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Nuclear receptor FXR, bile acids and liver damage: Introducing the progressive familial intrahepatic cholestasis with FXR mutations☆

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ABSTRACT

The nuclear receptor farnesoid X receptor (FXR) is the master regulator of bile acids (BAs) homeostasis since it transcriptionally drives modulation of BA synthesis, influx, efflux, and detoxification along the enterohepatic axis. Due to its crucial role, FXR alterations are involved in the progression of a plethora of BAs associated inflammatory disorders in the liver and in the gut. The involvement of the FXR pathway in cholestasis development and management has been elucidated so far with a direct role of FXR activating therapy in this condition. However, the recent identification of a new type of genetic progressive familial intrahepatic cholestasis (PFIC) linked to FXR mutations has strengthen also the bona fide beneficial effects of target therapies that bypass FXR activation, directly promoting the action of its target, namely the enterokine FGF19, in the repression of hepatic BAs synthesis with reduction of total BA levels in the liver and serum, accomplishing one of the major goals in cholestasis. This article is part of a Special Issue entitled: Cholangiocytes in Health and Diseaseedited by Jesus Banales, Marco Marzioni and Peter Jansen.

1. Nuclear receptor FXR is the master regulator of bile acids homeostasis

The farnesoid X receptor (FXR) belongs to a family of proteins known as nuclear receptors (NRs). 48 NR genes have been identified in the human genome and 49 in the mouse genome [71]. NRs are a group of ligand-activated transcription factors that mediate a wide range of physiological processes, including development, metabolism, and reproduction [71]. NRs mediate hormonal, metabolic and nutritional signals by promoting gene transcription in order to drive precise and coordinated functional responses and orchestrating a close cooperation between different organs. The structure of these receptors usually consists of an N-terminal DNA binding domain (DBD) and a C-terminal ligand-binding domain (LBD). The DBD is the most conserved region, containing two zinc finger motifs that allow the NR binding to a consensus AGGTCA-like DNA sequence (called responsive elements, REs). NRs bind to regulatory regions of target genes as a homodimer or heterodimer with the retinoid X receptor (RXR), acting in concert with co-activators and co-repressors in order to activate or repress gene expression [71]. In the absence of ligand, the NRs are often complexed on the chromatin with co-repressor proteins. Upon ligand binding to the NR, the co-repressor complex dissociates, and co-activator proteins are recruited. Among the NR ligands fatty acids, oxysterols and BAs are included. This integration of environmental stimuli with specific

Abbreviations: NR, nuclear receptor; FXR, farnesoid X receptor; RXR, retinoid X receptor; PXR, pregnane X receptor; VDR, Vitamin D receptor; TGR5, G protein–coupled bile acid receptor; Lrh1, liver receptor homolog 1; Mdr, multidrug resistance protein; Abcb4, ATP Binding Cassette Subfamily B Member 4; Bsep, bile salt export pump; Abcb11, ATP Binding Cassette Subfamily B Member 11; BACS, BA CoA synthase; BAAT, BA-CoA-amino acid *N*-acetyltransferase; SHP, small heterodimer partner; CYP7A1, cytochrome P450 7A1; MRP2, multidrug related protein 2; DBD, DNA binding domain; LBD, ligand binding domain; BA, bile acids; CA, cholic acid; CDCA, chenodeoxycholic acid; OCA, obeticholic acid; UDCA, ursodeoxycholic acid; HCC, hepatocellular carcinoma; IBD, inflammatory bowel disease; PFIC, progressive familial intrahepatic cholestasis; ICP, cholestasis of pregnancy; DIC, drug-induced cholestasis; BRIC, benign recurrent intrahepatic cholestasis; FGFR4, fibroblast growth factor receptor 4; FGF15/19, fibroblast growth factor 15/19; TNFα, tumor necrosis factor α; BAK1, BCL-2 antagonist killer 1; FADD, Fas-associated death domain; BCL2, B cell lymphoma gene-2; NF-κB, nuclear factor-κB; HSC, hepatic stellate cells; CTGF, connective tissue growth factor; TGF-β, transforming growth factor-β; PDGF, platelet-derived growth factor; NTCP, sodium (Na)-Taurocholate Cotransporter Protein; OSTα/β, organic solute transporter; OATP, organic anion transporting polypeptide

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transcriptional responses makes NRs central for whole-body physiology. Several of NRs were initially categorized as 'orphan' receptors because their natural ligands were unknown. Over the past 20 years, novel ligands have been matched with their orphan parents, leading to the identification of various homeostatic pathways [109].

FXR was first described in 1995 by Forman and co-workers [30], as a product of NR1H4 gene. FXR was described as a NR activated by the farnesol derivative, a metabolic intermediate of the mevalonic pathway. FXR is involved in the biosynthesis of cholesterol, BAs, sterol compounds, porphyrin, dolichol, ubiquinone, carotenoids, retinoids, vitamin D, steroid hormones, and farnesylated proteins. In following years, it became clear that FXR was activated by BAs and that it should be considered as the master regulator of bile acids homeostasis [70,95]. BAs are amphipathic molecules synthesized exclusively in the liver in order to allow the efficient digestion and absorption of lipids, cholesterol, and fat-soluble vitamins after food ingestion. BAs are cholesterol derivatives and their synthesis is a multistep reaction that involves several enzymes in different hepatic cellular compartments, such as the cytosol, mitochondria, endoplasmic reticulum and peroxisomes [106]. This is the "classical" pathway and leads to an equal amount of cholic acid and chenodeoxycholic acid (CA and CDCA, respectively). The first step in the 'classical' pathway is the 7α-hydroxylation of cholesterol by the rate-limiting enzyme cytochrome P450 7A1 (CYP7A1). Alternative production of BAs occurs by 27-hydroxylase and it is responsible for the production of oxidized cholesterol that is converted predominantly to CDCA in the liver [9]. Before being release from the liver, BAs are conjugated with taurine or glycine to form less toxic and more hydrophilic bile salts [132]. Normally, under fasting conditions, BAs are stored in the gallbladder and after the postprandial stimulus, BAs are secreted into the small intestine, where they participate in the digestion of food. Ultimately, they are reabsorbed back through the portal circulation into the liver [62] thereby reducing the requirement for de novo BA synthesis. Through this pathway, 95% of the BAs are recycled, and only 5% of them are newly synthesized in the liver daily in order to sustain a proper BA pool in the organism and to bypass an over expenditure of energy required for their synthesis. In mouse models, altered BA signaling in the liver is associated with severe diseases, including the development of cholestasis and hepatocellular carcinoma (HCC) [18,81]. The regulatory function of BAs is mainly a result of BA activation of various intracellular ligand-activated NRs, such as pregnane X receptor (PXR), the vitamin D receptor (VDR), the G protein–coupled bile acid receptor (TGR5) and FXR that orchestrate a tight control of BA production and circulation.

