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The role of canalicular ABC transporters in cholestasis

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ABC transporters and cholestasis

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Abbreviations:

6E-CDCA, 6E-chenodeoxycholic acid; ABC, ATP-binding cassette; ABCB1, ATP-binding cassette, subfamily B, member 1; ABCB11, ATP-binding cassette, subfamily B, member 11; ABCB4, ATP-binding cassette, subfamily B, member 4; ABCC2, ATP-binding cassette, subfamily C, member 2; ABCC3, ATP-binding cassette, subfamily C, member 3; ABCC4, ATP-binding cassette, subfamily C, member 4; ABCG2, ATP-binding cassette, subfamily G, member 2; ABCG5/8, ATP-binding cassette, subfamily G, members 5/8; AE2, anion exchanger 2; AMPK, AMP activated protein kinase; ASBT, apical sodium dependent bile acid transporter; ASCOM, activating signal cointegrator-2-containing complex; BCRP, breast cancer resistance protein; BRIC, benign recurrent intrahepatic cholestasis; BSEP, bile salt export pump; c-Src, proto-oncogene tyrosine-protein kinase; CAR, constitutive androstane receptor; CYP2B10, cytochrome P450 2B10; CYP3A4, cytochrome P450 3A4; CYP7a1, cytochrome P450 7A1, cholesterol 7α-hydroxylase; CYP8B1, cytochrome P450 8B1, sterol 12α-hydroxylase; EHBR, mutant Eisai hyperbilirubinemic (Sprague Dawley) rat; ERK, extracellular signal-regulated kinase; FGF15/19, Fibroblast growth factor 15/19; FOXO1, forkhead box O1A; FXR, farnesoid X receptor; GR, glucocorticoid receptor; GY/TR-, ABCC2-deficient (Wistar) rat strain; HNF, hepatocyte nuclear factor; ICP, intrahepatic cholestasis of pregnancy; IL, interleukin; IR-1, inverted repeat-1; LKB1, liver kinase B1; LPAC, low phospholipid associated cholelithiasis syndrome; LRH-1, liver receptor homolog-1; LXR, liver X receptor; MDR1, multidrug resistance protein 1, P-glycoprotein; MDR2 (rodents)/MDR3 (human), multidrug resistance protein 2 (rodents)/3 (human); MRP2, multidrug resistance-associated protein 2; norUDCA, norursodeoxycholic acid; NR, nuclear receptor; NRF-2, nuclear factor erythroid 2-related factor 2; NTCP, sodium/taurocholate co-transporting polypeptide; OATP, multispecific organic anion transporting polypeptide; p38MAPK, p38 mitogen-activated protein kinase; PBC, primary biliary cirrhosis; PC, phosphatidylcholine; PFIC, progressive familial intrahepatic cholestasis; PKC, protein kinase C; PPARα, peroxisome proliferator-activated receptor alpha; PPARγ, peroxisome proliferator-activated receptor gamma; PSC, primary sclerosing cholangitis; PXR, pregnane X receptor; RARα, retinoic acid receptor alpha; RXRα, retinoid X receptor alpha; SHP, short heterodimer partner; SNP, single-nucleotide polymorphism; SR-B1, scavenger receptor B1; SRC-2, steroid receptor coactivator-2; SULT2A1, sulfotransferase 2A1; TNF-α, tumor necrosis factor alpha; TPN, total parenteral nutrition; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid; UGT1A1, UDP glucuronosyltransferase 1A1; UGT2B4, UDP glucuronosyltransferase 2B4; VDR, vitamin D receptor.

Please note that abbreviations for transporters and nuclear receptors were capitalized throughout this article when symbols were identical for human and rodents.

Abstract

Cholestasis, a hallmark feature of hepatobiliary disease, is characterized by the retention of biliary constituents. Some of these constituents, such as bile acids, inflict damage to hepatocytes and bile duct cells. This damage may lead to inflammation, fibrosis, cirrhosis, and eventually carcinogenesis; sequelae that aggravate the underlying disease and deteriorate clinical outcome. Canalicular ATP-binding cassette (ABC) transporters, which mediate the excretion of individual bile constituents, play a key role in bile formation and cholestasis. The study of these transporters, and their regulatory nuclear receptors, has revolutionized our understanding of cholestatic disease. This knowledge has served as a template to develop novel treatment strategies, some of which are currently already undergoing phase III clinical trials. In this review we aim to provide an overview of the structure, function, and regulation of canalicular ABC transporters. In addition, we will focus on the role of these transporters in the pathogenesis and treatment of cholestatic bile duct and liver diseases.

Introduction

Hepatic ATP-binding cassette (ABC) transporters play a key role in cholestatic disease, and are expressed at the basolateral and apical membrane of liver cells (hepatocytes). Canalicular ABC transporters are responsible for the formation of bile, and secrete bile acids (ABCB11) (Gerloff *et al.*, 1998), bilirubin (ABCC2) (Paulusma *et al.*, 1997), phosphatidylcholine (ABCB4) (Smit *et al.*, 1993), cholesterol (ABCG5/G8) (Berge *et al.*, 2000), and drugs (ABCB1, ABCC2, ABCG2) across the bile canalicular membrane. ABCB11 transports bile acids against a steep (1000-fold) concentration gradient. This gradient attracts water into the bile canalicular lumen, and thereby drives bile flow. Mixed micelles of phosphatidylcholine (ABCB4) and cholesterol (ABCG5/8) incorporate these bile acids, and thereby mitigate their detergent effects (reviewed by Trauner *et al.*, 2008). Other canalicular ABC transporters (mainly ABCB1, ABCC2, and ABCG2) play a key role in the biliary excretion of xenobiotics, which has important implications for drug-drug interactions and the development of multi-drug resistance (reviewed by Ecker and Chiba, 2009; Keppler, 2011a). The important function of canalicular ABC transporters is underlined by their role in cholestatic disease. Hereditary and acquired ABC transporter defects may decrease bile flow, increase the biliary toxicity, and/or contribute to the development of drug-induced cholestasis (Oude Elferink *et al.*, 2006). Basolateral ABC transporters (e.g. ABCC3 and ABCC4) transport bile acids into the blood, which protects hepatocytes from bile acid-induced damage (Keppler, 2011a). The activity of ABC transporters, in short, can either protect or damage cells of the hepatobiliary system. Their expression is consequently tightly regulated, both by nuclear receptors (NRs) at the transcriptional level, and by various post-transcriptional modifications, such as insertion/retrieval of the transporter at the cell membrane (reviewed by Halilbasic *et al.*, 2013). These regulatory mechanisms ensure bile acid homeostasis and coordinate the adaptive response to cholestatic conditions.

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This review discusses the role of canalicular ABC transporters (ABCB11, ABCC2, ABCB1, ABCG2, ABCB4, ABCG5/8) in bile formation and cholestasis. To provide a basis for this undertaking, we will commence with a brief overview of bile acid metabolism. Subsequently, we will turn our attention to the individual canalicular transporters, and review their structure, function, associated substrates, and regulation in health and disease. In the last part of the review, we will focus on the potential role of these transporters, and the NRs that regulate their transcription, as drug targets in cholestatic disease. Many of the studies described in this review were performed in mice, which have a significantly different bile acid pool compared with humans. The direct extrapolation of animal data to human physiology is therefore not possible without their verification in human models. Although the animal studies discussed in this review were invaluable for our understanding of bile metabolism, their interpretation thus needs careful appreciation of interspecies discrepancies.

Bile acid metabolism, and its regulation

Bile acids are synthetized from cholesterol in the liver. This synthesis requires 17 enzymatic steps, of which the conversion of cholesterol into 7α-hydroxycholesterol by 7α-hydroxylase (CYP7a1) is considered to be rate limiting. Most (>99%) bile acids are directly conjugated (either with taurine or with glycine), which necessitates their active secretion (via ABCB11 and ABCC2) across the bile canalicular membrane. The secreted bile acids then enter the intestinal lumen and are efficiently (>95%) reabsorbed, mostly by the apical sodium dependent bile acid transporter (ASBT) in the terminal ileum (Dawson *et al.*, 2003). The reabsorbed bile acids return to the liver via the portal circulation, from where they are extracted by the basolateral uptake transporters of the hepatocyte. The sodium/taurocholate co-transporting polypeptide (NTCP) transports the majority (~90%) of these bile acids,

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while multi-specific organic anion transporters (OATPs) play a comparably modest role in hepatocellular bile acid uptake (Hagenbuch and Meier, 1994; Kullak-Ublick *et al.*, 1994).

NRs regulate the transcription of hepatic genes that are involved in bile acid homeostasis (Figure 1; reviewed by Halilbasic *et al.*, 2013). These receptors act as intracellular sensors, and prevent the accumulation of toxic biliary compounds. Activated NRs change conformation, recruit co-activators (and/or dissociate from co-repressors), and induce/repress transcription either by binding the DNA of their target genes, or by interacting with other NRs. The role and function of NRs is exemplified by the farnesoid X receptor (FXR), which acts as an intracellular sensor for bile acids (Figure 2) (Makishima *et al.*, 1999; Parks *et al.*, 1999; H Wang *et al.*, 1999). Bile acid-activated FXR forms a heterodimer with the retinoid X receptor (RXR), which then binds an inverted repeat-1 (IR-1) sequence (or other response elements) in the promoter of its target genes (Seol *et al.*, 1995; Forman *et al.*, 1995; Laffitte *et al.*, 2000). The resulting gene transcription decreases hepatocellular bile acid uptake (NTCP) and synthesis (CYP7A1/CYP8B1), while promoting canalicular (ABCB11, ABCC2) and basolateral bile acid excretion in rodent and human hepatocytes (Denson *et al.*, 2001; Ananthanarayanan *et al.*, 2001; Gerloff *et al.*, 2002; Plass *et al.*, 2002; Kast *et al.*, 2002; Eloranta and Kullak-Ublick, 2005). These effects are partly mediated by the FXR-induced activation of the short heterodimer partner (SHP), which represses the transcription of *NTCP*, *CYP7A1* and *CYP8B1* (Figure 1) (Gupta *et al.*, 2002; Brendel *et al.*, 2002; Abrahamsson *et al.*, 2005; Kir *et al.*, 2012). FXR also induces bile acid detoxification via *CYP3A4*, *SULT2A1*, and *UGT2B4*, which further protects the hepatocyte from bile acid-induced damage (reviewed by Zollner *et al.*, 2006). Finally, FGF19, which is expressed in the human liver and intestine, can also be induced by FXR (Holt *et al.*, 2003; Inagaki *et al.*, 2005; I Kim *et al.*, 2007; Schaap *et al.*, 2009). This last mechanism represents a negative feedback loop, which can be induced by an increased intestinal or hepatic bile acid concentration (e.g. after a meal) (Choi *et al.*, 2006). Other NRs such as the pregnane X

