transcripts without exon 3 (37.1 ± 7.7%) were calculated. Percentages are given as mean values with standard deviations, *significantly different with $p < 0.0001$ proved by the student's t-test. ex: exon, excl.: excluding, incl.: including.

Discussion

Progressive familial intrahepatic cholestasis (PFIC) represents a group of genetically diverse cholestatic liver diseases due to defects of distinct transporter proteins. Three different genes associated with PFIC are *ATP8B1* (FIC1), *ABCB11* (BSEP) and *ABCB4* (MDR3)[24–26], leading to PFIC type 1, 2 or 3, respectively. Recently, another PFIC-contributing gene was identified by Sambrotta and colleagues[27]. Mutations in the tight junction protein 2 gene (*TJP2*) can lead to PFIC type 4. The severity and possible treatment options of PFIC phenotypes are determined by the molecular consequences of particular mutations, and may, for example, depend on residual targeting and transport activity[28–30]. Hence, a detailed knowledge of the molecular effects of causative mutations is desirable for optimal disease management of individual PFIC patients. In the past, ursodeoxycholic acid (UDCA) has been shown to be effective in treatment of some PFIC-2 patients[31–34]. More specifically, UDCA increases BSEP activity of the BRIC-2-associated mutation p.A570T *in vitro*[35]. Furthermore, expression of BSEP with the common mutations p.E297G and p.D482G at the cell surface could be increased by the chaperone 4-phenylbutyrate[30,36]. Further studies showed improved liver function using 4-phenylbutyrate in patients[37–39]. The majority of known BSEP mutations are missense mutations due to nucleotide exchanges within exons[9,12], which may affect pre-mRNA splicing[40] or result in a non-functional, misfolded, mistargeted or instable protein with increased turnover[30]. Furthermore, several nonsense and frameshift mutations of BSEP are known. These mutations implicate PTCs, which may entail NMD, a mechanism for mRNA quality control[20–22]. During mRNA

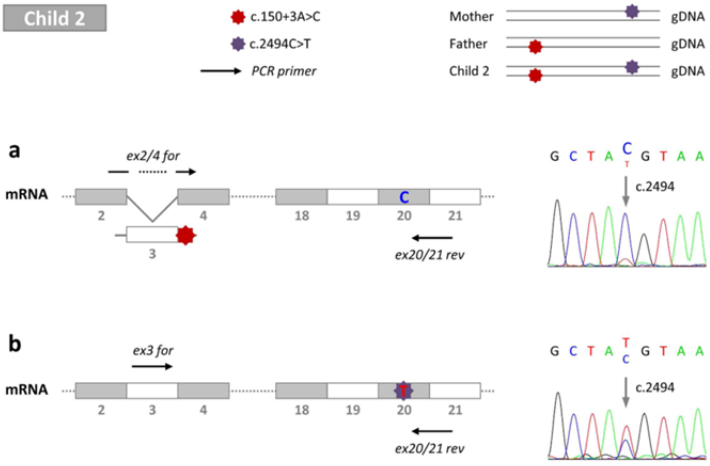

Figure 5. BSEP mRNA analysis of splicing extent due to c.150 + 3A > C in liver of child 2. The splice-site mutation c.150 + 3A > C on the paternal allele is shown in red. The missense mutation c.2494C > T (p.R832C) inherited by the mother is displayed in purple. RNA from the liver biopsy was used for reverse transcription. (**a**,**b**) PCR forward primers *ex2/4_for* and *ex3_for* were combined with *ex20/21_rev*. Exon 20 was sequenced from both PCR products. Appearance of cytosine (C) or thymine (T) at position 2494 indicates the extent of exon-skipping caused by c.150 + 3A > C. (**a**) When primer *ex2/4_for* was used and hence exon 3 was absent, the main sequencing peak at c.2494 results from C. (**b**) In contrast, presence of exon 3 is associated with T to larger and C to lower amounts at that position. Occurrence of both alleles using *ex3_for* confirms that c.150 + 3A > C causes only partial exon-skipping in the patients' liver tissues. for: forward primer, gDNA: genomic DNA, mRNA: messenger RNA, rev: reverse primer.

maturation, introns are excised from pre-mRNA and exon/exon borders are tagged by exon junction complexes (EJCs). These complexes are normally removed by the ribosome during the first translation cycle. A PTC stops the ribosome so that downstream EJCs persist. A remaining EJC triggers the recruitment of a termination complex of surveillance factors, which finally initiate mRNA degradation by exoribonucleases[20]. A prerequisite for NMD to occur is the presence of at least one retained EJC downstream to the PTC[41]. This is fulfilled in child 1 with BSEP c.2783_2787dup5. The PTC arises in exon 22 (c.3022–3024) upstream of five exon/exon borders with remaining EJCs. The duplication of GAGAT is detectable on gDNA but not on mRNA level from the same liver sample of child 1 (Fig. 3). Thus, it can be assumed that the corresponding mRNA transcripts are effectively removed by NMD. Moreover, absent BSEP protein expression is reported for a patient homozygous for c.2783_2787dup5[42]. Child 1 has been described earlier by Noe *et al.*[43], and the mutations were referred as intron 4(+3)A > C for c.150 + 3A > C and K930X for p.K930Efs79X. Interestingly, in a liver biopsy taken from the patient at the age of 20 months BSEP was not detectable by immunohistochemical staining[43]. Our immunofluorescent staining of the explanted liver obtained at the age of 3 years showed a detectable but reduced canalicular BSEP expression (Fig. 6)[44]. The discrepancy of BSEP staining in this patient's liver tissue may have different reasons. It is known that the expression of hepatobiliary transporters increases during development[45] with lower expression of BSEP, MRP2, MDR3 and FIC1 during the fetal period. Therefore, immunoreactivity may have exceeded a threshold at 3 years (this study) as compared to 20 months[43]. In both studies, BSEP antibodies directed against the same C-terminal epitope were used[46], however, they were raised in two unrelated rabbits resulting in different affinities. Lastly, the sensitivity of immunohistochemistry (Noe *et al.*[43]) as compared to immunofluorescence (this study) differs to some extent and may also influence the detection limit. Finally, canalicular BSEP protein immunoreactivity in child 1 is in line with the detection of correct BSEP mRNA transcripts containing exon 3 but not the duplication. Exon 3 consists of 52 bp, therefore skipping of this exon results in a frameshift with the first PTC at codon position 44. Nevertheless, mRNA transcripts with skipped exon 3 were detectable (Figs 3 and 4). Not only mRNAs with a PTC in the last exon are insensible to NMD because of a lacking downstream EJC, but also mRNAs containing a PTC close to the translation initiation site may escape NMD, when an alternative start codon is present[47,48]. Indeed, an alternative in-frame start codon is found at codon 62 of BSEP wildtype cDNA. Initiation of translation at this position would allow the ribosome to translate the sequence between exon 4 and 27 (resulting in a protein with 1260 amino acids), removing all EJCs and thereby protecting BSEP mRNA from NMD. Splice-site variants also have an impact on mRNA processing. About 6–7% of more than 380 known *ABCB11* variants (affecting exons or the adjacent 15 intronic nucleotides) are intronic splice-site variants (Table 1). Most of them concern the terminal intronic nucleotides (+1/+2 or −1/−2). These core or obligate nucleotides are essential for splice-site recognition[15,49] explaining the detrimental effects of these *ABCB11* splice-site mutations.

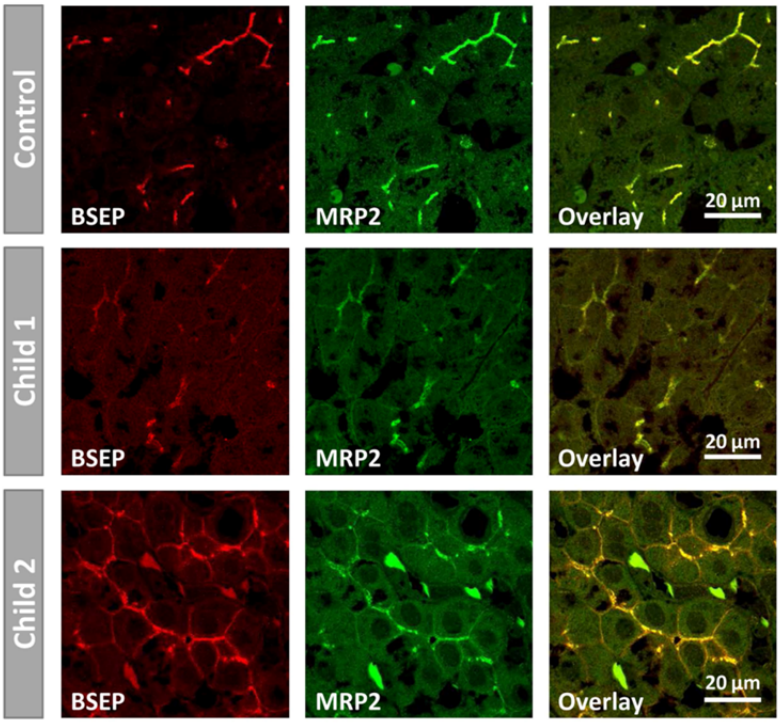

Figure 6. Detection of BSEP and MRP2 in human liver by immunofluorescence. BSEP and MRP2 are clearly stainable at the canalicular membrane of hepatocytes in normal human liver tissue (control). Both proteins are also detectable in liver tissue of child 1 and 2. For child 1, BSEP and MRP2 expression in the explanted liver is detectable to a lesser extent and slightly inhomogeneous as compared to control tissue. For child 2, both proteins show a distinct staining pattern. Additionally, a weak signal for BSEP and MRP2 at the basolateral membrane was observed in hepatocytes of child 2.

ABCB11 mutations affecting	donor splice-site		acceptor splice-site	
obligate dinucleotides	c.611 + 1G > A	c.2178 + 1G > T	c.77-1G > C	c.2179-2A > G
	c.908 + 1delG	c.2343 + 1G > T	c.99-1G > T	c.2611-2A > T
	c.908 + 1G > A	c.2343 + 2T > C	c.390-1G > A	c.3057-2A > G
	c.908 + 1G > T	c.3213 + 1delG	c.1639-1A > G*	
	c.2178 + 1G > A		c.2012-2A > G*	
distal positions	c.76 + 3G > T	c.3213 + 4A > G	c.77-7C > A#	c.2012-8T > G
	c.150 + 3A > C	c.3213 + 5G > A	c.1435-13_-8del	

Table 1. Clinical relevant *ABCB11* mutations affecting intronic splice-site consensus sequences. For *ABCB11*, 17 intronic mutations concerning the obligate dinucleotides (+1/+2 and −1/−2) are known. Additionally, seven intronic variants located in vicinity to exon/intron borders (+/−10) are described. New mutations recently identified in patients analysed in Düsseldorf are marked with an asterisk (*), others are referenced in[12], # is a variant of child 1.

Within this work, we present a detailed characterisation of the more distally located donor splice-site mutation c.150 + 3A > C associated with a PFIC-2 phenotype making use of RNA analysis obtained from the patients' liver samples. We found this variant in two out of 140 samples of children with a suspected BSEP deficiency analysed in our laboratory. In this highly selected cohort, the allele frequency is 0.7% (2/2 × 140). Otherwise, its allele frequency is most likely <0.2%, because there is no positive hit for this variant in more than 6000 sequences which

are available on Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) [February 2016 accessed]. While *in silico* analyses as well as the minigene assay suggests a complete exon-skipping, mRNA data from the patients' liver tissues clearly demonstrate that exon-skipping is only partial in the presence of c.150 + 3A > C. Therefore, results obtained from minigene constructs as used in our study, by Byrne *et al.*[40] or by van der Woerd *et al.*[50] should be interpreted carefully because they do not fully represent the situation in human tissue. Taking into account that in child 1 BSEP mRNA transcripts from the paternal allele are completely removed by NMD, BSEP mRNA entirely originates from the maternal allele with c.150 + 3A > C. Analysis of the overlapping sequences of exon 2 and 3 revealed an amount of 63% transcripts lacking exon 3 (Fig. 4), in other words, one third of available BSEP mRNA is correctly spliced despite c.150 + 3A > C. While Byrne *et al.* demonstrated the impact of exonic variants of *ABCB11* on mRNA processing[40], our data show that interference of splice-site variants with mRNA abundance may be variable. Our finding of partial exon-skipping is important in view of potential therapies, since modulation of splice processes represents a therapeutic approach for some genetic diseases. Analyses with modified U1 small nuclear RNA adapted to mutated donor splice-site sequences of *ATP8B1* were recently described to increase the amount of correctly spliced products *in vitro*[50]. Such approaches require detailed knowledge of the effects of individual mutations on splicing[51,52]. For example, glucocorticoids have been described to interfere with transcription and mRNA processing on different levels, including alternative promoter usage[53], induction of shortened protein variants[54] or inhibition of pre-mRNA splicing[55]. Interestingly, child 2 of our study was successfully treated by glucocorticoids[16]. His second mutation p.R832C is clearly associated to PFIC-2[9]. Although it is under suspicion to cause aberrant splicing[40], normal protein expression of $BSEP^{R832C}$ was observed[9], and loss of function is the likely reason for its severity. Although the beneficial effect of glucocorticoids may be due to a recovered transport activity of $BSEP^{R832C}$, it must be considered that glucocorticoids improve splicing efficiency of $BSEP^{150+3A>C}$. In conclusion, different mechanisms of defective BSEP mRNA processing cause BSEP deficiency; their recognition provide individual therapy targets.

Patients, Materials and Methods

Patients. The study was performed according to the guidelines of the Declaration of Helsinki and written informed consent from each patient (or parents) was obtained. Research on hepatobiliary transporters in human tissue or blood samples as presented in this study is approved by the ethical review committee of the Medical Faculty of the Heinrich Heine University Düsseldorf (approval number 2875).

Genomic DNA sequencing. Whole blood was used for genomic DNA (gDNA) extraction using MagNA Pure LC 2.0 DNA Isolation Kit I (Roche, Mannheim, Germany). gDNA from formalin-fixed paraffin-embedded (FFPE) liver tissue was isolated using EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) after pre-treatment of 10 µm slices with xylene and ethanol. *ABCB11* coding exons with flanking intron regions were amplified by polymerase chain reaction (PCR; primer: Supplementary Table S2), and sequenced[6,7]. Reference sequence was NM_003742.2 (Gene ID: 8647, Entrez Gene). Genetic variants were termed according to the rules of the Human Genome Variation Society[56]. First *ABCB11* coding exon is counted as exon 1, adenine of ATG is denoted as c.1, IVS4 indicates intervening sequence among coding exons 3 and 4.

Minigene construct preparation. gDNA was isolated from whole blood of a healthy person. *ABCB11* exon 3 and additional intronic 800 bp 5′ and 500 bp 3′ were amplified by PCR using *Taq* Polymerase (Qiagen). TOPO TA cloning and transformation into TOP10 *Escherichia coli* (Invitrogen, Carlsbad, CA, USA) were performed. Plasmid DNA was isolated from appropriate clones using HiSpeed Plasmid Purification Maxi Kit (Qiagen). The human immunodeficiency virus (HIV)1-based long terminal repeat heterologous splicing reporter (pHSR) has been described in Betz *et al.*[18]. Purified TOPO plasmid containing BSEP exon 3 and minigene pHSR were cut with *Eco*RI (NEB, Ipswich, MA, USA). Linearised dephosphorylated minigene (~4.5 kB) and BSEP exon 3 with intronic sequences (~1.3 kB) were ligated using T4 DNA Ligase (Promega, Mannheim, Germany). An eligible clone of minigene containing BSEP exon 3 with a wildtype (WT) donor splice-site (pHSR-BSEP_ex3_WT) was extended and used for mutagenesis (QuikChange Multi Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA). Final constructs pHSR-BSEP_ex3_WT and pHSR-BSEP_ex3_c.150 + 3A > C were verified by sequencing.

Cell culture and transfection. Hepatocellular carcinoma (Hep)G2 cells[57] were cultivated in DMEM Ham's F12 (Biochrom, Berlin, Germany) supplemented with 10% (v/v) FCS (PAA, Coelbe, Germany) at 37 °C in 5% CO_2. Cells were transfected 24 h after seeding in 6-well plates with 1 µg DNA of pHSR, pHSR-BSEP_ex3_WT or pHSR-BSEP_ex3_c.150 + 3A > C, using X-tremeGENE HP DNA transfection reagent (Roche).

Splicing analysis using a minigene assay. 48 h after transient transfection, total RNA was extracted from cells via the Maxwell16 system (LEV simplyRNA Tissue Kit, Promega). RNA was transcribed into complementary DNA (cDNA) using QuantiTect Reverse Transcription Kit (Qiagen). 3 µl of each cDNA were amplified by Phusion Polymerase (Thermo Fisher, Waltham, MA, USA) with primers surrounding the exon of interest insertion site (Supplementary Table S2). PCR products were analysed by agarose gel electrophoresis and proven by sequencing of extracted DNA. Gel was containing 1.5% agarose in Tris-acetate-EDTA buffer. Smart Ladder (Eurogentec, Kaneka Corporation, Osaka, Japan) was used as a marker.

Splicing analysis in human liver tissue. Liver tissue of both patients and controls was used for splicing analysis. For child 1, RNA was extracted from FFPE explanted liver tissue using LEV Blood DNA Kit with additional buffers (Promega). Two 10 µm slices were incubated in 200 µl RNA incubation buffer at 80 °C for 10 min and afterwards at 56 °C for 45 min. After adding 500 µl RNA lysis buffer, samples were processed in the Maxwell16 system (Promega). RNA was isolated from a snap frozen liver biopsy of child 2 and snap frozen

normal liver specimens of 14 patients who underwent surgery because of liver metastasis. cDNA synthesis was performed with GoScript Reverse Transcription System (Promega) at 50 °C using primers *ex0_for* and *ex23/24_rev* (Supplementary Table S2). Subsequent PCR reactions were conducted differing in their forward primers: (1) *ex0/1_for*, (2) *ex2/4_for*, and (3) *ex3_for*, each in combination with reverse primer *ex22/23_rev* for child 1 or *ex20/21_rev* for child 2 and controls. Samples of PCR (1) were sequenced with *ex0/1_for* and *ex4/5_rev*. BSEP mRNA exons 1 to 4 were analysed by reverse sequencing because the readout of forward sequencing was limited by an alternative coding exon 2 of isoform BSEP-B[23]. In PCR (2) *ex2/4_for* spans the junction of exon 2 and 4 and attaches to the template only when exon 3 is skipped. In (3) *ex3_for* just binds in the presence of exon 3. PCR products of (2) and (3) were sequenced with *ex18_for* and *ex22/23_rev* (child 1) or *ex20/21_rev* (child 2) to carry out the allelic assignment by the second mutation.

Immunofluorescence of liver tissue. Immunofluorescence was performed according to standard protocols[58,59] using Advalytix Slide Booster (Implen, Munich, Germany) for antibody incubation. A polyclonal rabbit antibody was raised against 13 C-terminal amino acids of BSEP compliant with Noe *et al.*[46]. A mix of monoclonal mouse antibodies for multidrug resistance-associated protein 2 (MRP2; M2I-4 and M2III-6, 1:25, Enzo Life Sciences, Lörrach, Germany)[59] and BSEP antibody (K24, 1:25) were applied for 2 h at 29 °C. Tissue samples were incubated with secondary antibodies conjugated to Alexa Flour 488 (goat anti mouse, A11029, green) or 546 (goat anti rabbit, A11035, red) (1:250, Invitrogen) for 1 h at 27 °C. Specimens were analysed with a LSM510 confocal laser scanning microscope (Zeiss, Jena, Germany).

Statistical analysis. To quantify the ratio of exon 3 inclusion and exclusion, sequencing traces for each base were separated by CodonCode Aligner (V.4.2.5, CodonCode Corporation, Dedham, MA, USA). The area underneath each peak within the overlap was measured using ImageJ[60] and calculated as a fraction of the combined signal at each position. Values were assigned to exon 2 or 3 and averaged. For detailed proceeding see Supplementary Fig. S2. Paired student's t-test was used with $p < 0.0001$ considered as significantly different.

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Author Contributions

C.D. and R.K. designed the experiments and wrote the manuscript; C.D. performed the experiments; C.D., H.S. and R.K. analysed the data; G.E. and D.W. treated the patients and provided patient data; all authors critically revised the manuscript.

Additional Information

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Supplementary Information for:

Exon-skipping and mRNA decay in human liver tissue: molecular consequences of pathogenic bile salt export pump mutations

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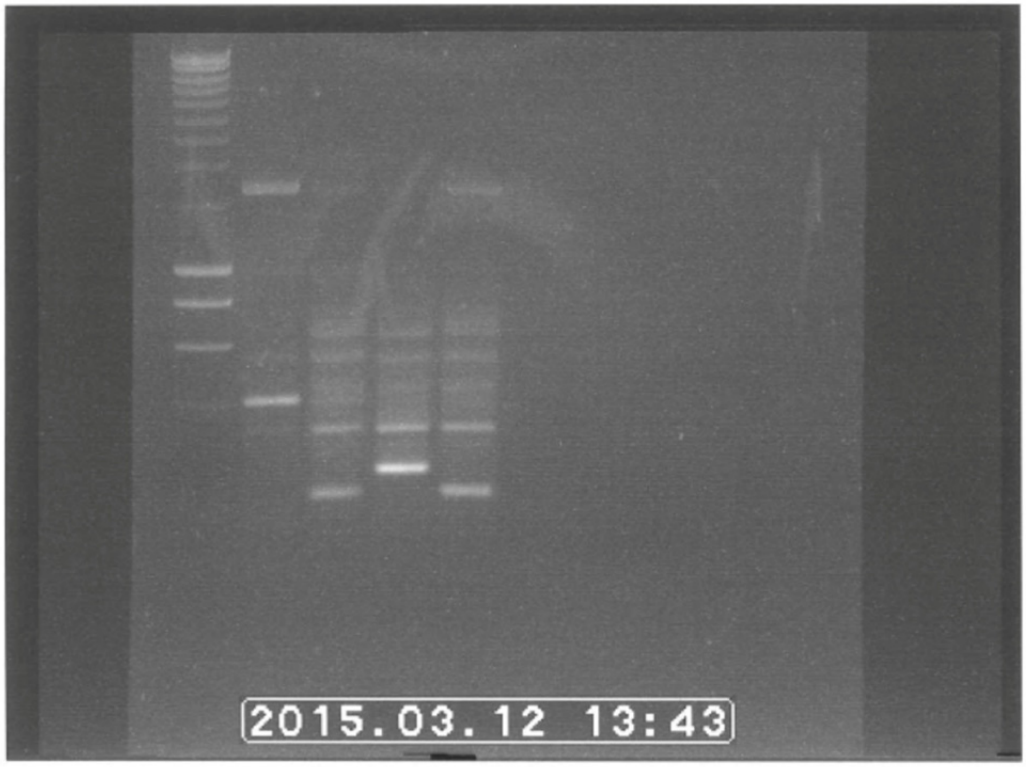
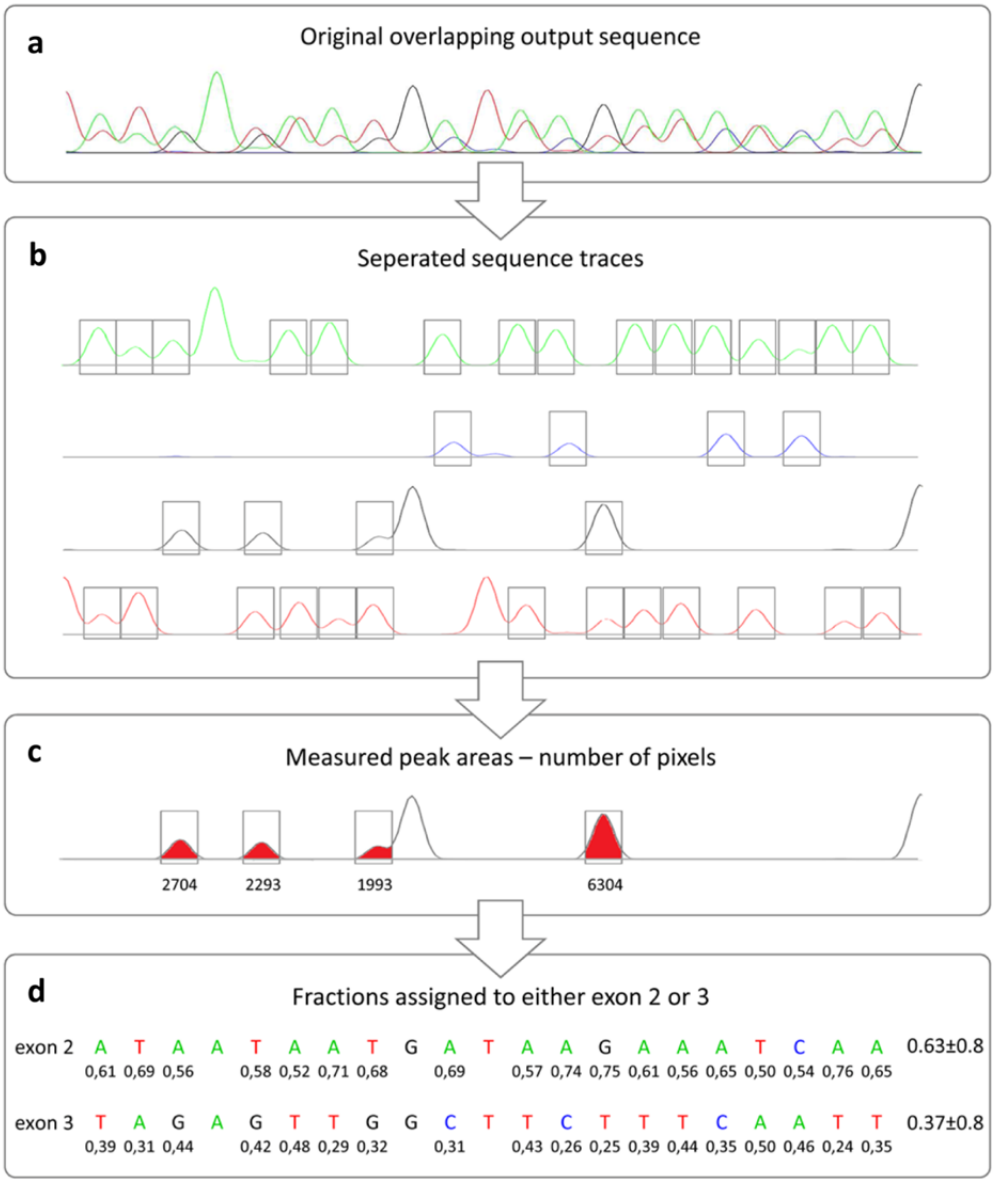
Supplementary Fig. S1

Splicing analysis of c.150+3A>C by a minigene assay (Original uncropped gel).



Supplementary Fig. S2

Determining relative amounts of mRNA transcripts with or without exon 3.



The method is elucidated based on the overlapping sequence from mRNA analysis of child 1. **a** Original sequencing output consisted of overlapping peaks. **b** Sequencing traces were separated for each base using CodonCode Aligner. Single peaks were encircled with boxes of the same width. **c** Areas underneath each peak (marked in red) were quantified by ImageJ, given as the number of pixel. **d** Values of area measurements were calculated as a fraction of the combined signal at each nucleotide position and assigned to exon 2 or 3. Mean values and standard deviation for signals related to exon 2 or 3 were calculated.

Supplementary Table S1

ABCB11 variants detected in two PFIC-2 patients.

Child 1					
exonic variants	**nucleotide change (c.)**	**protein effect (p.)**	**reference SNP cluster ID**	**zygosity**	**shown RNA effect**
coding ex 12	1331C>T	V444A	rs2287622	heterozygous	
coding ex 21	2783_2787dup5	K930Efs79X	-	heterozygous	mRNA degradation (NMD)
coding ex 23	3084A>G	A1028A	rs497692	homozygous	
intronic variants	**nucleotide change (c.)**		**reference SNP cluster ID**	**zygosity**	**shown RNA effect**
IVS2	77-7C>A		-	heterozygous	
IVS4	150+3A>C		rs387906354	heterozygous	exon skipping
IVS9	909-15A>G		rs2287618	heterozygous	
IVS13	1434+70C>T		rs2287623	heterozygous	
IVS14	1638+32T>C		rs2241340	heterozygous	
IVS18	2179-17C>A		rs853772	homozygous	

Child 2					
exonic variants	**nucleotide change (c.)**	**protein effect (p.)**	**reference SNP cluster ID**	**zygosity**	**shown RNA effect**
coding ex 12	1331C>T	V444A	rs2287622	heterozygous	
coding ex 20	2494C>T	R832C	-	heterozygous	
coding ex 23	3084A>G	A1028A	rs497692	heterozygous	
intronic variants	**nucleotide change (c.)**		**reference SNP cluster ID**	**zygosity**	**shown RNA effect**
IVS4	150+3A>C		rs387906354	heterozygous	exon skipping
IVS9	909-15A>G		rs2287618	heterozygous	
IVS13	1434+70C>T		rs2287623	heterozygous	
IVS18	2179-17C>A		rs853772	heterozygous	
IVS19	2344-17T>C		rs853789	heterozygous	

Supplementary Table S2

Primers used to characterise the relevant *ABCB11* (BSEP) mutations of two PFIC-2 patients.

gDNA analyses from blood	**primer: 5' → 3'**
seq_ex3_for	TCACCACCTAGGGAGAATTTC
seq_ex3_rev	AGTTATTCAGCATGTTATCAC
seq_ex20_for	CAGTAAGTGATTTAGTTATTTTTC
seq_ex20_rev	CACTGGTCCCTATTCCATAG
seq_ex21_for	TGAATTGTACACTTAAGAATG
seq_ex21_rev	GGCTGACAGCTTCCTTCAGTC
gDNA analyses from liver	**primer: 5' → 3'**
seq_liv_ex3_for	ATCACCACCTAGGGAGAATTTCCC
seq_liv_ex3_rev	TGCCAATATGACTAAAGATTTAACACTCCC
seq_liv_ex21_for	TGTCTGAGACGGGTTGATTGC
seq_liv_ex21_rev	TACTCGTTTTTAACAGTTTGTCTGATAGCC
minigene assay	**primer: 5' → 3'**
mini_ex3_for	ACTTCGAACTTTCCTAGATAGAGTAGGG
mini_ex3_rev	GTTTCGAATAATTGAGTGGCAGAGTTCG
mut_c.150+3A>C	AGAGTTGGCTTCTTTCAATTGGTCATAAAACTGTGTTGATTTATGGC
pHSR_ex1_for	CCGAACAGGGACTTGAAAGCG
pHSR_ex2_rev	TGCAGGAACAAGCAAGGCC
mRNA analyses from liver	**primer: 5' → 3'**
ex0_for	AACTCTCCACAGTGGAGTCC
ex23/24_rev	CCCCTGGAAGTTGTCCCATTT
ex0/1_for	TCACAGGGTCGTTGGCTGTGGGTTGC
ex2/4_for	TGATGGTTTTGAGTCAGATAAATCATATAATAATGATAAGAAATCAAGTTTCGG
ex3_for	AGATGAGAAGAAAGGTGATGGCGTTAGAGTTGGC
ex22/23_rev	AACTGCAGAGATCACCCTGAACACATAGC
ex20/21_rev	ATCTGAGAGCCGGCAGCCCCTTGAACTTGG
ex4/5_rev	TCTCGATGTTCAGCAACCCAC
ex18_for	AACGGGACAGTCACACC

3.6 A novel mutation within a transmembrane helix of the bile salt export pump (BSEP; *ABCB11*) with delayed development of cirrhosis

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CIRRHOSIS AND LIVER FAILURE

A novel mutation within a transmembrane helix of the bile salt export pump (BSEP, *ABCB11*) with delayed development of cirrhosis

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Keywords
ABC transporter – *ABCB11* – benign recurrent intrahepatic cholestasis – bile salt export pump – BRIC – BSEP – PFIC – progressive familial intrahepatic cholestasis

Abbreviations
BRIC, benign recurrent intrahepatic cholestasis; BSEP, bile salt export pump (*ABCB11*); CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; FIC1, familial intrahepatic cholestasis 1 gene product (*ATP8B1*); LCA, lithocholic acid; MDR3, multidrug resistance protein 3 (*ABCB4*); PFIC, progressive familial intrahepatic cholestasis; TCDC, taurochenodeoxycholate; TC, taurocholate; UDCA, ursodeoxycholic acid.

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Abstract

Background & Aims: The bile salt export pump (BSEP, *ABCB11*) is essential for bile salt secretion at the canalicular membrane of liver cells. Clinical phenotypes associated with BSEP mutations are commonly categorized as benign recurrent intrahepatic cholestasis (BRIC-2) or progressive familial intrahepatic cholestasis (PFIC-2). *Methods:* The molecular basis of BSEP-associated liver disease in a sibling pair was characterized by immunostaining, gene sequencing, bile salt analysis and recombinant expression in mammalian cells and yeast for localization and *in vitro* activity studies respectively. *Results:* Benign recurrent intrahepatic cholestasis was considered in a brother and sister who both suffered from intermittent cholestasis since childhood. Gene sequencing of *ABCB11* identified the novel missense mutation p.G374S, which is localized in the putative sixth transmembrane helix of BSEP. Liver fibrosis was present in the brother at the age of 18 with progression to cirrhosis within 3 years. Immunofluorescence of liver tissue showed clear canalicular BSEP expression; however, biliary concentration of bile salts was drastically reduced. In line with these *in vivo* findings, HEK293 cells showed regular membrane targeting of human $BSEP^{G374S}$, whereas *in vitro* transport measurements revealed a strongly reduced transport activity. *Conclusions:* The novel mutation p.G374S impairs transport function without disabling membrane localization of BSEP. While all other known BSEP mutations within transmembrane helices are associated with PFIC-2, the new p.G374S mutation causes a transitional phenotype between BRIC-2 and PFIC-2.

Bile salt export pump (BSEP) is the major canalicular bile salt transporter in humans and is responsible for the bile salt-dependent bile flow. It mainly transports monovalent bile salts including taurine and glycine conjugates of primary bile acids (cholic and chenodeoxycholic acid) as well as secondary bile acids (deoxycholic acid) and ursodeoxycholic acid (1). 'Severe' mutations of BSEP cause progressive familial intra hepatic cholestasis (PFIC) type 2 (2). PFIC-2 is similar in presentation to PFIC-1, which is caused by mutations of the *ATP8B1* gene (3). *ATP8B1* encodes the 'familial

All authors contributed to the final manuscript.

intrahepatic cholestasis 1′ protein (FIC1), a P-type ATPase involved in the flipping of aminophospholipids from the outer to the inner leaflet of the canalicular membrane (4). Some mutations of *ATP8B1* and *ABCB11* cause milder forms of cholestatic liver diseases, which are termed benign recurrent intrahepatic cholestasis (BRIC) type 1 and 2 (5–7), respectively. Here, we report on a novel BSEP mutation identified in a sibling pair that is initially associated with a typical BRIC-2 phenotype with intermittent cholestasis, but eventually progresses towards cirrhosis. We attribute its severity to its localization close to the putative translocation pore within one of the transmembrane alpha helices which commonly harbour PFIC-2 mutations.

Materials and methods

Analysis of serum bile salts

Bile salt analysis was performed by tandem mass spectroscopy. The system consisted of an HPLC Waters Alliance 2795 separation module (Waters, Milford, UK) coupled to a Quattro Micro triple quadrupole mass spectrometer (Micro Mass, Manchester, UK). Electrospray ionization was performed in the negative ionization mode. Chromatographic separation was performed on an analytical HPLC Phenomenex Luna C18 column coupled to guard column Phenomenex Gemini C18 (Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of water containing 0.01% formic acid and 5 mM ammonium acetate (Eluent A) and methanol (Eluent B). Sample elution was run isocratically in a ratio of 18:82 (A:B, v/v) over 15 min at a flow rate of 0.2 ml/min. Unconjugated bile acids were detected unfragmented. Taurine- and glycine-conjugated bile salts were analyzed using their specific fragment ions at mass-to-charge (*m/z*) 80 and 74. Analytes were detected in the multiple reaction mode. Quantification analysis was performed using standard calibration curves in five different concentrations (0.1, 0.5, 1.0, 2.5, 5.0 μg/ml) including the deuterated internal standard (d4–CA). Concentrations were calculated using the QuanLynx (MassLynx 4.1; Waters) software. A solid phase extraction (Chromabond HR-X cartridge; Macherey-Nagel, Düren, Germany) procedure was applied for serum samples prior to HPLC-MS/MS analysis. Normal bile samples were collected during surgery from patients who underwent partial liver resection for hepatic metastasis because of colorectal or breast cancer and whose livers were otherwise healthy. Their bile ducts were canulated before routine removal of the gall bladder.

Immunofluorescence of liver tissue and transfected cells

Immunofluorescence staining and microscopy were performed as described recently (7, 8) and by the use of the transporter-specific antibodies K24 for BSEP (9), P3II26 for MDR3 (Thermo Scientific, Schwerte, Germany) and A276 for the Na^+/K^+-ATPase (clone M7-PB-E9; Sigma-Aldrich, Munich, Germany).

Cloning and mutagenesis of human BSEP

For large-scale expression in the methylotrophic yeast *Pichia pastoris*, the BSEP coding sequence was PCR-amplified from the mammalian expression vector pEYFP-N1-hBSEP (10) using primers BSEP-HR-PP-S1 (5′-ATCAAAAAACAACTAATTATTCGAACGAGGTAAAA GAATGTCTGACTCAGTAATTCTTCGAAGTATA-3′) and BSEP-HR-PP-S2 (5′-ACGTTTGGACCTTGGAAAAGACT TCTAAGGAGTTGGAGGCACTGATGGGGGATCCAGT GGTGACTAGTTT-3′). The amplified coding sequence was cloned into a yeast-compatible derivative of the custom *P. pastoris* expression vector pSGP18 (11) (kindly provided by M. Dumont, Rochester) via homologous recombination as described in Stindt *et al.* (12). The integrity of the coding sequence on the resulting plasmid pSGP18-2μ-$BSEP^{WT}$ was verified by DNA sequencing of the entire gene. In addition to the removal of the cryptic prokaryotic promoter motif (a TATA box motif was removed by the silent modifications c.78T>C and c.81T>C), four silent nucleotide exchanges were found to be present with respect to the BSEP reference sequence NM_003742.2, with the first nucleotide of the start codon being numbered as 1: c.2064G>A, c.2559C>T, c.3084A>G, and c.3390T>C as described (10).

For transfection experiments, a yeast-compatible version (pEYFP-N1-OriLeu-BSEP) of the mammalian expression vector pEYFP-N1-hBSEP was generated as described recently (12). The mutation c.1120G>A (p.G374S) was introduced into the two plasmids pSGP18-2μ-BSEP and pEYFP-N1-OriLeu-BSEP by DREAM mutagenesis (12). The mutagenesis primers used were BSEP-G374S-HR-S1 (5′-CTTTAAATCTT AGCAATGCCTCTCCTTGTTTGGAAGCCTTTGCAAC T-3′) and BSEP-G374S-HR-S2 (5′-AGGCATTGCTAA GATTTAAAGCTCCTACTATGACACTGAGGAAAATC TG-3′). The plasmid pEYFP-N1-OriLeu-BSEP containing the p.G374S variant was found to have one additional silent nucleotide exchange with respect to the parent construct (c.1246G>A).

Expression of human BSEP in *P. pastoris*

Electrocompetent *P. pastoris* strain X33 cells (Invitrogen, Karlsruhe, Germany) were transformed with the linearized pSGP18-2μ-$BSEP^{WT}$, pSGP18-2μ-$BSEP^{G374S}$, or the empty pSGP18-2μ construct according to the manufacturer's instructions. Positive clones were selected on YPDS plates containing 100 μg/ml Zeocin. Small-scale expression screens of the obtained *P. pastoris* clones were performed as described by Lerner-Marmorosh *et al.* (13). For large-scale expression, BSEP-expressing clones were fermented in a 15 litre table-top glass fermentor (Applikon Biotechnology,

Schiedam, the Netherlands) according to the Invitrogen *Pichia* fermentation guidelines. Protein expression was induced by constant methanol feeding for 48 h. Cells were then harvested by centrifugation at 4°C for 10 min at 5000 × g, snap-frozen in liquid nitrogen and stored at −80°C until further use.

Analysis of BSEP^{G374S} expression in mammalian cells

Wildtype BSEP-EYFP as well as BSEP^{G374S}-EYFP were transiently transfected into HEK293 (human embryonic kidney) cells by the use of X-treme Gene HP (Roche Applied Science, Mannheim, Germany) according to the manufacturer's guidelines. After transfection cells were grown for 48 h, fixed with methanol (−20°C, 30 s) and were immunostained for the Na^+/K^+-ATPase. The secondary antibody was goat anti-mouse-Cy3 (1:500; Dianova, Hamburg, Germany). Cells were visualized with a LSM 510 Meta confocal laser scanning microscope (Zeiss, Jena, Germany). The excitation wavelength was 488 nm for the enhanced yellow fluorescent protein (EYFP) and 543 nm for Cy3. Emission was detected by 505–530 nm (green) and 560–615 nm band pass filters (red).

Plasma membrane preparation from *P. pastoris* yeast cells

Plasma membranes of fermented *P. pastoris* cells were prepared as described for *Saccharomyces cerevisiae* by Ernst *et al.* (14). All steps were carried out at 4°C and in the presence of protease inhibitors (Complete™ without EDTA, Roche Applied Science). Briefly, approximately 30 g of respective *Pichia* cells were resuspended in 50 mM Tris-acetate, pH 7.5 and 0.2 mM EDTA and disrupted in a bead beater with acid-washed glass beads. Unbroken cells and cellular debris were removed by two centrifugation steps at 1000 × g and one step at 3000 × g for 5 min each. The supernatant was centrifuged for 45 min at 20 000 × g, while the membrane pellet was resuspended in 10 mM Tris-acetate, pH 7.5 and 0.2 mM EDTA. After determination of protein concentration with the Coomassie Plus Protein Assay reagent (Thermo Scientific), the suspension was adjusted to 5 mg/ml with the same buffer and mitochondrial membranes were precipitated by lowering the pH to 5.2 and removed by centrifugation at 7000 × g for 5 min. The supernatant was re-adjusted to pH 7.5 and plasma membranes were harvested by ultracentrifugation for 1 h at 140 000 × g. After resuspension in buffer A (50 mM Tris-HCl, pH 8.0, 75 mM NaCl, 30% glycerol) protein concentration was determined and membranes were snap-frozen in liquid nitrogen and stored at −80°C.

Western blotting analysis of BSEP

Five micrograms of *P. pastoris* plasma membrane proteins were separated on a 7% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (PALL, Dreieich, Germany). For comparative expression analysis of wildtype and mutant BSEP, HEK293 cells were transiently transfected with 10 µg of the appropriate BSEP expression plasmids. After 48 h, they were lysed with 1% SDS in PBS (4°C) and were incubated for 10 min at RT. To reduce viscosity, 0.5 µl of Benzonase (Merck, Darmstadt, Germany) were added per 100 µl of lysate. Ten micrograms of these lysates were used for SDS-PAGE and Western blotting. Membranes were probed with the monoclonal BSEP antibody F6 (Santa Cruz Biotechnology, Heidelberg, Germany).

^{3}[H]-taurocholate transport assays

Transport activity of human BSEP was measured as described (15). *Pichia* plasma membranes were thawed on ice, diluted four-fold in ice-cold buffer (50 mM Tris/HCl pH 7.5, 250 mM sucrose) and vesiculated by 20 passages through a 26 gauge needle. Twenty micrograms of this vesicle preparation were assayed in a reaction volume of 20 µl with 0.1 mg/ml rabbit muscle creatine kinase (Sigma-Aldrich), 10 mM creatine phosphate and 10 mM $MgCl_2$ as an ATP-regenerating system. After addition of ^{3}[H]-taurocholate (Perkin Elmer, Rodgau, Germany) or ^{3}[H]-taurochenodeoxycholate (Hartmann Analytic, Braunschweig, Germany) to a final concentration of 2 µM, reactions were allowed to equilibrate on ice for 5 min. Bile salt transport was then initiated by addition of ATP to a final concentration of 4 mM. Control reactions contained buffer instead of ATP. The reactions were then immediately shifted to 37°C for 5 min. Bile salt transport was stopped by addition of 1 ml of ice-cold buffer containing 1 mM of unlabelled taurocholate (TC), followed by rapid filtration through 0.2 µm cellulose membranes (type GSTF; Millipore, Schwalbach, Germany) pre-wetted with the same buffer. After filtration, the membranes were immediately washed twice with 5 ml of the same ice-cold buffer and once with 5 ml of ice-cold TC-free buffer. The radioactivity retained on the filter discs was counted in a liquid scintillation counter (Packard Instruments, Frankfurt, Germany) using Ultima Gold liquid scintillation cocktail (Canberra Packard, Frankfurt, Germany).

Case description and results

A 22-year-old woman (=sister) of Moroccan origin presented with intermittent jaundice for several years and recurrent pruritus of increasing intensity for 5 months. Her body mass index was 12.5 kg/m^2. Erythematous macules on the back and abdomen with discrete desquamation accompanied by generalized xerosis cutis were observed. Alanine aminotransferase was mildly elevated with 64 U/L (normal: <45 U/L), alkaline phosphatase was 132 U/L (<108 U/L), while aspartate aminotransferase and gamma-glutamyltransferase (γGT) were not increased. Because of the history

of recurrent jaundice, a hepatic cause of the pruritus was taken into account. While anti-nuclear antibodies were detected at a titre of 1:80, all other parameters related to liver diseases (anti-mitochondrial antibodies, anti-smooth muscle antigen antibodies, liver kidney microsomal antibodies, anti-neutrophil cytoplasmatic antibodies, coeruloplasmin, α1-antitrypsin, ferritin, serology for hepatitis B and C and porphyrins) were within their normal ranges.

On abdominal ultrasound the liver appeared normal. A single bile stone was detected. Liver stiffness was normal at 6.2 kPa as determined by Fibroscan™ (Echosens, Paris, France) (16). To control pruritus, the patient was topically treated with polidocanol-containing emollients and received narrow band ultraviolet B (UVB, 311 nm) therapy up to a dose of 0.26 J/cm^2 with limited improvement of symptoms. Finally, the patient received ursodeoxycholic acid (UDCA, 1000 mg/d) as well as cholestyramin (12 g/d). Relief of pruritus was only moderate at the beginning, but improved consecutively because of continued intake of UDCA.

Her brother experienced intermittent jaundice and pruritus since the first year of life and presented with increased serum bile salt levels. Increased levels of aminotransferases completely normalized between the pruritic episodes, whereas γGT levels were normal at all times, leading to the diagnosis of BRIC. Symptoms were moderately controlled by UDCA, which was taken sporadically during longer periods. He presented again at the age of 18 because of progressive jaundice. Bilirubin reached a level of 10.6 mg/dl (normal: <1 mg/dl). Liver stiffness was 11.6 kPa (Fibroscan™, normal range <7.5 kPa) suggesting the presence of fibrosis. In line with this, a liver biopsy showed portal fibrosis stage 1, ductopenia and cytokeratin 7 positive metaplasia of hepatocytes.

