

## Mechanisms of Cholestasis

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Cholestasis can result from either a functional defect in bile formation at the level of the hepatocyte or from impairment in bile secretion and flow at the bile duct level (Fig. 1) [1,2]. Bile formation under physiological conditions is mediated via a broad range of specific uptake and export systems localized to the basolateral (sinusoidal) and canalicular (apical) membrane of hepatocytes and cholangiocytes (Fig. 2). In hereditary cholestasis syndromes, such as progressive familial intrahepatic cholestasis (PFIC), mutations in transporter genes are the primary cause of cholestasis (Table 1). However, such hereditary transporter defects are extremely rare and are of limited relevance in adults. Nevertheless, these defects have provided valuable insights into the physiology and pathophysiology of bile formation. Transporter defects may be incomplete and mild under physiological conditions and can only become evident later in life when the patient is challenged with a cholestatic agent (eg, drugs, sex hormones, cytokines released by inflammation). Therefore, hereditary transporter mutations may also predetermine the individual susceptibility to acquired cholestatic liver injury (see Table 1) [3]. However, in most cholestatic disorders, transporter alterations are the consequence rather than the cause of cholestasis and largely represent an attempt to adapt to accumulating biliary constituents in cholestasis and to protect hepatocytes from intracellular accumulation of toxic bile acids. Such adaptive mechanisms include the induction of bile acid detoxification systems and the recruitment of alternative export pumps for cholephiles at the basolateral membrane, finally leading to increased urinary bile acid elimination. These changes are mediated by specific nuclear receptors, which in turn can be activated by accumulating bile acids, proinflammatory cytokines, drugs, and hormones. In addition to transcriptional

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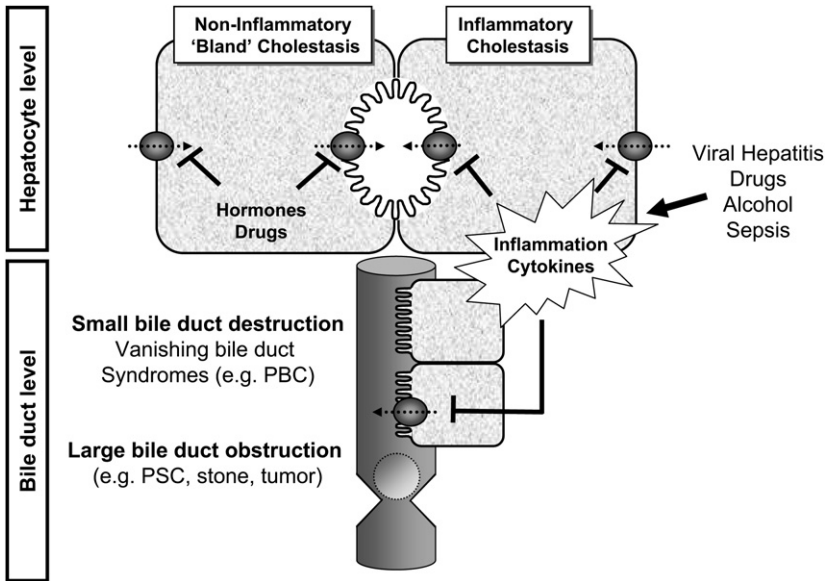


Fig. 1. Principle mechanisms of cholestasis. Cholestasis can result either from a functional defect in bile formation at the level of the hepatocyte or from impairment in bile secretion and flow at the bile duct level. At the hepatocellular level, hormones and drugs can cause “bland,” noninflammatory cholestasis (eg, by inhibition of transporter function or by retrieving transport proteins from the membrane). Pro-inflammatory cytokines (induced by sepsis, viral hepatitis, alcohol, or drugs) repress transporter expression and function, causing inflammatory cholestasis (“cholestatic hepatitis”). At the bile duct level, progressive bile duct destruction leading to bile duct paucity (vanishing bile duct syndromes, such as primary biliary cirrhosis [PBC]) and bile duct obstruction by stenoses (eg, in primary sclerosing cholangitis [PSC]), stones, or tumors interrupt bile flow. Pro-inflammatory cytokines produced by Kupffer cells and the bile duct epithelium itself can impair cholangiacellular and hepatocellular transporter expression and function in inflammatory cholestasis. Moreover, genetic defects (not shown) can cause cholestasis at the hepatocellular and cholangiacellular level (see Table 1).

changes, reduced transporter protein insertion to or increased retrieval from the cell membrane as well as other mechanisms, such as altered cell polarity, disruption of cell-to-cell junctions, membrane perturbations, and cytoskeletal changes can also be involved in the pathogenesis of cholestasis [1,2]. This review focuses on hepatocellular changes. See the article by Reau and Jensen elsewhere in this issue for further discussion of the mechanisms of cholangiopathies and ductopenic syndromes.

### Hereditary transporter defects as cause of cholestasis

Several hereditary cholestasis syndromes can now be understood and classified by linking genetic analysis to the clinical phenotype. While these PFIC syndromes are rare, the underlying molecular defects facilitate our understanding of the molecular mechanisms leading to cholestasis. Mutations in three important canalicular transporter genes cause PFIC-1, PFIC-2, and

