Genetic variations of bile salt transporters as predisposing factors for drug-induced cholestasis, intrahepatic cholestasis of pregnancy and therapeutic response of viral hepatitis

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AREAS COVERED: This article provides an introduction into the physiology of bile formation followed by a summary of the current knowledge on the key bile salt transporters, namely, the sodium-taurocholate co-transporting polypeptide NTCP, the organic anion transporting polypeptides (OATPs), BSEP and the multi-drug resistance protein 3. The pathophysiologic consequences of altered functions of these transporters, with an emphasis on molecular and genetic aspects, are then discussed.

EXPERT OPINION: Knowledge of the role of hepatocellular transporters, especially BSEP, in acquired cholestasis is continuously increasing. A common variant of BSEP (p.V444A) is now a well-established susceptibility factor for acquired cholestasis and recent evidence suggests that the same variant also influences the therapeutic response and disease progression of viral hepatitis C. Studies in large independent cohorts are now needed to confirm the relevance of p.V444A. Genome-wide association studies should lead to the identification of additional genetic factors underlying cholestatic liver disease.
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Areas covered in this review: An introduction into physiology of bile formation is followed by a summary on the current knowledge on the key bile salt transporters, namely the sodium-taurocholate cotransporting polypeptide NTCP, the organic anion transporting polypeptides (OATPs), BSEP and the multidrug resistance protein 3 (MDR3). The pathophysiologic consequences of altered functions of these transporters, with an emphasis on molecular and genetic aspects are covered.

Expert opinion: Knowledge on the role of hepatocellular transporters, especially BSEP, in acquired cholestasis is continuously increasing. A common variant of BSEP (p.V444A) is now a well established susceptibility factor for acquired cholestasis and recent evidence suggests that the same variant also influences the therapeutic response and disease progression of viral hepatitis C. Studies in large independent cohorts are now needed to confirm the relevance of p.V444A. Genome wide association studies should lead to the identification of additional genetic factors underlying cholestatic liver disease.
Article highlights

- The bile salt export pump BSEP is the key bile salt exporter in hepatocytes and drives the enterohepatic circulation of bile salts.

- Interference with the function of BSEP, e.g. by inhibition with drugs or steroid metabolites leads to an accumulation of bile salts within hepatocytes, which if sustained will lead to acquired liver disease.

- The c.1131T>C polymorphism in the gene coding for BSEP has been identified as a susceptibility factor for acquired cholestatic liver disease in independent cohorts.

- The c.1131T>C polymorphism and subsequent bile acid retention have been identified as host factors affecting fibrosis progression and therapy response in chronic viral hepatitis C infection.

- Additional genetic factors need to be identified for increasing the knowledge on pathogenetic mechanisms of drug induced cholestasis.

- The interplay of genetic variants of uptake transporters with genetic factors regulating BSEP expression and function needs to be investigated.

1. Physiology of bile formation

1.1. Introduction

The exclusive entry site for food and the major entry site for xenobiotics into our body is the oral route. Therefore, the small intestine plays a key role in the absorption of nutrients and for the delivery of many drugs. From the small intestine, substances are transported via the portal blood to the liver. In the liver, they are either retained or let into the systemic circulation. Hence, the liver is strategically located between the gut and the systemic circulation and constitutes, after the gut, a second barrier controlling access of dietary components to the systemic circulation [1]. In the small intestine, dietary components need to be processed for absorption, whereby fat requires bile salts for its digestion and absorption. Bile is produced by
the liver and delivered via the bile ducts and the gall bladder to the small intestine [2, 3]. Major components of bile are bile salts, lipids (phosphatidylcholine and cholesterol), organic anions (conjugated bilirubin and others) and small ions [4]. Hepatic bile flow is composed of bile salt-dependent bile flow, driven by bile salt secretion and bile salt-independent bile flow, which is maintained by hepatocellular secretion of organic anions and the secretory action of the bile duct epithelium [5, 6].

Overall, bile salts are absorbed to more than 90% in the small intestine and shipped back to the liver via the portal circulation, where they are taken up into hepatocytes for a new secretion into the biliary tree [7]. This cycling between the gut and the liver is named enterohepatic circulation of bile salts. Hepatocytes are equipped with an elaborate tool box for an efficient uptake of bile salts from the sinusoids and their export into the canaliculi as well as for tightly controlling intracellular concentrations of bile salts [6-15] (Figure 1). Bile salts are amphipathic and have detergent properties [3]. Disturbed bile salt secretion by hepatocytes is called cholestasis and has far reaching consequences. If bile salts accumulate within hepatocytes they become due to their cytotoxicity damaging or even lethal to the cells [16-18].