According to its major function as master regulator of BA homeostasis, FXR has been shown to have a specific tissue distribution; it is expressed along the entire gastrointestinal tract with a peak in the liver and ileum, as well as in the kidney, and adrenal glands [30,66,135]. Low FXR expression profiles have been detected in the heart, adipose tissue [148] and in some hormone-responsive tissues, such as the breast [122]. The important role of FXR in regulating BA synthesis was initially shown in $FXR^{-/-}$ mice. These transgenic animals exhibit an increased BA pool size combined with an increased expression of proinflammatory cytokines, resistance to apoptosis and cell hyperproliferation that lead to spontaneous HCC development between 12 and 15 months of age [51,55,111,142].

2. FXR in the gut-liver axis: orchestrating the bile acids metabolism

The regulation of BA concentrations within hepatocytes, enterocytes and in the enterohepatic circulation is orchestrated by tissue-specific FXR activities via an intensive molecular cross-talk between the liver and the intestine. FXR influences BA flux via various feedforward and feedback loops: it decreases BA de novo synthesis in the liver, while it increases BA secretion into the small intestine. In the liver, FXR-activation induces the expression of the small heterodimer partner (SHP), which interacts with the liver receptor homologous 1 (LRH-1), repressing its activity [36,61,65]. As a consequence, SHP-LRH-1 interaction results in a CYP7A1 reduced expression, ultimately decreasing BA synthesis. In a similar manner, via SHP-mediated repression through the hepatocytes nuclear factor 4α (HNF4α), FXR inhibits another critical cytochrome, the CYP8B1, which regulates the ratio between CA and CDCA [146]. Newly-synthesized BAs are conjugated with taurine or glycine, and FXR regulates these important processes inducing the BA CoA synthase (BACS) and BA-CoA-amino acid *N*-acetyltransferase (BAAT) [114], two enzymes responsible for BAs conjugation. Conjugated BAs are then actively secreted in the canaliculi by the bile salt export pump (BSEP/ABCA11) and the multidrug related protein 2 (MRP2/ABCC2) and stored in the gallbladder. These transporters belong to the ABC transporter family and are both induced by FXR at transcriptional level. Moreover, FXR activation also induces the expression of the multidrug protein 3/2 (MDR3/Mdr2, ABCB4/Abcb4), another ABC transporter involved in the biliary secretion of phosphatidylcholine [79].The regulation of these ABC transporters is crucial in order to avoid BA accumulation in the liver and, consequently, hepatic injury. Indeed, mutations in the BSEP and MDR3 proteins are responsible for two different type of progressive familial intrahepatic cholestasis (PFIC) [119] [17]. The FXR-dependent concomitant activation of BSEP and MDR3 has an extensive and protective physiological role avoiding BA cytotoxicity by their incorporation into phospholipid micelles.

After fat ingestion, the hormone cholecystokinin is released from the proximal intestinal tract, causing gallbladder contraction and bile delivery into the small intestine. The bile travels along the intestine and at the distal ileum, the majority of the BAs are actively absorbed and returned to the liver through the portal vein, to be re-secreted into the bile [64], in a process called enterohepatic circulation. During this event, in the distal intestine conjugated BAs undergo a de-conjugation process by bacterial enzymes, allowing unconjugated BAs to cross the plasma membrane via passive diffusion. In addition, BAs are also actively reabsorbed by the Apical Sodium-dependent Bile Acid Transporter (ASBT) [141]. In the ileum, BA-dependent FXR activation induces the fibroblast growth factor FGF15/19 (mouse and human, respectively), a hormone secreted in the portal circulation that is able to reach the liver and to bind to the fibroblast growth factor receptor 4 (FGFR4)/β-Klotho complex. FGF15-FGFR4/β-Klotho binding triggers the c-jun N-terminal kinase-dependent pathway [43], which ultimately leads to CYP7A1 repression. This mechanism constitutes an important crosstalk between intestine and liver in the regulation of BA synthesis. Moreover, the use of tissue-specific liver- or intestine-$FXR^{-/-}$ mice displayed the relative contribution of hepatic and intestinal FXR in the repression of CYP7A1 and indicated a more determinant role for the intestinal FXR [50]. In the enterocytes, BAs are shuttled from the apical to the basolateral membrane by the intestinal BA binding protein (IBABP) [35,123,124], although the precise biological function of IBABP is not clear [85].

Subsequently, BAs are secreted in the portal vein by the heterodimeric organic solute transporter OSTα/β [15]. At this level, BAs are transported back to the liver, where the great majority is reabsorbed by the sodium (Na)-Taurocholate Cotransporter Protein (NTCP) and organic anion transporting polypeptide (OATP), both negatively regulated by FXR, thereby limiting the increase of hepatic BA levels. Notably, FXR directly induces IBABP and OSTα/β expression at promoter level, while the expression of human ASBT is negatively regulated via SHP [86]. Finally, BAs are re-secreted into the bile [64], closing up the BAs enterohepatic cycle.

3. Defining the FXR role in gut-liver axis diseases

BAs are potentially toxic for the organism, and the excessive increase in BA levels has been depicted in different pathological contexts. Therefore, as shown in several mouse models, it is not unexpected that

FXR dysfunctions may concur to the progression of associated inflammatory disorders within the gut-liver axis, ranging from inflammatory bowel disease (IBD) to colorectal cancer in the gut [32,72,80,88] and from gallstones diseases to fibrosis and hepatocellular carcinoma in the liver [60,67,121].