receptor (PXR) and the constitutive androstane receptor (CAR) are also involved in bile acid metabolism. Both receptors are best known for their role in phase I (CYPs), phase II (conjugation), and phase III (transport proteins) drug elimination. PXR and CAR are, however, also activated by hydrophobic bile acids (PXR) and bilirubin (indirect activation; CAR) in rodent and human hepatocytes (Staudinger *et al.*, 2001; Xie *et al.*, 2001; W Huang *et al.*, 2003). This activation induces hepatocellular bile acid excretion (ABCC2, ABCC3, ABCC4) and detoxification (CYP3A4/CYP2B10/SULT2A1) (Xie *et al.*, 2000; Marschall *et al.*, 2005; Chai *et al.*, 2011; 2012), and stimulates bilirubin conjugation (UGT1A1) and excretion (ABCC2) (W Huang *et al.*, 2003; Marschall *et al.*, 2005). PXR also represses bile acid synthesis (via CYP7A1) (Staudinger *et al.*, 2001). The vitamin D receptor (VDR) is activated by secondary bile acids such as lithocholic acid (Makishima *et al.*, 2002). The impact of VDR activation on bile acid metabolism and cholestatic disease is difficult to predict, since it inhibited FXR-dependent gene transactivation *in vitro*, but also had anti-fibrotic effects in a rat model of liver fibrosis (Honjo *et al.*, 2006; Abramovitch *et al.*, 2011). VDR does not seem to have a significant impact on the expression of canalicular ABC transporters. Several non-bile acid activators, such as peroxisome proliferator-activated receptors (PPARs) and the glucocorticoid receptor (GR), are also involved in bile acid detoxification and elimination (Figure 1), but an extensive discussion on their role in bile acid metabolism falls beyond the scope of this review.

ABCB11

ABCB11 (BSEP) acts as the canalicular bile salt export pump and transports conjugated monovalent bile acids from the hepatocyte into the bile. This transport not only protects the liver from bile acid-induced toxicity, but also represents the major driving force for (bile acid dependent) bile flow. As the major canalicular bile acid transporter in humans, ABCB11 plays a key part in bile formation and (hereditary) cholestasis.

ABCB11 is a 160-kD member of the of the B sub-family (ABCB) of ABC transporters, and has a structure that consists of two nucleotide-binding and two 6-helical transmembrane domains (Figure 3) (Kubitz *et al.*, 2012). ABCB11 is, like ABCC2, ABCB4, ABCB1, and ABCG5/8, an exclusively apical transporter. Its expression pattern is restricted to hepatocytes, which supports its role in canalicular bile acid transport and bile formation. Human ABCB11 transports conjugated/amidated monovalent bile acids (Table 1) in the following order of clearance: taurochenodeoxycholic acid > glycochenodeoxycholic acid > taurocholic acid > glycocholic acid (Hayashi *et al.*, 2005). ABCB11 thus clears chenodeoxycholic acid, which is the most toxic of these bile aids, with the greatest efficacy (Hayashi *et al.*, 2005; Song *et al.*, 2011). Interestingly, some *in vitro* reports suggested that ABCB11 might also transport drugs (e.g. vinblastine, taxol, and pravastatin) (Childs *et al.*, 1998; Lecureur *et al.*, 2000; Hirano *et al.*, 2005). The impact of ABCB11 on drug transport, however, has not been established.

A decrease in ABCB11 activity leads to bile acid accumulation, and plays an important role in the pathogenesis of acquired and hereditary cholestatic disease. Prescription drugs, inflammation, and total parental nutrition (TPN), for example, can all lead to acquired cholestasis. Drugs, such as cyclosporine A, glybenclamide, rifampicin, and rifamycin, can repress ABCB11 activity via competitive inhibition. The resulting decrease in canalicular bile acid transport can lead to drug-induced cholestasis, which will generally resolve quickly after drug withdrawal (Stieger *et al.*, 2000). Inflammation and TPN repress canalicular ABCB11 expression in rodents (Nishimura *et al.*, 2005; Recknagel *et al.*, 2012). This decrease, which occurs via various (post-) transcriptional mechanisms, can contribute to the development of inflammatory/septic or TPN-induced cholestasis. *ABCB11* polymorphisms can predispose to acquired cholestatic disease, and the single nucleotide polymorphism (SNP) rs2287622 has a relatively high prevalence in patients with drug-induced cholestasis, intrahepatic cholestasis of pregnancy, liver fibrosis, and cholangiocarcinoma (reviewed by

Stieger and Beuers, 2011). Severe ABCB11 mutations can lead to the development of hereditary cholestasis, which covers a mild to severe phenotypical spectrum. Progressive familial intrahepatic cholestasis type 2 (PFIC2) leads to severe cholestasis, and is generally associated with a non-functional ABCB11 protein (reviewed by Jacquemin, 2012). This disease usually manifests itself within the first 6 months of life. Patients typically suffer from cholestasis, fat malabsorption, growth retardation, and an increased risk for hepatocellular carcinoma (Knisely *et al.*, 2006). The initial treatment usually consists of ursodeoxycholic acid (UDCA), fat-soluble vitamins (D, K), cholestyramine (for pruritus), and biliary diversion (Emond and Whitington, 1995). Most patients, however, will require liver transplantation in the first 2 decades of life. Some transplanted patients develop rebound cholestasis, due to formation of anti-ABCB11 antibodies (Keitel *et al.*, 2009). Treatment options for PFIC2 patients with a non-functional protein remain limited in the absence of gene therapy. Patients with residual ABCB11 activity, however, may benefit from ABCB11 activation via chaperones in the future. Treatment of MDCK cells harboring a (E297G or D482G) mutant form of ABCB11 with 4-phenylbutyrate, for example, led to an increase in apical ABCB11 incorporation (Hayashi and Sugiyama, 2007). PFIC2 patients with residual ABCB11 activity are also more likely to benefit from UDCA, because TUDCA relies on a functional protein for its transport (Gerloff *et al.*, 1998). UDCA shifts the bile acid pool to a more hydrophilic (i.e. less toxic) composition and promotes apical ABCB11 insertion (see below), which induces choleresis (Kurz *et al.*, 2001; Dombrowski *et al.*, 2006). BRIC 2 belongs to the same phenotypical continuum as PFIC2, and is characterized by mild and self-limiting episodes of cholestasis (C-W Lam *et al.*, 2006). Notably, *ABCB11* knockout mice display a significantly milder phenotype compared with their human PFIC2 counterparts (P Lam *et al.*, 2005). This discrepancy could partly be attributed to the formation of less toxic polyhydroxylated bile acids in mice (Perwaiz, 2002).

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These hydrophilic bile acids could, in theory, be excreted via alternative hepatocellular bile acid transporters, such as ABCC2 and ABCB1.

The activity of ABCB11 is tightly regulated at the level of its transcription, and by several post-transcriptional modifications. *ABCB11* transcription is mainly regulated by FXR, as stated above. Other transcriptional factors, however, influenced the interaction of FXR with the *ABCB11* promoter in *in vitro* and in rodents. VDR activation, via 1,25-dihydroxyvitamin D3, inhibits FXR-induced *ABCB11* transactivation (Honjo *et al.*, 2006). Activating signal cointegrator-2-containing complex (ASCOM) recruitment, by chenodeoxycholic acid, increases FXR-induced transactivation, because this co-activator complex methylates the *ABCB11* promoter histones (Ananthanarayanan *et al.*, 2011). Steroid receptor coactivator-2 (SRC-2) activation, by liver kinase B1 (LKB1) and AMP activated protein kinase (AMPK), also promotes FXR-induced transactivation, by acetylation of promoter histones (Chopra *et al.*, 2011). The liver receptor homolog-1 (LRH-1) and the oxidative stress sensor nuclear factor erythroid 2-related factor 2 (NRF-2), finally, transactivate *ABCB11* by binding to specific response elements in the *ABCB11* promoter (Weerachayaphorn *et al.*, 2009). The rapid, short-term, adaptation of canalicular ABCB11 expression is mainly regulated at the post-transcriptional level. This regulation involves the shuttling of ABCB11 between its intracellular pool and the canalicular membrane, and may be triggered by hormones (Crocenzi *et al.*, 2003), oxidative stress (Pérez *et al.*, 2006), hydration (Schmitt *et al.*, 2001), and cell swelling (Häussinger *et al.*, 1993), as demonstrated *in vitro* and in rodent studies. Cell swelling can occur in response to a meal and lead to a rapid canalicular insertion of ABCB11, which increases the postprandial excretion of bile acids. UDCA treatment, in addition, also increases bile flow partly via (post-transcriptional) canalicular ABCB11 insertion. The regulation of these post-translational mechanisms involves the induction of integrins by cell swelling, which triggers focal adhesion kinase, c-Src, mitogen activated protein kinases, ERKs, and p38MAPK (Kurz *et al.*, 2001; Häussinger *et al.*, 2003; Schliess

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et al., 2004). TUDCA acts via the same pathway, but also via the activation of various protein kinase C (PKC) isoforms. PKCα recruitment by estradiol-17β-D-glucuronoside decreases canalicular ABCB11 expression in rodents, which could partly be responsible for its cholestatic properties (Crocenzi *et al.*, 2008). Inflammation-induced cholestasis, finally, can lead to a decreased ABCB11 insertion into the canalicular membrane *in vitro* and in rodents. Inflammatory cytokines (e.g. IL-1, IL-6), however, can also decrease *ABCB11* (and *ABCC2*) transcription, by their inhibitory effect on key transcriptional networks (e.g. retinoic acid receptor-α -RARα-, RXRα, FXR, PXR, CAR) (reviewed by Wagner *et al.*, 2010), and (especially in human ABCB11) via post-transcriptional mechanisms (MGL Elferink *et al.*, 2004).