1.2. hepatocellular bile salt transporters

Uptake of bile salts into hepatocytes occurs predominantly in a sodium-dependent manner and to a minor extent via sodium-independent processes [11, 15, 19]. Sodium-dependent uptake of bile salts is mediated by the sodium-taurocholate cotransporting polypeptide NTCP (SLC10A1). This transporter prefers conjugated over unconjugated bile salts [7, 20]. Organic anion transporting polypeptides or OATPs (SLCOs) foster sodium-independent uptake of bile salts and show a marked preference for unconjugated bile salts [8, 9, 21]. In human liver, three OATPs are expressed: OATP1B1 and OATP1B3 transport uncojugated and conjugated

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bile salts in vitro, while OATP2B1 does not mediate transport of conjugated bile salts [8, 21]. The intracellular transport of bile salts from the basolateral membrane to the canalicular membrane is not understood in detail, but cytoplasmic binding proteins are almost certainly involved [22, 23]. The rate limiting step in bile salt transport from the sinusoidal blood plasma into the canaliculus is located at the canalicular plasma membrane [8, 24]. Canalicular export of bile salts occurs against a steep concentration gradient and is mediated by the bile salt export pump BSEP (ABCB11), which is a member of the ATP-binding cassette (ABC) transporter super family [7, 11, 15, 25, 26]. As a consequence, proper activity and regulation of BSEP is essential for keeping the potentially cytotoxic bile salts at a low intracellular level in hepatocytes. Given their high rate of canalicular secretion and their high concentration in bile, bile salts are a crucial driving force for canalicular bile flow. As it is the sole bile salt export system, BSEP constitutes the rate-limiting step of hepatocellular bile salt secretion [15, 25]. Bile flow is further maintained by the secretion of organic anions by the multidrug resistance associated protein 2 (MRP2), which contributes to bile salt independent bile flow [4]. Any reduction of total bile flow represents a pathophysiologic situation and is called cholestasis.

As bile salts are potentially cytotoxic, NTCP expression and function is controlled at the transcriptional and posttranscriptional level for regulating the influx of bile salts into hepatocytes. The bile salt level in hepatocytes is monitored by the nuclear transcription factor farnesoid X receptor (FXR, NR1H4) (table 1) [12]. Increasing intracellular bile salt levels, e.g. as a consequence of cholestasis, lead via FXR to an upregulation of the small heterodimer partner 1 (SHP-1, NR0B2) (table 1), which in turn represses SLC10A1 (Figure 1). The molecular mechanism of SHP-1 mediated SLC10A1 repression is species-dependent [12]. NTCP is a phosphoprotein and its state of phosphorylation regulates recycling of the transporter between an endosomal compartment and the basolateral plasma membrane.
Increasing cAMP induces dephosphorylation of NTCP in the endosomal compartment. From here, NTCP is translocated to the basolateral domain of hepatocytes in a process involving rab4 [27, 28].

OATPs display a broad and overlapping substrate pattern and transport endogenous substrates such as conjugated and unconjugated bile salts and metabolic end products. Examples of endogenous substrates and metabolic end products are bilirubin, estrone-3-sulfate, thyroxin and thyroxin sulfates [21, 29].

Hepatic OATPs are regulated at the transcriptional and posttranscriptional level. FXR controls the transcription of SLCO1B3 directly and the transcription of SLCO1B1 indirectly by a SHP-1 induced repression of hepatocyte nuclear factor 1α (HNF1α) (table 1). The latter transcription factor is the major activator of the SLCO1B1 promotor and also stimulates SLCO1B3 [12, 30]. Even though, OATP are key hepatocellular drug transporters (see below), only little information is available on the role of ligands for transcription factors activated by xenobiotics, such as the constitutive androstane receptor (CAR) or the pregnane X receptor (PXR). Expression of SLCO1B3 is downregulated the CAR ligand phenobarbital (table 1) [30]. Recently, modulation of the transport activity of OATP1B1 and OATP1B3 has been demonstrated for a variety of PXR ligands [31] and by physiologic substrates such as prostaglandins [32] or estrone-3-sulfate [33]. OATPs are subjected to phosphorylation, leading in the case of OATP1B1 to an increased plasma membrane localization [34] and in the case of OATP2B1 to an internalization from the plasma membrane [35].

BSEP transports almost exclusively monoanionic, conjugated bile salts, but displays species-dependent substrate specificity [15, 25, 26]. Rat Bsep barely transports unconjugated bile acids [36]. The same holds true for BSEP, as patients with defective bile acid conjugation
have very little unconjugated bile salts in their bile [37]. Mutations in the gene coding for BSEP lead often to severe liver disease [38].

At the transcriptional level, BSEP is under strong positive control by FXR [12, 26], which needs to interact with its nuclear receptor retinoid X receptor (RXR, NR2B1) forming a heterodimeric complex (Figure 1, table 1) [12, 39]. The activation of FXR is more pronounced with hydrophobic than with hydrophilic bile salts [12, 40] with chenodeoxycholate having an EC$_{50}$ value of 50 µM for human FXR [41]. At the posttranscriptional level, short term regulation of BSEP, involves the modulation of the carrier density of BSEP via endo- and exocytosis as well as regulation of its activity by changes of the phosphorylation state [42-44]. Additional elements of regulation may involve ubiquitinylation and protein-protein interaction [38].

1.3. Canalicular lipid secretion

Phosphatidylcholine is the major biliary lipid and requires functional MDR3 (ABCB4) for its canalicular secretion [45]. Phosphatidylcholine is released from the canalicular membrane into bile by the detergent action of bile salts [46]. Mutations in the gene coding for MDR3 lead to severe liver disease [47]. In canalicular bile, phosphatidylcholine and bile salts form mixed micelles, which then act as acceptors for poorly water-soluble substances, e.g. cholesterol [46]. Release of cholesterol from the canalicular membrane into the aqueous canalicular bile is facilitated by the heterodimeric ABC transporter ABCG5/ABCG8 [48]. Taken together, canalicular lipid secretion requires the coordinated action of three ABC transporters, namely BSEP, MDR3 and ABCG5/ABCG8. In addition to providing a biliary vehicle for poorly water soluble compounds, mixed micelles are instrumental for lowering the toxicity of bile salts against the epithelial cells lining the bile ducts [49].
1.4. Canalicular secretion of organic anions