3.1. The FXR beneficial role in intestinal disease is independent of BA levels

Several studies have highlighted the FXR-BA interaction in the pathophysiology of a plethora of gastrointestinal diseases. At the intestinal level, FXR activation is linked to decreased inflammation and preservation of the intestinal barrier integrity. Indeed, $FXR^{-/-}$ mice display a compromised epithelial barrier and increased intestinal permeability [43]. Consequently, the lack of FXR has been associated with bacteria infiltration, and subsequently, inflammation due to activated macrophages and neutrophils recruitment [80,131]. On the contrary, FXR activation reverses the inflammatory phenotype by decreasing cytokine production and preserving the architecture of intestinal epithelial barrier, thus limiting bacterial overgrowth [43,131]. Furthermore, it has been shown that FXR is able to control the immune reaction and colon inflammation-driven fibrosis in different murine models [32]. Interestingly FXR is not only able to inhibit inflammation in the gut, but its activation has been also observed being inhibited by proinflammatory stimuli in different contexts [31]. Moreover, FXR expression is decreased in patients with Crohn's colitis and colitis-associated neoplasia, and several genetic variations in the FXR gene sequence has been linked to IBD, thus confirming the FXR protective role in the molecular pathogenesis of IBD in human [1,88,125].

The regulation exerted by FXR on intestinal BAs mediates a wide range of enterohepatic diseases. Low levels of BA results in bacterial overgrowth and possible systemic infections, while excessive BA concentration leads to chronic diarrhea, IBD, and intestinal tumorigenesis. Although increased intestinal exposure to BA predisposes to tumor development, FXR oncosuppressive role in intestinal carcinogenesis appears not to be associated with BAs tumorigenic effects. Indeed, the susceptibility of $FXR^{-/-}$ mice to intestinal tumor development is not associated with the increased BA concentrations, as shown by treatment with cholestyramine (a BA sequestrant resin), further revealing that the absence of FXR per se, and not merely high BA levels, promotes intestinal tumorigenesis [80]. The high susceptibility to colorectal cancer displayed by the $FXR^{-/-}$ mice is mainly related to the promotion of WNT pathway via tumor necrosis factor α (TNFα) released by macrophages infiltrating the compromised intestinal mucosa [43,80,91]. On the contrary, FXR activation decreases intestinal cell proliferation and enhances the apoptosis process by upregulation of the mRNA levels of pro-apoptotic genes, such as P21, BCL-2 antagonist killer 1 (BAK1), FAS, Fas-associated death domain (FADD), and by decreasing the expression of anti-apoptotic genes, such as B cell lymphoma gene-2 (BCL2) and TNFα [80]. It has also been shown that FXR gene expression is downregulated in familial adenomatous polyposis (FAP) patients and in different murine models of colorectal cancer [72,80]. Moreover, in vivo and in vitro studies have revealed an inverse correlation between the FXR expression in the intestinal mucosa and the rate of carcinoma along the gut, with low FXR levels detectable in intestinal carcinoma cells compared to normal ones [16,125]. Furthermore, in the ileum, the high degree of FXR expression correlates with low carcinoma incidences, whereas in the colon carcinomas occur concurrently with very low FXR expression.

Overall, these evidences suggest a beneficial role of FXR in the intestinal pathophysiology. The extent of FXR expression could possibly be considered as a new prognostic marker for colorectal cancer progression and aggressiveness. Moreover, this will offer novel perspectives on therapeutic insights aimed at promoting the tumor suppressive role of FXR.

3.2. The FXR protective role in liver disease

The ability of BAs to affect liver regeneration and growth has been extensively elucidated [4]. Mice fed with a low dose of BAs displayed liver regeneration while reducing BA levels by cholestyramine delayed liver regeneration [42]. FXR plays a key role in mediating the BA-induced liver regeneration, exerting at least two roles. On one hand it suppresses Cyp7a1 and reduce BA synthesis through the coordinated activation of FGF15/19 in the intestine and SHP in the liver, whereas on the other FXR supports hepatocyte proliferation promoting the Foxm1b gene expression, a regulator of hepatic cell cycle progression [41,115,144]. In spite of the impaired liver regeneration ability observed in liver- and intestine-specific $FXR^{-/-}$ mice, FXR activation in wild type animals through the synthetic ligand GW4064 was unable to sustain hepatic growth, possibly due to the normal BA pool size that blunt its pro-proliferative effects [42,145].

FXR is not only able to foster liver regeneration and growth, but it also demonstrates effective repair ability in liver damage. Without the FXR contribution to liver repair after injury, the liver will be more prone to start endless cycles of injury and repair characterized by production of inflammatory cytokines and growth factors that are potential tumor promoters [6,42]. Most recent findings clearly indicated that FXR provides protection against hepatocarcinogenesis. The tight control of BA homeostasis exerted by FXR prevents the neoplastic transformation of hepatic cells due to oxidative damage, inflammation, and resistance to apoptosis induced by cytotoxic chronic high accumulation of BAs [99]. Moreover, FXR has also been shown to antagonize nuclear factor-κB (NF-κB), negatively regulating hepatic inflammation, thus contributing to hepatoprotection and suppression of hepatocarcinogenesis [139]. $FXR^{-/-}$ mice displayed high levels of the proinflammatory cytokine IL-1β, protooncogene β-catenin, and the β-catenin target gene c-Myc at 3 months of age, and developed spontaneous adenomas and carcinomas after 12 months [51,140,142]. Interestingly, selective intestinal FXR reactivation in $FXR^{-/-}$ mice protects against hepatocellular carcinoma development. Indeed, intestinal FXR activation prevents liver tumor development through a tight control of BA synthesis due to the restoration of the FGF15 axis, which protects from BA overload, limiting in this manner hepatic inflammation and proliferation while preserving intestinal epithelium integrity [18].