Three years later, symptoms deteriorated, liver stiffness was 25.7 kPa and incomplete cirrhosis, signs of chronic cholestasis, ductular proliferation and hepatocellular injury were detected in a subsequent liver biopsy. 4 months later, endoscopic retrograde cholangioscopy was performed because of progressive jaundice. Bile and serum were collected for analysis of bile salt profiles. At that point, liver stiffness was 39.1 kPa, indicating advanced cirrhosis (16, 17).

Gene sequencing of *ABCB11* reveals a novel BSEP mutation

Based on the history of recurrent pruritus with intermittent jaundice, the positive family history and consanguinity of the parents, an inherited form of cholestasis was considered. In view of low γGT levels despite high serum bile salt concentrations, mutations of the familial intrahepatic cholestasis 1 (FIC1) gene product (gene symbol: *ATP8B1*) or of the bile salt export pump (BSEP/*ABCB11*) were the most likely cause of cholestasis in both patients. Because bile stones are much more common in patients with BSEP mutations (6), the entire coding sequence and flanking intronic regions of *ABCB11* were sequenced (informed consent was obtained from all family members). In comparison with the reference sequence, NM_003742.2 nine homozygous genetic variants were detected in the siblings. All six intronic variants (c.77-17delA, c.99-18T>C, c.477+16G>A, c.909-17G>A, c.909-15A>G, c.3766-34G>A) were located at longer distances from

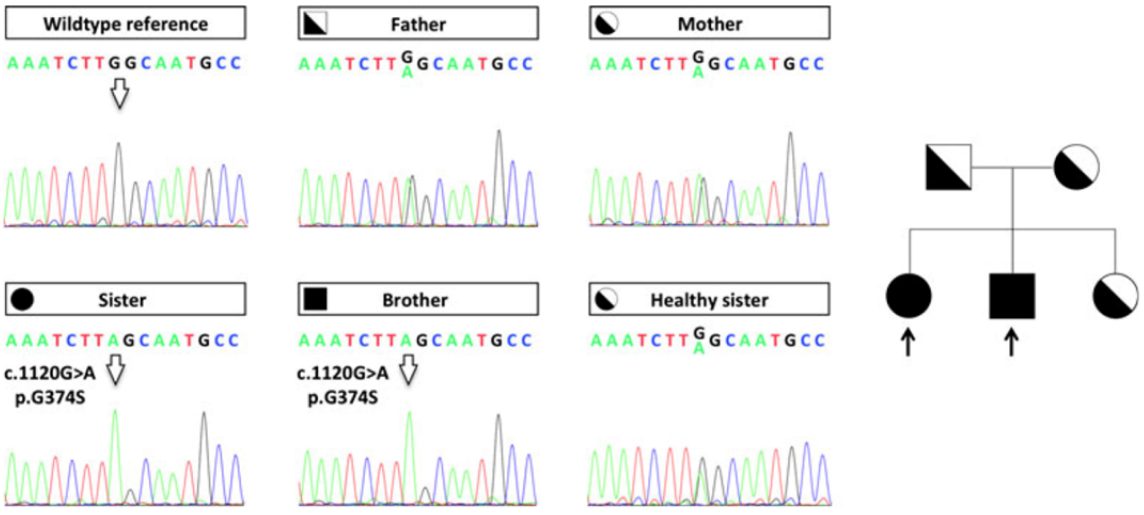

Fig. 1. Genetic analysis of *ABCB11*. Gene sequencing of bile salt export pump (BSEP, *ABCB11*) revealed a homozygous nucleotide exchange at position 1120 of the coding sequence from guanine to adenine (c.1120G>A) in the two patients (sister and brother), resulting in an amino acid change from glycine to serine at position 374 of the polypeptide sequence of BSEP (p.G374S). Both parents and the younger sister were heterozygous. The genotypes correlate with the phenotypes: heterozygous, phenotypically healthy family members are represented by half-filled symbols.

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splice sites. Furthermore, two synonymous exonic variants (c.402C>T; p.I134I and c.957A>G; p.G319G) were found. Most importantly, a homozygous genetic variant was detected at position 1120 at the level of coding DNA. Here, a guanine was replaced by an adenine (c.1120G>A), leading to an amino acid substitution from glycine to serine (p.Gly374Ser) at the protein level (Fig. 1). The parents, both of whom were symptom-free were heterozygous for this and all other variants. Likewise, the younger, healthy sister was heterozygous for p.G374S. In a control cohort of 6 Moroccans and of 148 unrelated persons p.G374S was not detected.

The p.G374S mutation does not impair BSEP trafficking to the plasma membrane

To investigate the pathophysiological relevance of this newly discovered mutation, a liver biopsy (taken at the age of 21) of the brother was stained with transporter-specific antibodies. The phospholipid floppase MDR3 (*ABCB4*) and the bile salt export pump BSEP were both detected at the canalicular membrane of hepatocytes as in healthy livers (Fig. 2A). To analyze intracellular trafficking of mutated BSEP, the mutation p.G374S was introduced into a plasmid encoding BSEP fused to the enhanced yellow fluorescent protein (EYFP) at the C-terminus. After transfection into HEK293 cells both the wildtype and the mutant protein were targeted to the plasma membrane (Fig. 2B) as compared with the Na^+/K^+-ATPase as a plasma membrane marker. Western blot analysis showed an equal expression level of wildtype and mutated BSEP in HEK293 cells (Fig. 2C). In line with the finding from the liver biopsy, these results clearly demonstrate that the amino acid exchange in $BSEP^{G374S}$ has no major impact on intracellular trafficking of $BSEP^{G374S}$ *in vivo* and *in vitro*.

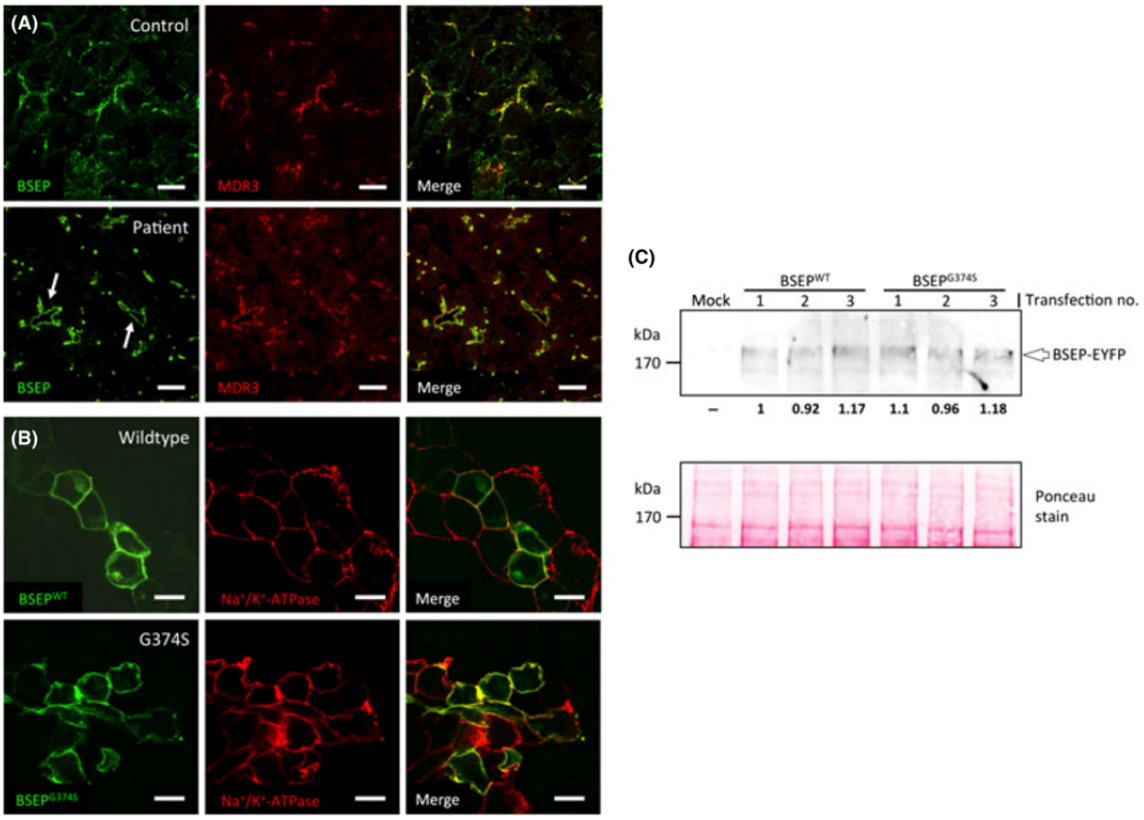

Fig. 2. $BSEP^{G374S}$ expression in the patient's liver and in cell culture. (A) Liver biopsy of the brother (21 years) and of a normal human liver (control) were immunostained for bile salt export pump (BSEP) (green) and the phospholipid floppase multidrug resistance protein 3 (MDR3) (red). Both proteins are localized at the canalicular membrane. Some canaliculi are considerably enlarged in the liver of the patient (arrows, affected brother; white bars = 20 μm). (B) Human BSEP was fused to yellow fluorescent protein (green). The mutation p.G374S was introduced by directed recombination-assisted mutagenesis (DREAM) (12). Transfection of human embryonic kidney (HEK293) cells showed targeting of $BSEP^{WT}$ as well as of $BSEP^{G374S}$ to the plasma membrane. Na^+/K^+-ATPase as a plasma membrane marker is shown in red (white bars = 10 μm). (C) Wildtype and mutated BSEP are equally expressed in HEK293 cells as indicated by densitometric analysis of three independent lysates of transfected HEK293 cells (for details see materials and methods). The Ponceau stain shows equal sample loading.

Bile salt analysis confirms disturbed bile salt secretion

Bile acids (CA, CDCA, DCA, UDCA and LCA) and their taurine and glycine conjugates were analyzed from sera of the sister, her brother and 40 controls as well as from the bile of the brother and seven persons with normal liver function by HPLC-MS/MS (high performance liquid chromatography coupled to tandem mass spectrometry). Although bilirubin concentration in the sister's serum was only 0.66 mg/dl (normal <1 mg/dl), serum bile salts were significantly elevated to 28.2 μM and in the brother's serum to 47.8 μM (normal: 1.4 ± 1.2 μM, n = 40). Relative amounts of derivatives of cholic (CA) and chenodeoxycholic acid (CDCA) were within normal ranges as compared with controls (Fig. 3). Deoxycholate, which is derived from CA by bacterial dehydroxylation within the large bowel, was slightly elevated in the sister's serum (0.82 μM; normal 0.27 ± 0.20 μM) but absent in the brother's serum. Absolute and relative amounts of unconjugated bile acids were decreased. Most notably, total bile salt concentration in the bile of the brother was as low as 0.061 mM as compared with 35.1 ± 18.0 mM in patients with healthy livers (n = 7). Thus, the brother's liver was unable to generate a sufficient bile salt gradient between serum and bile while showing a normal canalicular pattern of BSEP expression.

Bile salt transport by BSEP^{G374S} is strongly impaired

According to a homology model of BSEP, the mutation is located in the interior of the first transmembrane domain near the putative translocation channel (Fig. 4A–C). To analyze the functional consequence of this amino acid substitution, bile salt transport was measured in plasma membrane vesicles from *P. pastoris* expressing either BSEP^{WT} or BSEP^{G374S} (Fig. 4D). Vesicles from *P. pastoris* cells transformed with the empty vector (=control) showed unspecific binding of ^{3}[H]-TC or ^{3}[H]-TCDC; however, this was not significantly increased upon addition of ATP, excluding energy-dependent bile salt transport in these vesicles. When ATP was added to vesicles with BSEP^{WT}, ^{3}[H]-TC transport increased 1.65-fold (n = 3) compared with TC-transport in the absence of ATP. Likewise, transport of ^{3}[H]-TCDC increased 1.55-fold (n = 3) upon addition of ATP. ^{3}[H]-TC as well as ^{3}[H]-TCDC transport was strongly reduced in BSEP^{G374S}-containing vesicles (Fig. 4D). In summary, the data indicate that the amino

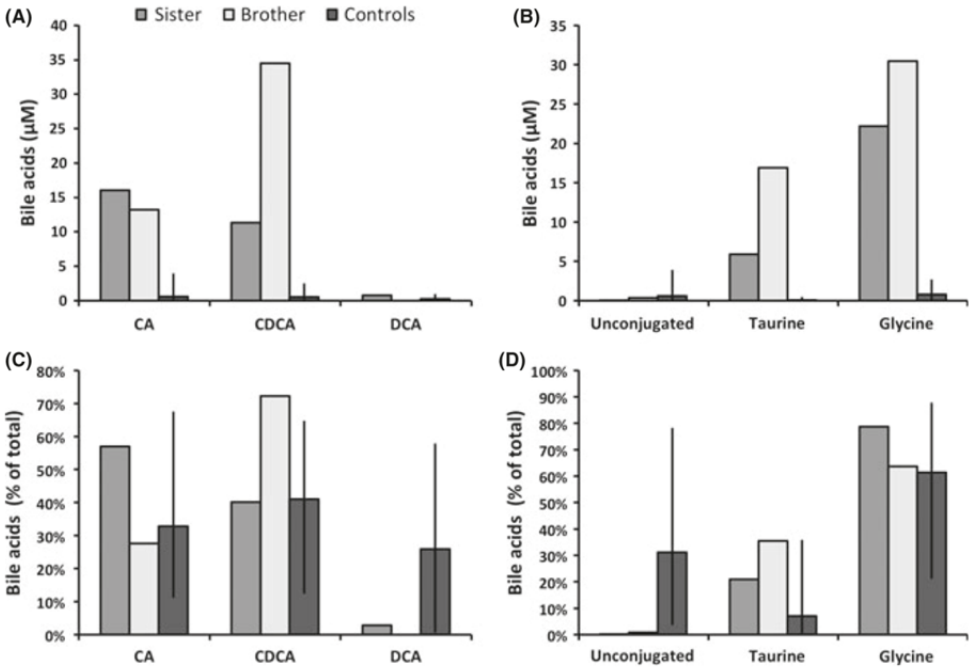

Fig. 3. Serum bile salt profiles of both patients. (A, B) The sister and brother had significantly elevated serum bile salt concentrations with absent deoxycholate (DCA)-derivatives in the brother's serum. (C) The relative amounts of the primary bile salts cholic acid (CA) and chenodeoxycholic acid (CDCA) were within the normal ranges. (B, D) Unconjugated bile acids were (almost) absent in their sera. Individual bile salt/acid concentrations were compared with measurements of 40 control persons (dark grey bars). Mean values are represented by bars; full ranges (minima til maxima) are represented by vertical lines.

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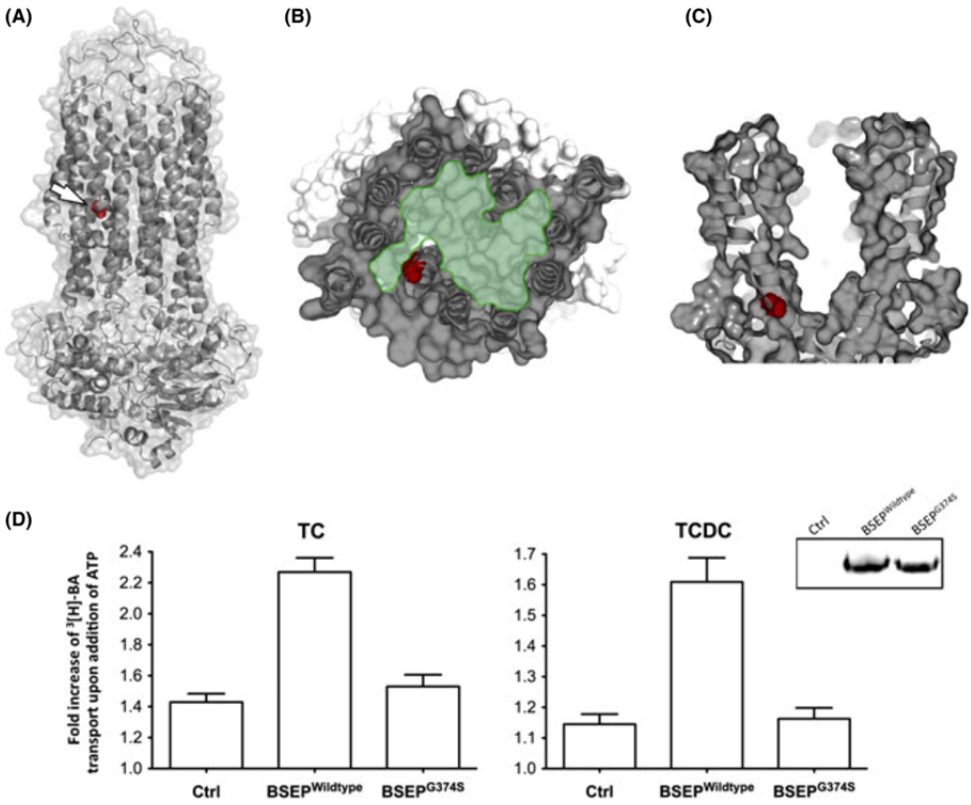

Fig. 4. Strongly reduced activity of BSEP^{G374S} and position of G374. (A-C) Based on the structure of the multidrug resistance protein (MDR) transporter Sav1866 (pdb entry: 2HYD) (26) a homology model of bile salt export pump (BSEP) was constructed. The position of G374 in the BSEP model is shown in red. (A) Side view (arrow indicates G374 position). G374 lies in the transmembrane part of the predicted sixth helix of the first transmembrane domain. (B) Top view on optical section (transporter channel in green). (C) Side view on optical section. (D) Transport of ^{3}[H]-labelled bile acids into yeast plasma membrane (PM) vesicles by BSEP^{G374S} is strongly reduced (mean and SEM, n = 3) as compared with BSEP^{WT}. Ctrl: PM vesicles without BSEP. Right inset: Western blot analysis of equal total PM protein amounts.

acid substitution p.G374S severely impairs bile salt transport by BSEP.

G374S is the first BRIC-associated mutation in a transmembrane helix of BSEP

To relate the p.G374S mutation to its localization within a transmembrane helix, all available missense mutations of BSEP were searched from databases and literature as reviewed recently (18). A total of 112 missense mutations related to PFIC-2 and 21 mutations associated with BRIC-2 were found. The HMMTOP 2.0 topology tool (19) predicts 12 putative transmembrane helices (TMH) for human BSEP. Interestingly, none of the previously identified 21 BRIC-2 mutations are localized within a TMH in this model, whereas 20 per cent (i.e., 22 of 112) of the PFIC-2 mutations affect the TMHs of BSEP (Fig. 5). Accordingly, p.G374S is the first BRIC-2-associated mutation that influences a transmembrane helix, a domain otherwise exclusively affected by PFIC-2 mutations.

Discussion

In this study, we describe the molecular basis of the novel BSEP mutation p.G374S, which was found in a pair of siblings. The initial clinical presentation of the siblings was consistent with a BRIC-2 phenotype. In particular, the complete remission of symptoms and laboratory abnormalities during childhood is in line with the definition of BRIC-2. Recently, 'BSEP-disease' has been recognized as a continuum of cholestasis of varying severity (6). Mutations such as p.E297G or p.A570T have been associated with an intermediate phenotype between BRIC-2 and PFIC-2. Furthermore, a patient who primarily presented with a BRIC-2 phenotype developed cirrhosis at the age of 8 years, which was also suggested to represent a phenotypic transition between BRIC-2 and PFIC-2 (20). In contrast to these reports, in our patient cirrhosis was diagnosed much later, both by transient elastography and liver histology. At that time, the ability of the liver to accumulate serum bile salts into bile was severely impaired as the ratio of

(A)

TMH/loops	BRIC-2 (n = 22)	PFIC-2 (n = 112)
Inside		M1V, G19R, L50S
59-83		M62K, C68Y
Outside	E135K, E137K	C107R, I112T, W114R
145-168		Y157C, A167T, A167V
Inside	E186G, L198P	I182K, M183T, M183V, G188W
217-236		M217R, R223C, S226L
Outside		G238V
241-264		T242I, A257G
Inside	E297G	V284L, E297G, R303G, R303K, Q312H, R313S
319-341		G327E, W330R, C336S
Outside		W342G
362-385	G374S	A382G
Inside	A390P, R432T, I498T, A570T, T586I, G648V, T655I	R387H, A390P, G410D, L413W, I420T, D440E, G455E, K461E, T463I, Q466K, R470Q, Y472C, V481E, D482G, R487H, R487P, N490D, I498T, G499E, I512T, N515T, R517H, F540L, I541L, I541T, F548Y, D549V, G556R, G562D, A570T, L581F, A588V, S593R, I627T, E636G, R698C, S699P, E709K
754-777		G758R, G766R
Outside		
798-821		Y818F
Inside		R832C, R832H, T859R, A865V, Q869P
876-895		G877R
Outside		
900-917		S901R
Inside	T923P, A926P, R948C	R948C, N979D
980-999		G982R
Outside	G1004D	G1004D
1020-1042		T1029K, G1032R
Inside	R1050C, G1116R, R1128H, L1197G, R1231Q	A1044P, L1055P, C1083Y, A1110E, S1114R, G1116E, G1116F, G1116R, S1120N, R1128C, S1144R, R1153C, R1153H, S1154P, N1173D, T1210P, N1211D, V1212F, R1231Q, R1231W, L1242I, D1243G, R1268Q, A1283V, G1292V, G1298R

(B)

Fig. 5. Localization of all known benign recurrent intrahepatic cholestasis (BRIC)- and progressive familial intrahepatic cholestasis (PFIC)-associated BSEP missense mutations. (A, B) All known missense mutations of BSEP were grouped according to their associated clinical phenotypes (BRIC-2 or PFIC-2) and to their localization in relation to the putative transmembrane helices (TMHs, grey boxes in A). All mutations within TMHs are related to PFIC-2 except p.G374S (red), which is the only BRIC-2 mutation so far localized in a TMH. (B) All known mutations within the predicted transmembrane segments are shown in the model of the BSEP transmembrane domain pair. The parts of the α-helices predicted to be membrane-integral are shown in dark grey. Amino acid positions of PFIC-2 mutations are coloured in green.

bile salt concentrations between bile and serum was less than 10 (μM/μM) as compared with more than 5000 (μM/μM) in healthy individuals (Herebian, Mayatepek, Kubitz and Häussinger, unpublished).

Because BSEP targeting was apparently normal, reduction in transporter activity was likely to explain the phenotypic presentation of the patients. While in other studies transport activity of BSEP was analyzed in membrane vesicles from insect cells (9, 21), we established heterologous BSEP expression in *P. pastoris* and performed transport assays using highly enriched plasma membrane vesicles. Transport of both primary bile salts (taurine conjugates of CA and CDCA) was reduced by the same order of magnitude *in vitro* (Fig. 4D), explaining their reduced concentration in bile despite an unchanged ratio of TC to TCDC in both serum and bile *in vivo*. Although this *in vitro* transport assay may not exactly reflect the residual activity of the mutated transporter *in vivo*, it strongly suggests a drastic reduction in $BSEP^{G374S}$ activity. The remaining BSEP transport activity may account for the stable BRIC-2 condition of the sister who closely followed the UDCA treatment.

A frequent finding in BSEP-disease is the reduction in BSEP expression at the canalicular membrane (8, 22), which has been estimated to occur in more than 90% of PFIC-2 patients (23). In our male patient, expression of $BSEP^{G374S}$ at the canalicular membrane was apparently unchanged as shown in his liver biopsy (Fig. 2A). This *in vivo* finding was supported *in vitro* by a comparable membrane localization of $BSEP^{WT}$ or $BSEP^{G374S}$ in transfected HEK293 cells (Fig. 2B). In contrast, many other disease-related mutations such as p.G238V, p.D482G, p.G982R, p.R1153C and p.R1268Q impair membrane targeting of BSEP in transfected cells (10, 24), and it was suggested that the degree of membrane expression inversely correlates with the severity of cholestasis (PFIC-2 vs. BRIC-2 vs. ICP) (25). However, the p.G374S mutation does not fit into this scheme. Systematic analysis revealed that all other missense mutations of BSEP within putative transmembrane α-helices (TMH) are linked to PFIC-2, the more severe form of BSEP-disease.

Transmembrane helices may be particularly sensitive to missense mutations because substrate recognition, binding and translocation occur within these protein

domains. p.G374S is the first missense mutation within a TMH that initially presents as 'benign recurrent cholestasis' before ultimately leading to the development of PFIC-2-like disease if not treated properly. Stable, BRIC-like liver disease in the sister but development of cirrhosis in the brother who took UDCA discontinuously indicates that p.G374S is a more severe BRIC-2 mutation which predisposes to a PFIC-2 phenotype.

In summary, p.G374S is, to the best of our knowledge, the first mutation associated with a BRIC-2 phenotype, which affects a transmembrane helix and strongly reduces transport without influencing canalicular expression of BSEP. The severity of this mutation may be due to its of its localization in a transmembrane helix of BSEP.

Acknowledgements

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Conflict of interest: The authors state no conflict of interest.

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3.7 Two case reports of successful treatment of cholestasis with steroids in patients with PFIC-2

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Analysis of the patient's genetic data obtained from gDNA sequencing

Management and analysis of the patient's clinical data

Writing text sections

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Revising the manuscript

Figures 1 and 3 are of low quality in the published version and therefore added in the originally form at the end.

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Two Case Reports of Successful Treatment of Cholestasis With Steroids in Patients With PFIC-2

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abstract

Mutations in the gene encoding the canalicular bile salt export pump (BSEP) can result in progressive familial intrahepatic cholestasis type 2 (PFIC-2). Treatment options are limited, and PFIC-2 often necessitates liver transplantation. We report on a young woman and a boy who clinically presented with PFIC-2 phenotypes and dramatically improved with steroid treatment. Gene sequencing of *ABCB11* encoding for BSEP revealed 2 relevant mutations in both patients. The young woman was compound heterozygous for p.T919del and p.R1235X. At the age of 5 years, partial biliary diversion was performed and rescued liver function but left serum bile salt levels elevated. At age 23 she developed systemic lupus erythematosus. Unexpectedly, steroid therapy normalized serum bile salt levels, with a strong correlation with the steroid dose. She is currently in clinical remission. The boy was compound heterozygous for the *ABCB11* mutations c.150+3A>C and p.R832C and presented with intractable pruritus. When he developed colitis, he was treated with steroids. The pruritus completely disappeared and relapsed when steroids were withdrawn. To date, with low-dose budesonide, the boy has been symptom-free for >3 years. In conclusion, the clinical courses suggest that patients with BSEP deficiency and residual BSEP activity may benefit from steroid-based therapy, which represents a new treatment option.

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Familial intrahepatic cholestasis syndromes comprise a group of liver diseases that are caused by the failure of hepatobiliary transporter proteins. Certain mutations in the *ABCB11* gene[1–3] lead to deficient expression of the canalicular bile salt export pump (BSEP), causing hepatocellular bile salt (BS) accumulation and subsequent liver cirrhosis. The residual transport activity of BSEP inversely correlates with the severity of the patient's phenotype. Complete absence of BSEP activity is associated with progressive familial intrahepatic cholestasis type 2 (PFIC-2). Patients with PFIC-2 suffer from continuously evolving pruritus, wasting jaundice, and sequelae of liver cirrhosis, often necessitating liver transplantation[4] in early childhood. In large studies, PFIC represented 10% to 15% of causes of childhood cholestasis and is the reason for up to 15% of liver transplantations in children. Treatment focuses on symptom relief and normalization of liver function tests. It is based on ursodeoxycholic acid (UDCA), with improvement of symptoms in 75% of cases,[5,6] rifampicin, and partial biliary diversion. We here report on 2 patients with PFIC-2 phenotypes and sustained clinical relief of symptoms and unexpected improvement in hypercholemia in response to oral steroids.

CASE REPORT

PATIENT PRESENTATION

Patient 1

A girl (born 1989) presented with vitamin K deficiency (8 weeks of age), pruritus (8 months), followed by jaundice (10 months) with a BS concentration of 583 μmol/L (normal: <8 μmol/L). Aminotransferases and gamma-glutamyl transferase were normal. A liver biopsy revealed severe intrahepatic cholestasis and PFIC-2 was suspected. UDCA treatment was started, and a partial external biliary diversion procedure was performed at the age of 5 years, which led to good control of pruritus for 15 years.

Sequencing of *ABCB11* (BSEP) revealed compound heterozygosity for the deletion of a coding triplet (c.2756_2758delCCA), resulting in the loss of a single threonine at amino acid position 919 (p.T919del, inherited by the mother) and the non-sense mutation c.3703C>T/p.R1235X, resulting in a premature stop-codon at amino acid position 1235 (inherited by the father) (Fig 1). The initial liver biopsy revealed an apparently normal expression of BSEP (Fig 1). The anti-BSEP antibody, which was used for immunofluorescence, was directed against the ^{13}C-terminal amino acids of BSEP.[7] BSEP immunoreactivity in the patient's liver biopsy sample must therefore have been due to expression of the allele carrying the T919del variant, because in the presence of p.R1235X (premature stop codon) the 86 C-terminal amino acids are lacking.

At the age of 23 years the patient's general condition deteriorated, and she presented with polyarthritis, polyserositis, splenomegaly, anemia, leukocytopenia, and pruritus. Antinuclear antibody titer was >1:5120, and anti-double-strandedDNA antibodies were 6738 U/mL (ELISA). Systemic lupus erythematosus was diagnosed.

Treatment was started with prednisolone at a dose of 30 mg/day followed by complete symptom relief. Serositis recovered, and the arthritis improved thereafter. Steroids were tapered to 7.5 mg/day, with a flare up of arthritis and increasing anti-double-strandedDNA antibodies to 4549 U/mL. Prednisolone was increased to 15 mg/day, once again resulting

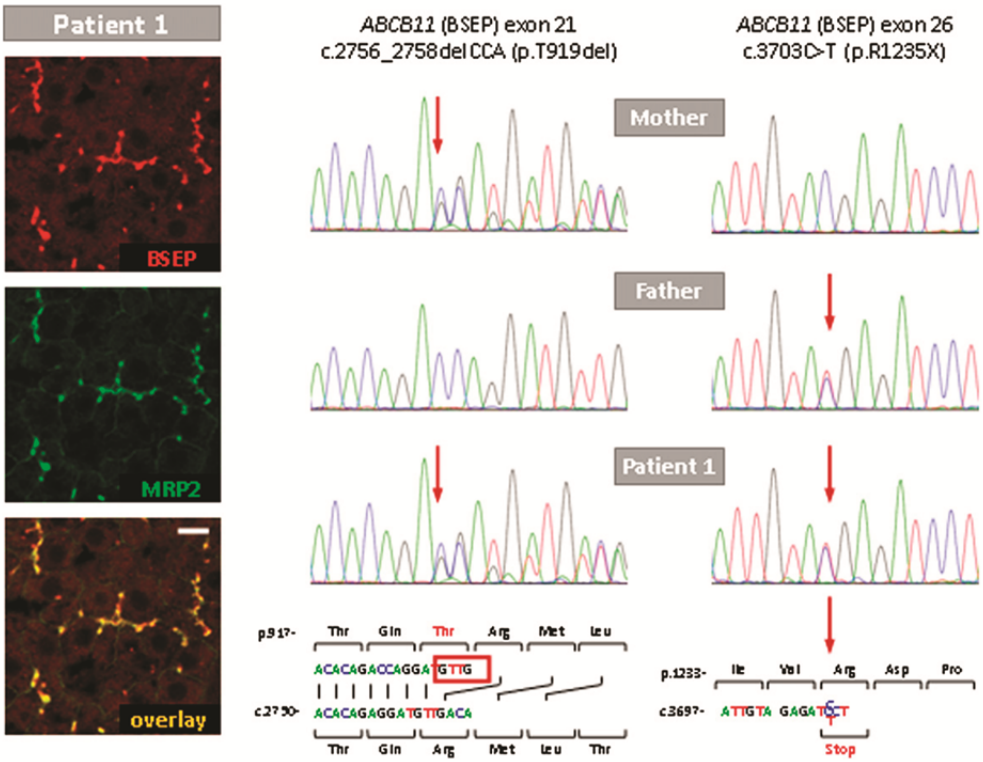

FIGURE 1
Immunofluorescence of liver tissue and DNA sequencing in patient 1. Liver tissue of patient 1 (taken at the age of 10 months) reveals a clear canalicular staining pattern for BSEP and MRP2 (scale bar = 10 μm), as visualized by immunofluorescent staining and confocal laser scanning microscopy. The young woman is compound heterozygous for a triplet deletion in exon 21 c.2756_2758delCCA (p.T919del) and a non-sense mutation c.3703C>T (p.R1235X) in coding exon 26 of *ABCB11*. Sequences are shown on the nucleotide and protein level.

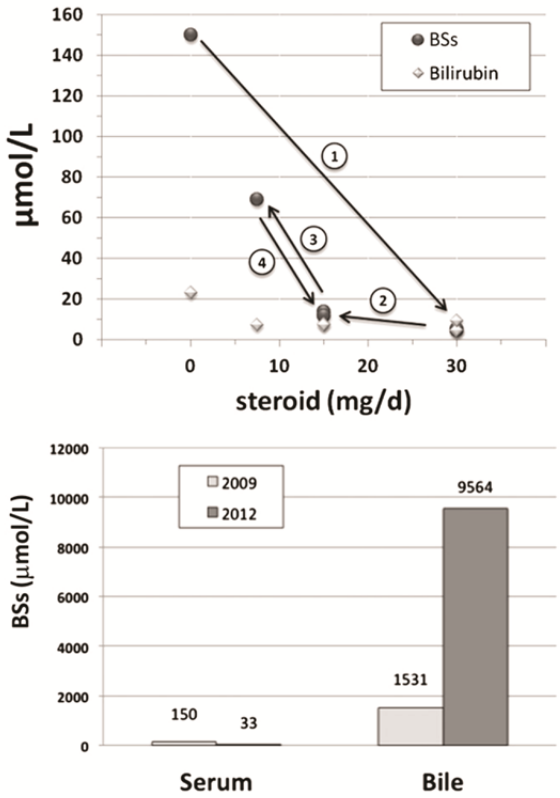

FIGURE 2

Serum BS and bilirubin levels in correlation with steroid therapy in patient 1. Upper panel: (1) steroid therapy (30 mg/day) led to normalized BS levels and a decline in bilirubin; (2) dose reduction to 15 mg/day resulted in slightly increased BSs; (3) further reduction to 7.5 mg/day entailed a distinct increase in BSs; (4) redoubling the dose led again to a decrease in BSs. Lower panel: BSs were measured in serum and bile before (2009) and during (2012) steroid therapy. Although BS concentration in serum decreased by a factor of 4.5, the concentration in bile (collected from the biliostoma) increased by a factor of 6.2, from 1.5 to 9.6 mmol/L, suggesting a strong stimulation of bile acid secretion during steroid therapy.

in clinical improvement in arthritis and pruritus.

Serum BS levels normalized during the initial higher dose of systemic steroid therapy (Fig 2). Interestingly, total BS concentration in bile collected from the partial external biliary diversion increased to 9.56 mmol/L (August 2012) compared with 1.53 mmol/L before steroid treatment (May 2009). Three years after the introduction of steroids (November 2011), she is still free of symptoms of cholestasis.

Patient 2

Patient 2 (born 2004) presented at an age of 2.5 years with severe pruritus despite adequate therapy with rifampicin, phenobarbital, and naloxone; interrupted night sleep; and fatigue. Blood examinations revealed a mild cholestasis with total bilirubin of 23.4 μmol/L and direct bilirubin of 9 μmol/L. Serum BS concentration was 148.6 μmol/L, whereas aminotransferases, gamma-glutamyl-transferase, alkaline phosphatase, and cholesterol were within normal ranges. PFIC-2 was suspected, and a liver biopsy was obtained. Histology revealed chronic cholestasis, disorganization of portal structures, and degenerative bile duct alterations. Treatment with UDCA and naltrexon improved pruritus.

At the age of 4.5 years pruritus and jaundice worsened, with a direct bilirubin of 230 μmol/L. In addition, diarrhea and painful bowel movements developed. Endoscopic retrograde cholangiography revealed no evidence of primary sclerosing cholangitis but showed a secretion of pale-appearing bile. On colonoscopy, a left-sided colitis with superficial granulocyte infiltration of the mucosa in the proximal parts of the colon was diagnosed.

Genetic analysis revealed mutations in the *ABCB11* gene. In the 20th coding exon a heterozygotic missense mutation c.2494C>T (p.R832C, inherited by the mother) was detected together with the heterozygotic splice site mutation c.150+3A>C distally to exon 3 (inherited by the father). No mutations of the *ATP8B1* gene or of the *JAG1* gene (Alagille syndrome) were detected. Immunofluorescence revealed normal distribution of BSEP and of the bilirubin transporter multidrug resistance-associated protein 2 (MRP 2) used as a canalicular marker (Fig 3).

The course of the disease was progressive, with increasing hepatomegaly and advancing liver fibrosis (FibroScan [Echosense, Paris, France] was 10.4 kPa at the age of 4.75 years and 14.6 kPa at the age of 5 years). The patient was listed for liver transplantation and a living related transplantation was considered.

The administration of prednisolone led to relief of the diarrhea and pruritus. BSs and bilirubin normalized completely (Fig 4). Budesonide had the same benefit but without side effects. Liver stiffness improved to almost normal values[8] (6.3 kPa, age 7 years). The patient was taken off the transplant list and is currently free of symptoms (as of 2014).

DISCUSSION

We here report on complete symptom relief and sustained normalization of

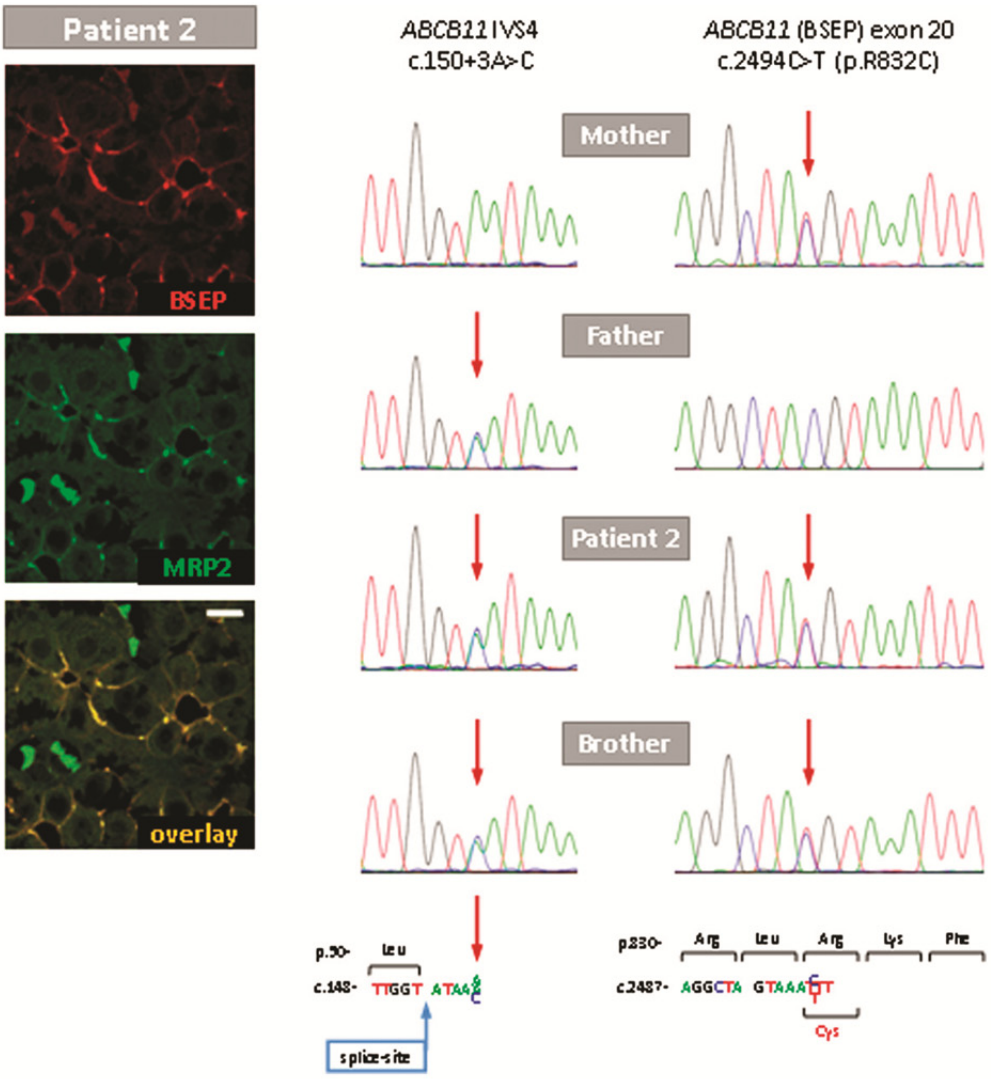

FIGURE 3
Immunofluorescence of liver tissue and DNA sequencing in patient 2. BSEP and MRP2 show a clear canalicular and a weak basolateral hepatocyte staining (scale bar = 10 μm) as uncovered by immunofluorescence and confocal laser scanning microscopy. Patient 2 and his brother (our patient's younger brother with the same phenotype but much lesser symptoms has not been treated with steroids so far) are compound heterozygous for the splice site mutation c.150+3A>C in intervening sequence 4 (IVS4) and the missense mutation c.2494C>T (p.R832C) in *ABCB11* coding exon 20. Sequences are shown on the nucleotide and protein level.

liver function tests in 2 unrelated patients with PFIC-2 phenotypes. In most PFIC-2 patients, BSEP expression is absent or severely reduced in liver tissue.[9] However, our patients showed clear immunoreactivity for BSEP in their livers. Detection of significant amounts of BSs (between 1.5 and 9.5 mmol/L) in the bile of patient 1 suggests residual BSEP activity, which may be an important requirement for successful steroid therapy. It is believed that the extent of expression inversely correlates with phenotype severity (reviewed in ref 10), with some exceptions.[5]

In patient 2, the course of liver and intestinal disease could be positively influenced by prednisolone. Tapering of prednisolone was associated with worsening of cholestasis and intestinal disease. Eventually, budesonide at a minimal effective dosage of 3 mg every second day resulted in complete clinical and biochemical remission within 6 months for >3 years.

The mechanism of action of the steroids in the context of PFIC-2 is

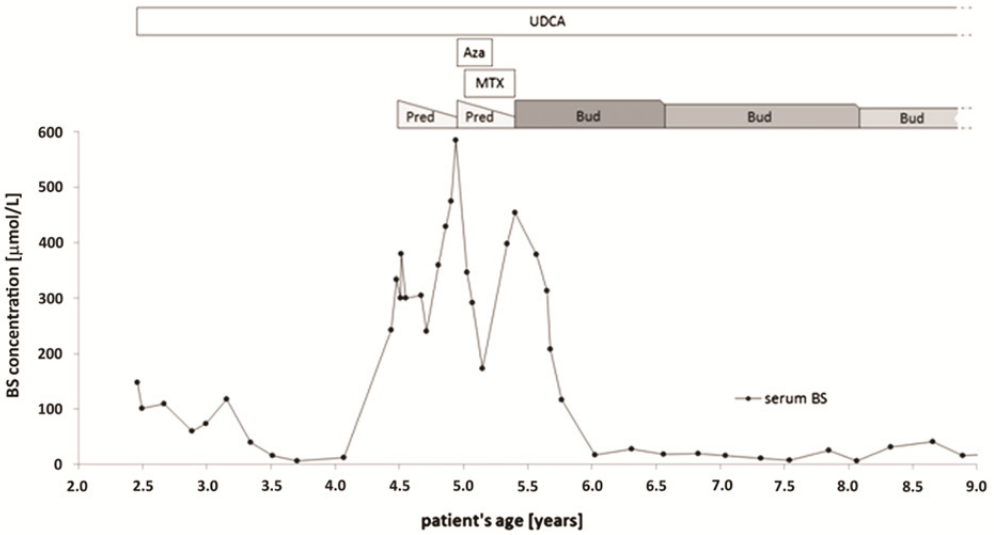

FIGURE 4
Progression of serum BS concentrations in patient 2. Prednisolone (Pred) administration was started at 2 mg/kg per day. Decreasing dosages of prednisolone led to an increase in BS concentrations. Repetitive prednisolone treatment improved serum BS concentrations. Treatment with azathioprine (Aza; 2 mg/kg) and methotrexate (MTX; 15 mg/m^2) were inefficient. Budesonide (Bud; 6 mg/day) induced a sustained remission.

not yet clear but could possibly involve upregulation of BSEP transporter activity, although experimental data are conflicting. In primary rat hepatocytes, BSEP-mRNA as well as Mrp2-mRNA and protein expression were upregulated by dexamethasone in a concentration-dependent manner.[11–13] In line with this finding, treatment of rats with glucocorticoids including budesonide was associated with the upregulation of BSEP, Mrp2, and cytochrome P450 oxidase.[14] On the other hand, Liu et al[15] showed that dexamethasone administration to neonatal rats in vivo had a stimulating effect on Mdr2 (the ortholog to the human MDR3) and Na^+-taurocholate cotransporting polypeptide (Ntcp) gene expression, but decreased the gene expression of Bsep, Mrp2, and Fic1, a P-type ATPase protein. However, most differences were not significant in that study. The effect of steroids on canine bile flow has been demonstrated by Brems et al.[16] Dogs given high dosages of hydrocortisone at 5 mg/kg per hour showed a significant increase in bile flow (from 258 μl/minute to 357 μL/minute) independent of BS concentration.

The *ABCB11* splice site mutation c.150+3A>C results in partial exon skipping of exon 3 in vivo, and approximately one-third of mRNA transcripts are correctly spliced (C.D., R.K., D.H., unpublished data). Therefore, in patient 2, steroids may upregulate transcription and could potentially improve correct mRNA splicing or recover transport activity of $BSEP^{R832C}$.

Another steroid-dependent mechanism of regulated BS transport may involve Na^+-dependent cellular bile acid uptake via NTCP, which is localized at the sinusoidal membrane of hepatocytes.[17] It has been shown that, under cholestatic conditions such as biliary atresia,[18] inflammation-induced cholestasis,[19,20] primary biliary cirrhosis,[21] or progressive familial intrahepatic cholestasis,[22] NTCP is downregulated in human livers. This downregulation may serve to protect hepatocytes from toxic BS concentration but may contribute to the retention of BSs in the blood under cholestatic conditions. An essential glucocorticoid response element (GRE) has been identified within the NTCP promoter at a nucleotide position of −32 to −12 relative to the transcription start site.[23] Therefore, glucocorticoids may improve transepithelial BS transport by upregulation of NTCP, providing an increased gradient for BSEP, thus counteracting cholestasis.