ABCC2

ABCC2 (MRP2) is expressed at critical sites of uptake and elimination, and is involved in the excretion and detoxification of endo- and xenobiotics. Hepatic ABCC2 plays an important role in the canalicular excretion of glutathione and conjugated bilirubin. ABCC2 mutations can cause the Dubin-Johnson-syndrome, which is characterized by a mild conjugated hyperbilirubinemia.

ABCC2 is a 190-kD member of the C sub-family (ABCC) of ABC transporters. Its structure consists of two nucleotide-binding and three (instead of the normal two) transmembrane domains (Figure 3). The function of the third transmembrane domain, which consists of 5 instead of 6 helices, is still investigated (Fernández *et al.*, 2002; Westlake *et al.*, 2005). ABCC2 is expressed at the apical membrane of intestinal epithelial cells (Fromm *et al.*, 2000; Sandusky *et al.*, 2002), hepatocytes (Keppler and Kartenbeck, 1996), renal proximal tubule epithelial cells (Schaub *et al.*, 1997; 1999), gallbladder epithelial cells (Rost *et al.*, 2001), and placental syncytiotrophoblast cells (Keppler, 2011b). This expression pattern, at major barrier sites, results in a decreased uptake (i.e. bioavailability) and an increased

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excretion of its various endo- and exogenous substrates. Although these mechanisms protect the body, they may also decrease treatment efficacy and/or lead to the development of multi-drug resistance. The development of drug resistance, however, has mainly been associated with the overexpression of other multi drug transporters (i.e. ABCB1 and ABCG2) (Gerhard Ecker, 2009; Marquez and Van Bambeke, 2011).

ABCC2 transports various amphiphilic anions, but displays a preference for phase II (e.g. glucuronic acid, sulfuric acid, or glutathione conjugated) metabolites (Table 1). Its endogenous substrates include tetrahydroxylated bile acids (Megaraj *et al.*, 2010), divalent bile acids (Kuipers *et al.*, 1988), glutathione (Oude Elferink *et al.*, 1990), bilirubin glucuronosides (Paulusma *et al.*, 1997), eicosanoids (prostaglandin E2, leukotriene C_4) (Cui *et al.*, 1999), and conjugated steroids (estrone 3-sulfate (Kopplow *et al.*, 2005), estradiol-17β-glucuronate (Cui *et al.*, 1999)). Exogenous ABCC2 substrates are mostly conjugated, either with glucuronic acid (e.g. phytoestrogens (Krumpochova *et al.*, 2012), acetaminophen (Xiong *et al.*, 2000), indomethacin (Kouzuki *et al.*, 2000), morphine (van de Wetering *et al.*, 2007)), sulfuric acid (e.g. acetaminophen (Zamek-Gliszczynski *et al.*, 2005), resveratrol (Kaldas *et al.*, 2003)), or with glutathione (e.g. acetaminophen (Chuan Chen *et al.*, 2003), bromosulfophthalein (Jansen *et al.*, 1987), dinitrophenyl (RP Elferink *et al.*, 1989)). However, ABCC2 also transports unconjugated anionic drugs, such as pravastatin (Yamazaki *et al.*, 1997), ampicillin (Verkade *et al.*, 1990), and methotrexate (Hooijberg *et al.*, 1999). In addition, it transports uncharged (vinblastine, sulfinpyrazone) (Evers *et al.*, 2000) or positively charged (Cd^{2+} and Zn^{2+}) (Houwen *et al.*, 1990; Dijkstra *et al.*, 1996) substrates that require glutathione-complex formation to obtain a negative charge, which is necessary for ABCC2-mediated transport.

Hepatic ABCC2 plays an important role in the development of acquired and hereditary jaundice. Sepsis, inflammatory cholestatic disease (e.g. alcoholic hepatitis, chronic hepatitis C), TPN, and obstructive cholestasis are all associated with a decrease in canalicular

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ABCC2 expression in rodents (Hinoshita *et al.*, 2001; Denson *et al.*, 2002; MGL Elferink *et al.*, 2004; Nishimura *et al.*, 2005). This decrease in ABCC2, which occurs via several (post-) transcriptional mechanisms, provides a molecular explanation for the conjugated hyperbilirubinemia that can be observed under inflammatory conditions (Hinoshita *et al.*, 2001; Zollner *et al.*, 2001; Denson *et al.*, 2002). Septic hyperbilirubinemia, for example, is largely induced by a cytokine-mediated decrease in ABCC2 expression, and is considered to be a poor prognostic sign in critically ill patients (Trauner *et al.*, 1997; Recknagel *et al.*, 2012). Hepatic ABCC2 also transports glutathione and bile acids. ABCC2-mediated glutathione transport helps to create an osmotic gradient in the bile canalicular lumen, and is mainly responsible for the instigation of the bile acid-independent bile flow (Chu *et al.*, 2006; Vlaming *et al.*, 2006). The ABCC2-mediated transport of divalent bile acids complements the monovalent bile acid transport by ABCB11, but plays a minor role in bile flow. Animal models that lack a functional ABCC2 transporter, such as GY/TR$^-$ rats, EHBR rats, and *ABCC2* knockout mice, fail to secrete glutathione and bilirubin into the bile (Büchler *et al.*, 1996; Paulusma *et al.*, 1996; Chu *et al.*, 2006; Vlaming *et al.*, 2006). Their phenotype is consequently characterized by a 30% decrease in bile flow and a permanent conjugated hyperbilirubinemia. The important role of ABCC2 in bilirubin metabolism is further illustrated by the Dubin-Johnson syndrome, which is caused by mutations that result in an inactive form of ABCC2. These patients are unable to excrete glucuronidated bilirubin into the bile and consequently develop a permanent isolated conjugated hyperbilirubinemia (DUBIN and JOHNSON, 1954; Paulusma *et al.*, 1997). ABCC2 deficiency is partly compensated by the activity of alternative transporters, which may be responsible for the absence of a severe (liver) phenotype in Dubin-Johnson patients. Basolateral ABCC3, for example, decreases various intracellular ABCC2 substrates such as bilirubin to non-toxic levels (Konig *et al.*, 1999; Johnson *et al.*, 2006). *ABCC2* SNPs, which can reduce ABCC2 activity, occur in a higher frequency in patients with non-fatty alcoholic liver disease

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(rs17222723 and rs8187710) (Sookoian *et al.*, 2009), intrahepatic cholestasis of pregnancy (rs3740066) (Sookoian *et al.*, 2008), bile duct cancer (rs3740066) (Hoblinger *et al.*, 2009), and diclofenac-induced hepatotoxicity (rs717620) (Daly *et al.*, 2007). Several of these SNP's are also associated with altered pharmacokinetics of ABCC2 substrate drugs, such as methotrexate and pravastatin. *ABCC2* polymorphisms also lead to a decreased biliary excretion of toxic metabolites during irinotecan treatment, which protects patients from irinotecan-induced diarrhea (de Jong *et al.*, 2007; Gradhand and RB Kim, 2008; Megaraj *et al.*, 2011).

ABCC2 gene transcription is regulated by FXR, PXR, and CAR. These NRs heterodimerize with RXR after their activation, and subsequently bind a shared 26 bp sequence hormone response element (ER-8) in the *ABCC2* promoter (Kast *et al.*, 2002). FXR (e.g. chenodeoxycholic acid), PXR (e.g. rifampicin) and CAR (e.g. phenobarbital) agonists thus increased ABCC2 expression in human and rodent livers (Fardel *et al.*, 2005). Inflammatory cholestasis, sepsis, and obstructive cholestasis can decrease ABCC2 expression by a cytokine-induced repression of transcriptional networks *in vitro* and in rodents (RARα-, RXRα, FXR, PXR, CAR) (reviewed by Wagner *et al.*, 2010). Bile duct ligation or lipopolysaccharide (LPS) treatment resulted in an IL-1β-mediated RARα/RXRα down regulation, which in turn decreased *ABCC2* transcription in rats (Denson *et al.*, 2002). Oxidative stress (e.g. via toxic bile acids) can increase *ABCC2* transcription via NRF-2 in rodents (Maher *et al.*, 2007; Okada *et al.*, 2008). Post-transcriptional mechanisms fine-tune the canalicular ABCC2 expression. LPS treatment, cytokines, estradiol-17β-D-glucuronoside, and hyperosmolar conditions all decreased the canalicular ABCC2 expression via post-transcriptional mechanisms in rodent models (Kubitz *et al.*, 1999; Paulusma *et al.*, 2000; Dombrowski *et al.*, 2000; Mottino *et al.*, 2002; Crocenzi *et al.*, 2003; Fickert *et al.*, 2006). These post-transcriptional modifications were associated with membrane retrieval and cytoplasmic accumulation of ABCC2, which was indicated by a

"fuzzy" immune-staining pattern. A similar fuzzy pattern was observed in cholestatic patients (e.g. in primary biliary cirrhosis and obstructive cholestasis) (Zollner *et al.*, 2001; Kojima *et al.*, 2003).

ABCB1

ABCB1 (MDR1; MDR1a/MDR1b in rodents) protects the body from a broad variety of hydrophobic drugs, and plays a key role in the development of multi-drug resistance. ABCB1 also interacts with several biliary constituents (e.g. cholesterol, bile acids, phospholipids), but its contribution to bile formation and cholestasis remains to be established.

ABCB1, a 170kDa member of the B sub-family (ABCB) of ABC transporters, consists of two nucleotide-binding and two 6-helical transmembrane domains (Figure 3). ABCB1 is expressed at the apical membrane of intestinal epithelial cells, hepatocytes, renal tubular epithelial cells, endothelial vascular cells of the blood-brain and blood-testis barrier, and in cells of the adrenal gland, pancreas, lung, and placenta (Thiebaut *et al.*, 1987; Sugawara *et al.*, 1988). This expression pattern allows ABCB1 to inhibit the uptake of drugs from the intestinal lumen (bioavailability), decrease their entry in sanctuary organs, such as the brain and testes (distribution), and increase their renal and biliary elimination.

