Molecular overview of progressive familial intrahepatic cholestasis

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Abstract

Cholestasis is a clinical condition resulting from the imapairment of bile flow. This condition could be caused by defects of the hepatocytes, which are responsible for the complex process of bile formation and secretion, and/or caused by defects in the secretory machinery of cholangiocytes. Several mutations and pathways that lead to cholestasis have been described. Progressive familial intrahepatic cholestasis (PFIC) is a group of rare diseases caused by autosomal recessive mutations in the genes that encode proteins expressed mainly in the apical membrane of the hepatocytes. PFIC 1, also known as Byler's disease, is caused by mutations of the *ATP8B1* gene, which encodes the familial intrahepatic cholestasis 1 protein. PFIC 2 is characterized by the downregulation or absence of functional bile salt export pump (BSEP) expression *via* variations in the *ABCB11* gene. Mutations of the *ABCB4* gene result in lower expression of the multidrug resistance class 3 glycoprotein, leading to the third type of PFIC. Newer variations of this disease have been described. Loss of function of the tight junction protein 2 protein results in PFIC 4, while mutations of the *NR1H4* gene, which encodes farnesoid X receptor, an important transcription factor for bile formation, cause PFIC 5. A recently described type of PFIC is associated with a mutation in the *MYO5B* gene, important for the trafficking of BSEP and hepatocyte membrane polarization. In this review, we provide a brief overview of the molecular mechanisms and clinical features associated with each type of PFIC based on peer reviewed journals published between 1993 and 2020.

Key Words: Progressive familial intrahepatic cholestasis; ATP8B1/familial intrahepatic cholestasis 1; ABCB11/bile salt export pump; ABCB4/multidrug resistance class 3; Intrahepatic cholestasis; Bile

Core Tip: Progressive familial intrahepatic cholestasis (PFIC) is an expanding group of genetically-based diseases caused by various autosomal recessive mutations which result in the inability to appropriately form and excrete bile from hepatocytes. There are three classic types of PFIC. Type 1 is caused by mutations of the *ATP8B1* gene, encoding familial intrahepatic cholestasis 1. PFIC 2 is the result of mutations in the *ABCB11* gene, reducing the apical membrane expression of bile salt export pump. PFIC 3 is caused by variations in the gene that encodes the multidrug resistance class 3 glycoprotein. With advances in DNA sequencing, new forms of this disease have been documented. Alterations in the *NR1H4*, *tight junction protein 2*, and *MYO5B* genes have been linked with new phenotypes of PFIC. Here, we provide a molecular and clinical overview of PFIC.

INTRODUCTION

Among the liver functions, bile production is one of the most important. Hepatocytes and cholangiocytes work together in order to produce a mixture of organic and inorganic compounds that aid in the digestion process and excrete metabolites[1]. The formation of the final product, bile, is divided into two main processes: The formation and conjugation of the bile acids by the hepatocytes and the alkalization and dilution by the cholangiocytes. The bile can be stored in the gallbladder and secreted into the duodenum after a meal by a cholecystokinin stimulus. The detergent properties of bile give it the ability to break lipids into small particles, helping digestion[1].

The impairment of bile flow is responsible for generating a clinical condition called cholestasis[2]. Alterations in the bile formation process, at the hepatocyte and/or cholangiocyte level, lead to some complications, potentially resulting in liver tumors or necessitating liver transplantation (LT)[2]. Several point mutations and pathways that contribute to this clinical condition have been elucidated. In hepatocytes, mutations and polymorphisms of the genes responsible for bile acid transporters, as well as farnesoid X receptor (FXR), an important transcription factor for the bile formation, were described[3,4]. In cholangiocytes, defects of the cystic fibrosis transmembrane conductance regulator (CFTR) and alterations in calcium (Ca^{2+}) signaling contribute to cholestasis establishment and development[5,6].

Nowadays, several cholestasis-related diseases have been described. At the biliary level, there are two such diseases: Primary sclerosing cholangitis and primary biliary cirrhosis. These diseases both result in nonfunctional cholangiocytes, leading to viscous bile[7-9]. Problems in the hepatocyte machinery are also related to cholestatic diseases. Through an unclear genetic, hormonal, and environmental mechanism, some women in late term pregnancy develop intrahepatic cholestasis due to the downregulation of some bile transporters of the hepatocyte membrane[10]. Some drugs, such as antimicrobials or acetaminophen, may also induce the impairment of bile flow through hepatocellular damage, resulting in cholestasis[11,12].

Some autosomal recessive mutations of the bile acid membrane transporters that affect hepatocytes result in a group of diseases known as progressive familial intrahepatic cholestasis (PFIC). Historically, three types of PFIC were described: Type 1 due to mutations of the *ATP8B1* gene, type 2 resulting from mutations of the *ABCB11* gene, and type 3 caused by variations in the *ABCB4* gene. Recently, new types of this disease have been reported. Here, we will discuss the recent molecular and clinical findings of not only PFIC 1, PFIC 2 and PFIC 3, but also of the new types that have recently been reported. This group of hereditary diseases causes severe cholestasis in infants and patients regularly have to undergo LT (Table 1).