Budesonide is a steroid with rapid intestinal reabsorption and a high first-pass clearance of ~90%.[24,25] Special formulations of budesonide, however, may reach the distal colon.[26] In line with this, colitis in patient 2 remained in remission under alternating dosages of 3 mg budesonide. It is possible that cholestasis might be perpetuated due to extrahepatic inflammation as the primary insult. On the other hand, patient 1 had a slight decrease in BS coincident with active systemic lupus erythematosus even before initiation of steroid therapy. It is well known that lipopolysaccharides induce cholestasis[27] and downregulate BSEP in a model of human liver slices.[19] However, primary sclerosing

cholangitis in the context of ulcerative colitis in general is not sensitive to steroid therapy,[28,29] a fact that demonstrates the completely different pathomechanisms of BSEP deficiency and primary sclerosing cholangitis. Clearly, steroid therapy in patients with PFIC is only indicated if prompt effect (within 4 weeks) is detected because the steroid therapy itself has relevant side effects if given for a long time.[30]

To our knowledge, this is the first clinical report in which, in vivo, a beneficial effect of steroid administration on the disease course of BSEP deficiency/PFIC-2 was shown. For future patient management it would be worth further classifying the PFIC population via genetic analysis, immunohistochemistry or immunofluorescence, and clinical profile to determine those patients who would benefit from steroid administration.

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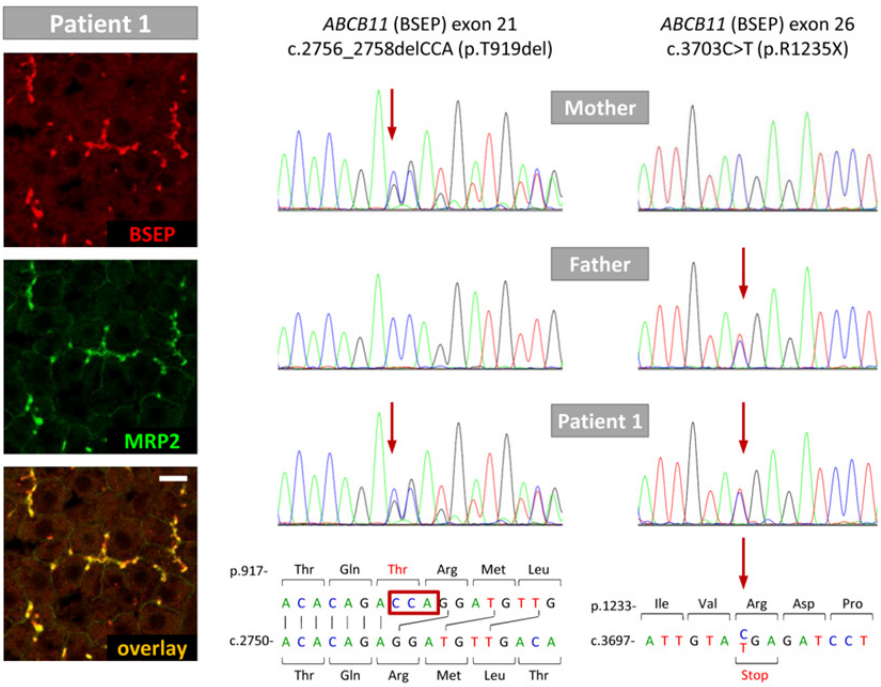
Original version of Figure 1:



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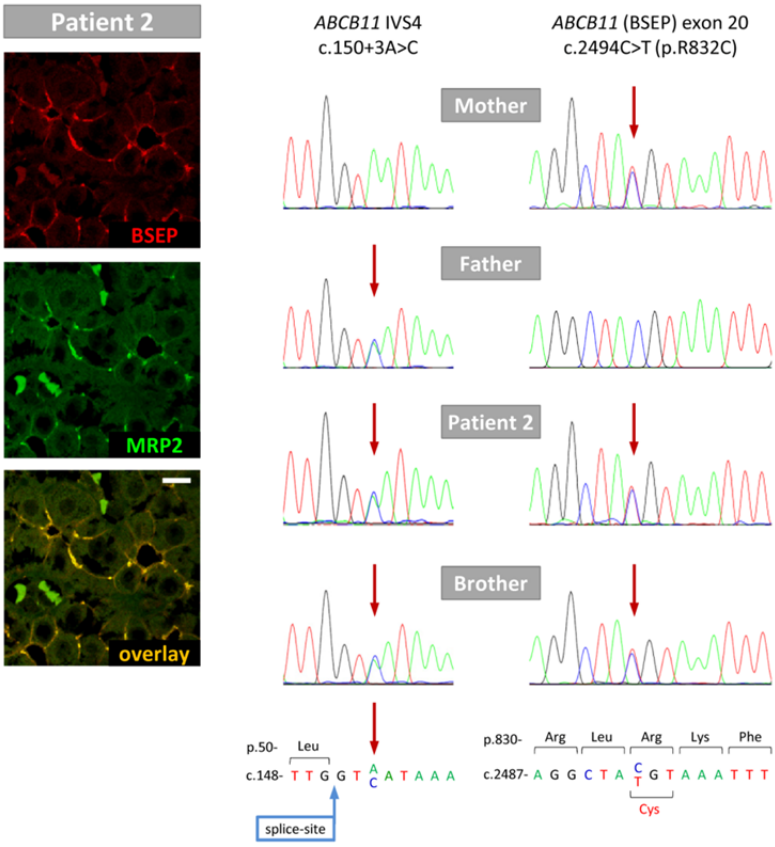

3.8 Autoimmune BSEP disease: Disease recurrence after liver transplantation for progressive familial intrahepatic cholestasis

Ralf Kubitz, Carola Dröge, Stefanie Kluge, Claudia Stross, Nathalie Walter, Verena Keitel, Dieter Häussinger, Jan Stindt

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Own contribution: Summary of the transporter-associated diseases

Development of a diagnostic algorithm

Evaluation of possible antibody trafficking routes

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Preparing the figures

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Autoimmune BSEP Disease: Disease Recurrence After Liver Transplantation for Progressive Familial Intrahepatic Cholestasis

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Abstract Severe cholestasis may result in end-stage liver disease with the need of liver transplantation (LTX). In children, about 10 % of LTX are necessary because of cholestatic liver diseases. Apart from bile duct atresia, three types of progressive familial intrahepatic cholestasis (PFIC) are common causes of severe cholestasis in children. The three subtypes of PFIC are defined by the involved genes: PFIC-1, PFIC-2, and PFIC-3 are due to mutations of P-type ATPase *ATP8B1* (familial intrahepatic cholestasis 1, FIC1), the ATP binding cassette transporter *ABCB11* (bile salt export pump, BSEP), or *ABCB4* (multidrug resistance protein 3, MDR3), respectively. All transporters are localized in the canalicular membrane of hepatocytes and together mediate bile salt and phospholipid transport. In some patients with PFIC-2 disease, recurrence has been observed after LTX, which mimics a PFIC phenotype. It could be shown by several groups that inhibitory anti-BSEP antibodies emerge, which most likely cause disease recurrence. The prevalence of severe BSEP mutations (e.g., splice site and premature stop codon mutations) is very high in this group of patients. These mutations often result in the complete absence of BSEP, which likely accounts for an insufficient auto-tolerance against BSEP. Although many aspects of this "new" disease are not fully elucidated, the possibility of anti-BSEP antibody formation has implications for the pre- and posttransplant management of PFIC-2 patients. This review will summarize the current knowledge including diagnosis, pathomechanisms, and management of "autoimmune BSEP disease."

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Keywords Autoimmune disease · Autoreactive antibodies · Bile salt export pump · BSEP · PFIC-2

Abbreviations

BRIC	Benign recurrent intrahepatic cholestasis
PFIC	Progressive familial intrahepatic cholestasis
BSEP	Bile salt export pump
LTX	Liver transplantation

Introduction

Progressive familial intrahepatic cholestasis (PFIC) represents a group of inherited cholestatic liver diseases, which typically manifests during early childhood [1, 2] and eventually leads to liver cirrhosis, often necessitating liver transplantation (LTX) [3]. The incidence of PFIC is considered to be about 1 to 2/100,000 births [4, 5]. To date, three subtypes have been defined and are termed PFIC-1, PFIC-2, and PFIC-3, and their characteristics are summarized in Table 1 (Fig. 1). PFIC-1 is caused by mutations of *ATP8B1*, which encodes for the transporter protein "familial intrahepatic cholestasis 1" (FIC1). FIC1 functions as an aminophospholipid flippase and maintains the membrane asymmetry required for proper function of other transporter proteins [6]. PFIC-1 has formerly been termed Byler's disease [7] as it has been first described in an Amish family named Byler.

PFIC-2 is due to mutations in the bile salt export pump (BSEP, *ABCB11*) [8]. BSEP is specifically expressed at the canalicular membrane of hepatocytes and is responsible for bile salt secretion into the biliary system [9]. So far, more than 150 BSEP mutations have been linked to PFIC-2 [10]. A

Table 1 Characteristics of the three subtypes of progressive familial intrahepatic cholestasis

	PFIC-1 (Byler's disease)	PFIC-2	PFIC-3
Etiology			
Gene	*ATP8B1*	*ABCB11*	*ABCB4*
Transmission	Autosomal recessive	Autosomal recessive	Autosomal recessive
Chromosomal locus	18q21-22	2q24	7q21
Protein	FIC1	BSEP	MDR3
Function	Phospholipid flippase: maintains membrane asymmetry required for function of other transporter proteins	Bile salt export: essential for bile salt-dependent bile flow	Phospholipid flippase: essential for formation of mixed micelles
Substrates	Phosphatidylserine	Monovalent bile salts	Phosphatidylcholine
High expression level	Liver, intestine, pancreas	Liver	Liver
Clinical characteristics			
Pruritus	+++	+++	+
Serum			
Cholesterol	Occasionally ↑	↑	Normal
gGT	Normal	Normal	↑
ALT	Mildly ↑	>5× normal	Mildly ↑
AFP	Normal	↑	Normal
Lipoprotein X	Present	Present	Absent
Albumin	↓	Usually normal	Normal
Bile salts	↑	↑	↑
Bile composition	↓ Primary bile salts; coarse granular	↓↓ Primary bile salts; amorphous, filamentous bile	↓ Phospholipids; needle-like cholesterol crystals
Liver histology	Bland canalicular cholestasis, mild lobular fibrosis	Canalicular cholestasis, lobular fibrosis, giant cell hepatitis	Ductular proliferation, biliary fibrosis
Progression to cirrhosis	2–7 years	6 months–10 years	5 months–20 years
Extrahepatic symptoms	Diarrhea, vitamin deficiency, pancreatic disease, hearing impairment, pneumonia, failure to thrive	Diarrhea, cholelithiasis, coagulopathy (vitamin K deficiency)	Cholelithiasis
Risks of malignancy	Not known	↑↑	↑
Treatment			
Medical therapy	UDCA	UDCA, steroids	UDCA
Invasive therapy	PEBD, OLT	PEBD, OLT	OLT

ALT alanine aminotransferase, AFP alpha-fetoprotein, *gGT* gamma-glutamyltransferase, *OLT* orthotopic liver transplantation, *PEBD* partial external biliary diversion, *UDCA* ursodeoxycholic acid (according to references [5, 6, 11, 12, 15, 81–85])

characteristic of PFIC-1 and PFIC-2 is a normal serum level of gamma-glutamyltransferase (gGT) during cholestatic episodes, which is uncommon in other forms of cholestasis (Table 1).

PFIC-3 is based on mutations of the multidrug resistance protein 3 (MDR3, *ABCB4*), which acts as a phospholipid flippase. MDR3 flops phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane, where it is released into the bile for subsequent formation of mixed micelles. In contrast to PFIC-1/PFIC-2, patients with PFIC-3 have elevated gGT serum levels. Apart from differences in biochemical parameters, the three PFIC subtypes differ in respect to clinical and histological characteristics [11, 12] (Table 1). For example, extrahepatic symptoms such as diarrhea, hearing impairment, or significant growth failure are more common in PFIC-1 patients, whereas PFIC-2 patients may have a higher incidence of hepatocellular carcinoma [13, 14]. On the histological level, PFIC-3 patients typically show bile duct proliferation, which is not common in PFIC-1 or PFIC-2 [15], whereas liver histology in PFIC-2 often encloses multinucleated giant cells [16]. Further symptoms of all PFIC subtypes are jaundice, hepatosplenomegaly, and severe pruritus. Deficiency of fat-soluble vitamins may develop with subsequent coagulopathy due to a lack of vitamin K or vitamin D-dependent bone disease (Table 1).

Disease recurrence after LTX is a common challenge in certain subgroups of transplanted patients. It is a known problem not only in patients with cholestatic liver diseases

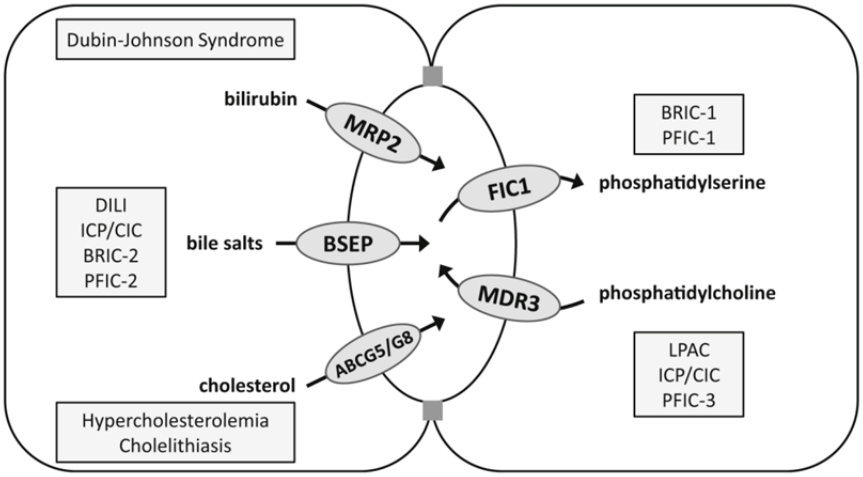

Fig. 1 Canalicular transporters, their substrates, and transporter-associated diseases. A major substrate of the multidrug resistance-associated protein 2 (*MRP2*) is conjugated bilirubin. Mutations of MRP2 can lead to Dubin-Johnson syndrome characterized by increased levels of conjugated bilirubin in the serum. The familial intrahepatic cholestasis 1 (*FIC1*) transporter acts as an aminophospholipid flippase to maintain membrane asymmetry. Mutations are associated with benign recurrent intrahepatic cholestasis type 1 (*BRIC-1*) or progressive familial intrahepatic cholestasis type 1 (*PFIC-1*). Bile salt export pump (*BSEP*) mutations are linked to cholestatic diseases of varying severity ranging from drug-induced liver injury (*DILI*), intrahepatic cholestasis of pregnancy (*ICP*) or contraceptive-induced cholestasis (*CIC*) via BRIC-2 to PFIC-2. The multidrug resistance protein 3 (*MDR3*) flops phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane. MDR3 mutations are related to low phospholipid-associated cholelithiasis (*LPAC*), ICP, CIC, or PFIC-3. Genetic variants of the heterodimeric cholesterol transporter ABCG5/G8 can lead to hypercholesterolemia (reduced cholesterol secretion) or cholelithiasis (increased cholesterol secretion into bile). The substrates of BSEP (bile salts), MDR3 (phospholipids), and ABCG5/G8 (cholesterol) form mixed micelles in bile

such as primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC) [17] but also in hepatitis C, hepatitis B, and hepatocellular carcinoma patients. In children with autoimmune sclerosing cholangitis, there is a high rate of recurrence after LTX, especially when inflammatory bowel disease is associated and poorly controlled [18, 19]. Furthermore, de novo autoimmune hepatitis is a frequent problem after LTX in children [20], necessitating immunosuppressant therapy which differs from the antirejection therapy [21]. Children who are transplanted because of PFIC-2 have a special risk for developing disease recurrence: the de novo appearance of anti-BSEP antibodies, which was first described in 2009 by us [22] and others [23] (Table 2). This special "autoimmune disease" will be discussed in this review in detail.

BSEP: Processing and Function

The bile salt export pump belongs to ATP-binding cassette (ABC) transporter subfamily B (*ABCB11*). BSEP, like other ABC transporters, is fueled by adenosine triphosphate (ATP). It mediates the transmembrane flux of bile salts (BS). BSEP is localized in the canalicular membrane of hepatocytes and concentrates BS in the biliary fluid up to ~8000-fold as compared to serum concentrations. Within bile, cholesterol is solubilized by BS and phosphatidylcholine (lecithin), which together form mixed micelles. Phosphatidylcholine is transported into bile by MDR3, whereas cholesterol is effluxed by the heterodimeric ABC transporter ABCG5/ABCG8, also localized in the canalicular membrane (Fig. 1).

Genetic variants and mutations of BSEP may directly impair transport function [24, 25] without affecting protein expression. However, many mutations apparently reduce transporter expression [2, 16, 22, 26–28] and consecutively decrease transport capacity for BS (Fig. 2). The reduction in protein expression may result from defects in messenger RNA (mRNA) splicing due to splice site mutations [24] or nonsense-mediated decay in the context premature termination codons (Dröge et al., submitted). Furthermore, protein processing may be defective on different levels and may involve the step of quality control by the endoplasmic reticulum (ER)-associated degradation (ERAD) of misfolded proteins [22, 28, 29]. It has been shown that, BSEP with the common PFIC-2-related mutation E297G is trapped in the ER in a core-glycosylated state [29]. The three mutations/variants Y818F, G982R, and V444A have been found on the same allele in a PFIC-2 patient. BSEP with Y818F together with G982R is poorly expressed. The combination with the common BSEP polymorphism V444A results in complete degradation by ERAD, which can only be unmasked by proteasomal inhibition [22].

BSEP, like other integral membrane proteins, becomes complex glycosylated within the Golgi apparatus, and exits the Golgi towards the canalicular (apical) membrane [30]. The

Table 2 Summary of patients with autoimmune BSEP disease (AIBD)

Patient [Ref.]	Sex	Age at LTX (years)	Zygosity	Nucleotide change	Predicted (protein) effect	BSEP expression (before LTX)	BSEP autoantibodies detected	Symptom recurrence–time after LTX (years)	Triggering factors
1 [22, 63]	F	3.5/3.5/4.9	hom hom	c.2453A>T c.2944G>A	p.Y818F p.G982R	Absent (exp)	Yes	1/0.4	Graft rejection after first LTX
2 [23]	M	5.2	hom	c.907A>G	p.R303G	Absent	Yes	12	Unknown
3 [23]	F	3.7	com het	c.1741C>T IVS12+1G>T[a]	p.L581F abb splic	Absent	Yes	3.5/5.2/8.1/12.1/13	EBV infection; ↓ corticosteroids
4 [23]	F	2.2	hom	IVS17+1T>A[a]	abb splic	Absent	Yes	2.1/4	Low CsA, switch from Tac to CsA
5 [62, 63]	F	9	com het	c.301delCA c.2944G>A	p.Q101Dfs8X p.G982R	Absent	NA	3.3/17	↓ IS, pregnancy?
6 [62]	M	2.8	hom	c.77-19T>A	p.Y26Ifs7X	Absent	Yes (rBsep)	4.8	↓ IS
7 [63]	–	2.4	hom	c.908+1G>A	abb splic	Absent	NA	0.8	Unknown
8 [63]	–	3	com het	c.1145_1165del c.2012-8T>G	p.A382_388del abb splic	Absent	NA	3/8.6	Unknown
9 [63]	–	1.8	com het	c.1941delA c.2012-8T>G	p.G648Vfs6X abb splic	Absent	NA	3.2/3.6	Unknown
10 [63]	–	0.8	com het	c.2783_2787dup5 c.1442T>A	p.K930Efs79X p.V481Q	Absent	NA	1/1.4	Unknown
11 [61]	M	0.8/3.5	hom	c.2783_2787dup5	p.K930Efs79X	Absent	YES	1.7/5	Unknown
12 [61]	M	5	hom	c.1639-2A>C	abb splic	Absent	YES	12	Unknown

abb splic aberrant splicing predicted, *com het* compound heterozygous, *CsA* cyclosporin A, *EBV* Epstein-Barr virus, *exp* experimental, *F* female, *hom* homozygous, *IS* immunosuppression, *IVS* intervening sequence, *M* male, *NA* not assessed, *Tac* tacrolimus

[a] Sequence position not precisely defined

myosin II regulatory light chain (MLC2) is required for trafficking to the apical membrane [31]. MLC2 is associated to the Golgi and trans-Golgi networks. Impairment of protein trafficking may substantially obstruct the arrival of BSEP at its target membrane, the canaliculus [29, 32]. Protein stability, trafficking, and activity at the canalicular membrane of rat Bsep depends on N-linked glycosylation. At least two of four potential glycosylation sites (Asn 109, Asn 116, Asn 122, or Asn 125 [33], which are all present in human BSEP) need to be glycosylated for proper processing and subsequent function of Bsep. Short-term regulation of Bsep may be achieved by retrieval of Bsep from the canalicular membrane, which can be induced by phorbol esters, 17-beta-estradiol, and Ca^{2+}-dependent PKC isoforms [34, 35]. Retrieval is regulated in part by the Src kinase family member Fyn [36]. On the other hand, the anti-cholestatic drug tauroursodeoxycholate stimulates insertion of Bsep into the canalicular membrane [37].

Variable Severity and Treatment of BSEP Deficiency

Almost 300 genetic variants of BSEP have been described including missense, nonsense, frameshift, or splice site mutations [10]. These variants have been associated to cholestatic liver diseases of variable severity ranging from progressive familial intrahepatic cholestasis type 2 (PFIC-2) [2, 8] and benign recurrent intrahepatic cholestasis type 2 (BRIC-2) [25, 38, 39] to intrahepatic cholestasis of pregnancy (ICP) [40, 41]. More recently, BSEP polymorphisms have been identified as risk factors for the development of drug-induced liver injury (DILI) [42–44] (Fig. 1), showing that BSEP deficiency is a clinical continuum. This emphasizes the importance of BSEP and intact BS excretion for liver function. Overall, it was suggested that the severity of BSEP-related cholestasis inversely correlates to the expression of BSEP at the canalicular membrane [45], although there are exceptions to this rule [25]. Altogether, there are several distinct mechanisms which cause reduced transport on the one hand or diminished or even absent expression on the other. These mechanisms are depicted in Fig. 2.

PFIC-2 is the severest form of BSEP deficiency. It is characterized by early onset of cholestasis starting within the first months of life or during early childhood. Jaundice, pruritus, and growth retardation are typical symptoms and coagulopathy is present to a variable degree. Untreated PFIC-2 progresses towards liver fibrosis, cirrhosis, and finally end-stage liver disease [2, 8, 11, 46]. BRIC-2 presents with milder symptoms, at later ages, and with self-limiting courses. ICP only manifests during pregnancy and is characterized by pruritus and elevated BS. In patients with a predisposition for ICP, contraceptives may result in similar symptoms (termed contraceptive-induced cholestasis, CIC).

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Milder forms of BSEP deficiency (especially BRIC-2 and ICP) often respond well to therapy with ursodeoxycholic acid (UDCA), whereas a substantial number of patients with a PFIC-2 phenotype do not benefit from UDCA at all. It is likely that responsiveness depends on residual activity, inducibility, or potential stabilization of BSEP at the canalicular membrane [10, 45, 47]. Complete absence of BSEP is an adverse prognostic factor for the course of BSEP disease.

Alternatively to medical treatment, partial external biliary diversion (PEBD) or ileal bypass surgery may improve the outcome in some PFIC patients. The aim of these surgical procedures is to circumvent the intestinal reabsorption of BS in the terminal ileum [11, 46, 48, 49]. As for medical therapy, the success of these procedures depends on the residual activity of BSEP. When these procedures fail, orthotopic liver transplantation is the remaining treatment modality with reasonable to excellent clinical outcome in these patients [1, 5, 11, 46, 50].

Disease Recurrence and Development of BSEP Autoantibodies After Liver Transplantation

The risk of disease recurrence after liver transplantation depends on the underlying cause of liver failure. For example, hepatitis C virus infection invariably recurs after LTX, although with different severity depending on the host immune system, the virulence of the virus, and the regimen of immunosuppression [51, 52]. Disease recurrence after LTX for primary biliary cirrhosis (PBC) is less frequent [53]. Antimitochondrial antibodies (AMA) persist after LTX in these patients, but histologically proven PBC recurrence affects "only" 20 to 50 % of transplanted PBC patients [54, 55]. The impact of PBC recurrence on the overall survival in these patients is unclear [53]. Recurrence of primary sclerosing cholangitis (PSC) after liver transplantation affects between 1 and 33 % [56–59] and seems to be higher in living donor liver transplantation [60].

In 2009, we reported on the first child with recurrence of a PFIC phenotype [22]. The indication for transplantation in this child was PFIC-2 due to three homozygous genetic BSEP variants. Because of primary non-function of the first transplanted liver, a second LTX was necessary. Thereafter, a cholestatic situation developed within 1 year. Because there was no evidence for rejection, other causes of cholestasis were considered. The clinical presentation was consistent with a PFIC-2 phenotype. Serum BS levels were >600 μmol/l, whereas gGT was almost normal. Finally, canalicular antibody deposits were detected in a biopsy of the second transplanted liver. Subsequent analysis of the child's posttransplant serum revealed antibodies directed against human BSEP, which were not present in the pre-transplant serum. These two findings of recurrent cholestasis and de novo antibodies directed against human BSEP suggested a causal relation. This causality was further supported by the observation that, reduction of serum antibodies by plasmapheresis and anti-CD20 antibodies (rituximab) temporarily alleviated cholestasis [22]. Following this report, similar observations were published

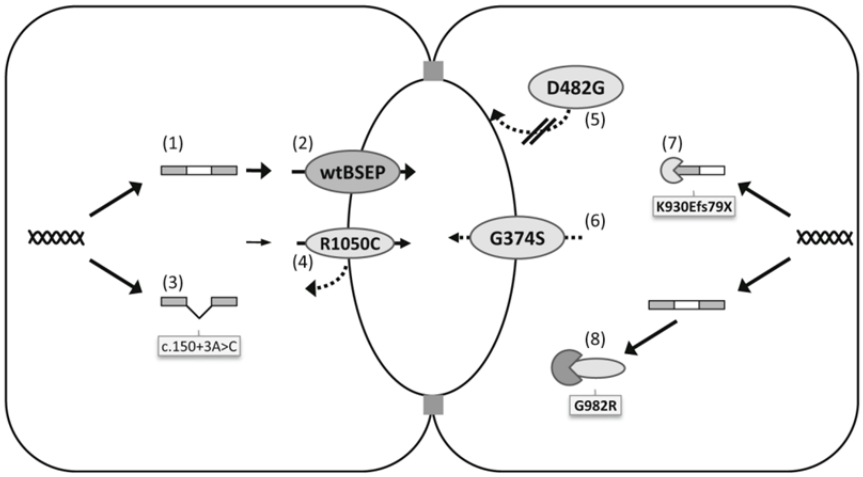

Fig. 2 Different effects of genetic *ABCB11* variants on processing and function of BSEP. Under normal conditions, splicing (*1*) of BSEP-mRNA is followed by trafficking of BSEP to the canalicular membrane (*2*). Splice site mutations may lead to reduction or absence of BSEP-mRNA (*3*). Missense mutations may decrease membrane expression by a reduced targeting or diminished half life (*4*). Other missense mutations are retained intracellular due to impaired insertion into the canalicular membrane (*5*). Mutations such as G374S have no effects on expression but display impaired or absent transport activity (*6*). Premature stop codons (e.g., in the context of insertions/deletions with frameshifts or splice site mutations with exon skipping) can lead to nonsense-mediated decay of mRNA molecules (*7*). Lastly, mutations can result in instable proteins which are eliminated by proteasomes through the mechanisms of ER-associated degradation (*8*)

by other groups [23, 61–63]. Meanwhile, we were able to detect anti-BSEP antibodies in seven more patients [64]. Overall, it is estimated that up to 8 % of transplanted PFIC-2 patients develop anti-BSEP antibodies [63]. Typical laboratory findings in these patients are elevated serum BS concentrations, which may exceed 500 μmol/l. Furthermore, increases in bilirubin levels (between 2 and ~36 times of the upper limit of normal) and liver function tests (alanine transaminase and aspartate transaminase) are observed, whereas gGT levels remain normal during cholestasis, in line with a PFIC-2-like phenotype [22, 23, 62, 63].

Diagnostic Work-up for Anti-BSEP Antibodies

The development of anti-BSEP antibodies needs to be taken into account in patients with genetically proven PFIC-2, who become cholestatic after LTX. When acute or chronic graft rejection has been excluded (e.g., by histology) and signs of cholestasis prevail (e.g., bile plugs), anti-BSEP autoantibodies should be considered.

Liver tissue obtained by biopsy can be analyzed by immunofluorescence (IF) or immunohistochemistry (IHC), using fluorochrome- or peroxidase-tagged anti-human immunoglobulin antibodies, respectively. These antibodies will bind to the patient's immunoglobulins (Ig) within the tissue. A canalicular staining pattern indicates the deposition of the patient's antibodies at the canalicular membrane in vivo and is the first hint for the presence of anti-BSEP antibodies (Fig. 3). Because the sinusoids within the liver biopsy are filled with the patient's serum and therefore contain large amounts of antibodies, a strong sinusoidal signal is typical and constricts the visibility of canalicular staining to some extent. For that reason, IF with co-staining for a canalicular marker protein such as multidrug resistance-associated protein 2 (MRP2), DPPIV, or gGT may simplify the identification of canalicular Ig deposits.

After detection of canalicular antibody deposition, the patient's serum should be further analyzed, when anti-BSEP antibodies are considered. A cryosection of a normal liver can be used as a template. In our experience, anti-human BSEP antibodies are almost always cross-reactive to rodent Bsep; therefore, rat, mouse, or human liver slices may be used with equal success. In this setup, a canalicular staining pattern produced by the patient's serum indicates the presence of antibodies directed against a canalicular epitope and would again be consistent with the presence of anti-BSEP antibodies (Fig. 3).

For the final diagnosis of anti-BSEP antibodies, cloned (human) BSEP is required. Transfected cell lines and their lysates can be utilized for immunostaining or Western blot analysis. A positive staining or a 150 kDa signal in lysates of BSEP-transfected versus non-transfected cells finally confirm the diagnosis of anti-BSEP antibodies (Fig. 3).

Finally, immunofluorescence in combination with serial dilutions of patient's serum can be employed in order to determine an antibody titer. More accurate and reproducible methods for antibody quantification such as enzyme-linked immunosorbent assay (ELISA) have not been used so far in the context of anti-BSEP antibodies but would be advantageous for a better correlation with the clinical presentation of the patient and for the management of therapy (Fig. 3).

The functional relevance of anti-BSEP antibodies can be tested in different systems of increasing complexity. Commercially available BSEP-containing vesicles can be used, bearing in mind that the readout is BS uptake from the exterior into the vesicle lumen, mediated by BSEP molecules with an inside-out configuration (cytosolic part of BSEP facing the exterior). Anti-BSEP antibodies, which bind to extracellular epitopes of BSEP, cannot enter the interior of the vesicles unless a special pretreatment (e.g., freeze-thaw cycles) is applied. More complex systems include cultured primary hepatocytes. In our initial report, we could show that antibodies reach the (pseudo-)canaliculi of isolated rat hepatocytes within minutes, followed by an inhibition of fluorescent substrates transport into the canaliculi [22]. Furthermore, polarized cell lines which are double-transfected with BSEP and the basolateral Na^+-taurocholate cotransporting polypeptide (NTCP) can be used. When such cells are grown to confluence, vectorial transport of BS from a basal compartment into cells and then into the apical compartment can be measured. The advantage of this system is the selective accessibility of the extracellular part of BSEP. Finally, anti-BSEP antibodies can be used in isolated perfused rodent livers or after intravenous injection [23].

Possible Mechanisms of Canalicular Deposition of Anti-BSEP Antibodies

BSEP is localized at the canalicular (apical) membrane of hepatocytes, a domain which is delimited from the blood by tight junctions. In view of the functional relevance of anti-BSEP antibodies, it is therefore of interest how these antibodies reach the canalicular space. In principal, three pathways are possible: (1) by passive transport across tight junctional complexes between liver cells and by fluid phase processes [65, 66], (2) by a receptor-mediated pathway, or (3) by direct binding to BSEP at the basolateral (sinusoidal) membrane followed by transcytosis of the BSEP/antibody complex.

When fluorescently labeled antibodies are applied to cultured primary hepatocytes, which already have established pseudocanaliculi, no enrichment of these antibodies can be observed (own unpublished data). However, anti-BSEP antibodies accumulate within less than 30 min in the

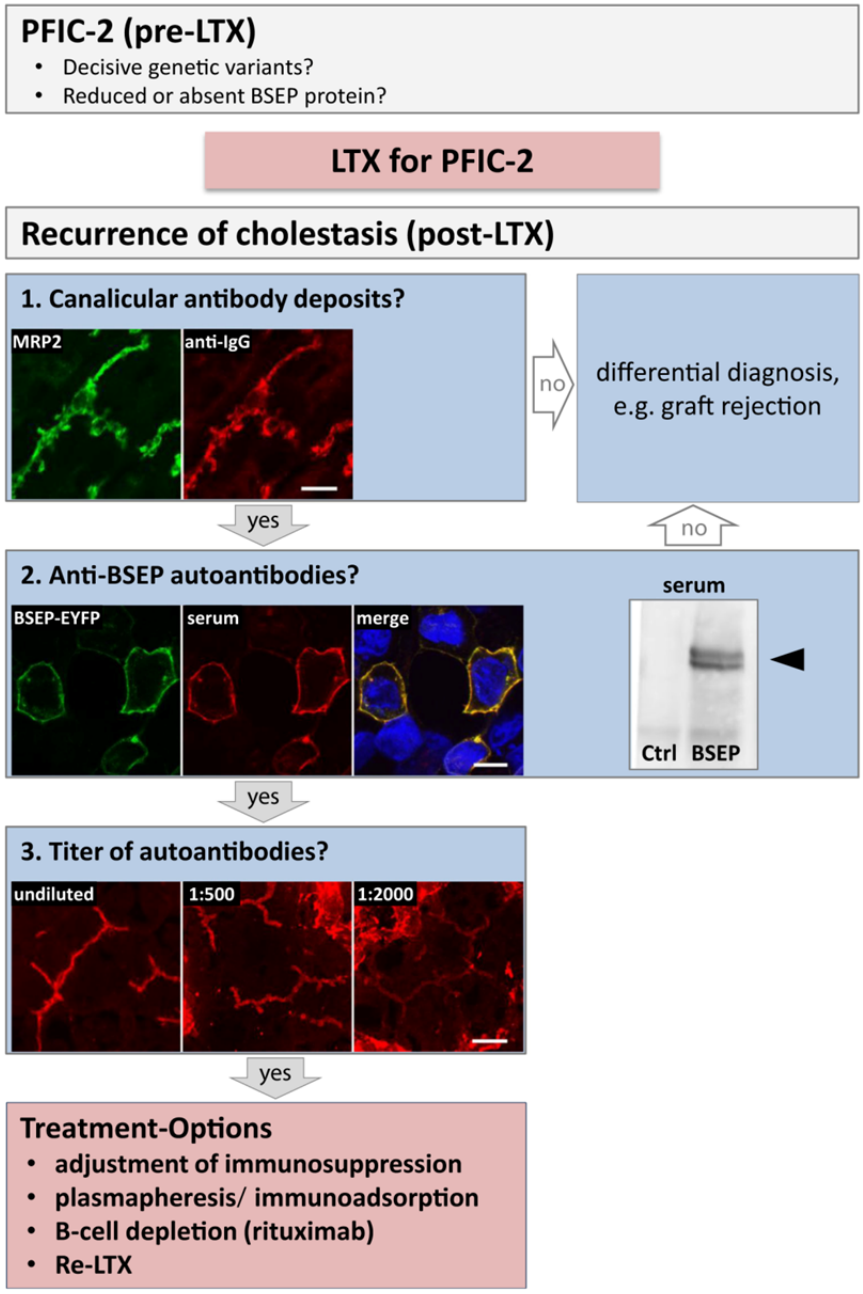

Fig. 3 Algorithm for the diagnosis of anti-BSEP antibodies. Patients with a PFIC phenotype, who are considered for liver transplantation, should be genotyped. If PFIC-2 is diagnosed, the expression level of BSEP protein in the original liver should be determined. When a PFIC-2 patient develops cholestasis after LTX and anti-BSEP antibodies are considered, they can be determined by (*1*) detection of canalicular IgG deposits in a biopsy of the transplant, followed by (*2*) proof of BSEP-specific antibodies in the patient's serum, using cloned BSEP (e.g., BSEP tagged to a fluorescent protein such as enhanced yellow fluorescent protein, EYFP). Finally (*3*), a titer of anti-BSEP antibodies should be measured for further clinical guidance before and during treatment. When no IgG deposits (step 1) or BSEP-specific antibodies (step 2) are detected, other causes of cholestasis need to be considered. The choice of treatment depends on the severity of symptoms and may range from simple adjustment of the immunosuppressive regimen to re-transplantation in cases of re-occurrence end-stage liver disease

pseudocanaliculi of cultured hepatocytes. This observation strongly suggests that passive diffusion of antibodies into the canaliculi is not the major pathway.

A receptor-mediated pathway is well described for IgA antibodies: The polymeric immunoglobulin A receptor (pIgAR) is expressed on the basolateral surfaces of enterocytes, hepatocytes, and biliary duct cells. pIgAR transports polymeric IgA (and to some extent IgM) to the apical surfaces of these cell types. Polymeric IgA is thereby actively secreted into human bile [67, 68], reaching concentrations of ~55 μg/ml, corresponding to ~22 % of serum concentrations, whereas IgG concentrations in bile was ~90 μg/ml, corresponding to ~0.8 % of serum concentrations [69]. Normally, the abundance of IgM in bile is low; however, when IgM is increased in serum

(e.g., in the presence of a plasmocytoma), IgM may replace IgA and can be detected in (rat) bile in significant amounts [70]. Because anti-BSEP antibodies belong to the IgG subclass (own unpublished data), it is less likely that they reach the canalicular domain by the pIgAR pathway. Apart from pIgAR, the Brambell receptor (FcRB or FcRn for neonatal Fc receptor) is expressed in hepatocytes. FcRn binds to the constant fragment region of antibodies. It has been suggested that, biliary IgG may be transported back from the apical to the sinusoidal membrane by FcRn [67]. Other studies suggested that FcRn has a protective role for endocytosed IgG, by preventing the lysosomal degradation of endocytosed IgG molecules [71]. However, there is no evidence that this receptor mediates sinusoidal-to-canalicular translocation of antibodies.

Finally, specific binding of anti-BSEP antibodies to BSEP at the sinusoidal membrane may be considered (Fig. 4). It has been debated if BSEP is directly targeted from the Golgi apparatus to the canalicular membrane [72, 73] or if BSEP reaches the canaliculus indirectly by a route including a stop-over at the basolateral membrane [74]. Hepatocytes, unlike other epithelial cells, have been suggested to utilize the indirect pathway for apical protein delivery, because they lack the myelin and lymphocyte (MAL) protein, which acts as a regulator of direct and indirect apical delivery [75]. When BSEP is transfected in non-polarized HEK293 cells [22, 25], BSEP is found at the plasma membrane (which corresponds to the basolateral membrane), showing that this membrane compartment is easily accessible for BSEP. In the context of anti-BSEP antibodies, the indirect pathway with residence of transporter molecules at the sinusoidal membrane would allow specific binding of anti-BSEP antibodies to BSEP at the interface of hepatocytes and blood and would explain how functionally relevant amounts of these antibodies could find access to bile canaliculi (Fig. 4).

The exact mechanism how anti-BSEP antibodies inhibit BS transport is not yet known. It is possible that anti-BSEP antibodies bind to functionally relevant extracellular epitopes and simply obstruct the channel pore which mediates BS flux. Likewise, the two binding sites of anti-BSEP antibodies may crosslink BSEP molecules, which in turn could disturb the structure and function of canalicular membranes and microdomains. This latter possibility might explain why autoimmune BSEP disease not only increases BS levels but also leads to increases of bilirubin, a substrate of the MRP2.

Possible Mechanisms of Anti-BSEP Antibody Formation

Recurrence of PFIC-1 or PFIC-3 on the basis of antibody formation against FIC1 or MDR3, respectively, has not been

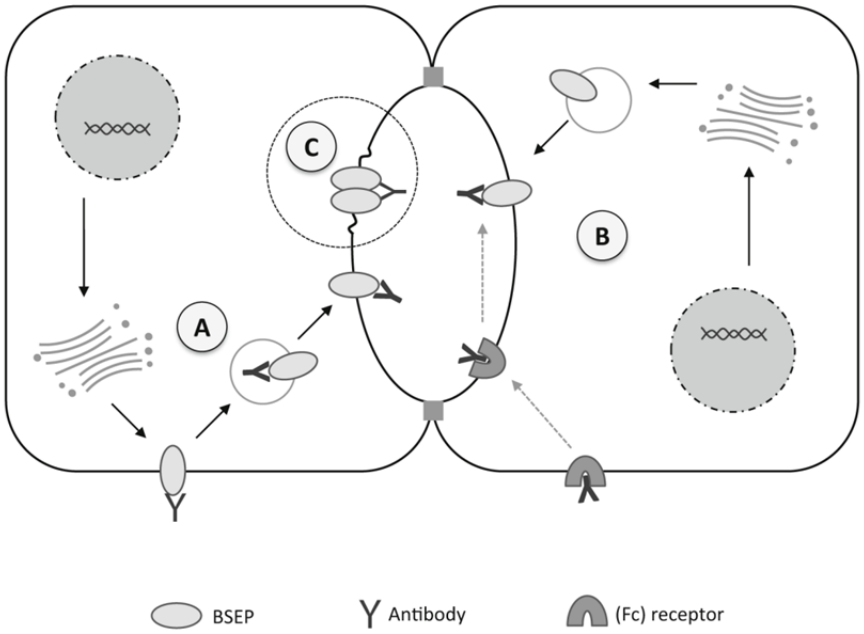

Fig. 4 Possible trafficking routes of anti-BSEP antibodies to the canalicular membrane. *A* Anti-BSEP antibodies may bind to BSEP at the sinusoidal membrane followed by the joined trafficking to the canalicular membrane, assuming that BSEP follows the indirect targeting route of apical proteins via the basolateral membrane of hepatocytes. *B* Single anti-BSEP antibodies may be transported to canalicular space by a (not fully defined) receptor pathway. After release of antibodies in the canalicular lumen, anti-BSEP antibodies can bind to extracellular epitopes of BSEP. *C* Apart from a one-to-one inhibition (one antibody blocks one BSEP molecule, see *A*/*B*), anti-BSEP antibodies can possibly induce cross-linking of BSEP molecules, thereby interfering with membrane topology and resulting in inhibition of bile salt transport

described so far (own unpublished data). It is of interest why anti-BSEP antibody development seems to be common in transplanted PFIC-2, but not in PFIC-1 or PFIC-3 patients.

It may be speculated that auto-tolerance towards BSEP is not achieved in PFIC-2 patients. Auto-tolerance is attained by antigen-presenting cells (APC) in the thymus and the peripheral tissue [76]. Thymic cells present only dominant epitopes from a given protein, whereas the peripheral tissue presents several different peptides of organ-specific antigens [76]. Therefore, strongly reduced or even complete absence of BSEP expression, which has been regularly described in PFIC-2 patients [2, 26, 27, 77], may account for an insufficient presentation of BSEP peptides in the liver, thus obstructing sufficient elimination of BSEP-reactive T lymphocytes. After organ transplantation, the massive availability of BSEP may then stimulate these BSEP-reactive T lymphocytes, which lead to clonal expansion of anti-BSEP antibody-producing B cells. On the basis of this proposed mechanism, it was suggested that the absence of BSEP may be a requirement sine qua non for the development of antibodies against BSEP [22, 63]. In line with this, the proportion of severe mutations is very high in this group of patients. In the group of the 12 patients published so far, 9 alleles carried splice site mutations, 8 alleles carried mutations associated with premature stop codons, and only 7 alleles had missense mutations (Table 2).

Clinical Course of Anti-BSEP Antibody-Associated Cholestasis

The severity of anti-BSEP antibody-mediated cholestasis varies considerably. Typically, recurrence of PFIC-2 phenotype becomes clinically evident through refractory pruritus [22, 23, 62, 63], which is difficult to treat by common drugs. Possible precipitating factors may be reduction or changes in immunosuppression [23, 62]; primary dysfunction of liver transplant; and re-transplantation [22], Epstein-Barr virus (EBV) infection [23], or pregnancy [62]. Reported time periods between LTX and apparent recurrence of a PFIC phenotype range between 9 months and 17 years [22, 62, 63].

In our own cohort, we observed courses of recurrence of moderate severity, which responded to adjustment of immunosuppression. In one patient, antibiotics (metronidazole) alleviated symptoms and induced temporary remission (personal communication, R. M.). The mechanism of improvement in this case remains unclear.

One patient experienced long-lasting recovery after a combined treatment with immunoadsorption and repetitive treatment with rituximab for B cell depletion (G. Engelmann, R. Kubitz, unpublished). In line with this, a successful treatment with rituximab, intravenous immunoglobulins, and plasmapheresis of two patients with recurrence after LTX for PFIC-2 has been reported [61]. In our own cohort, two patients experienced only transient improvement following these treatment regimens [64] and successive re-transplantation was necessary.

The occurrence of anti-BSEP antibodies often leads to re-transplantation: the first published case was re-transplanted after 1.5 years [22]. In another report, the rate of re-transplantation after disease recurrence was 66 % (four/six) of patients with a median time between the transplantation and re-transplantation of 25 months. Indications for re-transplantation were cholestasis with intractable pruritus, in combination with end-stage liver disease, refractory ascites, bone disease, or growth failure [63]. All patients again developed progressive cholestasis with low gGT in the subsequent transplant [22, 63].

Treatment Options for Anti-BSEP Autoantibodies

The development of anti-BSEP antibodies needs to be considered as a differential diagnosis to acute rejection in transplanted PFIC-2 patients. Although both forms of posttransplant liver disease are triggered immunologically, it has been pointed out that the condition of anti-BSEP antibodies is refractory to medication which is effective in the treatment of allograft rejection [61]. Single patients may successfully be treated by changes of immunosuppressive therapy, e.g., a combination of cyclosporine, prednisone, and azathioprine [62], or by changes to tacrolimus, prednisolone, and mycophenolate mofetil [23].

Autoimmune hepatitis often develops in children after LTX. Antinuclear antibodies are diagnostically important, but do not directly affect the course of disease. Anti-BSEP antibodies, however, are pathophysiologically relevant. This is supported by the observation that the severity of anti-BSEP antibody-related cholestasis correlates to antibody titers [23, 64] and that BSEP function is inhibited by these antibodies [22, 23, 64]. Therefore, lowering antibody titers represents a logical therapeutic aim. Depletion of anti-BSEP antibodies may be achieved by plasmapheresis (separation and removal of blood plasma from blood cells) [22, 61] or immunoadsorption (removal of antibodies from blood) (Engelmann, Kubitz, unpublished), which improves cholestasis and jaundice within days. In order to avoid infectious complications after antibody reduction, substitution of normal human immunoglobulins is rational.