ABCB1 is a highly promiscuous transporter that interacts with nearly half of all registered pharmaceutical compounds (Nicolaou *et al.*, 2012). ABCB1 transports mainly neutral or positively charged amphipathic compounds, although transport of negatively charged compounds (e.g. methotrexate) has been reported (Table 1) (de Graaf *et al.*, 1996; L Huang *et al.*, 1998; Gerhard Ecker, 2009). Its unusual promiscuity has made it hard to find compounds that are not substrates. Accordingly, ABCB1 has been implicated in the transport of various endogenous compounds, such as cholesterol (SD Lee *et al.*, 2013), steroids (e.g. cortisol, aldosterone, ethinylestradiol, estrone, estriol ((Ueda *et al.*, 1992; WY

Kim and Benet, 2004)), short-chain (not long-chain) phospholipids (van Helvoort *et al.*, 1996; Morita *et al.*, 2007), opioid peptides (Oude Elferink and Zadina, 2001), unconjugated bilirubin (Jetté *et al.*, 1995; Watchko *et al.*, 2001), and tetrahydroxylated bile acids (Megaraj *et al.*, 2010). Most of these compounds were only investigated *in vitro* and/or showed a low affinity for ABCB1. For several of these substrates (e.g. phospholipids, unconjugated bilirubin, tetrahydroxylated bile acids) it consequently remains to be determined if ABCB1 actually contributes to their *in vivo* metabolism. Exogenous ABCB1 substrates include chemotherapeutics (e.g. paclitaxel (Fellner *et al.*, 2002), topotecan (H Li *et al.*, 2008), etoposide (Takeuchi *et al.*, 2006), teniposide (Vasanthakumar and Ahmed, 1989), doxorubicin (Ueda *et al.*, 1987), vincristine (Cisternino *et al.*, 2001), vinblastine (Cisternino *et al.*, 2001), daunorubicin (Takeuchi *et al.*, 2006), docetaxel (Shirakawa *et al.*, 1999), mitomycin C (Hayes *et al.*, 2001)), cytotoxic drugs (e.g. colchicines)(Cisternino *et al.*, 2003), antihypertensives (e.g. losartan (Soldner *et al.*, 1999), diltiazem (Katoh *et al.*, 2006)), antiarrhythmics (e.g. verapamil (Soldner *et al.*, 1999), digoxin (Pauli-Magnus *et al.*, 2000)), antibiotics (e.g. erythromycin (Schuetz *et al.*, 1998)), HIV-protease inhibitors (e.g. indinavir, ritonavir) (CG Lee *et al.*, 1998), and various other xenobiotic compounds (rhodamine 123 (Bachmeier *et al.*, 2005), Hoechst 33342 (A Y Chen *et al.*, 1993), calcein-AM (Holló *et al.*, 1994)).

The physiological function of ABCB1 has been extensively studied in mice. Mice possess, in contrast to humans, two genes that code for two ABCB1 proteins, namely *ABCB1a* and *ABCB1b*. Together, these proteins fulfill the same function as ABCB1 in humans. The deletion of these genes in mice did, somewhat surprisingly, not lead to a severe phenotype. *ABCB1a/ABCB1b* compound knockout mice were fertile, displayed a normal biliary composition and flow, and showed a normal lifespan under laboratory conditions. The absence of ABCB1a and ABCB1b, however, did result in an altered pharmacological profile of substrate drugs. This altered profile generally led to an increased bioavailability, an

increased distribution volume (mainly to the brain), and a decreased renal/biliary elimination of ABCB1a/b substrates (Schinkel, 1998; Cuiping Chen *et al.*, 2003). As a consequence, these animals displayed higher plasma and tissue (e.g. brain) levels of ABCB1a/b substrate drugs, compared with their wild type controls. Human *ABCB1* mutations and polymorphisms have also been extensively investigated, and were (similarly) not associated with any severe phenotype (reviewed by Ieiri, 2012). *ABCB1* SNPs did affect the pharmacokinetic profile of several drugs, but results were equivocal and differed significantly between studies. Consequently, *ABCB1* genotype-directed drug dosing is not (yet) recommended in routine clinical practice (Wolf *et al.*, 2011; Ieiri, 2012). *ABCB1* SNPs have also been associated with an increased susceptibility to various diseases, such as inflammatory bowel disease and colorectal cancer (Schwab *et al.*, 2003; Andersen *et al.*, 2009). The validity of these associations, however, remains to be established and deserves further investigation. The above-mentioned considerations do not infer that alterations in ABCB1 expression are of no consequence. Indeed, drug resistance that results from intrinsic (e.g. untreated) and acquired (e.g. drug-induced) ABCB1 over-expression remains a major problem in brain-targeted therapies and in anti-cancer treatment (Chan *et al.*, 1991; Shukla *et al.*, 2011). An increased expression of ABCB1 in tumor cells, for example, confers drug resistance by promoting the efflux of anti-cancer drugs (Gottesman *et al.*, 2002; Sikic, 2006). Indeed, ABCB1 tumor over-expression has been associated with non-response to chemotherapy and a poor clinical prognosis in various cancers (Chan *et al.*, 1991; Penson *et al.*, 2004; Sikic, 2006). These considerations led to the development of ABCB1 inhibitors, which overcame drug resistance in animal models and tumor cell lines. Unfortunately, these inhibitors remained unsuccessful in clinical trials, due to side effects and toxicity (reviewed by Shukla *et al.*, 2011; Falasca and Linton, 2012). This lack of success may be due to the complexity of multi-drug transport, in which the inhibition of one transporter may lead to compensatory effects that can alter drug handling and promote toxicity.

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The role of ABCB1 in bile formation and cholestasis has yet to be elucidated. Bile formation seems unaffected in *ABCB1a/ABCB1b* knockout mice, as discussed above. ABCB1 is, however, significantly up-regulated in the liver of cholestatic animal models, and in liver specimens of patients with obstructive cholestasis, biliary atresia, and primary biliary cirrhosis (PBC) (Schrenk *et al.*, 1993; Shoda *et al.*, 2001; Zollner *et al.*, 2003; Barnes *et al.*, 2007). The reason for this up-regulation remains unclear, but it might result in an increased canalicular excretion of toxins under cholestatic conditions. Interestingly, ABCB1a/b was shown to transport tetrahydroxylated bile acids in mice, albeit with a much lower affinity than ABCC2 (Megaraj *et al.*, 2010). This transport could, as discussed in our section on ABCB11, mitigate the phenotype of *ABCB11* knockout mice. This hypothesis was supported by the observation that: (1) ABCB1 was markedly up-regulated in *ABCB11* knockout mice, and (2) that *ABCB11/ABCB1a/ABCB1b* compound knockout mice displayed a more severe cholestatic phenotype than single *ABCB11* knockouts (R Wang *et al.*, 2009). ABCB1 may also protect hepatocytes against apoptosis under cholestatic conditions, by exporting toxins (Sakaeda *et al.*, 2002). Taken together, these observations support a compensatory role for ABCB1 during cholestasis. Its role in bile acid transport, however, is likely more important in mice than in humans, since only mice are able to generate hydrophilic tetrahydroxylated bile acids as part of their adaptive response to cholestasis (Perwaiz, 2002).

ABCB1 transcription is mainly regulated via PXR, CAR, VDR, and FXR. PXR induced *ABCB1* transcription in the intestine, liver, and kidney. Its agonists (e.g. rifampicin) consequently decreased the intestinal uptake (bioavailability) and increased the (biliary/renal) elimination of ABCB1 ligands in healthy volunteers (Taosheng Chen, 2010). CAR agonists (e.g. CITCO) induced ABCB1 expression in brain capillary cells (Taosheng Chen, 2010; Lemmen *et al.*, 2013). VDR activation, via 1,25-dihydroxyvitamin D3, induced ABCB1 in the kidney and brain of mice (Chow *et al.*, 2011). Chenodeoxycholic acid, a

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potent FXR agonist, induced ABCB1 expression in HepG2 cells (Martin *et al.*, 2008). *FXR* knockout mice showed almost no increase in hepatic ABCB1 after bile duct ligation, which demonstrates that cholestatic up-regulation of ABCB1 is largely FXR-dependent in this animal model (Stedman *et al.*, 2006). ABCB1 (post-) transcriptional regulation is certainly not the exclusive domain of these NRs. The tumor suppressor protein p53, for example, down-regulates ABCB1a and ABCB1, and may influence drug resistance in cancer (Bush and G Li, 2002). Rat ABCB1b is up-regulated during endotoxin-induced cholestasis via TNF-α, which requires NFκB signaling (Ros *et al.*, 2001). P53 actually increases ABCB1b and endotoxin treatment does not affect ABCB1a, which illustrates that the two rodent *ABCB1* genes are differentially regulated. Indeed the (post-) transcriptional regulation of human ABCB1 is highly complex and influenced by epigenetic methylation, micro-RNA expression, and various other mechanisms (reviewed by Labialle *et al.*, 2002; Baker and El-Osta, 2004; Toscano-Garibay and Aquino-Jarquin, 2012).

ABCG2

ABCG2 (BCRP) is the final canalicular multi-drug transporter that will be discussed in this review. Its main function is similar to that of ABCC2 and ABCB1, namely the protection of the body against xenobiotics. ABCG2 does not seem to have a significant role in the adaptive response to cholestasis in the liver, although recent studies suggest that it is capable of bile acid transport. This transport, however, is likely more relevant in the placenta than in the liver.

ABCG2 is a 72kDa member of the G sub-family (ABCG) of ABC transporters. Its structure consists of one N-terminal nucleotide-binding domain, and one C-terminal (6-helical) transmembrane domain (Figure 3) (McDevitt *et al.*, 2006; Ni *et al.*, 2010). This structure is somewhat aberrant, since in most ABC transporters the transmembrane domain is located at the N-terminal end and the nucleotide-binding domain at the C-terminal end of the protein.

ABCG2 is a half-transporter, like all members of the ABCG subfamily, and must at least dimerize to become functional. It is expressed at the apical membrane of intestinal epithelial cells (Gutmann *et al.*, 2005), hepatocytes (Hilgendorf *et al.*, 2007), renal tubular epithelial cells (Huls *et al.*, 2008), endothelial vascular cells of the blood-brain and blood-testis barrier (Cooray *et al.*, 2002; Fetsch *et al.*, 2006), and cells of the placenta and mammary gland (Allikmets *et al.*, 1998; Robey *et al.*, 2011). Its expression pattern, at critical sites of uptake and elimination, resembles that of ABCB1. ABCG2 has consequently a similar effect on the bioavailability, distribution, and elimination of its ligands as ABCB1 (Vlaming *et al.*, 2009; Agarwal *et al.*, 2011). Since ABCG2 and ABCB1 are often co-localized, and because they share many substrates, they can team up at critical barrier sites (Agarwal *et al.*, 2011). This cooperation protects sanctuary organs, such as the brain, but may also prevent entry of chemotherapeutic drugs, which can lead to treatment failure (e.g. in brain cancer) (Agarwal *et al.*, 2011).

