Review

Molecular mechanisms of cholestasis: causes and consequences of impaired bile formation

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Abbreviations: ABC, ATP-binding cassette; BSDF, bile salt-dependent bile flow; BSIDF, bile salt-independent bile flow; BSP, bromosulphthalein; BRIC, benign recurrent intrahepatic cholestasis; CYP7A, gene encoding cholesterol 7α-hydroxylase; CYP27, gene encoding sterol 27-hydroxylase; DBSP, dibromosulphthalein; EE, ethinylestradiol; EHBR, mrp2-deficient Sprague-Dawley rat; γGT, gamma-glutamyltransferase; GY/TR′, Groningen Yellow, mrp2-deficient Wistar rat; IBST/ibst, ileal sodium-dependent bile salt transporter; ICP, intrahepatic cholestasis of pregnancy; LPS, lipopolysaccharide (endotoxin); MRP/mrp, multidrug resistance protein, encoded by MRP/mrp genes 1–6; NTCP/ntcp, Na+-taurocholate co-transporting polypeptide; OATP/oatp, organic anion transporting polypeptide; PFIC, progressive familial intrahepatic cholestasis; Pgp, P-glycoprotein, encoded by MDR/mdr genes 1–3; SPGP/spgp, sister of P-glycoprotein; TPN, total parenteral nutrition. Transporters indicated by capitals refer to human proteins, lowercase letters to animal proteins. Italic lettering refers to gene, upright lettering to protein

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1. Introduction

Hepatic bile formation and bile flow serve many important physiological functions. In the first place, cholesterol is excreted from the body almost exclusively via bile, either as the free compound or after conversion to bile salts. Therefore, bile formation is just as essential for maintaining cholesterol homeostasis as dietary intake and synthesis of the sterol, even though the latter processes have received much more attention. Secondly, biliary bile salts, in addition to being end-products of cholesterol catabolism, are essential for efficient biliary cholesterol secretion and for the solubilization of dietary fats in the intestine. Bile flow also has important toxicological and pharmacological ramifications. A large number of 'waste products' of endogenous origin and xenobiotics are secreted into the bile, often after preceding oxidative or conjugative metabolism by hepatic phase I and phase II detoxifying systems, respectively. For instance, the majority of bilirubin is excreted in the form of its diglucuronide by the biliary route, and disturbance of any step in this process results in hyperbilirubinemia (jaundice) and the associated clinical manifestations. Heavy metals may serve as an example of toxic xenobiotics excreted into bile.

The biochemistry of bile formation has been focus of research in numerous laboratories, due both to its intrinsic mechanistic interest, relevant to fundamental principles of bioenergetics and membrane biochemistry, and to the serious clinical consequences resulting from disturbances of the process, i.e., cholestasis. Cholestasis, functionally defined as a cessation or impairment of bile flow, can lead to nutritional problems related to malabsorption of dietary fats and fat-soluble vitamins as well as to (irreversible) liver damage caused by accumulation of toxic compounds. Cholestasis is a frequently observed clinical condition; treatment, however, is often hampered by insufficient knowledge of underlying causes and difficulties in distinguishing the primary events from secondary consequences. On a mechanistic basis, cholestasis usually is divided into ‘extrahepatic’ and ‘intrahepatic’ forms. The first refers to obstruction of large bile ducts outside the liver, for instance due to gallstones. Bile duct ligation in rodents serves as an experimental model for this form of cholestasis. The causes of intrahepatic cholestasis lie within the liver, either at the level of the liver parenchymal cells, also known as hepatocellular cholestasis, or within the canaliculi/intrahepatic bile ductules (cholangioles) and/or portal ducts. Experimental models exist for both, e.g., 17α-ethinylestradiol cholestasis [1] for the first and sulfated glycolithocholate cholestasis for the second form [2].

Generation of bile flow is an osmotic process driven by ongoing active secretion of solutes into the minute bile canalicular space between adjacent liver parenchymal cells (hepatocytes), that are sealed from blood by tight junctions, followed by passive influx of water and electrolytes by trans- or paracellular pathways. Conceptually, the liver can therefore be considered equivalent to a secretory epithelium, with the canalicular membrane corresponding to the apical membrane in a typical epithelium. Bile salts, in quantitative terms the major organic constituents of bile, are responsible for generation of the main portion of bile flow in most species, including humans. This portion has been termed the bile salt-dependent fraction of bile flow (BSDF); the remainder, the bile salt-independent fraction (BSIDF), comprises the combined action of all other osmotically active bile constituents. Recent data indicate that glutathione is an important contributor to this fraction, at least in the rat [3,4] (Fig. 1).

In the past couple of years it has become clear that the cells lining the bile ducts, the cholangiocytes, significantly contribute to bile formation by (secretin-stimulated) secretion of HCO$_3^-$ and Cl$^-$. It has been estimated that cholangiocyte excretion accounts for up to 40% of human bile flow [5,6]. In addition, the intrahepatic bile ducts represent an important target of injury in several disease states, such as primary biliary cirrhosis, sclerosing cholangitis and liver transplant rejection. For an extensive overview of
the role of cholangiocytes in bile formation and cholestasis the reader is referred to recent review articles [7,8].