Table 1 Overview of progressive familial intrahepatic cholestasis subtypes

	PFIC 1	PFIC 2	PFIC 3	PFIC 4	PFIC 5	PFICAssociated with *MYO5B* defects
Gene	*ATP8B1*	*ABCB11*	*ABCB4*	*TJP2*	*NR1H4*	*MYO5B*
Molecular findings	Impaired hepatocyte bile salt secretion; canalicular cholestasis; giant cell transformation; portal and lobular fibrosis; lack of ductular proliferation	Increased intracellular bile salt concentrations; canalicular cholestasis; lobular and portal fibrosis; hepatocellular necrosis	Absence of PC from the biliary canaliculi; increased free bile salts in canaliculi; cholangiocyte damage; increased cholesterol crystallization nonspecific portal inflammation; portal fibrosis; giant cell hepatitis	Abnormal CLDN1 localization; normal CLDN2 localization; compromised tight junctions; bile salt leakage into the paracellular space; hepatocyte and cholangiocyte damage; giant cell transformation	Undetectable expression of BSEP in the bile canaliculi; intralobular cholestasis with ductular reaction; hepatocellular ballooning; giant cell transformation	Possibly mislocalized BSEP and MDR3 upon staining; portal and lobular fibrosis; giant cell transformation. Intestinal MVID findings (mislocalized apical brush border proteins, villus atrophy, presence of microvillus inclusion bodies)
Clinical findings	Low GGT cholestasis; jaundice caused by hyperbilirubinemia; pruritus; hepatosplenomegaly; diarrhea; stunted growth; exocrine pancreatic insufficiency; progressive sensorineural hearing loss; fat-soluble vitamin deficiencies; can lead to cirrhosis and end stage liver disease	Low GGT cholestasis; elevated transaminases; elevated serum bilirubin; elevated AFP; jaundice; pruritus; scleral icterus; hepatomegaly; chronic skin picking; reduced growth	Elevated GGT cholestasis; pruritus; hepatosplenomegaly; variceal bleeding; portal hypertension; jaundice; acholic stools; stunted growth; reduced bone density; learning disabilities; elevated transaminases; elevated bilirubin; elevated AP	Low GGT cholestasis; neurological and respiratory symptoms	Neonatal onset of normal GGT associated cholestasis; elevated serum bilirubin; elevated serum AFP; vitamin K independent coagulopathy; fibrosis progressing into micronodular cirrhosis	Normal GGT cholestasis; jaundice; pruritus; mildly elevated ALT and AST; elevated serum BS; hepatomegaly
Clinical outcomes	Medical management is the first line of defense, but if it is insufficient, surgical is next. Bilary diversion has been effective in around 80% of patients. The last resort is LT in patients who develop cirrhosis and liver failure. Extrahepatic symptoms can persist (or worsen) after LT	There is a 15% chance of cirrhosis developing into HCC or cholangiocarcinoma. Genetic defect(s) can aid in determination of success of biliary diversion. LT in PFIC 2 patients might lead to development of BSEP specific allo-reactive antibodies (in approximately 8% of PFIC 2 patients who undergo LT). In these cases, a second LT may be needed if BSEP deficiency develops in the new liver	HCC and cholangiocarcinoma have been associated with PFIC 3. The severity varies, with those retaining MDR3 expression responding better to medical treatment. Biliary diversion procedures aren't as effective due to severity upon presentation, but LT is curative	There have been some reports of HCC occurrence in PFIC 4 patients, though not much is known about the mechanism. LT has been successful, with no reported recurrence of the PFIC 4 phenotype after LT	PFIC 5 is very rare (only 8 cases reported in the literature so far). LT has been used, but steatosis in the transplanted liver has been reported in some cases	MVID has been associated with MYO5B defects. Lifelong TPN is required but is associated with increased risk of sepsis and small bowel transplant. Combined bowel-liver transplants may reduce the risk of post transplant onset of cholestasis
Treatments	Medical: Vitamin supplementation; UDCA; Rifampin; Cholestyramine; CFTR folding correctors	Medical: 4PBA. Surgical: Biliary diversion procedures; LT (might need increased immunosuppression)	Medical: UDCA; Rifampin. Surgical: Biliary diversion; LT	Surgical: LT	Medical: UDCA; Rifampin; ASBT inhibitors; OCA. Surgical: LT	Medical: UDCA; rifampin; Cholestyramine. Surgical: PEBD; isolated or Combined liver-bowel transplant

PC: Phosphatidylcholine; CLDN1: Claudin-1; CLDN2: Claudin-2; BSEP: Bile salt export pump; MDR3: Multidrug resistance class 3 glycoprotein; MVID: Microvillus inclusion disease; GGT: Gamma-glutamyl transferase; AFP: Alpha-fetoprotein; AP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase; BS: Bile salt; HCC: Hepatocellular carcinoma; MYO5B: Myosin VB; TPN: Total parenteral nutrition; UDCA: Ursodeoxycholic acid; CFTR: Cystic fibrosis transmembrane conductance regulator; 4PBA: 4-phenylbutyrate; ASBT: Apical sodium-dependent bile acid transporter; OCA: Obeticholic acid; PEBD: Partial external biliary diversion; PIBD: Partial internal biliary diversion; IE: Ileal exclusion; LT: Liver transplant; PFIC: Progressive Familial Intrahepatic Cholestasis; TJP2: Tight junction protein-2.

BILE FORMATION

Prior to describing the molecular and clinical features of PFIC, it is important to comprehend the bile formation process in order to understand how complex it is and how alterations could lead not only to hepatic disease, but also to dermatologic and neurologic symptoms.

The main primary bile acids produced by the human liver are cholic acid (CA) and chenodeoxycholic acid (CDCA), generated by cholesterol metabolism[13]. In the hepatocytes, these compounds are conjugated with taurine and glycine in order to form bile salts, increasing their solubility. After the bile is secreted into the duodenum, the bile salts are reabsorbed by the brush border membrane of the terminal ileum. These reabsorbed bile salts return to the liver through portal blood circulation. A small amount of bile salts are lost in the feces but are replaced by de novo synthesis in the liver. Daily, the human liver produces around 200 to 600 mg of bile acids, replenishing the bile acid pool in the enterohepatic circulation[13].

The hepatocellular part of bile formation can be divided into four stages. Stage 0 is characterized by the uptake of the compounds that will be processed to form the bile. This occurs at the basolateral hepatocyte membrane. Stage I is characterized by the activity of cytochrome P450 which turns the lipid compounds into soluble substrates for the next stages. Stage II is marked by the reactions that conjugate the bile acids. Finally, stage III is when the bile salts are secreted into the biliary canaliculus, the virtual space between two hepatocytes[1,13]. There are several different proteins and enzymes related to each stage, as shown in Figure 1.

The two main pathways to produce bile acids involve 17 different enzymes located in the cytoplasm, endoplasmic reticulum, mitochondria, and peroxisomes[13]. In the classic pathway, also known as the neutral pathway, cholesterol is first metabolized by CYP7A1, generating CDCA. In the presence of CYP8B1, CDCA is converted into CA. The alternative pathway, known as the acid pathway, begins with cholesterol side chain metabolism by CYP27A1, which is expressed in the mitochondrial membrane[14]. The alternative pathway is important for bile acid production in neonates as well as in diseased livers.