In addition to bile salts, organic anions such as bilirubin diglucuronide or glutathione as well as drug metabolites conjugated to glucuronide or sulfate are secreted into bile. This secretion is maintained by two additional ABC transporters. The multidrug resistance protein 2 (MRP2, \textit{ABCC2}) excretes bilirubindiglucuronide and has a preference for glucuronidated drug metabolites [50, 51]. ABCG2 (also called breast cancer resistance protein or BCRP) mediates secretion of a variety of metabolites of endogenous and xenobiotic compounds and displays a preference for sulfated compounds [51, 52]. It should however be noted that the substrate specificities of \textit{ABCC2} and \textit{ABCG2} show some overlap.

In summary, bile salt secretion is mediated by BSEP and the coordinated interplay between several ABC transporters is necessary to keep intracellular bile salt concentrations at a nontoxic level and to protect bile ducts against the toxic action of bile salts. Any dysfunction of these processes caused either by inherited or acquired functional impairment of these canalicular transporters leads to a potential pathophysiologic state of the liver or even to clinically relevant liver disease.

2. Bile Salt Transporters as Drug Transporters

While NTCP is predominantly a bile salt transporter it can handle in addition bromosulfophthalein and sulfated steroid metabolites [7, 11, 20, 53]. In addition, human NTCP mediates transport of iodothyronine T4 and sulfated thyroid hormone metabolites [54] as well as recently demonstrated indocyanine green [55]. Very interestingly, human NTCP but not rat Ntcp can transport the statin rosvastatin [56]. Using isolated human hepatocytes, Ho et al. were able to demonstrate that NTCP may mediate up to 35\% of total rosvastatin uptake into hepatocytes, the remainder being imported by OATPs [56]. NTCP may therefore also contribute to hepatocellular handling of steroid metabolites and of some drugs.
In contrast to NTCP, OATPs are rather drug than bile salt transporters mediating the uptake of a multitude of drugs and xenobiotics. The hepatocellular OATPs have a considerable overlapping substrate pattern [21, 29, 57, 58]. Among the many drugs transported by OATPs, statins are among the most widely used drugs [59]. In heterologous expression systems, where transporters can be studied individually, OATPs clearly display overlapping substrate specificity. However, prediction of the contribution of individual OATPs to in vivo hepatocellular drug uptake is up to now difficult. For example, vitro assays have shown that all three hepatocyte OATPs can transport rosuvastatin [60]. Importantly, a recent genome-wide single nucleotide polymorphism association study however identified only OATP1B1 variants as a risk factor for myopathy in a patient population receiving a high-dose simvastatin treatment [61]. This can be interpreted that the functionally dominant simvastatin uptake system in human hepatocytes is OATP1B1. Part of the uncertainty in extrapolating in vitro findings to the in vivo situation may in addition be due to the fact that the transport mechanism of OATPs has not been worked out in molecular detail. In general, they are believed to be organic anion exchangers working bidirectionally [62]. In addition, recent evidence shows that transport activity of many OATPs is enhanced by a low extracellular pH [63]. Understanding the exact transport mechanism and the driving forces of OATPs is however instrumental for determining the role of individual OATPs in hepatocellular drug uptake. Knowing the driving force will also allow to predict whether OATPs can accumulate drugs within hepatocytes to a concentration higher than in blood plasma.

BSEP has a rather narrow substrate specificity and transports predominantly bile salts and in a species dependent manner tauroliothocholate-3-sulfate [15, 64]. So far, only pravastatin has been identified as a BSEP substrate different from bile salts [15, 65]. Hence, BSEP seems to play practically no role in hepatocellular handling of drugs.
3. Drug Induced Cholestasis

Drug induced liver injury is a form of acquired liver disease, is a clinically relevant entity and leads to a considerable number of hospitalizations [66-69]. Drug induced liver injury spans a wide spectrum of injuries from bland cholestasis to hepatitis including mixed forms [69-72]. Bland cholestasis is a consequence of drug induced inhibition of bile salt secretion with consequent reduction of bile flow [69, 73]. Any drug leading to cholestasis needs to be taken up into hepatocytes, prior to its interference with bile formation. Hence, hepatocellular uptake systems, such as for example OATPs are part of the cascade leading to drug induced cholestasis.