Bile salts are maintained in a so-called enterohepatic circulation, implying that, after being expelled into the intestinal lumen, these compounds are effectively reabsorbed and transported back to the liver via the portal system for uptake and re-secretion into the bile. Thus, in this case, as well as in the case of biliary secretion of other blood-bound compounds, three consecutive transport events occur and have to be considered: sinusoidal uptake, intracellular transport and canalicular secretion. Kinetic analyses have revealed that, in most cases, the canalicular secretion step is rate-limiting for the overall process. Therefore, canalicular transport events are thought to constitute the molecular basis for bile production. In the past few years, a number of important canalicular transport systems have been identified and characterized (summarized in Table 1). Most canalicular transport systems involved in bile formation are members of the ATP-binding cassette (ABC) transporter superfamily [9–12]. Relevant transporters identified so far belong either to the P-glycoprotein (Pgp) or the multidrug resistance protein (MRP) clusters of this superfamily [9,11]. It is now recognized that absence or malfunction of specific transporters due to mutations in their encoding genes underlie specific, inherited forms of cholestatic liver disease. On the other hand, the regulation of these systems and their dysregulation as an underlying cause of disturbed bile formation is, at the moment, only marginally understood.

The aim of this review is to give a short overview of inborn errors and other conditions in humans leading to disturbed hepatobiliary transport and cholestasis. In combination with observations in experimental models related to these clinical states, these will be integrated to define the current state of knowledge about primary and secondary events in cholestasis.

2. Inborn errors of hepatobiliary secretion

In the past couple of years considerable progress

![Graph](image)

Fig. 1. Relationship between bile flow and biliary bile salt secretion. Rats were equipped with permanent catheters in bile duct and duodenum and both catheters were immediately connected to each other to maintain the enterohepatic circulation. Eight days after surgery the enterohepatic circulation was interrupted and bile was collected in 30-min fractions, in both normal Wistar and mutant GY/TR<sup>−</sup> Wistar rats. The bile flow at the hypothetical zero value of bile salt secretion represents the bile salt independent fraction of bile flow. This BSIDF is decreased in GY/TR<sup>−</sup> rats, in part caused by reduced biliary GSH secretion in these mutant rats.

<table>
<thead>
<tr>
<th>Name&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Species</th>
<th>Localization&lt;sup&gt;b&lt;/sup&gt;</th>
<th>M&lt;sub&gt;r&lt;/sub&gt;</th>
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Adapted from Ref. [9].

<sup>a</sup>See text for full name.

has been made in characterization and cloning of hepatic transporters involved in bile formation, and mutations affecting their transport activity have been identified. This knowledge has significantly contributed to our understanding of inherited cholestatic syndromes and provides the tools for detailed analysis of regulation of transport as well as for development of diagnostic procedures.

2.1. Bile salt transport

Efficient translocation of bile salts at the canalicular membrane is crucial in bile formation and maintenance of the BSDF. Processes involved in this important function of bile salts include their translocation through the hepatocyte, secretion into the bile canalculus, reabsorption in the intestine and uptake from the portal blood at the sinusoidal membrane followed by resecretion into bile. In addition, hepatic bile salt synthesis is required to compensate for the small loss of bile salts via the feces to maintain an adequate bile salt pool size. Any disturbance in this complex sequence of events may underlie changes in BSDF.

2.1.1. Uptake systems

Uptake of bile salts by the hepatocytes is mediated by the Na\textsuperscript{+}-taurocholate cotransporting protein (ntcp) localized exclusively at the sinusoidal membrane [13,14]. The ntcp gene contains a sequence identical to a bile salt responsive element present in the gene encoding cholesterol 7α-hydroxylase (CYP7A) [15,16], the rate-limiting enzyme in the bile salt synthesis, suggesting ntcp down-regulation by bile salts similar to the situation observed for CYP7A. Yet, variations in hepatic bile salt flux ranging from 0% to 300% of normal do not alter activity, protein levels or mRNA levels of ntcp in rats [17], indicating that this protein is constitutively expressed under non-cholestatic conditions. The uniform zonal distribution of ntcp [18] supports this view, since bile salts are normally transported mainly by perportal hepatocytes. On the other hand, ntcp is rapidly down-regulated at transcriptional and posttranscriptional level in experimental models of cholestasis, such as bile duct ligation [19], endotoxin administration [20–22] and ethinylestradiol treatment [23]. This down-regulation has been ascribed to accumulation of bile salts in the hepatocytes: in fact, Gartung et al. [24] were able to demonstrate a significant negative correlation between plasma bile salt levels, presumably reflecting intracellular concentrations, and hepatic ntcp expression in rats. Similarly, ntcp is also rapidly down-regulated in cultured hepatocytes [25] and ntcp protein is virtually absent in hepatoma cell lines [25,26], i.e., cells experiencing dedifferentiation. Whether this down-regulation is due to bile salts that accumulate in media of the cultured cells or to the dedifferentiation-related phenomena remains to be established. While high intracellular bile salt levels may be important for regulation of ntcp under cholestatic conditions, recent data indicate that other factors are also involved. We were able to show that ethinylestradiol (EE) cholestasis also leads to a strong reduction of ntcp levels in livers of long-term bile diverted rats (Fig. 2). Since in these rats the bile salt pool is depleted and plasma bile salts remain...
below detectable levels, it is highly unlikely that this
effect is mediated by bile salts. Rather, it is likely that
the estrogen itself or some factor related to the oc-
currence of cholestasis per se is involved. The con-
sequences of absent or impaired ntcp activity is not
yet clear. So far, functional mutations in NTCP/ntcp
have not been reported and the generation of ntcp-
deficient animals is eagerly awaited for. It should be
noted that down-regulation of ntcp in EE-treated rats
[23] and in ferrochelatase-deficient mice (unpublished
results) is not associated with impaired biliary bile
salt secretion. This may be due to overcapacity of
the ntcp system or, alternatively, to the presence of
other systems capable of bile salt uptake. Data avail-
able so far suggest that down-regulation of ntcp is a
secondary effect of cholestasis that may be aimed at
protection of the hepatocyte against the toxic actions
of high intracellular bile salt concentrations. In this
respect it is important to note that reconstitution of
bile salt transport by transfection of cDNA encoding
ntcp under the control of a cytomegalus virus in the
MC Arde RH-7777 cell line, that normally does not
express ntcp, leads to profound structural changes
and mitochondrial abnormalities when cells were in-
cubated with physiological concentrations of bile
salts [27].