After bile acid synthesis and conjugation with the amino acids taurine and glycine, the bile salts are secreted into the canalicular region, a major step for bile flow. Most of the transporters belong to the ATP-binding cassette transporters (ABC transporters) superfamily[1]. As shown in Figure 1, there are several proteins involved in these steps and each one is responsible for transporting one compound in order to form the final product, the bile. Bile salt export pump (BSEP), multidrug resistance class 1 (MDR1), MDR3, MRP2, Breast Cancer Resistance Protein, sterolins 1 and 2, familial intrahepatic cholestasis 1 (FIC1) and others are examples of these transporters.

After the bile salts are secreted into the canalicular space, the cholangiocytes are responsible for alkalinizing and diluting the bile by secreting bicarbonate (HCO_3^-) and water[1,15,16]. This process is initiated by the stimulation of secretin, a hormone produced in the S cells of the duodenum in response to the change in pH and presence of peptides after a meal. When this hormone binds to its receptor at the cholangiocyte basolateral membrane, it leads to a cytosolic increase in cAMP which activates CFTR, thereby releasing chloride (Cl^-) into the bile duct lumen. This Cl^- is exchanged with HCO_3^-, mediated by the chloride-bicarbonate exchanger which is expressed on the apical membrane of cholangiocytes[15]. The release of HCO_3^- is enhanced by local increases in Ca^{2+} signaling that activate the M3 muscarinic receptor. When acetylcholine binds to the M3 muscarinic receptor, $InsP_3$, a second intracellular messenger, is produced. This $InsP_3$ mediates the release of a Ca^{2+} signal *via* the type 3 inositol 1,4,5-trisphosphate receptor. The Ca^{2+} release near the apical membrane activates the Ca^{2+}-dependent chloride channel, which releases more Cl^- into the luminal space to be exchanged by the chloride-bicarbonate exchanger[6]. The activation of purinergic receptors also increases bicarbonate secretion, resulting in a pH increase. The presence of bicarbonate in the luminal space creates an osmotic gradient that is important for the flux of water from the cholangiocyte cytosol to the extracellular space. This transport is mediated by small integral membrane proteins known as aquaporins. The water makes the bile less viscous and leads the bile to the gallbladder, where it will be stored until a cholecystokinin stimulus occurs after a meal[1,15,16].

Mutations in genes that encode proteins expressed in the hepatocyte canalicular membrane and involved in Phase III of bile formation are responsible for the development of a cholestatic disease known as PFIC. Now, we will provide a molecular and clinical overview of the three classic types of PFIC and discuss the newer types of this disease.
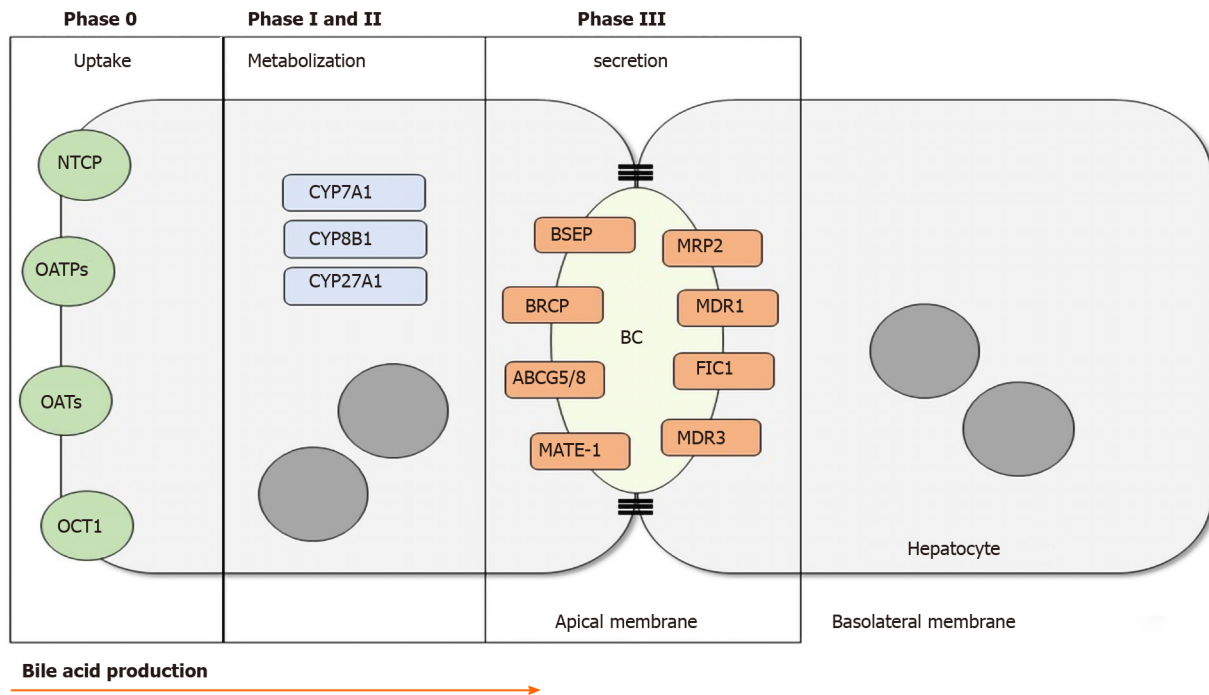
Figure 1 Bile acid production. In phase 0, bile acids and other organic products are transported through the basolateral membrane of the hepatocyte. Phase I and II take place in the cytoplasm through activation and metabolization of differents CYPs. In phase III the bile acids are secreted into the biliary canalicus by the transmebrane transporters. NTCP: Na$^+$-taurocholate cotransporting polypeptide; OATPs: Organic anion transporting polypeptides; OATs: Organic anion transporter; OCT1: Organic cation transporter 1; CYP7A1: Cytochrome P450 Family 7 Subfamily A Member 1; CYP8B1: Cytochrome P450 Family 8 Subfamily b Member 1; CYP27A1: Cytochrome P450 Family 27 Subfamily A Member 1; BSEP: Bile salt export pump; BRCP: Breast cancer resistance protein; ABCG5/8: ATP-binding cassette sub-family G member 5/8; MATE-1: Multidrug and toxin extrusion 1; MRP2: Multidrug resistance-associated protein 2; MDR1: Multidrug resistance class 1 glycoprotein; FIC1: Familial intrahepatic cholestasis 1; MDR3: Multidrug resistance class 3 glycoprotein; BC: Biliary canaliculus.