The ability of the FXR pathway to counterbalance NF-κB-mediated transcription of pro-inflammatory cytokines is pivotal to explain the FXR involvement in the prevention and resolution of liver fibrosis [31,76,139]. The progressive fibrosis accumulation within the liver parenchyma as a consequence of sustained liver injury is mainly due to Hepatic Stellate Cells (HSCs) activation. Pro-inflammatory and pro-fibrotic cytokines such as connective tissue growth factor (CTGF), transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) contribute to HSC activation, therefore promoting liver fibrosis, and ultimately cirrhosis, through extracellular matrix deposition [75]. It has been shown that FXR activation decreases the HSCs sensitivity to TGF-β [24]. Moreover, FXR activation in human and rats HSCs reduces fibrosis at early stages and it is able to prevent hepatic inflammation and fibrosis in different mice models of non-alcoholic steatohepatitis [26,28,143,147]. On the contrary, the ablation of FXR has been correlated with increased hepatic inflammation and fibrosis [139,142].

FXR plays also a protective role in cholesterol gallstone disease, in which the development of cholesterol gallstone is the ultimate result of disrupted homeostasis between cholesterol, bile salt, and phospholipid levels in the bile. It has been demonstrated that $FXR^{-/-}$ mice are more susceptible to gallstone formation when fed a lithogenic diet, whereas FXR activation in wild type mice in the same conditions prevents gallstone formation, thus indicating a crucial role of FXR in maintaining the proper solubilisation of cholesterol into bile [83].

Taken together these studies highlight the pivotal role played by FXR in counteracting hepatic inflammation and carcinogenesis, thereby

suggesting a possible benefit for patients upon therapeutic modulation of FXR.

3.3. The FXR pathway in cholestasis: finding new PFIC with FXR mutations

Cholestatic liver disorders consist of a wide spectrum of diseases with different etiologies. Cholestasis is characterized by impairment or reduction of bile flow, ascribable to either acquired or hereditable defects of bile formation process in hepatocytes or cholangiocytes or caused by a physical obstruction in bile ducts. The interruption of BA enterohepatic circulation leads to the accumulation of toxic hydrophobic biliary compounds that play a key role in cholestasis-associated liver damage.

Genetic studies have revealed that defects in several components of FXR pathway account for different clinical type of pediatric and adult cholestasis, such as intrahepatic cholestasis of pregnancy (ICP), drug-induced cholestasis (DIC) or progressive familial intrahepatic cholestasis (PFIC) [12,133]. ICP is a pathological condition occurring in 1/200 pregnancies in Caucasian women, that can lead to intrauterine fetal death [40,103]. Four different heterozygous variants of FXR (−1 > g, M1V, W80R, M173T) found in or near the transcribed sequence of FXR were described in ICP patients, thus resulting in defects on translational efficiency or activity of the NR, as indicated by the downregulation of FXR target genes upon bile salt stimulation [73,128].

PFIC is a heterogeneous group of liver disorders of autosomal recessive inheritance, characterized by an early onset of the disease (usually during infancy) with pruritus and malabsorption, which may progressively result in liver failure. All PFIC are characterized by impaired bile secretion from the hepatocytes to the canaliculi. Nowadays, it is possible to distinguish 5 different types of PFIC, with the latest one (PFIC with FXR mutations) recently identified. Molecular and genetic studies have shown that mutations in hepatocellular genes involved in bile formation are responsible for the onset of the disease. Furthermore, metabolic defect in BA synthesis can be consider as a type of PFIC. For example, in humans, a deficiency in CYP7A1 enzyme causes severe cholestasis, cirrhosis, and liver failure [110].

The first PFIC type, is associated with ATP8B1 (FIC1) mutations (chromosome 18) [7,52]. The protein FIC1 functions are still unknown, but evidences suggest that it is a flippase for aminophospholipid transport, involved in maintaining asymmetric distributions of lipids in the membrane bilayer, playing a protective role against high bile salt concentration in the canalicular lumen [97,117]. The genotype-phenotype association in this PFIC patients is complex since ATP8B1 mutations are also present in patients with milder manifestation, as benign recurrent intrahepatic cholestasis (BRIC1) and ICP, where FIC1 protein function is only partially impaired [84,93,130]. It has been described that FIC1 deficiency results in a decreased FXR activity, that could be implied in the pathogenesis of the disease [54].

Another type of PFIC is caused by ABCB11 mutations (chromosome 2). The ABCB11 gene encodes for BSEP, a pump expressed at the hepatocyte canalicular membrane, which mediates the active transport of BAs into the canalicular lumen, generating bile flow. Genetic mutations (insertion, deletion, nonsense and splicing mutations) result in either premature protein truncation or total failure of protein production, with consequent non-detectable BSEP on immunohistological analysis [14,46,56,119,136]. However, detectable BSEP expression is not indicative, since missense mutations affecting protein processing and trafficking, as well as protein folding, have also been identified [120]. In the milder form of cholestasis (BRIC2, ICP), missense mutations in the less conserved region of the gene are more common than those leading to failure of protein production [49,96,129].

Moreover, the third type of PFIC, associated with high GGT levels, is caused by defects in the adenosine triphosphate-binding cassette, subfamily B, member 4 (ABCB4) gene encoding multidrug resistance class III (MDR3) protein [45]. MDR3 is a floppase, predominantly expressed in the canalicular membrane of hepatocytes, responsible for phospholipids excretion within the bile (mainly phosphatidylcholine) [82,113]. The mechanism of liver damage in these patients is likely related to the absence of biliary phospholipids. Specifically, cholestasis results from the toxicity of bile in which detergent bile salts are not inactivated by phospholipids, leading to bile canaliculi and biliary epithelium injuries. Moreover, the absence of phospholipids destabilizes micelles and induces lithogenicity of bile with crystallization of cholesterol, which could further increase liver injury favouring small bile duct obstruction. Mutations in ABCB4 gene were present in both alleles in most cases. In one third, mutations resulted in no expression of the MDR3 protein, ascribable to a rapid destruction of truncated protein after synthesis or to a premature stop codon causing instability or decay of the ABCB4 mRNA [17,19,149]. The remaining two third of patients presented missense mutations, associated with a reduction of protein expression or in functional defects in the transporter ATPase activity [5,37,53,94]. Milder phenotypes of PFIC with ABCB4 mutations present ICP, DIC, cholesterol gallstone diseases, adult idiopathic cirrhosis and transient neonatal cholestasis [13,21,44,49,68,104]. In some patients, the absence of MDR3 may present a clinical continuum, starting with cholesterol gallstone disease, followed by PFIC and ending with biliary cirrhosis.

