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Review Article

Familial intrahepatic cholestasis: New and wide perspectives

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ABSTRACT

Background: Progressive familial intrahepatic cholestasis (PFIC) includes autosomal recessive cholestatic rare diseases of childhood.
Aims: To update the panel of single genes mutations involved in familial cholestasis.
Methods: PubMed search for "familial intrahepatic cholestasis" alone as well as in combination with other key words was performed considering primarily original studies and meta-analyses.
Results: PFIC1 involves ATP8B1 gene encoding for aminophospholipid flippase FIC1. PFIC2 includes ABCB11 gene, encoding for protein functioning as bile salt export pump. PFIC3 is due to mutations of ABCB4 gene responsible for the synthesis of class III multidrug resistance P-glycoprotein flippase. PFIC4 and PFIC5 involve tight junction protein-2 gene and NR1H4 gene encoding for farnesoid X receptor. Benign Intrahepatic Cholestasis, Intrahepatic Cholestasis of Pregnancy and Low-phospholipid-associated cholelithiasis involve the same genes and are characterized by intermittent attacks of cholestasis, no progression to cirrhosis, reversible pregnancy-specific cholestasis and cholelithiasis in young people. Blood and liver tissue levels of bile-excreted drugs can be influenced by the presence of mutations in PFIC genes, causing drug-induced cholestasis. Mutations in PFIC genes might increase the risk of liver cancer.
Conclusion: There is a high proportion of unexplained cholestasis potentially caused by specific genetic pathophysiologic pathways. The use of next generation sequencing and whole-exome sequencing could improve the diagnostic process in this setting.

1. Introduction

Progressive familial intrahepatic cholestasis (PFIC) represents a heterogeneous group of autosomal recessive disorders of childhood, characterized by intrahepatic cholestasis due to defects of both bile synthesis and transport [1,2]. PFIC can evolve rapidly leading to portal hypertension, liver failure, liver cancer and/or extrahepatic manifestations within the first ten years of life.

Three different types of PFIC have been previously identified [3–5]:

- PFIC1, which is associated to the mutation of ATP8B1, a gene encoding an amino-phospholipid flippase (FIC1 protein).
- PFIC2, also known as bile salt export pump (BSEP) deficiency, involves the ABCB11 gene encoding the BSEP.
- PFIC3, known as class III multidrug resistance P-glycoprotein (MDR3) deficiency, involves the ABCB4 gene encoding MDR3.

The same genes have been implicated in many phenotypes such as benign recurrent intrahepatic cholestasis (BRIC), intrahepatic cholestasis of pregnancy (ICP), drug induced cholestasis (DIC) and low-phospholipid-associated cholelithiasis (LPAC). These phenotypes represent a continuum of the same disease. They are due to the single or the compound heterozygous mutations, most frequently in ABCB4 gene. Other factors, such as variants in more than one of the cholestasis-associated genes, epigenetic regulation and environmental factors, contribute to the specific phenotype observed [6–8].

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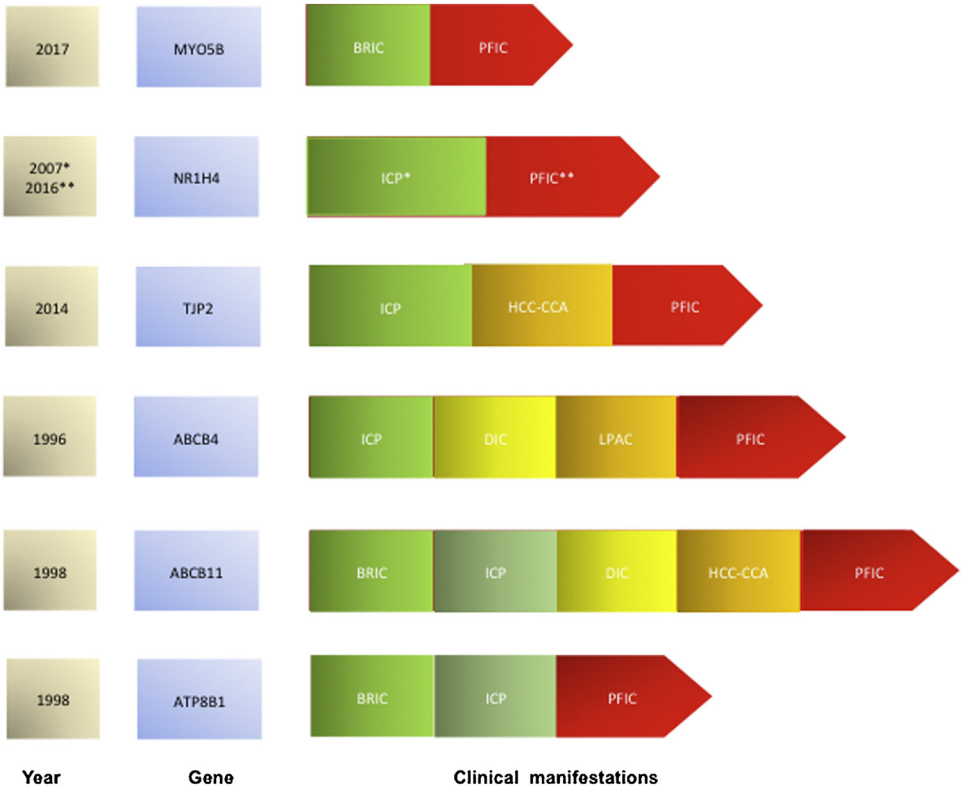