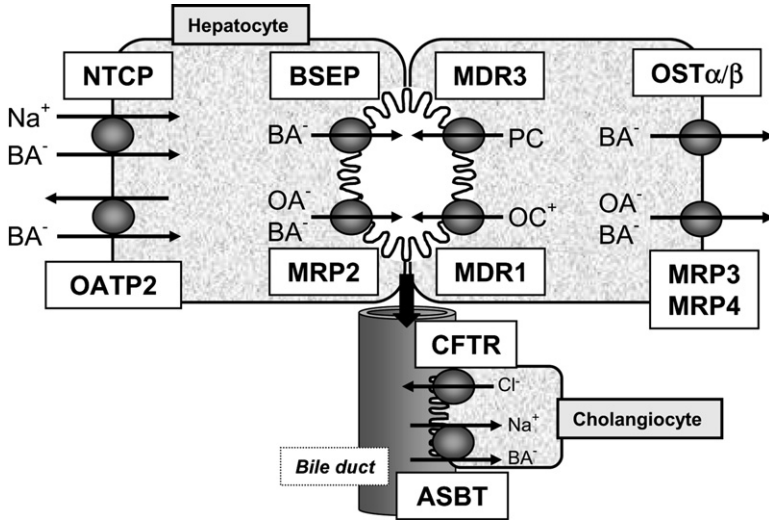


Fig. 2. Hepatobiliary transport systems in hepatocytes and cholangiocytes. Bile acids ( $\text{BA}^-$ ) are taken up by the  $\text{Na}^+$ /taurocholate cotransporter (NTCP) and organic anion transporting proteins (OATP2/OATP1B1) at the basolateral membrane of hepatocytes and exported into bile by the canalicular bile salt export pump (BSEP) and the canalicular conjugate export pump (MRP2). MRP2 also mediates excretion of various anions ( $\text{OA}^-$ ), such as bilirubin, into the canaliculus. The phospholipid export pump (MDR3) mediates excretion of phosphatidylcholine (PC), which forms mixed micelles together with  $\text{BA}^-$  and cholesterol in bile. Cationic drugs ( $\text{OC}^+$ ) are excreted by the multidrug export pump (MDR1). At the basolateral membrane of hepatocytes, MRP3, MRP4, and the heteromeric organic solute transporter (OST)  $\alpha/\beta$  provide an alternative excretion route for  $\text{BA}^-$  and other  $\text{OA}^-$  into the systemic circulation. Cholangiocytes express apical sodium-dependent bile acid transporter (ASBT) for bile acid reabsorption and a chloride channel, which is the cystic fibrosis transmembrane regulator (CFTR).

PFIC-3, which are autosomal recessively inherited disorders manifesting in neonates, infants, and children (see Table 1) [3]. A fourth subtype, PFIC-4, is not a transporter defect, but is caused by defects in the bile acid synthetic pathway and is not discussed in this review.

PFIC-1 (also known as Byler's disease) is caused by a mutation of the putative aminophospholipid transporter *FIC1/ATP8B1* and leads to the development of liver cirrhosis in early childhood (see Table 1) [4,5]. Patients usually present in the neonatal period with elevated levels of serum bile acids, bilirubin, and transaminases, but levels of gamma-glutamyl transpeptidase (GGT) are low. PFIC-1 rapidly progresses to end-stage liver disease requiring liver transplantation at an early age. Mutations in the *FIC1* gene also cause benign recurrent intrahepatic cholestasis (BRIC-1, Summer-skill syndrome), which is characterized by recurrent episodes of cholestasis not necessarily leading to liver cirrhosis [4]. BRIC-1 represents the milder spectrum of this disease. However, BRIC-1 may also progress to a form that is indistinguishable from PFIC-1 [6]. One might speculate that in BRIC-1 patients, residual activity of *FIC1* may still be present, while in

Table 1  
Genetic defects causing hereditary cholestasis syndromes and associations with acquired cholestatic diseases

Gene	Function	Hereditary cholestasis syndrome	Associated acquired cholestatic diseases (susceptibility)
<i>FIC1</i> ( <i>ATP8B1</i> )	Aminophospholipid flippase, maintaining phospholipid asymmetry of the canalicular membrane	<ul style="list-style-type: none"> <li>• PFIC-1 (Byler's disease)</li> <li>• Benign recurrent intrahepatic cholestasis type 1 (Summerskill syndrome)</li> </ul>	<ul style="list-style-type: none"> <li>• Intrahepatic cholestasis of pregnancy</li> </ul>
<i>BSEP</i> ( <i>ABCB11</i> )	Canalicular bile salt export pump	<ul style="list-style-type: none"> <li>• PFIC-2 (Byler's syndrome)</li> <li>• Benign recurrent intrahepatic cholestasis type 2</li> </ul>	<ul style="list-style-type: none"> <li>• Intrahepatic cholestasis of pregnancy</li> <li>• Drug-induced cholestasis</li> <li>• Potential role for disease progression of primary biliary cirrhosis</li> </ul>
<i>MDR3</i> ( <i>ABCB4</i> )	Canalicular phospholipid flippase	<ul style="list-style-type: none"> <li>• PFIC-3</li> </ul>	<ul style="list-style-type: none"> <li>• Intrahepatic cholestasis of pregnancy</li> <li>• Primary sclerosing cholangitis</li> <li>• Drug-induced cholestasis</li> <li>• Low-phospholipid-associated cholelithiasis syndrome</li> </ul>
<i>MRP2</i> ( <i>ABCC2</i> )	Canalicular organic anion conjugate export pump	<ul style="list-style-type: none"> <li>• Dubin-Johnson syndrome</li> </ul>	<ul style="list-style-type: none"> <li>• Intrahepatic cholestasis of pregnancy</li> </ul>
<i>CFTR</i> ( <i>ABCC7</i> )	Cholangiocyte apical chloride channel	<ul style="list-style-type: none"> <li>• Cystic fibrosis</li> </ul>	<ul style="list-style-type: none"> <li>• Primary sclerosing cholangitis</li> </ul>
Tight junction protein 2	Tight junction protein	<ul style="list-style-type: none"> <li>• Hypercholanemia</li> </ul>	—
Claudin-1	Tight junction protein	<ul style="list-style-type: none"> <li>• Neonatal sclerosing cholangitis associated with ichthyosis</li> </ul>	—

PFIC-1 patients, this activity is completely lost [3]. The pathomechanism by which *FIC1* deficiency leads to cholestasis has recently been elucidated in *Fic1* mutant mice. *Fic1* deficiency causes loss of the usual phospholipid asymmetry of the canalicular membrane, which in turn renders the canalicular membrane less resistant to hydrophobic bile acids [7]. The loss in phospholipid asymmetry may further reduce bile acid transport, causing cholestasis [7]. PFIC-1 and BRIC-1 are associated with extrahepatic manifestations, such as diarrhea, bile acid malabsorption, pancreatitis, and nephrolithiasis, which can be explained by wide expression of *FIC1* in these

tissues. Liver transplantation resolves cholestasis, but the extrahepatic manifestations persist [8].

Mutations of the bile salt export pump gene (*BSEP/ABCB11*), which encodes the major canalicular bile acid export system, causes PFIC-2 (see Table 1) [9]. The clinical course is similar to that for PFIC-1, while extrahepatic manifestations are absent because BSEP expression is restricted to the liver. Serum bile acid and transaminase levels are elevated but levels of GGT are low. PFIC-2 is also associated with hepatocellular carcinoma development in young children [10]. Hepatocarcinogenesis in these patients might be due to the mutagenic potential of bile acids [11]. Like in PFIC-1, more moderate courses of this disorder cause a variant of benign intrahepatic recurrent cholestasis (BRIC-2) [12]. BRIC-2 also seems to be associated with a high risk for the development of gallstones [12].