Recently, also mutations in TJP2 (tight junction protein 2) gene (chromosome 9) were associated with PFIC. Differently from the other PFIC associated with mutations in transporter genes, TJP2 codifies for a protein involved in the organization of epithelial and endothelial intercellular junctions. The TJP2 mutations found abolished the protein translation [107]. Alterations in TJP2 coding sequence producing a non-functional protein were described in hypercholanemia [8].

Despite the clear involvement of the FXR pathway in the pathogenesis of cholestasis, for many years no mutations in NR1H4 gene were described. However, the protective role of FXR activation has been extendedly investigated using cholestasis mouse and rat models. Systemic activation of FXR by specific synthetic ligands such as GW4046 and 6ECDCA (INT-747) was able to protect animal models from BDL-, ANIT-, and ethinyl estradiol–induced cholestasis, lowering CYP7A1 and NTCP expression while inducing MRP2 and BSEP protein [27,63]. Moreover, induction of FGF15 by specific intestinal FXR overexpression was found to protect against cholestasis along with reduction of the BA pool size subsequent CYP7A1 downregulation [81]. These findings are further supported by the increased FGF19 plasma content, consistent with a reduction of CYP7A1 mRNA levels, observed in patients with extrahepatic cholestasis [108].

Recently, Gomez-Ospina and colleagues presented for the first time evidence for NR1H4 mutations driving cholestasis, now classified as a new type of PFIC (Fig. 1) [34]. This PFIC is an autosomal recessive severe liver disorder characterized by an onset of intralobular cholestasis in the neonatal period. The disease is rapidly progressive, leading to liver failure and death unless liver transplantation is performed. Analysis of four pediatric patients suffering from cholestatic liver disease and associated complications identified homozygous loss of function mutations in the FXR coding gene. Whole-exome sequencing of the patients revealed a c.526C > T (p.Arg176*) mutation in NR1H4 gene that prematurely terminates the protein without the DNA- and ligand-binding domains and an in-frame insertion variant p.Tyr139_Asn140insLys located in the first zinc-binding module of the DBD resulting in a non-functional FXR protein probably due to a disruption of DNA binding capability. In both types of mutations, FXR protein was immunohistochemically undetectable. The absence of FXR expression-binding domain in all patients results in complete absence of BSEP expression in bile canaliculi (without any deleterious mutations in the BSEP gene), early onset severe vitamin K-independent coagulopathy, rapid progression to end-stage liver disease, low-to-normal serum GGT, and elevated serum alpha-fetoprotein [34].

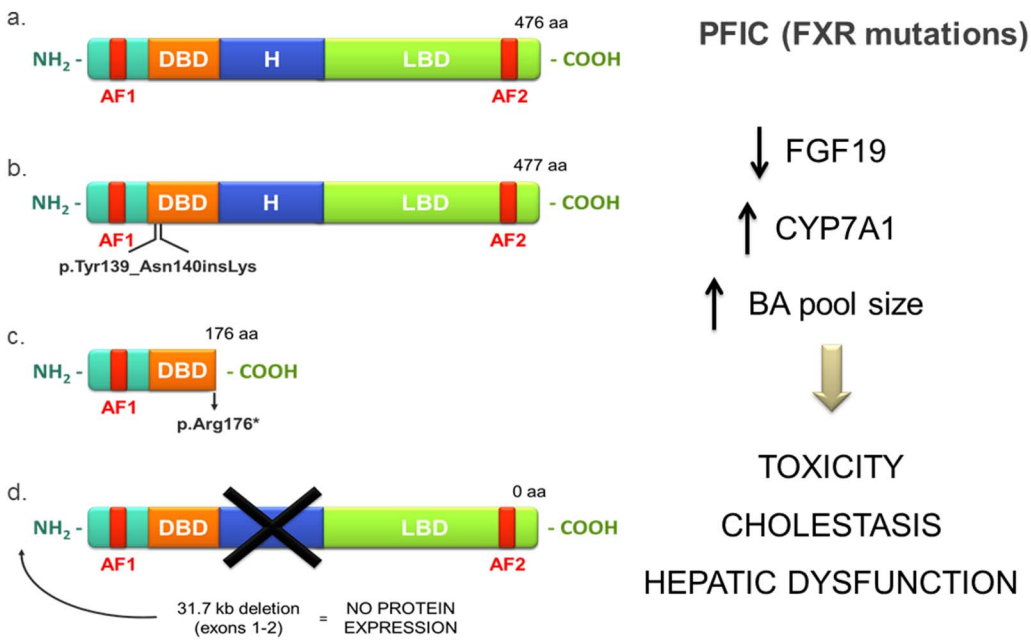

Fig. 1. FXR mutation in PFIC with FXR mutations. Recently, PFIC with FXR mutations has been identified in two families with homozygous loss of FXR function and severe neonatal cholestasis. a) Schematic representation of functional FXRα protein structure. b) The in-frame insertion in p.Tyr139_Asn140insLys in the first zinc-binding module of the FXR DBD results in a non-functional FXR protein maybe due to a disruption of DNA binding capability. c) The p.Arg176* mutation in the DBD binding domain causes the expression of a truncated protein (176aa) lacks both DBD and LBD. d) The 31.7Kb deletion spanning the first two coding exons of FXR transcript leads to a complete loss of FXR protein expression. The non-functional FXR protein leads to FGF19 decreased levels together with hepatic CYP7A1 up-regulation that collectively enhance bile acids pool size. These results in hepatic toxicity and cholestasis that ultimately drive severe hepatic dysfunction. DBD, DNA binding domain; LBD, ligand binding domain; AF1/2, ligand-independent and –dependent transactivation domains 1/2; H, Hinge region.