ABCG2 is, like ABCB1, somewhat promiscuous when it comes to its exogenous substrates. In addition, it has been implicated in the transport of several endogenous compounds, including heme (Jonker *et al.*, 2002), porphyrins (Jonker *et al.*, 2002), folates (mono-, di- and tri-glutamates of folic acid) (Lemos *et al.*, 2009), urate (Woodward *et al.*, 2009), sulfated steroids (Suzuki *et al.*, 2003), and bile acids (Blazquez *et al.*, 2012) (Table 1). Exogenous ABCG2 substrates include sulfuric acid- (e.g. E3040S (Suzuki *et al.*, 2003)), glucuronic acid- (e.g. E3040G (Suzuki *et al.*, 2003)) or glutathione-conjugated (e.g. dinitrophenyl glutathione (Suzuki *et al.*, 2003)) compounds. ABCG2 also transports various unconjugated drugs, sometimes in co-transport with glutathione. It is, however, best known for its ability to transport chemotherapeutics, such as methotrexate (Zhe-Sheng Chen *et al.*, 2003), topotecan (Maliepaard *et al.*, 1999), mitoxantrone (Doyle *et al.*, 1998), and the SN-38 metabolite of irinotecan (Maliepaard *et al.*, 1999).

ABCG2 knockout mice did, much like *ABCC2* and *ABCB1a/b* knockout mice, not display a severe phenotype. This may well be because multi-drug transporters have a considerable overlap in their substrates and sites of expression. If one gene is deleted, other transporters can compensate for its loss. A single gene deletion will therefore only have a limited phenotypic effect. *ABCG2* knockout mice did accumulate endogenous (i.e. protoporphyrin X) and dietary (i.e. pheophorbide) porphyrins, which induced protoporphyria (via protoporphyrin X) and phototoxic skin lesions (via pheophorbide) (Jonker *et al.*, 2002). These mice also showed an increased bioavailability, an increased distribution volume (e.g. to the brain), and a decreased biliary/urinary elimination of ABCG2 substrate drugs (reviewed by Vlaming *et al.*, 2009). *ABCG2* gene mutations and polymorphisms were (similarly) not associated with a severe phenotype in humans. *ABCG2* SNPs, however, were associated with an altered pharmacological profile of ABCG2 substrate drugs (e.g. sulfalazine, topotecan, statins) (reviewed by Ieiri, 2012). Interestingly, recent studies have demonstrated an association between *ABCG2* SNPs (e.g. rs2231142) and the development of gout (Dehghan *et al.*, 2008; Woodward *et al.*, 2009). These studies also identified uric acid as an ABCG2 substrate. ABCG2, like ABCB1, has been implicated to promote the efflux of anti-cancer drugs in tumor cell lines. Its role in drug resistance, however, remains to be established in a clinical setting, and clinical trials with ABCG2 inhibitors are currently not advisable (Falasca and Linton, 2012).

The role of ABCG2 in bile formation and cholestasis has been extensively debated. Mennone et al. failed to find a liver phenotype in bile duct ligated or sham operated *ABCG2* knockout mice. This result pleaded against a significant role of hepatic ABCG2 in the adaptive response to cholestasis (Mennone *et al.*, 2010). A recent study in pregnant *ABCG2* knockout mice by Blazquez *et al.*, 2012, suggested that ABCG2 might affect bile acid transport in the placenta, but not in the liver. This study also demonstrated bile acid transport by recombinant ABCG2 in WIF-B9/R cells, in CHO cells, and in Xenopus laevis

oocytes. Other *in vitro* studies have shown ABCG2-mediated bile acid transport in bacteria (Janvilisri *et al.*, 2005), liver flukes (Kumkate *et al.*, 2008), and transfected plasma membrane vesicles (Imai *et al.*, 2002). Some *in vitro* studies, however, have failed to demonstrate a role of ABCG2 in bile acid transport (Suzuki *et al.*, 2003; Vaidya and Gerk, 2006). However, the majority of the available data from *in vitro* and animal studies suggest that ABCG2 is capable of bile acid transport. The importance of this transport may depend on the relative co-expression of other bile acid exporters (e.g. ABCB11, ABCC2) in the apical membrane (Mennone *et al.*, 2010; Blazquez *et al.*, 2012). The relative contribution of ABCG2 to bile acid transport will consequently be minimal in the liver, due to the presence of ABCB11 (and ABCC2). Placental ABCC2, however, has no (significant) co-expression of ABCB11, and may consequently play a major role in (local) bile acid transport (P Patel *et al.*, 2003).

ABCG2 transcription is regulated via CAR and PXR. CAR (phenobarbital, CITCO) and PXR (rifampicin and 2-acetylaminofluorene) ligands can thus increase ABCG2 expression *in vitro* (Jigorel *et al.*, 2006; Lemmen *et al.*, 2013). Other transcription factors can also induce *ABCG2*, and its promoter contains hypoxia, estrogen, progesterone, PPARγ, and aryl hydrocarbon receptor response elements (Ebert *et al.*, 2005; Szatmari *et al.*, 2006; Robey *et al.*, 2011; To *et al.*, 2011). Cytokines, growth factors, and micro-RNAs affected gene expression in various ways, while promoter methylation increased ABCG2 expression *in vitro* (Vee *et al.*, 2009; Robey *et al.*, 2011).

ABCB4

ABCB4 (MDR3; MDR2 in rodents) plays a key role in bile formation. While ABCB11 transport bile acids, ABCB4 secretes phosphatidylcholine (PC). PC and cholesterol form mixed stable micelles with bile acids, which protects the biliary tree from their detergent effects.

ABCB4, a 170-kD member of the B sub-family (ABCB) of ABC transporters, consists of two nucleotide-binding and two 6-helical transmembrane domains (Figure 3) (Zhang, 1996). ABCB4 is predominantly expressed in the apical membrane of hepatocytes (Yoshikado *et al.*, 2011; Pasmant *et al.*, 2012), although low levels of mRNA transcripts have been detected in the adrenal glands, heart, striated muscles, tonsils, placenta, and brain (Smit *et al.*, 1994; P Patel *et al.*, 2003; Augustine *et al.*, 2005; WS Kim *et al.*, 2008; Cui *et al.*, 2009). This expression pattern supports its role as the major canalicular PC transporter in humans. ABCB4, a so-called floppase, translocates (”flops”) PC from the inner to the outer leaflet of the canalicular membrane, from where it is extracted by bile acids (Smit *et al.*, 1993). The association of PC with bile acids (and cholesterol) results in the formation of mixed and stable micelles (DQ-H Wang *et al.*, 2009). These micelles protect the epithelial lining of the biliary tree from bile acid-induced toxicity and phospholipid extraction (reviewed by Trauner *et al.*, 2008). Although ABCB4 is a particularly specific PC transporter, it has a weak affinity for some ABCB1 substrate drugs (e.g. digoxin, paclitaxel, vinblastine; Table 1) (Smith *et al.*, 2000). The clinical relevance of this transport, however, has not been established. Other drugs, such as oral contraceptives and itraconazole, can inhibit ABCB4 activity, which may result in drug-induced liver damage (Yoshikado *et al*., 2011; Pasmant *et al.*, 2012).

A loss in ABCB4 function is not readily compensated, and leads to severe hepatobiliary pathology in animal models and patients. *ABCB4* knockout mice are unable to excrete PC, and consequently produce toxic bile. This toxicity is due to the relatively high non-micellar (”free”) bile acid concentration and leads to an increased permeability of the biliary epithelium, bile leakage, pericholangitis, periductal fibrosis, sclerosing cholangitis, and finally (in older mice) to hepatocellular carcinoma (Mauad *et al.*, 1994; Fickert *et al.*, 2002; 2004; Katzenellenbogen *et al.*, 2007). The micro- and macroscopic damage observed in these animals closely resembles that of (primary) sclerosing cholangitis in humans (PSC).

The impaired PC/bile acid micelle formation also decreases the canalicular extraction (i.e. secretion) and solubility of cholesterol. The latter results in the recurrent formation of cholesterol gallstones (Trauner *et al.,* 2008). Patients with progressive familial intrahepatic cholestasis type 3 (PFIC3) are the human counterparts of *ABCB4* knockout mice. PFIC3 usually has a similar clinical presentation as PFIC2 (see ABCB11 section), but may also present with recurrent choledocholithiasis in older children and adults (reviewed by Jacquemin, 2012). Although UDCA treatment can be helpful in the presence of a partial ABCB4 defect, hepatic transplantation will remain the only definitive therapy before gene therapy becomes available in most patients (Deleuze *et al.*, 1996; De Vree *et al.*, 1998). Patients with misfolding of the transporter, such as the reported PFIC3 heterozygous mutation I541F, may benefit from chaperone treatment to correct these folding defects in the future (Delaunay *et al.*, 2009; Gautherot *et al.*, 2012). Cyclosporine A was indeed able to restore a correct maturation of the endoplasmic reticulum (ER) sequestered I541F mutant *in vitro* (Gautherot *et al.*, 2012). Less severe ABCB4 mutations can lead to the low phospholipid associated cholelithiasis syndrome (LPAC) and intrahepatic cholestasis of pregnancy (ICP). LPAC is characterized by the formation of cholesterol gallstones, and may lead to progressive fibrosing cholestatic liver disease and portal hypertension (Zakim *et al.*, 2011). ICP usually manifests in the second or third trimester of pregnancy and is associated with itching, abnormal liver biochemistry, and jaundice. Although it usually resolves spontaneously after delivery, it is associated with fetal risk (e.g. prematurity, neonatal respiratory distress syndrome) (Dixon *et al.*, 2000). Both LPAC and ICP are treated with UDCA, which prevents gallstone formation in LPAC, and improves symptoms and liver biochemistry in ICP. Bile duct ligation or partial hepatectomy only slightly enhanced ABCB4 expression in mice (Stedman *et al.*, 2006; Csanaky *et al.*, 2009), while TPN decreased ABCB4 expression in rats (Nishimura *et al.*, 2005). Several other cellular stress

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conditions (e.g. endotoxin treatment) were not associated with an altered ABCB4 expression in animal studies (Vos *et al.*, 1998).