Treatment with rituximab targets in the same direction of antibody reduction. Rituximab binds to and inhibits CD20, a receptor exclusively expressed on B cells. CD20 is essential for the clonal expansion of B cells and their differentiation to antibody-producing plasma cells. Most B cell subpopulations express CD20, but early stages (pro-B cells) and fully differentiated plasma cells are CD20-negative [78]. Apart from the treatment of B cell malignancies, rituximab is used for the therapy of antibody-dependent autoimmune diseases [79]. It has been reported that, autoantibodies decline more rapidly

than other antibodies, and it was suggested that autoantibodies are produced by more short-lived plasma cells [78, 80]. Rituximab has been used for the depletion of anti-BSEP antibody-producing plasma cells in combination with plasmapheresis [61] or immunoadsorption (own data). If rituximab is effective as a monotherapy has not been investigated so far.

Quite a few patients with anti-BSEP antibodies need a second (or third) liver transplantation. All re-transplanted patients again developed recurrence of cholestasis, indicating the difficulties of controlling anti-BSEP antibody production. If under these circumstances simultaneous bone marrow transplantation is rational and justified needs to be further evaluated in the future.

Concluding Remarks

The recently described posttransplant occurrence of anti-BSEP antibodies in PFIC-2 patients represents a novel disease which is clinically difficult to handle and which opens interesting questions. Awareness of this complication, which we suggest to term autoimmune BSEP disease (AIBD), should help to avoid its development in the special group of PFIC-2 patients. In this context, it seems further advisable that a genetic diagnosis should be made in children with a PFIC phenotype in order to estimate the risk of AIBD occurrence in cases of PFIC-2.

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3.9 Bile salt export pump-reactive antibodies form a polyclonal, multi-inhibitory response in antibody-induced bile salt export pump deficiency

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- Collection and analysis of the patient's clinical and sequencing data
- Confocal laser scanning microscopy of liver tissue of patient 1
- Sequencing analysis of patient 5 including PCR and gel electrophoresis
- Writing sections
- Preparing and revising the manuscript

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Bile Salt Export Pump-Reactive Antibodies Form a Polyclonal, Multi-Inhibitory Response in Antibody-Induced Bile Salt Export Pump Deficiency

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Progressive familial intrahepatic cholestasis type 2 (PFIC-2) is caused by mutations in *ABCB11*, encoding the bile salt export pump (BSEP). In 2009, we described a child with PFIC-2 who developed PFIC-like symptoms after orthotopic liver transplantation (OLT). BSEP-reactive antibodies were demonstrated to account for disease recurrence. Here, we characterize the nature of this antibody response in 7 more patients with antibody-induced BSEP deficiency (AIBD). Gene sequencing and immunostaining of native liver biopsies indicated absent or strongly reduced BSEP expression in all 7 PFIC-2 patients who suffered from phenotypic disease recurrence post-OLT. Immunofluorescence, western blotting analysis, and transepithelial transport assays demonstrated immunoglobulin (Ig) G-class BSEP-reactive antibodies in these patients. In all cases, the N-terminal half of BSEP was recognized, with reaction against its first extracellular loop (ECL1) in six sera. In five, antibodies reactive against the C-terminal half also were found. Only the sera recognizing ECL1 showed inhibition of transepithelial taurocholate transport. In a vesicle-based functional assay, transport inhibition by anti-BSEP antibodies binding from the cytosolic side was functionally proven as well. Within 2 hours of perfusion with antibodies purified from 1 patient, rat liver showed canalicular IgG staining that was absent after perfusion with control IgG. *Conclusions:* PFIC-2 patients carrying severe BSEP mutations are at risk of developing BSEP antibodies post-OLT. The antibody response is polyclonal, targeting both extra- and intracellular BSEP domains. ECL1, a unique domain of BSEP, likely is a critical target involved in transport inhibition as demonstrated in several patients with AIBD manifest as cholestasis. (HEPATOLOGY 2016;63:524-537)

Progressive familial intrahepatic cholestasis (PFIC) is a group of inherited cholestatic diseases of hepatocellular origin often starting in early infancy.[1,2] PFIC has an estimated incidence of 1-2 per 100,000 births[3] and can be divided into three subtypes with differences in clinical, biochemical, and histological features (PFIC-1-3). PFIC-1, also termed Byler's disease, is characterized by normal gamma-glutamyl

Abbreviations: ABCB11, adenosine triphosphate binding cassette transporter superfamily, subfamily B, member 11; AIBD, antibody-induced BSEP deficiency; ATP, adenosine triphosphate; BSEP, bile salt export pump; CsA, cyclosporin A; Cy3, cyanine 3; ECL1, first extracellular loop of BSEP; EYFP, enhanced yellow fluorescent protein; FIC1, familial intrahepatic cholestasis 1; GGT, gamma-glutamyl transpeptidase; Ig, immunoglobulin; MDR, multidrug resistance; mRNA, messenger RNA; mTECs, medullary thymic epithelial cells; NTCP, Na^+/taurocholate cotransporting polypeptide; OLT, orthotopic liver transplantation; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PFIC, progressive familial intrahepatic cholestasis; SDS, sodium dodecyl sulfate; TC, taurocholate; TCR, T-cell receptor; TSAs, tissue-specific antigens; WT, wild type.

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transpeptidase (GGT) levels and is associated with extrahepatic features such as pancreatitis, diarrhea, and hearing impairment. It is caused by mutations in the *ATP8B1* gene[4] encoding familial intrahepatic cholestasis 1 (FIC1), an aminophospholipid flippase. PFIC-2, like PFIC-1, is associated with low GGT levels and caused by mutations in the bile salt export pump (BSEP) expressed by the *ABCB11 (*adenosine triphosphate binding cassette transporter, subfamily B, member 11) gene. PFIC-2 is also referred to as BSEP deficiency.[5] PFIC-3 is caused by mutations in the *ABCB4* gene, which encodes the multidrug resistance protein 3, a phosphatidylcholine (PC) floppase.[6,7] When PC is reduced in bile, formation of mixed micelles is disturbed.[8] The resulting increase in free bile salts leads to injury of the biliary tree, resulting in elevated GGT levels.[7]

BSEP is exclusively expressed in the microvillar domains of the canalicular hepatocyte membrane[9]; it is the principal transporter of bile salts from hepatocytes into bile.[9,10] Missense, nonsense, indel, or splice-site mutations of *ABCB11* may impair bile salt transport as a result of reduced or absent protein expression, altered splicing, disturbed protein processing and trafficking, or reduced transmembrane transport.[10] Milder forms of BSEP deficiency are termed, depending on the clinical setting, benign recurrent intrahepatic cholestasis type 2, intrahepatic cholestasis of pregnancy, and drug-induced liver injury.[5]

PFIC-2 manifests in infancy with jaundice, severe intractable pruritus, diarrhea, complications of fat-soluble vitamin deficiency, and growth failure.[11] Serum alanine aminotransferase activity and bile salt levels are high; GGT levels are lower than expected for the degree of conjugated hyperbilirubinemia. Histological features of PFIC-2 at presentation are lobular and portal fibrosis and inflammation, with intralobular cholestasis accompanying hepatocellular disarray, giant-cell change, and necrosis, ascribed to retention of bile salts within hepatocytes.[3,12] In many patients, BSEP expression is absent or reduced on immunohistochemical or immunofluorescent study of liver tissue.[13-15] Without treatment, PFIC-2 progresses to end-stage liver disease, sometimes with hepatobiliary malignancy,[15,16] leading to death in childhood.[17] Only orthotopic liver transplantation (OLT) yields good outcome in most PFIC-2 patients.[5,11]

In some patients with PFIC-2, a phenotype suggesting recurrence of BSEP deficiency develops post-OLT. In 2009, we described a girl with PFIC-2 caused by lack of BSEP expression resulting from three homozygous nucleotide substitutions in *ABCB11*. After OLT, her serum contained high-affinity antibodies directed against the first extracellular loop (ECL1) of BSEP, with strong canalicular immunoglobulin (Ig) G deposits in two consecutive liver allografts.[18] We and others proposed that BSEP-reactive antibodies cause cholestasis by inhibiting BSEP transport activity.[18,19] Since then, only a few further cases of antibody-induced BSEP deficiency (AIBD) have been reported.[19-22] We here present a detailed analysis of the BSEP-reactive antibody repertoire causing AIBD, and of these antibodies' functional effects, in sera from 7 more AIBD patients.

Patients and Methods

Additional method descriptions can be found in the Supporting Information.

Patients. Charts of all patients in this study were reviewed. Table 1 summarizes patient details, genetic information, course of disease recurrence, and treatment regimens. Serum samples for antibody detection were stored at -20°C. A liver sample from a noncholestatic patient undergoing partial hepatectomy because of liver metastasis of a colon carcinoma was used for detecting antibodies in patients' serum with canalicular reactivity. Serum of a healthy 35-year-old man was used as a non-OLT control throughout this study. All patients or their parents gave written consent for investigation. This

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Table 1. Data of AIBD and Non-AIBD Control Patients

Patient	1	2	3	4	5	6	7	Control (8)	Control (9)
AIBD	Yes	Yes	Yes	Yes	Yes	Yes	Yes	PFIC-2 non-AIBD	non-PFIC-2 non-AIBD
YOB, sex Born in	2002, male Germany	2004, male Saudi Arabia	2002, female USA	2009, male Lithuania	2008, male Russia	2007, male Japan	1999, female Saudi Arabia	2009, male Germany	1996, male Germany
BSEP mutations (protein effect)	c.150+3A>C (aberrant splicing) c.2783_2787dup5 (p.K930Efs79X)	c.2371C>T (p.Q791X)	c.1723C>T (p.R575X) [2] c.2178+1G>T (aberrant splicing)	c.2926C>T (p.Q976X)	Coding exons 6-9 missing	c.953-954delAA (p.K318Rfs33X) c.1425T>A (p.C475X)	c.3213+1delG (p.D1072Tfs25X)	c.1259T>A (p.I420N)c.1445A>G (p.D482G)	None
Zygosity	comp het[1]	homozygous	comp het	homozygous	homozygous	comp het	homozygous	comp het	
BSEP expression before OLT	Reduced	Likely absent*	Absent	Absent	Absent	Absent	Absent	Likely present*	Normal
Age at OLT, years	3.1	1.9	1.6	1.4	1.3	1.9	3.1	1.6	3.3
Recurrence after OLT, months	45	37	3	9	42	20	108	2	24
Symptoms and signs at time of recurrence	Diarrhea, pruritus	Diarrhea, poor weight gain, progressive jaundice/ pruritus	Pruritus, diarrhea, abdominal pain, vomitus, fatigue, fever	Jaundice, pruritus, ↑transaminases	Jaundice, pruritus, ↑INR, poor weight gain	Jaundice	Jaundice, pruritus	Vomiting, dehydration. diarrhea ↑transaminases	Recurrent acute rejection ↑transaminases
GGT at time of recurrence, U/L	16	43	Not available	36	31	108	10	86	Not available
Potential AIBD triggering factors	None identified	Episode 1: Tac dose↓, *de novo* HBV infection Episode 2: change of MMF formulation	High doses of antibiotics (Cotrim, azithromycin, amoxicillin)	Low EBV titer w/o clinical symptoms, fluctuation in CsA level, acute rejection with toxic injury	Fluctuation in CsA level, mild rejection	Immunoadsorption + Tac + steroid↓	None identified	–	–
Initial IS	Basiliximab + CsA + steroid	Tac + steroid	Tac + steroid	Basiliximab	Basiliximab + CsA + steroid	Tac + steroid	Tac + steroid	CsA	CsA
IS at time of recurrence	Tac	↑Tac dose + steroid	Tac + steroid	CsA + steroid	CsA	Tac + steroid	Tac	Tac	CsA
IS for treatment of recurrence	Trim	MMF + Tac	↑Tac dose + steroid	Tac + azathioprin	CsA + everolimus	Tac + steroid + mizoribine	steroids + MMF + Tac	Tac	CsA + steroid (later + Tac)
Additional treatment	Immunoadsorption + rituximab	Entecavir	Metronidazol	Steroid pulse + immunoadsorption, plasmapheresis + rituximab	Immunoadsorption, plasmapheresis + rituximab	Steroid pulse + plasmapheresis + rituximab	–	–	Plasmapheresis
IgG deposits in transplant biopsies	Yes	None detected	Yes	Yes	Yes	Not available	Yes	None detected	None detected
Maximum anti-canalicular antibody titer	1:16,000	1:1,000	1:10,000	1:8,000	1:8,000	1:16,000	1:6,400	1:100	1:50

Abbreviations: comp het, compound heterozygous; Cotrim, trimethoprim/sulfamethoxazole; CsA, cyclosporin A; EBV, Epstein-Barr virus; HBV. Hepatitis B virus; INR, international normalized ratio; IS, immunosuppression; MMF, mycophenolate-mofetil; Tac, tacrolimus; Trim, trimethoprim; YOB, year of birth.

*Native liver biopsies were not examined for BSEP expression.

study was performed according to the guidelines of the declaration of Helsinki.

ABCB11 ***Sequencing From Genomic DNA.*** Genomic DNA was extracted using the QIAamp DNA Blood Kit (Qiagen, Hilden, Germany). All 27 coding exons of *ABCB11*, with exon-intron transitions, were amplified and sequenced as previously described.[23] Data were compared with reference sequence NM_003742.2 (Gene ID: 8647); variations were verified by resequencing and designated according to the Human Genome Variation Society guidelines.[24]

Immunofluorescence Staining of Liver Cryosections. As previously described,[14,23] sera diluted up to 1:16,000 were tested for immunoreactivity using methanol-fixed cryosections of noncholestatic human liver. A murine monoclonal antibody against multidrug resistance-associated protein 2 (M2I-4; Alexis, Grünberg, Germany) served as a canalicular marker (used at 1:25). Goat anti-human IgG/cyanine 3 (Cy3; Jackson ImmunoResearch Laboratories, West Grove, PA) and goat anti-mouse IgG/Alexa Fluor 488 (Invitrogen, Karlsruhe, Germany) were used as secondary antibodies (each at 1:500). For detection of canalicular IgG deposits, fixed cryosections of patient transplant liver biopsies and perfused rat liver were immunostained as described above. BSEP expression in native liver of patient 1 was analyzed using the K24 antiserum raised against the C-terminus of human BSEP.[25] Specimens were analyzed with a LSM 510 Meta confocal laser scanning microscope (Zeiss, Jena, Germany).

Analysis of Patient Sera for anti-BSEP Reactivity in Transiently Transfected HEK293 Cells. HEK293 (human embryonic kidney) cells were transiently transfected with wild type (WT) BSEP-EYFP (enhanced yellow fluorescent protein), BSEP-1-659-EYFP, or BSEP-659-1321-EYFP[18] using X-treme Gene HP (Roche Applied Science, Mannheim, Germany). After 48 hours, cells were fixed with ice-cold methanol for 30 seconds and stained with human sera (1:50) for 1 hour using goat anti-human IgG/Cy3 as the secondary antibody (1:500). The monoclonal anti-BSEP antibody, F-6 (Santa Cruz Biotechnology, Heidelberg, Germany), served as a positive staining control (1:100), with goat anti-mouse IgG/Cy3 as secondary antibody (1:500). Cell nuclei were stained with Hoechst 34580 or 4′,6-diamidino-2-phenylindole (Invitrogen). Cells were visualized by confocal laser-scanning microscopy.

Cloning, Expression, and Purification of the First Extracellular Loop of Human BSEP. The first extracellular loop of human BSEP, with an N-terminal hexahistidine tag for purification, was expressed as a C-terminal EYFP fusion protein (ECL1-EYFP) in *Escherichia coli* (see Supporting Information for details of cloning strategy, expression, and purification).

Analysis of Patient Sera for Anti-BSEP Reactivity by Western Blotting. Ten micrograms of *Sf*9 membrane vesicles (either empty control vesicles or vesicles containing human BSEP; Solvo Biotechnology, Budapest, Hungary) or 0.5 μg of purified ECL1-EYFP were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. For detection of BSEP-reactive IgG, membranes were first incubated for 1 hour with patient or control serum (1:100) followed by goat anti-human IgG/horseradish peroxidase (1:10,000) as the secondary antibody. The anti-BSEP antibody F-6 was used at 1:1,000 as a positive control.

Transepithelial Transport Assays. Generation of LLC-PK_1 cells stably expressing BSEP and/or NTCP (the Na^+/taurocholate cotransporting polypeptide, here facilitating cellular uptake of taurocholate [TC]) is described in the Supporting Information. For transepithelial transport studies, cells were seeded at high density onto ThinCert™ cell-culture inserts (0.4-μm pore size; Greiner Bio One, Frickenhausen, Germany) and cultured for 2 days. Cell monolayers were routinely used at a net transepithelial electric resistance of at least 500 Ωcm^2. To compensate for the lack of CO_2-enriched atmosphere during the transport inhibition studies, both apical and basal medium were exchanged for 1 and 2 mL, respectively, of 25 mM of HEPES buffered medium (pH, 7.5). Then, 100 μL of apical medium were replaced by either patient or control serum (thus diluted 1:10) for overnight incubation of the apical face of the cell monolayer. Alternatively, 4 μg of F-6 antibody, which does not recognize ECL1, was used as a negative control. A mixture of 1 mM of unlabeled sodium TC (Sigma-Aldrich, St. Louis, MO) and 200 μM of [^{3}H]-TC (PerkinElmer, Waltham, MA) at a ratio of 1:100 was added to the basal medium to a final concentration of 5 μM, and cells were incubated for 1 hour at 37°C. Then, cell-culture inserts were removed, apical and basal media were collected, and inserts were gently washed three times on their apical and basal sides with 1 and 2 mL of ice-cold phosphate-buffered saline (PBS), respectively. Finally, the cell monolayer was lysed in 500 μL of 0.5 M of NaOH with 0.1% (w/v) SDS. Then, 250 μL of each cell lysate (or 500 μL of medium) was mixed with 4 mL of liquid scintillation fluid (Ultima Gold; BD Biosciences, Heidelberg, Germany) for 20 seconds before measurement in a scintillation counter (Packard Instruments, Frankfurt, Germany).

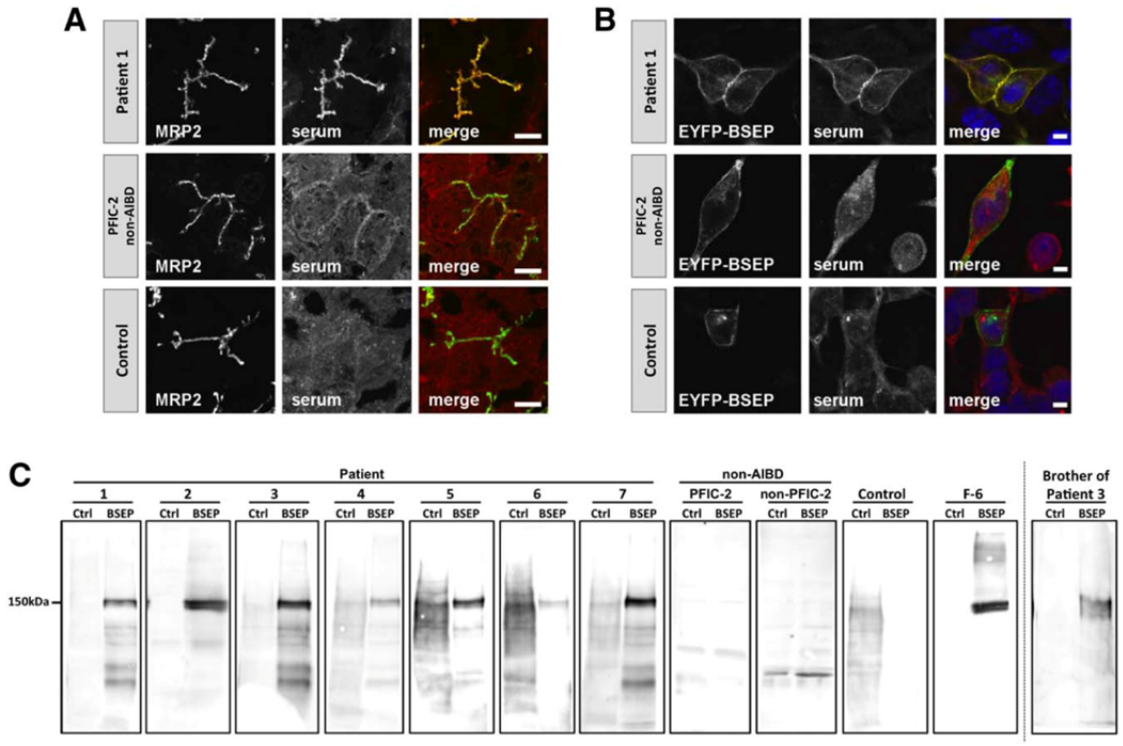

Fig. 1. AIBD sera specifically recognize human BSEP. (A) Post-OLT sera from AIBD patient 1 and a non-AIBD, PFIC-2 control yield canalicular staining patterns in cryosections of healthy human liver whereas naïve, non-OLT control serum does not. MRP2 was immunostained as a canalicular marker. The non-AIBD control sera only stain up to dilutions of 1:100, whereas all AIBD sera stain at maximal dilutions ranging from 1:1,000 up to 1:16,000. (B) All AIBD sera recognize human full-length BSEP-EYFP expressed in HEK293 cells (patient serum 1 shown). In contrast, non-AIBD sera do not recognize BSEP (non-AIBD PFIC-2 control shown). Bars = 10 μm. (C) Likewise, only the seven AIBD sera detect recombinant human BSEP (expressed in *Sf*9 cells) on nitrocellulose membranes (Ctrl, empty control vesicles; BSEP, vesicles containing human BSEP). The non-AIBD, PFIC-2 control likely had some BSEP expression before OLT and did not develop a subsequent humoral response whereas the non-AIBD, non-PFIC-2 control had normal BSEP expression and suffered from an unrelated liver condition. Notably, the asymptomatic brother of patient 3 who had the same homozygous PFIC-2 mutation had serum reactivity against BSEP. F-6, monoclonal BSEP antibody. Apparent molecular weight of BSEP is 150 kDa. The full data set of all patients is shown in Supporting Figs. 1 and 2.

BSEP Inhibition by Patient IgG in Vesicular Transport Assays. Transport activity of human recombinant BSEP was measured in *Sf*9 membrane vesicles. For incorporation of antibodies into the vesicular lumen, an adapted freeze-thaw technique[26] was applied. Briefly, purified patient IgG was added to thawed vesicles by gentle pipetting, and the preparation was repeatedly snap-frozen and allowed to thaw slowly on ice. In order to study BSEP transport inhibition from the "intracellular" (extravesicular) side alone, IgG was added as described above and samples were kept on ice as long as those subjected to freeze-thaw cycles.

Rat Liver Perfusion. Animals received care according to the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23, revised 1985; and Australian Psychological Society guidelines). The study protocol was approved by the local authorities. Livers of male Wistar rats (body weight approximately 150 g) were perfused in a nonrecirculating system as previously described.[27] Liver weight was estimated to be 3.5% of total body weight; flow rate was adjusted to 3.7 mL/(g liver*min). TC was perfused throughout the experiment at a concentration of 100 μM in order to saturate bile salt transport. After 30 minutes, Krebs-Henseleit buffer containing 6.57 mg/L of either control or patient IgG was perfused. After 120 minutes, the right ventral liver lobe was harvested and snap-frozen in liquid nitrogen for cryosectioning and immunostaining.

Statistical Analysis. Column diagrams were prepared using Prism (version 5.0a, GraphPad) and significances shown in Fig. 6 were calculated using the Student *t* test function (unpaired; two-sided *P* values).

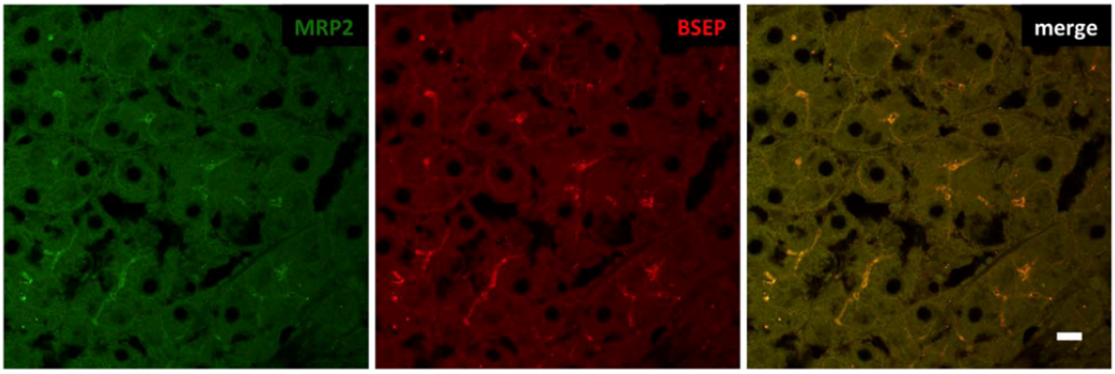

Fig. 2. Canalicular wildtype BSEP expression is clearly detectable in the native liver of AIBD patient 1. The K24 antiserum used for BSEP detection was raised against the carboxy-terminus of BSEP and thus only recognizes the full-length, wildtype gene product from the splice-site-mutated *ABCB11* allele. MRP2 was immunostained as a canalicular marker. Bar = 10 μm.

Results

AIBD Sera Specifically Recognize Human BSEP With a High Titer. We examined sera from 9 patients (1) diagnosed with PFIC-2 or low-GGT intrahepatic cholestasis, (2) that had undergone OLT, and (3) who showed symptoms compatible with disease recurrence. Their clinical and laboratory data are summarized in Table 1. Serum samples taken at the time of symptom recurrence were tested for canalicular immunoreactivity with histopathological sections from normal human liver (Fig. 1A; see Supporting Fig. 1 for the complete data set). All nine post-OLT sera, but not naïve control serum, yielded a canalicular staining pattern, albeit at very different titers (Table 1). Anticanalicular antibodies were detectable at serum dilutions from 1:1,000 up to 1:16,000 for 7 patients (numbered 1 to 7), whereas serum titers of canalicular reactivity were very low with a maximum of 1:100 for 2 patients (numbered 8 and 9). To test for specific reactivity against BSEP, serum samples were then analyzed by immunofluorescence using HEK293 cells transiently transfected with human BSEP-EYFP. All seven high-titer sera recognized recombinant BSEP in transfected cells (Fig. 1B and Supporting Fig. 2), that is, they contained anti-BSEP antibodies. This was further confirmed by immunoblotting analysis, in which only the sera 1-7 specifically detected recombinantly expressed human BSEP (Fig. 1C). Therefore, AIBD was diagnosed in 7 of 9 patients collected by the three criteria stated above (patients 1 to 7).

In contrast, sera from patients 8 and 9 did not recognize recombinant BSEP (immunofluorescence and immunoblotting) and were classified as non-AIBD cases. Patient 8 was a case of PFIC-2 caused by two missense mutations (Table 1) treated by OLT. He suffered from a cholestatic episode with elevated GGT-levels obviously not caused by a humoral anti-BSEP response. Consequently, his serum was used as a PFIC-2, non-AIBD control, which is representative of the majority of tranplanted, asymptomatic PFIC-2 patients.[18] Patient 9 was initially diagnosed as having low-GGT PFIC and was soon after transplanted because of his severe condition. A biopsy of his native liver showed normal canalicular expression of FIC1, BSEP, and multidrug resistance (MDR) 3 (not shown). PFIC-1 and PFIC-2 were eventually excluded by sequencing of the *ATP8B1* and *ABCB11* genes. Hence, his serum was used as a non-PFIC-2, non-AIBD control. Notably, patient 3 had a brother who suffered from the same PFIC-2 mutations as his sister and therefore had to be transplanted at the age of 1.5 years. Although he remained asymptomatic, anti-BSEP antibodies were detected in his serum at a titer of 1:1,600 (western blotting in Fig. 1C) 3 years post-OLT.

AIBD Patients Lack Normal BSEP Expression in Native Liver. Absence of BSEP expression in the native liver has been postulated to be a prerequisite for the development of anti-BSEP antibodies.[18-20,22] In line with this, BSEP was not detectable by immunohistochemistry in native livers of patients 3-7 (Table 1). Patient 2 was homozygous for the nonsense mutation p.Q971X, thus absence of BSEP in his native liver can be assumed. Only in patient 1 was a residual wildtype BSEP expression clearly demonstrated (Fig. 2), as shown by the use of the K24 antibody, which recognized the carboxy (C)-terminus of BSEP. In this patient, one *ABCB11* allele was affected by a c.150+3A>C splice-site mutation, whereas a five-nucleotide insertion

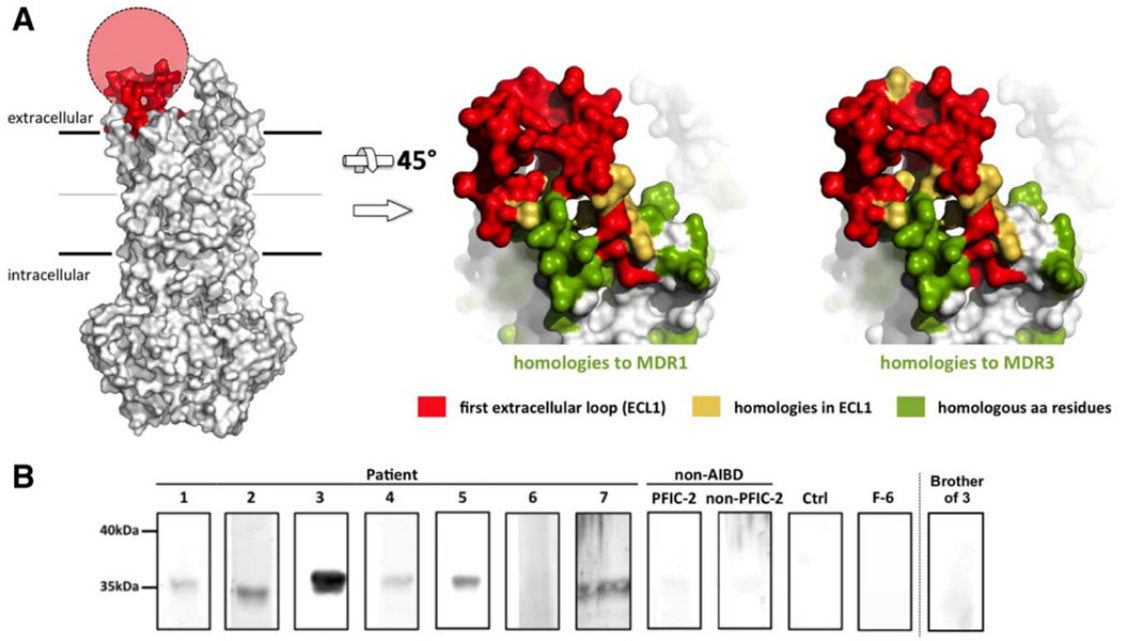

Fig. 3. The first extracellular loop of BSEP is a recurrent, likely critical target of the AIBD response. (A) A homology model of BSEP based on the crystal structure of $SAV1866_2$ suggests that ECL1 (61 amino acid residues [aa]; aa that could be modeled are shown in red; the red circle is intended to give an approximate impression of its actual size) is both a large and potentially accessible epitope. It is unique to BSEP and not found in the closely related MDR3 (ABCB4) or MDR1 (ABCB1). Only a few aa at the edges of ECL1 are conserved between ECL1 and the corresponding loops of MDR1/MDR3 (yellow), whereas a comparison of the remainder of BSEP with its homologs shows higher conservation (green). (B) The ECL1 of BSEP was expressed as a C-terminal EYFP fusion protein in *E. coli* and purified by its N-terminal hexahistidine tag. The epitope is frequently recognized by the immune response of AIBD patients; six of the seven BSEP-reactive antisera detected it when blotted onto nitrocellulose membranes. Interestingly, ECL1 was not detected by the BSEP-reactive serum response from the transplanted, asymptomatic brother of patient 3.

(c.2783_2787dup5) on the other resulted in a frameshift causing the hypothetical protein truncation p.K930Efs79X (Table 1). The remainder of BSEP detected by K24 in his native liver was therefore expressed from the allele carrying the intronic splice-site mutation, encoding the WT protein with its intact C-terminus.

Changes in Immunosuppression, Viral Infections, and Episodes of Acute Rejection May Trigger AIBD. We reviewed the clinical history of all seven AIBD cases in order to identify triggering factors for the development of BSEP-reactive antibodies post-OLT (summarized in Table 1). Cholestasis began to recur from 3 to 108 months post-transplantation. In four of the seven cases, this coincided with a change or decrease of immunosuppressants. In two of these, subsequent transplant rejection was reported. Also, two cases suffered from viral infections (Epstein-Barr virus, hepatitis B virus) at the time of phenotypic disease recurrence. In contrast, neither ischemia reperfusion injury nor biliary injury was documented for any of the AIBD patients or non-AIBD controls, while bouts of acute rejection preceding any potential trigger events were only reported for 1 AIBD patient (see Supporting Table 1).

The First Extracellular Loop of BSEP Is a Common Target of the Antibody Response in AIBD. Because the first AIBD case study[18] identified a sequence within ECL1 as the target of the patient's BSEP-reactive antibody response, we screened all AIBD sera for specific reactivity toward ECL1. Topological considerations predict that ECL1 is the largest extracellular domain of BSEP, with 61 amino acid residues[18,28]; by comparison, ECL2 to ECL6 comprise 4, 20, 20, 4, and 4 residues, respectively. This domain is unique to BSEP and has only minimal homologies to the corresponding loop of MDR1 and MDR3 (MDR1: 18%; MDR3: 21% identity; Fig. 3A). ECL1 was recombinantly expressed in *E. coli* as a fusion protein with a hexahistidine and an EYFP tag (Supporting Fig. 3). Using the purified recombinant epitope, we found ECL1-reactive IgG in nearly all AIBD sera, with the exception of patient 6. In

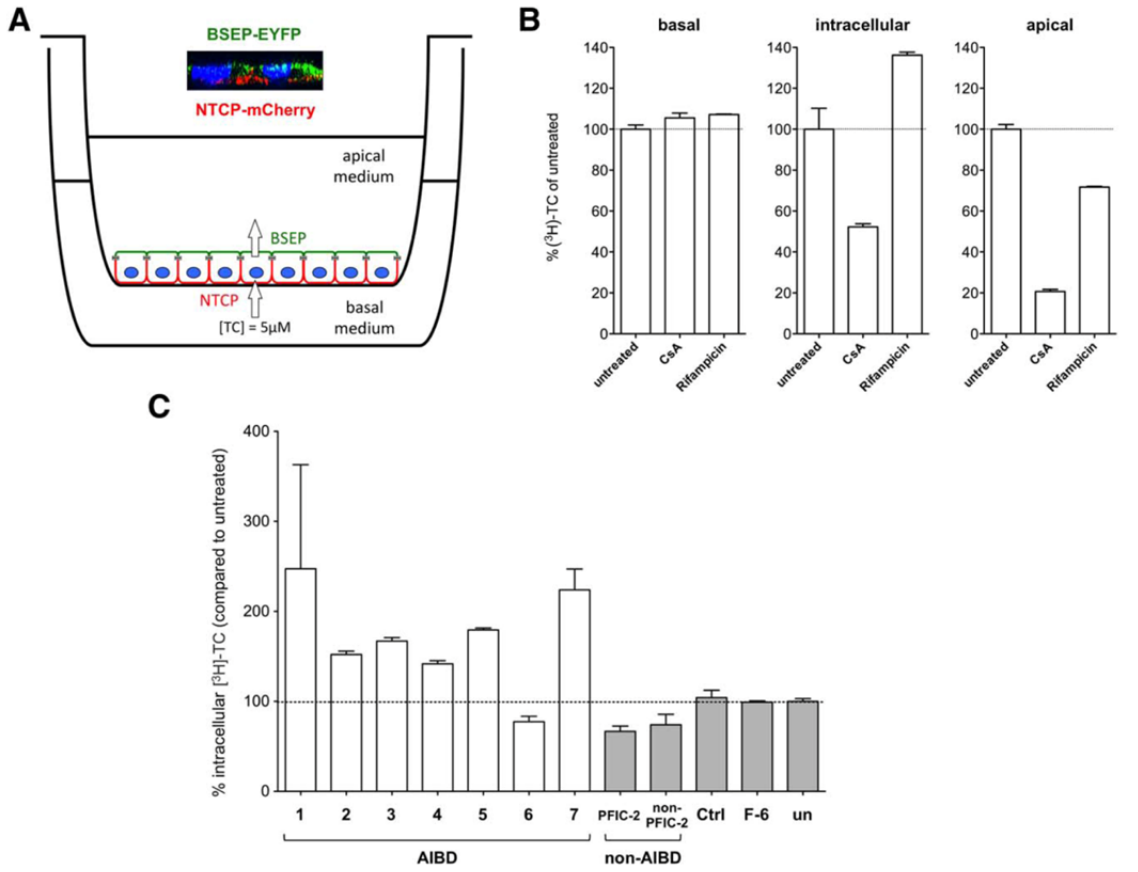

Fig. 4. AIBD sera inhibit BSEP-mediated transport in a polarized cell culture model. (A) Concept of the transepithelial transport assay. Inset, X/Z-section of the NTCP-mCherry (red)/BSEP-EYFP (green) double-transduced LLC-PK$_1$ cell line used in this assay. The intracellular volume of the monolayer is estimated to be around five orders of magnitude less than those of the apical and basolateral compartments. Accordingly, the intracellular equilibrium generated by influx from the source (basal compartment) and efflux to the sink (apical compartment) is much more sensitive to extracellular inhibition of TC efflux by BSEP whereas both the source and the sink compartment do not show significant changes upon apical serum incubation. (B) Inhibition of BSEP and NTCP/BSEP activity by rifampicin and CsA, respectively, indicates functionality of both transporter proteins. Cell monolayers were preincubated for 1 hour with these agents, applied apically. (C) Inhibition of BSEP-mediated transepithelial transport of TC in LLC-PK$_1$ cells by overnight apical incubation with AIBD and non-ABID control sera. Only incubation of cell monolayers with AIBD sera led to an increase in intracellular TC (imported by NTCP from the basal compartment). Notably, serum from patient 6, showing no BSEP inhibition, was the only AIBD patient serum incapable of detecting ECL1 (see Fig. 3B). Columns and error bars represent the mean and standard error of two independent measurements. un, untreated.

contrast, both non-AIBD control sera failed to recognize ECL1 (Fig. 3B). Strikingly, the transplanted (PFIC-2), asymptomatic brother of patient 3 had no ECL1 reactivity in his serum sample whereas his serum antibodies recognized full-length BSEP. The lack of reactivity in all controls also demonstrates that the human IgG pool is devoid of unspecific reactivity against the EYFP domain of the fusion protein.

AIBD Sera Inhibit Human BSEP From the Extracellular Side. The inhibitory capacity of anti-BSEP antibodies was analyzed in a cell- and a vesicle-based transport assay. We generated a double-transduced LLC-PK$_1$ cell line expressing human NTCP at the basolateral membrane and BSEP at the apical membrane (Fig. 4A), resulting in the vectorial transport of [^{3}H]-TC from the basal into the apical compartment (Fig. 4A,B). Untransduced LLC-PK$_1$ cells, as with cells expressing exclusively NTCP or BSEP, showed no significant transepithelial [^{3}H]-TC transport (Supporting Fig. 4). In double-transduced cells, inhibition of both BSEP and NTCP activity by 50 μM of cyclosporin A (CsA) resulted in a reduced intracellular uptake of [^{3}H]-TC, whereas inhibition of BSEP activity alone by 50 μM of rifampicin led to an increase in intracellular [^{3}H]-TC content (Fig. 4B). When the apical face of the double-transduced LLC-PK$_1$ cells was preincubated overnight

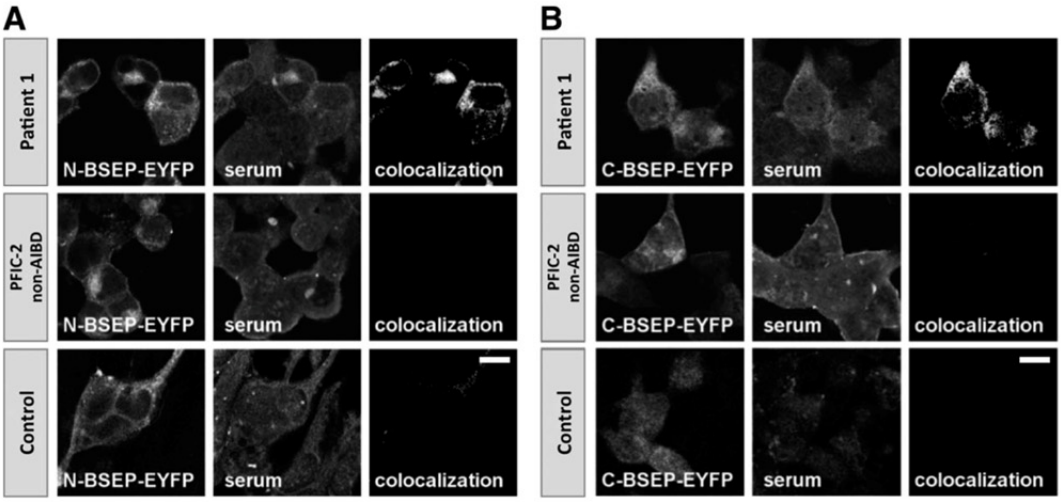

Fig. 5. AIBD sera stain HEK293 cells transiently expressing the N- or C-terminal halves of BSEP. (A and B) As exemplified by patient 1, all AIBD sera except those from patients 2 and 5 recognize both halves; the exceptions show little or no reactivity against the C-terminal half. In contrast, the non-AIBD controls and naïve control serum fail to detect either half of BSEP. Likely owing to its lack of the native N-terminal signal anchor sequence, the C-terminal half is largely retained intracellularly. Bars = 10 μm.

with AIBD sera, intracellular amounts of [^{3}H]-TC increased; this was ascribed to antibody-mediated inhibition of BSEP activity (also see Supporting Fig. 4 for an illustrative comparison of the different transgenic LLC-PK$_1$ cell lines). This BSEP inhibition was observed in the presence of all AIBD sera except patient 6 (Fig. 4C), who lacked serum reactivity against ECL1 (see Fig. 3B). In contrast, BSEP activity was not inhibited by the non-AIBD controls, naïve control serum or the monoclonal anti-BSEP antibody, F-6, which was raised against the N-terminal 180 amino acid residues but did not recognize ECL1.

AIBD Antibodies Detect Multiple Epitopes of BSEP and Inhibit Its Function From Intra- and Extracellular Sides. Whether AIBD antibodies react with one or several distinct domains of BSEP is unknown. To investigate this, we stained HEK293 cells transiently expressing either the N- or C-terminal half of BSEP[18] with patients' sera. As shown in Fig. 5A for patient 1, all AIBD sera recognized the N-terminus of BSEP, which contains ECL1 (for the complete set, see Supporting Figs. 5 and 6). The majority of sera also recognized the C-terminal half of BSEP (Fig. 5B), with two sera (2 and 5) showing little or no reactivity. Non-AIBD sera and the naïve control failed to recognize either the N- or C-terminal BSEP half.

Where sample material sufficed, total IgG from AIBD patient serum was purified for *in vitro* inhibition studies using human recombinant BSEP expressed in insect cell membrane vesicles (Fig. 6). To differentiate between intra- and extracellular inhibition, we measured transport activity in vesicles prepared with or without freeze/thaw cycles (Fig. 6A). By applying repeated freeze/thaw cycles, the antibodies were incorporated into the vesicular lumina, thus exposing both the intra- and extracellular sides of BSEP to patients' IgGs. In vesicles, which were not prepared by freeze/thaw cycles, BSEP was only accessible from the extravesicular side. The variable IgG concentrations reflect variable amounts of available serum, which, in some cases, was limiting (Fig. 6B; see IgG concentrations). Using this assay, we could confirm that all tested AIBD sera demonstrated IgG-mediated BSEP inhibition (Fig. 6B, white bars), ranging from approximately 6.9% to 21.6% per mg/mL total patient IgG. In contrast, the low-concentration IgG from patient 7 only showed a slight inhibition. IgG amounts from patient 6 allowed only for one assay mode, so the freeze/thaw variant was chosen for maximal accessibility of BSEP. In the remaining three AIBD IgG pools (patients 2-4), BSEP inhibition was significantly more effective when the transporter was accessible from both sides (white bars) than when it was accessible from the "intracellular" (extravesicular) side only (gray bars). Taken together, our findings demonstrate that BSEP recognition and inhibition are indeed aspects of a polyclonal antibody response directed against both intra- and extracellular portions of BSEP.

