

## REVIEW

## Genetics of familial intrahepatic cholestasis syndromes

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Bile acids and bile salts have essential functions in the liver and in the small intestine. Their synthesis in the liver provides a metabolic pathway for the catabolism of cholesterol and their detergent properties promote the solubilisation of essential nutrients and vitamins in the small intestine. Inherited conditions that prevent the synthesis of bile acids or their excretion cause cholestasis, or impaired bile flow. These disorders generally lead to severe human liver disease, underscoring the essential role of bile acids in metabolism. Recent advances in the elucidation of gene defects underlying familial cholestasis syndromes has greatly increased knowledge about the process of bile flow. The expression of key proteins involved in bile flow is tightly regulated by transcription factors of the nuclear hormone receptor family, which function as sensors of bile acids and cholesterol. Here we review the genetics of familial cholestasis disorders, the functions of the affected genes in bile flow, and their regulation by bile acids and cholesterol.

During the last decade, many loci underlying intrahepatic cholestasis have been mapped, the genes have been identified, and protein functions have been studied. Taken together with the recent exciting discovery of nuclear hormone receptors that act as bile acid sensors by transcriptional regulation of key enzymes and transporters in bile acid metabolism, these studies have allowed a detailed understanding of the molecular mechanisms and regulation of bile formation. This review summarises recent insights into the genetics of familial intrahepatic cholestasis syndromes and the functions and regulation of the affected proteins. We have divided most familial cholestasis syndromes in classes comprising bile acid synthesis defects and bile salt transport defects because the genetic disorders in these different classes have common aspects in disease outcome and possible treatment, which will be discussed below. We have classified a defect in bile acid conjugation as a synthesis defect and we tentatively consider familial intrahepatic cholestasis type 1 (FIC1) disease as a bile salt transport defect, although the exact mode by which FIC1 disease causes intrahepatic cholestasis is unknown.

Each day, approximately 500 mg of bile acids are synthesised from cholesterol in the adult human liver. Newly synthesised bile acids are conjugated with either glycine or taurine and subsequently secreted into bile and stored in the gallbladder. Biliary secretion of bile salts against a concentration gradient requires the hydrolysis of adenosine triphosphate (ATP) and this process provides the driving force for bile flow. Because of their detergent properties, bile acids are inherently cytotoxic, and hence it is important that intracellular levels of bile acids are tightly regulated. This is largely accomplished by transcriptional regulation of genes encoding proteins involved in bile acid synthesis and transport.

Cholestasis, or impaired bile flow, is one of the most common and devastating manifestations of liver disease. Cholestasis is clinically characterised by elevated plasma concentrations of biliary constituents, resulting in jaundice, malabsorption of fats and fat-soluble vitamins and, in many cases, progressive liver damage. Both acquired and hereditary forms of cholestasis have been described. Familial intrahepatic cholestasis syndromes can be caused by a deficiency either in bile acid synthesis or in the transport of bile salts into bile. Elucidation of the gene defects underlying these hereditary cholestasis syndromes is of critical importance for our understanding of hereditary cholestasis and, consequently, for our knowledge of the process of bile acid synthesis and transport.

## PHYSIOLOGY AND CELL BIOLOGY OF BILE FORMATION

Biliary excretion of different compounds is an important function of the liver. Synthesis and

**Abbreviations:** ABC, ATP-binding cassette; AKR1D1,  $\Delta^4$ -3-oxosteroid-5 $\beta$  reductase; AMACR, 2-methylacetyl-CoA racemase; ASBT, apical sodium-dependent bile salt transporter; ATP, adenosine triphosphate; BAAT, bile acid CoA:amino acid *N*-acyltransferase; BRIC, benign recurrent intrahepatic cholestasis; BSEP, bile salt export pump; C27 3 $\beta$ -HSD, 3 $\beta$ - $\Delta^5$ -C27-hydroxysteroid oxidoreductase; CA, cholic acid; CDCA, chenodeoxycholic acid; CEA, carcinoembryonic antigen; CTX, cerebrotendinous xanthomatosis; DCA, deoxycholic acid; DHCA, dihydroxycholestanic acid; FHCA, familial hypercholelanaemia; FIC1, familial intrahepatic cholestasis type 1; FXR, farnesoid X receptor; GFC, Greenland familial cholestasis; GGT, gamma-glutamyl transpeptidase; GPI, glycosyl phosphatidyl inositol; HSD3B7, 3 $\beta$ - $\Delta^5$ -C27-hydroxysteroid oxidoreductase; IBABP, ileal bile acid binding protein; ICP, intrahepatic cholestasis of pregnancy; LCA, lithocholic acid; LCS, lymphoedema-cholestasis syndrome; LXR $\alpha$ , liver X receptor  $\alpha$ ; mEH, microsomal epoxide hydrolase; NAICC, North American Indian childhood cirrhosis; NTCP, sodium-dependent taurocholate protein; OATP, organic anion transporter; PBAM, primary bile acid malabsorption; PC, phosphatidyl choline; PFIC, progressive familial intrahepatic cholestasis; PXR, pregnane X receptor; SHP, short heterodimeric partner; tASBT, truncated form of ASBT; THCA, trihydroxycholestanic acid; TJP2, tight junction protein 2; UDCA, ursodeoxycholic acid; VDR, vitamin D nuclear receptor

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transport of bile acids are essential for efficient bile flow. Bile acids are synthesised in the liver and stored in the gallbladder. Upon digestion of a meal, bile salts are delivered to the lumen of the small intestine where they act as emulsifiers of dietary lipids, cholesterol, and fat-soluble vitamins. Bile salts are converted by intestinal bacteria, transported from the intestine to the liver via the portal circulation, and subsequently resecreted into bile. Approximately 95% of bile salts are recovered in the gut during each cycle of this enterohepatic circulation, and the 5% that are lost via the faeces are replaced by de novo synthesis in the liver (fig 1A).

In fig 1B, some important transport proteins involved in the enterohepatic circulation of bile salts are depicted schematically. As can be seen from this figure, the enterohepatic circulation of bile salts requires regulated vectorial transport of bile salts in polarised epithelial cells, such as the hepatocyte and the enterocyte. The bile salt export pump (BSEP) mediates secretion of bile salts from the apical domains of hepatocytes into bile. In the intestine, the apical sodium-dependent bile salt transporter (ASBT) is responsible for reabsorption of bile acids at the brush border, while the ileal bile acid binding protein (IBABP) is thought to transport bile acids through the cytoplasm of the enterocytes to the basolateral membrane. A truncated form of ASBT (tASBT) transports bile acids from the enterocytes into the blood. At the basolateral membrane of the hepatocytes, the sodium-dependent taurocholate protein (NTCP) and to a lesser extent the organic anion transporter family (OATP) are needed for the uptake of bile acids. It is incompletely understood how the biliary epithelium, which consists of cholangiocytes, functionally participates in bile salt transport. It is known that ASBT is also expressed at the apical membranes of cholangiocytes. In cultured cholangiocytes, taurocholate is transported from the apical to the basolateral membrane in a sodium dependent fashion. These findings provided indirect evidence for a cholehepatic shunt pathway for bile salts, which is further discussed elsewhere.<sup>1,2</sup>

## BILE ACID SYNTHESIS

Bile acids are formed from cholesterol in the hepatocytes. In humans two bile acids are formed: cholic acid (CA), a trihydroxy-bile acid with hydroxyl groups at C-3, C-7, and C-12; and chenodeoxycholic acid (CDCA), a dihydroxy-bile acid with hydroxyl groups at C-3 and C-7 (fig 2). Bile acid biosynthesis is quite complex and synthesis of the full complement of bile acids requires 17 enzymes, which involves the addition of hydroxyl groups to the ring structure of cholesterol and the oxidation and shortening of the side chain<sup>3</sup> (figs 2 and 3). In order to survive the low pH in the intestine, bile acids are conjugated to glycine or taurine, forming bile salts. The enzymes required for bile acid biosynthesis are located in different cell organelles; the addition of hydroxyl groups mainly occurs in the endoplasmic reticulum, whereas further ring structure modifications are performed in the cytoplasm. Side chain modification and conjugation are mainly performed in the peroxisomes.<sup>3</sup> The hepatocyte, therefore, faces an enormous challenge in transporting intermediates throughout its interior, but the responsible transport mechanisms are unknown.

CA and CDCA are termed primary bile acids because they are synthesised de novo in hepatocytes. There are two main pathways that lead to the formation of bile acids: the classical pathway, which results in CA formation, and the acidic pathway, which is responsible for CDCA formation. In the intestine, bile salts undergo deconjugation by bacterial enzymes to form an unconjugated bile acid and glycine or taurine. Some of these unconjugated bile acids are reabsorbed in the ileum and returned to the liver. When primary

bile acids enter the colon, the hydroxyl group at C-7 is removed by anaerobic bacteria, and 7-deoxy bile acids are formed. By this process, CA is converted to deoxycholic acid (DCA) and CDCA is converted to lithocholic acid (LCA). DCA and LCA are called secondary bile acids because they are formed from primary bile acids in the intestine. Both are reabsorbed in the colon by an unknown mechanism and returned to the liver. In the liver, DCA is conjugated with glycine or taurine and circulates with the primary bile acids. In contrast, LCA is conjugated with glycine or taurine and additionally sulfated at the C-3 position. These sulfated bile salts are secreted into bile but are not efficiently absorbed from the intestine and are therefore eliminated from the body via the faeces.<sup>4,5</sup>

Bile acids are cytotoxic when their concentrations increase to abnormally high levels intracellularly or extracellularly and this cytotoxicity is strongly dependent on their physicochemical properties. CA is the most hydrophilic bile acid with three hydroxy groups and is therefore less cytotoxic, whereas DCA and CDCA are more harmful because they only have two hydroxyl groups. LCA is the most cytotoxic naturally occurring bile acid with only one hydroxyl group. UDCA, a naturally occurring bile acid in black bears but detected in humans only in trace amounts, is devoid of cytotoxic properties; this bile acid is relatively hydrophilic, although it is a di-hydroxy bile acid, but the hydroxyl group at C-7 is in the  $\beta$  rather than the  $\alpha$  configuration as in CDCA.<sup>6</sup> UDCA is used as a therapeutic agent in many forms of cholestasis in order to partly replace the relatively cytotoxic bile salt pool in human patients. The biliary bile salt pool consists mainly of equal amounts of conjugated CA and CDCA (80%), and more than 10% consists of conjugated DCA. Normally, only trace amounts of the conjugated forms of LCA and UDCA are present in bile. In the faeces, only DCA (70%) and LCA (30%) are detected.