ABCB4 regulation is still poorly understood, but occurs partly via FXR and PPARα. FXR agonists (cholate, GW4064) transactivate the human *ABCB4* gene *in vitro*, which results in an increased maximal biliary PC secretion (L Huang *et al.*, 2003). FXR thus regulates both biliary bile acid (ABCB11) and phospholipid (ABCB4) excretion. PPARα agonists (fibrates) also increased ABCB4 expression in human hepatocytes (Ghonem *et al.*, 2012).

ABCG5/8

ABCG5/8 is the main sterol transporter, and plays a key role in the biliary excretion of cholesterol and plant sterols (i.e. phytosterols). Mutations in the *ABCG5* or *ABCG8* gene lead to the development of sitosterolemia, which is characterized by sterol accumulation and atherosclerosis.

ABCG5 (73kDa) and ABCG8 (76kDa) are both members of the G sub-family of ABC transporters. Members of this transporter family are half-transporters, as mentioned in our section on ABCG2. ABCG5 and G8, which each consist of one nucleotide-binding and one 6-helical transmembrane domain, consequently need to combine in order to become functional (Figure 3) (Graf *et al.*, 2002). The ABCG5/8 heterodimer transports sterols (i.e. phytosterols and cholesterol; Table 1), and is expressed in the apical membrane of hepatocytes and enterocytes (Berge *et al.*, 2000). This expression pattern allows ABCG5/8 to promote sterol excretion in the bile, and to prevent sterol uptake from the intestinal lumen. *ABCG5/8* knockout mice displayed a 75% decrease in biliary cholesterol excretion, which showed a large but not exclusive role for ABCG5/8 in biliary cholesterol transport (the remaining 25% was partly transported by canalicular SR-B1) (Yu, Hammer, *et al.*, 2002; Klett *et al.*, 2004; Wiersma *et al.*, 2009; Dikkers *et al.*, 2013). These mice do not display a severe cholestatic phenotype like *ABCB4* knockout mice, which indicates that

mixed micelle formation remains adequate in the absence of this transporter (Yu, Hammer, *et al.*, 2002; Klett *et al.*, 2004; Wiersma *et al.*, 2009; Dikkers *et al.*, 2013). Other studies in mice showed that ABCG5/8 over-expression protected against atherosclerosis. This protective effect was only present in mice that overexpressed this transporter both in the bile canaliculus and in the intestine, which illustrated the complementary effect of canalicular and intestinal ABCG5/8-mediated sterol transport (Yu, Li-Hawkins, *et al.*, 2002; Wilund *et al.*, 2004). The role of ABCG5/8 in sterol transport was first discovered in sitosterolemia, which is characterized by an increased dietary absorption and a decreased biliary excretion of sterols (Berge *et al.*, 2000; MH Lee *et al.*, 2001). Patients with this rare inherited disease consequently accumulate phytosterols (e.g. sitosterol, stigmasterol, campesterol, 5α-cholestanol, 5α-campestanol, 5α-sitostanol, 22-dehydrocholesterol, brassicasterol, and 24-methylene cholesterol) and cholesterol in their blood, and suffer from premature development of atherosclerosis (Berge *et al.*, 2000). Since sitosterolemia is caused by mutations in the *ABCG5* or *ABCG8* gene, it was concluded that cholesterol and the above-mentioned plant sterols are ABCG5/G8 substrates. *ABCG5/8* polymorphisms, such as the common SNP rs11887534, also increase the risk of cholesterol gallstones (and lead to obstructive cholestasis), likely by increasing the biliary cholesterol content (Grünhage *et al.*, 2007). Apart from its role in gallstone formation, ABCG5/8 does not seem to be a major contributor to cholestatic disease, as illustrated by the absence of a cholestatic phenotype in *ABCG5/8* knockout mice and sitosterolemia patients.

ABCG5/8 transcription is mainly regulated via the liver X receptor (LXR) and FXR (Janowski *et al.*, 1996; Lehmann *et al.*, 1997; Janowski *et al.*, 1999; Gupta *et al.*, 2002; Freeman *et al.*, 2004). LXR is activated by oxysterols and promotes sterol excretion (ABCG5/8) and the conversion of cholesterol into bile acids (CYP7A1) in rodents (Gupta *et al.*, 2002). FXR inhibits LRH-1 (via SHP), which decreases ABCG5/8 expression in human liver and intestinal cell lines (Freeman *et al.*, 2004). FXR also inhibits CYP7A1 and

CYP8B1, which leads to a reduced bile acid synthesis (Gupta *et al.*, 2002). FXR and LXR thus have opposite effects on ABCG5/8 and bile acid synthesis. Several other transcription factors also play a role in *ABCG5/8* transactivation. GATA-binding protein 4 (GATA4), GATA6, and hepatocyte nuclear factor (HNF) 4-α synergistically induce human *ABCG5/8* transcription *in vitro* (Sumi *et al.*, 2007). Thyroid hormone also increased biliary cholesterol excretion in animal models by increasing ABCG5/8 expression, although the exact mechanism remains to be elucidated (Gälman *et al.*, 2008; Bonde *et al.*, 2012). Treatment with thyroid hormone and its liver specific agonists (e.g. eprotirome, sobetirome) significantly lowered cholesterol in various animal models, although its use in humans will be limited due to potential side effects, and the safety and efficacy of statin treatment. Insulin resistance can, finally, increase ABCG5/8 expression in mice via disinhibition of the forkhead box O1A (FOXO1) transcription factor by insulin (Biddinger *et al.*, 2008).

Canalicular ABC transporters, and their regulatory NRs, as drug targets

Canalicular ABC transporters, and their NRs, play a key role in bile formation and cholestasis. As such they are attractive targets for the treatment of cholestatic disease. We will therefore briefly discuss the effect of several important (experimental) treatment strategies on their expression.

UDCA, the only FDA approved drug for cholestasis, promoted the canalicular insertion of ABCB11, ABCC2, and ABCB4 in rodents (Beuers *et al.*, 2001; Fickert *et al.*, 2001; Kurz *et al.*, 2001). This post-transcriptional modification stimulated bile flow (ABCB11, ABCC2) and promoted the excretion of various biliary constituents (e.g. bile acids, glutathione, phospholipids) (reviewed by Poupon, 2012). Although UDCA has limited transcriptional effects, it also acts as a weak FXR and (after intestinal conversion to lithocholic acid) PXR agonist in *in vitro* and animal studies (Staudinger *et al.*, 2001; Lew *et al.*, 2004). The

activation of these NRs increased the canalicular (e.g. ABCB11, ABCC2) and basolateral (e.g. ABCC3, ABCC4) expression of bile acid exporters (reviewed by Poupon, 2012; Halilbasic *et al.*, 2013). UDCA has, in addition, various other beneficial effects, such as increasing the hydrophilicity of the circulating bile acid pool, cytoprotection against bile acids and cytokines, immune-modulation, and anti-inflammatory effects (reviewed by Poupon, 2012). In PBC patients, UDCA combined with budesonide (but not UDCA or budesonide alone) restored the activity of cholangiocyte anion exchanger 2 (AE2), which mitigated the impaired choleresis in these patients (Arenas *et al.*, 2008). UDCA also induced the antimicrobial peptide cathelicidin in PBC patients, presumably via VDR activation (D'Aldebert *et al.*, 2009).

The development of norUDCA, a side chain shortened UDCA analogue, represents a promising new treatment strategy for cholestatic bile duct diseases. NorUDCA does not exert its primary therapeutic effects via canalicular ABC transporters, although it did increase ABCB11 activity *in vitro* (Kagawa *et al.*, 2013). Nevertheless, it almost completely reversed sclerosing cholangitis in the *ABCB4* knockout mouse model for PFIC3/PSC (Fickert *et al.*, 2006). Its suggested therapeutic mechanisms include: an increased hydrophilicity of the circulating bile acid pool, protection of injured bile ducts by a bicarbonate-rich choleresis, a decreased hepatocellular bile acid load by the induction of basolateral bile acid efflux transporters and bile acid detoxification pathways (phase I and II enzymes), and various anti-inflammatory and anti-fibrotic properties (reviewed by Trauner *et al.*, 2008). NorUDCA supposedly has an intrinsic capacity to undergo cholehepatic shunting, which is essential for several of its beneficial effects (e.g. biliary $HCO3^-$ output) (Halilbasic *et al.*, 2009). The above-mentioned beneficial effects clearly favor its therapeutic potential, and norUDCA treatment is currently evaluated in PBC and PSC patients.

The past years have witnessed the development of several synthetic FXR activators. These activators have a far higher affinity for FXR than natural bile acids, and can be either bile

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acid- or non-bile acid-derived. The hepatoprotective effects of these activators have been convincingly demonstrated in animal studies. FXR activation in rodents promoted bile formation via ABCB11, ABCC2, ABCB1, and ABCB4. FXR also repressed hepatocellular bile acid uptake and synthesis, and promoted bile acid elimination and detoxification, as discussed in our section on bile acid metabolism. GW4064, a non-bile acid-based FXR activator, and 6E-chenodeoxycholic acid (6E-CDCA, INT747), a synthetic bile acid analogue, ameliorated obstructive and chemically induced cholestasis in rats (Liu *et al.*, 2003; Fiorucci *et al.*, 2005). INT767, another synthetic bile acid analogue, mitigated biliary fibrosis and portal inflammation in the *ABCB4* knockout mouse. INT767 increased, among others, the biliary bicarbonate content in these animals, which decreased biliary toxicity (Baghdasaryan *et al.*, 2011). FXR activation also has anti-inflammatory properties, since chenodeoxycholic acid treatment induced the expression of the antimicrobial peptide cathelicidin in the human biliary epithelium (D'Aldebert *et al.*, 2009). FXR activation via GW4064, finally, counteracted bacterial overgrowth in bile duct ligated rodents (Ogata *et al.*, 2003). FXR activation thus promotes bile formation, decreases the hepatocellular bile acid load, decreases biliary toxicity, and has anti-inflammatory and anti-microbial effects. In recent phase II clinical trials, INT747 with or without UDCA co-treatment ameliorated the biochemical markers of liver damage in PBC patients that were non-responsive to UDCA alone. Results of a multi-center INT747 trial in UDCA-responsive PBC patients are currently awaited (Mason *et al.*, 2010; Hirschfield *et al.*, 2011; Kowdley *et al.*, 2011).