Often, the underlying pathogenetic mechanisms of drug induced liver injury remain obscure and to some extent unpredictable, in particular for idiosyncratic reactions [72, 74]. Elucidation of the mechanism is complicated by the fact that both predisposing and environmental factors are important. Once rat Bsep was cloned and identified as the most important, if not the sole canalicular bile salt export system [36, 75], interaction of drugs causing cholestatic liver injury, e.g. cyclosporine [76, 77] could be tested specifically for the interaction with Bsep. It was found that cyclosporine, rifamycin SV, rifampicin, glibenclamide inhibit rat and human BSEP in a concentration dependent and competitive manner [75, 78, 79]. Thereby, the $K_i$ values of Bsep inhibition in the Sf9 cell expression system are comparable with the $K_i$ values obtained in isolated rat liver canalicular plasma membrane vesicles [75]. Inhibition of Bsep by such drugs leads to intracellular retention of bile salts in hepatocytes with ongoing and increasing elevation exerting cytotoxic reactions to hepatocytes [16]. Bosentan is a dual endothelin receptor antagonist and is predominantly eliminated by biliary excretion [80, 81]. Hepatocellular uptake of bosentan and its metabolite occurs via OATP1B1 and OATP1B3 [82]. Bosentan caused in clinical trials, asymptomatic,
reversible transaminase elevations in some patients with a dose dependent incidence [83]. Importantly individuals, who were on glyburide and bosentan showed a higher incidence of liver injury compared to patients with bosentan monotherapy. Bosentan is a competitive inhibitor of rat and human BSEP expressed in Sf9 cell vesicles [79, 83]. Experiments with rats, which were treated with bosentan and glyburide together provide strong in vivo evidence that a specific interaction of bosentan with BSEP is responsible for the observed liver injuries [69, 73, 83]. Interestingly, a follow-up study in rats it demonstrated that canalicular expression of functional Mrp2 is necessary for the cholestatic action of bosentan [84]. In rats, bosentan stimulates via Mrp2 canalicular bile flow, which in turn impairs canalicular lipid secretion [85]. In vitro studies with rat and human Mrp2/MRP2 displayed a stimulation of MRP2 transport activity and confirmed the inhibition of rat and human Bsep/BSEP by bosentan [86]. To summarize, the example of bosentan shows that drugs do not only impair Bsep function via direct inhibition, but can affect its activity also indirectly in the canalicular membrane by processes involving Mrp2 [69, 73] Indirect drug interaction with BSEP has also been shown for estradiol-17β-glucuronide and for the HER1/HER2 inhibitor PKI166 [75, 87].

Other examples of MRP2 activation include sulfinpyrazone, penicillin G or indomethacin [88]. The consequence of drug-induced activation of MRP2 may be a lowering of the bile salt concentration in the canaliculus below a (yet unknown) threshold value followed by an alteration in canalicular phospholipid and cholesterol secretion [85]. Taken together, BSEP can either be inhibited directly by drugs form the cytoplasm or indirectly, most probably from the canalicular side. This latter process seems to need the presence of functional MRP2.

Bland cholestasis caused by BSEP inhibition is rapidly reversible upon discontinuation of the drug, as exemplified with bosentan [83]. On the other hand, if additional toxic mechanisms are involved, liver injury may persist after cessation of the drug or display a delayed onset. An
example for this is troglitazone, which is together with a metabolite inhibits BSEP and exerts via metabolites toxicity to mitochondria [69, 73, 89, 90].

In conclusion, an increasing number of drugs or their metabolites is being identified as having the potential to interfere with transport activity of BSEP [15, 73, 91]. As alteration of BSEP activity includes direct and indirect mechanisms (figure 1), the actual molecular mechanism of drug induced reduction of BSEP activity may be complex and hard to predict for a given substance.

4. Intrahepatic Cholestasis of Pregnancy

Intrahepatic cholestasis of pregnancy occurs usually in the second or third trimester of pregnancy in otherwise healthy women and belongs to the acquired forms of cholestasis [92-95]. The incidence shows a wide geographic variability and is averaging between 0.5 to 1.5 % in Caucasians in Europe and the United States [95]. Clinical evidence shows a link between serum levels of estrogens and in particular progesterone and its metabolites and the pathogenesis of cholestasis of pregnancy [96-100]. In addition, oral contraceptives can cause cholestasis, too [99, 101]. Treatment of rats with the steroid metabolites estradiol-17β-glucuronide or progesterone sulfate leads to an acute cholestasis, supporting a role of steroid metabolites in cholestasis of pregnancy [102, 103]. Animal studies, summarized in [69] revealed that treatment of the animals with high doses of ethinyestradiol over 5 days lead to cholestasis, which is paralleled by a down-regulation of hepatocellular uptake transporters for bile salts and of the canalicular efflux transporters Mrp2 and Bsep [104, 105] and reduced transport activity [106]. The acute choletic effect of estradiol-17β-glucuronide requires functional Mrp2 in the canalicular membrane of rats [107]. In addition, a bolus injection of estradiol-17β-glucuronide leads to a rapid retrieval of part of Bsep and Mrp2 from the canalicular membrane into a subapical compartment [108] (Figure 1). Finally, estradiol-17β-
glucuronide and progesterone-sulfate are indirect inhibitors of Bsep requiring the coexpression of Mrp2 [75, 102, 109]. As an alternate and/or additional mechanism, Bsep inhibition via a physical interaction between Mrp2 and Bsep in the canalicular membrane, which is stimulated by estradiol-17β-glucuronide has been suggested [107]. Hence, the pathogenesis of estrogen induced cholestasis as a model for cholestasis of pregnancy is clearly multifactorial ranging from the transcriptional to the post-transcriptional level and ending with indirect transporter inhibition.

5. Pharmacogenetics of transcription factors

So far, a limited number of studies on the pharmacogenetics of FXR have been performed. A study involving Americans from European, African, Hispanic and Chinese descent identified a polymorphism at c.-1G>T with frequencies ranging from 2.5 to 21.1 % [110]. Analysis of gene expression revealed that this polymorphism leads to a significantly lower expression of the FXR target genes coding for SHP-1, OATP1B1 and OATP1B3. In addition, four non-synonymous polymorphisms with frequencies of less than 1 % were reported in this study. An independent study also identified the c.-1G>T polymorphism [111] and demonstrated that this polymorphism leads to a reduced transcriptional activity in a luciferase reporter assay. In addition, three non-synonymous polymorphisms different from the other study were reported. Lately, additional polymorphisms in the FXR gene were identified and an intronic polymorphism was associated with the body mass index [112] and a haplotype was associated with gallstone prevalence [113].