BSEP-Reactive IgG Rapidly Decorates Canalicular BSEP in Perfused Rat Liver. So far, strong IgG deposits in the canalicular space of liver from an AIBD

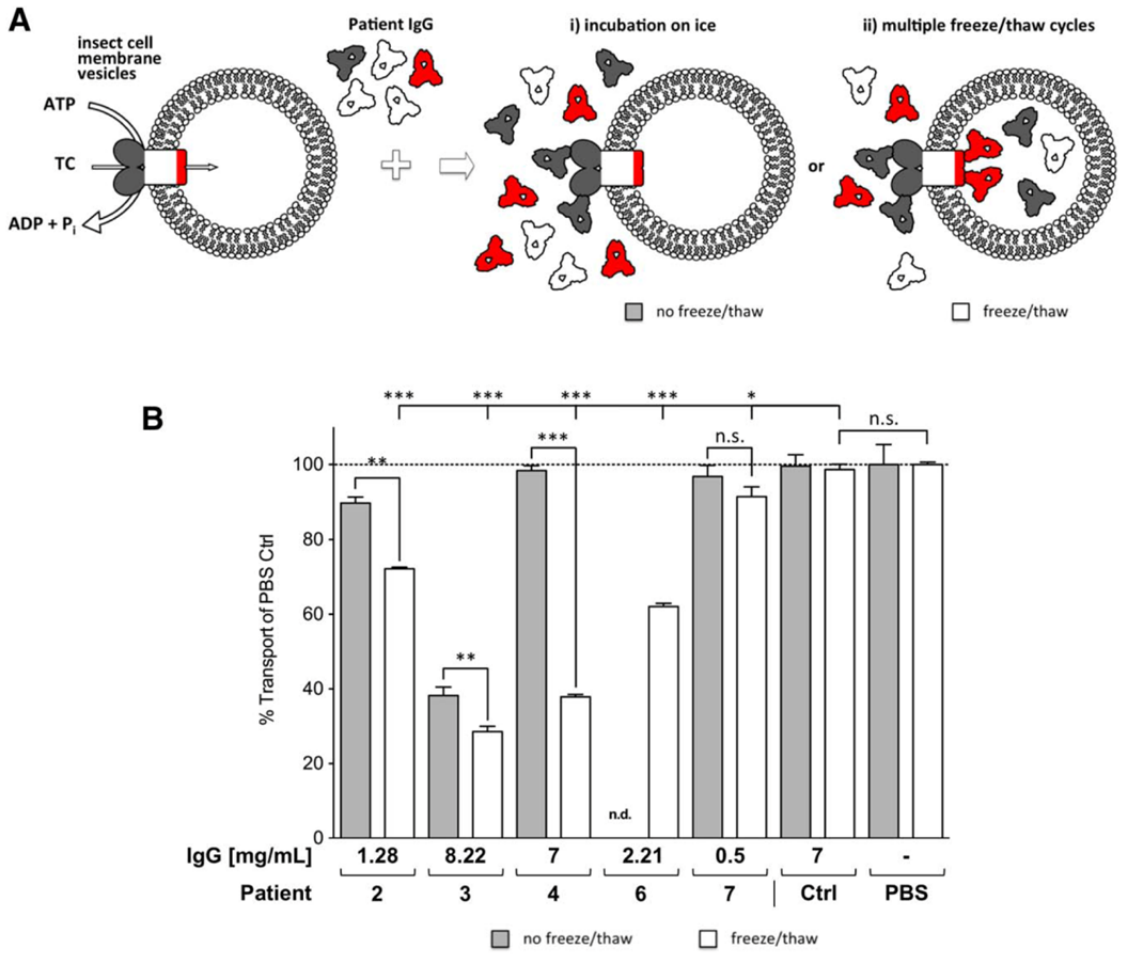

Fig. 6. BSEP-mediated TC transport in a vesicular assay is inhibited from the extra- as well as the intraluminal side. (A) Concept of the different vesicle incubation methods used in this assay. The red and dark gray antibodies recognize extra- and intracellular epitopes of BSEP, respectively. In essence, BSEP may be inhibited either (1) solely from its intracellular side or (2) from both its intracellular and its extracellular side. [^{3}H]-TC uptake by rapid filtration can be measured only using inside-out vesicles such as those shown here, which expose nucleotide-binding domains to ATP-containing buffer. (B) Purified patient IgGs were incorporated into the lumina of *Sf*9 membrane vesicles containing human BSEP by multiple freeze/thaw steps (white columns). Thus, BSEP-reactive antibodies can bind their targets on the outside of assayable (i.e., inside-out) vesicles as well as on the intraluminal ("extracellular") side (see (A, ii). Alternatively, BSEP vesicles were incubated on ice for the same duration with same amounts of patient IgG (gray columns). Here, only extravesicular ("intracellular") parts of BSEP are accessible to patient antibodies (see A, i). Data in (B) represent mean and standard error of three independent measurements and were analyzed using the Student *t* test (unpaired; two-sided *P* values). $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$; n.s., not significant.

patient have only once been described.[18] In our cohort, biopsy materials from allograft livers were available for all AIBD cases except patient 6. We could detect a clear canalicular IgG staining pattern using an anti-human IgG antibody in all available AIBD liver samples (Supporting Fig. 7). In contrast, no IgG was detected in the canaliculi of transplant biopsies from the non-AIBD controls. To find out whether and how fast BSEP-reactive IgG reaches the canalicular space in intact organs, rat livers were perfused *in situ* for 2 hours (Fig. 7). Rat Bsep is highly homologous to human BSEP and it has been shown previously that human AIBD sera recognize rat Bsep.[18,19] Using purified total IgG from patient 4 (16 mg), we observed a distinct, patch-like canalicular staining (Fig. 7B) that was absent when using an equal amount of naïve control IgG (Fig. 7A). This indicates that anti-BSEP IgG antibodies rapidly reach the canalicular space. Of note, we did not observe a

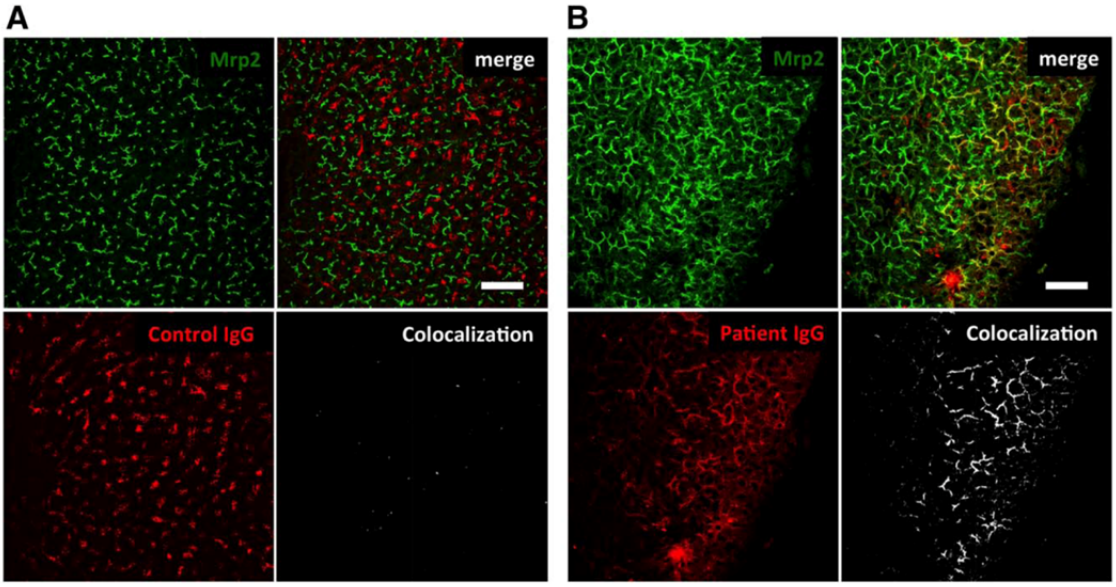

Fig. 7. BSEP-reactive IgG rapidly decorates canalicular BSEP in perfused rat liver. Rat livers were perfused *in situ* for 2 hours with equal amounts of total IgG purified from either (A) a naïve control or (B) AIBD patient 4 (from whom sufficient IgG could be obtained by purification from serum retained on immunoadsorption columns). Only perfusion with BSEP-reactive IgG resulted in a canalicular pattern of colocalization of variable extent (yellow in the merge panels) of human antibodies (red) with the canalicular marker Mrp2 (green). Naïve IgG stained only the sinusoids. Colocalized pixels are shown in white in the lower right panels. Bars = 50 μm.

change in bile salt-dependent bile flow (not shown), which likely is owing to incomplete antibody saturation of canalicular BSEP during the short time of perfusion.

Discussion

The development of anti-BSEP antibodies in PFIC-2 patients post-OLT was first described in 2009 by us and others.[18,19] This study demonstrates, for the first time, the polyclonal nature of the BSEP-reactive antibody repertoire, which targets epitopes on both N- and C-terminal halves of BSEP. Furthermore, anti-BSEP antibodies bind to both intra- and extracellular domains, inhibiting bile salt transport (Fig. 6). However, on intact hepatocytes only extracellular epitopes are accessible to antibodies from the canalicular lumen. Accordingly, only antibodies directed against extracellular structures can inhibit BSEP function *in vivo* and are thus of pathophysiological importance. Notably, Jara et al. demonstrated *in vitro* inhibition of BSEP-mediated transport after incubation of insect cell membrane vesicles with AIBD patient sera.[19] In the rapid filtration assay that they used, the buffer solution bathing the vesicles contained adenosine triphosphate (ATP), magnesium, and the inhibitory antibodies. Only when vesicular membranes are oriented inside-out, exposing the intracellular nucleotide-binding domains of BSEP to ATP, BSEP can transport the radiolabeled substrate into the vesicular lumen (compare Fig. 6A). Hence, the inhibition that Jara et al. demonstrated for three BSEP-reactive sera could only result from binding of IgG reactive toward intracellular BSEP domains. Upon inclusion of BSEP-reactive IgG within the vesicular lumen, we found a significantly higher decrease in transport, indicating that BSEP was inhibited from both the intra- and extracellular sides (Fig. 6B).

Unlike isolated vesicles, the closed polarized LLC-PK_1 cell monolayer only permits binding of antibodies to extracellular epitopes on intact cells. Preincubation of the apical cell surface with AIBD sera led to an increase in intracellular TC concentrations in this transport assay (Fig. 4C). That this effect was moderate may be owing to the small amounts of patient IgGs used, resulting in only partial or incomplete BSEP inhibition. Additionally, the extracellular matrix of the dense LLC-PK_1 cell monolayer may also reduce antibody access to BSEP. However, because the intracellular volume of the monolayer is much smaller than that of both medium-containing compartments, even small changes in TC influx or efflux are expected to result in a measurable change in cellular [^{3}H]-TC content.

A BSEP homology model based on the $SAV1866_2$ ABC transporter structure[29] indicates that ECL1 is the largest domain of BSEP accessible to antibodies from

the extracellular space (Fig. 3A). This domain is exclusively found in BSEP (ABCB11, also called sister of P-glycoprotein), but not in any other of its close relatives such as MDR1 (ABCB1, P-glycoprotein) and MDR3 (ABCB4; Fig. 3A, right panels) and likely has some unique functional role yet to be uncovered. We previously identified this domain as the target of an AIBD patient's anti-BSEP response.[18] Indeed, ECL1 was found to be a recurrent and likely critical target of the AIBD antibody response (Fig. 3). This is also supported by our finding that the serum of patient 6, devoid of reactivity against ECL1, failed to inhibit BSEP from the extracellular side in the transepithelial transport system (Fig. 4). Unfortunately, no biopsy material was available to check this patient's allograft for canalicular IgG deposits. Ultimately, some uncertainty remains whether this patient suffered from "true" AIBD because he eventually died from severe bleeding complications caused by a necrotizing pancreatitis 1.5 years after the detection of anti-BSEP antibodies. The notion of ECL1 as a critical extracellular epitope is further supported by the finding that serum from the asymptomatic, transplanted brother of patient 3 lacked reactivity against this domain while recognizing full-length BSEP (Fig. 3B). Clearly, he was not affected by AIBD. Taken together, which epitopes are recognized determines differences in severity and outcome of AIBD in individual patients.

After activation and proliferation of BSEP-reactive B cells, anti-BSEP antibodies reach the canalicular space of the allograft liver where they exert their inhibitory function. We could previously show that only BSEP-reactive IgG decorates the canalicular membrane.[18] In the study by Jara et al., livers of Wistar rats injected with BSEP-reactive patient serum at 48 and 24 hours before sacrifice showed canalicular staining specific for human IgG that was absent when using normal serum.[19] Strikingly, this canalicular antibody deposition is already detected after 2 hours of perfusion in rat liver (Fig. 7B). Moreover, BSEP-reactive IgG deposits may be considered a general diagnostic feature of AIBD, given that we could find these exclusively in allograft liver biopsy specimens from AIBD patients (Supporting Fig. 7).

Four findings overall strongly suggest a pathophysiological role of BSEP-reactive antibodies in AIBD: (1) recognition of extracellular BSEP epitopes; (2) capacity of antibodies binding to extracellular BSEP epitopes to inhibit BSEP function; (3) targeting of anti-BSEP antibodies to the canalicular membrane *in vivo*; and (4) canalicular antibody deposits in AIBD patients, but not in patients with intrahepatic cholestasis after OLT who lack anti-BSEP antibodies. The relevance of these antibodies as mediators of AIBD is also underlined by the clinical observation that AIBD symptoms are temporarily alleviated by immunoadsorption,[18] which removes antibodies from the circulation, and by treatment with rituximab,[22] a chimeric monoclonal therapeutic anti-CD20 antibody that induces B-cell depletion and is used for the treatment of autoimmune diseases. Rituximab treatment induced remission of cholestasis in patients 1, 4, and 5 in this study. Patient 6 passed away from unrelated complications and thus could not benefit from similar treatment. Successful treatment by rituximab also suggests that affected bile canaliculi must eventually undergo antibody clearance.

It has been speculated that complete absence of BSEP expression is a prerequisite for development of BSEP-reactive antibodies post-OLT.[18,20,30] In line with this, all our patients who developed post-transplant anti-BSEP antibodies carried mutations with predicted severe effects on both *ABCB11* alleles. Specifically, no missense mutations were found among our AIBD patients, but instead only nonsense, frameshift, or splice-site mutations (Table 1). Notably, one AIBD case (patient 1) had demonstrable, albeit strongly reduced, canalicular WT BSEP expression in his native liver (Fig. 1), yet post-OLT developed anti-BSEP antibodies. In general, central tolerance against tissue-specific antigens (TSAs) such as BSEP is obtained by clonal deletion (by apoptosis) of autoreactive T cells in the thymus, whereas autoreactive B cells in bone marrow are controlled both by clonal deletion and receptor editing.[31-33] Whereas the B-cell repertoire is known to contain low-affinity, autoreactive B-cell receptors, which normally broaden the initial humoral immune response, any BSEP-reactive B cell requires a "fitting" T-helper cell for coactivation, which recognizes the same antigen by its T-cell receptor (TCR). In thymus, medullary thymic epithelial cells (mTECs) stochastically express an abundant variety of peptides derived from TSAs. Any TCR binding to these peptide-MHC complexes is removed from the TCR pool along with its T cell by apoptosis. Although small amounts of mature, fully spliced messenger RNA (mRNA) in the presence of the splice-site mutation, c.150+3A>C, could be detected in the native liver of patient 1 (Dröge et al., in preparation), it is well conceivable that mRNA processing within mTECs was ineffective for sufficient peptide production, resulting in impaired clonal deletion of BSEP-reactive T cells. Peripheral tolerance against transplanted BSEP on the other hand would require constant antigen exposure.[34,35] In this context, BSEP may be regarded as a conditionally sequestered antigen. Residing in the canalicular membrane, it remains inaccessible to cellular

components of the immune system and is only released into the circulation upon acute hepatocellular damage, which then may trigger antibody production. Taken together, AIBD should also be considered in transplanted PFIC-2 patients with a residual BSEP expression before OLT upon symptom recurrence.

Some important pathomechanistic questions of AIBD remain to be addressed. It is still unclear how the BSEP-reactive antibodies reach the canalicular space,[30] as is by what mechanism(s) the polyclonal antibody repertoire inhibits BSEP function *in vivo*. Binding to the extracellular side of BSEP may occlude the translocation pore[18] or impose other conformational restrictions that prevent transporter function. Additionally, antibody-mediated cross-linking of BSEP molecules in the canalicular membrane may contribute to functional inhibition by sequestering BSEP into larger aggregates that, in turn, may disrupt the unique structure and asymmetry of this membrane environment. Answers to these questions hold the key to a better understanding of AIBD pathogenesis and to find improved means of treatment or altogether prevent this severe type of acquired cholestasis.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.28311/suppinfo.

Supplementary information for

Bile Salt Export Pump-reactive Antibodies Form a Polyclonal, Multi-inhibitory Response in Antibody-induced BSEP Deficiency

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Supplementary Table and Figure Legends

Supplementary Table 1. Details of orthotopic liver transplantations and associated pre-trigger complications. Abbreviations: ALT - alanine aminotransferase; AST - aspartate aminotransferase; BI - biliary injury; γGT - gamma-glutamyl transpeptidase; IRI - ischemia reperfusion injury; OLT - orthotopic liver transplantation.

Supplementary Figure 1. Serum samples of the candidate patients showing signs of post-OLT disease recurrence yield canalicular staining patterns on cryosections of healthy human liver while the naive control serum does not. MRP2 was used as a canalicular marker. Notably, two sera (from patients 8 and 9) stain only at dilutions up to 1:100 and 1:50, respectively, while the other sera stain at dilutions up to 1:16,000. For ease of reference, these are consistently designated as PFIC-2, non-ABID (patient 8) and non-PFIC-2, non-AIBD (patient 9) throughout all Supplementary Figures. Bar = 10µm.

Supplementary Figure 2. HEK293 cells transiently expressing human full-length BSEP-EYFP are stained by AIBD patient sera, but not by any non-AIBD or the control serum. Bar = 10µm.

Supplementary Figure 3. The ECL1-EYFP fusion protein used for detection of anti-ECL1 reactivity of patient sera. **A** The first extracellular loop of human BSEP is fused to an N-terminal hexahistidine tag and a C-terminal EYFP via a flexible linker consisting of three glycines. **B** Purification of ECL1-EYFP by immobilized metal cation affinity chromatography. The band at approximately 20kDa in the eluate most probably corresponds to an endogenous protein contaminant from the *E. coli* expression strain.

Supplementary Figure 4. The intracellular equilibrium represented by the content of radiolabelled taurocholate (TC) is shifted in the presence of NTCP, of BSEP, and of both together by cellular influx of TC (mediated by NTCP) and/or efflux (by BSEP). Inhibition of BSEP-mediated transepithelial transport of TC in LLC-PK_1 cells by overnight incubation of their apical surface with AIBD serum from patient 3 increases the intracellular [^{3}H]-TC content. **Lower panels** X/Z-sections of the four LLC-PK_1 cell lines used in this experiment. Red, NTCP-mCherry; green, BSEP-EYFP; blue, nuclei (stained with Hoechst 34580).

Supplementary Figure 5. HEK293 cells transiently expressing the N-terminal half of BSEP are also stained by all AIBD sera but not by the non-AIBD controls or the naive control serum. Bar = 10µm.

Supplementary Figure 6. HEK293 cells transiently expressing the C-terminal half of BSEP are stained by most of the AIBD sera while showing no staining by non-AIBD or naive serum controls. Profound BSEP staining is observed for patient sera 1, 3, 4, 6, and 7. In contrast to the N-terminal half of BSEP, the C-terminal half is largely retained intracellularly. This is likely due to its lack of the native N-terminal signal anchor sequence. Bar = 10µm.

Supplementary Figure 7. Five of six available AIBD transplant biopsies show canalicular IgG deposits (indicated by white arrows) which are not detected in transplant biopsies of the non-AIBD controls.

Detailed Materials and Methods

Cloning, expression, and purification of the 1st extracellular loop of human BSEP

The first extracellular loop of human BSEP and the coding region of enhanced yellow fluorescent protein (EYFP) were amplified via PCR using the primer pair 1st-loop-HR-S1/S2 (5´-CAT CAT CAT CAC AGC AGC GGC ATA GAG GGT CGG GGC ACA ATG ACA GAT GTT TTT A-3´ and 5´-CCC GGG CCC GCG GTA CCC ATC CTC CTC CGT AAC TGG CAA ATT TGA TCA T-3´) for the ECL1 and loop-EYFP-HR S1/S2 (5´-TTG CCA GTT ACG GAG GAG GAT GGG TAC CGC GGG CCC GGG ATC CAC CG-3´ and 5´-GGA TCT CAG TGG TGGTGGTGGTGG TGT TAC TTG TAC AGC TCG TCC ATG CCG AGA GTG-3´) for EYFP, thus generating an overlap of 39 nucleotides between both product strands and overlaps of 39 and 49 nucleotides to the ends of the *Nde*I- and *Xho*I-digested pET28b(+) expression plasmid (Merck Millipore, Darmstadt, Germany). Digested and gel-purified vector and both fragments were combined using the InFusion HD Cloning Kit (Clontech) following the manufacturer´s instructions. 4L of LB medium were inoculated with 40mL from an overnight culture of *E. coli* BL21 DE3 containing the pET28b(+)-ECL1-EYFP construct and allowed to grow to an OD_{600nm} of 0.6 at 37°C and 200rpm shaking before induction with 1mM IPTG and transfer to 30°C. Cells were harvested after 2h of expression by centrifugation, snap-frozen in liquid nitrogen and stored until further use. For purification, cells were thawed on ice, resuspended in 50mM Tris pH7.5 and 150mM NaCl, and homogenized by four passages through a TS series cell disruptor at 2.7kbar (Constant Systems, Daventry, UK). Cellular debris and remaining unbroken cells were removed by centrifugation for 100.000g at 4°C for 1h, and the resulting supernatant was adjusted to 5mM imidazole. The supernatant was loaded onto a 1mL Zn^{2+} IMAC column (GE Healthcare, Darmstadt, Germany) at 1mL/min, and after extensive washing protein was eluted with 200mM imidazole in resuspension buffer. The eluate was concentrated with a 10kDa cutoff centrifugal concentrator (Sartorius Stedim Biotech, Göttingen, Germany) unit while exchanging back into resuspension buffer without imidazole. This purification yields a

protein preparation of high purity as judged by SDS-PAGE separation and coomassie staining (see supplementary figure 3).

Generation of cell lines stably expressing human NTCP and BSEP for transepithelial transport assays

HEK293T cells were transfected using polyethyleneimine transfection reagent (Sigma-Aldrich, Heidelberg, Germany) with 6µg of an HIV1 helper plasmid pCD/NL-BH expression construct for HIV1 gag/pol/rev (pCD/NL-BH; 1), 6µg of the envelope vector pczVSV-G (2; kindly obtained from Jakob Reiser, New Orleans), and 6µg of either puc2CL6EGIP-NTCP-mCherry or -BSEP-eYFP vector plasmid (3, 4). Viral supernatants were harvested 48h after transfection, filtered through a 0.45mm filter (Sartorius Stedim Biotech, Göttingen, Germany) and used to transduce LLC-PK_1 cells with either human NTCP-mCherry, human BSEP-EYFP, or both. After 24h, the transduced cells were selected with 2µg/mL puromycin (Gibco, Karlsruhe, Germany).

Purification of patient IgG from immunoadsorption columns or serum samples

IMMUSORBA TR-350 (L) columns (Asahi Kasei Medical Co., Tokyo, Japan) used during immunoadsorption treatment of an acute AIBD episode were available for patient 4. From these, the retained serum was expelled with PBS using custom adaptor fittings on a ÄKTA Basic FPLC system (GE Healthcare). The collected serum was diluted twofold into PBS and then purified in steps using two 5mL HiTrap Protein G HP columns in tandem (GE Healthcare). Diagnostic serum samples were treated likewise, with the exception that IgG was purified using 1ml HiTrap Protein G HP columns. After extensive washing with PBS up to a stable baseline at 280nm wavelength, antibodies were eluted in 100mM glycine (pH2.7)

under direct neutralization of fractions with 1/5 volume of 1M Tris/HCl (pH9). Eluted fractions were pooled, then were concentrated and exchanged into PBS using centrifugal filter devices with a 50kDa molecular weight cutoff (Sartorius Stedim Biotech, Göttingen, Germany). Glycerol was added to a final concentration of 50%, and the concentrated IgG was stored at -20°C.

BSEP inhibition by auto-reactive patient IgG in vesicular transport assays

Transport activity of human recombinant BSEP was measured in *Sf*9 membrane vesicles according to the manufacturer's instructions (Sigma-Aldrich, Heidelberg, Germany). For incorporation of antibodies into the vesicular lumen, an adapted freeze-thaw technique was applied (5). Purified patient IgG was added to 30µL of thawed vesicles to a maximal final concentration of 7mg/mL (or less, depending on the amount of serum available for purification; for actual final concentrations see Figure 4A). The samples were brought to a final volume of 70µL with the buffer contained in the vesicle suspension. Samples were gently mixed, then flash-frozen, and finally allowed to thaw slowly on ice for 30min. This was repeated twice. In order to study BSEP transport inhibition from the intracellular (extravesicular) side, IgG was added as described above and samples were kept on ice for 1.5h after mixing. Vesicles were then diluted fivefold into ice-cold assay mix (2mM HEPES/Tris pH7.4, 100mM KNO_3, 10mM $Mg(NO_3)_2$, 50mM sucrose). After 5min of pre-incubation at 37°C, transport of 2µM [^{3}H]-TC (Perkin Elmer, Rodgau, Germany) was initiated by addition of ATP to a final concentration of 4mM (Sigma Aldrich, Deisenhofen, Germany) or assay mix only for control reactions. The reactions were kept at 37°C for 5min and bile salt transport was stopped by addition of 1mL of ice-cold washing mix (10mM Tris/HCl pH7.4, 100mM KNO_3, 50mM sucrose, 0.1mM sodium TC) followed by rapid filtration through pre-wetted 0.2µm cellulose membranes (type GSTF; Merck Millipore). After filtration, the

membranes were immediately washed three times with 4mL washing mix and allowed to dry. Retained radioactivity was measured in a liquid scintillation counter (Packard Instruments, Frankfurt, Germany) using UltimaGold liquid scintillation cocktail (Canberra Packard, Frankfurt, Germany).

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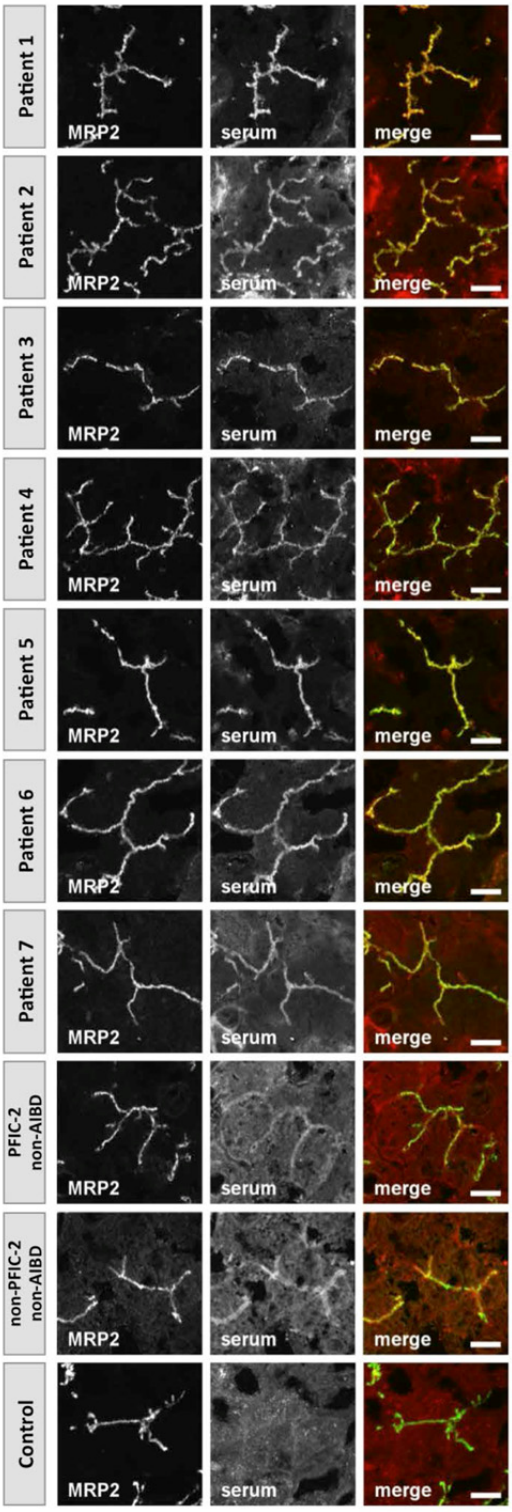
Supplementary Table 1

Details of orthotopic liver transplantations and associated pre-trigger complications.

Patient	Donation	OLT type	IRI	BI	Prior bouts of rejection (before potential trigger events)
1	cadaveric	full left split	no	no	no
2	cadaveric	left lateral lobe	no	no	no
3	cadaveric	full	no	no	no
4	living	left lateral lobe	no	no	no
5	living	Segments II and III	no	no	no
6	living	right lateral lobe	no	no	**yes (max. values: ALT=226; AST = 213, γGT = 41)**
7	cadaveric	full	no	no	no
8	living	Segments II and III	**yes (max. values: ALT = 1638; AST = 1724; γGT = 73)**	no	no
9	cadaveric	Segments II and III	no	no	no

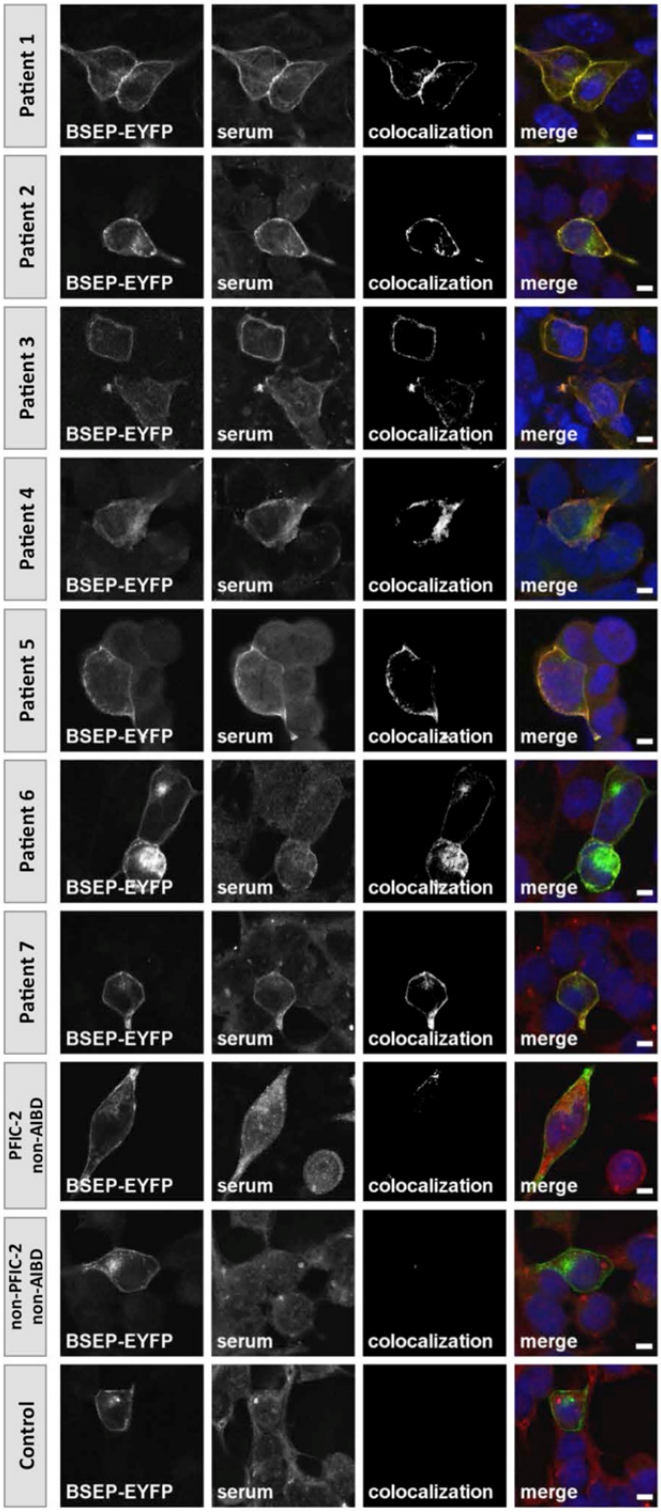
Supplementary Figure 1

Serum samples of the candidate patients showing signs of post-OLT disease recurrence yield canalicular staining patterns on cryosections of healthy human liver while the naïve control serum does not.



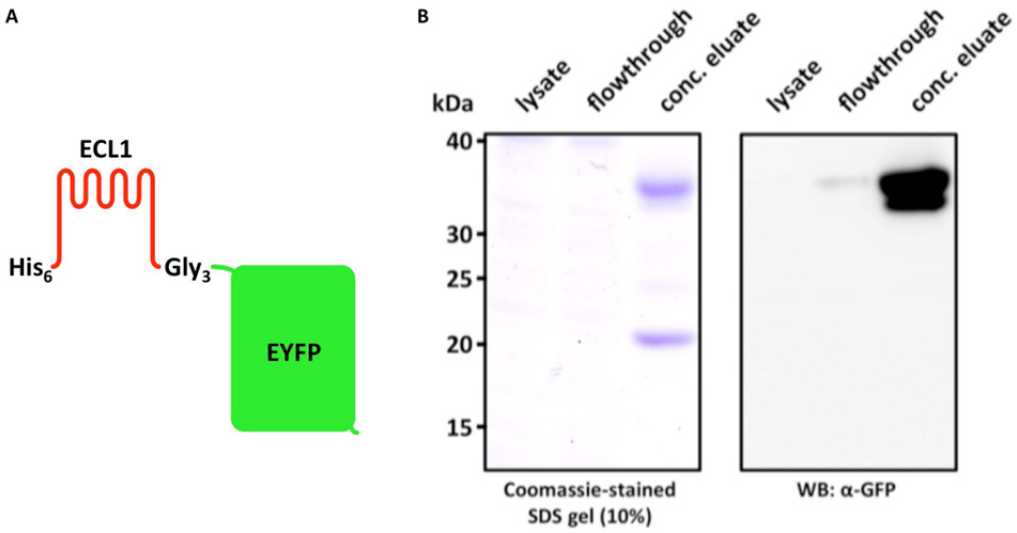
Supplementary Figure 2

HEK293 cells transiently expressing human full-length BSEP-EYFP are stained by AIBD patient sera, but not by any non-AIBD or the control serum.



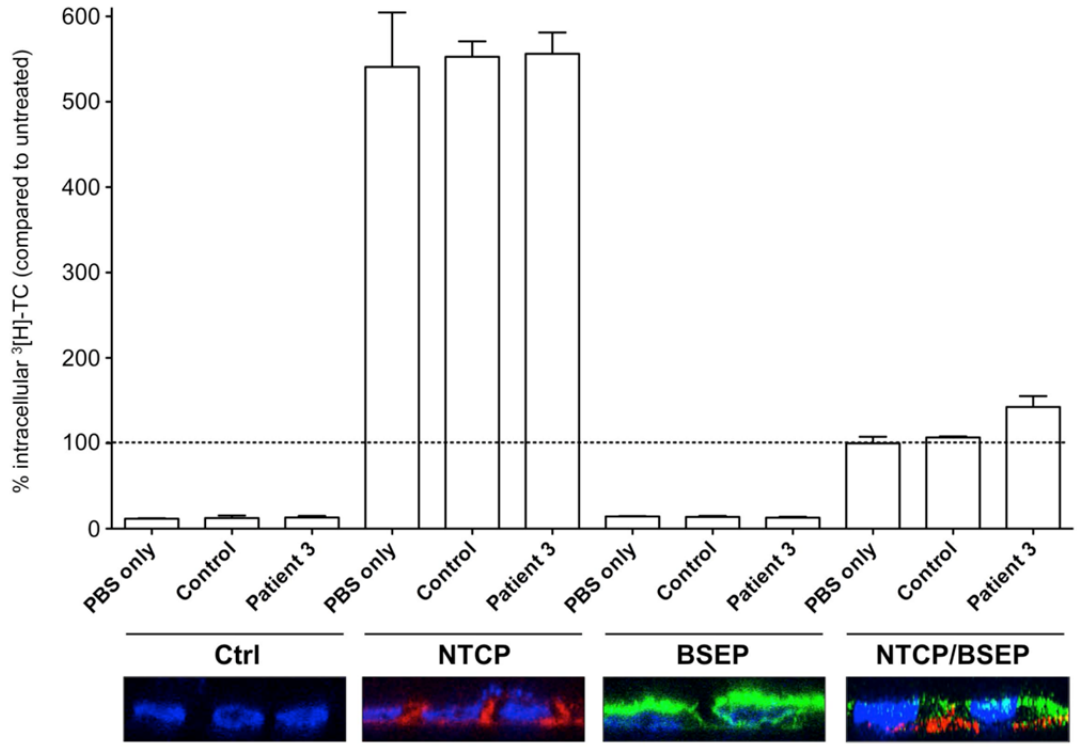
Supplementary Figure 3

The ECL1-EYFP fusion protein used for detection of anti-ECL1 reactivity of patient sera.



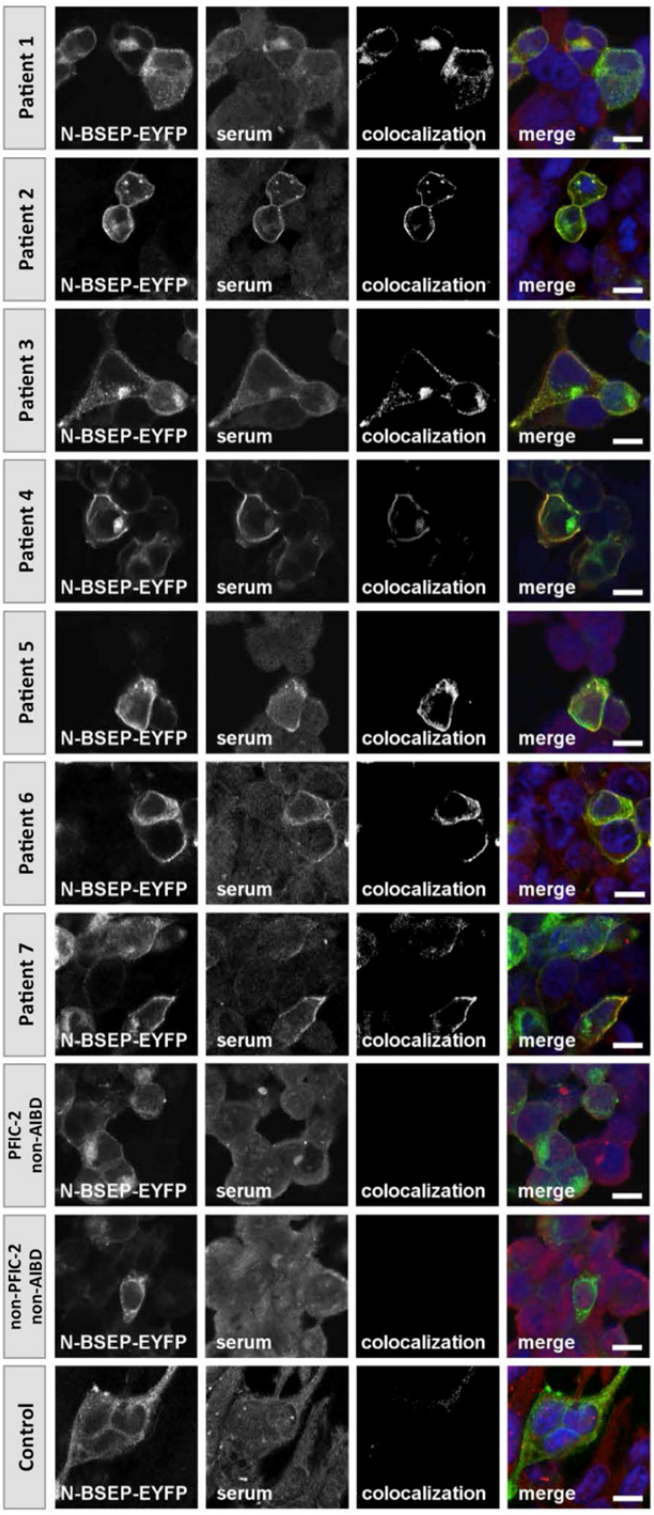
Supplementary Figure 4

The intracellular equilibrium represented by the content of radiolabelled taurocholate (TC) is shifted in the presence of NTCP, of BSEP, and of both together by cellular influx of TC (mediated by NTCP) and/or efflux (by BSEP).



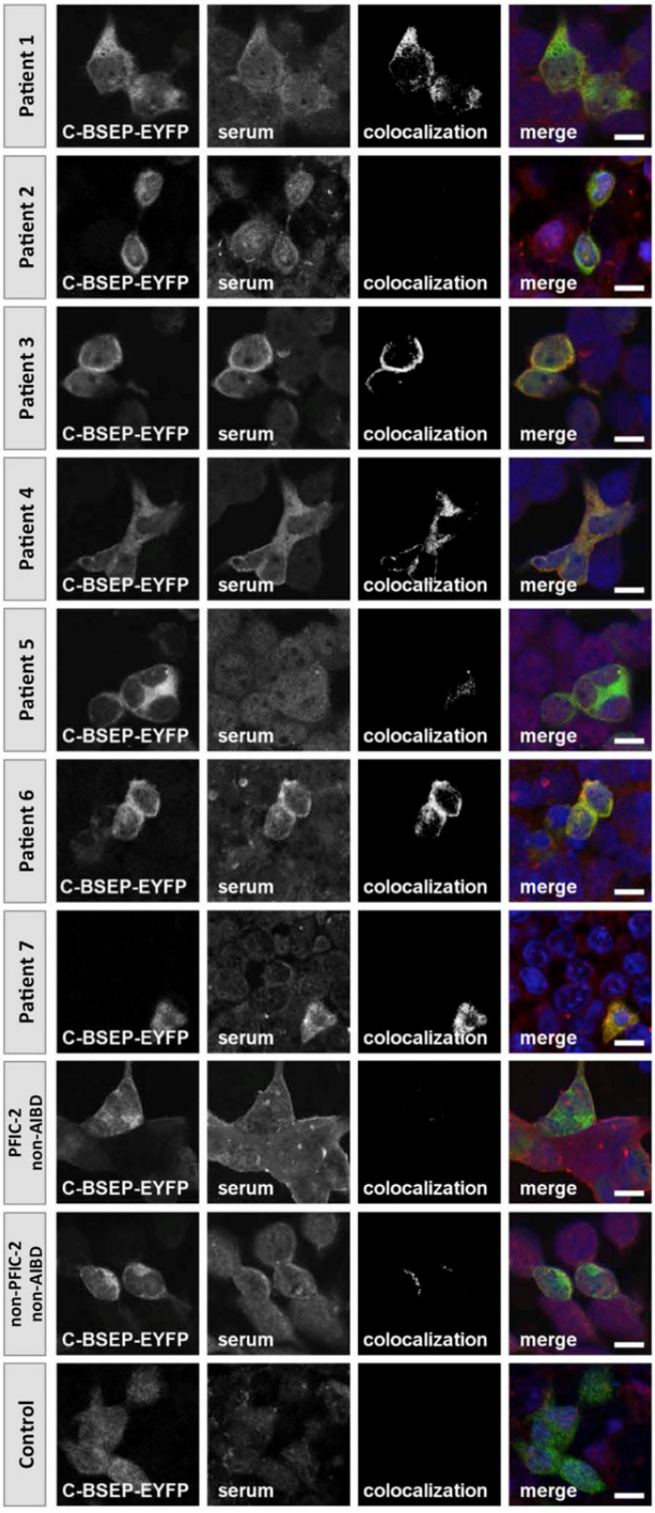
Supplementary Figure 5

HEK293 cells transiently expressing the N-terminal half of BSEP are also stained by all AIBD sera but not by the non-AIBD controls or the naïve control serum.



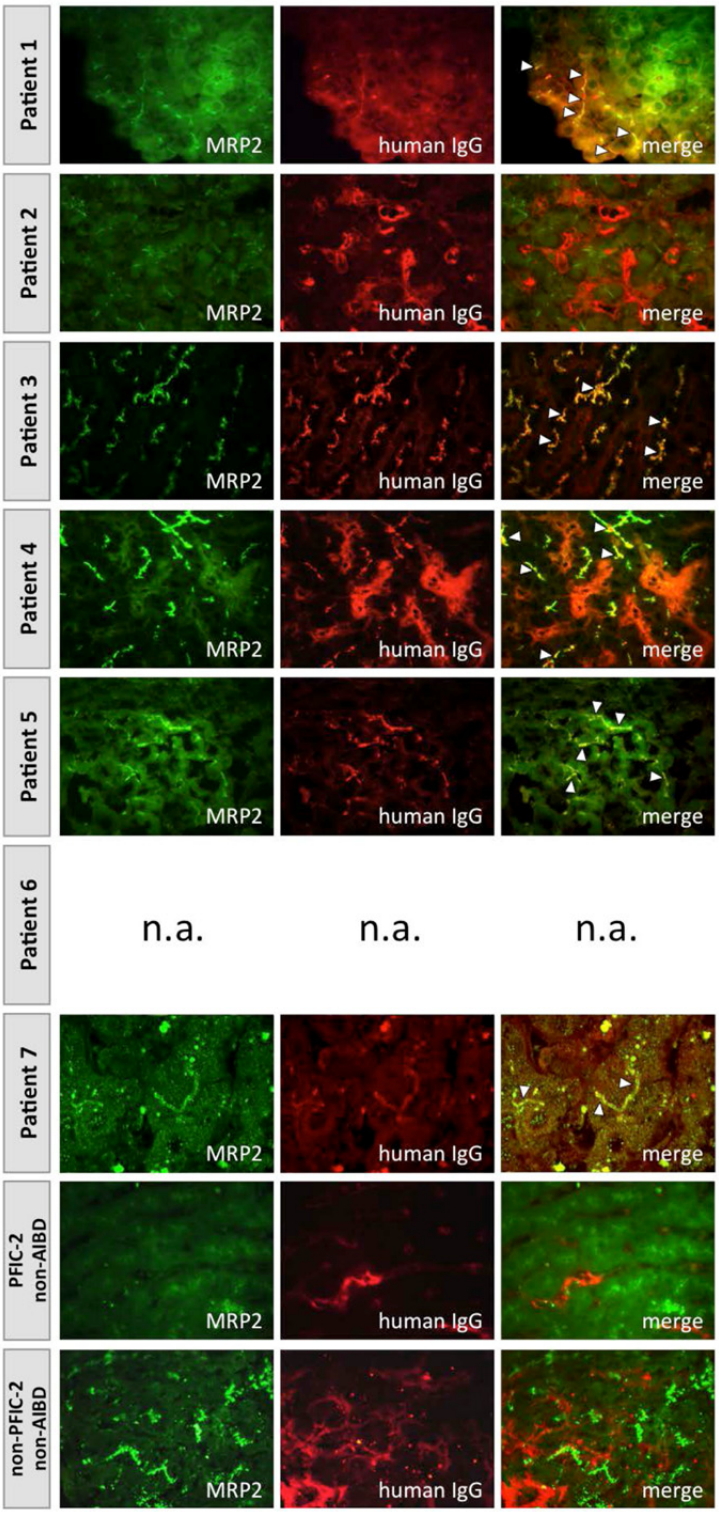
Supplementary Figure 6

HEK293 cells transiently expressing the C-terminal half of BSEP are stained by most of the AIBD sera while showing no staining by non-AIBD or naïve serum controls.



Supplementary Figure 7

Five of six available AIBD transplant biopsies show canalicular IgG deposits (indicated by white arrows) which are not detected in transplant biopsies of the non-AIBD controls.



3.10 High affinity anti-BSEP antibodies after liver transplantation for PFIC-2 – successful treatment with immunoadsorption and B-cell depletion

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Own contribution: Collection and analysis of the patient's clinical data

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High affinity anti-BSEP antibodies after liver transplantation for PFIC-2 – Successful treatment with immunoadsorption and B-cell depletion

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Abstract: PFIC due to BSEP mutations (PFIC type 2) often necessitates OLT. It has recently been recognized that some PFIC-2 patients develop phenotypic disease recurrence post-OLT due to the appearance of anti-BSEP antibodies. Here, we describe a boy who became cholestatic four yr after OLT during modification of immunosuppression. Canalicular antibody deposits were detected in biopsies of the transplant and antibodies specifically reacting with BSEP were identified at high titers in his serum. These antibodies bound extracellular epitopes of BSEP and inhibited BS transport and were assumed to cause disease recurrence. Consequently, anti-BSEP antibody depletion was pursued by IA and B-cell depletion by anti-CD20 antibodies (rituximab) along with a switch of immunosuppression. This treatment resulted in prolonged relief of symptoms. Depletion of pathogenic anti-BSEP antibodies causing AIBD after OLT in PFIC-2 patients should be considered as a central therapeutic goal.

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Key words: PFIC recurrence – children – rituximab – canalicular antibodies – *ABCB11* gene

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Inherited defects in BS transport can cause severe liver diseases in early childhood. Bile acids are synthesized *de novo* from cholesterol within hepatocytes. Here, they are conjugated to taurine or glycine before export into the canalicular lumen by the BSEP, which is exclusively expressed in hepatocytes (1). In the small intestine, BS act as emulsifiers for lipids and fat-soluble vitamins. In the terminal ileum, more than 90% of BS are reabsorbed and transported back to the liver via the portal vein within the enterohepatic circulation.

BSEP is encoded by the *ABCB11* gene. Mutations of *ABCB11* can cause intrahepatic cholestasis of varying severity (termed "BSEP deficiency") including PFIC type 2 (PFIC-2 or Byler syndrome [2, 3]; for review, see [4, 5]).