Fig. 1. Genes correlated with progressive familial intrahepatic cholestasis with year of discovery and associate phenotype. (*BRIC, benign recurrent intrahepatic cholestasis; PFIC, progressive familial intrahepatic cholestasis; ICP, intrahepatic cholestasis of pregnancy; HCC–CCA, hepatocellular carcinoma-cholangiocarcinoma; DIC, drug induced cholestasis; LPAC, low-phospholipid-associated cholelithiasis).*

Interestingly, some genes responsible for PFIC are involved in the genesis of liver tumors such as hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) [9,10].

The development of diagnostic methods such as next generation sequencing (NGS) and whole-exome sequencing (WES) has allowed the detection of new genes responsible for PFIC4 and PFIC5 [11–13], such as tight junction protein-2 gene (TJP2) [14] and NR1H4 gene [15], respectively.

Finally, mutations in Myosin 5B gene (MYO5B), responsible for a PFIC-like form without microvillus inclusion disease, have been reported [16,17].

Herein, we reviewed the available data on FIC classification, genetics, pathophysiology, histological features, natural history and treatment options (Fig. 1 summarizes genes, year of discovery and main phenotypes associated with PFICs). Literature search included e-Pub published articles (peer reviewed original articles, reviews and meta-analyses) with the following search terms: "progressive familial intrahepatic cholestasis", "Drug Induced Cholestasis", "Benign Intrahepatic Cholestasis", "Intrahepatic Cholestasis of Pregnancy", "Low-phospholipid-associated cholelithiasis", "Hepatocellular Carcinoma", "PFIC", "FIC", "ATP8B1", "ABCB11", "ABCB4", "TJP2", "NR1H4", "MYO5B", "BSEP", "MDR3".

2. Progressive forms

2.1. Progressive familial intrahepatic cholestasis 1

PFIC1 is a sporadic autosomal recessive liver disorder caused by homozygous or compound heterozygous mutations in the gene ATP8B1 (chromosome 18q21) that precludes protein expression. ATP8B1 acts as lipid flippase, carrying phosphatidylserine and phosphatidylethanolamine from ectoplasmic to the cytoplasmic leaflet of the hepatocyte canalicular membrane (Fig. 2A).

ATP8B1 maintains the asymmetry between inner and outer leaflet of plasma membrane, having a protective function against high concentrations of bile salts (BS) [4]. Abnormal ATP8B1 expression interferes also with BS secretion through down-regulation of farnesoid X receptor (FXR). The FXR is expressed in the liver and in the intestine and is encoded by the NR1H4 gene, implicated in PFIC5 (Fig. 2D). In healthy liver, elevated BS levels mediated activation of FXR. The FXR induces BSEP expression, thereby stimulating biliary BS output. In the intestine, FXR represses apical sodium-dependent bile acid transporter (ASBT) expression reducing intestinal BS reabsorption. Reduced FXR expression in PFIC1 represents a consequence, rather than a cause, of cholestatic phenotype in ATP8B1 deficiency [18].

Traditionally, PFIC1 was identified with the name of Byler's disease and of Greenland Familial Cholestasis [19,20].