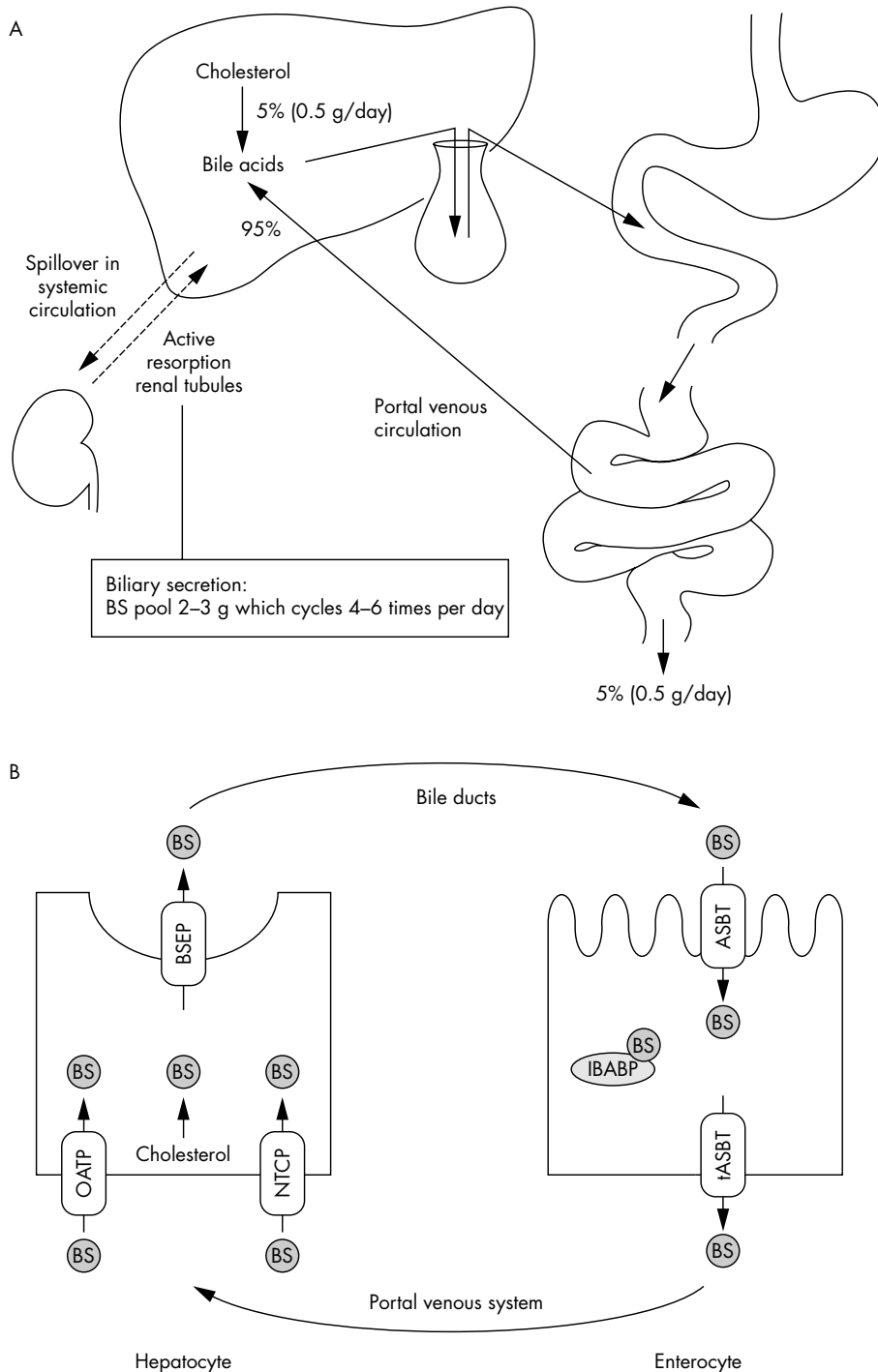
Because of the potential cytotoxicity of bile acids, the expression of selected enzymes in the bile acid biosynthesis pathway is tightly regulated by feedback inhibition and feed forward induction processes. It is now known that the main regulatory responses are at the level of transcription and are largely mediated by nuclear hormone receptors and other transcription factors, which ensure a continuous supply of bile acids in an always-changing metabolic environment.

## BILE ACID SYNTHESIS DEFECTS

Inherited mutations that impair bile acid synthesis cause a spectrum of human disease ranging from liver failure in early childhood to progressive neuropathy in adults. As discussed above, the ATP-dependent secretion of bile salts provides a hyperosmotic environment in hepatic canaliculi, which is responsible for the subsequent secretion of water, eventually resulting in bile flow. A decrease in bile acid availability, secondary to biosynthesis defects, is therefore predicted to impair bile flow. In this review, we focus on disorders that result in intrahepatic cholestasis. In fig 3, we depicted a simplified schematic representation of the bile acid synthesis pathway. Only enzymes that are implicated in disease are depicted in this figure. (For reviews of the total bile acid synthesis pathway, see Russell<sup>3</sup> and Chiang.<sup>7</sup>)

### Deficiency of 7 $\alpha$ -hydroxylases

Initiation of bile acid synthesis begins with the conversion of cholesterol to 7 $\alpha$ -hydroxycholesterol by CYP7A1 and CYP7B1 and this is the rate-limiting step in bile acid biosynthesis. CYP7A1 is used in the classical pathway, while CYP7B1 is used in the acidic pathway and is preceded by 27 $\alpha$ -hydroxylation of cholesterol (fig 3 and reviewed in Russell<sup>3</sup> and Chiang<sup>7</sup>).



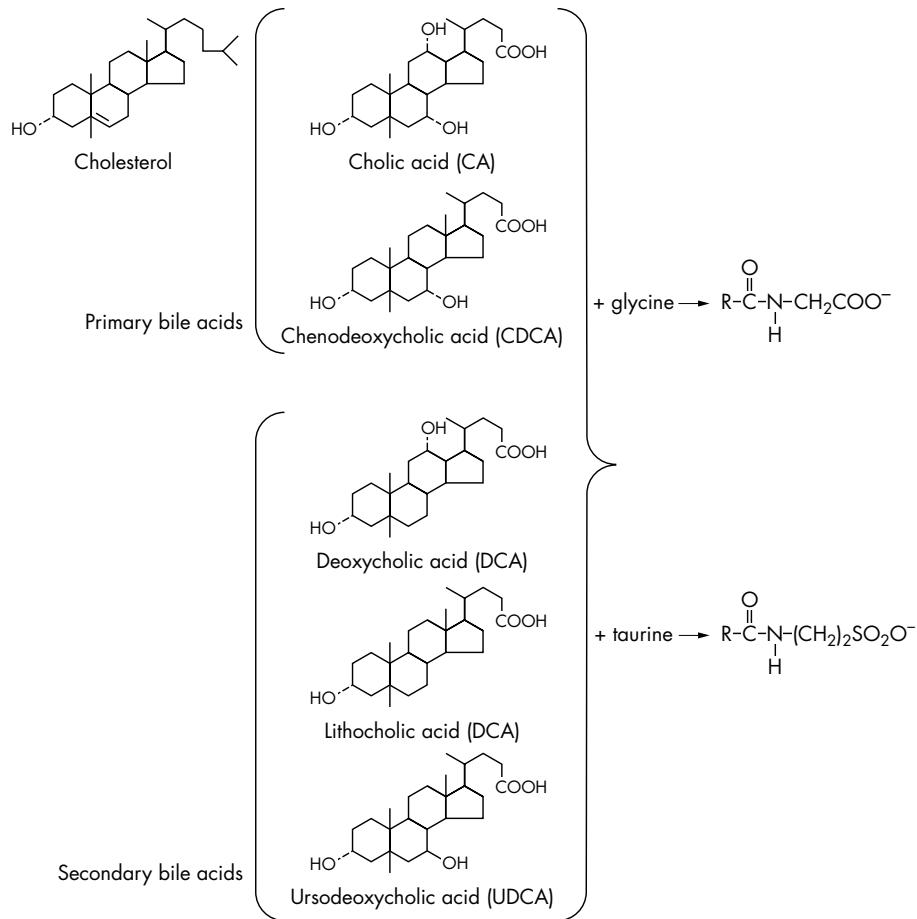
**Figure 1** Schematic representation of the enterohepatic circulation of bile salts. The organs (A) and cell types and transporters (B) that are involved in the enterohepatic circulation of bile salts are depicted. For simplicity, the cholangiocytes are not presented. For further details, see text. ASBT, apical sodium-dependent bile salt transporter; BS, bile salts explained; BSEP, bile salt export pump; IBABP, ileal bile acid binding protein; NTCP, sodium-dependent taurocholate protein; OATP, organic anion transporter; tASBT, truncated form of ASBT.