PFIC 1

PFIC type 1, also known as Byler's disease, is caused by homozygous or compound heterozygous mutations of the *ATP8B1* gene on chromosome 18 (18q21), which encodes the FIC1 protein[17]. FIC1 is part of the type 4 subfamily of P-type adenosine triphosphatases, which are involved in translocating phospholipids in membranes[18]. This protein, present at the apical membrane of hepatocytes, is thought to function as an aminophospholipid translocase, carrying phospholipids, specifically phosphatidylserine (PS) and phosphatidylethanolamine (PE), from the outside, ectoplasmic leaflet of the canalicular membrane, to the inside, cytoplasmic leaflet in hepatocytes[17-19]. When functioning normally, FIC1 protects the hepatocyte from high bile salt concentrations[17]. By maintaining the asymmetry of the plasma membrane, FIC1 guards the canalicular membrane from the detergent nature of hydrophobic bile salts present in the canalicular lumen[17,18].

FIC1 was found to be successful at flopping phospholipids only when it was coexpressed with CDC50 proteins, specifically CDC50A[18]. This coexpression allows the FIC1-CDC50A heterodimer to leave the endoplasmic reticulum and localize to the apical membrane[18]. Mutations in CDC50A may also lead to the PFIC 1 phenotype if FIC1 fails to localize as a result[18]. In CDC50A-depleted intestinal cells, not only the apical membrane expression of FIC1 is reduced, but also the expression of the Solute Carrier Family 10 Member 2 (SLC10A2) also known as apical sodium-dependent bile acid transporter (ASBT)[20]. This transporter is responsible for the reuptake of bile acids in the intestines, so mutations in either the *ATP8B1* gene or the *CDC50A* gene will impair localization of ASBT, reducing bile uptake[20]. This suggests that the FIC1-CDC50A heterodimer is involved in the localization of SLC10A2 to the apical membrane, resulting in bile salt malabsorption, which may be the cause of the diarrhea that PFIC 1 patients experience[20].

There is also evidence that FIC1 is involved in the organization of the apical membrane of polarized cells[21]. In *ATP8B1*-deficient intestinal Caco-2 cells, the apical actin cytoskeleton was found to be disorganized, there was a loss of microvilli, and the translation of apical membrane protein mRNA was disrupted[21]. The reduced

microvilli in the *ATP8B1*-deficient intestinal cells mirrors the loss of stereocilia on the apical membrane of inner hair cells in mice with an *ATP8B1* mutation, which is linked to hearing loss, an event that is also observed in humans[21,22]. These examples support the case for the function of FIC1 in forming or stabilizing microvillar structures throughout the body[21].

In the disease phenotype, the lipid flipping process does not occur, interfering with the secretion of bile salt from the hepatocytes[23]. Lower expression of FIC1 downregulates FXR expression, encoded by the *NR1H4* gene[17]. The function of FXR is two-fold. In hepatocytes, elevated bile salts concentrations activate FXR, which induces the expression of the BSEP[17,19]. BSEP allows for the pumping of bile salts out of the hepatocyte into the canalicular lumen[17,19]. Additionally, when expressed in the intestines, FXR represses the expression of ASBT. By doing so, bile salt reabsorption is reduced in the intestines. The bile salt transport defect is secondary in the case of the *ATP8B1* mutation. The accumulation of bile salts inside the hepatocytes, coupled with the absence of the protective lipid flipping by FIC1 has a cytotoxic effect, harming the cells and leading to the cholestatic phenotype[17,19].

With the lack of lipid flipping, PS is freely present on the outer leaflet of the canalicular membrane[23]. This gives hydrophobic bile salts that exist in the canalicular space the opportunity to extract the PS from the membrane[23]. In normal hepatocytes, FIC1's lipid flipping activity is necessary in maintaining the asymmetric and liquid ordered state of the outer leaflet of the canalicular membrane[23]. In diseased hepatocytes with an *ATP8B1* mutation, the liquid ordered state becomes disordered because FIC1 is not able to flip the excess aminophospholipids from the outer to the inner leaflet[23]. This makes the membrane more susceptible to phospholipid, cholesterol, and ectoenzyme extraction[23]. Reduced cholesterol presence in the outer leaflet is vital in the functioning of certain integral membrane proteins such as BSEP (implicated in PFIC 2) and MRP1[23].

Each specific mutation comes with its own presentation of cholestatic disease. The G308V mutation was first identified in the Amish population and in homozygotes with this mutation, the FIC1 protein is undetectable in the liver[24]. The D554N mutation was found in Greenland familial cholestasis and also resulted in reduced expression of FIC1[24]. The G1040R mutation, first found in two Saudi families, did not result in a significantly decreased FIC1 expression when compared to the wild-type[24]. As expected, the interaction between FIC1 and CDC50A was undetectable in both the G308V and D554N mutations[24]. It was, however, still present in the G1040R mutation, although at a much lower level than the wild-type[24]. This is likely the reason why these three FIC1 mutant proteins are not localized to the canalicular membrane in WIF-B9 cell models[24].

Clinically, PFIC 1 presents with low gamma-glutamyl transferase (GGT) cholestasis[19]. Recurrent episodes of jaundice caused by hyperbilirubinemia, along with uncontrollable itching, or pruritus also occur[19]. Transaminases, as well as serum bile acids are elevated[19]. In infants with this disease, jaundice with pruritus, as well as hepatosplenomegaly develop early in life[19]. In severe cases, a progressive cholestasis presents along with portal hypertension[19]. Histologically, canalicular cholestasis, giant cell transformation, portal and lobular fibrosis, as well as lack of ductular proliferation are most common[17,19]. Because FIC1 is also expressed in cholangiocytes, enterocytes, and in the pancreas, there are extrahepatic manifestations of PFIC 1, setting it apart from the other types of intrahepatic cholestasis[17,19]. Some of these symptoms include diarrhea, stunted growth, exocrine pancreatic insufficiency, and progressive sensorineural hearing loss[17,19]. Nutritional deficiencies are also common, especially in fat-soluble vitamins A, D, E, and K[19].