PFIC1 is the only one, in contrast to the other PFICs, that can present with extra-hepatic manifestations since the protein is also expressed in apical membrane of cholangiocytes, enterocytes and acinar cells of the pancreas. Clinical manifestations are diarrhea, growth retardation, pancreatitis and deafness. The main occurrences are the appearance of cholestasis, usually in the first few months of life, recurrent episodes of jaundice associated with uncontrollable itching with low gamma-glutamyl transferase (GGT) [4]. The histopathology and immunohistochemistry (IHC) are not pathognomonic. However, the most common histological findings are canalicular cholestasis, absence of true ductular proliferation, portal and lobular fibrosis with inflammation and giant cell transformation [4] (Table 1).

The ursodeoxycholic acid (UDCA) represents a milestone for the early therapeutic management in children [21], while pancreatic enzymes and fat-soluble vitamins are indicated if gut related symptoms (including pancreatitis) are present [4]. Cholestyramine and rifampicin have also been used for the treatment of the itch-

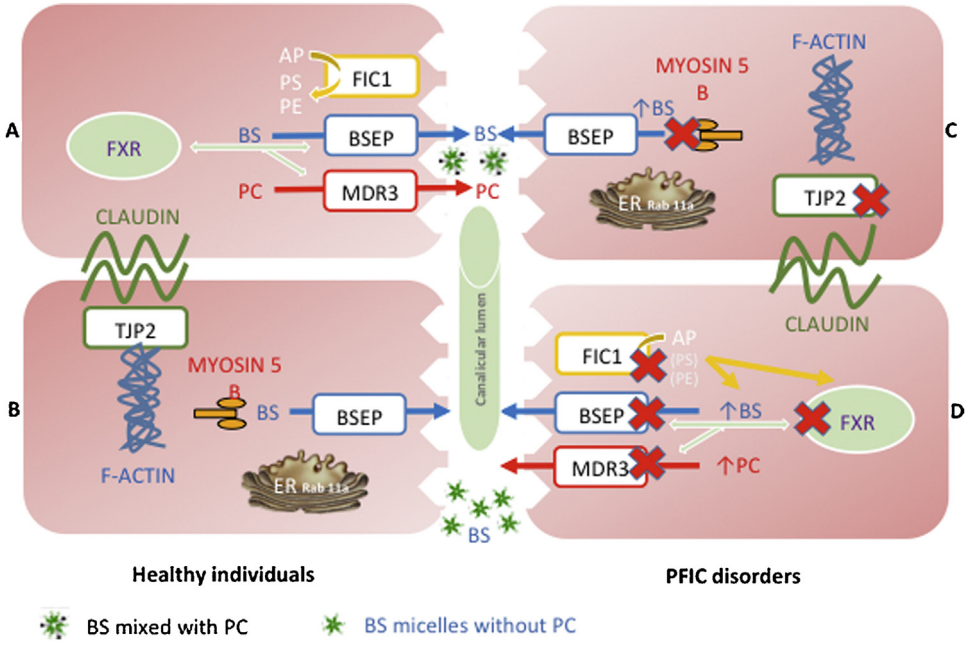

Fig. 2. (A,B) Simplified representation of the proteins expressed by the genes involved in cholestasis in healthy subjects. (B,C) Simplified representation of the altered mechanisms involved in PFIC disorders. (*AP, amino-phospholipides; BS, bile salts; BSEP, bile salt export pump protein; ER, endoplasmic reticulum; PC, phosphatidylcholine; FIC 1, familial intrahepatic cholestasis deficiency type 1 protein; FXR, farnesoid X receptor; MDR3, multidrug resistance protein 3; PS, phosphatidylserine; PE, phosphatidylethinolamine; PFIC, progressive familial intrahepatic cholestasis; TJP2, tight junction protein 2 gene*).

ing [22]. When medical therapy fails, the partial biliary diversion (PBD) should be considered and the naso-biliary drainage may help to select potential responders to surgery. The PBD can be external, when the jejunal conduit between gall bladder and abdominal wall is created by a stoma, or internal if jejunal conduit between gall bladder and colon or anastomosis between gall bladder and anti-reflux loop of colon is performed [23]. In approximately 80% of patients with PFIC1 and 2, the PBD results in improved growth, improvement/normalization of liver function, and reduction of fibrosis [24]. If the latter are ineffective, the liver transplantation (LT) is the only option [4] but the development of the non-alcoholic fatty liver disease and growth retardation can worsen the quality of life after surgery [25].