**CYP7A1 deficiency**

Mice deficient in 7 $\alpha$ -hydroxylase activity were produced by targeted disruption of *Cyp7a1*.<sup>8</sup> Homozygous animals appeared normal at birth but died within the first 18 days of life, unless their diet was supplemented with vitamins and CA. The newborn *Cyp7a1*<sup>-/-</sup> mice developed neonatal cholestasis and, due to vitamin deficiencies, oily coats, hyperkeratosis, vision defects, and behavioural irregularities. Cyp7b1 starts to be expressed after weaning (at 21 days after

birth) and this enzyme can probably compensate for *Cyp7a1* deficiency after weaning, because *Cyp7a1*<sup>-/-</sup> mice that survived through day 17 became healthy and had life spans that approximate those of wildtype mice.<sup>8</sup>

Human *CYP7A1* is located at chromosome 8q21.13 and currently only one human family with *CYP7A1* deficiency has been described.<sup>9</sup> In three adult patients a nonsense mutation was detected in homozygous form, which resulted in the truncation of the last 91 amino acid residues. Lack of CYP7A1



**Figure 2** Chemical structure of the major bile acids present in human bile and faeces. Primary bile acids are formed in the liver, and secondary bile acids are formed in the intestine by bacterial 7-dehydroxylation of their primary bile acid precursor (deoxycholic acid and lithocholic acid). Ursodeoxycholic acid (UDCA) is formed by bacterial epimerisation of the hydroxyl group at C-7. (Adapted from Hofmann<sup>4</sup>)

activity *in vitro* was confirmed using a mutant protein lacking the carboxy terminal 91 amino acid residues. Patients have hyperlipidaemia, premature coronary and peripheral vascular disease, and premature gallstone disease, together with a markedly reduced bile acid synthesis rate. The acidic bile acid synthesis pathway is upregulated. However, the severe malnutrition and neonatal cholestasis observed in mice were not present in this family. This may reflect differences in how bile acid synthesis is regulated between these two species.<sup>10</sup>

The disorder appears to be inherited in an autosomal codominant fashion because heterozygous family members had increased serum LDL cholesterol levels in comparison with their unaffected relatives.

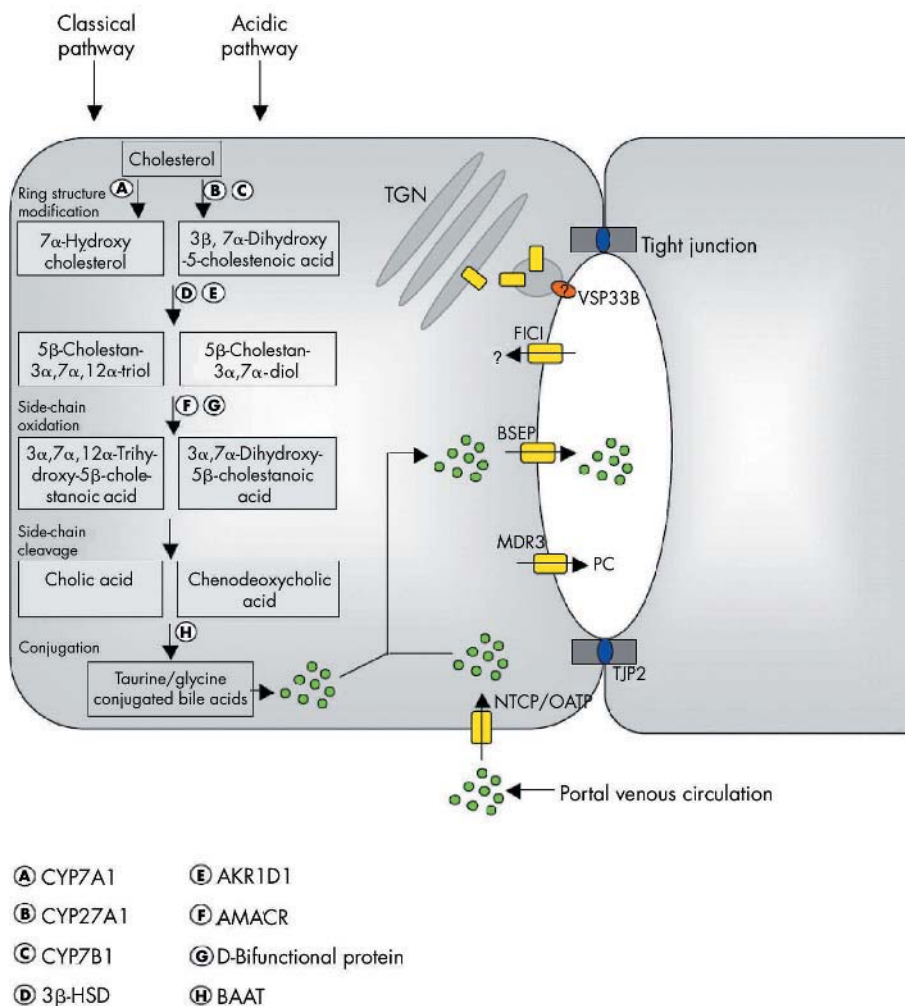
### CYP7B1 deficiency

*CYP7B1* is located at chromosome 8q21.3 and encodes CYP7B1, which is localised in the endoplasmic reticulum in the cells of many tissues and hydroxylates cholesterol in the acidic pathway. Setchell *et al*<sup>11</sup> reported the only known case of CYP7B1 deficiency. A child of consanguineous parents presented with neonatal cholestasis and cirrhosis at 6 days of age. Primary bile acid conjugates were absent in serum, whereas the concentration of the highly toxic mono-hydroxylated 27 $\alpha$ -hydroxy cholesterol was 4500-fold higher than normal. Strikingly, there were no 7 $\alpha$ -hydroxylated bile acids. Neither CYP7A1 nor CYP7B1 hydroxylase activities were detectable in the liver of this patient, although 7 $\alpha$ -hydroxylase protein was present as evidenced by immunoblotting.

Sequence analysis revealed a nonsense mutation in exon 5 (of 6) of *CYP7B1* but no mutations in *CYP7A1*. The truncated CYP7B1 protein was inactive when expressed in cells. Presumably, the accumulated 27 $\alpha$ -hydroxysterol reduced *CYP7A1* expression or activity and resulted in a complete loss of bile acid synthesis. Oral bile acid therapy did not improve the patient's condition and a liver transplantation was performed at 4.5 months of age. Acute allograft rejection occurred and the patient died 20 days after transplantation. Setchell *et al*<sup>11</sup> speculate that *CYP7B1* mutations generally cause prenatal or early neonatal lethality, and that this single reported case represents an exception to fetal death. Li-Hawkins *et al*<sup>12</sup> observed that *Cyp7b1* knockout mice were phenotypically indistinguishable from healthy littermates. They concluded that the major physiological role of *Cyp7b1* is to inactivate oxysterols and that loss of this enzyme in the liver of mice is compensated for by an increase in the synthesis of bile acids by other pathways. Again, the differences between mice and man are presumably the result of differences in regulation of bile acid synthesis between these species.<sup>10</sup>

### 3 $\beta$ - $\Delta$ 5-C27-Hydroxysteroid oxidoreductase (C27 3 $\beta$ -HSD or HSD3B7) deficiency

Clayton and colleagues first reported progressive intrahepatic cholestasis due to C27 3 $\beta$ -HSD enzyme deficiency in 1987<sup>13</sup> (fig 3 and table 1). A child presented with neonatal jaundice, liver enlargement, fat-soluble vitamin deficiency, and steatorrhoea. Mass spectrometry analysis of urine and plasma



**Figure 3** Schematic representation of proteins that are involved in bile flow in the hepatocyte. On the left side of the hepatocyte, a simplified representation of bile acid synthesis is depicted; only proteins that are known to be implicated in familial disease are depicted. At the canalicular membranes of the hepatocytes, transporters in which mutations cause cholestasis are depicted. MDR3 transports phosphatidyl choline (PC) from the inner to the outer leaflet of the plasma membrane, the BSEP transports bile salts into the bile canaliculi, and FIC1 is thought to transport aminophospholipids. Mutations in TJP2 and VSP33B are also associated with cholestatic disease. See text for further explanation.

revealed accumulation of hepatotoxic C24 and C27 steroids. C27 3β-HSD enzyme activity was absent in cultured fibroblasts of this patient. Since then, several patients have been diagnosed with C27 3β-HSD deficiency.<sup>13-15</sup> It was only in 2000 that *HSD3B7*, which encodes C27 3β-HSD, was cloned and mapped to chromosome 16p11.2-12, and a homozygous mutation was found in *HSD3B7* of the patient previously described by Clayton and his colleagues.<sup>13, 16</sup> This mutation resulted in truncation of the last 23 amino acid residues and elimination of enzyme activity in transfected cells. Bile acid therapy leads to increased well being, a decrease in pruritus, and a normalisation of urinary steroids in these patients.<sup>17</sup>

**Δ4-3-Oxosteroid-5β reductase (AKR1D1) deficiency**  
 Severe intrahepatic cholestasis was observed by Setchell *et al* in two identical twin siblings in 1988.<sup>18</sup> The parents were not consanguineous. Jaundice, pale stools, and dark urine were noted on the first day of life. Mass spectrometry analysis revealed markedly reduced primary bile acid synthesis and concomitant accumulation of Δ4-3-oxo and allo bile acids. These biochemical findings, which were identical in both infants, indicated a defect in bile acid synthesis catalysed by Δ4-3-oxosteroid-5β reductase (AKR1D1). The accumulating

sterols are hepatotoxic and cause liver failure over time, a situation which is effectively treated with oral bile acid therapy.