Children with PFIC 1 often require vitamin supplementation, as well as other dietary aids in order to manage malnutrition[19]. Ursodeoxycholic acid (UDCA) is the first-line of medication used to treat pruritus[19]. It is a hydrophilic bile acid that is hypothesized to induce the expression of BSEP and MDR3, improving cholestatic symptoms[19]. Rifampin and cholestyramine have also been used in order to reduce pruritus, but have not been as effective as UDCA[19]. In addition, CFTR folding correctors may be a promising future therapy to enhance the trafficking of FIC1 in the hepatocytes[19].

In cases where medical management is inadequate, surgery is the next option. The purpose of these surgeries is to bypass the enterohepatic circulation, thereby lowering the amount of bile salts that are reabsorbed by the digestive system. Partial external biliary diversion (PEBD), partial internal biliary diversion (PIBD), and ileal exclusion (IE) have led to improvement in some cases[19]. PEBD has been shown to improve liver function, reduce serum bile acid levels, and slow progression of liver fibrosis in 80% of patients with either PFIC 1 or PFIC 2[25]. In this procedure, an external stomal conduit

is created to divert bile flow outside of the body[19]. PIBD diverts bile flow from the gall bladder to the colon in order to bypass the terminal ileum, where bile salt reabsorption occurs[19]. This procedure has been shown to improve pruritus, as well as decrease bilirubin and plasma bile acid levels[19]. IE bypasses the distal 15% of the ileum to prevent bile acid reabsorption[19]. It provides relief from pruritus and may be used as a bridge to transplant[26]. Ileal adaptation may occur, reducing the effectiveness of this surgery[25].

When no other treatment is successful, liver transplant is the final option. Lobular fibrosis may develop into cirrhosis and end stage liver disease[19]. There is no known association between FIC1 deficiency and tumorigenesis[19]. At this point, LT is indicated, which will improve cholestatic symptoms[19]. However, since FIC1 is expressed outside of the liver, extrahepatic symptoms may persist, or worsen[19]. Refractory diarrhea, coupled with the development of fatty liver changes in the allograft and fibrosis, may lead to re-transplant[27]. Ileal diversion during transplant may be used to potentially prevent these developments[19].

PFIC 2

Homozygous or compound heterozygous mutations in the *ABCB11* gene (chromosome 2q31), which encodes the BSEP, results in PFIC 2[17]. BSEP is an adenosine triphosphate-binding cassette transporter involved in actively transporting bile salts out of hepatocytes into biliary canaliculi[17]. It is crucial in maintaining enterohepatic circulation of bile salts[17]. Mutations in BSEP interrupt the process of pumping bile out of hepatocytes, leading to increased intracellular bile salt concentrations which consequently damage the hepatocytes[17].

FXR, activated in a heterodimer with retinoid X receptor α (RXRα), has been linked to upregulating enzymes and bile salt transporters when bound to bile salts in the cell[28]. BSEP is one of these bile salt transporters that FXR activates[28]. CDCA and the FXR/RXRα heterodimer collectively work together to transactivate the *ABCB11* promoter[28]. The level of activation of the *ABCB11* gene is dependent on the concentration of CDCA[28]. When more bile acids are present in the hepatocyte, more BSEP is made to maintain the equilibrium.

There are over 200 mutations that have been linked with causing PFIC 2[19]. Some of these mutations cause splicing defects while most cause protein processing defects[29]. The majority of missense mutations resulted in a reduction of BSEP levels *in vitro*[29]. Some common mutations — G238V, D482G, G982R, R1153C, R1286Q, and ΔGly — cause the mutant protein to be retained in the endoplasmic reticulum[30]. Proteosomes are the main cellular component that breaks down these mutant proteins, but the D482G mutant protein can also be broken down by lysosomes[30]. The degradation occurs through Endoplasmic Reticulum Associated Degradation, part of the quality control system of the endoplasmic reticulum[30]. This process involves ubiquitination, retro-translocation out of the endoplasmic reticulum, and degradation by the proteasome[30].

PFIC 2 presents with low GGT cholestasis as well as highly elevated transaminases, serum bilirubin, and alpha-fetoprotein (AFP) levels[17,19]. Jaundice, pruritus, scleral icterus, hepatomegaly, chronic skin picking, and reduced growth are also common[17,19]. Liver histology shows canalicular cholestasis, lobular and portal fibrosis, and hepatocellular necrosis[17]. Unlike PFIC 1, there is up to a 15% chance of cirrhosis developing into either hepatocellular carcinoma (HCC) or cholangiocarcinoma in patients with PFIC 2[19].

Treatment of PFIC 2 is similar to PFIC 1. A promising therapy includes treatment with cell surface BSEP-enhancer molecules such as 4-phenylbutyrate (4PBA)[19]. 4PBA has been shown to improve liver function and pruritus in patients with certain mutations of BSEP[19]. Bile salt excretion was improved, although not completely[31]. 4PBA helps increase cell surface expression of the mutant BSEPs, likely altering a post-transcriptional mechanism[32]. This therapy works best when the mutation results in normal transport ability, but impaired membrane trafficking[32,33]. Biliary diversion procedures have a better response in cases where there is still some BSEP function than in cases where function is nonexistent[19].

LT is the ultimate therapeutic option, but there is an additional risk in patients with PFIC 2. In severe cases, patients might develop allo-reactive antibodies specific to BSEP in the allograft, causing BSEP deficiency in the transplanted liver[19]. This happens in up to 8% of PFIC 2 patients who undergo LT[27]. Increased immuno-suppression may prevent these antibodies from developing, but if the disease recurs,

plasmapheresis to remove anti-BSEP antibodies, B-cell depleting antibody therapy, or allogeneic hematopoietic stem cell transplant may help[19,27]. If none of these therapies succeed, a second LT might be required[27].