2.2. Progressive familial intrahepatic cholestasis 2

PFIC2 is caused by homozygous or compound heterozygous mutations in the ABCB11 gene, a form of FIC caused by BSEP deficiency. The gene is located on chromosome 2q31.

BSEP is a liver-specific adenosine triphosphate-binding cassette transporter that mediates the excretion of monovalent BS from hepatocyte to canaliculi against a concentration gradient [26,27]. It is the main transporter of BS with a critical role in physiologic maintenance of enterohepatic BS circulation (Fig. 2A). A loss of BSEP leads to accumulation of BS in the hepatocytes with consequent cellular damage and alterations to FXR signalling (Fig. 2D).

BSEP deficiency can lead to cholestatic jaundice and itching in the neonatal period. The GGT and serum cholesterol levels are low or normal while serum primary BS, serum aminotransferase concentrations and alpha-fetoprotein can be very high in comparison with PFIC1 (Table 1). Notably, patients develop increased risk of early HCC [10].

Liver histology shows the canalicular cholestasis, severe lobular injury, more pronounced lobular/portal fibrosis and inflammation, more severe hepatocellular necrosis and more evident giant cell transformation respect to PFIC1. A reduced expression of BSEP protein at IHC can help the diagnostic process [4].

The therapeutic options are the same used in PFIC1. Notably, post-transplant recurrence has been described [28–31]. Recently, preliminary reports have described benefit from use of 4-phenylbutyrate. This drug, previously used in patients with urea cycle defects, seems to be able to improve bile secretion, liver function and itching by inducing *de novo* canalicular BSEP expression [32–34]. Conversely, no data are available about use of FXR agonists [35].

2.3. Progressive familial intrahepatic cholestasis 3

Patients with PFIC3 display a late onset disease [1]. The autosomal recessive illness is caused by mutations of the MDR3 glycoprotein, which is coded by the ABCB4 gene, on chromosome 7q21. The causative mutations include insertions, missense mutations and nonsense mutations [7,8]. The protein is a phosphatidylcholine flippase, located in the canalicular membrane of hepatocytes. It transports phosphatidylcholine from the hepatocytes into the bile canaliculus. Phosphatidylcholine is a key component of micelles of BS as it reduces their detergent activity and thus protects the cholangiocytes from cellular damage (Fig. 2A) [19]. The patients may present less aggressive jaundice and the itching is often triggered by certain drugs [36]. Other clinical features are hepatomegaly, growth retardation and acholic stools [1].

The patients with PFIC3 show high levels of GGT and alkaline phosphatase (AP). The BS and cholesterol values can be normal while biliary phospholipids are significantly reduced [1]. The age of the onset of PFIC3 ranges from 1 month to over 20 years (Table 1). Liver histology is characterized by non-specific portal inflammation, extensive portal fibrosis, cholestasis with ductular proliferation and by the loss of MDR3 protein expression at IHC [37,38].

The complete absence of canalicular staining of MDR3 protein is associated with mutations leading to a truncated protein form (Fig. 2D) while a faint or normal MDR3 canalicular expression is observed in patients with missense mutations. The abnormal MDR3

Table 1
Laboratory, clinical, genetic, and histological characteristics of different PFIC classes.

	Progressive familial intrahepatic cholestasis					
	PFIC 1	PFIC 2	PFIC 3	PFIC 4	PFIC 5	PFIC[b]
Locus/gene/protein	18q21-22/ATP8B1/FIC1	2q24/ABCB11/BSEP	7q21/ABCB4/MDR3	9q21.11/TJP2/ZO-2	12q23.1/NR1H4/FXR	18q21.1/MyosinVB/MYO5B
Clinics	Early onset; severe jaundice/itching; growth retardation; diarrhea, pancreatitis, deafness; leads to LT;	Early onset, severe jaundice/itching; leads to LT; potential post-LT recurrence	Childhood/young adulthood onset; can be drug-triggered; hepatomegaly, growth retardation, HCC risk;leads to LT	Early severe cholestasis onset; Progression to liver failure in childhood; No post-LT recurrence; HCC risk	Neonatal onset, rapid progression to ESLD, vitK-independent; coagulopathy	Onset < 2 years: ± MVID, jaundice/itching; hepatomegaly
Laboratory[a]						
BA	High	Very high	High	High	High	High
GGT	Low or normal	Low or normal	High	Normal or mild elevation	Normal	Normal
AST/ALT	Mild elevation	Moderate elevation	Mild elevation	Elevation	Moderate elevation	Mild or moderate elevation
AFP	Normal	High	Normal	High	High	Normal
Histology	Mild cholestasis, mild lobular fibrosis and inflammation with giant cells	Canalicular cholestasis, lobular/portal fibrosis and inflammation with giant cells	Loss of MDR3 expression, portal inflammation, portal fibrosis, cholestasis, ductular proliferation	Centrolobular cholestasis; mislocalization of claudin	Cholestasis, loss of BSEP expression	Cholestasis, Inflammation with giant cells, BSEP and MDR3 tissue expression, MYO5B and RAB11 A canalicular staining