Kondo *et al*<sup>19</sup> cloned the human gene for Δ4-3-oxosteroid-5β reductase (*AKR1D1*), which is located at chromosome 7q32-33.<sup>20</sup> Mutation analysis in the twin patients has not been reported and currently there is no mouse model available.

**Other bile acid biosynthesis defects not involving intrahepatic cholestasis**

Patients have been described with Cyp27A1 deficiency (fig 3 and table 1) which causes the neuropathological disorder cerebrotendinous xanthomatosis (CTX).<sup>21</sup> Bile acid synthesis is reduced but it is the buildup of cholesterol and cholestanol in the blood and tissues that causes xanthomata together with cardiovascular problems. In the brain, these sterols gradually disrupt the myelin sheaths surrounding neurons resulting in progressive neurological dysfunction. If diagnosed at an early age, CTX is treated efficiently with oral bile acid therapy.

Patients with 2-methylacyl-CoA racemase (*AMACR*) (fig 3 and table 1) deficiency accumulate pristanic acid and the bile

**Table 1** Familial intrahepatic cholestasis syndromes

Disorder	Chromosome	Gene	Phenotype
<b>Bile acid synthesis disorders</b>			
CYP7A1 deficiency	8q21.13	<i>CYP7A1</i>	Hyperlipidaemia, premature coronary and peripheral vascular disease, and premature gallstone disease
CYP7B1 deficiency	8q21.3	<i>CYP7B1</i>	Neonatal cholestasis and cirrhosis
3 $\beta$ - $\Delta$ 5-C27-Hydroxysteroid oxidoreductase deficiency	16p11.2-12	<i>HSD3B7</i>	Neonatal jaundice, fat-soluble vitamin deficiency, and steatorrhea
$\Delta$ 4-3-Oxosteroid-5 $\beta$ reductase deficiency	7q32-33	<i>AKR1D1</i>	Intrahepatic cholestasis, jaundice, pale stools, and dark urine
Cerebrotendinous xanthomatosis (CTX)	2q33-qter	<i>CYP27A1</i>	Xanthomas and cardiovascular problems
2-Methylacyl CoA racemase deficiency	15p13.2-5q11.1	<i>AMACR</i>	Adult-onset sensory motor neuropathy, accumulation of pristanic acids and bile acid intermediates
D-bifunctional protein deficiency	5q2	<i>HSD17B4</i>	Hepatomegaly, developmental defects, hypotonia, and seizures
<b>Bile acid synthesis/tight junction defect</b>			
Familial hypercholesterolaemia (FHCA)	9q12-13 9q22-23	<i>TJP2</i> <i>BAAT</i>	Fat malabsorption, vitamin K-deficiency, sometimes cholestasis and chronic hepatitis
<b>Canalicular transport defects</b>			
FIC1 disease	18q21-22	<i>ATP8B1</i>	PFIC1: progressive cholestasis, cirrhosis BRIC1: recurrent attacks of cholestasis GFC: as in PFIC1
BSEP disease	2q24	<i>ABCB11</i>	PFIC2: progressive cholestasis, cirrhosis BRIC2: recurrent attacks of cholestasis
MDR3 disease	7q21	<i>MDR3</i>	PFIC3: extensive bile duct proliferation and fibrosis
Intrahepatic cholestasis of pregnancy (ICP)	18q21-22, 2q24, 7q21	<i>ATP8B1</i> <i>ABCB11</i> <i>MDR3</i>	Pruritus during third trimester of pregnancy, resolves after delivery
<b>Other familial intrahepatic cholestasis syndromes</b>			
ARC syndrome	15q26.1	<i>VSP33B</i>	Arthrogryposis, renal tubular dysfunction, and cholestasis
Lymphoedema-cholestasis syndrome (LCS)/ Aagenaes syndrome	15q	?	Neonatal intrahepatic cholestasis and lymphoedema
North American Indian childhood cirrhosis (NAICC)	16q22	<i>Cirhin</i>	Neonatal jaundice, biliary cirrhosis

acid intermediates di- and trihydroxycholestanic acid (DHCA and THCA) and present with adult-onset sensory motor neuropathy, but liver function appeared normal in these patients. Interestingly, two brothers are described with THCA syndrome (OMIM 214950); these patients only accumulate THCA and present with cholestasis and obstructive jaundice.<sup>22</sup> A disease locus has not yet been identified.

Finally, loss of D-bifunctional protein activity (fig 3) is associated with the accumulation of C27 bile acid intermediates and pristanic acid. Patients present with hepatomegaly, developmental defects, hypotonia, and seizures. All patients manifest the neurological deficiencies but only some patients have symptoms of liver failure,<sup>23</sup> probably due to excretion of C27 bile acid intermediates into bile.<sup>24-26</sup> Effective therapy for D-bifunctional protein deficiency has not yet been reported.

### Common denominators of bile acid biosynthesis defects

In general, bile acid synthesis defects that affect early biosynthetic steps cause neonatal cholestasis because bile acid intermediates accumulate in the liver. These disorders can generally be treated with oral bile acid therapy. It is hypothesised that bile acid therapy causes suppression of cholesterol 7 $\alpha$ -hydroxylase preventing further synthesis of cholestatic intermediates (see "Regulation of bile acid homeostasis" section below). One exception is CYP7B1 deficiency, with concomitantly reduced CYP7A1 activity and expression, probably because of feedback inhibition of CYP7A1 by C-27 hydroxylated cholesterol. This exception could be explained by absence of feedback regulation of bile acids on CYP27A, which is responsible for the first step in bile acid synthesis via the acidic pathway, leading to further accumulation of C-27 hydroxylated cholesterol.

Why do some bile acid synthesis disorders not manifest with liver disease but with neuropathology? AMACR and

D-bifunctional protein are both localised in peroxisomes and both cause neurological defects but liver disease only sometimes. It is thought that the bile acid intermediates that are substrates for these enzymes can function as bile acids.<sup>24</sup> Both enzymes are not only involved in bile acid synthesis but also act on branched chain fatty acids such as pristanic acid. This explains the accumulation of pristanic acid in both disorders. Pristanic acid, which is neurotoxic, accumulates in the circulation and causes the progressive neuropathy in these patients. A similar phenotype of progressive peripheral neuropathy is characteristic for peroxisome biogenesis disorders such as Refsum disease and Zellweger syndrome. In such generalised peroxisomal disorders, these enzymes are not functional; pristanic acid accumulation is thought to contribute to the neurologic problems in these disorders.<sup>27</sup>

Hyperlipidaemia is observed in some of the biosynthesis disorders. The relationship between defective bile acid synthesis and hyperlipidaemia has long been recognised, but its mechanism is poorly understood. It was suggested that bile acids have an effect on the synthesis of VLDL triglycerides, but there is also evidence that the reduced bile acid pool causes a decrease in the activity of the nuclear hormone receptor FXR (farnesoid X receptor) and a corresponding decline in the expression of apolipoprotein C-II which normally induces lipoprotein lipase activity.<sup>3 28 29</sup>

The different bile acid biosynthesis defects can easily be distinguished from each other and from bile salt transport defects by mass spectrometry analysis of blood and urine; all have a characteristic pattern of accumulating bile acid intermediates. In the past, mass spectrometry analysis has proven to be a powerful technique for elucidating this class of cholestasis disorders, even before their genetic basis was known. Future studies should address whether mass spectrometry analysis of blood, urine, and bile samples could also distinguish between BSEP and FIC1 disease (see below),

because genetic testing currently remains the only strategy to accurately discriminate between these conditions.

## BILE ACID SYNTHESIS/TIGHT JUNCTION DEFECT

### Familial hypercholanemia (FHCA)

FHCA is a recently resolved genetic defect, which was diagnosed in 12 families of Old Order Amish descent in Lancaster County in the USA.<sup>30</sup> Patients manifest fat malabsorption, vitamin K deficiency, and rickets, indicating a shortage of bile salts in the intestine. Serum bile salt concentrations were usually elevated and cholestasis and chronic hepatitis were sometimes observed. Symptoms could often be reversed by UDCA treatment. A whole genome screen in these families revealed a chromosomal region (9q12–9q13) shared identically by descent in eight FHCA patients. This region contained the candidate gene *TJP2*. Genomic sequencing of this gene identified a homozygous valine to alanine substitution (V48A) in these eight patients. As three unaffected siblings were also homozygous for this mutation, penetrance of FHCA was incomplete.

Tight junction protein 2 (*TJP2*) belongs to a family of membrane-associated guanylate cyclase homologues that are involved in the organisation of tight junctions.<sup>31–32</sup> In the hepatocytes, tight junctions have an important role in separating bile from plasma and the canalicular membrane domain from the sinusoidal membrane domain. *TJP2* is presumed to affect tight junction structure by binding to claudins and occludin. Carlton *et al.*<sup>30</sup> proposed that in patients with the V48A mutation in *TJP2*, bile salts enter the bile but subsequently leak into plasma through dysfunctional tight junctions, resulting in high serum bile salt concentrations.