PFIC-3 is caused by mutations of the multidrug resistance protein *MDR3/ABCB4* gene, which encodes a phospholipid export pump (see Table 1) [13]. PFIC-3 patients present with progressive cholestasis and 50% of patients require liver transplantation. In contrast to PFIC-1 and PFIC-2, PFIC-3 is characterized by high levels of GGT. MDR3 is a phospholipid flippase translocating phospholipids from the inner to the outer leaflet of the canalicular membrane where they can be extracted by bile acids [14]. Phospholipids in bile are required for the formation of mixed micelles with bile acids and cholesterol to protect the bile duct epithelium from the detergent properties of bile acids. Reduced or absent phospholipid excretion into bile in PFIC-3 causes bile duct injury in these patients. The pathophysiologic consequences have been established in mice lacking *Mdr2* (the rodent homolog of human *MDR3*) [15], which develop sclerosing cholangitis [16,17].

Another important example of how a transporter mutation can impair biliary excretory function is Dubin-Johnson syndrome. Dubin-Johnson syndrome is caused by mutations of the canalicular bilirubin export pump (*MRP2/ABCC2*) [18] leading to reduced biliary excretion of various endogenous and exogenous compounds (eg, conjugated bilirubin, oral cholecystography agents) (see Table 1). However, the patients are not cholestatic, do not develop pruritus, and have normal serum bile acid levels.

Mutations in transporter genes in cholangiocytes can also cause cholestasis. The cystic fibrosis transmembrane regulator (CFTR), a cyclic adenosine monophosphate (cAMP)-dependent chloride channel, is mutated in cystic fibrosis (see Table 1) [19]. Increased life expectancy has led to an increasing recognition of hepatobiliary complications in cystic fibrosis patients, including biliary tract complications, such as stones and sclerosing cholangitis [20]. Other examples of hereditary transporter defects that do not cause cholestasis are mutations of the sitosterol and cholesterol transporter *ABCG5/G8* causing sitosterolemia [21,22].

## Transporter defects in acquired cholestatic liver diseases

Alterations of hepatobiliary transport systems in acquired forms of cholestasis are caused by pro-inflammatory cytokines (eg, sepsis-induced cholestasis), hormones (eg, intrahepatic cholestasis of pregnancy [ICP], oral contraceptive-induced cholestasis), or drugs (ie, drug-induced cholestasis). These factors can influence transporter mRNA or protein expression, lead to retrieval of transporter proteins from the membrane to submembranous compartments, or directly inhibit transporter protein function [1,23–25]. Most of these concepts have been established in animal models of cholestasis. Only a few studies have investigated transporter protein expression in humans, probably because of the limited amount of human tissue available for molecular analysis, since in many cholestatic disorders liver biopsy is no longer performed for diagnostic work-up. The focus of this article is on transporter alterations in acquired forms of cholestasis in humans and genetic factors predisposing to cholestatic liver injury.

### *Intrahepatic cholestasis of pregnancy*

ICP occurs in the third trimester of pregnancy and is characterized by pruritus and elevated serum bile acid levels, which usually fully resolve after delivery [26]. Apart from causing maternal pruritus, ICP is associated with increased incidence of fetal distress, premature birth, and stillbirth [26]. Furthermore, the risk for the development of cholesterol gallstones and of associated or even subsequent chronic liver diseases may be increased in patients who have ICP [26–30]. ICP patients can be divided into two groups according to GGT levels. Levels of GGT are in the normal range in 70% and elevated in 30% of patients, suggesting different mechanisms causing cholestasis [31]. Hormonal factors play a key role in the pathogenesis of ICP. Levels of estrogens and progesterone are highest in the third trimester of pregnancy, the time of onset of ICP, and return to normal values after delivery, when ICP resolves [26,32]. The procholestatic effects of estrogens and their metabolites have been established in various animal experiments. Ethinylestradiol 17 $\beta$ -glucuronide exerts potent cholestatic properties in rats and induces an acute but reversible decrease in bile acid-dependent and bile salt-independent bile flow [33]. The decrease of bile acid-independent bile flow has been attributed to a marked endocytic internalization of Mrp2 from the canalicular membrane into intracellular membranes, to a decrease in Mrp2 transport activity [34], and to reduced Mrp2 protein expression [35]. Furthermore, ethinylestradiol 17 $\beta$ -glucuronide *cis*-inhibits the biliary secretion of glutathione [36]. Bile acid-dependent bile flow is reduced by *trans*-inhibition of Bsep by ethinylestradiol 17 $\beta$ -glucuronide after its secretion into bile via Mrp2 [37] and by endocytic internalization of Bsep [38]. Estrogen-induced cholestasis is also associated with alterations of uptake systems at the basolateral membrane (ie, reduced expression of basolateral

Na<sup>+</sup>/taurocholate cotransporter [Ntcp] and, to a lesser degree, of the organic anion transporting proteins Oatp1a1, Oatp1a4, and Oatp1b2) and reduces basolateral membrane fluidity in rats, which in turn may impair hepatobiliary transport [39–41]. Interestingly, the changes observed in transporter expression in estrogen-induced cholestasis are very similar to those observed during normal pregnancy in the rat [42,43]. Animal models of estrogen-induced cholestasis were established using very high doses of single hormones. Whether similar changes occur during pregnancy or in ICP in humans remains to be determined. Abnormalities in progesterone metabolism may also play a major role in the pathogenesis ICP. This is emphasized by the observed association of ICP with progesterone treatment in the third trimester [44]. In addition, the levels of sulfated progesterone metabolites are increased in ICP and the profiles of sulfated progesterone metabolites differ between normal pregnancy and ICP [45,46]. These data clearly indicate a defect in progesterone metabolism in ICP and *in vitro* data suggest that several progesterone metabolites are able to *trans*-inhibit Bsep [47]. However, it remains unclear whether these alterations in progesterone metabolism are primary events or secondary changes due to cholestasis [46]. Roles have been suggested for other factors, such as selenium deficiency, hepatitis C virus infection, or translocation of bacterial endotoxin [29,48].