Down-regulation during extrahepatic cholestasis in
rats of the organic anion transporting polypeptide-1
(oatp1), a sodium-independent uptake system that
also accommodates bile salts, may contribute to a
decreased uptake of potential toxic bile salts in this
situation [28]. Alternatively, increased OATP mRNA
in livers of patients with chronic cholestatic liver dis-
ease and up-regulation of OATP expression in trans-
fected HepG2 cells by low concentrations of tai-
urocholate suggest that OATP may serve to enhance
sinusoidal e¡ux of toxic compounds during choles-
tasis [29], as OATP has been shown to function bi-
directionally as an anion exchanger [30].

Another important step in the maintenance of the
enterohepatic circulation of bile salts involves their
uptake from the intestine. While unconjugated bile
salt can be reabsorbed passively to a certain extent,
uptake of conjugated species is mediated by the ileal
sodium-dependent bile salt transporter (IBST/ibst)
[31,32]. In contrast to ntcp [17], ibst appears to be
regulated by physiological bile salt flux through the
intestinal cells [33]. Patients with mutations in IBST
that affect function have been described. These pa-
tients suffer from diarrhea, steatorrhea and bile salt
malabsorption, and, as a consequence of interruption
of the enterohepatic circulation, they are thought to
have a decreased bile salt pool size [34]. Two mis-
sense mutations have been identified in one allele of
the IBST gene (also termed SLC10A2) at two con-
served amino acid positions in a family with primary
bile salt malabsorption. These mutations did not re-
sult in altered expression of the protein. Kinetic stud-
ies, however, revealed that transport of bile salts was
defective when the mutant protein was transfected
into COS cells [34]. Interestingly, ibst has also been
found in apical membranes of rat cholangiocytes, the
cells lining the hepatic bile ducts [35,36]. The phys-
iological functions of the protein at this location re-
main to be revealed. It has been suggested that its
presence enables an intrahepatic short-circuit for bili-
ary bile salts, reminiscent of the cholehepatic shunt
proposed by Hoffman and coworkers [37,38] for un-
conjugated bile salts. A major goal in the near future
will be to establish the role of IBST in liver function
during cholestasis, a condition frequently associated
with marked bile duct proliferation.

2.1.2. Secretory systems

Functional studies in the early 1990s revealed that
there are two ATP-dependent systems involved in
bile salt transport localized in the canalicular mem-
brane [9]. The first, recently identi¢ed as MRP2/mrp2
[39–41], accommodates bivalent, i.e., sulfated and
glucuronidated bile salts, that normally comprise
only a small fraction of biliary bile salts. The other
system accommodates monovalent, amino acid con-
jugated bile salts, for experimental purposes usually
exemplified by taurocholate. The nature of this latter
transporter, having a Kₘ in the low micromolar con-
centration range, has not been clarified with cer-
tainty. It has been claimed, based on in vitro work,
that the canalicular ecto-ATPase or C-CAM 105
could ful¢ll this function [42]. However, strong argu-
ments against this possibility have been put forward
[43]. Very recently, it has been claimed that a novel
member of the Pgp family, originally cloned by
Childs et al. [44], may actually be ‘the’ bile salt trans-
porter. This protein, called sister of P-glycoprotein
(SPGP/spgp), is highly abundant in rat liver and ex-
clusive present at the canalicular membrane [45,46].
Most importantly, it has been shown in spgp-cRNA injected oocytes and in membranes isolated from Sf9 insect cells infected with a recombinant baculovirus containing spgp-cDNA, that spgp transports taurocholate in an ATP-dependent fashion. Kinetic analysis revealed that in these systems this taurocholate uptake is saturable with a $K_m$ value similar to that obtained in isolated canalicular membrane vesicles isolated from rat liver [46]. Therefore, data available so far strongly indicate that spgp is the canalicular bile salt transporter. At this point, genetic studies in human cholestatic syndromes have recently provided important clues about the nature of bile salt transporting systems.