PXR and CAR induce bile acid detoxification, bile acid elimination, and bilirubin glucuronidation, as discussed in our section on bile acid metabolism. Several PXR and CAR ligands have been used to treat pruritus or jaundice long before their mode of action became known. Rifampicin, a classic PXR agonist, is used to treat pruritus in cholestatic patients, and ameliorated biochemical markers of liver damage in PBC patients (Bachs *et al.*, 1989; Cançado *et al.*, 1998; Yerushalmi *et al.*, 1999). Rifampicin induced bile acid and bilirubin

elimination via canalicular ABCC2. In addition, it induced bile acid detoxification (CYP3A4) and bilirubin conjugation (UGT1A1) in rodents (Marschall *et al.*, 2005). Its antipruritic effect may partly involve PXR-mediated transactivation of autotaxin, a recently identified mediator of pruritus (Kremer *et al.*, 2012). Phenobarbital, a potent CAR agonist, was used to treat neonatal jaundice in the 1960s, and exerts its hypobilirubinemic effect by inducing ABCC2 and UGT1A1 (reviewed by Cuperus *et al.*, 2009).

PPARs, finally, are fatty acid activated NRs that play an important role in lipid homeostasis. These NRs, however, also play a role in bile formation and cholestasis. Treatment with the PPARα agonist fenofibrate increased the canalicular expression of ABCB4 in human hepatoma cells, which may be beneficial in patients with inherited ABCB4 defects (i.e. PFIC3, LPAC, and ICP) (Ghonem *et al.*, 2012). In addition, PPARα decreased bile acid synthesis (CYP7A1), and induced bile acid detoxification (SULT2A1, UGT2B4, UGT1A3) in animal models (DD Patel *et al.*, 2000; Jung *et al.*, 2002; Barbier *et al.*, 2003; Fang *et al.*, 2005). The PPAR agonist bezafibrate showed beneficial effects in PBC patients in pilot trials, although these results need to be confirmed by larger randomized-controlled clinical trials (Honda *et al.*, 2013).

Conclusion and perspectives

Canalicular ABC transporters, and their regulatory transporters, play a key role in the pathogenesis and pathophysiology of cholestatic disorders. The study of these transporters has provided researchers and clinicians with a molecular framework that allows the development of novel treatment strategies. The clinical implementation of some of these treatments (e.g. FXR-agonists, norUDCA) will likely benefit cholestatic patients in the near future.

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Footnote section

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Figure legends

Figure 1. Nuclear receptors as key regulators of bile homeostasis in the liver.

Hepatic FXR represses bile acid uptake (NTCP) and synthesis (CYP7A1), and induces bile acid elimination (ABCB11, ABCC2, ABCC3, ABCC4) and detoxification (CYPs, SULTs, UGTs). FXR also stimulates the biliary excretion of phospholipid (ABCB4), but decreases canalicular ABCG5/8 activity (via SHP). PXR and CAR induce bile acid (ABCC2, ABCC3, ABCC4) and conjugated bilirubin (ABCC2) excretion. PPARα increases ABCB4-mediated phospholipid secretion (ABCB4), and induces bile acid detoxification. LXR promotes ABCG5/8-mediated cholesterol excretion. GR, finally, decreases bile acid uptake (NTCP) and increases basoalteral bile acid excretion (OSTα/β). For simplicity other uptake systems for organic anions and cations are not shown.

Black arrows: stimulatory effects; grey lines: suppressive effects on target genes. (BAs, bile acids; Bili-glu, bilirubin glucuronide; ABCB11, bile salt export pump; CAR, constitutive androstane receptor; CYP7A1, cholesterol-7a-hydroxylase, CYPs, cytochrome P450 enzymes; FXR, farnesoid X receptor; GR, glucocorticoid receptor; LXR, liver X receptor; ABCB4, multidrug resistance protein 3; ABCC2, multidrug resistance-associated protein 2; ABCC3, multidrug resistance- associated protein 3; ABCC4, multidrug resistance-associated protein 4; NTCP, sodium tauro-cholate co-transporting polypeptide; OSTα/β, organic solute transporter α and β; PC, phosphatidylcholine; PXR, pregnane X receptor; PPARα, peroxisome proliferator-activated receptor α; PPARγ, peroxisome proliferator-activated receptor γ; SHP, small heterodimer partner; SULTs, sulfatation enzymes; UGTs, glucuronidation enzymes).

Figure 2. The principal structure and function of nuclear receptors, as exemplified by FXR.

The structure and function of NR can be exemplified by FXR. Bile acid (⬡)-activated FXR heterodimerizes with RXR, recruits co-activators/ dissociates from co-repressors, and induces transcription of its target genes. The upper left panel shows the general structure of a nuclear receptor, consisting of an activation function domain-2 (AF-2), a ligand-binding domain, a hinge region, a DNA-binding domain, and an AF-1. The DNA- and ligand-binding domains recognize (promoter) DNA and NR ligands, and AF-1 and AF-2 induce ligand-independent nuclear receptor

transactivation.

Figure 3. The principal structure of canalicular ABC transporters.

The structure of canalicular ABC transporters can consist of 1, 2, or 3 transmembrane domains (for details kindly refer to the text). The ABCB (B1, B11) and ABCG (G2, G5, G8) transporter family members mentioned in the text have comparable structures, and are therefore not shown separately in this figure.

Table 1 Selected endogenous and exogenous canalicular ABC transporter substrates (bold face: demonstrated in human ABC transporter studies)

ABCB11	ABCC2	ABCB1	ABCG2	ABCB4	ABCG5/G8
Endogenous substrates					
Glycocholic acid	**Bilirubin mono- and diglucuronide**	**Aldosterone**	**Cholic acid**	**Phosphatidylcholine**	**Cholesterol**
Taurocholic acid	**Cholecystokinin-8-sulfate**	**Cholesterol**	**Estradiol-17β-glucuronide**		
Glycochenodeoxycholic acid	**Estradiol-17β-glucuronide**	**Cortisol**	**Estrone-3-sulfate**		
Taurochenodeoxycholic acid	**Estrone-3-sulfate**	**Estradiol-17β-glucuronide**	**Folic acid glutamates**		
Glycodeoxycholic acid	**Glutathione disulfide**	**Estrone**	**Glycocholic acid**		
Taurodeoxycholic acid	**Hyodeoxycholic acid glucuronide**	**Ethinylestradiol**	**Heme**		
Tauroursodeoxycholic acid	**Leukotriene C_4**	**Opioid peptides**	**Protoporphyrin IX**		
Taurolithocholate-3-sulfate	**Prostaglandin E2**	**Short-chain phospholipids**	**Taurocholic acid**		
	Taurolithocholatic acid sulfate	**Unconjugated bilirubin**	**Taurolithocholic acid sulfate**		
	Tauroursodeoxycholic acid	6α-OH-taurocholic acid	**Urate**		
	6α-OH-taurocholic acid				
Exogenous substrates					
Calcein-AM	Acetaminophen glucuronide	**Calcein-AM**	**4-Methylumbelliferone**	**Digoxin**	**24-Methylene cholesterol**
Pravastatin	Acetaminophen glutathione	Colchicine	**glucuronide**	**Paclitaxel**	**Brassicasterol**
Taxol	Acetaminophen sulfate	**Daunorubicin**	**4-Methylumbelliferone sulfate**	**Vinblastine**	**Campesterol**
Vinblastine	Ampicillin	**Digoxin**	**Albendazole sulphoxide**		**5α-Campestanol**
	Arsenite	**Diltiazem**	**Anthracenes**		**5α-Cholestanol**
	Bromosulfophthalein glutathione	**Docetaxel**	**Anthracyclines**		**22-Dehydrocholesterol**
	Cadmium	**Doxorubicin**	**Camptothecin derivates**		**Sitosterol**
	Carboxydichlorofluorescein-diacetate	**Erythromycin**	**Daunomycin**		**5α-Sitostanol**
	Ceftriaxone	**Ethidium bromide**	**Dinitrophenyl glutathione**		**Stigmasterol**

	Dibromosulfophthalein **Dinitrophenyl glutathione** Indomethacin glucuronide **Methotrexate** **Morphine glucuronide** Mycophenolic acid glucuronide **Paclitaxel** Phenobarbital glucuronide Phenolphthalein sulfate 2-amino-1-methyl-6- Phenylimidazo[4,5b]pyridine (PhIP) Phytoestrogen glucuronides **Pravastatin** **Probenecid** **Resveratrol sulfate** **Sulfinpyrazone** **Vinblastine** Zinc	**Etoposide** **Gramicidin D** **Hoechst 33342** Indinavir **Ivermectin** Losartan **Methotrexate** **Mitomycin C** **Opioid peptides** **Paclitaxel** **Rhodamine 123** **Ritonavir** **Saquinavir** **Teniposide** **Topotecan** **Valinomycin** **Verapamil** **Vinblastine** **Vincristine**	**Doxorubicin** E3040-glucuronide **Hoechst 33342** **Irinotecan (SN-38 metabolite)** **Imatinib** **Lysotracker green** **Methotrexate** **Mitoxantrone** **Nucleoside analogues** **Pheophorbide α** **PhIP** **Pitavastatin** **Rhodamine 123** **Topotecan**		