6. Pharmacogenetics of Drug Induced Cholestasis

Investigation of the contribution of genetics to drug induced cholestasis and other forms of liver injury is challenging due to the low incidence of this disease. Progress in this area as
well as in genetics of drug response has however in recent years began to speed up by using the candidate-gene approach or by genome-wide association studies [69, 72, 114-117].

Recently, an elegant genome wide association study with patients treated with a high dose simvastatin identified with highest statistical significance a polymorphism located in intron 11 of the SCLO1B1 gene associated with myotoxicity [61] as well as additional polymorphisms in the same gene. Of note, the p.V174A polymorphism showed reduced transport activity for the prototypic OATP substrates estrone-3-sulfate and estradiol-17β-glucuronide in a model expression system [118]. Hence, the increased incidence of myotoxicity of simvastatin in these patients can be explained by a reduced hepatocellular uptake of this drug with subsequent higher serum levels. Numerous reviews have summarized the impact of OATP polymorphisms on pharmacokinetics and adverse drug reactions [57, 58, 119-124]. In contrast to OATPs, little information on the physiologic consequences of NTCP is available. This may be due to the fact that the role of NTCP on hepatocellular drug transport is minimal. Nevertheless, two nonsynonymous polymorphisms (p.I223T and p.S267F) were identified in different ethnicities, whereby very interestingly the p.S267F variant does practically not transport taurocholate but displays unaltered transport of estrone-3-sulfate [125]. Later, the p.S267F variant was found to be a gain of function variant for rosuvastatin transport [56]. This may, in the case of rosuvastatin have an impact on hepatocellular uptake of this drug, as NTCP may account for up to 35 % of rosuvastatin uptake into human hepatocytes [56].

At the efflux side, the impact of genetic alterations in \textit{ABCB11} is currently being studied. For example, a study including a cohort of 36 patients with drug-induced cholestasis found the p.V444A polymorphism of BSEP to be significantly overrepresented in the patient group leading to a threefold increased risk of developing drug-induced cholestasis under treatment with various drugs [126] (Figure 1). The same study also identified a p.D676Y mutation in a
patient receiving fluvastatin. Interestingly, the drugs associated with cholestasis in this cohort are not inhibiting the transport activity of BSEP [69]. However, the p.V444A polymorphism has been shown to be associated with reduced BSEP protein levels in healthy human liver samples and in an in vitro expression system [128]. A recent study found a correlation between lower mRNA levels and the p.V444A coding variant of the BSEP gene in a patient population, but no correlation between mRNA and BSEP protein levels [129]. It is clear that the limited size of the cohorts so far studied does not allow extrapolation into the general population with respect to this BSEP polymorphism. In addition, it should be kept in mind that the frequency of individual BSEP polymorphisms is ethnicity dependent [130-133]. Polymorphisms of BSEP may, in addition to affecting hepatocytes, have systemic effects [26]. A large study aimed at correlating in a genome-wide study single nucleotide polymorphisms with fasting glucose levels identified two polymorphisms in intron 19 of ABCB11 [134]. This may reflect that fact that serum bile salt levels are tightly interconnected with energy homeostasis [135, 136]. The genetic study on drug induced cholestasis identified a mutation in the BSEP gene in one of the patients, whereby p.D676Y was present as a heterozygous allele but displayed normal taurocholate transport activity [126]. In the same study, a novel heterozygous mutation was identified in MDR3, p.I764L, which was not further characterized. This is to the best of our knowledge the only variant of the ABCB4 gene, which has been associated with drug induced cholestasis. Even though MRP2 has been shown to be necessary for certain substances to inhibit Bsep function, no clear evidence has been presented for a role of MRP2 in drug induced cholestasis in humans. Very interestingly however, ABCC2 haplotypes have been associated with diclofenac-induced hepatotoxicity [137], with toxic liver injury caused by herbal remedies [138] and with nonalcoholic fatty liver disease [139].

7. Pharmacogenetics of intrahepatic cholestasis of pregnancy
As the incidence of intrahepatic cholestasis of pregnancy shows regional clusters, genetic susceptibility factors are very likely to play a role in the pathogenesis of this disease [47, 69, 117]. Mutations and polymorphisms in the genes coding for BSEP and MDR3 have clearly been associated with this disease. The first evidence for a genetic trait in intrahepatic cholestasis of pregnancy came from a family, where female members displayed with intrahepatic cholestasis of pregnancy and had a mutation in the \textit{ABCB4} gene [140]. This finding was confirmed and extended in a cohort study comparing genetic variations of the \textit{ABCB4} gene in women with intrahepatic cholestasis of pregnancy and in women with uneventful pregnancies [141]. In this study, close to half of the patients had elevated \(\gamma\)-glutamyl transferase, which is a clinical hallmark for liver disorders caused by impaired MDR3 function. Of the women with elevated \(\gamma\)-glutamyl transferase, 77% were carriers of disease-specific mutations in the \textit{ABCB4} gene. In addition, multiple studies including case reports have previously and later reported a defective MDR3 associated with intrahepatic cholestasis of pregnancy [142-150]. Opposite to this, there are studies, which could not identify a role of mutations in \textit{ABCB4} in cholestasis of pregnancy, indicating the presence of additional susceptibility factors for intrahepatic cholestasis of pregnancy [151, 152].