In five patients with FHCA the V48A mutation in *TJP2* could not be detected. Carlton *et al.*<sup>30</sup> discovered a second FHCA locus at 9q22–9q23, which contains the candidate gene *BAAT* (bile acid CoA:amino acid *N*-acyltransferase), the key enzyme in conjugation of bile acids with glycine or taurine (fig 3 and table 1). Sequencing revealed a methionine to valine substitution (M76V) in *BAAT* in four individuals who did not harbour the V48A mutation in *TJP2*. Unconjugated bile acids cannot be secreted into bile by the bile salt export pump (BSEP).<sup>33</sup> Thus, unconjugated bile acids accumulated in the livers of these patients, with concomitantly reduced concentrations in bile and intestine. Strikingly, five of the eight individuals with the homozygous V48A mutation in *TJP2* additionally presented with a heterozygous *BAAT* mutation and, conversely, one patient with a homozygous M76V mutation in *BAAT* also had a heterozygous V48A mutation in *TJP2*. In one patient with FHCA, mutations were not detected in *TJP2* or in *BAAT* nor was this patient homozygous at either region at chromosome 9. Therefore, a third locus for FHCA probably exists. Interestingly in this respect, recessive mutations in microsomal epoxide hydrolase (*mEH*), which plays a central role in carcinogen metabolism but is also able to mediate sodium-dependent uptake of bile acids into hepatocytes, are also associated with hypercholanemia.<sup>34</sup>

Carlton *et al.*<sup>30</sup> speculate that manifestation of FHCA associated with homozygous *TJP2* mutations requires a concomitant heterozygous mutation in a second locus, such as *BAAT*, or the third locus postulated to be associated with FHCA. The population frequency of the *TJP2* V48A mutation is unexpectedly high (7%) for the number of FHCA patients detected with a homozygous *TJP2* mutation, indicating that this disease has an oligogenic mode of inheritance. Both *BAAT* and *TJP2* may represent important genetic modifiers of disease severity. Further studies should address whether there are differences in clinical and biochemical presentation in FHCA patients with distinct genetic backgrounds.

## TRANSPORTER DEFECTS AFFECTING BILE FORMATION

After biosynthesis of bile acids or reuptake of bile salts from blood into hepatocytes, conjugated bile acids are actively transported into the bile canaliculus, where they are the predominant components of bile. Bile formation in bile canaliculi is an osmotic process that largely depends on secretion of various compounds by ATP-dependent transporters in the canalicular membrane of the hepatocytes. In this way bile salts, and also cholesterol, bilirubin, bicarbonate, glutathione, heavy metals, various drugs, toxins, and phospholipids are secreted into bile. The protein pumps responsible for the transport of these compounds are ATP-binding cassette (ABC) transporters or transporters that belong to the P-type ATPase superfamily. Several of these transporter genes are associated with familial cholestasis syndromes or other hereditary disorders in man.

Progressive familial intrahepatic cholestasis (PFIC) and benign recurrent intrahepatic cholestasis (BRIC) are two long recognised autosomal recessive hereditary cholestasis disorders characterised by a defect in bile secretion. However, when these disorders were first described it was not known that PFIC and BRIC displayed genetic heterogeneity. Currently, PFIC is known to be associated with mutations in *ATP8B1* (PFIC1), *ABCB11* (PFIC2), and *MDR3* (PFIC3), while BRIC is associated with mutations in *ATP8B1* (BRIC1) and *ABCB11* (BRIC2). A subtype of BRIC with autosomal dominant inheritance is also described, but the gene mutated in this defect has not yet been identified.<sup>35</sup>

### FIC1 disease

FIC1 disease is defined by genetic criteria: all patients have mutations in *ATP8B1*. However, the disorder comprises at least three previously described clinical entities: progressive familial intrahepatic cholestasis type 1 (PFIC1), benign recurrent intrahepatic cholestasis type 1 (BRIC1), and Greenland familial cholestasis (GFC).

PFIC was first reported in an Amish family who were all descendants of a Jacob Byler, hence the former name Byler disease.<sup>36</sup> Seven patients of this family suffered the same symptoms: steatorrhoea, diarrhoea, jaundice, hepatosplenomegaly, and failure to thrive. From the Byler pedigree it was deduced that the disorder followed autosomal recessive inheritance. Because patients presented with very low biliary bile salts, but high serum levels of bile salts, a bile acid biosynthesis defect was ruled out. Therefore, these patients were thought to have a defect in bile salt transport.

Subsequently, homozygosity mapping identified a locus for PFIC at chromosome 18q21–q22,<sup>37</sup> the same locus previously attributed to BRIC.<sup>38</sup> BRIC was first described in 1959 by Summerskill and Walshe<sup>39</sup> and manifests with similar symptoms although with episodic attacks of cholestasis. Between attacks the patients are symptom-free and the progressive liver damage seen at an early age in PFIC is not seen in BRIC. Further studies revealed that both disorders were associated with mutations in the same gene, *ATP8B1*, which encodes FIC1, and currently these disorders are termed PFIC1 and BRIC1. One other syndrome of intrahepatic cholestasis in childhood, Greenland familial cholestasis (GFC), was described in 16 Inuit children from Greenland.<sup>40</sup> The patients presented shortly after birth with jaundice and pruritus. Homozygosity mapping revealed homozygosity for a conserved microsatellite haplotype on chromosome 18, containing *ATP8B1*.<sup>41</sup> In 2000, Klomp *et al.*<sup>42</sup> identified a homozygous missense mutation (D554N) in the *ATP8B1* gene as the cause of GFC.

All three clinical entities present with and are defined by normal or slightly elevated gamma-glutamyl transpeptidase (GGT) activity in serum. GGT is normally bound to the

canalicular membrane by a glycosyl phosphatidyl inositol (GPI) anchor, but when excessive amounts of bile salts are present in the canaliculus, the detergent activity liberates GGT, which is usually seen in obstructive cholestasis. In FIC1 disease, however, it is thought that the reduced concentration of bile salts in bile preserves GGT localisation at the canalicular membrane, hence the normal GGT activity in serum.

Mutation analysis has revealed 39 different mutations in *ATP8B1* in PFIC1 patients and 19 in BRIC1 patients.<sup>38, 42-47</sup> The *ATP8B1* I661T mutation is the most common mutation in FIC1 disease; it was detected in approximately 75% of BRIC1 patients but was associated with a remarkable variability in clinical presentation.<sup>44</sup> Compound heterozygosity for I661T and other different mutations can lead to PFIC1. Homozygosity for I661T is associated with recurrent phenotypes and even with a symptom-free life,<sup>44</sup> indicating incomplete penetrance. The differences in phenotypes of these patients with the same homozygous mutation suggest that environmental factors and/or modifier genes play a role in the clinical expression of FIC1 disease. Candidates for modifier genes of FIC1 disease could be *TJP2* and *BAAT* (described above). Interestingly, patients from the Faeroe Islands with the homozygous I661T mutation have similar genetic makeup, but this group of patients still shows marked heterogeneity in disease phenotype.<sup>47</sup>

It was postulated that BRIC1 and PFIC1 represent two ends of a phenotypic spectrum, because both are caused by mutations in the same gene. An intermediate form between BRIC1 and PFIC1 was described by Van Ooteghem *et al*<sup>48</sup> in two BRIC1 patients with a splice site mutation resulting in skipping of exon 24. These patients presented with recurrent cholestatic bouts initially but developed progressive cholestasis later in life. A difference in the severity of mutations is likely to be linked to this phenotypic continuum. Consistent with this notion, 63% of the *ATP8B1* mutations identified in BRIC1 patients were missense mutations, whereas only 41% of mutations associated with PFIC1 are missense mutations. PFIC1 patients more often have nonsense and frame shift mutations than BRIC1 patients; these mutations likely alter protein structure and/or function severely.<sup>44</sup> Residual activity of FIC1 protein could be the explanation for the relative mild phenotypes observed in BRIC1 patients. Future functional studies should systematically address the effects of *ATP8B1* mutations associated with mild and severe disease.

FIC1 is expressed at the apical membranes of different epithelial cells: at the apical membranes of enterocytes from the proximal to the distal intestine, of cells lining the gastric pits, of pancreatic acinar cells, and of cholangiocytes and hepatocytes.<sup>49-51</sup> Strikingly, the expression in hepatocytes is low compared to that in some other tissues. Pancreatitis and diarrhoea are commonly observed in FIC1 disease patients and do not resolve after liver transplantation.<sup>46, 47, 52</sup> These symptoms are probably explained by the absence of FIC1 function in pancreas and intestine in these patients. Mice with an engineered mutation in *Atp8b1*, the G308V mutation that was commonly detected in patients of Amish descent, displayed no cholestatic phenotype and unimpaired biliary secretion, even upon bile salt feeding, but these mice did present with serum bile salt accumulation, weight loss, jaundice, and hepatomegaly, suggesting that there is a defect in the regulation of resorption of bile acids in the cholangiocytes or enterocytes. Furthermore, *Atp8b1*<sup>G308V/G308V</sup> mice counteract bile salt accumulation by enhanced rehydroxylation of excess bile salts, indicating an important difference between mice and humans.<sup>53</sup>

During immediate postnatal development, FIC1 expression is hardly detectable in the intestinal tissue of newborn mice but is specifically induced at 3 weeks of postnatal life. In contrast, FIC1 is already expressed in liver, pancreas, and

stomach before weaning and is not further induced during postnatal development.<sup>49</sup>

It is presently unclear how all these observations fit into a working model of the function of FIC1 and how impairment of FIC1 activity causes intrahepatic cholestasis. The primary biochemical function of FIC1 is not yet known. However, it belongs to the P4 P-type ATPase subfamily and initial functional studies have suggested that this subfamily may have conserved functions in the transport of aminophospholipids from the outer leaflet to the inner leaflet of plasma membranes,<sup>54-56</sup> but this has not been unequivocally demonstrated.<sup>57</sup> Indeed, Ujhazy *et al*<sup>51</sup> were able to demonstrate aminophospholipid translocase activity in canalicular liver membrane preparations which contained FIC1 protein. Transfection of CHOK1 cells with *ATP8B1* cDNA resulted in the appearance of FIC1 in membrane preparations and energy-dependent translocation of a fluorescent analogue of phosphatidylserine, providing indirect evidence that FIC1 may actually represent an aminophospholipid transporter.<sup>51</sup> How this proposed function is related to bile salt transport is unclear.