Deficits in BSEP function lead to increased BS concentrations in hepatocytes and serum, which are potentially cytotoxic due to their detergent

Abbreviations: AIBD, antibody-induced BSEP deficiency; ALD, autoimmune liver diseases; ANA, antinuclear antibodies; anti-GBM, antiglomerular basal membrane; ASMA, antismooth muscle antibodies; BS, bile salts; BSEP, bile salt export pump; CsA, cyclosporine A; *de novo* AIH, *de novo* autoimmune hepatitis; EYFP, enhanced yellow fluorescent protein; gGT, gamma glutamyltransferase; HBV and HCV, hepatitis B and C virus; HEK293, human embryonic kidney 293 cells; IA, immunoadsorption; IVIG, intravenous IgG; MARS, molecular adsorbents recirculating system; MMF, mycophenolic acid; MRI, magnetic resonance imaging; OLT, orthotopic liver transplantation; PBC, primary biliary cirrhosis; PFIC, progressive familial intrahepatic cholestasis; PSC, primary sclerosing cholangitis; PTLD, post-transplant lymphoproliferative disorder; SPA, staphylococcal protein A; Tac, tacrolimus; UDCA, ursodeoxycholic acid.

properties. Thus, failure to excrete BS from hepatocytes due to BSEP deficiency results in liver cell damage, causing progressive liver disease early in life. Characteristics of BSEP deficiency include intractable pruritus and elevated serum BS concentrations accompanied by low gGT levels. So far, therapeutic options are limited and usually focus on symptom relief for pruritus and other sequelae of liver dysfunction. For patients without cirrhosis, biliary diversion is one option with unpredictable outcome (6, 7). Despite biliary diversion, PFIC-2 often leads to end-stage liver disease, necessitating OLT. At present, approximately 10% of pediatric OLT are performed due to PFIC (4). The overall long-term outcome after pediatric OLT is excellent. One-year graft survival rates have increased up to 90% in recent years (8, 9) and survival after liver transplantation in patients with PFIC does not differ from that in other indications for transplantation (10).

On a long-term scale, renal disease due to immunosuppression, PTLD (11) and *de novo* AIH (12, 13) cause relevant morbidity and mortality after OLT. In patients with *de novo* AIH-specific antibodies like ANA or ASMA can be detected. These are not directed against HLA receptors and thus do not fulfill criteria of classical humoral rejection (14).

In a review by Davit-Spraul et al. from 2009, it was speculated for the first time that "... possible 'recurrence of PFIC' on liver grafts due to alloimmunization of the recipient against the BSEP, MDR3 or FIC1 proteins of liver donor remains a theoretical matter of debate..." (4). In the same year, Keitel et al. (15) and Jara et al. (16) described the first four PFIC-2 patients who developed antibodies against BSEP after OLT together with the phenotypic recurrence of BSEP deficiency, which we recently termed AIBD (17, 18). Since the recognition of these antibodies in 2009, four further reports with 15 patients suffering from phenotypic recurrence of PFIC-2 after transplantation have been published (17, 19–21). Siebold et al. calculated a prevalence of about 8% (18, 20). Time from transplantation to onset of AIBD ranged between months and many years and therapeutic options are wanting (18). Retransplantation and modulation of immunosuppression did not induce sustained remission in some of these patients. In a recent report by Lin et al. (21), a possible treatment based on rituximab and plasma exchange was described for the first time.

Rituximab is a chimeric (mouse/human) monoclonal antibody directed against CD20, an activated-glycosylated phosphoprotein expressed on the surface of B lymphocytes. Since the introduction of rituximab in 1997 for the treatment of $CD20^+$ B-cell lymphoma (22), it was also proven to be effective in the treatment of various autoimmune diseases (23). It has been used in a few patients with AIH (24–31) and in ABO-incompatible OLT in adults (32, 33). In adults treated with rituximab, HBV or HBC reactivation is a severe side effect (34, 35), while in children, rituximab is regarded as a safe drug. In a retrospective study of 2875 children who received 375 mg/m^2 rituximab for treatment of autoimmune diseases (e.g., nephritic syndrome, systemic lupus erythematosus, autoimmune hemolytic anemia), primary immunodeficiency, malignancies, and transplantation-related diseases, the rate of opportunistic infections was not increased within one yr after administration (36).

In the first case of AIBD in a PFIC-2 patient (15), a combination of five courses of plasmapheresis or MARS dialysis was employed. This treatment temporarily relieved symptoms but failed to prevent early relapse of cholestasis. The child had to be retransplanted and ultimately died. In contrast to plasmapheresis which requires plasma replacement, IA efficiently removes antibodies directly from serum (37). In IA, SPA is employed as the antibody binding matrix (human IgG > IgA and IgM). We here report for the first time a successful treatment and sustained response of AIBD in a child by eight consecutive sessions of IA followed by a single dose of rituximab. The patient is currently in remission for more than five yr since diagnosis of AIBD.

Methods

This study was performed according to the guidelines of the Declaration of Helsinki and written informed consent was provided by the parents of the patient. All investigations on hepatobiliary transporters including human tissue or serum samples as presented in this study were approved by the ethical review committee of the Medical Faculty of the Heinrich Heine University Düsseldorf.

IA

Eight sessions of IA therapy using a protein A column (Immunosorba®; Fresenius Medical Care, Bad Homburg, Germany) and a 220–240% plasma turnover rate per session were performed within 10 days. Thereafter, a single dose of 375 mg/m^2 rituximab (MabThera; Roche Pharma AG, Grenzach-Wyhlen, Germany) was administered.

Immunofluorescence, Western blotting, and *in vitro* transport studies

Immunostaining of liver sections (38, 39), and transfection and immunostaining of transfected HEK293 cells (17) were performed as described previously. For Western blot detection of BSEP-reactive serum antibodies, protein lysates of un- or BSEP-transfected Huh7 cells were resolved by SDS-PAGE and transferred onto nitrocellulose membrane as

described for HEK293 cells (17). For detection of BSEP-reactive antibodies, membranes were cut into replicate sections and incubated with pre- and post-transplant sera (1:5000), respectively, followed by incubation with goat anti-human IgG-HRP (1:20 000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) as a detection antibody. *In vitro* BSEP transport inhibition was described previously (17).

Case description and results

The male patient of non-consanguineous parents was born in May 2002. He presented at two yr of age with severe pruritus refractory to medical therapy. BSs were in the range of 380 μmol/L (normal <22 μmol/L). Since liver cirrhosis was histologically evident, a biliary diversion was not considered for therapy. Bilirubin (34 μmol/L [normal: <17 μmol/L]) and aminotransferases were elevated (AST 313 U/L [<35 U/L]; ALT 265 U/L [<38 U/L]), and gGT was within the normal range (<35 U/L). Due to clinical presentation and laboratory parameters, PFIC-2 (MIM ID #601847) was suspected. Genetic analysis of *ABCB11* revealed two severe heterozygous mutations: a duplication of nucleotides (c.2783_2787dup5; p.K930Efs79X) and the splice site mutation c.150 + 3A>C. The asymptomatic parents were heterozygous for either c.2783_2787dup5 (father) or c.150 + 3A>C (mother). In June 2005, the patient received a split-liver transplant (deceased donor, segments 1–4). Immunosuppression was started with CsA (5 mg/kg td), methylprednisolone (15 mg/m^2 body surface), and basiliximab (Simulect; Novartis, Nürnberg, Germany; 10 mg on day 0 and day 4). Due to severe side effects, CsA was switched to Tac one yr after transplantation.

Forty-five months after OLT (March 2009), the boy developed slight pruritus. It was first associated with a skin infection but persisted after healing of the skin. Liver enzymes (AST 52 U/L; ALT 28 U/L; gGT 4 U/L) and bilirubin (17 μmol/L) were normal at that time, while BS were slightly elevated (38 μmol/L; Fig. 1a). Fifty-one months post-OLT, a liver biopsy showed no evidence of acute or chronic rejection. The bile ducts showed little regression, but there was no evidence of ductopenia. Tac was stopped and MMF (1200 mg/m^2 body surface) and prednisone (2 mg/kg) were introduced as pruritus was thought to be a side effect of Tac treatment. Within the next two months, liver enzymes (AST 130 U/L; ALT 131 U/L), bilirubin (48 μmol/L), and BS (263 μmol/L) increased (Fig. 1a). The patient developed diarrhea and pruritus

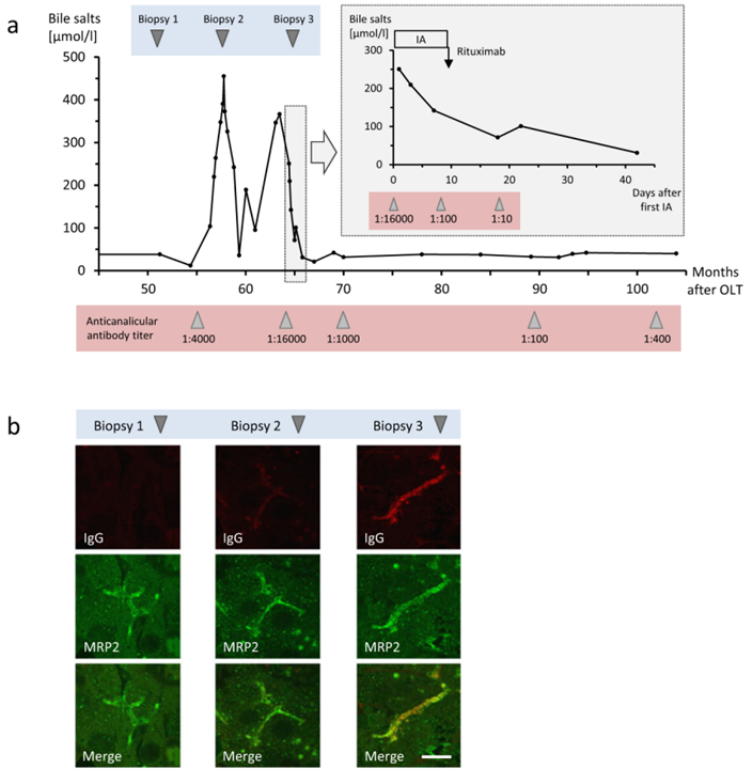

Fig. 1. (a) Serum BS levels were measured at various time points after OLT. The inset shows the course of serum BS concentration in the days after first IA and administration of rituximab (arrow) in detail. Time points of liver biopsies and serum samples taken for determination of anticanalicular antibody titers are indicated by gray triangles. (b) Immunofluorescence of the three liver biopsies obtained after OLT. In contrast to biopsy 1 and 2, IgG deposits were clearly detectable in biopsy 3 (red). MRP2 was stained as a canalicular marker (green). Scale bar = 10 μm.

worsened. Another liver biopsy (58 months after OLT) showed no evidence of rejection but severe chronic cholestasis and degeneration of small bile ducts while large bile ducts appeared normal on MRI scan. Tac was reintroduced in combination with prednisone (2 mg/kg) and symptoms of pruritus slowly disappeared while bilirubin and BSs decreased. With reduction of prednisone, itching returned and liver enzymes increased again. A third liver biopsy was performed 65 months post-OLT showing evidence of severe canalicular destruction and F3 fibrosis. In contrast to earlier liver biopsies, distinct canalicular antibody deposits were now clearly detectable (Fig. 1b). Post-OLT serum taken at the time of the third biopsy, but not control serum, specifically stained transiently expressed human BSEP-EYFP at a high titer of 1:16 000 (Fig. 2a). Furthermore, this serum inhibited BSEP-mediated transport of taurocholate into *Sf*9 membrane vesicles *in vitro* (Fig. 2b). Taken together, the patient clearly suffered from AIBD, due to BSEP-reactive and BSEP-inhibitory IgG-class antibodies.

After confirmation of anti-BSEP antibodies, IA using a protein A column (Immunosorba®; Fresenius Medical Care) was initiated in order to reduce serum levels of these pathogenic antibodies. Anti-BSEP antibody level was effectively decreased after each IA as shown by Western blot analysis and immunofluorescent staining (Fig. 3). This treatment was well tolerated. Pruritus and cholestasis disappeared already after two sessions of IA and liver enzymes normalized within four wk.

Since the completion of treatment with IA and rituximab, the patient has remained in excellent clinical condition for more than five yr (Fig. 1a). Current therapy consists of Tac (trough level 3–6 ng/mL), MMF (600 mg/m^2 body surface), prednisone 5 mg, UDCA (10 mg/kg), and sulfamethoxazole/trimethoprim (5 mg/kg). B lymphocytes did reappear in January 2012 but did not reach normal values. The range was between 4 and 7% of total lymphocytes (normal value: 9–24%). Serum IgG rapidly recovered after IA and remained in the normal range throughout; IVIG was not administered and no infections occurred. Although anti-BSEP antibodies were detectable at various time points (up to a titer of 1:1600 in January 2015) the patient remained symptom-free.

Discussion

Disease recurrence after liver transplantation is a risk factor for graft dysfunction and may restrict long-term graft survival. Recurrence is observed in patients transplanted for liver cirrhosis due to viral hepatitis or ALD like AIH, PSC, or PBC. The various mechanisms of recurrence include re-infection (HBV, HCV), reactivation of preexisting liver disease (PSC), or reappearance of immune-mediated liver damage (AIH, PBC) (40, 41).

In 2009, the first case of post-OLT phenotypic disease recurrence in a PFIC-2 patient due to BSEP-specific antibodies was described (15). Evidence was presented that these anti-BSEP antibodies are functionally relevant and directly cause disease recurrence (15–17); it has been regarded as a unique form of humoral rejection (42). The causal relation between anti-BSEP antibodies and phenotypic recurrence of symptoms is fully supported by the case presented here, because the appearance of anti-BSEP antibodies positively correlates with serum BS levels (Fig. 1a) and therapeutic reduction of these

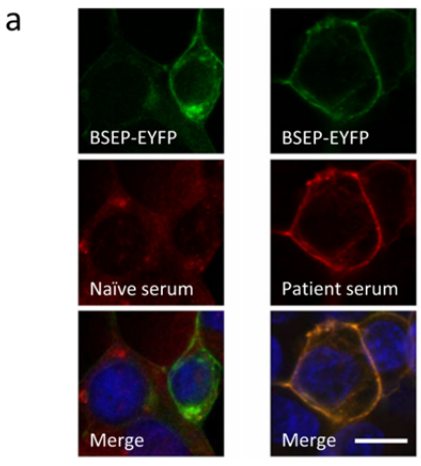

Fig. 2. (a) Compared to naïve control serum (left), the patient serum (right) specifically stained (red) human full-length BSEP-EYFP (green) transiently expressed in HEK293 cells. Nuclei (blue) were stained using Hoechst. Scale bar = 10 μm. (b) In contrast to the control serum, the patient's serum almost completely inhibited BSEP-mediated taurocholate transport into *Sf*9 membrane vesicles. Data represent the average and standard error of triplicate measurements.

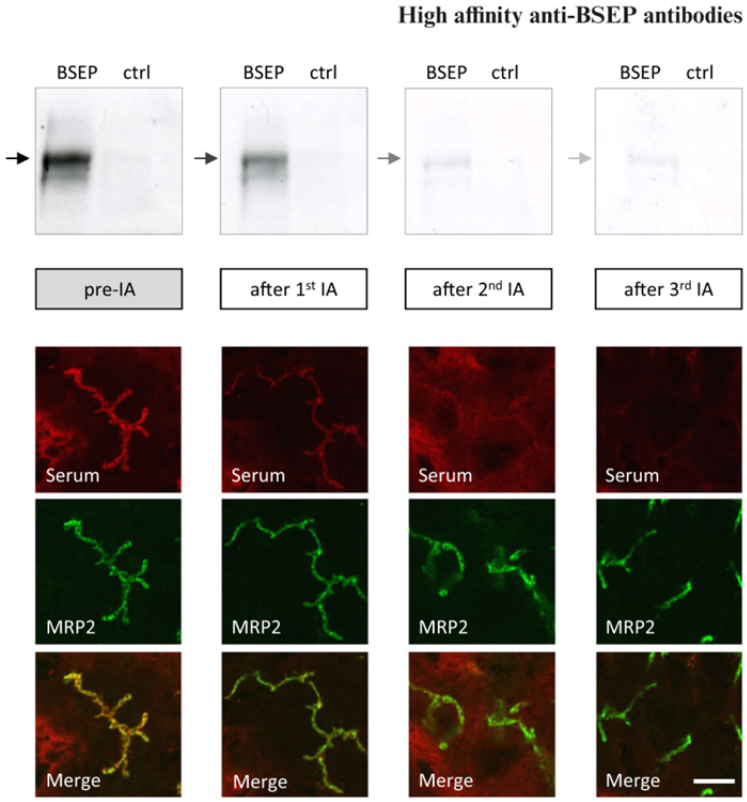

Fig. 3. Patient serum was obtained before (pre) and after three sessions of IA. In response to IA treatment, serum reactivity against BSEP-EYFP (BSEP) transiently expressed in Huh7 cells was diminished after each treatment as shown by Western blots using four replicates cut from the same nitrocellulose membrane containing equal amounts of BSEP/protein lysates. Concurrent with BSEP reactivity, anticanalicular staining of normal liver tissue by the serum samples (red) was also reduced with each round of IA. MRP2 was stained as a canalicular marker (green). Scale bar = 10 μm. Ctrl = protein lysates from untransfected Huh7 cells.

antibodies by IA and rituximab administration rapidly and profoundly improved the clinical condition of the patient.

It is believed that absent or markedly reduced BSEP expression in the native liver is a prerequisite for the formation of anti-BSEP antibodies after OLT and it has been observed that patients affected by anti-BSEP antibodies carry severe BSEP mutations (15–17). Other liver diseases such as tyrosinemia type 1 (43), primary hyperoxaluria (44), or bilirubin-UDP-glucuronyl transferase deficiency (Crigler–Najjar syndrome) may also require OLT (45). Like in PFIC-2, the respective proteins are reduced or absent in these liver diseases. Disease recurrence after OLT, however, has not been described in these patients. This most likely is due to the strict intracellular localization of the respective proteins, which makes these proteins fully sequestered antigens (28). Although these proteins may get into contact with the host's immune system, for example, during reperfusion cell injury or during acute rejection, with subsequent immunization, cells of the host's immune system or antibodies do not reach the proteins in intact hepatocytes, thus preventing the development of liver injury. In this context, it may be speculated that at least some of these transplanted patients have serum reactivity against the respective proteins introduced by OLT but remain asymptomatic.

Antibody formation against a specific protein, formerly unknown to the host's immune system, has been described in a patient with Becker's muscular dystrophy after heart transplantation (46). In this case, antibodies directed against the deleted part of dystrophin could be detected; however, their functional relevance remained uncertain (46). In patients with Alport syndrome, frequently due to mutations in type IV collagen α5 chain, anti-GBM nephritis develops in 3–5% after kidney transplantation, caused by anti-GBM antibodies directed against the carboxy-terminal non-collagenous domain of the mutated chain (47). Patients with post-transplantation anti-GBM nephritis are at risk of irreversible graft failure, the rate of recurrence in subsequent allografts is high, and treatment response to plasmapheresis and cyclophosphamide is limited (47), features which are similarly observed in several patients with AIBD (15–20).

Three patients with AIBD were reported by Jara et al. (16), who experienced one to five episodes of cholestasis. Therapy was based on steroid boluses (0.17–10 mg/kg) and temporal switches to a triple immunosuppression (MMF, Tac, steroids), which induced remission in two patients for up to six yr. Maggiore et al. (19)

described two patients who developed severe cholestasis with signs of *de novo* AIH. They were treated by increases in immunosuppression, yet pruritus resolved only in one patient. All six patients reported by Siebold et al. (20) (one was previously described by Keitel et al. [15]) suffered from pruritus and four of them required retransplantation while one died on the waiting list. None of these six patients was treated with IA or rituximab.

In 2013, a successful therapy using rituximab was reported for the first time by Lin et al. (21). Rituximab was administered 375 mg/m^2 weekly for six wk followed by four doses every six wk and repeated IVIG administration over 12 months. Plasmapheresis and IVIG alone had been used before without success in this patient. Another patient reported by Lin et al. received a combination of plasmapheresis and IVIG plus rituximab and was free of symptoms six months after first recurrence of PFIC-like symptoms (21).

IA is capable of removing large amounts of antibodies from the circulation. Its antibody clearing capacity is significantly higher than with plasmapheresis because in IA antibodies are specifically removed from the plasma (e.g., by direct binding to protein A as an absorbent), whereas in plasmapheresis only a fraction of the circulating plasma together with its contents is replaced by fresh frozen plasma or albumin. Our patients' drastic and sustained treatment response suggests that a combination of fast and efficient removal of antibodies from circulation in combination with a temporary block of new antibody formation and intense immunosuppression based on calcineurin inhibitors and MMF might be a more sustained therapeutic option for patients with severe phenotypic PFIC-2 recurrence. After IA and rituximab administration, anti-BSEP antibodies in the patient's serum were detectable to a significantly lower extent (Figs. 1 and 3). Further studies are needed to assess the effectiveness of IA in combination with rituximab and double immunosuppression in other PFIC-2 patients with AIBD.

In conclusion, we demonstrated that reduction of anti-BSEP antibodies correlates with clinical improvement of cholestasis in transplanted PFIC-2 patients with phenotypic disease recurrence. The combination of IA and B-cell depletion is safe and effective to achieve long-term remission in AIBD.

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3.11 Intrahepatic Cholestasis of Pregnancy (ICP): Case report and review of the literature

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Intrahepatic Cholestasis of Pregnancy (ICP): Case report and review of the literature

Intrahepatische Schwangerschaftscholestase (ICP): Kasuistik und Übersicht über die Literatur

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Key Words
Cholestasis, pregnancy, bile acids, MDR3, BSEP, polymorphisms

Abstract
Intrahepatic cholestasis of pregnancy (ICP) represents the most common pregnancy-related liver disease in women. Women frequently present in the third trimester with pruritus and elevated serum bile acid and/or alanine transaminase levels. Clinical symptoms quickly resolve after delivery however, recurrence in subsequent pregnancies has to be expected. ICP is associated with increased perinatal complications, such as premature delivery, meconium staining of the amniotic fluid, respiratory distress, low Apgar scores and even stillbirth. The risk for the fetus is significantly increased with maternal serum bile acid levels above 40 µmol/l, which characterize severe ICP. An important factor for ICP development is a rise of gestational hormones leading to cholestasis in genetically predisposed women. Variants in the bile salt export pump BSEP and the multidrug resistance protein 3 (MDR3) are most often identified in ICP. Here, we give an overview of the current literature on ICP and present the case of a woman with recurrent severe ICP. A common BSEP polymorphism as well as a rare MDR3 mutation may underlie the development of ICP in our patient. She had a premature delivery with meconium staining of the amniotic fluid. The neonate showed signs of respiratory distress with a low Apgar score. This case emphasizes that women with severe ICP have an increased risk for perinatal complications. Furthermore, severe ICP was associated with a MDR3 mutation, which has already been described in adult patients with liver cirrhosis. Thus, ICP may unmask an underlying MDR3 defect, which may predispose to development of hepatobiliary diseases such as gallstone disease, liver fibrosis/cirrhosis as well as hepatobiliary malignancies. Therefore, genetic testing should be considered in women with severe as well as early onset ICP. Furthermore, regular follow up should be discussed for women with genetic variants.

Zusammenfassung
Die intrahepatische Schwangerschaftscholestase (ICP) ist die häufigste Lebererkrankung bei schwangeren Frauen. ICP tritt üblicherweise im dritten Trimenon mit Pruritus und erhöhten Serumspiegeln für Gallensäuren und/oder Alanin-Aminotransferase auf. Die Symptome klingen zeitnah nach der Entbindung ab, ein Rezidiv in nachfolgenden Schwangerschaften ist jedoch zu erwarten. Die ICP ist mit einer erhöhten Wahrscheinlichkeit für perinatale Komplikationen, wie Frühgeburt, Mekonium im Fruchtwasser, Atemnot, niedrigem Apgar-Score bis hin zur Totgeburt assoziiert. Das Risiko für den Fetus steigt bei Serum Gallensäurespiegeln der Mutter oberhalb von 40 µmol/l, welche eine schwere ICP ausmachen, signifikant an. Die Erhöhung der Schwangerschaftshormone hat einen erheblichen Einfluss auf die Entwicklung einer ICP, insbesondere bei Frauen mit genetischer Prädisposition. Besonders häufig sind Varianten in der Gallensalzexportpumpe BSEP und der Phospholipidfloppase MDR3. Mit der vorliegenden Arbeit geben wir einen Überblick über die aktuelle ICP Literatur und berichten über eine Patientin mit wiederkehrender schwerer ICP. Der frühen Manifestation und der Schwere der ICP liegen bei

dieser Patientin ein häufiger BSEP-Polymorphismus und eine seltene MDR3-Mutation zugrunde. Die Patientin hatte eine Frühgeburt sowie Mekonium im Fruchtwasser. Das Neugeborene zeigte Zeichen von Atemnot und einen niedrigen Apgar-Score. Dieser Fall verdeutlicht, dass bei Frauen mit schwerer ICP ein erhöhtes Risiko für perinatale Komplikationen besteht. Außerdem ist die vorliegende schwere ICP mit einer MDR3-Mutation assoziiert, die bereits bei erwachsenen Patienten mit Leberzirrhose nachgewiesen wurde. So kann durch das Auftreten einer ICP ein zugrunde liegender MDR3-Defekt identifiziert werden, welcher für die Entwicklung hepatobiliärer Erkrankungen wie Gallensteinleiden, Leberfibrose und -zirrhose oder hepatobiliären Tumoren prädisponieren kann. Daher sollte eine Genanalyse bei Frauen mit schwergradiger, frühmanifester ICP in Betracht gezogen werden. Zudem sollte für Frauen mit nachgewiesenen genetischen Varianten Verlaufsuntersuchungen zur Früherkennung ICP assoziierter Erkrankungen angeboten werden.

Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a reversible form of cholestasis, which usually occurs in late pregnancy, persists until delivery and affects 0.1-2.0% of pregnancies in Europe (1-4). Thus, ICP is the most common liver disease in pregnant women (5, 6). ICP, which develops in up to 80% of patients after 28 weeks of gestation (third trimester) is characterized by pruritus, elevated serum bile acid (BA) levels and increased serum transaminase levels, especially elevation of alanine transaminase (ALT) (1, 2, 4). Jaundice with elevation of bilirubin levels is observed in about 10% of ICP patients (1, 4). Diagnosis of ICP is based on the presence of pruritus and elevated serum BA levels and/or ALT levels in the absence of other causes for cholestasis and pruritus. Both, pruritus and abnormalities of serum BA and transaminase levels resolve quickly after delivery, however, may recur in subsequent pregnancies (1, 4). ICP is associated with a higher incidence of adverse fetal outcomes, comprising spontaneous or iatrogenic premature delivery, meconium staining of the amniotic fluid, respiratory distress, low Apgar scores and even stillbirth (7-10). The risk for the fetus increases significantly once maternal BA levels exceed 40 µmol/l (7, 8). Women with ICP have a higher incidence of several liver, biliary, pancreatic, metabolic and immune-mediated diseases, including hepatitis C virus infection, nonalcoholic liver cirrhosis, cholelithiasis, cholecystitis and cholangitis, gallstone-associated pancreatitis as well as diabetes, cardiovascular diseases and thyroid disease (6, 11, 12). A recent study also found a significant increase in hepatocellular and biliary cancer in women with ICP (6). The pathogenesis of ICP is still not completely understood, however, gestational hormones, environmental factors but also a genetic predisposition contribute to the development of the disease. Here, we describe the case of a woman with recurrent early onset ICP due to a heterozygous mutation in the MDR3 (*ABCB4*) gene as well as a homozygous common polymorphism in the BSEP (*ABCB11*) gene.

Case Presentation

A 35-year old woman was referred to our outpatient clinic in the 15th week of her third pregnancy because of mildly elevated aspartate transaminase (AST) and ALT levels as well as mild pruritus. The patient had a prior history of severe ICP in the first trimester of her first pregnancy following ovarian hyperstimulation. In her first pregnancy, BA levels were as high as 252 µmol/l and ALT levels were raised to 732 U/l in the 7th week of gestation. Levels for alkaline phosphatase (ALP) were mildly elevated at 235 U/l, while gamma GT (GGT) levels were normal. Total bilirubin levels were elevated about 3-fold to 3,4 mg/dl. She received treatment with ursodeoxycholic acid (UDCA) from the first trimester onwards until delivery, which occurred spontaneously in the 36th week of gestation. The neonate was healthy and well developed. UDCA treatment was discontinued after delivery and liver enzyme and BA levels returned to normal within the next 6 weeks. She experienced a miscarriage in her second pregnancy. The third pregnancy was conceived spontaneously. Due to transient elevation of serum transaminase levels and mild pruritus during the first trimester, the patient was referred to our clinic. The first presentation in our clinic took place in the 15th week of gestation. Values for AST 20 U/l (normal range < 31 U/l), ALT 16 U/l (normal range < 35 U/l), GGT 15 U/l (normal range < 38 U/l), ALP 41 U/l (normal range 35-104 U/l), bilirubin 0,4 mg/dl (normal range < 1 mg/dl) and BA were all within the normal range at this time point. Pruritus was mild and well tolerated by the patient. No laboratory abnormalities suggestive of liver diseases despite ICP were detected. Ultrasound of the liver showed an organ of normal size and consistency without dilatation

of the intra- or extrahepatic bile ducts or signs for portal hypertension or vascular abnormalities. One gallstone was detected within the gallbladder, which was otherwise unremarkable. Because of the past history of severe ICP the patient and fetus were closely monitored in 2-4 week intervals both in the hepatology as well as in the gynecology outpatient departments. UDCA therapy was discussed with the patient, however, not initiated until BA levels rose above the upper limit of normal. At 29 weeks of gestation BA levels rose to 14 µmol/l, while transaminase and GGT levels were within the normal range (AST 27 U/l, ALT 32 U/l, GGT 19 U/l). UDCA was prescribed, however, not yet taken by the patient. At 31 weeks of gestation BA levels rose to 260 µmol/ and AST and ALT levels were elevated to 62 and 71 U/l, respectively. UDCA therapy was immediately started with 250 mg twice daily and increased to 13 mg/kg body weight daily. After initiation of UDCA therapy serum BA levels decreased to 27 µmol/l in the 33rd week of pregnancy and were below 40 µmol/l for the remainder of the pregnancy. Transaminase levels increased over the course of pregnancy with peak values of 86 and 110 U/l for AST and ALT, respectively at 35 weeks of gestation. Pruritus gradually worsened over the course of the pregnancy and persisted until delivery despite UDCA treatment. However, since pruritus was tolerable, total BA levels were below 40 µmol/l with UDCA administration and transaminase levels were below 100 U/l up to the 35th week of gestation, no additional treatment with rifampicin was initiated. At 35+2 weeks of gestation the patient had a preterm rupture of membranes with meconium stained amniotic fluid. The female neonate (2400 g, 47 cm) was well developed but showed signs of respiratory distress with tachypnea and had an Apgar score of 1/1/5. The neonate was taken to a pediatric intensive care unit and received continuous positive pressure ventilation (CPAP) for 2 days. The breathing improved quickly and the neonate could be transferred to a normal ward after 48 hours and discharged from the hospital 10 days after delivery. UDCA was discontinued after delivery and values for AST and ALT as well as BA normalized within the next 4 weeks.

Targeted resequencing of the genes encoding FIC1 (*ATP8B1*), BSEP (*ABCB11*), MDR3 (*ABCB4*) and FXR (*NR1H4*) was enabled by a custom designed AmpliSeq panel (IAD28759, Thermo Fisher Scientific). This gene panel covers 93.1% of the target region, comprising all coding exons including 20 bp flanking intron sequences and UTRs. The DNA sample was processed with the Ion AmpliSeq Library 2.0 Kit and sequenced on the Ion PGM sequencer using the 200 bp Hi-Q chemistry (Thermo Fisher Scientific). Resulting reads were analyzed with the Torrent Suite software 4.4.3 and variantCaller Plugin 4.4.2.2 with default 'germline low stringency' settings. Approximately 500,000 reads were generated per sample leading to a 2400x mean coverage. Identified exonic variants were confirmed by Sanger sequencing. In comparison to the reference sequence NM_005603 three homozygous exonic variants were detected in the *ATP8B1* gene: c.696T>C (p.D232D, rs319438), c.811A>C (p.R271R, rs319443) and c.3454G>A (p.A1152T, rs222581). All of these have an allele frequency of over 99% in Caucasians. In comparison to the reference sequence NM_003742 two homozygous exonic variants were detected in the *ABCB11* gene: c.1331T>C (p.V444A, rs2287622) and c.3084A>G (p.A1028A, rs497692) (Figure 1). Both variants represent common polymorphisms, which are found in about 50% of healthy individuals (allele frequencies from ExAc_Aggregated-Populations). In comparison to the reference sequence NM_000443 one homozygous exonic variant c.504C>T (p.N168N, rs1202283), which is a common polymorphism found in about 50% of healthy individuals (allele frequencies from ExAc_Aggregated-Populations), and one heterozygous exonic variant c.2932T>C (p.S978P) were detected in the *ABCB4* gene (Figure 1). No exonic variants were detected in the *NR1H4* gene (reference sequence NM_001206977). Mutations affecting intronic splice sites were not found in either of the four genes.

Discussion and Review of the Literature

ICP is the most frequent pregnancy-specific liver disease and diagnosis of ICP should be considered in pregnant women who present with pruritus and have elevated ALT serum levels (4). Increased serum BA levels in the absence of other hepatobiliary diseases confirm the diagnosis of ICP (4, 13).

Pathophysiology of ICP:

Gestational hormones and genetic predisposition are key factors in the development of ICP, while the role of environmental and alimentary factors as well as pregnancy-associated changes in the intestinal microbiome are less well understood (1, 14).

There are several lines of evidence, supporting the role of gestational hormones in the development of ICP (1, 3, 14). Serum levels of estrogen and progesterone as well as of their metabolites increase over the course of pregnancy with a peak in the third trimester and a rapid decline after delivery, which is reflected by the time of

presentation and disease course of ICP (1, 14). Twin pregnancies, which are associated with higher hormone levels, also have an increased risk of ICP development (1, 3, 15). Furthermore, a number of women experience pruritus and cholestasis after intake of hormonal contraception prior to ICP development (4, 13, 16). The mechanisms by which estrogens promote cholestasis, include trans-inhibition of the transport activity of the bile salt export pump BSEP (ABCB11), which excrets BA from the hepatocyte into the bile canaliculus (17) (Figure 2). Furthermore, transcription of BSEP is repressed by 17β-estradiol through estrogen receptor α (ERα), which interacts with the farnesoid X receptor (FXR, NR1H4) on the protein level, thus inhibiting the binding of FXR to the FXR response element in the BSEP promotor (18). FXR is a nuclear receptor responsive to BAs and the master regulator of BA synthesis, detoxification and transport in liver and the intestine (19-23). Under cholestatic conditions FXR is activated by BAs, which in turn leads to an upregulation of BA detoxification enzymes and hepatobiliary transport proteins such as BSEP, the organic anion transporter multidrug resistance-associated protein 2 (MRP2, ABCC2) as well as the phospholipid floppase multidrug resistance protein 3 (MDR3, ABCB4) (20, 24) (Figure 2). This mechanism protects hepatocytes from BA overload during cholestasis and stimulation of FXR with a synthetic agonist can protect rats from estrogen-induced cholestasis (25). However, BA mediated FXR activation and target gene expression is inhibited by sulfated progesterone metabolites, which are elevated in normal pregnancy and further raised in ICP patients (3, 14, 26, 27). Therefore, the susceptibility to hypercholanaemia, dyslipidemia and gallstone formation in pregnancy may be in part explained by the attenuated FXR signalling (26, 27). Furthermore, progesterone metabolites also inhibit hepatobiliary BA excretion through competitive inhibition of the basolateral BA uptake transporter NTCP (SLC10A1) as well as through trans-inhibition of canalicular BSEP (28, 29) (Figure 2). These mechanisms not only lead to retention of BAs within the hepatocyte but also to elevation of serum BA levels, which may in turn be responsible for the adverse fetal outcomes (7, 8). Elevation of estrogens, progesterones and their metabolites are a hallmark of pregnancy, however, only a small proportion of women develops ICP. This discrepancy indicates that other factors contribute to the pathogenesis of ICP. Genetic variants in genes encoding hepatobiliary transport proteins, such as BSEP and MDR3 have been identified as a key factor in ICP development (1, 14, 16, 30-37). The first report linking a heterozygous MDR3 mutation to ICP, came from the observation that the mothers of patients with progressive familial intrahepatic cholestasis type 3 (PFIC3), a severe from of cholestasis due to homozygous or compound heterozygous mutations in the MDR3 (*ABCB4*) gene, had recurrent episodes of cholestasis during pregnancy (37, 38). Heterozygositiy for MDR3 mutations is not only found in ICP and contraceptive-induced cholestasis (CIC), but also in hereditary gallstone disease, named low phospholipid-associated cholelithiasis (LPAC) as well as in drug-induced cholestasis (14, 39-43). Presence of MDR3 variants are detected in approximately 16% of ICP patients and have also been linked to severe forms of ICP with serum BA levels above 40 µmol/l and/or early onset of symptoms (13, 36, 44). Interestingly, besides rare heterozygous MDR3 variants, a common synonymous polymorphism (c.711A>T, p.I237I, rs2109505) shows a significant association with ICP development. The presence of this c.711A>T MDR3 variant, which has a minor allele frequency (MAF) of 0.21, leads to a two-fold increase in ICP development in affected individuals (31). However, the mechanism by which this synonymous variant contributes to ICP remains elusive (31).

Similar to MDR3, rare mutations as well as common polymorphisms in the BSEP (*ABCB11*) gene have also been identified (14, 33). The BSEP common variant c.1331T>C (p.V444A, rs2287622), which has a MAF of about 0.5, is significantly more common in patients with ICP (30, 31, 35). The amino acid exchange at position 444 may lead to lower BSEP protein levels, which may explain the association of this common variant not only to ICP but also to drug-induced liver injury and CIC (16, 42, 45). A recent extensive study in 563 ICP patients identified a highly significant association of ICP with an intronic common variant (MAF 0.4) upstream of the BSEP gene (rs7577650) (31). The mechanisms by which this intronic variant affects ICP development is unclear.

Besides MDR3 and BSEP, rare variants have also been detected in the FIC1 (*ATP8B1*) gene, which encodes a phosphatidylserine flippase located in the canalicular membrane and the FXR (*NR1H4*) gene in Caucasian ICP patients (46, 47). In contrast to MDR3 and BSEP, an association of common variants in the FIC1 and FXR genes with ICP could not be identified in a recent analysis of 563 Caucasian ICP patients (31). Furthermore, a link between variants in the MRP2 (*ABCC2*) gene and the gene encoding the pregnane X receptor (PXR, *NR1I2*) were only found in ICP patients from South America but not within the Caucasian ICP cohorts (14, 31).

Adverse events for the mother and the fetus:

The risk for adverse events for the fetus is associated with maternal serum BA levels (4, 7, 8). Fetuses from women with severe ICP, which is defined as serum BA levels above 40 µmol/l, have a significantly elevated risk for complications (7-10). In contrast, no increase in perinatal complications was observed in patients with mild ICP and serum BA levels below 40 µmol/l (8). Adverse perinatal outcomes in women with severe ICP comprise a higher risk for preterm delivery (adjusted odds ratio (OR) 5.4), for admission of the newborn to a neonatal unit (adjusted OR 2.7) for prematury or respiratory problems and for stillbirth (adjusted OR 2.6) (7, 8). In the large prospective study from the UK, there was also a trend for lower Apgar scores at 5 minutes (OR 1.9), however, this was not significant as compared to the control cohort (7). Furthermore, a higher rate of meconium stained amniotic fluid (up 24.8% in (8)) and abnormalities on cardiotocography (up to 28%) were reported in women with severe ICP (7). The molecular mechanisms responsible for adverse perinatal outcomes are not fully understood. However, animal studies and *in vitro* studies suggest that elevated BA levels may account for most of these fetal complications. It has been demonstrated that cholic acid not only induces myometrial contractions and preterm labor in rodents and sheep, respectively, but also stimulates intestinal motility and meconium staining of the amniotic fluid (for a review see (4)). Maternal cholestasis caused by targeted deletion of BSEP in mice results in perinatal lethality of well developed pups due to defective pulmonary surfactant and subsequent pulmonary failure (48). Lowering of maternal BA levels by additional deletion of PXR (*NR1I2*) in ABCB11 knockout mice improved neonatal survival significantly (48). Furthermore, BAs can induce arrhythmias in cultured neonatal cardiomyocytes, an effect which is abrogated by UDCA administration (49-52). Thus, sudden intrauterine death in severe ICP may be caused by fetal arrhythmias (4). The hypothesis that elevated BAs may be sufficient to cause sudden intrauterine death in late pregnancy is supported by a case report of a female patient with NTCP deficiency, a disorder which presents with elevated serum BA levels in absence of hepatic injury (F. M. Vaz et al., oral presentation at the XXIV Bile Acid Meeting, Düsseldorf 2016). Besides the immediate adverse outcomes maternal ICP seems to affect the body mass and metabolic profile of the adolescent children (53). This finding is supported by mouse studies, in which the female offspring of cholic acid-fed mothers developed increased body mass, impaired glucose tolerance and insulin sensitivity as well as steatohepatitis and inflammation of the white adipose tissue (53).

ICP not only poses a risk for the fetus but is also associated with a higher incidence of several liver, biliary, pancreatic, metabolic and immune-mediated diseases for the mother (6, 11, 12). The association of ICP with gallstone disease is well established and may be attributed in part to MDR3 variants, which are also associated with LPAC syndrome (11, 39). The increased prevalence of chronic hepatitis C infection in ICP patients may be responsible for the higher rate of liver fibrosis and cirrhosis development as well as the significant increase in hepatocellular and biliary cancer in ICP patients (6, 11). However, heterozygous rare MDR3 variants may also contribute to fibrosis and cirrhosis development (54-56) and may in part explain the higher risk for biliary cancer in women with ICP (6, 56). These findings suggest that ICP may be an indicator for subsequent diseases and that follow up of women with ICP for development of hepatobiliary, cardiovascular and metabolic disease should be considered and discussed with the patient (12).

Management of ICP:

Ursodeoxycholic acid (UDCA) is widely used in the therapy of ICP and is considered the first line treatment for ICP (57). Doses of UDCA range from 500 mg to 2 g/d (2, 4, 57-61). UDCA treatment has been shown to improve maternal serum biochemistry and pruritus (2, 4, 58, 60, 61). Whether UDCA treatment reduces fetal adverse events remains elusive, since a meta-analysis of UDCA trials only showed a trend towards improvement of fetal outcomes, however, the risk reduction was not statistically significant when compared to placebo (2, 4). Rifampicin has recently been successfully used to lower serum BA levels in combination with UDCA in women with severe ICP, who failed to respond to UDCA therapy alone (62). Besides UDCA, cholestyramine, dexamethasone and S-adenosyl-L-methionine have all been used with varying success in ICP (for an overview of drugs, which have been evaluated in ICP see (4)), however, are not considered as first line treatment (57).

Regular fetal monitoring using ultrasound and cardiotocography in women with ICP has not been shown to predict or reduce the risk of perinatal complications (4). In addition, there is also no clear recommendation for elective early delivery (4).

The patient described in this report had recurrent early onset ICP due to a combination of the ICP-associated homozygous common BSEP polymorphism p.V444A (rs2287622) as well as the heterozygous MDR3 mutation. Early onset ICP due to a combination of the common BSEP variant

p.V444A and a MDR3 missense mutation (p.S320F) has previously been described in a single patient (13). The MDR3 p.S978P variant detected in our patient has been reported in two female siblings, who were both heterozygous for this variant with no disease-causing mutation on the second allele. While one sister had a history of ICP, LPAC syndrome and liver fibrosis, the other sister suffered from cholangiocarcinoma (56). Furthermore, the p.S978P variant was also detected on one allele in a child with PFIC3, again in absence of a second disease-causing mutation (63). *In vitro* analysis of the p.S978P MDR3 variant demonstrated a normal localization of the mutated protein in the apical membrane but a complete loss of phosphatidylcholine floppase activity (63). Despite the lack of function of the mutated MDR3 protein, the liver disease of the affected child responded well to UDCA therapy (63), which may be explained by the heterozygous nature of the variant in this patient.

The elevation of liver enzymes and serum BAs was more pronounced in the first pregnancy of our patient, which may be attributed to the hormonal stimulation prior to this pregnancy. During the third pregnancy, serum BA levels were only once detected above 40 µmol/l despite initial monthly and later weekly controls. UDCA therapy was initiated at the time of highly elevated BA levels. Despite the UDCA treatment and serum BA concentrations below 40 µmol/l from 33 weeks of gestation onwards, the patient had a preterm spontaneous delivery with meconium staining of the amniotic fluid. The well developed female neonate had a low Apgar score, showed signs of respiratory distress and was admitted to a neonatal unit. The child recovered quickly and could be discharged from the hospital after 10 days. Serum biochemistry and BA levels of the mother returned to normal values within 4 weeks after delivery. Due to the heterozygous MDR3 mutation a yearly follow up with ultrasound, elastography as well as serum biochemistry was highly recommended. In case of elevated liver enzymes or cholestatic parameters, UDCA therapy should be commenced and continued life-long.

In summary, ICP is the most common pregnancy-associated liver disease. Elevated gestational hormone levels unmask a genetic predisposition in some women, leading to cholestasis development (14). Mutations in the MDR3 gene may be found in up to 16% of patients with ICP and may also be more common in severe ICP (36, 44). In adults MDR3 mutations are not only associated with ICP and hereditary gallstone disease but also with progressive liver disease leading to fibrosis and cirrhosis as well as cholangiocarcinoma development (55). Since MDR3 mutations may respond to UDCA therapy with stabilization of liver function, MDR3 gene sequencing may be recommended in ICP patients with gallstones as well as in patients with severe ICP. Furthermore, MDR3 gene sequencing could be considered in patients with a family history of cholangiocarcinoma. Women with ICP have a higher risk of hepatobiliary as well as metabolic and immune-mediated diseases, later in life, which may warrant regular follow ups. Administration of UDCA (500 mg/d up to 2 g/d) improves serum biochemistry and pruritus in ICP and may also reduce fetal adverse events and is therefore widely used as first line therapy. Serum BA levels should be monitored in ICP patients throughout pregnancy since concentrations above 40 µmol/l, which define severe ICP, are associated with increased fetal complications. Therefore, it is highly recommended that delivery of women with severe ICP takes place in a hospital which is equipped with a neonatal intensive care unit.