Indeed, BSEP came into the spotlight as a susceptibility factor for intrahepatic cholestasis of pregnancy [153]. Two studies indicated that the BSEP p.444A variant is significantly overrepresented in women with intrahepatic cholestasis of pregnancy [141, 154] (Figure 1). Of note, the c.1131T>C polymorphism of the \textit{BSEP} gene was associated with higher serum bile salt levels [154], which may be due to the tendency of lower protein expression levels of this BSEP variant [127]. The association of p.444A with intrahepatic cholestasis of pregnancy has since been confirmed in two independent cohorts [155]. Additionally, several mutations in BSEP have been reported in patients with intrahepatic cholestasis of pregnancy [47, 69, 156].
A cohort study identified in the FXR gene four heterozygous variants, one of which (p.M173T) is significantly associated with intrahepatic cholestasis of pregnancy [111]. This variant leads to a reduced activity of the BSEP promoter, which most likely will lead to a lower BSEP expression level. Finally, a recent report presented a patient suffering from intrahepatic cholestasis of pregnancy, which has a c.-1G>T variant of FXR, a p.S320F variant of MDR3 and a p.V444A in BSEP [157]. This patient clearly demonstrates that the genetic basis of cholestatic liver disease may be complex and in turn also the pathophysiological consequences of the altered genes.

8. Impact of ABCB11 variants on the course of viral hepatitis C

Recent evidence indicates that bile salts affect the replication of the hepatitis C virus (HCV) by suppressing interferon effects in vitro and the therapy response to pegylated interferon in patients with chronic HCV infection (Figure 1, table 2). Two independent studies demonstrated an activation of HCV replication by bile acids [158, 159]. In both studies, HCV RNA expression has been monitored after incubation of HCV genotype 1 replicon-harbouring cells with various bile acids. Addition of individual bile acids including deoxycholic acid and chenodeoxycholic acid into the medium increased the levels of HCV RNA five- to ten-fold while guggulsterone, an antagonist of bile acid receptor farnesoid X receptor (FXR), reduced the bile acid-mediated increase of HCV RNA [158, 159]. Furthermore, bile acids significantly reduced the anti-HCV effect of either IFN α or γ clearly demonstrating functional relevant alterations of the interferon signal [158, 159]. In one study, the specific FXR-dependent nature of the bile acid effect on hepatitis C replication has been investigated in more detail [159]. FXR ligands stimulated HCV replication while FXR silencing and FXR antagonism by guggulsterone blocked the up-regulation induced by bile acids.
The effects of bile acids on interferon α effects have also been investigated in patients with chronic hepatitis C infection. First observations in small and heterogenous HCV populations with different, inferior treatment regimens indicated that accumulation of bile salts may be a negative prognostic marker to predict sustained viral response (SVR) [160, 161]. Recently, the effects of plasma bile acid levels and a related BSEP gene polymorphism have been investigated in a large and well defined cohort of PEG-interferon treated hepatitis C patients [162]. Patients experiencing viral clearance had significantly lower bile acid concentrations than those without SVR. Significant differences in therapy response according to bile acid levels could be detected for a combined HCV genotype 2 and 3 subgroup and with marginal statistical significance also for genotype 1 patients. To determine whether BSEP genotypes, predisposing to a "cholestatic" phenotype [111, 126, 154, 155, 163], influence the outcome of interferon treatment similar to bile acid levels, the p.V444A of BSEP was analyzed. In line with elevated bile acid levels in non-SVR patients, an association between SVR and presence of the ABCB11 1331C allele (coding for 444A) was observed in the subgroup of HCV genotype 2 and 3 infected patients (OR 2.94) but not statistically significant for genotype 1. Besides this association to therapy response, a slight but significant difference in the frequency of the homozygous1331C BSEP genotype between patients with chronic hepatitis C compared to healthy control subjects was identified [162]. This finding supports the speculation that the host may also play a causal role via increased bile salt levels for a susceptibility to develop chronic HCV infection. On a first view, these in vivo findings are in contradiction to the replicon studies where bile acids did not affect replication of HCV genotype 2a-JFH1 replicons. However, the three tested replicons were differently sensitive to FXR modulation [158, 159]. Nevertheless, the findings in HCV patients are in line with an established negative effect of bile acids on interferon signalling and the induction of proteins involved in the antiviral activity in cell culture studies [164].
Hepatocellular accumulation of bile acids contributes to an up-regulation of inflammatory cytokines and chemokines such as tumor necrosis factor α and monocyte chemotaxis protein-1 which in turn results in hepatic stellate cell recruitment, and therefore may represent an early event in liver fibrogenesis [165]. Therefore, another HCV cohort study investigated whether BSEP or FXR genotypes are associated with fibrosis progression and the development of cirrhosis as they were with increased bile acid levels [166]. The main finding of this study is an association of the BSEP c.1331T>C polymorphism with progression to liver cirrhosis with a more than twofold carrier risk in patients with chronic viral hepatitis C. Of note, no genetic association of this polymorphism with the presence of advanced fibrosis and cirrhosis has been observed in a second independent cohort presenting with non-alcoholic fatty liver disease (NAFLD) as a non-viral disease. This leads to the speculation that the observed bile acid effects on the presence of cirrhosis could be HCV-specific and may not generally apply for chronic liver disease of other non-viral causes. Due to a low allelic frequency of the c.-1G>T variant of FXR in Caucasians only 9 heterozygous individuals (all without cirrhosis) could be identified carrying this variant, which does not allow definite conclusions [166].