As an aminophospholipid translocase, FIC1 might have a role in intracellular vesicular transport, thereby regulating proper expression or function of the canalicular transporters such as BSEP. Such a function would be consistent with the localisation of FIC1 at the canalicular membrane.<sup>50, 51</sup> A recent preliminary study revealed unremarkable BSEP expression at the canalicular membrane of hepatocytes in PFIC1 patients, but BSEP function was not addressed.<sup>58</sup> In addition, the expression of P-glycoproteins at the canalicular membrane was not affected in a PFIC1 patient.<sup>50</sup> There are additional hypotheses for the function of FIC1; it might be a bile salt transporter for hydrophilic bile salts or could play a role in ion transport, based on the 30% homology with P-type ATPases that function as Ca<sup>2+</sup> transporters. These hypotheses are explained in more detail in van Mil *et al*<sup>59</sup>; further studies are required to reveal how the biochemical function of FIC1 is related to bile salt homeostasis.

Recently, Chen *et al*<sup>60</sup> observed that in ileal biopsies from PFIC1 patients and in Caco2 cells treated with *ATP8B1* antisense oligonucleotides, *ASBT* expression was increased, whereas *FXR*, short heterodimeric partner (*SHP*), and *IBABP* showed reduced expression. The authors proposed that loss of FIC1 expression leads to diminished nuclear translocation of FXR. This hypothesis partly explains the pathophysiology of FIC1 disease as pathologic alterations in the transcriptional regulation of intestinal and hepatic bile acid transport expression. Cholestasis presumably develops because of both enhanced ileal uptake of bile acids via up-regulation of *ASBT* and diminished canalicular secretion of bile acids secondary to down-regulation of BSEP. Although this possible explanation of FIC1 disease pathophysiology is attractive, it raises a novel question as to how FIC1 expression regulates FXR activity. The observed changes in transporter expression might also be secondary to altered bile acid levels resulting from diminished FIC1 expression or function.

Mutations in *ASBT* are associated with primary bile acid malabsorption (PBAM), which manifests with chronic intractable diarrhoea, steatorrhoea, interruption of the enterohepatic circulation, and reduced plasma cholesterol levels.<sup>61</sup> Both FIC1 disease and PBAM result in chronic intractable diarrhoea, although there are elevated and diminished levels, respectively, of functional *ASBT* present, which indicates that the pathogenesis of the diarrhoea is different in these disorders.

### BSEP disease

In 1997, a second locus for PFIC with normal serum GGT activity was mapped to chromosome 2q24 by homozygosity



mapping and linkage analysis.<sup>62</sup> After locus refinement, mutations were detected in *ABCB11* (formerly called *SPGP* and *BSEP*),<sup>63</sup> which encodes BSEP, an ABC transporter that was first cloned in rat. In vitro studies revealed that it functions as a bile salt transporter and is expressed solely at the canalicular membranes of hepatocytes.<sup>33 64-66</sup>

The knockout of *Abcb11* in mice resulted in intrahepatic cholestasis but with significantly less severity than found in human patients with mutations in *ABCB11* (PFIC2 patients).<sup>67</sup> Biliary cholate excretion was reduced to 6% of that of wildtype mice, but there was excretion of tetrahydroxylated bile salts, which were not detected in healthy littermates. Hence, the bile salt output was 30% of that of wildtype mice, whereas bile salt output in PFIC2 patients is almost absent. These results suggest that hydroxylation and an alternative canalicular transport mechanism can compensate for the absence of *Abcb11* in mice and this may protect the mutant mice from liver damage.<sup>67</sup> Crossing these mice with several other mouse models might reveal the nature of this alternative canalicular transport mechanism.

A large number of different mutations have been described in patients with PFIC2.<sup>63 68</sup> Two mutations, E297G and D482G, are commonly detected and have been described in 25 and 16 families, respectively; at least one of these two mutations is present in 30% of PFIC2 patients of European descent.<sup>69</sup> Functional studies revealed that in the case of the E297G mutation, almost all bile salt transport activity was abolished, probably because the mutation caused misfolding of the newly synthesised protein.<sup>70</sup> For the D482G mutation, functional studies revealed conflicting results; Wang *et al* reported that a mutated rat BSEP was expressed normally at the apical membranes of MDCK cells but had diminished activity,<sup>70</sup> but Plass *et al*<sup>71</sup> reported that mouse BSEP with the D482G mutation was glycosylated inefficiently and was therefore retained intracellularly. The  $\Delta$ F508 mutation in the ABC transporter CFTR also leads to inefficient glycosylation of the mutant protein, and the processing and stability of mutant BSEP as well as CFTR were shown to be temperature sensitive.<sup>66 72 73</sup> These studies are important in elucidating the effects of the mutations and in providing new therapeutic strategies.

As in PFIC1, PFIC2 manifests with severe intrahepatic cholestasis in infancy and progresses to end-stage liver disease and death in childhood, unless a liver transplantation, a partial biliary diversion, or an ileal exclusion is performed. There are some variable differences between PFIC1 and PFIC2: extrahepatic features have been described exclusively in PFIC1 patients but not in PFIC2 patients. Also, PFIC2 often presents with non-specific giant cell hepatitis, whereas in PFIC1, the cholestatic liver disease is typically more bland. Despite these variable differences, it is currently only possible to accurately distinguish between PFIC1 and PFIC2 by genetic testing.

Recent genetic evidence implies a third locus for autosomal recessive PFIC, because 10 families were inconsistent with linkage to either *ABCB11* or *ATP8B1*<sup>74</sup> and no mutations in either *ABCB11* or *ATP8B1* could be detected in several PFIC patients.<sup>74</sup> In this last study, 83% of BRIC patients did not harbour mutations in *ATP8B1*, strongly suggesting that there is at least one additional locus underlying autosomal recessive BRIC.

Recently, it was discovered that 11 BRIC patients from eight different families harboured mutations in *ABCB11*; these individuals are currently denoted as BRIC2 patients.<sup>75</sup> None of these BRIC2 patients manifest with extrahepatic features, but seven out of 11 presented with cholelithiasis. It was speculated that supersaturation of cholesterol in bile occurs due to diminished bile salt secretion, secondary to impaired BSEP function,<sup>76</sup> and that this may account for the

high incidence of gallstone formation. Interestingly, *Abcb11* is one of two candidate genes of the *Lith1* locus, which is associated with increased risk of gallstone formation in inbred mice. Three BRIC2 patients manifest with progressive cholestasis in the third or fourth decade of life, as was also described for two BRIC1 patients. Therefore, it was proposed that *ABCB11* disease ranges along a phenotypic continuum, as was described previously for *ATP8B1* disease.<sup>59</sup> The E297G mutation that was commonly detected in PFIC2, was also observed in homozygous form in two BRIC2 families, indicating that other genetic and environmental factors play a role in the phenotypic expression of *ABCB11* disease. As in *ATP8B1* disease, there seems to be a correlation between the predicted effects of mutations and the severity of the phenotype. In this same study, 12 BRIC patients did not harbour mutations in either *ATP8B1* or *ABCB11*, providing additional evidence for a third locus for autosomal recessive BRIC and PFIC with low serum GGT activity.

### MDR3 disease

Individuals with MDR3 disease are called PFIC3 patients. PFIC3 is quite different from the other PFIC subtypes, because serum GGT activity is usually elevated in these patients and liver histology shows extensive bile duct proliferation and fibrosis.<sup>77-79</sup> The age of onset ranges from 1 month to 20.5 years of age and the most prominent features are portal hypertension, hepatosplenomegaly, jaundice, pruritus, and liver failure later in life.

The genetic background of PFIC3 was elucidated after mice with a disruption in the *Mdr2* gene, the murine orthologue of human *MDR3*, were found to develop hepatocyte necrosis, dilated canaliculi, cholangiopathy represented by portal tract inflammation, severe ductular proliferation in the first 3 months of life, and hepatocellular carcinoma.<sup>80</sup>

The bile of *Mdr2* knockout mice and PFIC3 patients is almost devoid of phosphatidylcholine, whereas bile salt secretion is normal. Normally, phosphatidylcholine is the predominant phospholipid in bile. Bile salts secreted into the bile canaliculi induce the extraction of phospholipids from the exoplasmic leaflet of the canalicular membrane. In the biliary tree phospholipids are of crucial importance in protecting the cellular membranes against the high concentrations of bile salts by the formation of mixed micelles. Ruetz and Gros<sup>81</sup> discovered that *Mdr2* translocates phosphatidylcholine from the inner to the outer plasma membrane leaflet. The phenotypic and biochemical similarities between PFIC3 patients and *Mdr2*<sup>-/-</sup> mice led Hadchouel, Oude Elferick, and coworkers<sup>77-79</sup> to investigate whether mutations in *MDR3* underlie PFIC3. This revealed 17 different mutations in *MDR3* (at chromosome 7q21) in 22 PFIC3 patients: 11 missense mutations and six mutations that were predicted to result in a truncated protein.<sup>77-79</sup> The truncated *MDR3* proteins could not be detected in the livers of these patients by immunochemical methodology and almost no biliary phospholipids were present in samples obtained from patients affected with such *MDR3* mutations, whereas residual expression of *MDR3* was observed concomitantly with low but detectable levels of biliary phospholipids in some patients with missense mutations in *MDR3*. Children with an *MDR3* missense mutation have relatively mild disease, with late onset and a slow progression. Missense mutations are likely associated with residual transport activity and treatment with UDCA was beneficial in most patients with missense mutations but not in patients with truncating *MDR3* mutations. It was hypothesised that, despite the reduced amounts of phospholipids in bile, partial replacement of endogenous hepatotoxic bile acids by the hydrophilic UDCA can realise a reduction of bile salt toxicity.<sup>82</sup> In conclusion, MDR3 disease can be associated

with mild and severe liver disease, as we described above for FIC1 and BSEP disease.

