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Hepatobiliary ABC transporters: physiology, regulation and implications for disease

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1. ABSTRACT

The liver plays a key role in the metabolic conversion and elimination of endo- and xenobiotics. Hepatobiliary transport of many of these compounds is mediated by several ATP-binding cassette (ABC) transporters expressed at the canalicular membrane of the hepatocyte. Impaired function of these ABC transporters leads to impaired bile formation or cholestasis and mutations in these genes are associated with a variety of hereditary cholestatic syndromes. At the transcriptional level, these ABC transporters and the metabolizing enzymes involved in processing of their substrates are coordinately regulated by members of the nuclear receptor (NR) family of ligand-modulated transcription factors. In this review we will focus on ABC transporters involved in hepatobiliary excretion and how they are associated with hepatic physiology and disease states. We will also examine how NRs, acting as intracellular sensors for lipophilic molecules, regulate these ABC transporters and maintain metabolic homeostasis.

2. INTRODUCTION

The body is continuously exposed to a wide variety of environmental chemicals (xenobiotics), endogenously synthesized compounds (endobiotics) and metabolic waste products arising from the metabolism of both sources of molecules. The liver plays a central role in their elimination and is equipped with a plethora of detoxification mechanisms. Central to this detoxification are the processes of metabolism and transport, both of which are carried out by superfamilies of specialized proteins the members of which exhibit substrate specificity. Hepatocytes, the most abundant cells in the liver, are polarized epithelial cells with a basolateral (sinusoidal) membrane facing the blood and an apical (canalicular) membrane facing the bile canaliculus (Figure 1). Hepatic uptake is initiated at the basolateral membrane, which is in direct contact with portal blood plasma via the fenestrae of the sinusoidal endothelial cells and the space of Disse. After uptake into hepatocytes, bile acids and other cholephiles reach the canalicular membrane by diffusion

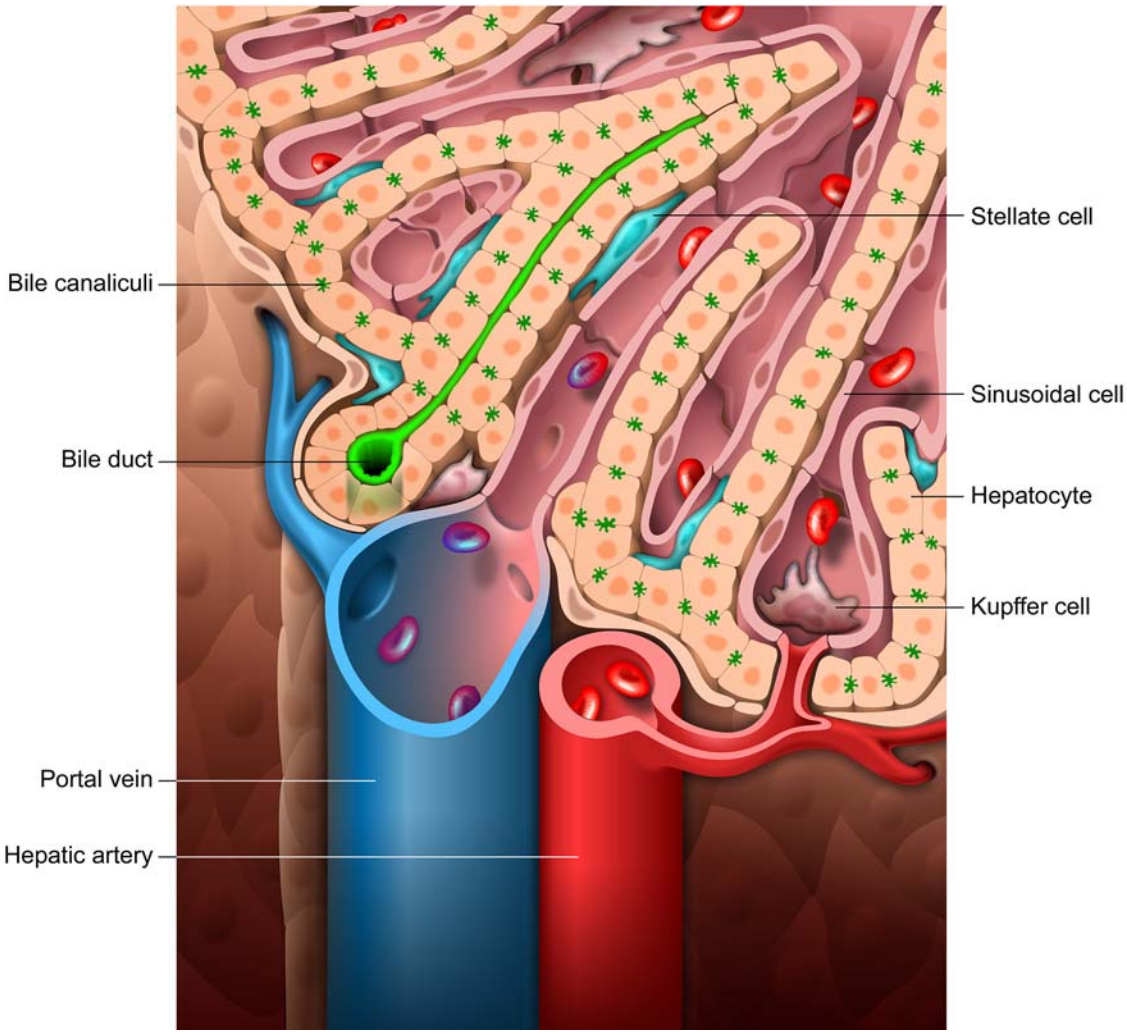

Figure 1. Schematic diagram of the normal liver. At the microscopic level, the liver consists of hexagonal shaped functional units called lobules. These are made up mostly of hepatocytes (the most common type of liver cell) arranged in thin layers that radiate from the central vein to the periphery of the lobule. Bile is produced by the hepatocytes and drains into many small bile ducts (canaliculi) that unite to form larger bile ducts which lead to the gallblader and ultimately drain into the duodenum. Between the radiating rows of hepatocytes are small blood vessels called sinusoids that are lined by sinusoidal endothelial cells (SEC). These receive oxygen-rich blood from the hepatic artery and nutrients from the intestines *via* the portal vein. Within the sinusoids are specialized macrophages called Kupffer cells that are involved in the recycling of erythrocytes. Hepatic stellate cells (HSC) are the primary cell type responsible for the production of collagen I, the key protein involved in the development of liver fibrosis. Adapted from Friedman (150).