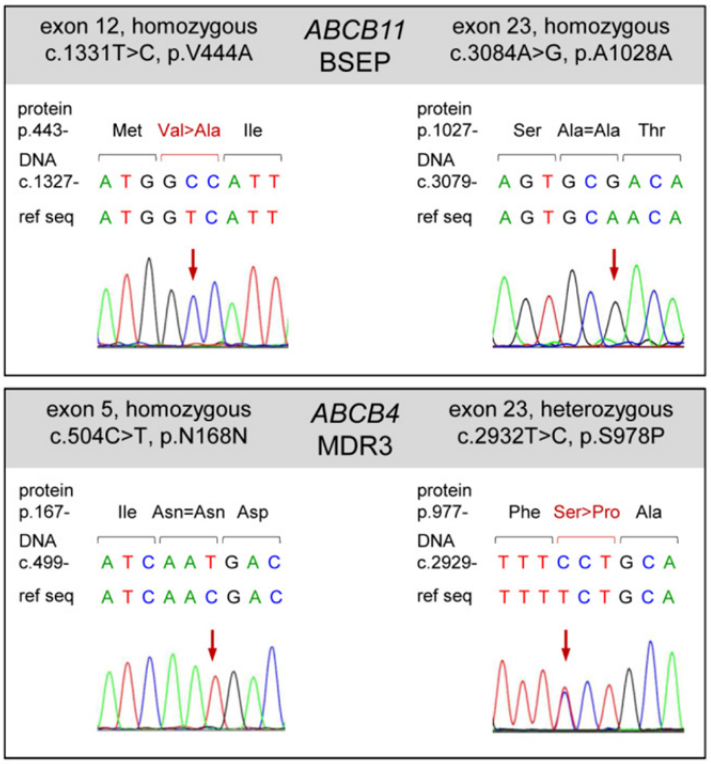

Figure 1. BSEP (*ABCB11*) and MDR3 (*ABCB4*) mutations detected in our ICP patient
Sequencing of all coding exons with adjacent intron regions of BSEP (upper panel) and MDR3 (lower panel) from genomic DNA revealed four exonic variants compared to reference sequences NM_003742 (BSEP) and NM_000443 (MDR3). The first translated exon was denoted as exon 1, adenine from ATG was counted as c.1. The woman had two common BSEP polymorphisms (p.V444A, p.A1028A) and a frequent MDR3 variant (p.N168N). Additionally, a heterozygous MDR3 missense mutation p.S978P was detected. Corresponding sequences on coding DNA (c.) and protein (p.) level are shown in detail. ref seq: reference sequence on nucleotide level.

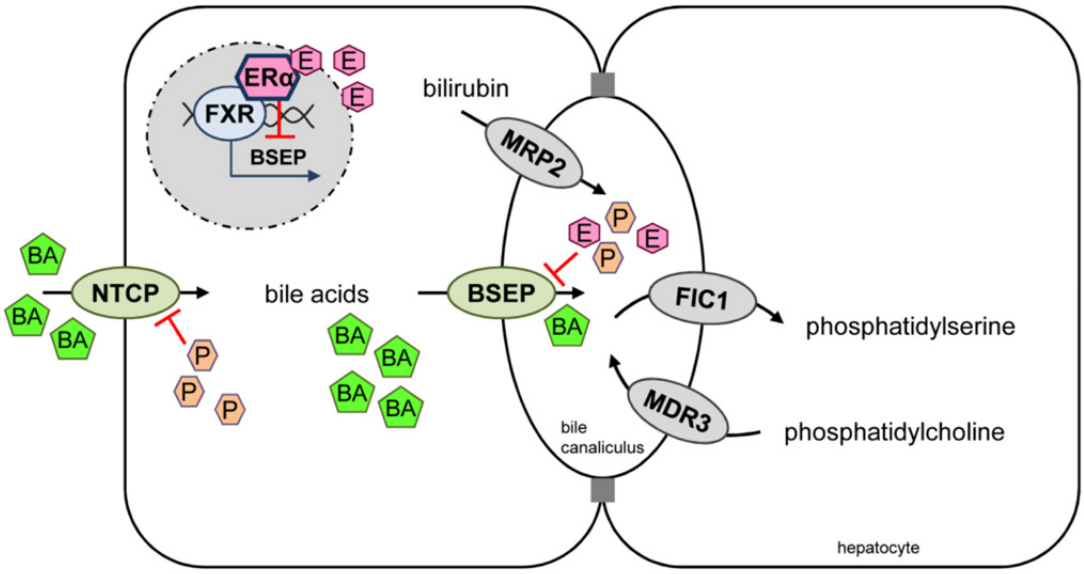

Figure 2. The gestational hormones estrogen and progesterone can trigger intrahepatic cholestasis through different mechanisms

Estrogen (E) can repress transcription of the bile salt export pump (BSEP) through direct interaction of estrogen receptor α (ERα) with the farnesoid X receptor (FXR). Therefore, the mechanism of FXR-mediated enhanced BSEP expression induced by elevated intracellular bile acid (BA) levels is blocked. Both estrogens as well as progesterones can also reduce BA excretion by trans-inhibition of BSEP resulting in an increase of hepatocellular BAs. Progesterone (P) may also inhibit BA uptake into the hepatocyte via the Na^+-taurocholate co-transporting polypeptide (NTCP), which further elevates serum BA levels in ICP patients. The multidrug resistance-associated protein 2 (MRP2) excretes bilirubin and organic anions into bile. The multidrug resistance protein 3 (MDR3) flopps phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane while the protein familial intrahepatic cholestasis 1 (FIC1) is responsible for membrane asymmetry by flipping phosphatidylserine. Mutations in the genes encoding BSEP, MDR3, FIC1 and FXR have been detected in Caucasian ICP patients.

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3.12 A mutation within the extended X loop abolished substrate-induced ATPase activity of the human liver ATP-binding cassette (ABC) transporter MDR3

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A Mutation within the Extended X Loop Abolished Substrate-induced ATPase Activity of the Human Liver ATP-binding Cassette (ABC) Transporter MDR3*

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Background: A mutation of the extended X loop of MDR3 caused hereditary liver cholestasis.
Results: Wild type MDR3 exhibited PC-induced ATPase activity, but the Q1174E mutant displayed no stimulation.
Conclusion: The glutamine preceding the ABC signature motif communicates substrate binding within the TMD to the extended X loop of the NBD.
Significance: This study provides evidence for a transmission interface coupling ATP hydrolysis to substrate transport.

The human multidrug resistance protein 3 (MDR3/ABCB4) belongs to the ubiquitous family of ATP-binding cassette (ABC) transporters and is located in the canalicular membrane of hepatocytes. There it flops the phospholipids of the phosphatidylcholine (PC) family from the inner to the outer leaflet. Here, we report the characterization of wild type MDR3 and the Q1174E mutant, which was identified previously in a patient with progressive familial intrahepatic cholestasis type 3 (PFIC-3). We expressed different variants of MDR3 in the yeast *Pichia pastoris*, purified the proteins via tandem affinity chromatography, and determined MDR3-specific ATPase activity in the presence or absence of phospholipids. The ATPase activity of wild type MDR3 was stimulated 2-fold by liver PC or 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine lipids. Furthermore, the cross-linking of MDR3 with a thiol-reactive fluorophore blocked ATP hydrolysis and exhibited no PC stimulation. Similarly, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin lipids did not induce an increase of wild type MDR3 ATPase activity. The phosphate analogues beryllium fluoride and aluminum fluoride led to complete inhibition of ATPase activity, whereas orthovanadate inhibited exclusively the PC-stimulated ATPase activity of MDR3. The Q1174E mutation is located in the nucleotide-binding domain in direct proximity of the leucine of the ABC signature motif and extended the X loop, which is found in ABC exporters. Our data on the Q1174E mutant demonstrated basal ATPase activity, but PC lipids were incapable of stimulating ATPase activity highlighting the role of the extended X loop in the cross-talk of the nucleotide-binding domain and the transmembrane domain.

The human multidrug resistance protein 3 (MDR3/ABCB4) belongs to the family of ATP-binding cassette (ABC)[2] transporters and is highly expressed in the canalicular membrane of hepatocytes. In the canaliculus, phosphatidylcholine (PC) lipids form mixed micelles with bile salts and cholesterol to reduce the destructive detergent activity of bile salts and to protect the biliary ducts (1). Because the flop of PC lipids from the inner leaflet of the lipid bilayer to the outer leaflet is a very slow process, PC lipids are translocated by the PC floppase MDR3 energized by ATP binding and hydrolysis (2–7).

The first evidence that MDR3 flops phospholipids (PLs) was obtained by the generation of homozygous knock-out mice for the murine *Mdr2* gene (*Mdr2*$^{-/-}$ mice), which is homologous to human MDR3 (2). These mice showed a complete absence of PLs from bile. The function of Mdr2 could be substituted in these mice by expressing the human *MDR3* gene, indicating that MDR3 acts as a PL floppase (3). Further studies used the lower hydrophobicity of short chain lipids (C_5-C_6 chain) to determine Mdr2/MDR3-mediated lipid translocation in yeast and cultured mammalian cells, respectively. Direct evidence that MDR3 translocates exclusively the PLs of the phosphatidylcholine family was obtained by van Helvoort and co-workers (5). They reported the translocation of fluorescently labeled short chain PC lipids in polarized pig kidney epithelial cells transfected with MDR3. Subsequently, it was demonstrated in HEK293 cells stably expressing MDR3 that PC lipids are excreted in a bile salt-dependent manner (6–8).

MDR3 is a 1279-amino acid glycoprotein and is composed of two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs), which are encoded on a single gene

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[2] The abbreviations used are: ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; MDR3/1, multidrug resistance protein 3/1; NBD, nucleotide-binding domain; PC, phosphatidylcholine; PFIC, progressive familial intrahepatic cholestasis; TAP, tandem affinity purification; TAP1/2, transporter associated with antigen processing; TMD, transmembrane domain; ICL, intracellular loop; EYFP, enhanced YFP; PL, phospholipid; BeF_x, beryllium fluoride; AlF_x, aluminum fluoride.

forming a so-called full-size ABC transporter (9). MDR3 shares up to 76% identity and 86% similarity in the amino acid sequence with the human multidrug resistance protein 1 (MDR1/ABCB1) while fulfilling a different physiological function (9–15). As of yet, no specific ATPase activity of MDR3 could be determined when the protein was expressed at high levels in insect (*Sf*9) cells or HEK293 cells, although MDR1 exhibited high ATPase activity in these systems (16, 17). Smith *et al.* (16) demonstrated vanadate-dependent nucleotide trapping of MDR3 in *Sf*9 plasma membranes, which could be inhibited by the MDR1 reversal agents verapamil and cyclosporin A. In addition, Ishigami *et al.* (17) ascertained the drug-stimulated ATPase activity of a chimera protein containing the TMDs of MDR1 and the NBDs of MDR3. They demonstrated that the purified chimera protein exhibited 10-fold lower drug-stimulated ATPase activity compared with MDR1. These findings confirmed that the NBDs of MDR3 bind ATP but that ATP hydrolysis takes place with an apparent low turnover number.

Previously, we demonstrated that human wild type MDR3 exhibited PC-induced ATPase activity, whereas the ATPase-deficient mutant of both Walker B motifs did not show stimulation (18). In this study, we characterized the ATPase activity of wild type MDR3 in terms of kinetic parameters, substrate spectrum, and the effect of phosphate analogues.

Furthermore, we analyzed the ATPase activity of the MDR3 Q1174E mutant *in vitro*, which was identified in a patient with progressive familial intrahepatic cholestasis type 3 (PFIC-3) and described previously in Kubitz *et al.* (19). This mutation is located in the extended X loop of NBD2 (TRVGDKGTQ). Based on structural and biochemical data, the NBDs dimerize in a head-to-tail orientation in the presence of ATP (20–22). Each ATP-binding site harbors highly conserved motifs, the Walker A (G*X*X*G*X*G*K(S/T), where *X* can be any amino acid residue), and the Walker B motif (ΦΦΦΦD, where Φ can be any hydrophobic residue) of one NBD, and the ABC signature motif (C loop, LSGGQ) of the opposing NBD (23). A highly conserved motif, the X loop (TEVGERG), which is located in close proximity to the ABC signature motif, was identified by Dawson and Locher (22) in ABC exporters. The X loop contacts the first intracellular loop (ICL1) of the TMD and likely transmits conformational changes generated by ATP binding and hydrolysis to the TMD (22, 24, 25). To date, the molecular function of this transmission interface with respect to coupling of the ATP hydrolysis cycle with substrate translocation is still not entirely clear. We expressed the Q1174E mutant in *Pichia pastoris* and purified the mutant via tandem affinity purification (TAP). The detergent-solubilized Q1174E mutant exhibited basal ATPase activity, which was demonstrated by modifying this mutant with a thiol-reactive fluorophore, but no substrate-stimulated ATPase activity in contrast to the wild type floppase. Thus, we suggest that glutamine 1174 is involved in the cross-talk of NBD and TMD.

EXPERIMENTAL PROCEDURES

Chemicals and Routine Procedures—Fos-choline 16 (FC-16) was obtained from Anatrace, and lipids were purchased from Avanti Polar Lipids. All other chemicals were from Sigma. The protein concentration was determined by a Bradford assay using the Coomassie Plus Assay (Pierce). The Mini-Protean 3 system (Bio-Rad) was used for SDS-PAGE on 7% gels. Immunoblotting was performed with a Tank blot system (Bio-Rad) using the monoclonal anti-P-gp C219 antibody (Merck) employing standard procedures.

Cloning of Human MDR3 and Site-directed Mutagenesis—We cloned human wild type *MDR3* (NM_000443.3) as described previously (18). Site-directed mutagenesis was carried out with the QuikChange® XL (Agilent Technologies) and the Phusion site-directed mutagenesis kit (Thermo Scientific), respectively. To generate the ATPase-deficient mutant, we exchanged Glu-558 and Glu-1207 of the conserved Walker B motif against Gln with the primer pair as described before (18). The Q1174E mutant was introduced into MDR3 with the Phusion site-directed mutagenesis kit (Thermo Scientific) using forward primer 5′-GATAAGGGGACTGAGCTCTCAGGAGGTCAAAAAC-3′ and the reverse primer 5′-CCCACTCTTGTTTCATATTTGTGGGGTAACG-3′. The sequences of all constructs were verified by DNA sequencing (GATC Biotech).

Transformation of P. pastoris and Expression Screening—*MDR3* expression constructs were transformed into electrocompetent *P. pastoris* X33 cells (Invitrogen) using standard procedures, and the expression level was analyzed as described in Ellinger *et al.* (18).

Fermentation of MDR3 Transformed P. pastoris Cells—For large-scale expression, *P. pastoris* cells containing the chromosomally integrated wild type *MDR3,* the E558Q/E1702Q double mutant, or the Q1174E mutant gene were fermented in a 15-liter table-top glass fermenter (Applikon Biotechnology) according to the *P. pastoris* fermentation guidelines from Invitrogen. Usually, a volume of 7 liters of basal salt media was inoculated with 1 liter of an overnight culture grown in MGY (1.34% yeast nitrogen base, 1% glycerol, and 4×10^{-5}% biotin) media. During the glycerol-fed batch phase, ~500 ml of 50% (v/v) glycerol added with 12 ml/liter PTM1 salts was fed for 4 h. Protein expression was induced by addition of 3.6 ml/h/liter of methanol for 48 h. During the complete fermentation, the temperature was set at 30 °C; oxygenation was kept above 20% O_2 saturation, and the mixer was set at 1000 rpm. Cells were harvested by centrifugation (5000 × *g*, 10 min, 4 °C), flash-frozen in liquid nitrogen, and stored at −80 °C until further use. Under these conditions, 1.4 kg of wet cell mass were routinely obtained.

Preparation of Crude Membrane Vesicles for Protein Purification—Generally, 100 g of frozen *P. pastoris* cells expressing the respective MDR3 protein (wild type or mutant) were thawed on ice, washed with 500 ml of ice-cold 50 mM Tris-HCl, pH 8.0, and resuspended at a concentration of 0.5 g of cells/ml in homogenization buffer (50 mM Tris-HCl, pH 8.0, 0.33 M sucrose, 75 mM NaCl, 1 mM EDTA, 1 mM EGTA, 100 mM 6-aminocaproic acid, 1 mM DTT) containing a protease inhibitor mixture (Roche Applied Science). Cells were disrupted by two passages through a pre-cooled TS Series Cell Disrupter (Constant Systems) at 2.7 kbar. After cell debris was removed by three centrifugation steps (10 min at 5000 × *g*, 4 °C, and two times for 30 min at 15,000 × *g*, 4 °C), crude membrane vesicles were prepared by ultracentrifugation for 1 h at 125,000 × *g* at 4 °C. Membrane vesicles were resuspended in buffer A (50 mM

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Tris-HCl, pH 8.0, 50 mM NaCl, 30% (v/v), glycerol) and flash-frozen in liquid N_2.

Solubilization and Purification of MDR3—The purification of wild type MDR3 or mutant was performed as described previously with a few modifications (18). All procedures were carried out at 4 °C. Crude membrane vesicles equivalent to 100 g of wet cells were diluted to a final concentration of 15 mg/ml total protein with buffer A and solubilized with 1% (w/v) of FC-16 for 1 h at 4 °C. Nonsolubilized membrane vesicles were removed by centrifugation at 125,000 × *g* at 4 °C for 1 h. All buffers typically contained 2× critical micelle concentration of FC-16 (0.0011% (w/v) FC-16) and were cooled to 4 °C. The supernatant supplemented with 20 mM imidazole was loaded onto a Ni^{2+}-loaded HiTrap chelating column (5 ml, GE Healthcare) and washed with 10 column volumes of buffer A supplemented with 20 mM imidazole. Proteins were eluted in one step with buffer B (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 200 mM imidazole, 20% (v/v) glycerol). The immobilized metal ion affinity chromatography eluate was diluted five times with $CaCl_2$ binding buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 20% (v/v) glycerol), applied to 4 ml of calmodulin affinity resin equilibrated in $CaCl_2$ binding buffer and incubated with the calmodulin resin overnight at 4 °C on a rotator. The resin was transferred into a gravity flow column and washed with 10 column volumes of $CaCl_2$ binding buffer. Protein was eluted with 5 column volumes of EGTA elution buffer (2 mM EGTA, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 20% (v/v) glycerol). The purified protein was directly used for ATPase activity measurements or aliquoted, snap-frozen in liquid nitrogen, and stored at −80 °C. Aliquots of the samples were analyzed by Coomassie Blue-stained SDS-PAGE and immunoblotting.

Labeling of MDR3 with BODIPY® FL Maleimide—Purified wild type MDR3 and mutants were incubated with 10-fold molar excess of BODIPY® FL maleimide (BODIPY® FL N-(2-aminoethyl))maleimide, Molecular Probes) at room temperature for 20 min. BODIPY®FL maleimide was added from a stock solution prepared in DMSO. The concentration of DMSO in the mixture did not exceed 0.2% (v/v). The reaction was terminated by the addition of a 20-fold molar excess of dithiothreitol, and samples were stored on ice until ATPase activity measurements. The labeling was analyzed by SDS-PAGE and fluorescence imaging at 488 nm excitation and 520 nm emission wavelength.

ATPase Activity Measurements of MDR3—The ATPase activity of MDR3 was examined with the malachite green assay by determination of released free inorganic orthophosphate as described previously with a few changes in the experimental procedures (18, 26, 27). Reactions were performed in a total volume of 100 μl in 50 mM Tris-HCl, pH 7.5 (at 37 °C), containing 2× critical micelle concentration of FC-16 and 10 mM $MgCl_2$. 5–10 μg of purified detergent-solubilized MDR3 proteins were used, and the reaction was started by adding 2 mM ATP at 37 °C and stopped after 0 and 40 min by the addition of 25 μl of the reaction mixture into 175 μl of 20 mM ice-cold H_2SO_4. Subsequently, 50 μl of dye solution (0.096% (w/v) malachite green, 1.48% (w/v) ammonium molybdate, and 0.173% (w/v) Tween 20 in 2.36 M H_2SO_4) was added. After 15 min, the amount of free phosphate was quantified by measuring the absorption at 595 nm. For substrate-stimulated ATPase activity, purified MDR3 was incubated at room temperature for 20 min with a defined volume taken from a 5 mM lipid stock solution and sonicated for 30 s to facilitate the incorporation of lipids into the detergent-protein micelles. The lipid-protein sample was directly used for ATPase activity measurement. For determination of kinetic parameters, ATP concentration was varied. The kinetic data were analyzed according to Michaelis-Menten kinetics as shown in Equation 1,

$$v = \frac{v_{max}[S]}{K_M + [S]} \quad \text{(Eq. 1)}$$

Here, v describes the reaction velocity; v_{max} is the maximal reaction velocity; S the substrate concentration, and K_m is the Michaelis-Menten constant.

Inhibition by phosphate analogues was assayed with stock solutions containing 100 mM $BeCl_2$ complemented with 500 mM NaF (100 mM BeF_x) and 100 mM $AlCl_3$ complemented with 500 mM NaF (100 mM AlF_x), respectively. Orthovanadate solutions (100 and 10 mM) were prepared from Na_3VO_4 at pH 10 and boiled for 2 min prior to use (28). To determine the IC_{50} values, the ATPase activity was plotted against the log of inhibitor concentration. The data were analyzed according to Equation 2,

$$y = y_{min} + \frac{y_{max} - y_{min}}{1 + 10^{((\log IC_{50} - x) \cdot slope)}} \quad \text{(Eq. 2)}$$

Here, y_{max} denotes the ATPase activity in the starting plateau, and y_{min} denotes the ATPase activity of the final inhibited plateau. y describes the ATPase activity value, and x represents the logarithmic concentration of the inhibitor. The IC_{50} value is calculated as the value of the inhibitor concentration used at an ATPase activity inhibition of 50%. This corresponds to the inflection point of the resulting curves.

For subsequent data evaluation, a reaction with EDTA (final concentration of 20 mM) was performed, and the autohydrolysis of ATP was subtracted. For calibration of free phosphate concentrations, a Na_2HPO_4 standard curve was used. All experiments were generally performed three times if not otherwise stated. Fitting was carried out using the GraphPad Prism Software (version 5.0a).

Immunofluorescence of Liver Tissue and MDR3-EYFP-transfected HEK293 Cells—Immunofluorescence staining and confocal microscopy were performed as described recently (29, 30). Briefly, snap-frozen sample liver tissue was cryo-sectioned and incubated with the transporter-specific antibodies K24 for bile salt export pump and P3II26 for MDR3 (Thermo Scientific) (31). Staining was then performed with secondary antibodies conjugated to Alexa Fluor 488 (green) and Alexa Fluor 546 (red). HEK293 cells were transiently transfected with either wild type MDR3-EYFP or $MDR3^{Q1174E}$-EYFP for 48 h using the pEYFP-N1 vector and polyethyleneimine ((average mass of 25 kDa; Sigma) as a transfection agent. After fixation for 30 s with ice-cold methanol, plasma membranes were immunostained with a monoclonal Na^+/K^+-ATPase antibody (clone M7-PB-E9, Sigma). Goat anti-mouse Cy3 (Dianova) was used as the

secondary antibody. Tissue and cells were visualized with an LSM 510 Meta confocal laser scanning microscope (Zeiss) using excitation wavelengths of 488 nm for EYFP and Alexa Fluor 488 or 543 nm for Cy3 and Alexa Fluor 546, respectively. 505–530-nm (green) and 560–615-nm band pass filters (red) were used for signal detection.

RESULTS

Expression and Purification of the Human ABC Transporter MDR3 in P. pastoris—Previously, we described the expression of wild type MDR3 and the ATP hydrolysis-deficient mutant (E558Q/E1207Q, later called the EQ/EQ mutant) in the methylotrophic yeast *P. pastoris*. To determine the influence of the Q1174E mutation located in the extended X loop on the ATPase activity, we mutated the Gln to Glu at position 1174 of MDR3 and expressed the mutant in *P. pastoris*. The fermentation of each *P. pastoris* strain, containing MDR3 chromosomally integrated, yielded about 1.4 kg of wet cell weight. We found that the detergent Fos-choline 16 (FC-16) solubilized MDR3 in large quantities, and fluorescence detection size exclusion chromatography analysis demonstrated an appropriate quality in terms of monodispersity of MDR3 solubilized in FC-16 (18). Therefore, we solubilized and purified all three MDR3 variants in FC-16 by means of tandem-affinity chromatography (TAP), which was established by Rigaut *et al.* (32). The purity of wild type MDR3, EQ/EQ, and Q1174E mutants was analyzed by Coomassie Brilliant Blue-stained SDS gels, and MDR3 was identified by immunoblot analysis using the C219 antibody, which is specific for the human ABC transporters MDR1 and MDR3 (Fig. 1*A*, *left* and *middle panels*). For wild type MDR3, we obtained 6.3 mg of highly purified (>90%) protein from 100 g wet cell weight in eight independent purifications compared with MDR3 EQ/EQ mutant with 3.4 mg of protein and the MDR3 Q1174E mutant with 2.1 mg of protein per 100 g wet cell weight (Table 1). The lower yield and purity of the mutants compared with the wild type were likely due to the reduced expression levels of the mutants in *P. pastoris* (data not shown).

ATPase Activity of Isolated Wild Type MDR3, EQ/EQ Mutant, and Maleimide-BODIPY-labeled MDR3—In a previous study, we demonstrated that MDR3 exhibits a PC-induced ATPase activity of 15 nmol min^{-1} per mg of purified MDR3 in comparison with the ATPase activity of the ATP hydrolysis-deficient Walker B (EQ/EQ) mutant in the presence of 2 mM ATP and 10 mM Mg^{2+} (18). We used mass spectrometry to analyze an FC-16 solubilized and purified sample of wild type MDR3. Here, we observed that high amounts of detergent molecules were attached to MDR3 (data not shown). We increased the ratio of MDR3 containing crude membranes to FC-16 during solubilization and reduced the amount of FC-16 during the purification protocol as described under "Experimental Procedures." Thus, we obtained a 6-fold higher stimulation of 105 ± 30 nmol min^{-1} mg^{-1} in the presence of DOPC lipids and 2 mM ATP (Fig. 1*B*). A similar effect was reported for the multidrug resistance protein LmrA from *Lactococcus lactis* (27).

In a first step of analyzing MDR3-related ATPase activity in detergent solution in the presence or absence of lipids, we determined the maximal reaction velocity (v_{max}) and the Michaelis-Menten constant (K_m) of ATP hydrolysis of wild type MDR3 and the EQ/EQ mutant in the absence and presence of DOPC lipids, respectively. Purified protein was first activated with 300 μM DOPC lipids, and the amount of released inorganic phosphate was measured at various ATP concentrations at 37 °C for 40 min.

The ATP hydrolysis-deficient EQ/EQ mutant exhibited an ATPase activity with a v_{max} of 240 ± 5 nmol min^{-1} mg^{-1}, but it could not be stimulated by adding DOPC lipids (v_{max} = 245 ± 5 nmol min^{-1} mg^{-1}) (Table 1). The exchange of the highly conserved Glu to Gln in the Walker B motif of ABC transporter prevents in general the hydrolysis of ATP in both nucleotide-binding sites (21). Groen *et al.* (7) reported an important cytotoxicity caused by expression of wild type MDR3 in HEK293T cells, which was counteracted by the single mutation E558Q of the Walker B motif of the first NBD resulting in an inactive floppase. This suggests that the observed ATPase activity EQ/EQ mutant is likely derived from co-purified contaminating ATPases. For wild type MDR3, we obtained an increased v_{max} value of 354 ± 13 nmol min^{-1} mg^{-1} in the absence of DOPC lipids corresponding to a k_{cat} value of 0.83 ± 0.03 s^{-1} (Fig. 1*B*, *left panel*; Table 1). ATPase activity of wild type MDR3 as well as the corresponding k_{cat} value were increased in the presence of DOPC lipids. We observed a v_{max} value of 536 ± 11 nmol min^{-1} mg^{-1} corresponding to a k_{cat} value of 1.26 ± 0.03 s^{-1} (Fig. 1*B*, *right panel*; Table 1). Furthermore, the activity of wild type MDR3 displayed a relatively high K_m value of 2.17 ± 0.20 mM in the absence and 1.78 ± 0.10 mM in the presence of DOPC lipids. Within experimental error, these values are more or less identical. The subtraction of the maximal ATPase activity of ATP-deficient EQ/EQ mutant from the measured v_{max} value of wild type MDR3 resulted in the maximal basal ATPase activity of 114 nmol min^{-1} mg^{-1} for wild type MDR3. However, a higher ATPase activity of wild type MDR3 cannot be ruled out, because of the differences in quality of the purified proteins. To get rid of contaminating ATPases, we performed additional purification steps such as ion exchange chromatography or size exclusion chromatography, but both purification steps resulted in no improvement (data not shown). This might be caused by the incorporation of contaminating proteins and MDR3 within the same detergent-lipid micelles. In addition, we varied ion strength, exchanged FC-16 with a number of commonly used detergents such as dodecyl β-D-maltopyranoside or octyl glucoside during the purification procedure, or elongated the washing step during immobilized metal ion affinity chromatography resulting in loss of ATPase activity or aggregation of protein, which might be caused by a de-lipidation of MDR3, for example.

Cross-linking of Cys in the Walker A motif of MDR1 with maleimide derivatives was demonstrated to block ATP hydrolysis, and the covalent modification was used to investigate the function of MDR1 (33–35). Because the Cys residues 433 and 1073 in the Walker A motifs are conserved in MDR3 and MDR1 and both proteins share high amino acid sequence identity, we reasoned that cross-linking of Cys of MDR3 using a maleimide-fluorophore might block ATP hydrolysis of MDR3. Thus, the difference in activity of wild type and modified MDR3 should reflect the basal ATPase activity. We labeled wild type MDR3

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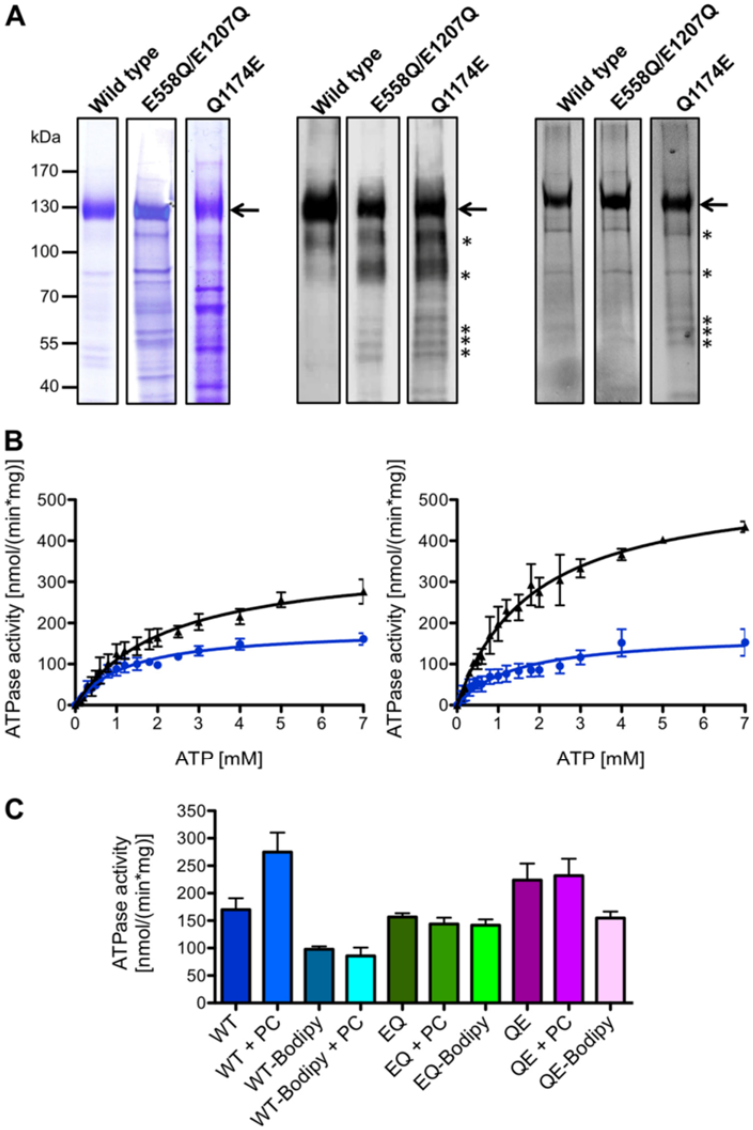

FIGURE 1. *A,* human wild type MDR3, the E558Q/E1207Q double mutant, and the Q1174E mutant purified from *P. pastoris*. The MDR3 variants containing a C-terminal His_6 tag and a calmodulin-binding peptide were expressed in the yeast *P. pastoris* and purified as described under "Experimental Procedures." 10 μg of purified MDR3 was resolved on a 7% SDS-PAGE and either stained with Coomassie Brilliant Blue (*left panel*) or detected by immunoblotting (*middle panel*) using the monoclonal anti-P-gp C219 antibody. MDR3 was cross-linked by the thiol-reactive maleimide-BODIPY fluorophore (MDR3-BODIPY) and analyzed by fluorescence imaging using excitation wavelength at 488 nm and emission wavelength at 520 nm (*right panel*). Molecular mass markers are shown on the *left*. MDR3 is indicated with an *arrow*, and degradation products of MDR3 are shown with *asterisks*. *B,* ATPase activity of purified wild type MDR3 (*black triangle*) and MDR3-BODIPY (*blue circles*) in the absence (*left panel*) or presence (*right panel*) of DOPC lipids. *C,* ATPase activity of purified wild type MDR3 (*blue*), the ATPase-deficient MDR3 EQ/EQ mutant (*green*), and the Q1174E mutant (*magenta*) and the corresponding cross-linked BODIPY derivatives in the presence of 2 mM ATP and 300 μM DOPC (*PC*).

and mutants with the thiol-reactive fluorophore maleimide-BODIPY (further called MDR3-BODIPY). Full-length MDR3 was predominantly labeled beside degradation products of MDR3 as determined by comparison of the fluorescence image and the immunoblot (Fig. 1*A*, *middle* and *right panels*). To exclude that labeling also inhibited the ATP hydrolysis of co-purified ATPases, we compared the ATPase activity of the EQ/EQ mutant with the corresponding labeled protein in the presence of 2 mM ATP and observed a slight decrease of the ATPase activity of 17 nmol min^{-1} mg^{-1} (Fig. 1*C*), whereas the ATPase activity of wild type MDR3-BODIPY was decreased by 67 nmol min^{-1} mg^{-1} in the presence of 2 mM ATP. This difference therefore reflects the basal activity of MDR3. To examine whether co-purified ATPases exhibited PC-induced stimula-

TABLE 1
Purification and ATPase activity of wild type MDR3, the EQ/EQ, and the Q1174E mutant

Purified protein	Yield	No. of purifications	K_m (MgATP)	v_{max}	k_{cat}
	mg/100 g wet cell weight		*mM*	*nmol min^{-1} mg^{-1}*	*s^{-1}*
Wild type	6.3 ± 1.2	8	2.17 ± 0.20	354 ± 13	0.83 ± 0.03
			1.78 ± 0.10[a]	536 ± 11[a]	1.26 ± 0.03[a]
Wild type-BODIPY			1.26 ± 0.10	186 ± 6	0.44 ± 0.01
			1.43 ± 0.19[a]	175 ± 10[a]	0.41 ± 0.02[a]
E558Q/E1207Q	3.4 ± 0.6	5	[b]	[b]	[b]
Q1174E	2.0 ± 0.2	5	1.04 ± 0.15	286 ± 16	0.64 ± 0.04
Q1174E-BODIPY			0.74 ± 0.10	198 ± 5	0.46 ± 0.01

[a] ATPase activity was in the presence of 300 μM DOPC lipids. The subtraction of the ATPase activity in the absence and presence of DOPC lipids revealed a Δv_{max} of 182 nmol min^{-1} mg^{-1} and Δk_{cat} of 0.43 s^{-1} for wild type, a Δv_{max} of −9 nmol min^{-1} mg^{-1}, and a Δk_{cat} of ≤ 0.03 s^{-1} for labeled wild type MDR3-BODIPY.
[b] The observed ATPase activity of the EQ/EQ mutant results from co-purified, contaminating ATPase. Please see text for further details.

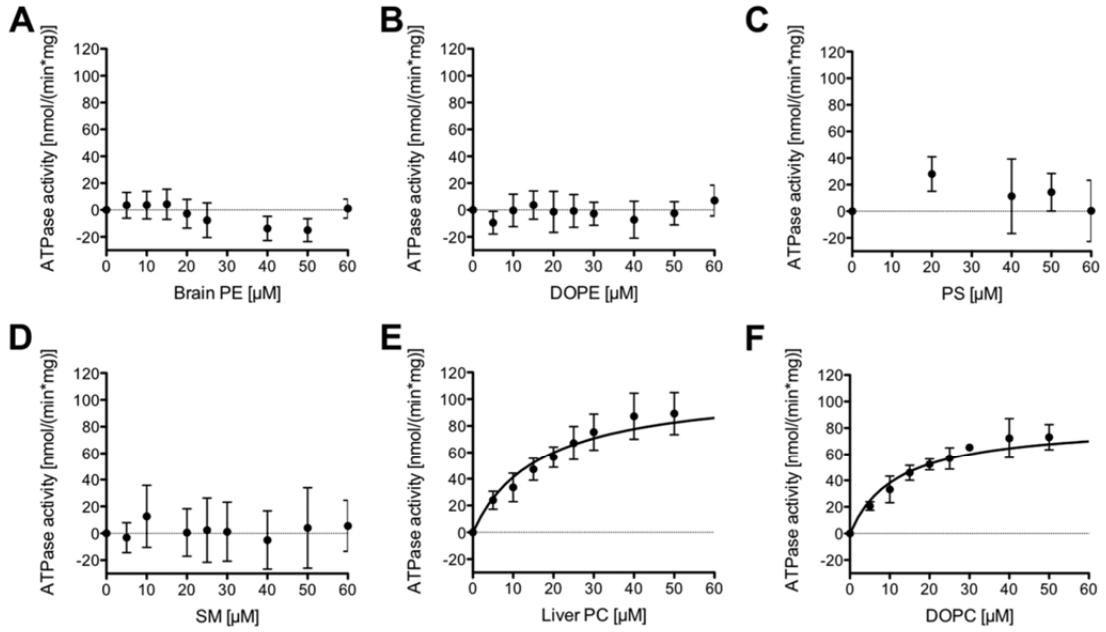

FIGURE 2. **Concentration dependence of the ATPase activity of MDR3 in the presence of different kinds of lipids: *A*, brain PE lipids; *B*, DOPE lipids; *C*, PS lipids; *D*, SM lipids; *E*, liver PC lipids; and *F*, DOPC lipids, respectively.** The ATPase activity was started by addition of 2 mM ATP and assayed for 40 min at 37 °C. The ATPase activity in the absence of lipids (159 ± 14 nmol min^{-1} mg^{-1}) was subtracted from the ATPase activity in the presence of lipids. The data represent the average of at least six independent experiments (mean ± S.D.).

tion, we determined the ATPase activity of MDR3-BODIPY in the presence of DOPC lipids and observed no stimulation (Fig. 1, *B* and *C*). For MDR3-BODIPY, we determined a v_{max} value of 186 ± 6 nmol min^{-1} mg^{-1} and a corresponding K_m value of 1.26 ± 0.10 mM in the absence of DOPC lipids, which was identical in the presence of DOPC lipids within the experimental error (v_{max} = 175 ± 10 nmol min^{-1} mg^{-1} and K_m = 1.43 ± 0.19 mM) (Fig. 1*B* and Table 1). These data demonstrated that MDR3 exhibited a basal ATPase activity between 114 and 168 nmol min^{-1} mg^{-1} and that the PC-induced ATPase activity exclusively reflected MDR3-specific ATP hydrolysis.

Headgroup of the Lipid Moiety Determines MDR3 Substrate Specificity—The physiological function of MDR3 is the flop of PC lipids into the canaliculus of hepatocytes. Transport experiments with MDR3-transfected polarized pig kidney epithelial cells demonstrated that MDR3 flops fluorescently labeled short chain PC lipids but not the corresponding PE variants. Furthermore, van Helvoort *et al.* (5) ascertained that MDR3 distinguished between PC and sphingomyelin (SM) lipids. These findings led to the conclusion that MDR3 binds predominantly phospholipids with a choline headgroup and a diacyl backbone.

To exclude that the increased ATPase activity in the presence of PC lipids is caused by an unspecific effect of these lipids on MDR3, we determined the ATPase activity of wild type MDR3 in the presence of brain PE, DOPE, PS, and SM lipids. Data derived for other human ABC transporters demonstrated that lipids might display a stabilizing effect on the conformation of the protein, which would result in an increased ATPase activity (36–39). We found that neither the presence of brain PE nor DOPE lipids increased MDR3 ATPase activity (Fig. 2, *A* and *B*). Furthermore, the addition of PS or SM lipids resulted in no significant stimulation (Fig. 2, *C* and *D*). To further investigate the influence of liver PC and DOPC lipids on the ATPase activity of wild type MDR3 in the solubilized state, K_m and v_{max} values were determined by Michaelis-Menten kinetic analysis (Fig. 2, *E* and *F*). Liver PC and DOPC lipids stimulated the

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ATPase activity in a concentration-dependent manner. The maximal velocity v_{max} value was slightly increased for liver PC (109.8 ± 5.9 nmol min^{-1} mg^{-1}) as compared with DOPC lipids (84.0 ± 3.4 nmol min^{-1} mg^{-1}) (Table 2). Assuming that 100% homogeneous and active MDR3 is present in the ATPase assay, approximately 4 s are required to catalyze the flop of one lipid molecule by one molecule of MDR3 in the case of DOPC and liver PC lipids. The K_m values were 16.6 ± 2.6 μM for liver PC lipids and 12.0 ± 1.6 μM for DOPC lipids.

Inhibition of MDR3 ATPase by Transition and Ground State Analogues—Next, we examined whether MDR3-specific ATP hydrolysis could be inhibited by transition and ground state analogues of phosphate. Phosphate analogues have been extensively used to analyze, for example, the catalytic mechanism of MDR1 and other human ABC transporters (36, 40–42). We determined the ATPase activity of wild type MDR3 after adding BeF_x, AlF_x, and orthovanadate in the presence and absence of DOPC lipids, respectively (Fig. 3*A*). BeF_x and AlF_x led to complete inhibition of ATP hydrolysis at a concentration of 1 mM and abolished the ATPase activity of MDR3 as well as the hydrolysis activity of the co-purified ATPases. No inhibition of the ATPase activity by orthovanadate was observed in the absence of DOPC lipids even at concentrations up to 3 mM. However, ATP hydrolysis in the presence of DOPC lipids is more strongly inhibited than in the absence of DOPC lipids (~20%) at a concentration of 3 mM orthovanadate. Additionally, the Q1174E mutant was not susceptible to vanadate inhibition (Fig. 3*B*). Hence, orthovanadate inhibited exclusively the PC-stimulated ATPase activity of MDR3.

Furthermore, we investigated the half-maximal inhibitory concentration (IC_{50}) of BeF_x, AlF_x, and orthovanadate in the absence and presence of DOPC lipids. The calculated IC_{50} values are summarized in Table 3. The IC_{50} value for BeF_x is slightly decreased in the presence of DOPC lipids (28.6 ± 1.0 μM) compared with the IC_{50} value of 38.0 ± 1.1 μM in the absence of DOPC lipids. In good agreement with this, the half-maximal ATPase inhibition of the close homologue MDR1 was achieved at a concentration of 23 μM BeF_x (43). AlF_x exhibited similar IC_{50} values of MDR3 ATPase activity of 199.8 ± 1.0 μM in the absence and 183.8 ± 1.0 μM in the presence of DOPC lipids, respectively. Interestingly, orthovanadate inhibited the MDR3-specific DOPC-stimulated ATPase activity, and we determined an IC_{50} value of 397.5 ± 2.0 μM. In contrast, purified human MDR1, which shares more than 86% amino acid similarity to MDR3, exhibited an IC_{50} of 2.3 μM (41, 44).

MDR3 Expression in a Liver Biopsy of a Patient with PFIC-3 and in Transfected HEK293 Cells—A 3-year-old girl with a PFIC-3 phenotype was compound heterozygous for a nucleotide exchange (c.3520C>G) in coding exon 26, resulting in the missense mutation Q1174E, as well as for the intronic donor splice site mutation c.286 + 1G>A of ABCB4 (Gene ID, 5244; mRNA reference NM_000443.3), which likely disrupts MDR3-mRNA expression from the related allele (19). Immunofluorescence staining of the patient's liver revealed an apparently normal immunoreactivity for MDR3 and the bile salt

TABLE 2

Kinetic parameters of MDR3 ATPase activity in the presence of different kinds of lipids

ATPase activity in the absence of lipids was determined and subtracted from the ATPase activity of MDR3 in the presence of liver PC, DOPC, brain PE, and DOPE lipids, respectively.

Lipids	K_m	v_{max}	k_{cat}
	μM	*nmol min^{-1} mg^{-1}*	s^{-1}
Liver PC	16.6 ± 2.6	109.8 ± 5.9	0.26 ± 0.01
DOPC	12.0 ± 1.6	84.0 ± 3.4	0.20 ± 0.01

TABLE 3

IC_{50} values of MDR3 ATPase activity inhibited by phosphate analogues in the absence or presence of 300 μM DOPC lipids

Phosphate analogue	IC_{50}	
	−DOPC	+DOPC
	μM	
Orthovanadate		397.5 ± 2.0
BeF_x	38.0 ± 1.1	28.6 ± 1.0
AlF_x	199.8 ± 1.0	183.8 ± 1.0

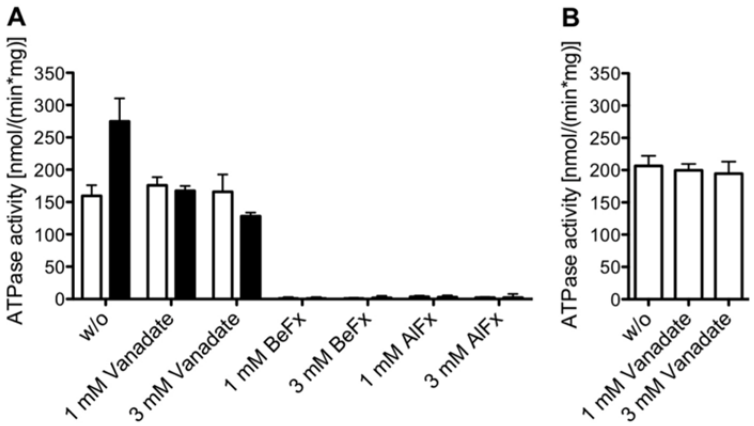

FIGURE 3. **Inhibition of MDR3 ATPase activity by phosphate analogues.** *A,* ATPase activity of purified wild type MDR3 was measured without (*w/o*, *white column*) and with 300 μM DOPC lipids (*black column*) in the presence of orthovanadate, BeF_x, and AlF_x. The reaction mixture contained 2 mM ATP and inhibitors at a final concentration of 1 or 3 mM. The data are means ± S.D. of at least four independent experiments. *B,* ATPase activity of Q1174E mutant in the presence of orthovanadate. The data represent the average of at least two independent experiments (mean ± S.D.).