2.1.3. Progressive familial intrahepatic cholestasis (PFIC)

From genetic studies it is now clear that PFIC, a heterogeneous group of autosomal recessive cholestatic disorders characterized by severe fibrosis, cirrhosis and hepatitis progressing to liver failure before adulthood [47–52], consists of at least three different disorders with completely separated underlying causes. This insight has led to the proposal to distinguish between PFIC-1, -2 and -3 [53]. PFIC-1 and -2 appear to be associated with impaired bile salt transport capacity and are characterized by low $\gamma$GT values in serum [51,52]. In contrast, the defect in PFIC-3, characterized by high $\gamma$GT levels in serum, has been pinpointed to defective phospholipid transport into bile (see below).

The first patients identified with PFIC-1 are descendants of Jacob Byler, an Amish settler from Pennsylvania; hence the ‘old’ name Byler disease [47]. Positional cloning studies have located the genetic defect of this disease to chromosome 18q21–q22 [54]. Interestingly, another type of inherited cholestatic disease, benign recurrent intrahepatic cholestasis (BRIC), was mapped to the same region of chromosome 18 [55]. In contrast to the situation in PFIC-1, BRIC is characterized by recurrent attacks of cholestasis separated by symptom-free periods that may last from months to several years [56–58]. In addition, increased fecal bile salt loss due to intestinal malabsorption in non-symptomatic BRIC patients [61] is compatible with a function of the protein in bile salt transport in the intestine.

Patients with almost identical clinical presentation, but unrelated to the Byler family have been referred to as Byler syndrome. In a number of these patients, the defect has also been mapped to chromosome 18, suggesting a similar etiology of the disease [62,63]. Yet, Arnell et al. [64], who studied eight Swedish families with PFIC, excluded linkage to 18q21–22, providing evidence for genetic heterogeneity in this disorder. Strautnieks et al. [53,65] studied six Middle East kindreds by homozygosity mapping and conventional linkage analysis, and mapped a locus for this subgroup to chromosome 2q24. As SPGP has recently been mapped to chromosome 2, it is attractive to speculate that the SPGP gene is affected in these patients with PFIC, now referred to as PFIC-2 and that defective canalicular bile salt transport underlies this disease [66].

2.1.4. Inborn errors of bile salt synthesis

Disturbed de novo bile salt synthesis from cholesterol may contribute to decreased bile formation. A cascade of enzymatic conversions of the cholesterol backbone leads via different pathways to the primary bile salts cholate and chenodeoxycholate [67,68]. An incomplete conversion by improperly functioning enzymes in this route can result in the formation of toxic bile salt intermediates or bile salt metabolites that interfere with transport processes and, as a consequence, to cholestasis. At least five inborn errors of the bile salt biosynthetic pathways have been described [69,70].

The Smitz-Lemli-Opitz syndrome is characterized by extremely low levels of cholesterol in the circula-
tion and very high concentrations of its precursor 7-dehydrocholesterol [71,72]. These patients share a mutation in the gene encoding 7-dehydrocholesterol 7α-reductase, the last enzyme in the cholesterol biosynthetic pathway. Drastically reduced bile salt secretion in feces have been reported [71], indicating reduced bile salt synthesis. Although not directly measured to the best of our knowledge, it is highly likely that BSDF is impaired in these patients.

Progressive liver disease and cholestatic jaundice are seen in patients with a defect in 3β-hydroxysteroid Δ5-dehydrogenase/isomerase [73–75]. The di- and trihydroxy cholanolic acids and their sulfates that accumulate are unable to generate BSDF and inhibit biliary secretion of other compounds. It was shown that these atypical bile salts act as cholestatic agents by inhibiting the ATP-dependent transport system for bile salts [76]. Deficiency for 3-oxo-Δ4-steroid 5β-reductase leads to accumulation of 3-oxo-Δ4-bile salts that are extremely hepatotoxic. These unusual bile salts were also shown to inhibit ATP-dependent bile salt transport [76]. This, together with decreased amounts of primary bile salts that are formed result in a decreased BSDF [77,78]. The gene encoding the latter enzyme has been cloned [79] and several mutations have been identified [70,79].

Cerebrotendinous xanthomatosis is a rare lipid storage disease [80] associated with disturbed formation of bile salts, especially of chenodeoxycholate. Increased concentrations of so-called bile alcohols are found in these patients [69,81]. This disease is caused by mutations in the CYP27 gene, encoding the mitochondrial enzyme sterol 27-hydroxylase, involved in the formation of chenodeoxycholate via both the neutral and acidic pathways of bile salt synthesis. Several mutations in this gene have been identified so far [82–85]. Finally, peroxisomal disorders are characterized by accumulation of dihydroxy and trihydroxy coprostanolic acids and their metabolites [86]. Effects of these compounds on bile formation per se have not been reported.

In general, treatment with primary bile salts or with ursodeoxycholic acid may be beneficial in these patients, by improving the formation of BSDF and in case of primary bile salts, by suppressing synthesis of the atypical bile salts [87]. Yet, in the case of a primary defect in bile salt transport, bile salt therapy is contraindicated by definition.