### Intrahepatic cholestasis of pregnancy (ICP)

Intrahepatic cholestasis of pregnancy (ICP) is a reversible form of cholestasis that may develop in the third trimester of pregnancy and persists until delivery. The main symptoms are pruritus and to a lesser extent, jaundice.<sup>83-84</sup> Serum bile salt levels are increased. The prognosis is good for the mother, but ICP can be associated with increased incidence of fetal distress, premature birth, and stillbirth.<sup>85</sup> Oestrogen and progesterone levels probably have important roles in the pathogenesis of ICP because the disease starts in the last trimester of pregnancy, when the hormone concentrations are high, and resolves 1 or 2 days after delivery, when levels of placenta-derived hormones return to normal.<sup>86-87</sup>

In ethinyloestradiol-induced cholestasis in rats, the expression of basolateral bile acid transport proteins (NTCP and different OATPs) is inhibited at the transcriptional level,<sup>88-89</sup> which could explain the etiology of cholestasis in ICP.

Besides hormonal factors, genetic factors play a role in ICP. A higher incidence of ICP has been observed in mothers of patients with PFIC1, PFIC3, or BRIC1,<sup>36-90-91</sup> indicating that heterozygous mutations in genes involved in bile formation predispose to ICP, thereby underscoring the contribution of genetic factors to the development of ICP. More frequently, ICP occurs in women with no known family history of PFIC. In some of these patients heterozygous mutations were detected in *MDR3*.<sup>92-93</sup> Savander *et al*<sup>94</sup> genotyped 16 individuals from two Finnish ICP families for markers flanking *ABCB11*, *ATP8B1*, and *MDR3*, but linkage to any of these genes was excluded, indicating that further genetic heterogeneity in ICP is expected. Known genes involved in the regulation of bile acid metabolism are good candidates for factors predisposing to ICP. Finally, environmental factors might also increase the risk for ICP. This is indicated by the fact that ICP recurs in less than 70% of pregnancies in multiparous women and by the decline in ICP prevalence rates in Chile over the last decade.<sup>95</sup> Furthermore, the incidence of ICP is reported to be higher in winter than in summer.<sup>96</sup>

During ICP, there is an increased flux of bile salts from the mother to the fetus and it is hypothesized that the placental transfer capacity for bile salts is reduced. This might result in decreased fetal elimination and retention of toxic bile salts by the fetus, which may explain the higher risk of premature birth and stillbirth in these pregnancies.<sup>97-98</sup> UDCA has been shown to be beneficial in ICP mothers and the incidence of fetal loss decreases.<sup>99</sup> Furthermore, because stillbirths occur late in pregnancy, labour is induced in these women before term.

### OTHER FAMILIAL INTRAHEPATIC CHOLESTASIS SYNDROMES

#### ARC syndrome/VPS33B deficiency

ARC syndrome refers to an autosomal recessive association between arthrogryposis, renal tubular dysfunction, and cholestasis. It has become apparent that there is notable clinical variability, even within the same family. Cases may even be undiagnosed as not all patients present with these three cardinal features.<sup>100-101</sup> Additional symptoms have been reported in some patients, including diarrhoea, cerebral malformation, nerve deafness, nephrogenic diabetes insipidus, and failure to thrive. Most patients die by the age of 7 months, but those who exceed this age have shown severe developmental delay.<sup>102</sup>

The GGT activity in serum in these ARC patients with neonatal cholestasis is low, indicative of decreased amounts or absence of biliary bile salts, and suggestive of a defect in bile salt transport. Because of the multiplexity of the ARC

syndrome, a general membrane transport defect was expected.<sup>103</sup> Since it is expressed in multiple tissues, including brain, liver, kidney, intestine, and skeletal muscle, *ATP8B1* was suspected as a candidate gene mutated in this disorder, but mutations in *ATP8B1* were excluded by Gissen *et al*.<sup>104</sup>

Recently, it was demonstrated that recessive mutations in *VPS33B* at chromosome 15q26.1 cause ARC syndrome.<sup>105</sup> Nine different mutations have been characterised, the majority leading to truncated *VPS33B* proteins. *VPS33B* encodes a homologue of the yeast *S cerevisiae* class C vacuolar protein sorting 33 gene (*Vps33*) which is important in the regulation of the fusion of intracellular vesicles with the plasma membrane. *VPS33B* interacts with members of the syntaxin family of t-SNAREs, thereby probably influencing vesicle SNARE to target SNARE complex assembly, an essential step in determining the specificity of heterotypic membrane docking and fusion. The clinical features of ARC syndrome are consistent with abnormal intracellular protein trafficking and defective membrane fusion mechanisms. The neonatal cholestasis with low GGT activity observed in these patients is probably caused by the absence of bile salt transport proteins at the canalicular membranes of these patients. Consistent with this observation, the polarised localisation of a canalicular marker protein, carcinoembryonic antigen (CEA), was markedly disturbed in liver biopsies of ARC patients.<sup>105</sup>

#### Lymphoedema-cholestasis syndrome (LCS) or Aagaens syndrome

Patients with LCS (lymphoedema-cholestasis syndrome), also called Aagaens syndrome, suffer from severe neonatal intrahepatic cholestasis and develop lymphoedema in their youth. In most patients, the cholestasis lessens during early childhood and becomes episodic, as in BRIC. A few patients, however, manifest with cirrhosis and death in early childhood, or develop cirrhosis as adults. The lymphoedema, which can start at birth or in early childhood, becomes chronic and severe, mainly affecting the lower extremities but also the hands, scrotum, and periorbital soft tissues.

Aagaens first described LCS in patients in an isolated population in the south west of Norway. To date, there have been many published cases: most are Norwegian but some are of other origin.<sup>106-107</sup> Bull *et al* mapped a locus for LCS to chromosome 15q in Norwegian patients,<sup>107</sup> but the gene has not yet been identified. Interestingly, the locus for LCS reported by Bull *et al* contains *VPS33B*, which is mutated in ARC syndrome, and both disorders manifest with developmental defects. Sequencing of *VPS33B* in LCS patients is therefore warranted. In a consanguineous Serbian Romani LCS family no linkage was detected at chromosome 15q, suggesting that locus heterogeneity exists for LCS.<sup>108</sup>

It is not known whether the normal GGT cholestasis in this syndrome is a direct or a secondary feature of the LCS gene defect. Cholestasis might develop due to abnormal function of the lymphatic system or both lymphatic vessels and bile ducts might develop abnormally.<sup>109-110</sup> Perhaps the livers of LCS patients are able to compensate for the gene defect in LCS later in life.<sup>107</sup>

#### North American Indian childhood cirrhosis/Cirrhin deficiency (NAICC)

North American Indian childhood cirrhosis (NAICC) is a rapidly evolving form of familial cholestasis with autosomal recessive inheritance found in Ojibway-Cree children from north western Quebec in Canada.<sup>111-112</sup> The disease manifests with transient neonatal jaundice, which progresses to biliary cirrhosis requiring hepatic transplantation in childhood or young adulthood. Histological samples show early bile duct proliferation and rapid development of portal fibrosis and biliary cirrhosis, suggesting involvement of bile ducts rather

than bile canaliculi. A missense mutation in *Cirhin* at chromosome 16q22 has been detected in these patients. The secondary structure predicts that this protein contains 10 WD repeats (also known as WD40 or beta-transducin repeats), which are short motifs of approximately 40 amino acids, often terminating in a Trp-Asp (WD) dipeptide. WD repeats are implicated in the organisation of proteins into complexes and are thus involved in many processes such as signal transduction, vesicular trafficking, cytoskeletal assembly, and transcription initiation complex assembly.<sup>113-115</sup> The elucidation of the function of *Cirhin* requires further studies. Furthermore, since all patients with *Cirhin* deficiency currently are from one isolated population, the clinical presentation of NAICC may expand when other unrelated patients with *Cirhin* deficiency are identified.

## REGULATION OF BILE ACID HOMEOSTASIS

Because of the intrinsic toxicity of bile acids, bile acid synthesis and transport must be tightly regulated. It is now apparent that members of the nuclear hormone receptor family of lipid-activated transcription factors are key regulators of these physiological processes. Nuclear hormone receptors, after sensing inappropriate oxysterol and bile acid levels, are transcription factors that initiate the genetic transactivation of DNA response elements in promoter regions of target genes to modulate the synthesis of bile acids and their enterohepatic circulation. The four key nuclear hormone receptors that regulate bile acid homeostasis are FXR, VDR, PXR/SXR, and LXR, although others have been described (for reviews, see Betard *et al.*,<sup>113</sup> Chagnon *et al.*,<sup>114</sup> Smith *et al.*,<sup>115</sup> Chawla *et al.*,<sup>116</sup> Goodwin *et al.*,<sup>117</sup> and Redinger *et al.*<sup>118</sup>). FXR, PXR/SXR, and VDR bind bile acids and are therefore sensors of excessive amounts of bile acids, whereas LXR is a cholesterol sensor and binds oxysterols. These nuclear hormone receptors act as heterodimers; RXR, the retinoid receptor, is an obligatory partner in this heterodimer.