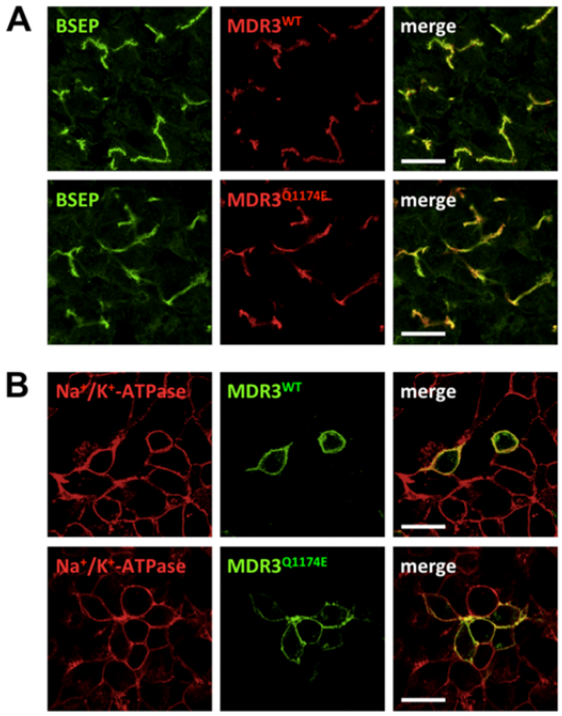

FIGURE 4. **MDR3 mutation Q1174E does not affect protein localization *in vivo* or *in vitro*.** *A*, sample from a liver with normal MDR3 expression (*upper panel*) and from the patient carrying the heterozygous Q1174E mutation (*lower panel*). The *green fluorescence* corresponds to bile salt export pump (*BSEP*), which acts as a canalicular marker, and the *red fluorescence* represents MDR3. *B*, transient expression of human wild type (*upper panel*) and Q1174E (*lower panel*) MDR3-eYFP (*green*) after transfection into HEK293 cells. Cells were fixed after 48 h and stained for the Na^+/K^+-ATPase as a plasma membrane marker (*red*). *Scale bars*, 20 μm.

export pump (used here as a canalicular marker protein) at the canalicular membrane as compared with a normal liver (Fig. 4*A*). HEK293 cells were transiently transfected with wild type MDR3-EYFP or MDR3 Q1174E-EYFP (Fig. 4*B*). Both MDR3 variants were equally targeted to the plasma membrane. This indicates that the amino acid exchange allows normal protein folding and trafficking and that the mutation more likely results in a functional defect of MDR3.

Extended X Loop Mutation Q1174E Abolished PC-induced ATPase Activity—In ABC exporters, the highly conserved X loop (TEVGERG) is localized in the helical subdomain of the NBD in direct proximity of the ABC signature motif. X-ray structures of two bacterial ABC exporters, Sav1866 from *Staphylococcus aureus* and MsbA from *Salmonella typhimurium*, and five eukaryotic ABC transporters, MDR1 from *Mus musculus*, *Cyanidioschyzon merolae*, and *Caenorhabditis elegans*, ABCB10 from *Homo sapiens*, and Atm1 from *Saccharomyces cerevisiae*, revealed an enlarged transmission interface of the NBD and the TMD (22, 45–50). This transmission interface includes the X loop, which contacts the intracellular loop 1 (ICL1) of the opposing TMD and transmits signals of ATP binding and hydrolysis by the nearby ABC signature motif to the TMD (22). Oancea *et al.* (24) substituted the conserved glutamate (Glu-602) of the X loop of the transporter associated with antigen processing (TAP1/2) and demonstrated that peptide binding was not affected; however, transport activity was reduced from 20% for the E602D mutant to complete disruption for the E602R mutant, suggesting a pivotal role in transmitting conformational changes generated by ATP hydrolysis and substrate translocation. However, the molecular function of this transmission interface in terms of the coupling of the ATP hydrolysis cycle and substrate translocation is still not entirely clear.

We focused on the interaction between the glutamine at position 1174 of the NBD2, which is located in the extended X loop (TRVGDKGTQ) next to the leucine of the ABC signature motif and conserved in human ABC transporters such as MDR1, TAP1/2, and the cystic fibrosis transmembrane conductance regulator (CFTR) (Fig. 5*A*) (24, 25, 46) We hypothesized that the glutamine (Gln-1174) is indispensable for the transmission of the conformational change of the NBD2 to the ICL1 of the TMD.

Previously, we generated the homology model of human wild type MDR3 based on the crystal structure of the homologue of multidrug resistance ABC transporters Sav1866 (Protein Data Bank code 2HYD) as a structural template and the amino acid sequence of the isoform B of MDR3 (Fig. 5*B*) (51). The isoform B of MDR3 contains seven additional amino acids within the NBD2 compared with the major isoform A, which was used in our experiments. Nevertheless, the Gln of isoform A at position 1174 and of isoform B at position 1181 did not differ in the homology model of NBD2, because the orientation of the side chain of Gln-1174 represents a rotamer of Gln-1181 (Fig. 5*C*). The NBD structures of both isoforms are well aligned and had a root mean square deviation value of 1.6 Å over 229 Cα atoms. Thus, we used this structural model of MDR3 to generate the structure of the MDR3 Q1181E mutant (Q1174E isoform A). The close-up view of the transmission interface clearly indicates that Gln-1181 of human MDR3 can be replaced by a Glu without large conformational rearrangement, but it prohibited hydrogen bonding between Gln-1181 (Q1174 of isoform A) and Asp-166 of the coupling helix of ICL1 (Fig. 5*D*).

To ascertain whether the mutation of Gln-1174 to Glu abrogates ATP hydrolysis *in vitro*, we purified MDR3 Q1174E via TAP and assayed ATPase stimulation with DOPC, 1,2-dipalmitoyl-phosphatidylcholine, and liver PC lipids (Fig. 6). These data clearly demonstrated that all tested PC lipids were incapable of stimulating ATPase activity of MDR3, a situation comparable with the ATPase-deficient EQ/EQ mutant and the labeled wild type MDR3-BODIPY sample (Fig. 1). To clarify whether the Q1174E mutant exhibits basal ATPase activity or abrogates the ATPase activity completely, we determined ATPase activity of the Q1174E mutant and the corresponding Cys-labeled Q1174E-BODIPY sample in the presence of 2 mM ATP (Fig. 1*C*). The ATPase activity of Q1174E was higher as compared with wild type MDR3 likely due to a higher degree of protein impurities in the purified sample. Notably, the inhibition of ATPase activity of Q1174E-BODIPY (69 nmol min^{-1} mg^{-1}) was comparable with wild type MDR3-BODIPY (67 nmol min^{-1} mg^{-1}) with 2 mM ATP indicating that the Q1174E

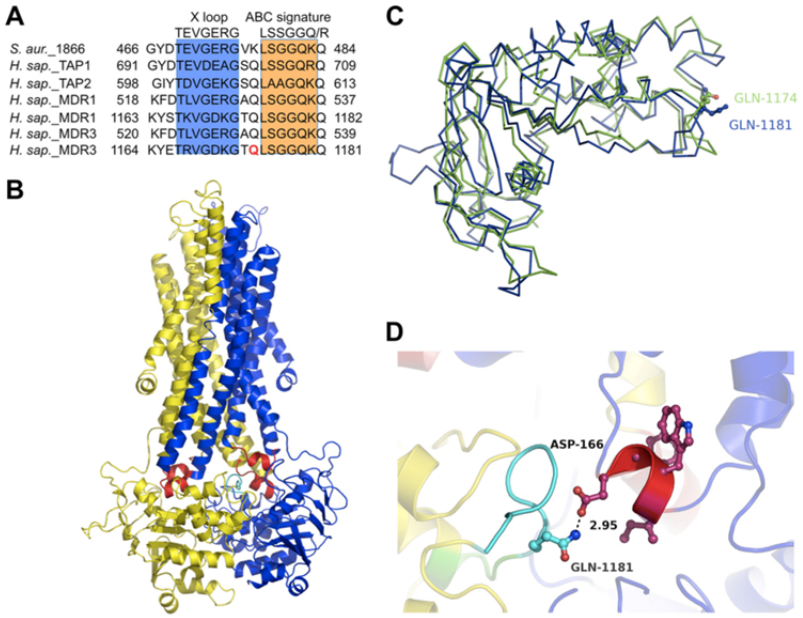

FIGURE 5. *A,* alignment of the amino acid sequence of MDR3 with selected human ABC transporters. The X loop motif is shaded in *blue* and the ABC signature motif in *orange*. Gln-1174 is colored in *red*. *B,* homology model of human MDR3 based on the structure of Sav1866 (Protein Data Bank code 2HYD) and the amino acid sequence of isoform B of MDR3 (51). One transporter half, consisting of transmembrane domain (*TMD*) and nucleotide-binding domain (*NBD*), is shown in *blue* and the other in *yellow*. The coupling helices are highlighted in *red,* and the X loop is colored in *cyan*. *C,* overlay of the MDR3 NBD2 of the isoform A (*green*) and isoform B (*blue*). Gln-1174 of the isoform A is a rotamer of Gln-1181 of the isoform B. *D,* close-up view of the interface between TMD and NBD2. Gln-1174 of the X loop is shown in *stick* representation (*cyan*).

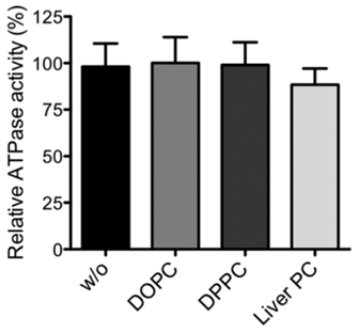

FIGURE 6. **ATPase activity of the purified Q1174E mutant was measured without (*w/o*) lipids and with DOPC, DPPC, or liver PC lipids as described under "Experimental Procedures."** None of these lipids stimulated the ATPase activity of the Q1174E mutant. One hundred percent activity represents 223 ± 25 nmol min^{-1} mg^{-1}.

mutant exhibited basal ATPase activity. In addition, we determined the kinetic parameters of Q1174E and Q1174E-BODIPY according to Michaelis-Menten (Table 1). We obtained a maximal velocity of 286 ± 16 and 198 ± 5 nmol min^{-1} mg^{-1} for Q1174E-BODIPY. The K_m values were similar for Q1174E (K_m = 1.04 ± 0.15 mM) and the corresponding labeled sample (K_m = 0.74 ± 0.1 mM) within experimental error. Taken together, the Q1174E mutant exhibited a higher affinity to ATP and a reduced basal activity of ~90 nmol min^{-1} mg^{-1} compared with wild type floppase (v_{max} = 168 nmol min^{-1} mg^{-1}; K_m = 2.17 ± 0.2 mM). On the basis of these data, we suggest that the signals of substrate binding and substrate transport are not transmitted properly in this mutant.

DISCUSSION

Recently, we established the expression of human *MDR3* in the yeast *P. pastoris* and purified the protein in amounts suitable for a detailed functional analysis with a purity of more than 90% in the case of wild type MDR3 (Fig. 1). In ABC transporters, binding of the substrate to the TMD typically stimulates ATP hydrolysis at the NBDs, and the energy from the hydrolysis is used to translocate the substrate across the membrane (52). The modulation of ATP hydrolysis upon substrate binding and translocation has been reported for several ABC transporters and is frequently used as a readout of transporter function and activity (36, 40, 44, 53, 54). However, no specific ATPase activity of MDR3 has been reported so far (16, 17). We demonstrated previously that wild type MDR3 exhibited a PC-stimulated ATPase activity (18). In this study, we characterized the ATPase activity of wild type MDR3 in terms of kinetic parameters, substrate spectrum, and the effect of phosphate analogues. We demonstrated that lower amounts of FC-16 increased the ATPase activity of MDR3. Former studies have shown that especially these zwitterionic detergents tend to deactivate proteins (55). The influence of the concentration of detergent on the ATPase activity was also demonstrated for MDR1 (37, 56). We observed a basal ATPase activity of wild type MDR3, but due to co-purified contaminating impurities that might act as ATPases, the observed basal ATPase activity is derived from MDR3 and impurities.

Cross-linking of wild type MDR3 with maleimide-BODIPY blocks basal and PC-induced ATPase activity as demonstrated for MDR1 (33–35), whereas the ATP hydrolysis-deficient EQ double mutant (E558Q/E1207Q) showed no PC stimulation, and ATPase activity of the labeled EQ/EQ mutant was only marginally reduced (Table 1 and Fig. 1). The PC-induced ATPase activity of wild type MDR3 was about 9-fold lower than the described ATPase activity of detergent-soluble mouse (1.5 μmol min^{-1} mg^{-1}) and human MDR1 (1.8 μmol min^{-1} mg^{-1}) in the presence of verapamil and lipids, but it was comparable with the substrate-induced ATPase activity of other members of the human liver ABC transporter family such as ABCC3 (v_{max} = 170 nmol min^{-1} mg^{-1} in the presence of 1 mM taurocholate) and ABCG5/G8 (v_{max} = 256 nmol min^{-1} mg^{-1} in the presence of taurocholate or taurodeoxycholate) (36, 37, 40, 44). Furthermore, purified chimera protein composed of MDR1 TMDs and MDR3 NBDs expressed in HEK293 cells exhibited drug-stimulated ATPase in the presence of vinblastine (v_{max} = 170 nmol min^{-1} mg^{-1}) and verapamil (v_{max} = 450 nmol $min^{-1}mg^{-1}$), respectively, but it did not mediate bile salt-dependent PC efflux (17). These findings confirm that MDR3 NBDs are able to accomplish substrate-stimulated ATP hydrolysis in the detergent-solubilized state. Despite the high degree of amino acid sequence identity between MDR1 and MDR3 (>85% homology to human MDR1 and 80% to mouse Mdr1a (previously called mouse MDR3)), they exhibited different maximal ATPase activities in the presence of the transport substrate but similar and relatively high K_m values for ATP (MDR3, $K_m = 1.90 \pm 0.27$ mM; MDR1, $K_m = 1.5$ mM (44)), which were in a range typical for ABC transporters (36, 40, 44, 57). Because PC lipids are present in high concentrations in the plasma membrane, Ishigami *et al.* (17) suggested that MDR3 is a low affinity transporter optimized for PC translocation. Our data confirm such a suggestion. Nevertheless, bile salts were shown to be the driving force for PC secretion, and currently, we cannot rule out that bile salts influence the ATPase activity of MDR3 (6, 7, 58).

Furthermore, we demonstrated that PC lipids specifically stimulated MDR3 ATPase activity, whereas brain PE, DOPE, PS, and SM lipids did not induce any stimulation (Fig. 2). This is consistent with previously reported data (3–6, 16). All experiments showed that MDR3 translocates fluorescently labeled short chain PC lipids or long chain derivatives but not PE, SM, or ceramides. In conclusion, MDR3 binds exclusively phospholipids with a choline headgroup. However, we cannot exclude that MDR3 translocates MDR1 substrates as well. There is evidence that MDR3 is able to transport digoxin, paclitaxel, and vinblastine and that this transport is inhibited by either verapamil, cyclosporin A, or PSC833 (16). Furthermore, MDR3 gene products were found in paclitaxel-, doxorubicin-, and vincristine-resistant cell lines, indicating that MDR3 might be involved in multidrug resistance (59). Further investigations are required to address this issue.

The ATPase activity of many ABC proteins, such as MDR1, is efficiently inhibited by phosphate analogues such as orthovanadate, aluminum, and beryllium fluoride (41, 44). For MDR3, it was shown that nucleotide trapping by orthovanadate is indeed possible (16). Here, we have demonstrated that aluminum fluoride and beryllium fluoride inhibited ATPase activity of MDR3 and co-purified contaminating NTPases. Importantly, orthovanadate inhibited exclusively PC-induced MDR3-specific ATPase activity at high concentrations (Fig. 3). In contrast, mouse Mdr1a was completely inhibited at a concentration of 200 μM orthovanadate, and human MDR1 showed an IC_{50} of 2.3 μM, ~165-fold lower than the IC_{50} value found for MDR3 (Table 3) (41, 44). Nevertheless, our data are in agreement with other studies on human ABC transporters. Orthovanadate did not inhibit ABCA1, and bile acid-stimulated ATPase hydrolysis of ABCG5/G8 was also only inhibited at high orthovanadate concentrations (40, 54).

Structural studies on the full-length ABC transporter Sav1866 have revealed that the NBDs of ABC exporters contain a conserved X loop motif, which transmits the conformational changes of ATP binding and hydrolysis to the ICLs of the TMD (22). The molecular function of this transmission interface relating coupling of the ATP hydrolysis cycle and substrate transport has not been extensively studied yet. To date, only a few studies dealt with the X loop of ABC transporters (22, 24, 25). Previously, the X loop Q1174E mutant of MDR3 in a young patient showing symptoms of PFIC-3 was described in Kubitz *et al.* (19). Thus, we substituted the conserved Gln in the extended X loop of MDR3 to Glu, expressed the Q1174E mutant in *P. pastoris,* and purified the protein employing the same procedure as described for wild type MDR3. We observed that the Q1174E mutant was purified with smaller yields and homogeneity, which is likely caused by the lower expression levels in *P. pastoris*. However, lower integration numbers of the gene expression cassette into the target chromosome cannot be ruled out. Here, we demonstrated that the Q1174E mutant was properly located in the plasma membrane of hepatocytes and MDR3-transfected HEK293 cells indicating a functional defect of MDR3 (Fig. 4). Cross-linking of the Q1174E mutant demonstrated that the Q1174E exhibited basal ATPase activity comparable with wild type in the presence of 2 mM ATP (Fig. 1). In contrast, the Q1174E exhibited a slightly increased affinity to ATP and a reduced maximal ATPase activity (Table 1) as the wild type floppase. Furthermore, all tested PC lipids were not capable of stimulating ATPase activity (Fig. 6). Based on these data and the structural model of MDR3, the exchange of the Gln at position 1174 of NBD2 prohibited the interaction with Asp-166 of the ICL1, and the conformational changes of the ICL as a result of PC lipid binding were not transmitted to the extended X loop of the NBD (Fig. 5).

This is in agreement with mutational and biochemical analysis of the ABC transporter TAP1/2 and CFTR and molecular dynamics simulation of human MDR1 (24, 25, 60). The mutational analysis of the conserved Glu within the X loop of TAP1/2 (TEVD**E**A(G/T)DVG**E**KG) demonstrated that substrate binding was not affected; however, substrate transport was drastically reduced (24). Moreover, Cys pair cross-linking experiments of the chloride channel CFTR showed that the X loops are in close proximity to ICLs connecting TMDs and also to the ATP-binding sites (25). He *et al.* (25) demonstrated that the interface between NBDs and ICLs of CFTR was involved in the stabilizing of interdomain contacts and regulation of the channel gating. Furthermore, Chang *et al.* (60) modeled the

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structure of human MDR1 based on mouse Mdr1a and investigated the transmission interface between NBDs and TMDs. They demonstrated that the amino acid residue Gln-1175 of NBD2, which is identical to Gln-1174 in MDR3, hydrogen bonds with Asp-164 (Asp-166 in MDR3) of the ICL1 and identified this residue pair Asp-164–Gln-1175 as key residue pair in the transmission interface. Furthermore, they suggested that the X loop plays an important role in formation of the outward-facing conformation of human MDR1 (60). Based on the biochemical data, we suggest that the conserved Gln next to the Leu of the ABC signature motif is crucial for the cross-talk between the extended X loop of the NBD and the coupling helices of the TMDs similar to the role of this region in MDR1. Further investigations are required to clarify the role of the individual amino acids, which are involved in signal transmission between the extended X loop and ICL1 of the TMDs, and should aid our understanding of how mutations relating to cholestatic diseases disrupt interdomain interactions of ATP binding and hydrolysis on a molecular level.

Taken together, we demonstrated exclusively that PC lipids stimulate the ATPase activity of detergent-solubilized human MDR3, which was exclusively inhibited by orthovanadate. For the first time, this study provides evidence that the glutamine next to the leucine of the ABC signature motif participates in the transmission of the substrate binding at the ICLs of the TMD to the extended X loop of the NBD.

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4 GENERAL DISCUSSION

Genetic variants are associated with the manifestation of a broad spectrum of liver diseases. Chapter 3.1 gives an overview of specific common genetic variants related to monogenetic liver diseases, different forms of intrahepatic cholestasis, gallstone formation, fatty liver disease, drug-induced liver injury, liver cirrhosis or even hepatocellular carcinoma (HCC). While Chapter 3.1 focuses on liver diseases with manifestation in adulthood in general, Chapter 3.2 summarizes genetic variants in the main bile salt transporters in the liver and intestine BSEP, NTCP, ASBT and OSTα/OSTβ. Furthermore, the molecular consequences of specific genetic variants affecting these transporters are listed as known to date and appropriate experimental approaches to analyze the impact of genetic variants are elucidated. Additionally, the consequences of a predicted BSEP transcript variant was experimentally verified to varying extents by sequencing analysis of mRNA obtained from human liver tissue.

ATP8B1, *ABCB11*, and *ABCB4* encode for the hepatobiliary transport proteins FIC1, BSEP, and MDR3, which are essential for hepatic bile formation. Chapter 3.3 summarizes FIC1, BSEP, and MDR3 variants detected in more than 400 patients with cholestatic phenotypes of varying severity. The impact of new identified variants was evaluated by the use of bioinformatics tools and 3D protein modeling. Chapter 3.4 provides insight into the role of BSEP as the main exporter of bile salts from hepatocytes into the bile canaliculi. All exonic and splice-site BSEP variants known so far are listed and linked to the cholestatic phenotypes of varying severity. The progressive familial intrahepatic cholestasis (PFIC) affects children and mostly necessitates liver transplantation, whereas the benign recurrent intrahepatic cholestasis (BRIC) or intrahepatic cholestasis of pregnancy (ICP) are characterized by a later onset of symptoms and a less severe disease progression.

In Chapters 3.5 and 3.6, the genetic variants of patients suffering from PFIC-2 and BRIC-2 were identified and moreover, these papers focus on the characterization of specific variants on a molecular level. Chapter 3.5 shows data of an intronic BSEP splice-site mutation that was investigated not only by a custom-made splicing reporter in a cell culture-based assay but also for the first time analyzed in the patients' liver tissue by RNA sequencing. The divergent results underline the importance of the investigation of mutational effects in the patient's liver tissue. Furthermore, a new BSEP missense mutation occurring in several members of one family was examined in Chapter 3.6. Since immunofluorescence staining showed a normal

BSEP protein expression in the canalicular membrane, a transport assay revealed an impaired BSEP function. Chapter 3.7 presents two PFIC-2-affected patients who benefited from steroid treatment. The underlying BSEP mutations were shown to result in a residual BSEP protein expression in the patients' liver tissue. These cases indicate that a detailed knowledge of the underlying mutations is helpful to select treatment options.

Patients liver transplanted due to PFIC-2 may suffer from recurrent symptoms, denoted as antibody-induced BSEP deficiency (AIBD). Chapter 3.8 summarizes the different forms of PFIC and what is known about the recurrence of a PFIC-2-like phenotype including a compilation of the published AIBD cases so far. Moreover, an algorithm for the diagnosis of AIBD is suggested. The genetic and clinical background of additional AIBD patients is listed in Chapter 3.9 to identify potential risk factors such as severe mutations with detrimental effects. Moreover, the occurring anti-BSEP-antibodies are further characterized in detail using the patients' sera. The antibodies form a polyclonal antibody response with intra- and extracellular BSEP domains as their targets. Since treatment options for AIBD are currently under investigation, the successful therapy of an AIBD patient is presented in Chapter 3.10. In this case, the patient improved receiving an immunoadsorption-based therapy in combination with a B cell depletion.

Besides BSEP variants, genetic variants in the multidrug resistance protein 3 (MDR3) also contribute to the manifestation of cholestatic liver diseases. Chapter 3.11 describes a woman suffering from ICP and also gives an overview of the current literature for ICP. In this case, a common BSEP polymorphism and an additional MDR3 missense mutation represent the underlying genetic variants. Chapter 3.12 depicts the impact of a new identified MDR3 mutation detected in a patient with a PFIC-3 phenotype. The missense mutation was analyzed not only in the patient's liver tissue and in cultured cells but also using an ATPase activity assay showing an impaired MDR3 function.

The results achieved, presented in Chapters 3.1 to 3.12, illustrate that a detailed knowledge of genetic variants underlying cholestatic liver diseases is important to identify a genetic predisposition, to predict the course of the disease or manifestation of recurrent symptoms and to estimate therapeutic options.

4.1 The impact of genetic variants as the basis of cholestasis

4.1.1 Diverging genetic variants related to cholestatic liver diseases

A variety of mutations in FIC1, BSEP, and MDR3 are associated with different forms of intrahepatic cholestasis. In contrast to rare mutations, genetic variants with minor allele frequencies (MAFs) of more than 1 % are denoted as polymorphisms (236). Single nucleotide polymorphisms (SNPs) can be found with AFs over 50 % often depending on the ethnic background. Nevertheless, some common SNPs are related to a variety of liver diseases. A common BSEP SNP is p.V444A (c.1331T>C) with an AF of about 55 % in the Caucasian population (1000 Genomes, (73, 170, 171)). However, the BSEP p.V444A is related to the manifestation of ICP and DILI (70-73, 172). Furthermore, a combination of BSEP p.V444A and the second common BSEP SNP p.A1028A was found in over 70 % of patients with a cholestatic phenotype in the absence of a disease causing mutation (Chapter 3.3). Although p.A1028A has no direct effect on the AA sequence, it was shown to influence mRNA splicing *in vitro* (176). This aspect will be further discussed in section 4.1.4 in the context of splice variations. Together with heterozygous decisive mutations in the same or even another gene, common SNPs may contribute to a cholestatic phenotype in terms of BRIC or ICP (75, 95, 107, 237). As presented in Chapter 3.11, a woman affected by ICP was homozygous for BSEP p.V444A and p.A1028A additionally to the heterozygous MDR3 mutation p.S978P. Nevertheless, parents of children with a cholestatic phenotype are often symptom-free when heterozygous for one severe mutation as depicted in Chapter 3.6. In this family, two siblings homozygous for the new identified BSEP missense mutation p.G374S showed a BRIC-2/PFIC-2-like phenotype while the healthy sister and parents were heterozygous for p.G374S. While the identification of genetic variants in siblings is relevant with regards to a potential disease manifestation, the genetic background of the patients' parents is of interest to verify that the patients' varying mutations are located on different alleles. In autosomal recessive diseases such as PFIC, the combination of one heterozygous mutation on each allele has the same impact as one homozygous mutation. (65, 238-240). This state of compound heterozygosity makes it difficult to assign the impact of a mutation to a distinct phenotype, which matters in regard to the detailed analysis of specific genetic variants. In Chapter 3.3, the FIC1, BSEP, and MDR3 variants detected in 427 patients with varying forms of intrahepatic cholestasis are composed. Nevertheless, it was difficult to match the variants and the phenotype as described by the referring clinicians. For one thing, a number of mutations appears in a

compound heterozygous status, for another thing, there is sometimes no clear distinction between the phenotypes especially of PFIC and BRIC.

As elucidated in section 1.4 in detail, there are different forms of intrahepatic cholestasis with varying severity. The forms of PFIC predominantly affect children and can result in end stage liver disease with manifestation in early childhood often necessitating liver transplantation. In contrast, the milder forms like BRIC, LPAC, and ICP mostly appear in adulthood with less severe courses of disease and episodes of recovery. The clinical manifestation of intrahepatic cholestasis in the examined siblings presented in Chapter 3.6 point out that there is no clear differentiation between PFIC and BRIC in some cases. Thus, these cholestatic syndromes represent more a disease continuum of varying severity (90, 91, 188).

4.1.2 A detailed knowledge of mutational effects is essential

As visualized in Chapter 3.8 (Figure 2), the different types of mutations can have effects on different levels from DNA to protein. For example, splice-site mutations can affect pre-mRNA processing and therefore either lead to mRNA degradation or a reduced mRNA expression with corresponding effects for protein expression. Premature termination codons also cause a nonsense-mediated mRNA decay. For example, the BSEP mutation based on a duplication of five nucleotides c.2783_2787dup5 (p.K930Efs79X) was detectable on gDNA but not on mRNA level in the patient's liver tissue indicating an mRNA degradation as depicted in Chapter 3.5 (Figure 3). Missense mutations may cause a decreased or even absent membrane expression of the protein, e.g. as known for the PFIC-2-associated BSEP mutations p.E297G and p.D482G (207, 241). Despite a normal membrane expression, also a functional defect can result in a useless protein as shown for the BSEP missense mutation p.G374S in Chapter 3.6 or the MDR3 missense mutation p.Q1174E in Chapter 3.12. The MDR3 mutation p.S978P associated with the manifestation of ICP as presented in Chapter 3.11 is also known to result in a dysfunctional protein (242).

Nowadays, a number of bioinformatics tools are freely accessible to predict the impact of genetic variants. Whereas the effects of mutations leading to a premature termination codon (nonsense and frameshift mutations) are obvious, the consequences of single nucleotide exchanges resulting in synonymous variants, missense mutations or intronic variations are often uncertain. Nucleotide substitutions affecting the splice-sites can be predicted by the matching of the sequence to the splice-site apparatus (243, 244). Mismatches can result in a

disruption of correct pre-mRNA splicing. The effects of AA substitutions, e.g. missense mutations, are mostly predicted by the adjustment of the degree of conservation of this AA position (161, 163). Nevertheless, using different tools can lead to varying results as depicted in Chapter 3.3, which illustrates that these predictions only give first impressions of the mutational impact. Moreover, the type of exchange plays a role for effects on protein structure or function. 3D protein modeling is another useful tool to gain insights of the localization and structural variation caused by an AA substitution. In Chapter 3.3, the new identified missense mutations in BSEP and MDR3 were integrated into a 3D homology model. Structural alterations and thus possible functional variations based on the AA exchanges could be predicted (Chapter 3.3). The combination of 3D protein modeling and analyzing the patient's clinical data and liver tissue if available was also used for another MDR3 missense mutation to estimate the impact of this variant (166).

Despite the possibilities of *in silico* prediction tools, a variety of *in vitro* assays are available to analyze the mutational effects. Specific genetic variants can be introduced into expression vectors and transfected into cultured cell lines. For example, the effects of mutations on protein trafficking, targeting or expression can be analyzed by immunofluorescent staining of the cultured cells as depicted in Chapter 3.6 for BSEP p.G374S or in Chapter 3.12 for MDR3 p.Q1174E. Recently, results of cell culture based experiments were published for a number of MDR3 variants to identify the class of the mutational effect (245). Furthermore, minigene constructs are available to gain information concerning splicing events as shown for a number of BSEP variants (176). As presented in Chapter 3.5, the intronic BSEP mutation c.150+3A>C was investigated using a custom-made splicing reporter. The mentioned *in vitro* techniques are useful instruments to gain an insight concerning the consequences of genetic variations. Nevertheless, the results shown in Chapter 3.5 emphasize the necessity of the investigation of the patients' liver tissue. The splicing assay resulted in a total exon-skipping due to the BSEP mutation c.150+3A>C, whereas the analysis of the mRNA obtained from the liver tissue as well as the immunofluorescent staining revealed a residual correct spliced BSEP expression. This result points out that the cell and tissue environment play an important role. Hence, a classification based on *in vitro* assays as published recently for MDR3 missense mutations only gives hint to the mutational effects but represents no absolute grading definitely applicable to the patients' mutations (245). Although the analysis of the patient's liver tissue would be the gold standard, the benefits and the risks of a liver biopsy have to be considered very carefully (246). However, especially samples of explanted livers should be

stored, whereby it is easier to make use of snap frozen liver tissue than of formalin-fixed paraffin-embedded (FFPE) liver tissue. Unfortunately, the FFPE tissue of the explanted liver of child 1 presented in Chapter 3.5 was not of sufficient quality for further analysis on a protein level despite for immunofluorescent staining. It was impossible to obtain appropriate protein amounts for Western blot analysis to determine the BSEP protein levels.

4.1.3 How to benefit from the knowledge of mutational effects

For the different forms of intrahepatic cholestasis, the course of the disease as well as potential treatment options are determined by the molecular consequences of certain mutations. In particular, a residual targeting or transport activity of the affected hepatobiliary proteins seems to be a prerequisite for a less severe phenotype and a better treatment outcome. Thus, a detailed knowledge concerning the molecular effects of causative mutations is desirable for optimal disease management of individual patients suffering from varying types of intrahepatic cholestasis.

The first-line treatment for various types of intrahepatic cholestasis is UDCA (189, 197, 198). The success of a UDCA therapy is probably determined by a residual (even intracellular) protein expression and less severe disease parameters like a later disease onset as seen in PFIC-2 patients (247). There, a retargeting of intracellular retained BSEP protein was demonstrable (247). An increased BSEP membrane expression by the use of 4-phenylbutyrate was also shown for the BSEP mutations p.E297G and p.D482G, which are known to cause an intracellular retained protein (206, 207). Moreover, the administration of 4-phenylbutyrate improved liver function in PFIC-2 patients (209, 210, 248). Additionally to UDCA, other therapeutic options are needed due to UDCA therapy failure in some cases. For ICP patients non-responding to UDCA, the administration of the antibiotic rifampicin in combination with UDCA led to a better outcome (203). In contrast, the application of dexamethasone resulted in no improvement of symptoms compared to UDCA therapy (199). Treatment of ICP patients is challenging in regard to fetal health and has to be carefully considered (95). Probable options for treating cholestasis are also targeting transcription factors as FXR with its important role in bile salt homeostasis, e.g. BSEP expression (249).

As presented in Chapter 3.7, two PFIC-2 patients were successfully treated with steroids. Besides the BSEP nonsense mutation p.R1235X, a deletion of one AA (p.T919del) was detectable in patient 1. Patient 2 had the BSEP missense mutation p.R832C, with probably a

functional defect, additional to the BSEP splice-site mutation c.150+3A>C (see also Chapter 3.5). In both patients' liver tissue, correct targeted BSEP protein was verifiable to a certain amount as shown by immunofluorescent staining (Chapter 3.7, Figure 1 and 3). Both patients received their steroid treatment due to the appearance of an inflammatory disease. Probably, the residual BSEP expression was helpful for the improvement based on steroid administration. An increased membrane expression after therapy was not provable because no further liver biopsy was obtained since both patients were in a good clinical condition.

Awareness of the underlying mutations of the forms of cholestasis is not only useful for treatment decisions but also for monitoring the patients and the disease progression. PFIC and even ICP patients have a higher incidence to develop CCC or HCC (79-82, 94, 250). Thus, the woman suffering from severe ICP as presented in Chapter 3.11 may benefit from a regular follow-up.

Some PFIC-2 patients are also predisposed for a specific form of recurrent symptoms after orthotopic liver transplantation (OLT). In 2009, the disease recurrence after OLT due to PFIC-2 was first described by Keitel *et al*. (144). Nowadays, to the best of our knowledge, the affected patients had a PFIC-2 phenotype mainly based on severe BSEP mutations like nonsense, frameshift and splice-site mutations as summarized in Chapter 3.8 (Table 2) and Chapter 3.9 (Table 1). These mutations mostly result in a premature termination codon and therefore in an aberrant non-functional BSEP protein (149). Patients with a diagnosed PFIC-2 especially based on these detrimental mutations should be monitored after OLT for an early identification of possibly occurring anti-BSEP antibodies in the patients' serum. In the case of AIBD, an adjustment of immunosuppression, the application of plasmapheresis, immunoadsorption, or B cell depletion or even a retransplantation has to be considered as summarized in Chapter 3.8.

B cell depletion by the administration of rituximab was described for other AIBD patients (144, 146, 148). The treatment of a single AIBD patient is depicted in detail in Chapter 3.10. This patient considerably improved under immunoadsorption combined with B cell depletion since the amount of anti-BSEP antibodies in the patient's serum decreased. Up to now, further treatment options for AIBD patients are still under evaluation.

4.1.4 Splicing events in BSEP and MDR3

About 15 % of single nucleotide exchanges in human genetic diseases concern the canonical splice-sites (173). Splice-site mutations affecting the terminal intronic or exonic dinucleotides have detrimental effects, since these nucleotides are crucial for splice-site recognition (173, 251). Approximately 6 % of the known relevant BSEP variants represent intronic splice-site mutations as summarized in Chapter 3.5 (Table 1). The impact of splice-site variants affecting more distal intronic nucleotides is often unclear. Therefore, the BSEP splice-site mutation c.150+3A>C of two PFIC-2 patients was analyzed in detail as presented in Chapter 3.5 with diverging results obtained by the cell culture-based splicing assay and the investigation of the patients' liver tissue. The importance of analyzing splicing alterations in human liver tissue due to intronic or silent BSEP variants was also emphasized by others (252).

Not only specific splice-site variants can lead to a reduced correct splicing, to exon-skipping or the activation of cryptic splice-sites. Even variants far away from the exon-intron transitions can affect splicing when for example located in exonic or intronic splicing enhancer (ESE/ISE) sequences, which interact with the SR protein during RNA splicing (253). As already mentioned in section 4.1.1, the impact of the common synonymous BSEP SNP p.A1028A (c.3084A>G) is not obvious. This SNP and other exonic nucleotide exchanges in the *ABCB11* gene, encoding for BSEP, were investigated using a minigene splicing assay. In this *in vitro* approach, p.A1028A caused severe exon-skipping (176). To further investigate the impact on splicing in liver tissue, as part of this thesis, a custom-made TaqMan assay was designed. This assay includes a reference probe detecting BSEP mRNA and moreover allele-specific probes for c.3084A or c.3084G. Based on this assay, it is possible to determine allele-specific BSEP mRNA transcripts. First findings from human liver tissue heterozygous for c.3084A>G indicate a lower amount of the mutated allele (G) compared with the wildtype allele (A).

Since splicing-defects may have detrimental effects, restoring correct splicing represents an interesting therapeutic target. For *ATP8B1* (FIC1) splice-site mutations associated with BRIC-1 or PFIC-1, a modified U1 small nuclear RNA complementary to the mutated splice-site was expressed and could improve splicing *in vitro* (254). Nevertheless, it remains to be determined which approaches are applicable for treating patients as reviewed by Havens *et al.* (255).

Despite splicing variations based on single nucleotide exchanges, naturally occurring alternative splicing events increase the variability of the genome. For BSEP, two alternative

isoforms including intronic sequences, respectively, are predicted (256). In human liver tissue, one alternate isoform (BSEP-B) could be experimentally verified, which is depicted in Chapter 3.2 (Figure 2). The impact of these splicing variants may be of interest, since the splicing isoforms were present to a varying extent in human healthy liver tissue. For MDR3, three isoforms were described in the late 1980s. In comparison with MDR3-A, MDR3-B includes an additional intronic sequence while MDR3-C is lacking one exon (174). A custom-designed TaqMan assay was used to elucidate the isoform distribution in human liver tissue. The preliminary results indicate that all isoforms are present while MDR3-A seems to constitute the major isoform.

4.2 Additional genes are related to intrahepatic cholestasis

Genetic variants in FIC1, BSEP, and MDR3 are the basis for the manifestation of intrahepatic cholestasis. Nevertheless, there are a number of cases in which the phenotype is not confirmed by mutations in FIC1, BSEP, or MDR3 (Chapter 3.3).

The nuclear receptor FXR is essential for bile acid homeostasis (257, 258). Thus, FXR mutations can also cause cholestasis as seen in patients with an ICP or a PFIC-like phenotype (109, 259). Recently, four children with low γGT PFIC and absent BSEP protein expression were described and whole-exome sequencing revealed no severe BSEP mutations but disease-causing FXR mutations (259). Since the underlying FXR mutations were all of severe impact, these cases indicate the relevance of a restored FXR function by FXR agonists as potential therapeutic options for cholestasis or fatty liver disease (260).

Sambrotta *et al*. identified genetic variants in the tight junction protein 2 (TJP2) to be associated with forms of low γGT cholestasis (86, 87). The progressive form was therefore denoted as PFIC-4. In the liver, TJP2 or zona occludens 2 (ZO2) separates the canalicular membrane from the sinusoidal membrane of hepatocytes. Previously, genetic variants in TJP2 were already known to be related to familial hypercholanemia (261). Recently, another gene was identified to cause a form of low γGT cholestasis: the myosin 5B (MYO5B) (88). Before, MYO5B mutations were associated to microvillus inclusion disease (MVID) leading to diarrhea (262). The interaction of MYO5B with RAB11 is known to be important for an appropriate function of epithelial cells including hepatocytes (263-265).

The latest findings emphasize that genetic variants in other genes can contribute to a cholestatic phenotype. In both studies, advanced sequencing techniques enabled the detection of an additional affected gene.

4.2.1 Advanced techniques to identify other cholestasis-relevant genes

Since developed by Frederick Sanger in the 1970s, Sanger sequencing was the method of choice to identify genetic variations (266). More recently, advanced sequencing techniques came up to reach a higher throughput and these new methods are denoted as next generation sequencing (NGS) (267, 268). NGS is already in use for the identification of underlying genetic variants in cholestatic patients as for the ICP patient depicted in Chapter 3.11 or other cases (259, 269, 270). The main advantage of NGS is the possibility to analyze a broader spectrum of genes or even the whole exome cost-effectively and in a short period of time. Nevertheless, using NGS techniques produces masses of data, which needs to be handled. The recent identification of genetic variants in TJP2 and MYO5B as contributors to forms of intrahepatic cholestasis underscores the utility of advanced sequencing techniques in the field of genetic based cholestatic liver diseases (86-88).

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6 ABBREVIATIONS

AA	amino acid
ABC	adenosine triphosphate binding cassette
ABCB4	ABC transporter, subfamily B, member 4
ABCB11	ABC transporter, subfamily B, member 11
ABCC2	ABC transporter, subfamily C, member 2
ABCG5/8	ABC transporter, subfamily G, heterodimer of members 5 and 8
ADP	adenosine diphosphate
ASBT	apical sodium-dependent bile acid transporter
ATP	adenosine triphosphate
ATP8B1	ATPase, class I, type 8B, member 1
BCRP	breast cancer resistance protein
BSEP	bile salt export pump
BRIC	benign recurrent intrahepatic cholestasis
c.	sequence on coding DNA level
cDNA	complementary deoxyribonucleic acid
CH	coupling helix
CIC	contraceptive-induced cholestasis
CSD	catalytic subdomain
C-terminus	carboxy-terminus
CYP7A1	cytochrome P450, family 7, subfamily A, polypeptide 1
DILI	drug-induced liver injury
DJS	Dubin-Johnson syndrome
e.g.	exemplia gratia
FFPE	formalin-fixed paraffin-embedded
FIC1	familial intrahepatic cholestasis 1
FXR	farnesoid X receptor
g	gram

gDNA	genomic deoxyribonucleic acid
γGT	gamma-glutamyltransferase
HAX1	HCLS1-associated protein X-1
HCC	hepatocellular carcinoma
HSD	helical subdomain
ICL	intracytosolic loop
ICP	intrahepatic cholestasis of pregnancy
Ig	immunoglobulin
kDa	kilodalton
kg	kilogram
Km	Michaelis-Menten constant
LPAC	low phospholipid-associated cholelithiasis
MAF	minor allele frequency
MDCK	Madin-Darby canine kidney cell line
MDR	multidrug resistance protein
mRNA	messenger ribonucleic acid
MRP	multidrug resistance-associated protein
MYO5B	myosin VB gene
NBD	nucleotide binding domain
NGS	next generation sequencing
NMD	nonsense-mediated mRNA decay
NTCP	Na^+-taurocholate co-transporting polypeptide
OAT	organic anion transporter
OATP	organic anion transporting polypeptide
OCT	organic cation transporter
OLT	orthotopic liver transplantation
OST	organic solute transporter
p.	sequence on protein level
PCR	polymerase chain reaction

PEBD	partial external biliary diversion
PFIC	progressive familial intrahepatic cholestasis
PBC	primary biliary cirrhosis
P-gp	permeability glycoprotein 1
Pi	inorganic phosphate
PKC	protein kinase C
P-loop	phosphate-binding loop
pp	pages
PSC	primary sclerosing cholangitis
SLC	solute carrier
SNP	single nucleotide polymorphism
TMD	transmembrane domain
TMH	transmembrane helix
TJP2	tight junction protein 2
UDCA	ursodeoxycholic acid
Vmax	maximum velocity
3D	three-dimensional

Nucleobases

A	adenine
C	cytosine
G	guanine
T	thymine
U	uracil

Amino acids, one and three letter code

A	Ala	alanine
C	Cys	cysteine
D	Asp	aspartic acid
E	Glu	glutamic acid
F	Phe	phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine
P	Pro	proline
Q	Gln	glutamine
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valine
W	Trp	tryptophane
Y	Tyr	tyrosine

7 LIST OF FIGURES AND TABLES

8 DANKSAGUNG

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9 CURRICULUM VITAE

PERSONAL DATA

Date of Birth:	15th January 1984 in Braunschweig, Germany
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EDUCATION

since 11/2010	**PhD student** at the Clinic of Gastroenterology, Hepatology and Infectious Diseases via Project 1 of CRG217 with Prof. Dr. med. Ralf Kubitz / Prof. Dr. med. Verena Keitel-Anselmino, University Hospital, Heinrich Heine University Düsseldorf
10/2003 - 06/2010	**Studies in Biology** at the Heinrich Heine University Düsseldorf Degree: Diploma
08/1990 - 06/2003	Elementary and secondary school in Lübeck, Germany Degree: Abitur

FURTHER TRAINING AND COMMITMENTS

since 08/2014	Contribution to lab diagnostics and training in the Hirsch Institute of Tropical Medicine (HITM), Heinrich Heine University/Arsi University, Asella, Ethiopia
10/2012 - 10/2015	Participant of the *SelmaMeyerMentoring* Program of the Heinrich Heine University Düsseldorf
since 11/2010	Associated member of the integrated Graduate School of CRC575 (till 2011) and CRC974 (since 2012)

AWARDS

03/2014	Poster prize winner of the *Deutsche Gesellschaft für Gastroenterologie in Nordrhein-Westfalen*

PUBLICATIONS

Papers

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Poster: Falk Symposium, XXIV International Bile Acid Meeting: Bile Acids in Health and Disease, Düsseldorf, Germany, 06/2016

Talk & Poster: Gordon Research Conference: Human Single Nucleotide Polymorphisms & Disease, South Hadley, MA, USA, 06/2016

Poster: Annual Meeting of the German Association for the Study of the Liver (GASL), Düsseldorf, Germany, 01/2016

Poster: Falk Workshop: Communication and System Relevance in Liver Damage and Regeneration, Düsseldorf, Germany, 01/2016

Talk: ESTHER Workshop: Scientific Methodology, Research and Publication in low-income settings and Clinical Research in OB/GYN, Asella, Ethiopia, 04/2015

Poster: Falk Symposium, XXIII International Bile Acid Meeting: Bile Acids as Signal Integrators and Metabolic Modulators, Freiburg, Germany, 10/2014

Talk: Annual Meeting of the *Deutsche Gesellschaft für Gastroenterologie, Verdauungs- und Stoffwechselkrankheiten* (DGVS), Leipzig, Germany, 09/2014

Poster: Annual Meeting of the *Gesellschaft für Gastroenterologie in Nordrhein-Westfalen*, Neuss, Germany, 03/2014

Poster: The International Liver Meeting of the American Association for the Study of Liver Diseases (AASLD), Washington, DC, USA, 11/2013

Poster: The International Liver Meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, USA, 11/2012

Poster: The International Liver Congress of the European Association for the Study of the Liver (EASL), Barcelona, Spain, 04/2012

10 ERKLÄRUNG

Ich versichere an Eides statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der "Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf" erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, 19. Oktober 2016 _____