4. Scouting for FXR roles in cholestasis: the contribution of mouse models

Several experimental murine models have been used in order to explore the pathophysiological mechanisms of cholestasis. Recently, the development of genetically modified mouse models provided new insights in understanding the involvement of the FXR pathway in chronic cholestasis. Many of these animal models are characterized by alterations of genes involved in the FXR pathway, and they have been extensively investigated to elucidate the mechanism driving cholestasis.

4.1. The Abcb4$^{-/-}$ mice

Abcb4$^{-/-}$ mice are characterized by the ablation of Abcb4 gene product, a canalicular phospholipid transporter, resulting in a deficiency in the excretion of phosphatidylcholine into bile [105,112]. Low biliary phospholipid levels promote bile regurgitation into the portal tracts, leading to a spontaneous development of periportal biliary fibrosis and liver injury [23,25]. Generally, Abcb4$^{-/-}$ animals can be considered as a mouse model for human MDR3 deficiency, ranging from progressive intrahepatic cholestasis type 3 to adult liver cirrhosis [45]. After 2–3 weeks of age, Abcb4$^{-/-}$ mice display inflammation, ductural proliferation, and fibrosis, leading to the presence of hepatocyte dysplasia at 4–6 months. Moreover, by 16 months of age virtually 100% of Abcb4$^{-/-}$ mice develop liver tumors, and lung metastasis start to appear around 18 months [74]. Conversely, the lack of P-glycoprotein encoded by Abcb4 results in a protection from intestinal tumor development, mainly due to the activation of the NR Lrh1 [101].

Abcb4$^{-/-}$ mice have been widely used as a model for the investigation of the pathological mechanism involving FXR pathway in cholangiopathies and hepatic tumors [2]. Taking advantage of Abcb4$^{-/-}$ mouse model, together with other different models of murine cholestasis, Modica et al. demonstrated that selective activation of intestinal FXR is sufficient to rescue cholestasis, including the PFIC with ABCB4 mutations phenotype. Indeed, transgenic mice constitutively expressing active FXR in the intestine (iVP16FXR) have been shown to be protected from cholestasis development via FGF15 induction, leading to Cyp7a1 repression, and finally resulting in a hepatic BA pool size reduction and different BA pool composition [81].

The characterization of precancerous lesions in the liver of Abcb4$^{-/-}$ animals revealed an increase of many oncogenes along with the upregulation of many anti-inflammatory and antioxidant genes [48]. Additionally, the cancerous stage of the liver disease in the Abcb4$^{-/-}$ model displayed lower cyclin D1 combined with a downregulation of multiple tumor suppressor genes, making this mouse a suitable model for β-catenin–negative subgroup of human HCCs [47]. Moreover, it has been described that both tumor necrosis factor α (TNF-α) and NFκB are elevated in inflamed portal tracts of Abcb4$^{-/-}$ mice, and that decrease or inactivation of one of both prevented tumor development, providing a rational link between inflammation and tumorigenesis in this HCC model [102].

Furthermore, these mice provide a valuable model for testing the effect of specific treatments aimed at promoting the beneficial FXR activation [2].

4.2. The FXR$^{-/-}$ mice

In line with the pivotal role that FXR plays to keep BA levels under control, the FXR$^{-/-}$ mice phenotype has been associated PFIC1, a hereditary form of cholestasis with reduced FXR expression. Nevertheless, a possible correlation with the FXR$^{-/-}$ mouse phenotype and the PFIC with FXR mutations, recently described, needs to be further addressed. FXR$^{-/-}$ mice [59,111] exhibit a phenotype of cholestatic liver disease, with hypercholanemia, impaired canalicular bile salt secretion and failure to thrive [111]. FXR$^{-/-}$ mice, in which functional Fxr is missed, are unable to correctly control BA synthesis, transport and metabolism. FXR$^{-/-}$ mice display lack of Cyp7a1 and Ibabp genes expression upon CA-supplemented diet. Therefore, they exhibit impaired BAs homeostasis, as indicated by inactive hepatic canalicular secretion, increased BA hydrophilicity, urinary and fecal BA loss, and deficiency of BAs hepatic uptake from the bloodstream, with consequent increase of BA pool size [111]. FXR$^{-/-}$ mice under conditions of bile acid loading, such as CA feeding and common bile duct ligation, had increased serum and liver BA levels and decreased fecal BA excretion due to reduced Bsep expression [89]. Metabolomic investigation on FXR$^{-/-}$ mice with CA loading revealed that several metabolites of corticosterone and CA were highly elevated due to the high induction of CYP3A11, the major compensatory defense mechanism to detoxify cholestatic bile acids in these mice [10]. FXR$^{-/-}$ mice show protection from BDL-induced liver injury through MRP4 up-regulation, suggesting that FXR may negatively regulate this hepatic basolateral transporter. The induction of MRP4 expression represents an important adaptive mechanism against

Table 1
ASBT inhibitors, FXR agonists and FGF19 mimetic clinical trials.