In addition to hormonal and environmental factors, mutations of several transporter genes may be associated with ICP (see Table 1). In analogy to PFIC-1, PFIC-2, and PFIC-3, normal or elevated GGT levels suggest the involvement of different transporters. The best characterized defects are *MDR3* mutations. Several studies have identified heterozygous *MDR3* mutations in ICP patients [27,30,49–56]. Similar to PFIC-3, most patients with ICP and *MDR3* variations had elevated GGT levels. However, a recent study identified three novel *MDR3* mutations in ICP patients with normal GGT [54]. Since *MDR3* defects are expected to cause bile duct injury and elevated GGT levels due to low biliary phospholipid concentrations, the functional relevance of these mutations are questionable. Two other studies, however, could not demonstrate a role for *MDR3* mutations in ICP, indicating the presence of considerable genetic variability [57,58].

Patients who experienced ICP are also prone to cholesterol gallstone formation and may also develop subsequent chronic liver disease [26–30]. One might speculate that compound heterozygosity together with another yet unidentified *MDR3* mutation or chronic intermittent biliary obstruction due to biliary stones might cause progressive liver disease [48]. Consistent with this line of reasoning, *MDR3* variants are also associated with syndromic cholesterol cholelithiasis, the low-phospholipid-associated cholelithiasis syndrome [59–61] characterized by cholesterol gallstones that recur after cholecystectomy, mild chronic cholestasis due to intrahepatic stones, and an increased incidence of ICP.

Other transporter defects may also play a role in the pathogenesis of ICP (see Table 1). *BSEP* polymorphisms were associated with ICP in a Finnish

study [62] and combined homozygous mutations of *BSEP* and *MDR3* have been described in one ICP patient with severe cholestasis [63]. In addition, a British study identified *FIC1* mutations in some ICP patients [64] and also *MRP2* mutations have recently been observed in ICP in Argentina [65]. Other candidate genes possibly involved in the pathogenesis of ICP are cytochrome P450 enzymes and their regulatory nuclear receptors [48,66–68]. The present data suggest that only a minority of ICP cases are caused by genetic transporter defects [3].

### *Drug-induced cholestasis*

Drugs can cause cholestasis by inhibiting hepatocellular transporter expression and function and, in rare cases, by inducing a vanishing bile duct syndrome, which can progress to biliary cirrhosis. Drug-induced liver injury is rare and less than 30% of cases are cholestatic. More often, drugs induce an idiosyncratic reaction, where hypersensitivity and aberrant metabolism are thought to represent the predominant pathway leading to inflammation. However, inflammation itself can interfere with bile secretion, causing inflammatory cholestasis (“cholestatic hepatitis”) [48,69]. Many cases of drug-induced cholestasis result from a functional inhibition of transporter proteins by the drug itself or its metabolites. Such drugs as cyclosporine, rifampin, bosentan, troglitazone, and glibenclamide *cis*-inhibit ATP-dependent taurocholate transport via Bsep in a competitive manner [37,70,71]. Estrogen and progesterone metabolites indirectly *trans*-inhibit Bsep function after their secretion into bile via Mrp2 [37]. It is possible that phospholipid secretion via MDR3 might also be impaired by verapamil, cyclosporin A, and vinblastine, because these compounds are transported by MDR3 *in vitro* [72]. In addition, inhibition of MRP2 by drugs, such as the antibiotic fusidate, causes jaundice [73]. Bosentan, on the other hand, stimulates Mrp2-dependent bile salt-independent bile flow and inhibits biliary lipid secretion, thus uncoupling biliary lipid secretion from bile acid secretion possibly causing cholestasis [74]. In addition to an inflammatory stimulus and direct inhibition of transporter function, genetic variations in transporter genes can also predispose to drug-induced cholestasis (see Table 1). *BSEP* and *MDR3* gene polymorphisms and mutations have recently been described in drug-induced cholestasis [75]. In addition to these changes in transporter function, drugs or their metabolites may also trigger an immune response targeted against the bile duct epithelium finally leading to a vanishing bile duct syndrome [76]. The detailed mechanisms of drug-induced cholestasis are reviewed elsewhere [69].

### *Inflammation-induced cholestasis*

Inflammation-induced cholestasis is the most common cause of jaundice in hospitalized patients [77]. However, most of our knowledge is derived from animal models. Lipopolysaccharide treatment inhibits transporter



expression and function at the basolateral and canalicular membrane of hepatocytes, reducing both bile acid-dependent and bile acid-independent bile flow [1,23,78]. Pro-inflammatory cytokines reduce expression of transporter genes and cause ultrastructural changes to the cytoskeleton and cell junctions [1,79]. Either direct cytokine-dependent down-regulation or reduced promoter binding reduces transcriptional activity of various nuclear receptors and transcription factors, leading to repression of basolateral uptake (Ntcp, Oatps) and canalicular export systems (Bsep, Mrp2) [79–82]. Simultaneous up-regulation of other export proteins (eg, Mrp1, Mdr1) is mediated by nuclear factor  $\kappa$ B and might confer resistance to cytokine-induced metabolic stress [83]. In humans, however, post-transcriptional mechanisms may play a more important role as demonstrated in lipopolysaccharide-treated human liver slices and in liver biopsies from patients with inflammatory cholestasis [84,85]. In addition to transcriptional changes, retrieval of transporters, such as Mrp2, Bsep, or Ntcp, from the membrane occurs hours before changes in mRNA or protein levels, representing a short-term mode of regulation [79,86]. In addition to hepatocellular alterations, pro-inflammatory cytokines increase nitric oxide production in cholangiocytes, which inhibits ductal bile secretion and contributes to ductular cholestasis [87,88]. See the article by Fuchs and Sanyal elsewhere in this issue for further review of the detailed mechanisms involved in sepsis-induced cholestasis.