either in the aqueous cytoplasm or within intracellular lipid membranes, depending on their hydrophobicity. The canalicular membrane represents the excretory pole of the hepatocyte and forms the border of the bile canaliculus (Figure 2). Canalicular excretion of biliary constituents is the rate-limiting step of bile formation since biliary constituents are excreted against high concentration gradients into bile. The basolateral and canalicular membranes differ in their biochemical composition and functional characteristics and are separated by tight junctions that seal off the bile canaliculi and hence form the only anatomical barrier maintaining the concentration gradient between blood and bile (1). The primary functions of bile are firstly, to act as a detergent for the emulsification of dietary lipids and lipid-soluble vitamins, thereby promoting their intestinal absorption; and secondly, the removal of endobiotics, xenobiotics and their metabolites. The main constituents of normal bile are bile acids (72% of solutes by weight), phospholipids (24%) and cholesterol (4%) (2). Each component is actively excreted into bile by specific ABC transporters expressed at the canalicular membrane and defects in these transporters have been associated with distinct clinical syndromes. Bile acids, which combined with phospholipids form mixed micelles and provide the detergent properties of bile, are conserved through extensive entero-hepatic recirculation. From the total bile acid pool in adult humans (3-4 g), only 1 to 2% per day is lost through fecal excretion and this loss is

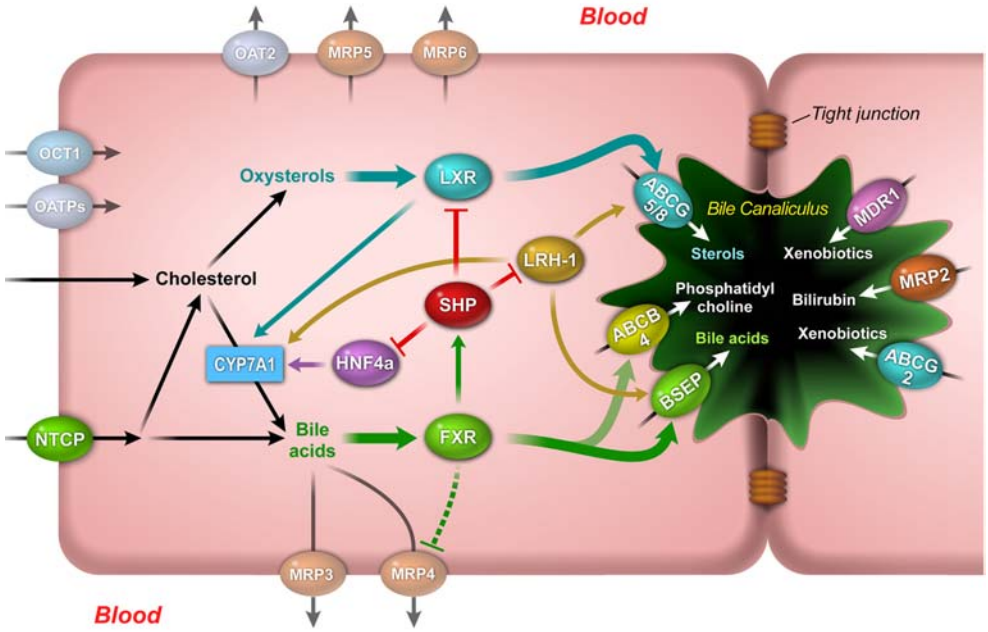

Figure 2. Nuclear Receptor mediated bile acid metabolism and elimination in the hepatocyte. Hepatic uptake of bile acids from the circulation takes place at the sinusoidal membrane of the hepatocyte and is mediated by the sodium-dependent bile acid uptake system NTCP (SLC10A1). Within the hepatocyte, bile acids can be converted to cholesterol or eliminated via bile. In addition, cholesterol can be converted to bile acids by CYP7A1. Bile acids can activate FXR which in turn induces the expression of the bile acid transporter, ABCB11 (BSEP). Negative feedback on bile acid metabolism and elimination is mediated by SHP, which is induced by FXR and inhibits the action of several NRs including LXR, LRH-1 and HNF4-α as well as CYP7A1. Via an alternative pathway, cholesterol is converted to oxysterols that can activate LXR which in turn induces the expression of the sterol transporters ABCG5/8 at the canalicular membrane. Other canalicular transporters are the phospholipid export pump ABCB4 (MDR2/3) which mediates excretion of phosphatidylcholine (PC); ABCC2 (MRP2) which mediates excretion of bilirubin; and the multidrug transporters ABCB1 (MDR1, P-glycoprotein) and ABCG2 (BCRP) which mediate excretion of a wide variety of xenobiotics. During cholestasis bile acids can also be excreted back into the circulation via the sinusoidal ABC transporters ABCC3 and -4 (MRP3,4).

compensated by de novo bile acid synthesis from cholesterol in the liver (3). Hepatic re-uptake of bile acids at the sinusoidal membrane is mediated by the sodium-dependent bile acid uptake system NTCP (SLC10A1) and by several members of the organic anion transporting polypeptide (OATP) family that facilitate sodiumindependent bile acid uptake (4). Within the hepatocyte, the majority of bile acids are bound to cytosolic proteins and traverse the cell by diffusion. At the canalicular membrane, active transport of bile acids results in canalicular bile acid concentrations that are about 100- to 1000-fold higher than in portal blood (3). This strong osmotic gradient attracts water and provides the driving force for bile flow. Impaired transport of bile acids or phospholipids will compromise bile flow and consequently lead to cholestasis.

Besides its role in digestion, bile also serves as a major excretory route for many endobiotics, such as cholesterol and the heme catabolite bilirubin, which like bile acids and phospholipids have specialized ABC transporters mediating their excretion. In addition, there are several polyspecific ABC transporters which have a broad substrate specificity and act as "vacuum cleaners" to eliminate a wide range of compounds, particularly xenobiotics such as therapeutic drugs. Altered expression of a subset of canalicular transporters can potentially impact on the pharmacokinetics and therefore the efficacy and toxicity of a broad range of medications. In this review we will focus on hepatocyte apical ABC transporters involved in biliary excretion and how they are associated with hepatic physiology and disease states. We will also examine how nuclear receptors, acting as intracellular sensors for lipophilic molecules, regulate these ABC transporters and maintain metabolic homeostasis.