### 2.2. Phospholipid translocation

Secretion of phospholipids into bile is essential for efficient removal of the cholesterol in bile [88] and for protection of cells lining the bile ductuli and ducts against the actions of free (hydrophobic) bile salts. Biliary phospholipids consist mainly of phosphatidylcholine with bile-specific acyl-chain configuration [89]. When phospholipid secretion is impaired, bile salts may disrupt membrane structures and membrane-bound enzymes appear in the circulation. Damage of epithelial cells leads to increased levels of γGT in the serum [90]. The mdr2 Pgp localized in the canalicular membrane acts as an ATP-dependent phosphatidylcholine translocator or ‘flipase’ [91,92]. Mdr2 knockout mice have been generated [88]: these animals are unable to secrete phospholipids into bile. As a consequence, biliary cholesterol secretion is also severely impaired. However, bile flow and biliary bile salt secretion are not affected. Consequently, these mice are not cholestatic when the condition is defined on the basis of bile flow alone [88]. Serum bile salt levels are increased in these animals [88], probably reflecting down-regulation of ntcp [93]. The toxic action of bile salts at the level of the bile ducts leads to development of characteristic pathology, including bile duct proliferation and fibrosis. The severity of liver pathology could be influenced by modulation of the hydrophobicity of the bile salt pool in these mice. Ursodeoxycholate largely prevented progression of disease while cholate, a relative hydrophobic species, aggravated liver pathology [94].

MDR3 is the human homologue of the rodent mdr2 [95]. Recently, inherited cholestatic liver disease in humans has been associated with MDR3 malfunction [96–98]. This subtype of PFIC, proposed to be termed PFIC-3, is characterized by progressive cholestatic liver disease with features similar as described for the mdr2 Pgp-deficient mice associated with elevated γGT levels in serum [97]. In a first paper a patient was described in which the MDR3 mRNA could not be detected in biopsy material [97]. More recently, analysis of genomic DNA encoding MDR3 in two PFIC patients with high γGT revealed respec-
atively a homozygous 7 bp deletion resulting in a frame shift, a premature stop codon and a nonsense mutation leading to a stop codon. Resulting truncated proteins may be rapidly degraded leading to absence of immunoreactive protein in the liver [99].

2.3. Organic anion transport

Secretion of organic anions into the canalicular lumen is mediated by an ATP-dependent organic anion transporting protein known as cmoat or mrp2. The human and rat genes encoding this transporter have been cloned [39,100]. Rat strains lacking mrp2 (GY/TR\(^{-}\) and EHBR) have generated insight into the function of this protein [101]. These mutant rats show defective secretion of a broad range of organic anions including bilirubin ditaurate [101], glutathione S-conjugates [102–105], reduced glutathione [105], glucuronidated and sulfated bile salts [106], and a variety of xenobiotics [102,107,108]. Despite the absence of the mrp2, some organic anion transport activity is maintained in these rats, suggesting the presence of alternative pathways for secretion. Bilirubin-diglucuronide, for example, is secreted at virtually normal rates in these mutant rats albeit in the face of elevated serum and hepatic bilirubin levels [101]. This may be due to the presence of an electrionic transporter for bilirubin diglucuronide [109]. Other members of the ABC superfamily [110] and more specifically the mrp family [111] whose functions are not yet known, may contribute and/or compensate. Finally, a possible additional hepatic transporter has been partially characterized in functional terms but not yet sequenced [112,113].

An assessment of its relationship to known transporters and its physiological role will have to await cloning.

The nature of the mutation in mrp2 which inherits an autosomal recessive fashion has first been analyzed in GY/TR\(^{-}\) rats by sequencing of the mutated mrp2 after PCR amplification of its mRNA [39]. A single base deletion was observed in the coding region, leading to an early stop codon and to formation of a truncated protein. In EHBR rats with a Sprague–Dawley background, showing an identical phenotype as Wistar GY/TR\(^{-}\) rats, another deletion was recently reported [100]. The decrease in bile flow in these mutant rats is thought to be caused by the decreased secretion of GSH into the bile [102]. In contrast to older reports [114], it was recently shown that mrp2 does mediate transport of reduced glutathione in rats [105].

The recently cloned human MRP2 [41,115] is the homologue of the canalicular mrp2 of rat liver. In the human counterpart of the GY/TR\(^{-}\) rat, i.e., the Dubin Johnson patient, MRP2 is lacking [41]. Recently, mutations in MRP2 resulting in production of a truncated MRP2-protein in Dubin Johnson patients have been identified [40]. Dubin Johnson patients show a mild conjugated hyperbilirubinemia, but like the mutant rat, do not have severe liver disease. Secretion of various organic anions has been found to be impaired in these patients [116].

2.3.1. Inborn errors in bilirubin conjugation

To allow efficient transport of bilirubin across the canalicular membrane, glucuronidation of this poorly soluble compound is necessary. The glucuronidation process is mediated by the bilirubin–UDP-glucuronosyl transferase (B-UGT) [117,118]. Defective glucuronidation leads to accumulation of unconjugated bilirubin in the liver and blood, manifesting as jaundice. The circulating high concentrations of unconjugated bilirubin in these patients may cause neurological damage. Two diseases with defects in bilirubin conjugation are known. Crigler–Najjar type I patients lack the B-UGT caused by mutations in the conserved region of the gene [119]. Milder forms of this disease, type II, are characterized by impaired activity of the enzyme. For both subtypes, several mutations are known [117]. Patients suffering Gilbert syndrome are homozygous for one additional A in the TATAA sequence of the promoter region of the B-UGT gene. As a result, the gene is less efficiently transcribed, leading to a decreased expression of the enzyme and mild hyperbilirubinemia [120].