### *Primary sclerosing cholangitis*

The exact pathogenesis of primary sclerosing cholangitis (PSC) is still unknown and is likely multifactorial. A potential role for a transporter defect (ie, *MDR3*) in the pathogenesis of PSC has been proposed, since mice lacking *Mdr2* (the rodent homolog of human *MDR3*) develop sclerosing cholangitis macroscopically and microscopically resembling PSC in humans [16,89,90]. However, because of the complex pathophysiology of PSC, a single *MDR3* mutation seems an unlikely cause. Pauli-Magnus and colleagues [91] could find no causative role for a single *MDR3* mutation when they observed no significant differences in the total number of *MDR3* (and also *BSEP*) variants or in the allele frequency of the common variable sites in controls and in PSC patients. Another study analyzing single *MDR3* polymorphisms also did not find evidence for a major role for *MDR3* mutations in the pathogenesis of PSC [92]. However, *MDR3* variants could play a role as modifier genes in specific subphenotypes of PSC (eg, pediatric PSC, female patients with gallstones) [48]. Sclerosing cholangitis is also increasingly encountered in adult patients with cystic fibrosis, raising the question for a role of *CFTR* variants in PSC (see Table 1) [20]. In a subset of PSC patients with inflammatory bowel disease, an increased prevalence of *CFTR* abnormalities was observed. In this study, the genetic variations were further confirmed by a reduced chloride secretory response [93]. The investigators speculated that *CFTR* might represent

a modifier gene for development of PSC in patients with inflammatory bowel disease. Interestingly, induction of colitis in *Cftr* knockout mice resulted in the development of bile duct injury [94]. Furthermore, a recent study observed a high prevalence of *CFTR* mutations and decreased CFTR function in childhood PSC [95]. However, other studies could not confirm an association of disease-causing *CFTR* mutations with PSC [96–98] and the role of *CFTR* in PSC is still under debate [93,98]. While transporter gene mutations do not seem to be causative in the pathogenesis of PSC, they could play an important role as disease modifiers [48]. These variants might alter bile composition, thus influencing the secondary response to any primary bile duct injury (eg, immune-mediated, ischemic injury) and thereby modifying the clinical course of disease [48,99]. The only study so far investigating transporter expression in PSC showed reduced expression of OATP1B1/*SLCO1B1* and MRP2 [100]. These alterations may contribute to reduced secretion of bile acids and conjugated bilirubin. However, these changes are rather secondary because of elevated levels of bile acids (and probably cytokines) (see below) but could play an important role in modifying disease course.

### *Primary biliary cirrhosis*

Primary biliary cirrhosis (PBC) is characterized by immune-mediated destruction of intrahepatic bile ducts leading to a vanishing bile duct syndrome. Genetic and environmental factors play key roles in the pathogenesis of PBC [101,102]. A primary role for transporter gene mutations (ie, *MDR3* and *BSEP*) was dismissed by Pauli-Magnus and colleagues [91], who found no differences in overall variant segregation or haplotype structure of these two genes between controls and PBC patients. Interestingly, a *BSEP* haplotype revealed association with higher Mayo Risk Scores, suggesting a possible role in disease progression [91]. As for PSC, variants in transporter genes may play a role in modifying disease course and progression (see Table 1) [48].

Several studies have found reduced expression and function of anion exchanger AE2 (*SLC4A2*), a  $\text{Cl}^-/\text{HCO}_3^-$  exchanger, in PBC, which may contribute to reduced bile flow and cholestasis in PBC [103–106]. Moreover, decreased AE2 expression in salivary and lacrimal glands could explain the frequently associated sicca syndrome in these patients [107]. Pro-inflammatory cytokines, which play a role in the pathogenesis of PBC, may also contribute to altered transporter expression [108]. However, reduced AE2 expression is not secondary to inflammation in PBC, as suggested by unchanged Ae2 expression in cytokine-treated rat cholangiocytes [87]. Whether AE2 repression is a primary mechanism in the pathogenesis of PBC is not clear and mutations of the *AE2* gene in PBC have not yet been reported.

Most other changes in hepatocellular transporter gene expression levels encountered in PBC represent adaptation to elevated bile acid levels. In late-stage PBC, basolateral bile acid uptake systems were repressed, while

alternative basolateral export pumps were induced [109,110]. In contrast, in early stage PBC patients with normal bile acid and bilirubin levels, no changes in transporter expression were observed, suggesting that the alterations observed in late-stage disease represent secondary mechanisms activated by increased bile acid levels [85]. In addition to transporter changes, regurgitation of biliary constituents through leaky tight junctions could also contribute to elevated serum levels of conjugated bilirubin and bile acids in PBC [111] (see below). Adaptive mechanisms are not only encountered in PBC but represent a general concept in cholestatic diseases and will now be discussed in more detail.

### **Adaptive mechanisms in cholestatic liver disease**

Cholestatic disorders where disturbed transporter function or expression are causing the disease are rare (ie, hereditary cholestasis syndromes, some forms of ICP, and drug-induced cholestasis). In most other acquired forms of cholestasis, alterations in transporter expression are secondary phenomena due to the retention of cholephiles. Most cholestatic diseases are caused at the bile duct level by obstruction (eg, extrahepatic biliary obstruction by stones and tumors) or destruction (ie, vanishing bile duct syndromes, such as PBC) (see Fig. 1). Alterations in transporter expression in these diseases may explain the impairment of transport function resulting in or maintaining cholestasis only to some extent. Most of these alterations represent compensatory mechanisms aimed at protecting hepatocytes from toxic bile acid overload by providing alternative excretory routes for accumulating cholephiles during cholestasis. Reducing basolateral bile acid uptake and simultaneously increasing basolateral bile acid excretion may be considered major hepatic defense mechanisms counteracting accumulation of toxic bile acid within hepatocytes [112]. In addition, bile acid metabolism and detoxification (ie, phase I bile acid hydroxylation and phase II conjugation with sulfate or glucuronidate) is increased while bile acid synthesis is repressed. A complex machinery of coordinated mechanisms is activated by bile acids to counteract cholestatic liver injury [113]. Adaptive mechanisms in response to cholestasis are not restricted only to the liver but also occur in kidney, intestine, and bile duct epithelia (Fig. 3) [112].

Limiting hepatic bile acid uptake during cholestasis may be considered a protective mechanism to reduce hepatocellular bile acid overload. Expression of the main basolateral bile acid uptake systems *NTCP/SLC10A1* and *OATP1B1/SLCO1B1* (formerly known as OATP-C or OATP2) is reduced in human cholestatic liver diseases [85,86,100,109,114] and in various rodent models of cholestasis and bile acid overload (Fig. 3A) [115–122].