3. HEPATOCYTE APICAL ABC TRANSPORTERS IN PHYSIOLOGY AND DISEASE

3.1. ABCB1 (MDR1/P-glycoprotein)

P-glycoprotein was discovered over 30 years ago by its capacity to confer multidrug resistance (MDR) in a Chinese hamster ovary cell line that was selected for resistance to colchicine (5). It was the first member of what turned out to be a superfamily of ATP-binding cassette (ABC) transport proteins, with 48 members in humans, many of which are associated with disease states (6-8). In 1999, P-glycoprotein was renamed ABCB1 by the Human Genome Nomenclature Committee. Throughout this review we will use the new nomenclature and, where necessary,

indicate alternative names for clarity. Ten years after its discovery, it was demonstrated that ABCB1 is encoded by the *MDR1* gene, which had been shown independently to be associated with multidrug resistance in cultured cells (9-10). Most of the work on ABCB1 in the two decades after its discovery focused on the characterization of its molecular and biochemical properties and its relevance to chemotherapeutic cytotoxic drug resistance in cells and tumors. ABCB1 is a large (approximately 1280 amino acids), *N*-glycosylated, membrane-spanning protein that functions as an ATP-driven efflux pump. It is localized in the plasma membrane of the cell, where it can actively extrude a variety of drugs. The polypeptide chain consists of two similar halves, each containing six putative transmembrane segments and an intracellular ATP-binding site. Hydrolysis of ATP provides the energy for active substrate export, which can occur against a large concentration gradient. Its most striking property is its broad substrate specificity for a wide range of structurally and functionally unrelated compounds, including a vast number of drugs covering many therapeutic applications. This broad substrate specificity also explains extensive cross-resistance observed in drug resistant cells and tumors that overexpress ABCB1.

ABCB1 is expressed in a variety of pharmacologically important epithelial barriers, such as the intestinal epithelium, the blood-brain and blood-nerve barrier, the blood-testis barrier, and the materno-fetal barrier formed by placental trophoblasts. ABCB1 is also expressed at the biliary canalicular membrane of hepatocytes and at the brush border of renal proximal tubule cells. The pharmacological and physiological significance of ABCB1, however, remained obscure until *Abcb1a* knockout mice were created (11). Humans have one gene encoding *ABCB1*, whereas mice have two genes, *Abcb1a* (*Mdr3, Mdr1a*) and *Abcb1b* (*Mdr1, Mdr1b*). The tissue distribution of mouse Abcb1a and Abcb1b suggests that together they fulfil the same function as ABCB1 in humans. Although, *Abcb1* knockout mice were viable and fertile and at first appeared phenotypically normal, a serendipitous event provided the first insight into one of its major functions. When these mice were treated with a topical spray of the anti-parasitic ivermectin, a routine procedure to treat mite infestation, almost all the knockout mice died whereas wild-type mice were unaffected (11). Further analysis revealed that the mice had died of neurotoxicity due to dramatically increased brain penetration of ivermectin. It turned out that Abcb1a has an important function in the blood-brain barrier. Abcb1a, expressed in luminal membrane (*i.e.* facing the blood) of the endothelial cells of small capillaries, prevents the entry of substrate drugs into the brain by actively pumping them back into the circulation. Absence of Abcb1a from the blood-brain barrier, can lead to up to 10- to 100-fold increased brain penetration of substrate drugs, thus turning normally harmless agents like ivermectin into lethal neurotoxins. Interestingly, a deletion mutation of the *ABCB1* gene was also shown to be responsible for the ivermectin sensitivity of a subpopulation of collie dogs (12). A few years later, Sparreboom *et al.* (13) were the first to demonstrate role of ABCB1 in limiting oral bioavailability and mediating biliary excretion of drugs. After intravenous or oral administration, plasma levels of the anticancer drug paclitaxel were 2- and 6-fold increased in *Abcb1* knockout mice as compared to wild-type mice, respectively. This increase was due to a combination of increased intestinal absorption and decreased elimination into bile. The precise role of ABCB1 in direct intestinal excretion and biliary transport was further elucidated in mice with a cannulated gallbladder allowing repeated bile sampling (13-15). Studies using *Abcb1* knockout mice have also demonstrated a protective role in a variety of other tissues and barriers including the materno-fetal barrier, blood-testis barrier and bone marrow (16). It has now become clear that ABCB1 is a key player in the defense of the body against xenotoxins with the result that the main focus of ABCB1 research has shifted from its role in drug resistant cancer to its pharmacological functions.

3.2. ABCB4 (MDR2/3 P-glycoprotein)

The search for additional proteins that could confer multidrug resistance independently led to the isolation of a gene highly homologous to *ABCB1* in two laboratories, and was named *MDR2* and *MDR3*, respectively (17-18). Nomenclature for this gene has been extremely confusing as rodents have two genes encoding MDR1 P-glycoprotein (*Abcb1*), one of which was originally also named *Mdr3*. For clarity, these genes have later been renamed to *Mdr1a* (*Mdr3*) and *Mdr1b* (*Mdr1*) and ultimately to *Abcb1a* and *Abcb1b*. *Mdr2* in rodents is the ortholog of human *MDR2/3* and is now officially renamed *ABCB4*. ABCB4 is expressed in a variety of tissues including liver, heart, skeletal muscle and B lymphocytes (18-19). However, unlike ABCB1, ABCB4 was not involved in conferring the MDR phenotype and its function remained unknown for several years until knockout mice were generated (20). *Abcb4* knockout mice displayed progressive liver damage at an early age, which was accompanied by hyperbilirubinemia and increased liver enzymes in plasma. Further analysis revealed the absence of phospholipids and dramatically reduced levels of cholesterol and glutathione in bile, whereas bile flow itself was about 2-fold increased (20). A link with a human disease was made when De Vree *et al.* (21) showed that *ABCB4* is mutated in patients with progressive familial intrahepatic cholestasis type 3 (PFIC3), a subgroup of PFIC characterized by reduced or absent phospholipid excretion into bile and increased serum levels of γGT. Later it was established that ABCB4 functions as a phospholipid flippase, promoting the transfer of phosphatidylcholine from the inner to the outer leaflet of the plasma membrane lipid bilayer (22). Phospholipids are essential constituent of the bile and act to reduce the detergent activity of bile acid micelles, thereby protecting the membranes of cells lining the biliary tree from damage. In the absence of phospholipids, bile acid toxicity results in damage to cholangiocytes and progressive cholestatic liver injury accompanied by increased serum levels of γGT, as seen in PFIC-3.