The rat homologue for the Crigler Najjar syndrome is the Gunn rat [121] that has a frame shift mutation leading to the formation of a truncated, inactive protein that is rapidly degraded [122].

3. Clinical conditions associated with cholestasis

In addition to inborn errors causing cholestatic liver disease, there is a large number of clinical con-
ditions associated with cholestasis. In this section, some of these common clinical conditions and experimental models hereof are discussed.

3.1. Neonatal cholestasis

After birth, the neonate has to make some important shifts in its excretory pathways. The maternal placenta largely covers the removal of waste products from the fetus by specific transporters in the trophoblasts [123]. After birth, this must be taken over by the newborn’s liver. This change requires a certain adaptive period in which hepatic transport systems have to be adjusted, bile salt synthesis and bile salt pool have to be developed, and hepatic blood flow has to be redirected. Bile salt synthesis and bile salt conjugation in the fetus differ from those in adults as indicated by the atypical bile salts found in meconium and urine of newborns [124–126]. As determined in rodents, BSDF is reduced in the neonatal state [127] and the BSIDF is completely absent [127]. The latter is associated with a complete absence of glutathione in bile [3,128]. This so-called physiological cholestasis usually spontaneously disappears when maturation of hepatic transport systems and bile salt metabolism has taken place. The ontogenetic development of hepatic transport systems has been studied mainly in rats. The oatp-1 mRNA levels appears at day 16 of gestation, remains stable until birth and then gradually increase in time [129]. The ntcp mRNA, however, can first be detected at day 20 of gestation and levels gradually increase in time during further fetal and neonatal life [130]. Recent studies on the developmental patterns of the canalicular organic anion transporter mrp2 and the bile salt transporter spgp showed a similar pattern. At gestation day 18–22, levels of mrp2 and spgp mRNA are about 2% of adult levels, then gradually increase during gestation and the postnatal period. Directly after birth the spgp levels are about 160% of adult levels and decrease to adult levels within weeks [131]. Studies on bile formation during postnatal development shows a gradual increase in both BSDF and BSIDF [127]. This is in agreement with the levels of respective transport proteins. In addition to the fact that the immaturity of biliary transport systems and hepatic metabolism cause physiological jaundice, it also renders the neonate extremely susceptible to various cholestasis-inducing factors, including total parenteral nutrition and sepsis [132,133]. Surprisingly, however, the neonatal rat [134,135] and guinea pig [136,137] are extremely resistant towards the cholestatic actions of the secondary bile salt lithocholate. The underlying mechanism of this protection remains to be elucidated.

3.2. Total parenteral nutrition

Total parenteral nutrition (TPN) is associated with hepatic dysfunction and cholestasis in a considerable number of patients [132,133,138]. Especially in young infants, there is a high prevalence of TPN cholestasis. Immaturity of the biliary system is probably an important factor contributing to hepatic problems associated with TPN in infants. Several mechanisms have been suggested to play a role. Absence of oral food intake and enteral stimulation affects the physiological stimuli for bile flow generation and bile salt secretion [139,140] which, together with the reduced influx of bile salts from the intestine, results in a compromised enterohepatic circulation [133]. The composition of TPN per se has been implicated. Solutions used for TPN contain glucose, amino acids and sometimes triglycerides. Both glucose and amino acids have been shown to decrease bile flow in animal models [141–144]. Non-protein caloric overload has been shown to contribute to the incidence of cholestasis in TPN-treated patients [144–148]. Other studies have implicated amino acids to be an important factor in the induction of cholestasis in humans [132,144]. In one study, the effect of TPN on the development of cholestatic jaundice was tested in 82 infants. The volume of amino acids infused was related with the incidence of cholestasis, with the higher relative amounts of amino acids giving a higher prevalence [145]. Another study pointed to a role for specific amino acids, in particular for methionine, as potential toxic agent [144]. Deficiencies for specific amino acids have also been proposed [132]. Since many of the TPN solutions given are hypertonic, TPN could give rise to cell volume regulatory responses in hepatocytes. For instance, swelling of hepatocytes induced by hypooosmolality of the perfusate has been shown to result in increased transport of bile salts in isolated perfused rat liver [149,150].
while hyperosmotic pressure leads to retrieval of mrp2 into pericanalicular vesicles in cultured hepatocytes [151].

Absence of intestinal stimulation during TPN leads to overgrowth of the small intestine with colonic bacteria. This enhances the intestinal production of lithocholate from chenodeoxycholate. Lithocholate administration leads to cholestasis with specific liver pathology in experimental animals [152,153]. Similarities between TPN and lithocholate-induced histological changes in the liver suggest a relationship between the two entities [154,155]. A role of secondary bile salts in the etiology of TPN cholestasis is supported by studies demonstrating a decreased prevalence of cholestasis in patients treated with antibiotics [156–158]. However, these results could not be confirmed in other studies [159].