Bile acid export via the canalicular membrane is mediated by BSEP and by MRP2 (see Fig. 3 [B]). Expression of these transporters must be tightly controlled to prevent bile acid accumulation within the hepatocyte. As observed in bile acid-fed mice, bile acids can induce their own efflux into bile

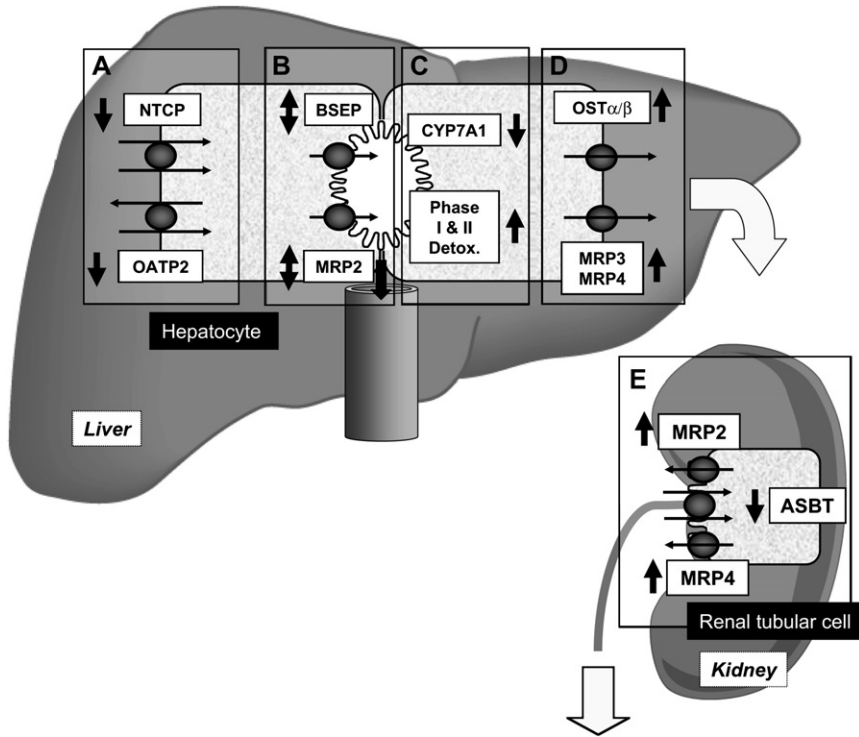


Fig. 3. Adaptive regulation of transport systems and of bile acid synthetic and metabolizing enzymes during cholestasis. (A) Down-regulation of the basolateral NTCP and OATP2/OATP1B1, together with (B) preserved expression of the canalicular BSEP and MRP2 reduces the hepatocellular retention of bile acids. (C) In addition, repression of bile acid synthesis reduces bile acid load and increased phase I (ie, hydroxylation) and phase II (ie, sulfation, glucuronidation) detoxification renders bile acid less toxic and more amenable to alternative basolateral excretion. (D) Furthermore, induction of overflow systems, such as MRP3, MRP4, and organic solute transporter (OST)  $\alpha/\beta$  at the basolateral membrane of hepatocytes provides an alternative route for the elimination of bile acids and bilirubin from cholestatic hepatocytes. Decreased basolateral bile acid uptake together with increased basolateral bile acid secretion into the systemic circulation facilitates bile acid delivery to the kidneys. (E) Passive glomerular filtration may be assisted by active tubular secretion of bile acids (via MRP2 and MRP4) and by reduced tubular re-uptake via repression of apical sodium-dependent bile acid transporter (ASBT), thereby increasing urinary bile acid elimination in cholestasis.

by increasing both Bsep and Mrp2 expression [121,123]. While bile acids are normally excreted into bile, alternative basolateral bile acid excretion into portal blood may become a major way for hepatic bile acid elimination during cholestasis. Alternative basolateral bile acid export is mediated by MRP3, MRP4, and the heteromeric bile acid transporter, the organic solute transporter OST $\alpha$ /OST $\beta$  (see Fig. 3 [D]) [124]. These export systems are normally expressed at very low levels at the basolateral membrane but are dramatically up-regulated after bile acid feeding and in experimental

cholestasis in rodents as well as in human cholestatic liver diseases [86,109,110,121,123,125–132]. Since MRP3, MRP4, and OST $\alpha$ /OST $\beta$  are able to transport sulfated and glucuronidated bile acids that are eliminated into urine during cholestasis, the induction of these transporters may explain the shift toward renal excretion of bile acids as a major mechanism for bile acid elimination in patients with chronic, long-standing cholestasis (see Fig. 3 [E]) [133–137]. In addition to bile acid elimination via the kidney, induction of basolateral efflux pumps and repression of basolateral uptake systems, together with disruption of tight junctions (see below), may also explain the appearance of conjugated bilirubin and development of jaundice and bilirubinuria in cholestasis.

Bile acids that either escape first-pass clearance by the liver or are actively excreted by hepatocytes into sinusoidal blood under cholestatic conditions are filtered at the glomerulus from plasma into urine [138]. Thereafter, bile acids are reabsorbed by apical sodium-dependent bile acid transporter (ASBT) localized to the apical membrane of proximal renal tubular cells and probably in turn excreted into systemic circulation by basolateral OST $\alpha$ /OST $\beta$  (see Fig. 3 [E]) [139–141]. Urinary bile acid elimination can be increased by reducing Asbt-mediated bile acid reabsorption in the proximal tubuli as observed in obstructive cholestasis in rats [142]. Passive glomerular filtration of bile acids might also be aided by active tubular secretion. Candidate transporters for export of bile acids are Mrp2 and Mrp4, since both are localized to the apical tubular membrane [143–145] and are induced by bile acid feeding (Fig. 3D) [126]. However, the relative contribution of active tubular excretion to passive glomerular filtration still remains to be determined.