3.3. ABCB11 (BSEP/S-PGP)

By low stringency screening for novel proteins that might confer MDR, another close homolog of P-

glycoprotein was identified and named sister of P-glycoprotein (s-Pgp) (23). The function of s-Pgp, which was later renamed ABCB11, remained unknown until Gerloff *et al.* (24) demonstrated that it represents the hepatocyte canalicular bile salt export pump (BSEP). Based on the earlier observation by Nishida *et al.* (25), that transport of bile acids is ATP-dependent, Gerloff *et al.* (24) demonstrated transport of bile acids by ABCB11 *in vitro*, using cRNA injected *Xenopus laevis* oocytes and vesicles isolated from transfected Sf9 cells (24). Shortly thereafter, the human ortholog was cloned and found to be mutated in progressive familial intrahepatic cholestasis type 2 (PFIC2) (26). PFIC2 is an autosomal recessive disorder characterized by early onset intrahepatic cholestasis, jaundice, pruritus and progression to hepatic fibrosis, cirrhosis and endstage liver disease before adulthood. PFIC2 patients exhibit a 100-fold reduction in bile acid secretion into bile resulting in the accumulation of bile acids within hepatocytes, liver injury and cholestasis. Unlike patients with PFIC3 (see § *3.2.*), serum levels of cholesterol and γGT are usually normal or only mildly elevated. Mutations in ABCB11 have also been associated with two milder cholestatic syndromes: 1) benign recurrent intrahepatic cholestasis type 2 (BRIC2), which is characterized by intermittent episodes of cholestasis without progression to liver disease and 2) intrahepatic cholestasis of pregnancy (ICP), which is associated with increased risk of intrauterine fetal death and prematurity (27-28). Although the functional consequences of most mutations in ABCB11 are still unknown, several mutations have been demonstrated to result in impaired activity, stability or trafficking to the membrane (29-30). The severity of the different cholestatic phenotypes has further been demonstrated to correlate with activity and levels of expression of ABCB11 (31). In contrast to humans with PFIC2, targeted inactivation of *Abcb11* in mice resulted only in a mild non-progressive cholestasis (32). Surprisingly, although secretion of cholic acid (CA), the major bile acid in mice, was greatly reduced (to 6% of wild-type), total bile acid output in mutant mice was still about 30% of wild-type. Also, secretion of an unexpectedly large amount of tetrahydroxylated bile acids, which were not present in wild-type mice, was observed. These results suggested that hydroxylation and an alternative canalicular transport mechanism for bile acids could compensate for the absence of Abcb11 and protect the mutant mice from severe cholestatic liver injury (33). *Abcb11* knockout mice fed with a diet supplemented with CA displayed a more severe PFIC2 phenotype, indicating that with bile acid loading this compensatory transport was not sufficient (33). Further analysis of the *Abcb11* knockout mice showed that expression of Abcb1 (Mdr1) was markedly increased, especially after CA feeding, while Abcb4 (Mdr2), Abcc2 (Mrp2), and Abcc3 (Mrp3) were increased only to a moderate extent (34). Moreover, plasma membrane vesicles isolated from a cell line overexpressing ABCB1 exhibited ATP-dependent bile salt transport, albeit with a 5-fold lower affinity compared to ABCB11. These findings suggested that, in mice Abcb1 may act as a compensatory bile acid transporter, and could explain the relatively mild phenotype of *Abcb11* knockout mice (34). In keeping with the more severe phenotype in humans, no upregulation of ABCB1 was found in PFIC2 patients (35). In addition to its role in PFIC2, *Abcb11* has been mapped to the *Lith1* locus for gallstone susceptibility in mice (36). Interestingly, the *Lith1* locus also harbors the nuclear receptor LXRα, which is associated with gallstone formation through the regulation of cholesterol transport by ABCG5/8 (see § *3.5.*). The role of Abcb11 in the formation of gallstones was confirmed by the finding that gallstone-susceptible C57L/J mice (Lith1 mice) displayed increased levels of Abcb11 as compared to gallstone-resistant AKR/J mice (37-38) and further by the fact that transgenic mice overexpressing hepatic Abcb11 rapidly developed cholesterol gallstones (39).

3.4. ABCC2 (MRP2/cMOAT)

ABCC2 (previously known as MRP2 or canalicular multispecific organic anion transporter (cMOAT)) was independently identified in two laboratories based on its similarity with ABCC1 and absence of its expression in homozygous MRP2-deficient rats and humans (40-41). The *in vivo* function of ABCC2 was elucidated prior to identification of its encoding gene, as ABCC2 is effectively deficient in two mutant rat strains (TR−/GY and EHBR), and in patients that suffer from the Dubin–Johnson syndrome (42-46). Affected individuals suffer from a recessively inherited conjugated hyperbilirubinemia, which can result in clinically apparent jaundice, but overall the phenotype of this disease is relatively mild. The cause of the defect is the absence of ABCC2, from the hepatocyte canalicular membrane, where it normally mediates the hepatobiliary excretion of (amongst others) mono- and bis-glucuronidated bilirubin. Although many mutations in the *ABCC2* gene have been identified, only some of them result in Dubin-Johnson syndrome. ABCC2 expression is highest in the canalicular membrane of the liver, but it is also expressed in the kidney, jejunum, and ileum, where it may also be involved in the elimination of toxic compounds from the body (47). Recently, *Abcc2* knockout mice have been generated (48-50). These mice display hyperbilirubinemia and reduced levels of biliary glutathione but the overall phenotype is relatively mild as compared to humans and rats.

3.5. ABCG5/ABCG8 (Sterolin 1 and 2)

ABCG5 and ABCG8 have been identified as the major sterol transporters (51-53). ABCG5 and -8 are half-transporters, primarily expressed in liver and intestine, where they function as an obligate heterodimer to limit the intestinal absorption and promote biliary excretion of dietary sterols. In this way, they provide the body with a mechanism to selectively limit systemic exposure to plant sterols while allowing absorption and retention of cholesterol. Mutations in these genes are associated with sitosterolemia (also known as phytosterolemia), a rare autosomal recessive disorder characterized by elevated plasma and tissue levels of plant sterols, xanthomatosis and increased risk for atherosclerosis (54). Disruption of *Abcg5/8* in mice resulted in a 2- to 3-fold increase in fractional absorption of dietary plant sterols, and was associated with a 30-fold increase in plasma sitosterol (55). The accumulation of plant sterols in *Abcg5/8* null mice was further associated with an overall decrease in cholesterol

production and secretion (55-56). This altered cholesterol homeostasis is suggested to be mediated by two critical regulatory pathways; first, *via* activation of LXR by selective plant sterols such as stigmasterol, resulting in increased elimination of cholesterol through upregulation of the cholesterol transporter ABCA1, and second, *via* inhibition of *de novo* cholesterol synthesis (56). Conversely, genetic overexpression or pharmacological induction of ABCG5/8 through LXR activation resulted in increased cholesterol secretion and reduced absorption (57-58). By quantitative trait locus (QTL) and genome wide SNP analysis, ABCG5/8 have also been linked to the formation of cholesterol gallstones in mice and humans, respectively (59-62). In humans, gallstone risk was specifically attributable to a G-to-C transversion corresponding to an Asp19His (D19H) substitution in the *ABCG8* gene (61-62). These findings suggest that D19H is a gain of function mutation resulting increased efficiency of cholesterol transport into the bile lumen, causing cholesterol hypersaturation of bile and promoting the formation of gallstones. This is in line with previous studies suggesting that this D19H variant results in increased ABCG8 activity (63-64).