9. Expert Opinion

The role of BSEP in acquired cholestasis is now well established. Inhibition of BSEP by drugs may lead to drug induced cholestasis [73, 75, 78, 79, 83]. Recently, an exhaustive screen of more than 200 compounds confirmed that substances inhibiting BSEP and having an IC₅₀ value below 25 µM are associated with liver problems in humans [91]. This work clearly demonstrates the value of inhibition experiments against BSEP heterologously expressed in membrane vesicles during drug development. It should however be pointed out that an interference of a new chemical entity with the transport activity of BSEP does not necessarily render such a compound obsolete. This point is supported by the fact that
cyclosporine is a very potent inhibitor of BSEP in the vesicular test system [75, 91], but
nevertheless made transplantation of solid organs possible. Another example of a BSEP
inhibitor is rifampicin (rifampin), which is still very important in treatment of active
pulmonary tuberculosis [167]. Observation of BSEP inhibition would rather rise a warning
sign to carefully monitor the function or BSEP later during development, e.g. by following
serum bile salt levels. From this perspective, clearly more information is needed on
similarities and/or differences of BSEP in different species [79, 168], such as rats or dogs,
which are frequently used animals in drug development.

Vesicle studies should be complemented with more complex systems, such as cultured cells,
displaying both transport and metabolic activity. BSEP inhibition leads to an intracellular
accumulation of bile salts, which by interfering with mitochondrial function become cytotoxic
[16]. The example of troglitazone has demonstrated that formation of toxic metabolites in
combination with BSEP inhibition may lead to severe liver problems. Metabolites of
troglitazone are directly toxic to mitochondria [89, 90]. Troglitazone and its sulfated
metabolites are both potent BSEP inhibitors [169]. Hence, in addition to toxic troglitazone
metabolites elevated bile salts may act on mitochondria and thus synergistically damage them
[170]. An established model system to investigate bile salt transport and drug metabolism
simultaneously are sandwich-cultured hepatocytes [171]. A combination of different
conceptual approaches and experimental model systems is clearly needed, in particular as
primary cultured hepatocytes tend to dedifferentiate and to develop a cholestatic phenotype
[172].

New cellular models are needed to study mechanisms of drug induced liver injury, including
drug induced cholestasis. In this area, new exciting developments hold great promise. Proof of
principle has been presented that hepatocyte-like cells can be produced from multipotent
mesenchymal stem cells isolated from human umbilical cord blood or from induced pluripotent stem cells [173-176]. The latter should allow to develop hepatocyte cell culture models using cells derived from patients with acquired, drug induced cholestasis or other forms of liver disease [177]. The advantage of such an approach is the possibility for studying the impact of a given genetic background of all systems involved in drug transport, metabolism on adverse drug reactions and putting them in perspective to the clinical phenotype.

While a lot of progress has already been made in understanding the impact of pharmacogenetics on drug and bile salt transport, as well as on the role of BSEP in susceptibility to acquired liver disease [69, 178], more work is needed in this area. First, while there is now considerable evidence that the p.V444A polymorphism is associated with an increased risk of acquired cholestasis, the identification of additional factors is necessary. Given the almost equal distribution of the two alleles with frequencies around 50% in different populations and the much lower incidence of drug-induced cholestasis, additional susceptibility factors are involved. One possible way to identify novel factors will be genome wide association studies in cohorts of patients suffering from drug-induced cholestasis. It is important to keep in mind that any drug interfering with cananlicular bile formation first needs to enter hepatocytes, before it reaches the canaliculus. Hence, more information is needed on the impact of genetic variability of hepatocellular uptake systems. While some transporters, such as for example OATP1B1 [57, 179] are well studied, information on others, such as for example OATP2B1 or OATP1B3 has just started to emerge [180, 181]. In addition, the driving force for OATPs has not yet been fully worked out [29]. Evidence has been presented that a large number of OATPs are stimulated by a slightly acidic extracellular pH [63]. This brings the importance of the microclimate of a transporter into the spotlight. Hence, more research is needed both at the mechanistic as well as at the genetic level of drug
transporters, not to mention that so far no structure of an uptake drug transporter has been resolved.

Finally, understanding the impact of BSEP polymorphisms on the natural course of acquired chronic liver disease is currently emerging. Recent evidence points to a relevant interference with the interferon therapy in patients with chronic hepatitis C infection [162]. Furthermore, data from this study with an overrepresentation of the respective BSEP polymorphism also point to a potential role of bile salt retention on the development of chronic viral disease during acute infection and have been confirmed by other groups. The molecular mechanism by which bile salts affect the HCV replication are still unknown. For future research, the differential response of distinct replicons to bile salt-mediated signals should provide a unique clue for mapping the region bearing HCV sensitivity to FXR on the viral genome [159]. However, bile salt-dependent effects on the natural course of chronic liver disease must be interpreted strictly in the disease-specific pathophysiological context since the ABCC11 c.1331T>C polymorphism is associated with fibrosis in chronic hepatitis C patients but not in those with fatty liver disease [166]. More information on the particular role of bile acid retention on insulin resistance accompanying fatty liver disease and other clinically relevant disease entities such as hepatocellular carcinoma are expected in the near future.