PFIC: progressive familial intrahepatic cholestasis; BRIC: benign recurrent intrahepatic cholestasis; ICP: intrahepatic cholestasis of pregnancy; DILI: drug induced cholestasis; LPAC: low-phospholipid-associated cholelithiasis; BSEP: biliary salt export pump; MDR-3: class III multidrug resistance P-glycoprotein; TJP-2: tight junctions protein-2; zo-2: zona occludens-2; FXR: fanesoid X receptor; MVID: microvillous Inclusion Disease; MYO5B: myosinVB protein; RAB11A: RAS-related protein RAB11 A; LT: liver transplantation; ESLD: end-stage liver disease; vitK: vitamin K; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutammyl transferase; AFP: alpha-1-fetoprotein; BA: bile acids.

[a] Alkaline phosphatase levels in pediatric population are strongly influenced by the levels of bone isoenzymes and are therefore not reported.

[b] MYO5 B is classified by OMIM, online mendelian inheritance in man, as the gene responsible for microvillus inclusion disease but not yet for PFIC6.

canalicular IHC, combined with low levels of biliary phospholipids, is highly suggestive for MDR3 deficiency [36].

Patients with residual phosphatidylcholine secretion and MDR3 expression, especially those with missense mutations, respond to treatment with UDCA in 70% of cases [21] while LT should be reserved to patients with PFIC3-associated liver failure. The mean age at LT is 9.6 years, ranging from 2 to 33 years [39].

There are no reports about the use of PBD in patients with PFIC3. However, drugs that increase MDR3 expression via FXR activation could be good candidates for PFIC3 therapy [40].

2.4. Progressive familial intrahepatic cholestasis 4

PFIC4 is a form of PFIC that has been recently described and is caused by homozygous mutations of the TJP2 gene (also known as ZO-2) on chromosome 9q21. This gene encodes for a protein able to create a link between the transmembrane tight junction proteins and the actin cytoskeleton forming tight junctions themselves (Fig. 2B–C) [11]. The recently described paediatric cases presented a severe cholestatic disease with low GGT levels and were free of mutations in ATP8B1 and ABCB11 genes. In a recent report described by Sambrotta et al. [11] the PFIC4 onset appeared within 3 years in twelve pediatric cases. Nine of twelve patients needed LT within 10 years. No recurrences after LT were observed (Table 1). The mutations were identified by a combination of WES and targeted sequencing of genes known to be associated with cholestasis. All homozygous mutations were predicted to abolish protein translation, consistent with a complete loss of function. Eighteen of the 29 families examined in this study were consanguineous.

2.5. Progressive familial intrahepatic cholestasis 5

The association between PFIC and mutations in NR1H4 on chromosome 12q23 has been described [15] being denominated PFIC5. The PFIC5 is an autosomal recessive severe liver disorder involving NR1H4 gene, which encodes FXR.

The FXR is a bile acid-activated nuclear hormone receptor involved in the bile acid metabolism and homeostasis. The genes ABCB11 and ABCB4 are direct targets of FXR (Fig. 2A). Gomez-Ospina et al. [15] described four patients, from 2 unrelated families, who presented neonatal onset with rapid progression to end-stage liver disease, vitamin K-independent coagulopathy, low-to-normal levels of GGT, elevated serum alpha-fetoprotein (AFP) in combination with undetectable ABCB11 expression (Table 1). The variants in the NR1H4 gene were found by WES and SNP analysis, resulting in homozygous or compound heterozygous loss of function mutations (c.526C > T and c.419_420insAAA/intragenic 31.7-kb deletion, respectively). The authors underlined the relation between the development of early progressive cholestasis and the role of FXR as a main regulator of bile acid homeostasis, thus identifying the features of PFIC5 (Fig. 2D).