3.6. ABCG2 (BCRP/MXR/ABCP)

Besides ABCB1 (MDR1) and ABCC1 (MRP1), ABCG2 was the third transporter found to confer multidrug resistance (MDR), and together they account for most if not all MDR activity observed in cell lines. The *ABCG2* gene was first cloned based on its overexpression in a highly doxorubicin-resistant MCF-7 breast cancer cell line (65-66). Despite reported expression in a variety of tumors, its role in clinical drug resistance is still unclear (67). ABCG2 is a half-transporter that functions as a homodimer in the plasma membranes of a variety of mostly epithelial cells (68). ABCG2 is present at strategic sites in the body, such as the intestine, placenta and blood-brain barrier, where it protects the organism by limiting the systemic and tissue penetration and hence toxicity of xenotoxins. In addition, ABCG2 is strongly induced in the mammary gland during lactation where it is responsible for the active secretion of substrates into milk (69-70). The *in vivo* function of ABCG2 was first illustrated by pharmacological inhibition of ABCG2 in mice, demonstrating its role in limiting oral absorption, mediating biliary excretion and fetal protection (71-72). ABCG2 has also been shown to be expressed in hematopoietic and many other stem cells, where it may have a protective function, or play a role in maintaining progenitor cells in an undifferentiated state (73-74). ABCG2 can transport a structurally and functionally diverse range of organic substrates, including hydrophobic compounds, weak bases, organic anions, and glucuronide-, sulfate-, glutamylate- and glutathione-conjugates of many endogenous and exogenous molecules. *Abcg2* knockout mice have been generated independently in two laboratories (75-76) being viable and healthy but displaying an extreme sensitivity to the dietary chlorophyll breakdown product pheophorbide A, resulting in severe, sometimes lethal phototoxic lesions on light-exposed skin (75). *Abcg2* knockout mice also display a unique type of porphyria, not caused by a defect in one of the enzymes of the heme biosynthetic pathway, in contrast to the typical hereditary porphyrias (75). Porphyrias are metabolic disorders characterized by increased intracellular levels of porphyrins, the precursors of heme, and a subset are associated with skin photosensitivity in affected patients (77). In addition, one of the hallmarks of erythropoietic protoporphyria (EPP) is the deposition of protoporphyrin IX (PPIX) in the liver, causing progressive and sometimes fatal liver damage (78). PPIX can only be removed from the liver *via* biliary excretion and recently ABCG2-mediated transport of conjugated PPIX has been implicated in this process (79). In this way, ABCG2 is believed to function as an overflow system, allowing the liver to eliminate excess PPIX by high-affinity transport of its conjugates, thereby preventing or reducing its cytotoxicity.

4. REGULATION OF HEPATIC TRANSPORT AND METABOLISM BY NUCLEAR RECEPTORS

4.1. Nuclear Receptors

The Nuclear Receptor (NR) superfamily is comprised of 48 individual transcription factors that serve as pleiotropic and prototypic regulators of cellular differentiation and function. This stems in part from their ability to function as ligand-dependent sensors for steroid hormones, fat soluble hormones and metabolic intermediates, as well as dietary lipids. NRs are widely implicated in disease states and encompass one of the most successful therapeutically and pharmacologically validated drug targets. NRs bind to sequence-specific DNA response elements on target gene promoters as homodimers, heterodimers, or monomers. Structural and functional analyses of the NR superfamily have demonstrated that the receptors are comprised of functional modular domains (80) (Figure 3). A highly variable N-terminal region contains a ligand-independent activation domain called AF-1. The central DNA-binding domain (DBD), consisting of two highly conserved zinc-finger motifs unique to NRs, targets the receptor to specific DNA sequences called hormone response elements (HRE). A typical HRE consists of two hexa-nucleotide motifs AGGTCA or its variants, separated by a gap of several nucleotides. Binding specificity by various receptors is largely achieved by the spacing (the 3-4-5 rule) and the orientation of two half-sites (direct-, inverted- or everted-repeat). The hinge region confers structural flexibility in the receptor dimers allowing a single receptor dimer to interact with multiple HRE sequences. The C-terminal ligand-binding domain (LBD) is functionally very unique to NRs and responsible for ligand recognition, receptor dimerization and cofactor interaction.

Through recent extensive NR-expression profiling studies we now know a great deal about the spatial and temporal expression profiles for the 49 murine NRs (81-82). Of interest to this review, it was found that a large number of the NR family members are expressed in the liver and gut, 37 and 41 respectively. Eight NRs are effectively limited in expression to these tissues, including the farnesoid X receptor (FXR, NR1H4), liver receptor homolog-1 (LRH-1, NR5A2), small heterodimer partner (SHP, NR0B2), hepatocyte nuclear factor-4α and -γ (HNF-4α,γ NR2A1,2), the vitamin D receptor (VDR, NR1I1), pregnane X receptor (PXR, NR1I2), and constitutive