### 3.3. Sepsis

Hyperbilirubinemia is frequently observed in septic patients [160–166]. Hepatic handling of the organic anion bromosulphthalein (BSP) is altered in human volunteers injected with endotoxin [167] and in septic patients [166]. In the latter study, canalicular excretion of the dye was found to be decreased. A reduced hepatic BSP clearance was also found in septic animal models [165,168–170]. It was shown that the presence of E. coli endotoxin (LPS) in the perfusate of recirculating perfused rat livers gives rise to an acute reduction of the biliary excretion of BSP by 36%, accompanied by a decrease in both BSIDF and perfusate flow [171]. Decreased glutathione secretion (−86%) and decreased HCO$_3^−$ (−25%) secretion are probably responsible for this decrease in BSIDF [171,172]. Both the basolateral uptake and canalicular secretion of the organic anions BSP and sulfated taurolithocholate, as measured in isolated perfused rat livers and in isolated basolateral and canalicular membrane vesicles from LPS-treated rats, were reduced [20]. In the same study, it was shown that the maximal transport rate of bile salts was decreased by 60–80%. In basolateral membranes isolated from LPS-treated rats, Na$^+$-dependent taurocholate transport was decreased by about 40%. ATP-Dependent bile salt transport measured in canalicular vesicles was decreased to an even greater extent [20,22]. In both cases a decreased $V_{\text{max}}$ was found, indicating a reduction in the number of transporters available. These effects of endotoxemia are probably mediated by tumor necrosis factor α (TNF-α) [22,173]: it was shown that the inhibition of bile salt transport induced by endotoxin is prevented, when rats are pretreated with anti-TNF-α antibodies [173]. In addition LPS, TNF-α and interleukin-1β (IL-1β) were all shown to reduce ntcp mRNA levels [21]. IL-6 did not affect ntcp mRNA, but taurocholate uptake in IL-6 incubated hepatocytes was reduced [21,174]. This indicates that reduced basolateral bile salt uptake in this case is caused by post-translational events. Furthermore, the activity of Na$^+/K^+$-ATPase responsible for maintenance of a proper sodium gradient is decreased by LPS [175], which could contribute to the decreased bile salt uptake at the sinusoidal membrane.

Canalicular excretion of the mrp2 substrate, leukotriene D$_4$ (LTD$_4$) is reduced by 80% in the endotoxemic treated rats [176]. This is also the case for bilirubin glucuronides [177]. Down-regulation of mrp2 activity during endotoxemia appeared to be a gradual process with a maximal inhibition of 66% at 12 h after endotoxin injection followed by a slow recovery during the subsequent 4 to 5 days [178]. Treatment of rats with LPS resulted in a down-regulation of the mrp2 protein and mRNA levels [179]. Protein levels of Pgp were not affected in this study [179]. Furthermore, the inhibition of canalicular organic anion transport during endotoxemia can be counteracted by pretreating the animals with dexamethasone, an established inhibitor of cytokine production [178]. LPS-Induced cytokines are also potent stimulators of systemic and hepatic nitric oxide (NO) production. NO donors stimulate BSIDF in rat liver [180]. In LPS-treated rats NO$_2^-$ and NO$_3^−$ levels were increased many-fold [172]. However, both stimulation of NO synthesis with L-arginine and inhibition of NO production by aminoguanidine did not affect bile flow in LPS-treated rats [172], indicating that NO itself is not involved in disturbed bile formation during endotoxemia. If cytokines are the main mediators of the down-regulation of hepatobiliary transport during sepsis, this may also be of great importance for other situations were cytokines are produced, e.g., to explain impaired hepatobiliary transport in transplanted livers or in liver diseases like hepatitis and primary biliary cirrhosis.
3.4. Cholestasis of pregnancy

Intrahepatic cholestasis of pregnancy (ICP) is characterized by pruritus in the second half of pregnancy, sometimes associated with elevated serum bilirubin and transaminases [181–185]. These symptoms generally disappear within a few hours to days after delivery. A geographic variation, with high incidences in Sweden and Chile, suggest a genetic component to be involved [182,184]. This disease has been related to high perinatal mortality, high incidence of meconium staining, abnormal intrapartum heart rate and preterm deliveries [181,185]. Patients susceptible for ICP often also develop cholestasis during the use of oral contraceptives [182]. The actual mechanism causing ICP, however, has not been revealed yet. Genetic studies provide evidence that it is transmitted as dominant trait which is sex-limited [183]. However, males from families with a history of ICP show a decreased biliary secretion of BSP [186]. This suggests that males can also transmit this trait to their female descendants [186].

The increased frequency of ICP in mothers of patients with PFIC and BRIC [47,52,99,187] suggests that heterozygotes for mutations in transport systems involved in these diseases remain asymptomatic under normal circumstances, but cannot deal with increased pressure on these systems induced by pregnancy-related factors, e.g., elevated estrogen levels. Data available so far therefore suggest that mutations in different canalicular transport proteins and/or regulatory proteins may underlie ICP. Additionally, altered metabolism of estrogens leading to formation of cholestatic metabolites can not be excluded [188]. The observations that slight elevations of serum bilirubin are present in 20% of normal pregnancies and that the secretion of BSP in EE-treated healthy volunteers is decreased, suggest that estrogens may also modulate canalicular secretion under ‘physiological’ circumstances [186, 189].