When bile acid levels increase in hepatocytes, the farnesoid X receptor (FXR) binds bile acids, which then activates transcription of short heterodimeric partner (SHP). SHP inhibits transcription of *LRHI*, the liver receptor homologue 1, which normally transactivates *CYP7A1* and *CYP7B1*. Consequently, bile acid synthesis is inhibited (fig 4).<sup>10</sup> SHP is also responsible for the inhibition of the transcription of *NTCP*, encoding the principal importer of bile salts at the basolateral membrane; this leads to a reduction of bile salt absorption into hepatocytes (fig 4).<sup>119-121</sup> On the other hand, FXR induces transcription of *BSEP* resulting in enhanced secretion of excessive bile salts into bile (fig 4).<sup>122</sup> Hence, FXR functions as a bile acid sensor to maintain low bile acid concentrations in hepatocytes. In the intestine, FXR facilitates bile salt secretion into the portal circulation through induction of the ileal bile acid binding protein (*IBABP*) gene (fig 4).<sup>123</sup> Mice lacking the nuclear bile acid receptor *Fxr* developed normally but are distinguished from wildtype mice by elevated serum and hepatic bile acid, cholesterol, and triglyceride levels. *Fxr*<sup>-/-</sup> mice also had reduced bile acid pools and reduced fecal bile salt excretion due to decreased expression of *BSEP*. These data demonstrate that FXR is critical for bile acid and lipid homeostasis.<sup>124</sup>

The pregnane X receptor (PXR) can be activated by the toxic bile acid LCA. PXR inhibits transcription of *CYP7A1*<sup>125</sup> but also stimulates transcription of *CYP3A4*, a cytochrome P-450 enzyme that detoxifies secondary bile acids by monooxygenation leading to formation of more hydrophilic bile acids (fig 4). The vitamin D nuclear receptor (VDR) binds vitamin D but also LCA at low concentrations, which also results in induction of *CYP3A4* expression in the enterocytes and cholangiocytes, which explains how the enteric system

and the biliary epithelium could protect themselves from the harmful effects of LCA (fig 4).<sup>126-127</sup> This may be the secondary defence protecting the liver against the accumulation of highly toxic bile acids.<sup>128</sup> When cholesterol levels increase in hepatocytes, oxysterols activate the liver X receptor  $\alpha$  (LXR $\alpha$ ) to induce *CYP7A1* to convert excess cholesterol into bile acids.<sup>129</sup> This provides a feed-forward mechanism to primarily regulate cellular cholesterol levels rather than bile acid levels.

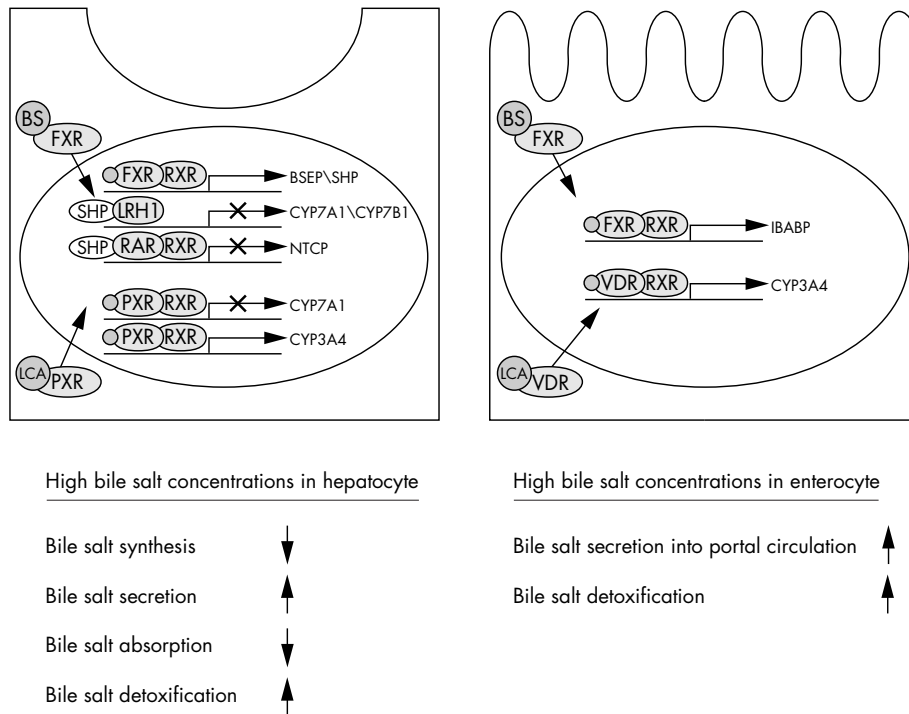
Bile salt homeostasis is also regulated at the posttranslational level. Oestradiol-17 $\beta$ -D-glucuronide has been shown to induce an acute and completely reversible cholestasis in the rat. The mechanism of pathogenesis of cholestasis is explained by the induction of endocytic internalisation of both Bsep and Mrp2 by oestradiol-17 $\beta$ -D-glucuronide, thereby rapidly reducing the number of canalicular transporters. Mrp2 is an ABC type transport protein that secretes glutathione but also the lithogenic sulfated and glucuronated bile salts across the canalicular membranes of hepatocytes.<sup>130-131</sup> Bile duct ligation in the rat, a well known experimental model of cholestasis, also resulted in decreased Mrp2 protein at the canalicular membrane accompanied by intracellular localisation of the protein in pericanalicular vesicular structures.<sup>130-132</sup> In any case, these events provide a rapid way to regulate the cell surface expression of bile salt transporters based on cellular demand and further studies of the molecular mechanisms governing this posttranslational regulation may permit the development of novel therapeutic approaches to prevent or ameliorate the clinical symptoms of cholestasis.

## CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Identification of genes involved in familial intrahepatic cholestasis has proven to be an important strategy to unravel the processes of bile acid synthesis and bile salt transport. The complexity of the mechanisms of bile flow clearly suggests that many more genetic abnormalities have yet to be identified. Consistent with this hypothesis, numerous unexplained familial cholestasis syndromes are known and the affected genes are likely to have a role in bile flow (for a list of these syndromes see Jansen *et al.*<sup>133</sup>). Identification of the genes involved in these syndromes will further enhance our insight into bile formation and cholestasis. Polymorphisms in these genes may cause an individual's response to changes in bile acid homeostasis to vary and may be genetic modifiers of disease severity. Genes involved in the regulation of bile acid homeostasis are candidate genes for idiopathic hereditary cholestasis syndromes.

Mass spectrometry analysis remains an important tool for future studies to elucidate enzyme defects in bile acid synthesis and further studies should reveal whether this application can also distinguish between specific transport defects. Microarray and proteomic approaches that compare RNA or protein expression patterns in livers from patients or mouse models with different forms of familial cholestasis, could identify new players in bile flow but may also be tools to recognise specific gene defects. Such approaches will also define common pathophysiological pathways responsible for liver damage in different forms of intrahepatic cholestasis.

Phenotype-genotype correlation studies are possible for those disorders in which many different mutations have been identified. In FIC1 disease, BSEP disease, and MDR3 disease, a correlation between the types and location of distinct mutations in the affected genes and the severity of the patient's phenotype has been observed. These observations need to be complemented by additional functional studies that allow determination of the impact of distinct mutations on protein localisation, expression, and function. Often, missense mutations cause relatively mild symptoms and it



**Figure 4** Role of nuclear hormone receptors in transcriptional regulation of bile acid synthesis and transport. In response to high concentrations of bile acids, bile acid synthesis and absorption in the hepatocyte are decreased, whereas bile salt secretion and detoxification are increased due to nuclear hormone receptor action. In the enterocyte, detoxification of secondary bile acids is increased and the secretion of bile acids into the portal venous system is eventually enhanced by transcriptional activation of *IBABP*. See text for further explanation.

is thought that residual activity of the mutated proteins explains this observation. Novel therapeutic approaches for these patients might be found by inducing the expression of the mutated proteins. Recent knowledge concerning the regulation of bile acid homeostasis by nuclear hormone receptors is promising in this respect, because specific agonists and antagonists of nuclear hormone receptors are now being developed. Not only might specific genetic disorders be treatable with such agonists and antagonists, but symptoms of patients with acquired forms of cholestasis might also improve using this approach. UDCA has recently been found to be effective in ameliorating intrahepatic cholestasis in some cases not only because it replaces the toxic endogenous bile acid pool by the less toxic, less hydrophobic bile acid UDCA, but also because it is a ligand for PXR. PXR transactivates *CYP3A4*, resulting in enhanced detoxification of bile acids.<sup>134</sup> Other PXR agonists such as rifampicin, often used for treatment of cholestasis, may also enhance *CYP3A4* expression. Furthermore, FXR agonist GW4064 has been shown to enhance *Bsep* expression resulting in increased bile salt export from mouse hepatocytes.<sup>135</sup> Combined therapy with PXR and FXR agonists holds great promise as a potential remedy for cholestasis symptoms.

Gene defects in bile acid synthesis are often treatable with bile acid supplements. For deficiencies in canalicular export, however, orthotopic liver transplantation or partial external biliary diversion are still the best treatment options. In addition to drug therapy, other remedies have to be explored. Genes that are exclusively expressed in the liver are good candidates for gene therapy: restoration of gene function should confer a selective advantage in bile acid handling to a population of hepatocytes.<sup>136</sup> However, these approaches to curative therapy may be far away.

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