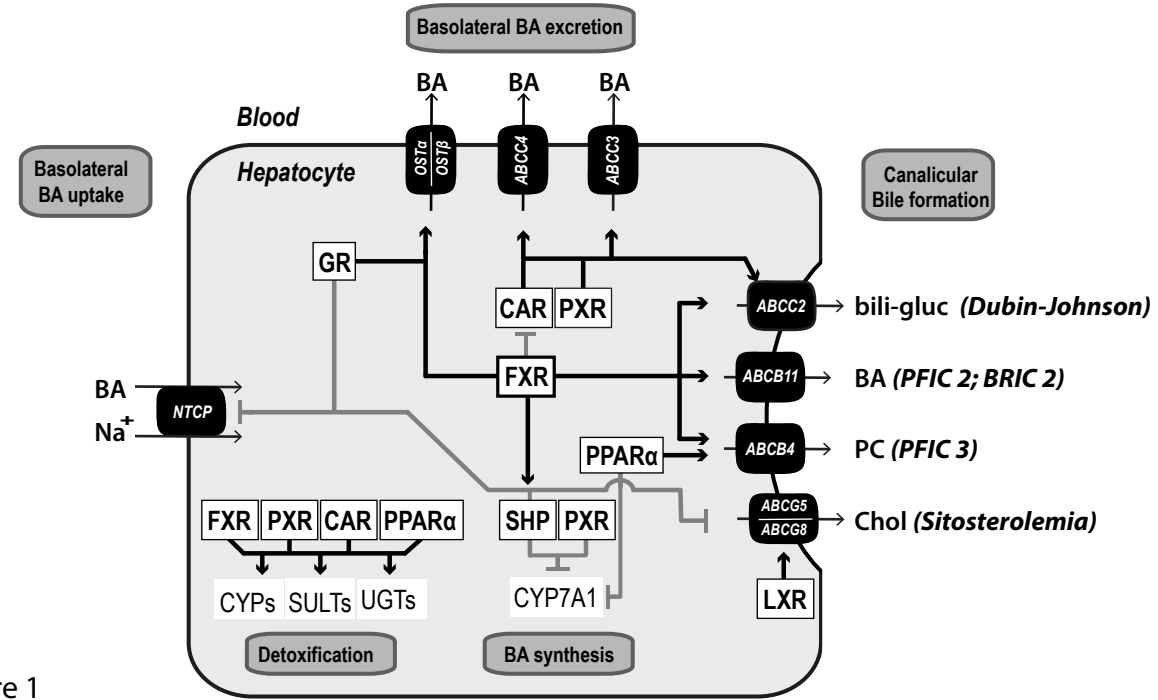
Basolateral BA excretion
BA
BA
BA
Blood
Hepatocyte
OSTα
OSTβ
ABCC4
ABCC3
Basolateral
BA uptake
Canalicular
Bile formation
GR
CAR
PXR
ABCC2
bili-gluc (Dubin-Johnson)
FXR
ABCB11
BA (PFIC 2; BRIC 2)
BA
NTCP
Na+
PPARα
ABCB4
PC (PFIC 3)
FXR
PXR
CAR
PPARα
SHP
PXR
ABCG5
ABCG8
Chol (Sitosterolemia)
CYPs
SULTs
UGTs
CYP7A1
LXR
Detoxification
BA synthesis

Figure 1

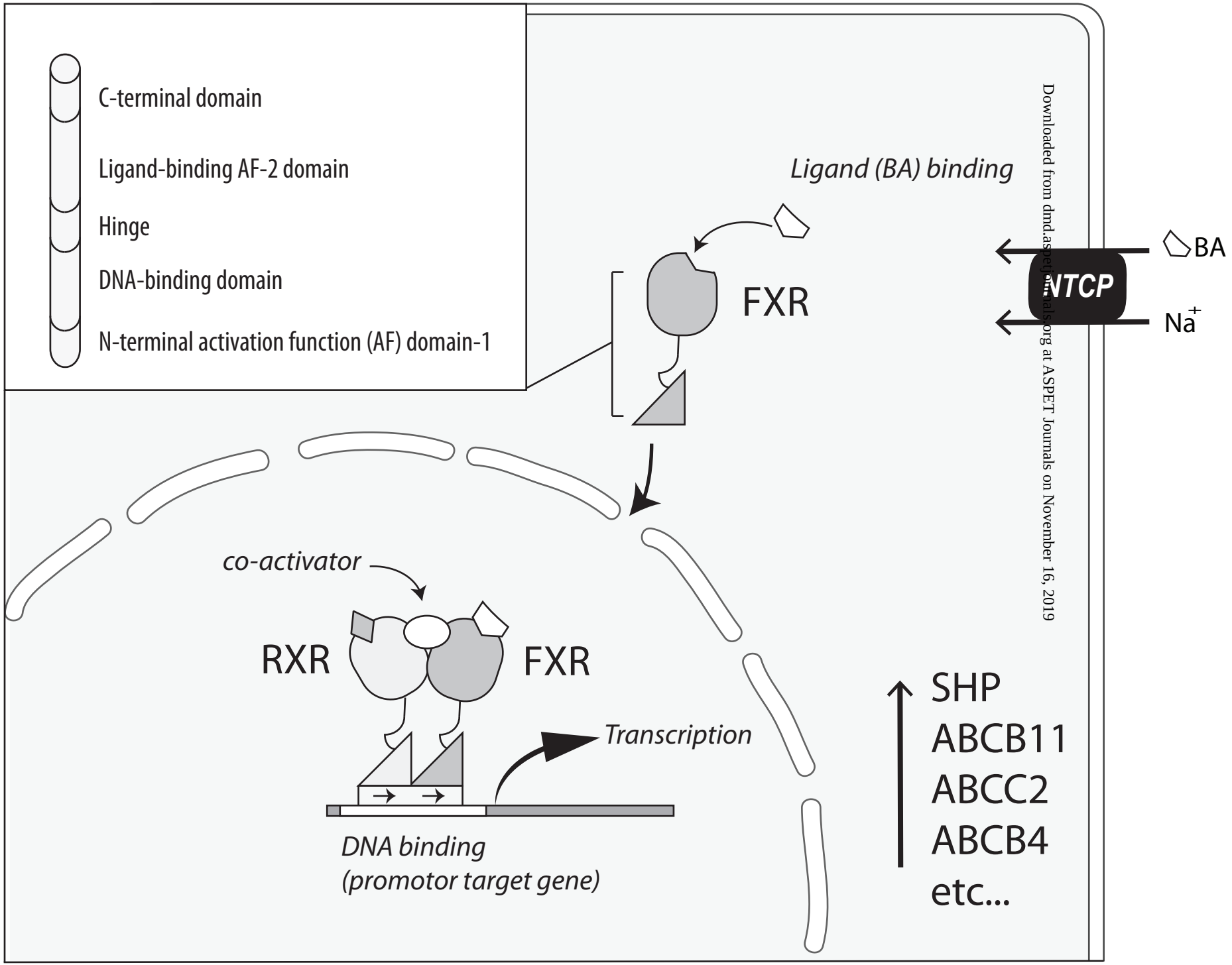

Figure 2

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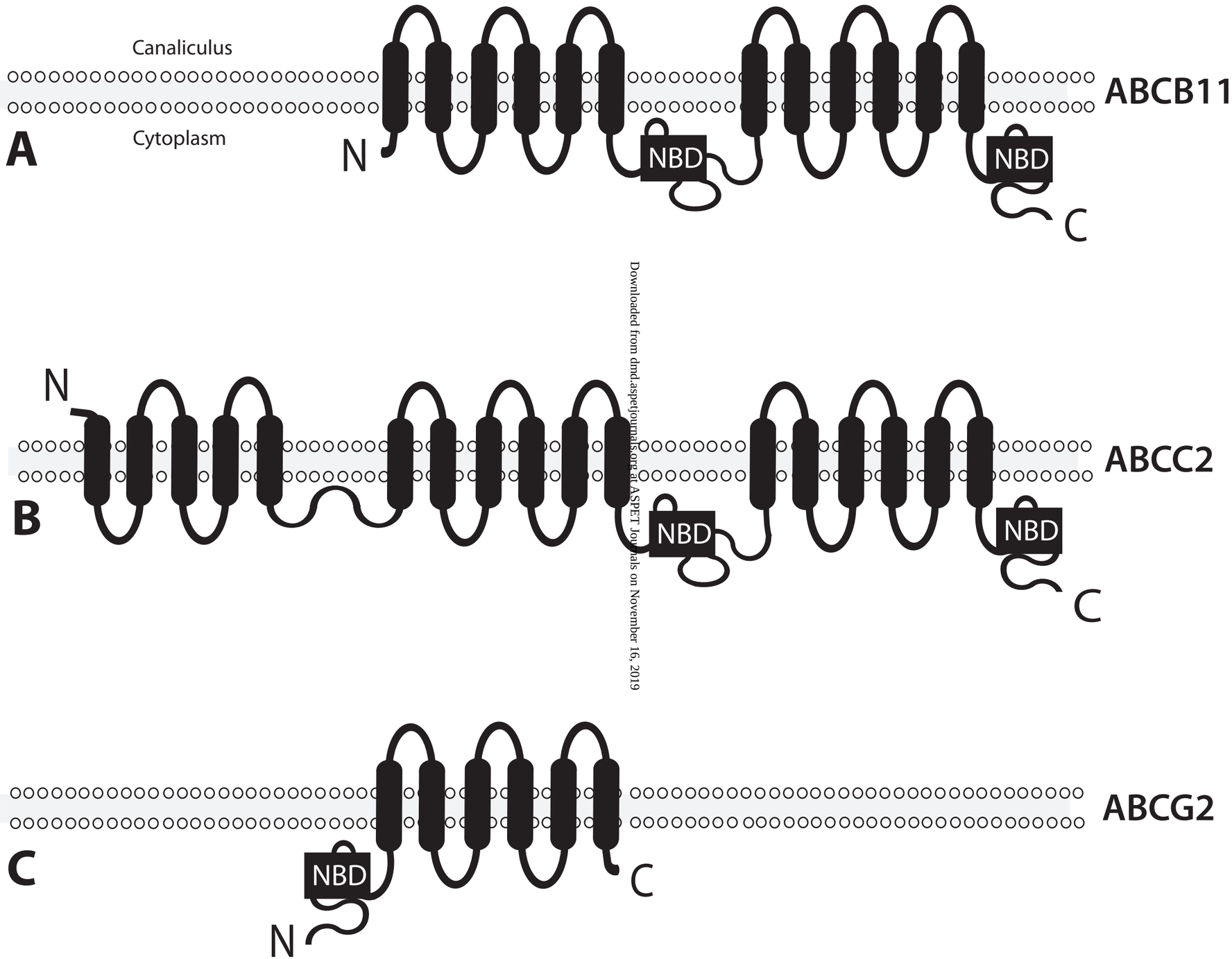

Figure 3