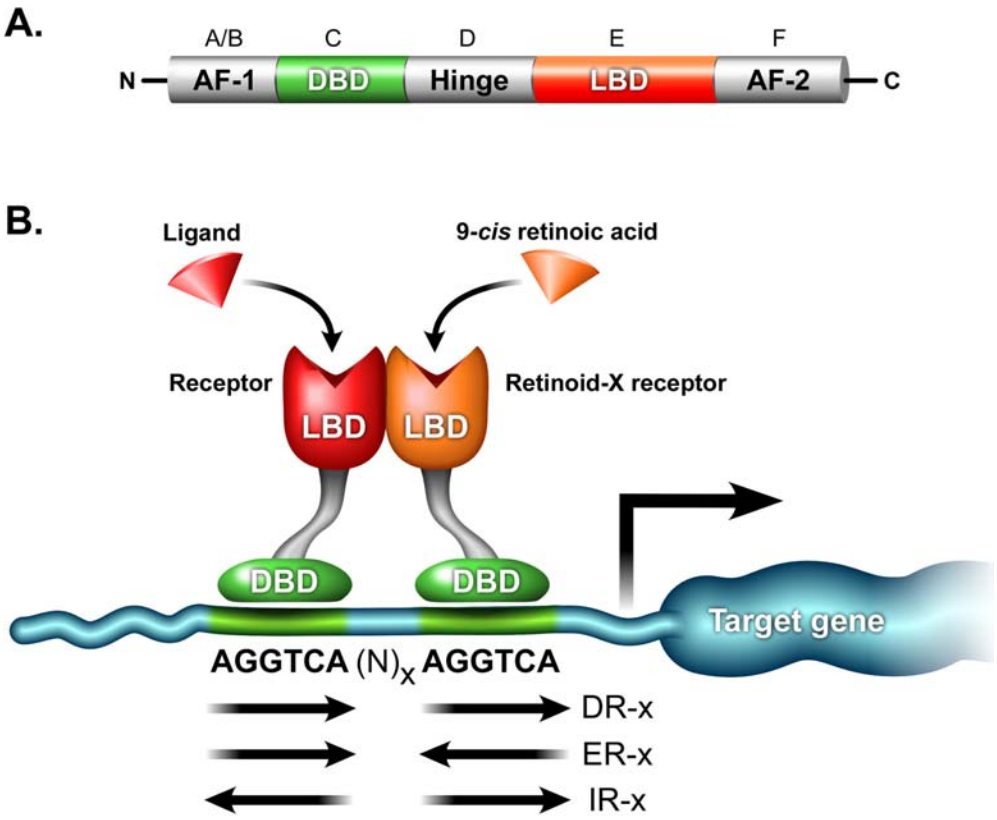

Figure 3. Structure and DNA binding of nuclear receptors. (A) Schematic diagram for a common domain structure of NRs which include N-terminal activation function 1 (AF-1), DNA binding domain (DBD) consisting of two zinc fingers (ZF), hingeregion (Hinge), ligand binding domain (LBD), and C-terminal AF-2. (B) Schematic diagram for NR dimerization and DNA binding. Nuclear receptors bind as heterodimers with RXR to repeats of the nucleotide hexamer AGGTCA with variable spacing. The hexamers can be arranged either as direct repeats (DR), everted repeats (ER), or inverted repeats (IR).

androstane receptor (CAR, NR1I3). In addition, liver X receptors (LXRα and β, NR1H3 and 2) are abundant in liver and play an important roles in hepatic cholesterol transport and metabolism. Numerous studies have now demonstrated that NRs play key roles in many of the diverse of signaling and homeostatic processes of the enterohepatic axis including digestion, lipid and energy homeostasis as well as inflammation (83). Perhaps one of the major ways by which NRs achieve this complex regulation is through their ability to regulate at the transcriptional level the synthesis of endobiotics and the degradation and transport of both endobiotic and xenobiotic molecules in the liver.

4.2. Regulation of bile acid transport and metabolism by FXR

Bile acids are endogenous ligands for several NRs that in turn regulate bile acid synthesis, hydroxylation, and transport into and out of the hepatocyte *via* the basolateral and apical membrane transporters. Specifically, bile acids directly activate FXR, PXR and VDR, with differing ligand specificities for individual bile acids. FXR (also known as BAR, bile acid receptor) was the first NR identified to have bile acids as endogenous and physiologically relevant ligands. FXR is closely related to LXR, and both belong to the NR1H subfamily of NRs (84). FXR is abundantly expressed in the liver and intestine as well as the kidney and adrenal gland (85). Bile acids function as endogenous ligands for FXR, with differing potency. *In vitro* studies indicate that chenodeoxycholic acid (CDCA) is a potent FXR ligand at physiological concentrations, whereas others such as lithocholic acid (LCA), deoxycholic acid (DCA), and CA are less effective, and muricholic acids do not activate FXR (86-87). Interestingly, although LCA can by itself act as a weakly agonistic ligand for FXR, it strongly antagonizes CDCA-stimulated activation of FXR (88). Synthetic agonists that mimic the ability of bile acids to activate the FXR include the isoxazole derivative GW4064 (89), and fexaramine (90). FXR transcriptionally activates *ABCB11* (91-93), and bile acids increase *ABCB11* expression in primary hepatocytes or HepG2 cells with the same rank order of potency that activates FXR (94). Conversely, the secondary bile acid LCA decreases *ABCB11* expression by antagonizing FXR activation (88). An important mechanism of drug-induced cholestasis is inhibition of ABCB11, with accumulation of bile acids in hepatocytes and subsequent liver injury. Examples of such drugs include cyclosporin A, rifampicin and glibenclamide. Reductions in ABCB11 have also been implicated in

sepsis-induced cholestasis (4). Recently, an LRH-1 response element (LRHRE) was identified in the human *ABCB11* promoter and overexpression of LRH-1 was shown to induce expression of ABCB11, suggesting that the NR LRH-1 supports FXR in the regulation of bile acid levels (95).

FXR also downregulates many target genes indirectly *via* transcriptional induction of another NR, small heterodimer partner (SHP, NR0B2) (96-97). SHP is an atypical member of the NR subfamily as it lacks a DNA-binding domain. SHP can interact with and negatively affect the transcriptional activity of several other members of the NR subfamily, including LXR, LRH-1, HNF-4α (Figure 2), as well as transcription factors belonging to the basic-helix-loop-helix family. It appears that SHP-mediated repression involves competition with transcriptional coactivators for access to DNA-bound transcription factors. SHP also contains a strong transcriptional repressor domain in its carboxy-terminus, which may contribute to the SHP-mediated repression (98-99).

4.3. Regulation of bile acid metabolism through CYP7A1

The first and rate-limiting enzyme in the neutral pathway of bile acid biosynthesis is cytochrome P450 7A1 (CYP7A1), a liver-specific microsomal cytochrome P450, catalyzing the formation of 7α-hydroxycholesterol from cholesterol. CYP7A1 regulation is controlled by a variety of factors, including hormones, oxysterols, bile acids, drugs and diurnal rhythms (100). In rodents, LXR binds to a direct repeat NR motif (DR-4) in the Cyp7a1 promoter when activated by oxysterols, and strongly induces *Cyp7a1* transcription (101). Interestingly, LXR does not activate human *CYP7A1* expression in the same way, which means that humans are more susceptible to developing hypercholesterolemia from a high cholesterol diet than rodents (102). The *CYP7A1* proximal promoter also contains a negative bile acid response element (BARE), that can bind two nuclear receptors: monomeric LRH-1, and homodimeric HNF-4α (103-104). PGC-1α is a versatile coactivator for many nuclear receptors, and has been shown to increase the HNF-4α-mediated transactivation of CYP7A1, as well as other genes (105). Bile acids negatively regulate bile acid synthesis *via* CYP7A1, by several mechanisms. By activating FXR, bile acids induce the expression of SHP, which in turn negatively interacts with LRH-1, and possibly HNF-4α to inhibit the CYP7A1 gene (96). However, bile acids are still able to repress Cyp7a1 expression in *SHP* knockout mice, suggesting the presence of redundant mechanisms (106). Bile acids can also activate PXR, and recent work has suggested that activated PXR interferes with HNF-4α signaling by competing for PGC-1α in hepatic cells, resulting in dissociation of PGC-1α, and suppression of the CYP7A1 gene (107). Additionally, FXR directly activates the transcription of the fibroblast growth factor 19 (FGF-19) (108) which, via the intracellular JNK (c-Jun N-terminal kinase) pathway, leads to reduced CYP7A1 expression (106).