There have been many scientific advances offering hope for potential treatments for BSEP deficiency. One group led by Jillian Ellis developed a zebrafish model of BSEP deficiency[34]. Treating these zebrafish with rapamycin partially restored bile excretion as well as lengthened their lifespans[34]. The rapamycin likely localizes other transporters, such as MDR1, to the canaliculus in order to compensate for the lack of BSEP[34]. Rapamycin is an autophagy inducer, so the increased trafficking of MDR1 in rapamycin treated zebrafish might be the result of increased autophagy[34]. Another group led by Imagawa *et al*[33] generated a BSEP-deficient cellular model using patient cells[33]. They generated induced pluripotent stem cells (iPSCs) from peripheral blood mononuclear cells[33]. Then, they were able to differentiate these iPSCs into hepatocyte-like cells that mimicked the BSEP deficiency of the patient from whom they were derived[33]. These cells had abnormal BSEP expression, as well as reduced biliary excretion[33]. When the cells were treated with 4PBA, there was an increase in membrane expression of BSEP[33]. *In vitro* models such as this one provide a practical way to test the effect of many different therapeutic agents on cells derived from individual patients themselves. Advances in stem cell research might make it possible to determine what drugs a patient will respond best to, based on their specific mutation, without having to test them all on the patient.

PFIC 3

Mutations in the *ABCB4* gene, located on chromosome 7q21, which encodes the MDR3 glycoprotein, cause not only PFIC 3, but also other cholestasis related diseases such as gallbladder disease 1/low phospholipid-associated cholelithiasis (GBD1/LPAC), parenteral nutrition-associated liver disease, transient neonatal cholestasis and intrahepatic cholestasis of pregnancy[17]. This phosphatidylcholine (PC) flippase is localized only to the canalicular membrane of hepatocytes and consists of two cytoplasmic nucleotide binding domains and two transmembrane domains, each with six transmembrane segments. This structure facilitates the transport of PC out of the hepatocyte and into the biliary canaliculi. This process is supported by the over-expression of MDR3 in polarized cells, increasing the rate of transport of fluorescently labeled PC, but not other phospholipids. MDR2, the mouse homolog of human MDR3, has been used in knockout (KO) mice in order to model MDR3 deficiency[35]. The absence of the PC flippase leads to hepatocyte necrosis, dilated biliary canaliculus, periportal inflammation and bile duct proliferation[36]. These MDR2 KO animals develop HCC earlier, within 4-6 mo, due to an accumulation of carcinogenic factors[37-39]. The expression of human MDR3 in the MDR2 KO animals restores the function and histological features of the hepatocytes[38,40].

Several variations in the *ABCB4* gene have been described. Delaunay and colleagues used site-directed mutagenesis in a cell line model to show that the mutations I541F, L556R, and Q855L result in protein retention in the endoplasmic reticulum[41]. Another four mutations have no effect on MDR3 function: T424A and N510S had a normal pattern of canalicular expression with lower protein stability, while T175A and R652G had no detectable defect. Degiorgio *et al*[42] analyzed *ABCB4* gene mutations in 68 PFIC 3 patients. The authors found 29 mutations in the coding region, including 23 missense, four nonsense, and two short insertion mutations in the gene. 10 of these mutations were present in the transmembrane domains (TM), specifically in TM7, involved in PC translocation[42]. Delaunay *et al*[41] devised a classification system for the various forms of these mutations[41]. Class I includes mutations that result in defective synthesis, class II covers variations that prevent protein maturation, class III mutants result in mature but defective proteins, class IV variations are unstable, and class V mutants have unknown pathogenicity[41]. These classifications are useful in determining potential therapies for patients, depending on their genotype.

When MDR3 does not function properly, PC is absent from the biliary canaliculi. The free hydrophobic bile acids in this space will damage cholangiocytes, leading to cholestasis[19]. In addition, cholesterol is more likely to crystallize into stones, damaging liver structures by obstructing small bile ducts[25]. PFIC 3 often presents later on in life than both PFIC 1 and PFIC 2, as late as adulthood in some cases[19]. In early onset, pruritus, hepatosplenomegaly, variceal bleeding caused by portal hypertension, alcoholic stools, stunted growth, jaundice, reduced bone density, and learning disabilities have all been associated with MDR3 deficiency[17,19]. Adult onset can present

anywhere from cholelithiasis to benign recurrent intrahepatic cholestasis (BRIC)[19]. Unlike PFIC 1 and 2, PFIC 3 presents with elevated GGT[19]. Transaminases, bilirubin, and alkaline phosphatase are elevated as well[17,19]. Although serum bile salt and cholesterol can be in the normal range, biliary phospholipids will be reduced[17]. Histological findings include nonspecific portal inflammation, portal fibrosis, cholestasis with bile duct proliferation, and slight giant cell hepatitis[17,19]. MDR3 expression upon staining will depend on the type of protein defect, with functional protein defects staining normally and truncated protein defects not staining[17,19]. Additionally, hepatocellular carcinoma and cholangiocarcinoma have been associated with PFIC 3[19].

UDCA treatment has been effective in 70% of cases where the patient retains MDR3 expression and residual PC secretion[17]. Rifampin could also improve pruritus[19]. Chemical chaperones could improve MDR3 trafficking, depending on the specific mutation[43]. Biliary diversion procedures are not as successful with MDR3 deficiency because of the severity upon presentation[19]. LT is currently the only known curative treatment[19]. Gene therapy *via* adeno-associated virus has shown promise in restoring phospholipid excretion and preventing liver injury in murine models of PFIC 3[44]. This may be an alternative therapy for MRD3 deficiency in the near future.

NEWER TYPES OF PFIC

PFIC 4
PFIC 4 is caused by a loss of function mutation in the tight junction protein 2 (TJP2), also called zona occludens 2, present on chromosome 9q21[17]. In the liver, this protein is involved in forming tight junctions by interacting with transmembrane tight junction proteins and the actin cytoskeleton[45]. Tight junctions are essential in the liver because they help prevent the leakage of biliary components into the liver parenchyma[46]. Claudin-1 (CLDN1) and claudin-2 (CLDN2) are two integral tight junction proteins that are expressed in the liver, aiding in the formation of tight junctions[45]. Normally, these two proteins are localized to the canalicular membrane, but in *TJP2* mutation, CLDN1 fails to localize, especially in the parenchyma of the hepatic lobule[45,47]. These compromised tight junctions then allow cytotoxic bile salts to leak into the paracellular space, causing damage to the surrounding hepatocytes and cholangiocytes[19,45].