Phase I bile acid hydroxylation renders bile acid more hydrophilic, less toxic, and more amenable for urinary excretion. Bile acids (and several drugs and xenobiotics) are metabolized by CYP3A4 (and by its rodent homolog Cyp3a11), converting them to more hydrophilic and less toxic compounds that can be eliminated more easily from the body [146–148]. Cyp3a11 levels are increased in rodent models of obstructive cholestasis and in bile acid-challenged mice [125,149–154]. Besides hydroxylation, phase II conjugation of bile acids with sulfate or glucuronidate represent important mechanisms of bile acid detoxification. Dehydroepiandrosterone-sulfotransferase (SULT2A1) catalyzes sulfo-conjugation of a broad range of endogenous compounds, including bile acids [155,156]. Upon sulfation, SULT2A1 substrates (such as toxic lithocholic acid) become polar, water soluble, and less toxic [157] and are more amenable to rapid excretion [158]. Glucuronide conjugation also renders bile acids less toxic and more readily soluble in water [159]. Glucuronidation of bile acids is catalyzed by the uridine diphosphate glucuronosyltransferases UGT2B4 and UGT2B7 [159,160]. Bile acids induce expression of these genes by activation of nuclear receptors (see below), thus regulating their own detoxification in a feed-forward fashion [113]. The importance of these detoxification

pathways is underlined by the appearance of hydroxylated, sulfated, and glucuronidated bile acids in urine of patients with cholestatic diseases, since these bile acid metabolites are absent under normal conditions [135–137,161–166]. A recent study from the authors' group reported preserved but not induced expression of phase I and II detoxification enzymes in patients with late-stage PBC [110]. One might speculate that the preserved expression of these phase I and II detoxification enzymes is sufficient to mediate bile acid detoxification, explaining increased levels of hydroxylated, sulfated, and glucuronidated bile acid metabolites in these patients.

Repression of bile acid synthesis is another important part of the adaptive response to cholestasis. CYP7A1, which mediates the rate-limiting step in bile acid synthesis, is negatively regulated in cholestasis by bile acids and by other bile acid-independent mechanisms (Fig. 3C) [167]. In studies of late-stage PBC patients, as well as those involving in vitro experiments and animal models, CYP7A1 expression was markedly reduced [110].

In summary, these adaptive alterations in transporter and phase I and II enzyme expression explain many biochemical hallmarks in cholestasis (eg, elevated serum and urinary bile acid levels; the appearance of hydroxylated, sulphated, and glucuronidated bile acid metabolites; elevated conjugated bilirubin) as well as many clinical hallmarks in cholestasis (eg, jaundice, bilirubinuria, potentially pruritus). However, these adaptive changes are not sufficient to fully prevent cholestatic liver injury.

### **Regulation of genes involved in hepatobiliary transport and metabolism in cholestasis**

Because most bile acids are cytotoxic and their accumulation can lead to liver injury, coordinated regulation of bile acid transport, metabolism, and synthesis is essential to maintain bile acid homeostasis. Bile acid homeostasis is the result of coordinated feedback and feed-forward regulation of genes involved in bile acid synthesis, detoxification, and transport. Liver-enriched transcription factors (eg, hepatocyte nuclear factors) and nuclear receptors play key roles in the transcriptional regulation of hepatobiliary transport systems and of enzymes involved in bile acid metabolism [113,168]. Nuclear receptors are activated by various biliary compounds retained during cholestasis (ie, bile acids, bilirubin). After binding their ligands, nuclear receptors undergo a conformational change and allow binding to their specific response elements in the promoters of their target genes, thus activating transcriptional activity [169].

Three bile acid-activated nuclear receptors have been identified. One is the farnesoid X receptor (FXR/*NR1H4*), which was the first bile acid receptor to be identified and is activated by a variety of primary and secondary bile acids. The other two are the pregnane X receptor (PXR/*NR1I2*) and the vitamin D receptor (VDR/*NR1I3*), which are activated by hydrophobic lithocholic acid and by their natural ligands (xenobiotics, including

rifampicin, phenobarbital, dexamethasone, and statins in the case of PXR, and  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> in the case of VDR). The constitutive androstane receptor (CAR/*NR1I3*) is activated by xenobiotics and bilirubin. Thus, binding of biliary constituents (eg, bile acids, bilirubin), lipid metabolites (eg, oxysterols), and xenobiotics (eg, drugs) to nuclear receptors facilitates the positive feed-forward and negative-feedback regulation of hepatic transport and the metabolism of these compounds under physiologic and pathologic conditions [112,168]. Endobiotic and xenobiotic compounds share hepatic transport and metabolic systems and their regulatory nuclear receptors. This “crosstalk” at the nuclear receptor level may therefore have important implications for the therapeutic modulation of bile acid transport and metabolism.

The observed changes in transporter expression in cholestasis are not only effects of retained biliary constituents but are also the result of a combination of many factors influencing nuclear receptor activity, depending on the type of cholestatic injury, including those from proinflammatory cytokines, hormones, and drugs (Fig. 4). In many cholestatic disorders (eg, obstructive cholestasis, some forms of drug-induced cholestasis, vanishing bile duct syndromes, “cholestatic hepatitis”), not only do retained bile acids and other cholephiles cause deregulation of transporter genes, but so do inflammatory cytokines. The detailed transcriptional mechanisms involved in the regulation of bile acid transporters and metabolizing enzymes have recently been reviewed elsewhere [113,168,170].

### **Cytoskeletal and other hepatocellular changes**

In addition to transporter changes, other hepatocellular alterations may interfere with bile secretion (Fig. 5). As such, cholestasis is associated with profound alterations of the cytoskeleton of hepatocytes, including disruption of the microtubular system, disturbance to the actin microfilament network, alterations in tight junctions, and increases in cytokeratin intermediate filaments [171]. Again, most of these cytoskeletal changes are secondary and nonspecific phenomena [1]. Alterations in actin microfilaments (caused by such drugs as chlorpromazine) impair bile canalicular contractility and contribute to cholestasis [172,173]. Reduced canalicular motility is also observed in response to pro-inflammatory mediators, such as nitric oxide, which could contribute to cholestasis of sepsis [174]. Tethering proteins, such as radixin, crosslink actin filaments to integral membrane proteins. Radixin is required for maintenance of structure of the bile canaliculus and for the polarized targeting and retention of canalicular transport systems [175,176]. Radixin is reduced and disrupted in late-stage PBC and in various other cholestatic diseases, which might cause altered localization of canalicular transporters, such as MRP2 [177,178].