4.4. Regulation of ABCC2, ABCB4 and ABCG5/8

ABCC2 can transport a variety of compounds including bilirubin diglucuronide, sulfates, some bile acids (*e.g.* conjugates of LCA), xenobiotics (*e.g.* cisplatin, anthracyclines, vinca alkaloids, methotrexate), as well as glutathione conjugates into bile, and is therefore a major determinant of bile acid-independent bile flow (109). Once bile acids have been excreted into bile, they stimulate the release of phosphatidylcholine (PC) and cholesterol from the outer leaflet of the canalicular membrane, which then form mixed micelles in bile. By doing so, bile acid toxicity to the bile duct epithelium is avoided, which would otherwise occur due to an unopposed detergent action. It has been suggested that bile acids can regulate ABCC2 expression, since CDCA, an FXR ligand, can induce the expression of *ABCC2* mRNA in human and rat hepatocytes (1). An atypical promoter everted repeat element (ER-8) has been identified within the rat *Abcc2* promoter that is involved in the ligand-mediated induction of Abcc2 by FXR, PXR and CAR in cultured cells (110). Subsequently, *in vivo* studies of mice with cholestasis induced by common bile duct ligation have found regulation of Abcc2 to be independent of FXR (111). Induction of Abcc2 in the liver of *PXR* wild-type but not knockout mice has been reported after administration of pregnenolone 16α-carbonitrile (PCN) or CA (112-113), implying a significant *in vivo* role for PXR in regulation of Abcc2.

In humans, ABCB4 is induced in cholestasis (114), and is regulated by FXR (115). Trans-activation of *ABCB4* by FXR has been demonstrated through direct binding of FXR/retinoid X receptor α (RXRα) heterodimers to a highly conserved inverted repeat element (FXR response element) at the distal promoter (115). In rats, Abcb4 is induced by the FXR agonist GW4064 (116), but can still be induced in *FXR* knockout mice fed a CA diet (117), suggesting that several bile acid-responsive regulatory mechanisms must be capable of inducing this gene. In mice, another NR, peroxisome-proliferator activated receptor α (PPARα, NR1C1) has also been shown to be involved in Abcb4 regulation (118).

ABCG5 and ABCG8 facilitate biliary removal of neutral sterols, and are coordinately upregulated at the transcriptional level by dietary cholesterol. These genes are direct targets of LXR (119), a NR that regulates the expression of may key genes involved in lipid metabolism and energy homeostasis. Additionally, a binding site for LRH-1 has been identified in the ABCG5/8 intergenic region necessary for the activity of both the ABCG5 and ABCG8 promoters (120).

4.5. Regulation of the multidrug transporters ABCB1 and ABCG2

The transcriptional regulation of ABCB1 is complex, and numerous transcription factors have been implicated in its regulation (121). A large number of drugs have been identified as either substrates or inhibitors of ABCB1, and a range of exogenous stimuli can increase transcription of the *Abcb1* promoter (122). Bile acids and their conjugated metabolites are not substrates for ABCB1; however certain substrates for ABCB1, including drugs, may also inhibit ABCB11 if they accumulate in the liver (1). Bile flow remains normal in *Abcb1a/b* knockout mice, and organic cation excretion is only modestly impaired. *In*

vitro studies have implicated rifampicin- and paclitaxel-mediated activation of PXR in the induction of ABCB1 expression in human colon carcinoma cell lines, and induction of *Abcb1b* by a range of xenobiotics has also been shown to be dependent on PXR (123-124). However, in a study involving administration of CA to mice, *Abcb1a* expression was induced independently of PXR and FXR (125), and levothyroxine was demonstrated to upregulate *Abcb1* independently of PXR (126), suggesting that induction by endobiotics differs from induction by xenobiotics. More recently, physiological concentrations of bile acids were shown *in vivo* to induce *Abcb1a* and *Abcb1b* via FXR, independently of PXR (127), demonstrating the complexity of *in vivo* regulation of these transporters.

ABCG2 has been shown to be transcriptionally regulated by the Estrogen Receptor α (ERα, NR3A1). An estrogen response element (ERE) was found in the ABCG2 promoter and 17β-estradiol (E2) enhanced the expression of ABCG2 mRNA in estrogen receptor ER-positive T47D:A18 cells and PA-1 cells stably expressing ERα (128). ABCG2 is abundant in the placenta and is highly induced in the mammary gland during pregnancy suggesting a physiological function for ER in the regulation of ABCG2 in these tissues. Recently, ABCG2 has been shown to be regulated by PPARγ (NR1C3) in human dendritic cells (129). Three PPAR/RXR binding sites were identified upstream of the *ABCG2* gene as well as an increased ABCG2 mRNA and protein expression following treatment with the PPARγ agonist rosiglitazone. The physiological function of this PPARγ-dependent upregulation of ABCG2, and whether it is restricted to cells of the myeloid lineage, is not known.

4.6. Implications for cholestatic liver disorders

Cholestatic liver disorders include a spectrum of hepatobiliary diseases of diverse etiologies that are characterized by impaired hepatocellular secretion of bile, resulting in accumulation of bile acids, bilirubin and cholesterol. Causes of cholestasis include extrahepatic biliary obstruction (*e.g.* stones, tumors), intrahepatic biliary obstruction (*e.g.* primary biliary cirrhosis, primary sclerosing cholangitis) and intrahepatic cholestasis (*e.g.* drugs, genetic transporter defects, or infections) (130-131). When there is complete absence of bile flow (as in biliary obstruction), there is an absence of bile acids in the small intestine, an increase in bile acids in the hepatocyte and in plasma, and an increase in the urinary excretion of bile acids. Phospholipids, cholesterol and conjugated bilirubin that were destined for biliary secretion also accumulate in the plasma in cholestasis (132). The major abnormalities in bile acid metabolism in patients with cholestasis are elevation of circulating levels of primary bile acids, increased formation of sulfated bile acids, and a shift to renal excretion as a major mechanism for bile acid elimination. In particular, relatively hydrophilic tetrahydroxy bile acids are formed and excreted in urine. The ratio of the serum concentration of CA to CDCA increases, the proportion of unconjugated bile acids is reduced, and concentrations of the secondary bile acid DCA acid decrease in advanced cholestasis (132). These changes have physiological consequences, with absence of intestinal bile acids resulting in fat maldigestion because of absence of micelle formation, and malabsorption of fat-soluble vitamins. Increased circulating bile acids may cause pruritis (132), and in the hepatocyte are likely to induce apoptosis or necrosis because of their detergent properties (133). Progressive hepatic fibrosis and cirrhosis can ensue leading to death due to hepatic failure or the complications from portal hypertension.