Trial identifier	Trial phase (status)	Disease	Intervention	Purpose
ASBT inhibitor				
NCT02963077	Phase I: completed	Orphan cholestatic liver diseases; primary biliary cirrhosis; PFIC; Alagille syndrome	A4250 or in combination with CRC (A3384) or Questran versus placebo	Evaluation of the safety, tolerability and pharmacokinetics of A4250 or in combination with cholestyramine after single or multiple oral doses in healthy subjects.
NCT02787304	Phase II: recruiting	NASH	SHP626 versus placebo	Evaluation of the effects of SHP626 with respect to safety and tolerability in patients with NASH.
NCT02061540	Phase II: completed	PSC	LUM001	Evaluation of the safety and tolerability of LUM001 in patients with PSC.
NCT02057718	Phase II: active not recruiting	PFIC	LUM001	Evaluation of the safety and tolerability of LUM001 and the efficacy on pruritus in children with PFIC.
NCT01904058	Phase II: completed	PBC	LUM001 and ursodeoxycholic acid versus placebo	Evaluation of the effects of LUM001 in combination with ursodeoxycholic acid in patients with PBC.
FXR agonist				
NCT03059537	Phase IV: recruiting	Bile acid malabsorption	chenodeoxycholic acid	Stimulation of the ileal bile acid transporter and farnesoid X receptor
NCT02855164	Phase II: recruiting	NASH	LJN452 versus placebo	Evaluation of the effects of different doses of LJN452 with respect to safety, tolerability, and on markers of liver inflammation in patients with NASH
NCT02808312	Phase I: recruiting	NASH	GS-9674	Evaluation of the single-dose pharmacokinetics of GS-9674 in adults with impaired hepatic function.
NCT02654002	Phase I: completed	NASH	GS-9674	Evaluation of the safety, tolerability, pharmacokinetics and pharmacodynamics of GS-9674 and the effect of food on GS-9674 pharmacokinetics and pharmacodynamics in healthy volunteers
NCT02308111	Phase IV: recruiting	PBC	OCA versus placebo	Evaluation of effect of OCA compared to placebo, combined with stable standard care, on clinical outcomes in PBC patients.
NCT02177136	Phase II: active not recruiting	PSC	OCA versus placebo	Evaluation of the effect of obeticholic acid on liver biochemistry and safety in patients with PSC.
NCT01999101	Phase II: completed	NAFLD	Px-104	Evaluation of the safety and tolerability of Px-104 in NAFLD patients and assessing of the influence of Px-104 on hepatic fat.
NCT01585025	Phase II: completed	Primary and secondary bile acid malabsorption; chronic diarrhea	OCA	A pilot study to investigate whether OCA can stimulate FGF19 in bile acid diarrhea patients.
NCT01473524	Phase III: active not recruiting	PBC	OCA versus placebo	Evaluation of OCA treatment effects on liver function in PBC patients.
NCT01265498	Phase II: completed	NAFLD, NASH	OCA versus placebo	Evaluation of OCA treatment effects on liver disease measured by changes in the NAFLD activity score (NAS) subjects with NASH.
NCT00501592	Phase II: completed	Diabetes mellitus, type II; fatty liver	INT-747 versus placebo	Evaluation of the safety and tolerability of multiple doses of INT-747 in patients with type 2 diabetes mellitus and presumed NAFLD. Evaluation of INT-747 effects on hepatocellular function.
FGF19 mimetic				
NCT02135536	Phase II: completed	PBC	NGM282	Evaluation of the safety, tolerability and activity of extended treatment with NGM282 in patients with PBC.
NCT02704364	Phase II: active not recruiting	PSC	NGM282 versus placebo	Evaluation of the safety, tolerability, and activity of NGM282 in patients with PSC.
NCT02026401	Phase II: completed	PBC	NGM282 versus placebo	Evaluation of the safety, tolerability and activity of extended treatment with NGM282 in patients with PBC.
NCT02443116	Phase II: recruiting	NASH	NGM282 versus placebo	Evaluation of the safety, tolerability and activity of extended treatment with NGM282 in patients with NASH.

PFIC, progressive familial intrahepatic cholestasis; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; NAFLD, nonalcoholic fatty liver disease; HCC, hepatocellular carcinoma; A4250, SHP626 and LUM001 are inhibitors of the apical sodium dependent bile acid transporter (ASBT); A3384 is a colestyramine; LJN452, GS-9674, Px-104, OCA, INT-747 are FXR agonists; NGM282 is an engineered variant of endogenous FGF19.

cholestasis, aimed to protect the liver from cholestasis-induced BA accumulation, by stimulating their efflux into the systemic circulation for final renal excretion. Therefore, MRP4 knock-out mice are susceptible to cholestatic-induced liver damage [77]. Moreover, a marked upregulation of MRP4 expression has been found in subjects with PFIC [46] and BDL mice [20,77].

In order to determine the role of FXR in intrahepatic cholestasis, Cui. Y. et al. administered alpha-naphthyl isothiocyanate (ANIT), a hepatotoxicant used in rodents to mimic human intrahepatic cholestasis, to FXR$^{-/-}$ mice [11]. FXR deficiency enhanced the susceptibility to ANIT-induced liver injury, likely due to impaired efflux transporters induction that carries out unconjugated BA accumulation within hepatocytes. FXR pharmacological activation by the synthetic agonist GW4064 protected the mice from ANIT-induced liver injury, therefore indicating that FXR has a beneficial role and may represent a good therapeutic target for intrahepatic cholestasis. The mechanism proposed for the protective effect of FXR observed in these studies focused solely on the liver and it was attributed to the reduced expression of CYP7A1 and NTCP, as well as to the induced expression of MRP2 and BSEP.

4.3. The *Abcb11*$^{-/-}$ mice

The bile salt export pump (Bsep), encoded by Abcb11, is the primary efflux transporter of BAs at the canalicular membrane of hepatocytes [126]. When Bsep function is impaired [38,57,90,118], defective BA export leads to progressive cholestasis. The development of the Abcb11$^{-/-}$ mice was expected to clarify the molecular mechanisms behind the development of PFIC with Abcb11 mutations. The Abcb11-deficient mouse model shows progressive accumulation of hepatic BA that leads to liver injury even under normal dietary conditions. Conversely, the Abcb11$^{-/-}$ mice generated on the mixed background only exhibited mild, non-progressive liver injury [138], explained by tetrahydroxylated BA accumulation [100] along with the up-regulation of compensatory BA efflux transporters [58]. In contrast, in Abcb11$^{-/-}$ on C57BL/6 mouse background, these compensatory mechanisms are not sufficient to alleviate the progressive hepatic damage induced by BA overload. Indeed, the elevated BAs in this model produces a change in the metabolic state by disrupting glycolysis and gluconeogenesis, and altering the fatty acids oxidation, finally resulting in liver damage.