10. References


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Table 1: Main transcription factors regulating the expression of hepatocellular transporter genes

<table>
<thead>
<tr>
<th>Transporter Gene (Transporter Protein)</th>
<th>Nuclear Receptor (Nuclear Receptor Gene)</th>
<th>Receptor Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC10A1 (NTCP)</td>
<td>SHP-1 (NR0B2) regulated by FXR</td>
<td>hydrophobic bile acids, GW4064</td>
</tr>
<tr>
<td></td>
<td>FXR (NR1H4)</td>
<td></td>
</tr>
<tr>
<td>SLC10A1 (NTCP)</td>
<td>SHP-1 FXR cascade</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHP-1 (NR0B2)</td>
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<tr>
<td></td>
<td>FXR (NR1H4)</td>
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<tr>
<td>SLC10A1 (NTCP)</td>
<td>HNF1α (TCF1) regulated by</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHP-1 FXR cascade</td>
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<tr>
<td></td>
<td>SHP-1 (NR0B2)</td>
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<td></td>
<td>FXR (NR1H4)</td>
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<tr>
<td>SLC10A1 (NTCP)</td>
<td>HNF1α (TCF1)</td>
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</tr>
<tr>
<td></td>
<td>FXR (NR1H4)</td>
<td>hydrophobic bile acids, GW4064</td>
</tr>
<tr>
<td></td>
<td>CAR (NR1I2)</td>
<td>bilirubin, Phenobarbital TCOBOP, androstenol (antagonist)</td>
</tr>
<tr>
<td>SLC10A1 (NTCP)</td>
<td>FXR (NR1H4)</td>
<td>hydrophobic bile acids, GW4064</td>
</tr>
<tr>
<td>SLC10A1 (NTCP)</td>
<td>FXR (NR1H4)</td>
<td></td>
</tr>
<tr>
<td>ABCB11 (BSEP)</td>
<td>FXR (NR1H4)</td>
<td>hydrophobic bile acids, GW4064</td>
</tr>
<tr>
<td>ABCB11 (BSEP)</td>
<td>RXR (NR2B1) (obligate heterodimeric partner for class II nuclear receptors)</td>
<td>9-cis retinoic acid, rexinoids</td>
</tr>
</tbody>
</table>
Table 2: Effect of bile salts on viral hepatitis C

<table>
<thead>
<tr>
<th>Effect</th>
<th>Study System</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>inhibiton of interferone response</td>
<td>HepG2 cells</td>
<td>[164]</td>
</tr>
<tr>
<td>lower levels of serum bile salts in sustained responders than in non-responders and relapsing patients</td>
<td>retrospective clinical study</td>
<td>[161]</td>
</tr>
<tr>
<td>bile acids promote expression of HCV</td>
<td>Huh7, GS41, HG23 cells</td>
<td>[158]</td>
</tr>
<tr>
<td>no effect of bile acids on HCV clearance but improvement of transaminases</td>
<td>relative risks, weighted mean differences of randomized clinical trials</td>
<td>[182]</td>
</tr>
<tr>
<td>FXR mediated enhancement of genotype 1 HCV replication by bile acids</td>
<td>Huh7, Huh7 Lunet cells</td>
<td>[159]</td>
</tr>
<tr>
<td>activation of pro-fibrogenic cells by bile acids</td>
<td>clinical study</td>
<td>[165]</td>
</tr>
<tr>
<td>association of c.1331T&gt;C of ABCB11 with fibrosis progression in HCV</td>
<td>retrospective clinical study</td>
<td>[166]</td>
</tr>
<tr>
<td>association of serum bile salts levels with sustained virological response</td>
<td>retrospective clinical study</td>
<td>[162]</td>
</tr>
</tbody>
</table>
Figure 1: Overview on key transporters involved in hepatocellular bile formation

Bile salts are predominantly taken up by the sodium taucholate cotransporting polypeptide (NTCP) and to a minor extent by the organic anion transporting polypeptides (OATPs), which work sodium-independent. OATPs are also key drug uptake systems. Bile salts are secreted into the canaliculus by the bile salt export pump (BSEP), while the biliary lipids phosphatidylcholine and cholesterol require the multidrug resistance protein 3 (MDR3) and the heterodimeric transporter ABCG5/ABCG8, respectively. In normal physiologic states the nuclear receptor FXR acts as bile salt sensod and downregulates the expression of NTCP indirectly and upregulates expression of BSEP directly. Drugs can inhibit BSEP from the cytoplasmic side directly and indirectly from the canaliculus in a process requiring functional MRP2 (trans-inhibition, right lower insert). Single nucleotide polymorphisms of the ABCB1 gene with clinical implications for drug-induced cholestasis, intrahepatic cholestasis of pregnancy and viral hepatitis C are displayed in a topographical scheme (right upper insert). In livers infected with hepatitis C virus (HCV), bile salts interfere with interferon signaling and promote HCV replication in an FXR-mediated manner (left insert).
BSEP (ABCB11) selected SNPs

Biliary canaliculus

Canalicular membrane

COOH

NH₂

ATP-binding

V444A

D676Y

ATP-binding

Hepatocyte

Canaliculus

NTCP

OATP2B1

OATP1B1

OATP1B3

Shp

Chol

PC

BSEP

ABCG5/G8

MRP2

MDR3

IFN-α

Bile acids, FXR and HCV

BSEP trans-inhibition

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