Pharmacological therapy for cholestasis is limited, and ursodeoxycholic acid (UDCA) is the only disease-modifying drug therapy with evidence of efficacy, improving symptoms, hepatic enzyme abnormalities, and reducing death and liver transplantation in patients with primary biliary cirrhosis (134-135), and improving both maternal and fetal outcomes in cholestasis of pregnancy (136). However a majority of patients are incomplete responders to UDCA (137), and UDCA has not been demonstrated to be efficacious in other forms of cholestasis, such as primary sclerosing cholangitis. There is therefore a need for novel therapies for treatment of cholestasis, both to delay progression of liver disease and relieve associated symptoms. The physiological response to cholestasis generally involves downregulation of the hepatocyte basolateral uptake transporters (114) and upregulation of the basolateral efflux transporters (Donner *et al.*, 2001). Interestingly, the apical transporter function is often preserved. Abcb11 expression is only modestly impaired, or preserved, both in animals with bile duct obstruction (111) and in humans with cholestasis (114, 139). ABCB4 and ABCB1 are both induced in humans with cholestasis (114), suggesting that bile acids that are specific ligands for FXR may help to maintain expression of these transporters during cholestatic injury. NR-mediated regulation has a marked impact on the development of hepatic damage in cholestasis, and this is most clearly demonstrated in *NR* knockout mice subjected to various models of cholestasis and/or bile acid overload. Mice with deletion of PXR or CAR have an increase in the areas of hepatic necrosis and bile infarcts after injection of LCA (140-141), or bile duct ligation (142). Mechanisms for this include loss of NR-mediated bile acid detoxification mechanisms, encompassing both metabolism and transport. Conversely, PXR activation by PCN protects wild-type mouse livers against necrosis caused by LCA (140-141). Similarly, the *FXR* knockout phenotype demonstrates altered bile acid and lipid homeostasis (117), and an altered response to various animal models of cholestasis. High concentrations of dietary CA cause a marked increase in serum, liver and urine bile acid concentrations, and severe hepatotoxicity in *FXR* knockout mice, associated with the loss of expression of Abcb11, and reduced biliary elimination of bile acids (117, 125, 143). However in a bile duct ligation (BDL) model of complete biliary obstruction, *FXR* knockout mice had a mortality and morbidity advantage, and were protected from developing hepatic bile infarcts, even with concurrent deletion of PXR. This protection is probably secondary to downregulation of the FXR-regulated apical transporters ABCB11, ABCB4 and ABCB1, reducing pressure in obstructed bile ducts, as well as other effects including upregulation of the sinusoidal

ABC transporter ABCC4 (MRP4) which mediates transport of bile acids back into the circulation (127, 144). These findings suggest a role for targeted therapy for different cholestatic syndromes, and specifically, a clinical role for FXR antagonists in the treatment of obstructive cholestasis, and PXR agonists in other cholestatic syndromes.

5. PERSPECTIVE

As covered in this review, the apical hepatocyte ABC transporters determine both the composition and flow of bile. It follows that any disease process in which either of these factors is important could potentially benefit from pharmacological manipulation of these transporters. The most obvious disease candidates are the cholestatic liver disorders, as covered in § *4.6*. In addition, gallstones, the most common of all biliary diseases, should be largely preventable in high-risk individuals if bile can be manipulated to reduce lithogenicity, particularly lowering of cholesterol content, lowering of deoxycholic acid concentration and maintenance of bile flow (145). Direct manipulation of the transporter proteins to achieve these therapeutic goals is likely to prove difficult. A more tantalizing approach is to target the NRs recognized as being responsible for the regulation of these transporters. Like their classical steroid hormone relatives, certain bile acids and oxysterols are signaling molecules that are sensed by NRs. These in turn regulate multiple aspects of hepatic metabolism and transport as well as contributing to communication between the intestine and the liver to coordinate digestion and energy homeostasis. Using both genetic abrogation as well as pharmacological manipulation in mice, FXR, PXR and CAR have been shown to influence cholestatic liver disease in animal models to the point of significantly impacting on survival after complete bile duct ligation. Given its role in regulating biliary cholesterol transport *via* ABCG5/8, it is not surprising that LXR activation increases bile lithogenicity causing cholesterol crystallization by increasing cholesterol and phospholipid concentrations while lowering bile acids (146). Given their pharmacological tractability, these and other closely related NRs such as the PPARs are presently of interest for a range of human diseases extending from cholestasis to fatty liver disease through to inflammatory bowel disease (147). Research priorities include the development of additional pharmacologic tools for the manipulation of these receptors and exploration of their effects in a more diverse range of *in vivo* models, particularly in view of the observed differences between rodents and man in some aspects of transporter regulation. If suitable nuclear receptor agonists and antagonists can be developed early phase human studies are likely to yield exciting results.

Finally, genetic variation in the apical hepatocyte ABC transporters is well recognized and has significant clinical implications. Loss of function mutations can lead to severe cholestatic liver disorders, as observed in PFIC2 and PFIC3. More subtle changes are associated with conditions such as BRIC2 and ICP. It is becoming apparent that variability in these transporters also contributes to gallstone susceptibility (61) and is associated with drug clearance phenotypes (148), however more extensive research is needed to further clarify these associations. An important question is whether genetic variability also contributes to idiosyncratic drug-induced liver disease, a spectrum adverse drug responses that affect the liver, often have a cholestatic component and represent a major issue in therapeutic drug development (149). Again, further studies are needed to determine if this is the case and if these often severe reactions to often commonly used drugs can be predicted and therefore avoided.

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Abbreviations: ABC: ATP-binding cassette; BDL: bile duct ligation; CA: cholic acid; CDCA: chenodeoxycholic acid; DCA:deoxycholic acid; LCA: lithocholic acid; MDR: multidrug resistance; NR: nuclear receptor; PFIC: progressive familial intrahepatic cholestasis; T-CDCA: taurochenodeoxycholic acid

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