Mounting evidences suggest that the P-glycoprotein Mdr1a/Mdr1b is also involved in this rescue pathway. Mdr1a/Mdr1b mRNA and protein expression are significantly increased in the canalicular membrane of these mice, mediating ATP-dependent taurocholate transport [58]. The triple knockout mice (Abcb11$^{-/-}$/Mdr1a$^{-/-}$/Mdr1b$^{-/-}$) display a reduced bile flow rate and severe cholestasis, suggesting a compensatory role of Mdr1 in Abcb11$^{-/-}$ [137]. However, much more information will be needed to identify the specific components of the BSEP dependent trafficking machinery in order to modulate their expression and to improve clinical outcomes.

5. Current therapies in cholestasis management: seeking for target the FXR pathway

Cholestasis, which is characterized by jaundice and pruritus, is the hallmark presentation of PFIC. Medical therapy is the first line of treatment in patients with all types of PFIC. The goals are to provide relief from pruritus, to improve the nutritional status, to correct vitamin deficiencies and to treat the complications of advanced liver disease like ascites and variceal bleeding. Dietary fat should mainly be provided as medium chain triglycerides, together with water and fat soluble vitamins administration. Adequate sunlight exposure and calcium dietary intake are also essential [116]. In order to relief pruritus and to prevent deficiencies two drugs can be used: ursodeoxycholic acid (UDCA) and rifampicin. UDCA stimulates hepatobiliary secretion of BA and enhances bile flow by stimulating the impaired targeting of transport proteins such as BSEP or the conjugate export pump MRP2 to the canalicular membrane through activation of a complex signaling network [7,22,116]. Treatment with UDCA is the first-line therapy and is effective in more than a half of patients with ABCB4 alterations [117]. However, patients with complete ablation of MDR3 gene expression are usually non-responders to UDCA therapy [45].

Rifampicin acts by upregulating detoxification enzymes and export pumps by mechanisms dependent of PXR [127]. Rifampicin induces the expression of CYP3A4 (an enzyme of drug metabolism) which increases 6-a hydroxylation of bile salts. These bile salts are thereafter glucuronidated and excreted in urine. The treatment also induces uridine diphosphate (UDP)-glucuronosyl transferase (UGT1A1), that leads to increased conjugation and excretion of bilirubin.

When treatment and surgical management fail, liver transplantation is required. However, this is associated with several complications as rejection, post-transplant hepatic steatosis, and disease recurrence. Therefore, the identification of improved strategies for those patients is mandatory. Currently, ASBT inhibitors, FXR agonists and FGF19 mimetics represent the most promising anti-cholestatic strategies and are being tested in several clinical trials (Table 1).

ASBT sustains the BA enterohepatic circulation by efficiently taking up almost 95% of BAs from the intestine, preventing their fecal loss. In the Abcb4$^{-/-}$ mice, ASBT inhibitors effectively decreases the BA pool size, biliary BA concentrations, and bile flow, which results in significant improvement of liver injury and biliary fibrosis [3,78]. In a human phase I trial, ASBT inhibitors have been found to reduce total serum BAs with increased fecal BA excretion. However, future clinical trials with ASBT inhibitors are necessary.

FXR agonists represent an attractive class of drugs for patients with PFIC. Synthetic and semi-synthetic FXR agonists, with higher affinity and potency to activate FXR, have been successfully tested in animal models of cholestasis. In these murine models, the semi-synthetic steroidal FXR ligand obeticholic acid (OCA, or 6-ethylchenodeoxycholic acid [6-ECDCA]) was able to restore reduced bile flow and improve cholestasis outcome [27,98]. In phase II clinical trials, significant improvements were observed in the levels of ALP, serum bilirubin, γ-glutamyl transpeptidase and ALT. However, the increased frequency of pruritus along with the dyslipidaemia, a well-known cardiovascular risk, among cholestatic patients included in the clinical trial raise concerns with regard to the clinical application of this compound [39]. Interestingly, in Abcb4$^{-/-}$ mice, OCA did not show beneficial anti-cholestatic effects, although ileal FGF15 was induced and hepatic Cyp7a1 repressed. The complete absence of biliary phospholipids in the Abcb4 model may explain these discrepancies. Moreover, the dual FXR/TGR5 agonist, INT-767, improved the cholestasis phenotype along with robustly induced bicarbonate-rich choleresis and reduction of biliary BA output [2]. Analysis of the liver gene expression in cholestatic rats treated with the non-steroidal FXR agonist GW4064 demonstrated a decreased expression of BA biosynthetic genes together with an increased upregulation of genes involved in BA transport, including the phospholipid floppase MDR2. Nevertheless, the short terminal half-life of GW4064 has restricted its clinical utility [29]. Notably, when FXR was selectively overexpressed in the intestine of various mouse models of intrahepatic and extrahepatic cholestasis, BA pool size was substantially reduced and cholestasis improved [33,63], suggesting that ileal FXR stimulation alone may be sufficient to counteract cholestasis.

Pharmacological FXR activation leads to increased production of the FGF19 hormone, which reduces hepatic BA synthesis. FGF19 overexpression in mice has been associated with the development of HCC, therefore the activation of FXR with FXR agonist therapy raises unproven safety clinical concerns due to potential predisposition to the development of liver cancer. Recently, a non-tumorigenic FGF19-like peptide, which lacks the proliferative potency of their endogenous mother compounds [69,87] was designed. Whereas it is not able to affect proliferation, it reduces BA production in humans [134]. Moreover, this peptide effectively reversed the cholestatic liver injury

phenotype in Abcb4$^{-/-}$ mice [92], through the suppression of Cyp7a1 and total BA pools; therefore, it could be potentially good not only in the treatment of PFIC with Abcb4 mutations, but also PFIC with Atp8b1 and Abcb11 mutations. In a phase I trial on human volunteers, FGF19 mimetics resulted in a robust suppression of endogenous BA synthesis without showing apparent side effects [69]. Finally, in a very recent phase II clinical trial in primary biliary cholangitis patients unresponsive to UDCA treatment, FGF19 mimetics was able to decrease total BA and AP levels. Overall, further studies are still required for effective treatment in the management of PFIC.

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Conflict of interest

The authors declare that they have no conflict of interest.

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