Drugs and toxins (eg, colchicin) that disrupt microtubules may cause cholestasis through impaired vesicular trafficking of transport proteins to

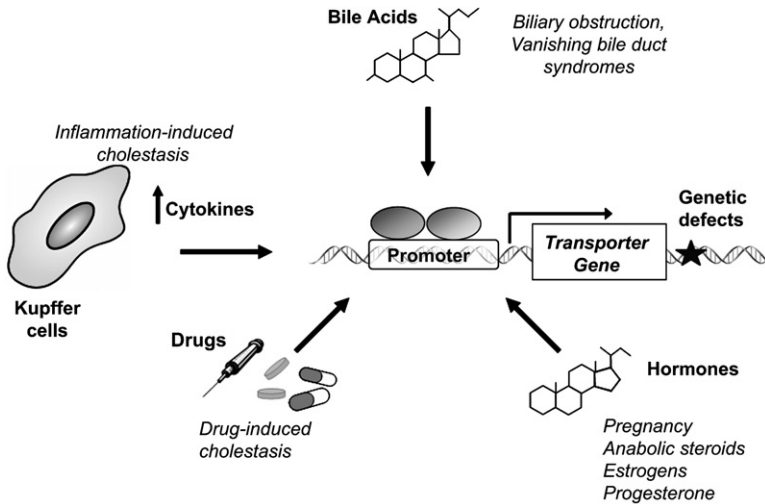


Fig. 4. Transcriptional regulation of hepatobiliary transporter systems in cholestasis. Many alterations in bile acid transport and metabolism in cholestasis are mediated at a transcriptional level. Accumulating bile acids, drugs, and hormones can act as nuclear receptor ligands, thus modifying the expression of respective target genes. Depending on the cause of cholestasis, effects of cytokines, bile acids, hormones, or drugs may dominate the picture. In everyday clinical practice, however, more than one of these mechanisms may alter transporter gene expression (eg, bile acid accumulation in obstructive cholestasis and cytokine induction due to concomitant cholangitis).

the canalicular membrane [1,2]. High concentrations of hydrophobic bile acids impair the function of microtubular motors (eg, kinesin) [179], suggesting that impaired vesicular targeting may be a universal finding as a result of bile acid accumulation in cholestasis. Furthermore, rapid retrieval of transporters from the canalicular membrane in response to cholestatic injury (eg, treatment with lipopolysaccharide and sex hormones, bile acid challenge, hyperosmolarity) may also contribute to cholestasis [1,86]. Finally, transporter gene mutations may also result in mistargeting of transport proteins with retention in the endoplasmatic reticulum and Golgi apparatus due to alterations in critical sorting sequences [180].

Disruption of tight junctions as observed in PBC and PSC results in increased paracellular permeability, regurgitation of biliary constituents into plasma, and reduction of the osmotic gradients in the bile canaliculi that normally constitute the driving force for bile secretion [111]. In addition, mutations in tight junction protein genes can cause cholestasis (see Table 1). Missense mutations in tight junction protein 2 have been identified in patients with familial hypercholanemia, an oligogenic disease requiring a mutation in a second gene (ie, bile acid amino acid transferase) for clinical manifestations [181]. Furthermore, mutations of claudin-1, which forms the backbone of tight junctions with occludin and junctional adhesion molecules, causes neonatal sclerosing cholangitis associated with ichthyosis [182].



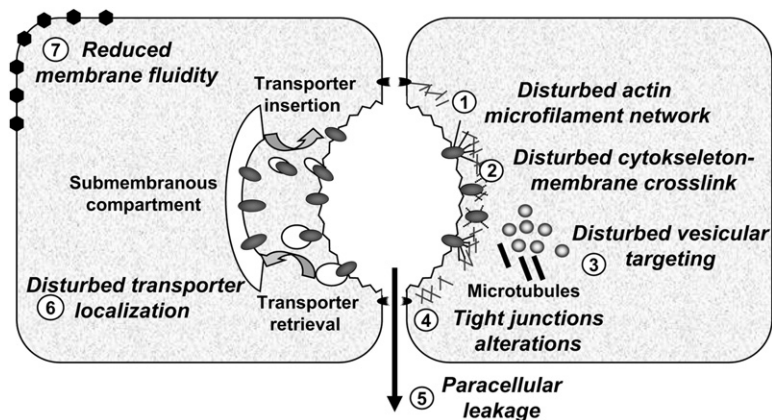


Fig. 5. Cytoskeletal and other hepatocellular changes in cholestasis. Cholestasis is associated with profound alterations of the cytoskeleton of hepatocytes. (1) Disruption of the actin microfilament network reduces contraction of the bile canaliculi. (2) Disturbed microfilament-membrane cross-linkers (eg, radixin) lead to impaired canalicular transporter localization. (3) Disruption of the microtubular system impairs transcellular transport processes, including vesicular targeting of transport proteins to the membrane. (4) Alterations of tight junctions result in (5) increased paracellular permeability, regurgitation of biliary constituents into plasma, and reduction of the osmotic gradients in the bile canaliculi that normally constitute the driving force for bile secretion. Transporter retrieval from the canalicular membrane to a submembranous compartment and reinsertion into the membrane is critical for rapid regulation of hepatocellular transport capacity. (6) Disturbed canalicular transporter localization due to reduced targeting of transporters to and enhanced retrieval of transporters from the canalicular membrane also results in reduced canalicular export capacity and may, thus, contribute to cholestasis. (7) Reduced basolateral membrane fluidity in response to drugs and hormones may be associated with reduced basolateral transporter function.

Last, but not least, alterations in membrane lipid composition and fluidity caused by hormones and drugs result in altered function of embedded enzymes, transporters, and ion channels and thus may contribute to cholestasis under these conditions (see above). Melum and colleagues [183] have demonstrated a role for hereditary *MDR3* variants (in epistatic interaction with polymorphisms of the *OST- $\alpha$*  gene) as disease modifying and susceptibility gene in primary sclerosing cholangitis, further supporting the concept that genetic transporter variants may contribute to the pathogenesis of sclerosing cholangitis in humans.

## Summary

The mechanisms leading to cholestasis are complex. Recent studies providing insights into the pathogenesis of hereditary cholestasis syndromes and of various acquired cholestatic liver diseases have increased our understanding of the molecular and hepatocellular mechanisms leading to cholestasis. While some of these changes result in reduced transporter function,

most of the observed changes are consequences but not causes of cholestasis. These secondary alterations in transporter (and also in detoxification enzymes) expression are aimed at protecting the hepatocyte from accumulating cytotoxic bile acids. Understanding the molecular mechanisms both leading to and counteracting cholestatic diseases is the prerequisite for a better understanding of the pathophysiology of these disorders and for development of novel therapeutic approaches.

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