This disease presents with severe cholestasis and low GGT levels[17]. Patients lack mutations in *ATP8B1* and *ABCB11* genes, excluding the diagnoses of PFIC 1 and PFIC 2. Extrahepatic symptoms have been reported in some patients, mainly in the neurological and respiratory systems[45]. This is likely related to the expression of TJP2 in every epithelial cell in the body[45]. The reason the liver is consistently affected by TJP2 deficiency is because of the particularly damaging nature of the bile acids present in the canaliculi[47]. Pathological findings include intracellular cholestasis, giant cell transformation, lack of TJP2 with staining, abnormal CLDN1 localization, and normal CLDN2 localization[19,47]. The lack of TJP2 could be tied to the nonsense mediated mRNA decay (NMD) process[45]. This process degrades mutant transcripts, such as TJP2 in the case of a mutation[45]. Even though the protein could have residual function, it is still degraded[45]. *TJP2* was found to have low mRNA expression in PFIC 4 patients[45]. Western blotting revealed no TJP2 expression, indicating a possible link between the mutation in *TJP2* and NMD[45].

There have been several reports of HCC occurrence in patients suffering with TJP2 deficiency[48-50]. The exact mechanism of tumorigenesis has not yet been elucidated. In terms of treatment, there have been no reported cases of recurrence after LT[17].

PFIC 5
Loss of function mutations in the *NR1H4* gene (chromosome 12q23), encoding FXR, result in PFIC 5[4,17]. FXR is a nuclear receptor activated by bile acids and directly involved in the expression of both BSEP and MDR3, proteins affected in PFIC 2 and PFIC 3 respectively[4]. This protein is activated by elevated bile acids levels in the ileum, inducing expression of fibroblast growth factor 19 (FGF19)[51]. In the liver, FGF19 binds to the fibroblast growth factor receptor 4/β-Klotho complex, which in turn represses cytochrome P450 7A1 (CYP7A1)[51]. Repression of this enzyme reduces de novo bile acid synthesis[51].

Features of this disease include neonatal onset of normal GGT associated cholestasis, elevated serum bilirubin, elevated serum AFP levels, undetectable expression of BSEP in the bile canaliculi, and vitamin K independent coagulopathy[4].

This vitamin K independent coagulopathy is unique to PFIC 5 and has been shown to be a direct result of FXR mutation[4]. Three fibrinogen genes, as well as some coagulation factors have been linked with FXR-dependent induction, which does not occur in PFIC 5 patients[4]. Histological findings are intralobular cholestasis with ductular reaction, hepatocellular ballooning, fibrosis progressing into micronodular cirrhosis, and giant cell transformation[19]. *NR1H4*/FXR associated PFIC is very rare as only eight cases have been reported in the literature[4,52,53]. LT has been used, but steatosis in the transplanted liver has been reported in some of these cases[4,53]. UDCA and rifampicin may be used in order to improve pruritus, but ASBT inhibitors, as well as FXR agonists such as obeticholic acid, both show promise as future medical therapies[51,54].

PFIC associated with MYO5B defects

The *MYO5B* gene (chromosome 18q21.1) encodes an actin-associated molecular motor known as MYO5B[17]. The interaction between MYO5B and RAS-related GTP-binding protein 11A (RAB11A) is essential for the polarization of epithelial cells, as well as localizing BSEP to the canalicular membrane[55]. Diminished activity of the MYO5B/RAB11A recycling endosome pathway is related to disrupting the localization of BSEP[56]. Mutations of this gene are associated with microvillus inclusion disease (MVID) which affects the enterocytes and leads to diarrhea, as well as malabsorption[57]. Mislocalized apical brush border proteins, villus atrophy, and the presence of microvillus inclusion bodies are all associated with MVID[57-60]. Total parenteral nutrition (TPN) is required throughout life, but it has been associated with a high risk of sepsis and small bowel transplant[61].

MVID has been associated with cholestatic liver disease, which possibly occurs as a result of TPN[57]. In fact, *MYO5B* gene mutations may account for 20% of the idiopathic low-GGT associated cholestasis in pediatric patients[60]. This cholestasis presents with low to normal GGT levels, jaundice, pruritus, mildly elevated Alanine transaminase and Aspartate transaminase, elevated serum BS levels, hepatomegaly, portal and lobular fibrosis, and giant cell transformation[17,19,60]. Staining of BSEP and MDR3 may show that they are present, but not localized to the canalicular membrane, although this may not occur in every patient[19,60].

There is evidence for different phenotypes resulting from the type of *MYO5B* gene mutation[55,60]. Mutations with limited effects on MYO5B functionality are linked with canalicular transport defects in hepatocytes but are not known to have as significant of an effect on enterocytes[60]. In more damaging mutations, the hepatocyte defects may be greater, but there will also be enterocyte disruption[60]. Enterocytes will not uptake bile acids as efficiently, reducing the amount of bile product taken back to the liver *via* enterohepatic circulation, potentially alleviating or protecting against cholestatic symptoms[60]. There seems to be an inverse relationship between cholestasis and intestinal function. UDCA, rifampin, and cholestyramine have been used to treat pruritus in these patients[19]. If medical treatment is not sufficient, PEBD may be successful[19]. When symptoms necessitate transplant, combined bowel-liver transplant should be considered in order to avoid post-transplant onset of cholestasis[60].

HOW TO STUDY PFIC?

After the identification of mutations in patients, several models have been created to better understand not only the pathophysiology of PFIC, but also to investigate drugs and treatments. The first model is the use of a cell line with mutations in PFIC related genes to study the mechanisms of these variants on both the molecular and clinical level. van der Velden *et al*[62], using different cell lines, studied some FIC1 mutations[62]. Delaunay *et al*[41], studied *MDR3* gene mutations using HepG2 as a model. A second option to study PFIC are knockout mice[63]. Some studies have reported the use of *ABCB11-/-* mice as a model for PFIC 2, however the clinical findings are less severe than those of humans[63]. MDR2 KO mice are used to model complications resulting from MDR3 deficiency in humans and are also used to study treatments for all MDR3-related diseases[35,40].