Administration of EE to rodents is commonly used as a model for estrogen-related cholestasis. EE-treatment of rats mainly affects BSIDF as well as the maximum secretory rate for taurocholate into bile [1,190–192]. Decreased fluidity of the sinusoidal membrane, decreased Na⁺/K⁺-ATPase activity and increased permeability of the tight junctions have been proposed as potential mechanisms [193–196]. More recently, it was shown that Na⁺ dependent sinusoidal transport and ATP-dependent canalicular transport of taurocholate in membrane vesicles isolated from EE-treated rats are decreased [197]. Protein content and mRNA levels of ntcp were clearly decreased by EE [23]. Both in rats and humans the maximal secretion rate of BSP is decreased by EE [179,186,197]. In rats this is associated with decreased protein levels of mrp2 [179]. The mrp2 mRNA levels were not affected in EE-treated rats, indicating posttranscriptional regulation [179]. We recently showed in mrp2-deficient GY/TR⁺⁻⁻ rats that alternative pathways for biliary secretion of organic anions, including DBSP and bilirubin diglucuronide, are also affected by EE, resulting in severe hyperbilirubinemia in GY/TR⁻⁻⁻ rats upon EE treatment. This finding shows that EE exaggerates symptoms in situations in which canalicular transport is already affected [198]. Besides canalicular organic anion transport, this could also be the case for other canalicular transport proteins such as spgp. It was shown that the transport maximum for both taurocholate and the organic anion BSP is decreased in neonatal rats born from mothers that underwent bile duct ligation during pregnancy [199]. This again underlines the fact that special care has to be taken with children when maternal cholestasis occurs during pregnancy.

4. Cholestasis, causes and consequences

The mechanisms that underlie development of cholestasis in patients can be extremely varied. In its most simple form, mechanical obstruction of the biliary tree due to gallstones or hepatic or biliary tumors causes stagnation of bile flow. In the newly identified inborn errors in bile formation, for example in PFIC-1 to -3, dysfunction of a crucial protein underlies impaired bile formation. At the other end of the spectrum, a complex interaction between subtle impairment of transport function, accumulation of substrates and/or their metabolites, damage to hepatocytes and subsequent inflammation may give rise to a vicious cycle leading to cessation of bile flow. In this situation, it is extremely difficult to distinguish primary events from secondary consequences. Yet, this
distinction can be crucial for designing optimal treatment for the patient.

In animal models, a decrease in bile flow is, in general, associated with elevated cholestatic serum markers, altered liver histology and changes in sinusoidal and canalicular transport as determined in vivo and in vitro systems. The regulation of hepatic transport systems in cholestatic states has been and will be a major focus of research. However, a clear distinction between the mechanisms that actually cause cholestasis and the cascade of changes that is the result of cholestasis is also difficult to make in most experimental systems, including the model of bile duct ligation and recently generated knockout mice. For instance, extrahepatic cholestasis induced by bile duct ligation in rats causes bile duct proliferation, mitochondrial dysfunction, disruption of tight junction structure and redistribution of membrane proteins [200–207]. In addition, down-regulation of canalicular ABC transporters and of sinusoidal transporters like ntcp and oatp-1 occurs [19,179] and serum bile salts, bilirubin and transaminases increase. These changes are clearly secondary to induction of cholestasis. A number of these effects are also seen in intrahepatic cholestasis induced by EE. Yet, in this case, down-regulation of ntcp [23] and mrp2 [179] have been implicated in the onset of cholestasis. In our hands, administration of EE (5 mg/kg) for 3 consecutive days caused a 30% decrease in bile flow but did not, or only minimally, affect the serum markers of cholestasis in normal rats. However, even with this treatment schedule we found reduced mrp2 levels in the canalicular membrane and reduced biliary GSH secretion rates. In the same study, we found a very similar decrease in bile flow in GY/TR rats that do not express mrp2 and do not secrete GSH into bile [198]. This example illustrates that a reduction of bile flow can be established without changes serum markers and that down-regulation of transporters are not always the cause of cholestasis but often a consequence. The down-regulation of ntcp in a number of cholestatic models probably provides a protective mechanism for the hepatocytes against intracellular accumulation of bile salts. Reversibly, serum bile salt levels and transaminases are increased and ntcp protein content and activity is decreased in mdr2-knock out mice, while bile flow is actually stimulated in these animals (Koopen et al., unpublished results). An even more striking example hereof is provided by ferrochelatase-deficient mice with deficient heme synthesis [208,209]. In these mice, we recently found an 80-fold increase in serum bile salts, increased transaminases and bilirubin and an almost complete absence of ntcp protein in the liver. Paradoxically, bile flow and biliary bile salt secretion in these animals are not decreased but increased. These findings illustrate very clearly that there may be differences between ‘clinical cholestasis’ diagnosed on the basis of serum values of bile salts, bilirubin and transaminases and ‘actual’ cholestasis, as defined by reduced bile flow. In this particular model, proliferation of bile ducts may modulate bile formation at non-hepatocytic level [7]. Clearly, more research is needed to clarify the relationships between transporter activities, transporter localization (membranous versus vesicular) and the actual process of bile formation.

In conclusion, therefore, the identification and cloning of a number of key proteins involved in biliary transport, and the description of common mutations herein, provide tools to diagnose the primary event in certain types of inherited cholestatic syndromes and allow for development of adequate treatment. For other, acquired types of cholestasis, treatment remains limited to palliative interventions until the real causes are defined.

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References