In 2012, when Shinya Yamanaka and John B. Gurdon were awarded The Nobel Prize in Physiology or Medicine for their discovery in pluripotency, a new method to study PFIC became available. The possibility of transforming any somatic cell into an iPSCs was revolutionary. Okita and colleagues used plasmids to express c-Myc, SRY-box transcription factor 2 (Sox2), Kruppel-like factor 4, and octamer-binding transcription factor 4 (Oct4) in reprogrammed mature cells[64]. This last set of genes is

known as Yamanaka's Factors. However, there are many different protocols to generate iPSCs. Si-Tayeb *et al*[65] used the expression of Oct4, Sox2, Nanog, and Lin-28 homolog A in a lentivirus delivery system to generate iPS cells[65]. In this way, Imagawa *et al*[33] generated a BSEP-deficient cellular model using patient cells and tested the effects of 4PBA treatment on protein expression and localization[33]. With this system of reprogramming cells, and access to patient cells with different mutations, a new model became available to better understand the various types of PFIC. The possibility of differentiating these cells into liver cells, such as hepatocytes and cholangiocytes, gives researchers the opportunity to test potential treatments *in vitro*. This can be extremely useful since very few patients have these rare diseases and are available to enroll in clinical trials. Large scale drug trials may not be feasible.

CONCLUSION

PFIC is a rare disease that affects the bile secretion process of the hepatocytes due to alterations in the proteins involved, as shown in Figure 2. Better understanding of the molecular alterations in this context is necessary to elucidate new targets and pathways that could be the focus of new treatments in the future. With advances in inducing pluripotent cells, a new model that is more precise and closer to humans has become available. Also, the progress in gene editing will help in the development of new models and potentially be used as a treatment method in the future.
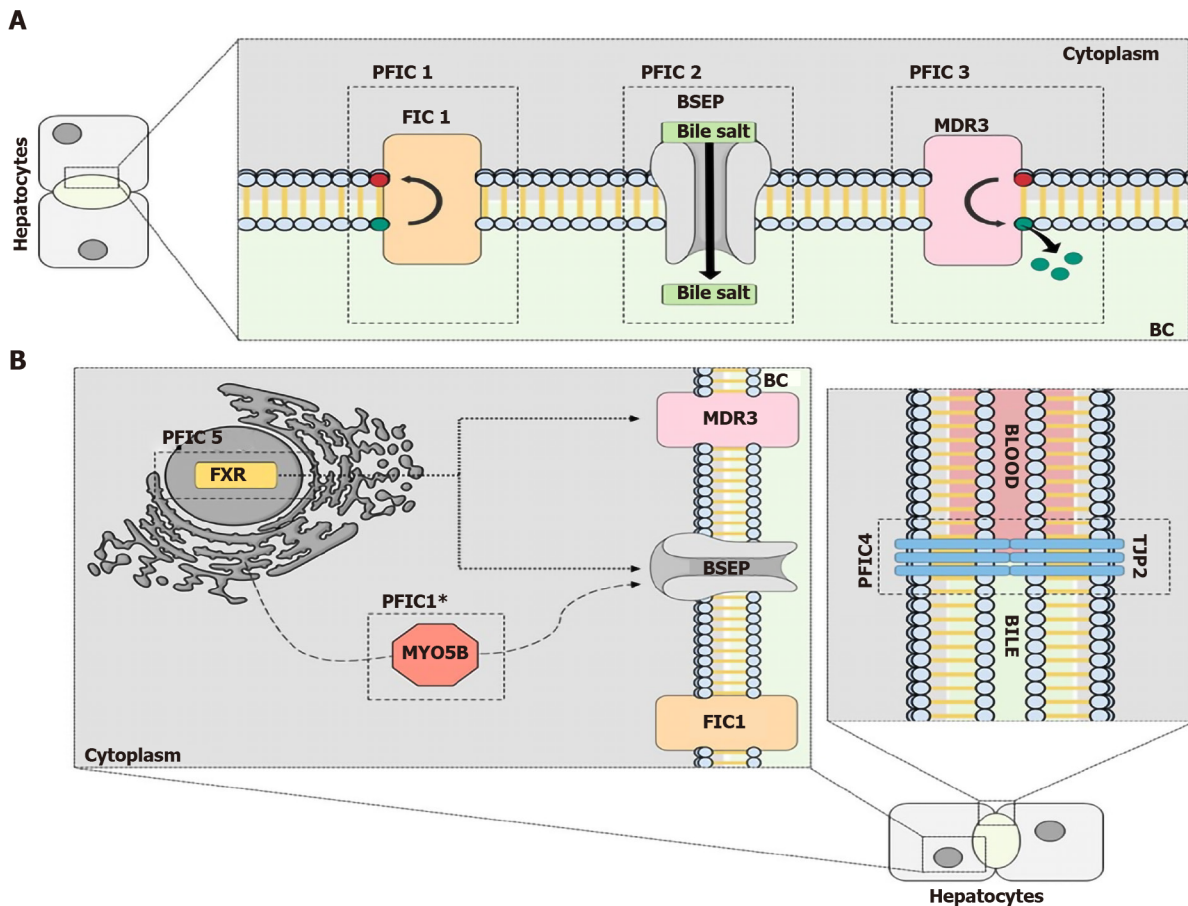
Figure 2 The molecular mechanisms behind progressive familial intrahepatic cholestasis. A: The classic types of Progressive Familial Intrahepatic Cholestasis (PFIC). PFIC 1 is related to mutations in the genes that encode the flippase familial intrahepatic cholestasis 1, which flips phospholipids in the plasma membrane. Mutations in the bile salt export pump (BSEP) protein, a bile salt transporter, results in PFIC 2. The third type of PFIC is caused by mutations in the gene that encodes the Multidrug resistance class 3 glycoprotein (MDR3) protein, another lipid flippase; B: The newer types of PFIC. Mutations in the tight junction protein-2 protein, which prevents the mixing of blood and bile acids, are responsible for PFIC 4. PFIC 5 is a result of mutations in the farnesoid X receptor protein, a transcription factor important for BSEP and MDR3 ecpression. Mutations in Myosin VB result in a PFIC phenotype because the trafficking of the BSEP protein from the endoplasmic reticulum to the plasma membrane is disrupted. PFIC: Progressive Familial Intrahepatic Cholestasis; FIC1: Familial intrahepatic cholestasis 1; BSEP: Bile salt export pump; MDR3: Multidrug resistance class 3 glycoprotein; TJP2: Tight junction protein-2; FXR: Farnesoid X receptor.

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