2.6. Progressive familial intrahepatic cholestasis linked to defects in MYO5B gene

Mutations of the MYO5B gene, on chromosome 18q21.1, are associated with the microvillus inclusion disease, a congenital disorder of the enterocyte leading to the intractable diarrhea. The onset of cholestasis is commonly associated with parenteral nutrition required for the treatment of the diarrhea [16]. The MYO5B, an actin-associated molecular motor, interacts with recycling endosome-associated RAB family proteins (particularly with RAS-related protein RAB11A) and this interface is essential for proper functioning of polarized epithelial cells, such as subcellular positioning of recycling endosomes and normal bile canaliculus formation (Fig. 2B) [17,41]. The MYO5B mutations alter MYO5B/RAB11A interaction, thus worsening cholestasis by a loss of expression of BSEP and MDR3 (Fig. 2C).

By the NGS of a panel of genes involved in genetic cholestasis (including RAB11A) and by WES, Gonzales et al. [17] identified, four compound heterozygous and one homozygous MYO5B mutation in five pediatric patients with normal-GGT PFIC phenotype without intestinal disease. *In silico* analysis (Sift, Mutation Taster, Polyphen2) predicted a deleterious effect of the non-synonymous MYO5B variants not previously reported in the single nucleotide polymorphism database.

The age of onset of symptoms was about 1 year and at a median age of 5 years none of the children have progressed to liver failure nor been listed for LT.

Interestingly, Qiu et al. [16] showed a prevalence of MYO5B deficiency in 20% of previously undiagnosed low-GGT cholestasis cases in a cohort of Chinese children. All patients showed elevated bilirubin, low GGT, mildly elevated Alanine transaminase (ALT) and Aspartate transaminase (AST) values, elevated serum total BS levels and impaired BSEP expression at IHC (Table 1). Among the ten MYO5B-mutated patients identified in this study, the phenotypes resembled a progressive, recurrent and transient cholestasis as observed in ABCB11 and in ATP8B1 mutations. Here, the absence of intestinal involvement suggests that cholestasis is a manifestation of mild MYO5B functional deficiency. Treatments used routinely in this study were UDCA, cholestyramine and fat-soluble vitamins. One patient was non-responsive to routine treatment and died before three years of life.

The MYO5B deficiency has not yet been recognized as PFIC6 by the Online Mendelian Inheritance in Man (OMIM®).

3. Non progressive forms

3.1. Benign recurrent intrahepatic cholestasis

The benign recurrent intrahepatic cholestasis (BRIC) is an autosomal recessive inherited disorder characterized by the intermittent occurrence of severe cholestasis (minimum of two episodes of jaundice) [42].

The clinical presentation is usually less aggressive than PFIC [1]. BRIC typically appears later than PFIC and is not progressive. Two genetic features of the recessive inherited forms of BRIC are known; BRIC1 is characterized by the mutations of the ATP8B1 gene while BRIC2 presents a mutation in the ABCB11 gene.

BRICs show a non-progressive course since the protein function is only partially impaired [23]. Therefore, the main mutations are missense type and are located in less conserved regions of the gene as described for ABCB11 gene [43].

The triggers can be the pregnancy, the infections or drugs such as oral contraceptives. The itching usually anticipates the appearance of jaundice. Between two cholestatic attacks the laboratory tests return into the normal range and the patient feels good. Patients with BRIC1 can present the extrahepatic manifestations (similarly to PFIC1) such as hearing loss, pancreatitis and diarrhea while BRIC2 is often associated to the cholelithiasis (Table 2). The laboratory tests in BRIC1 and BRIC2 show low or normal levels of GGT, and high levels of BS and of conjugated bilirubin. The differential diagnosis should be made versus the extrahepatic biliary obstructions, the acute viral hepatitis, the autoimmune hepatitis, and the immune-related cholestatic disorders [23].

Liver histology shows cholestasis without alterations of liver structure while tissue expression of BSEP is absent or reduced at IHC in BRIC2 [23].

Diagnostic criteria are the following: (a) at least two episodes of jaundice with asymptomatic interval up to several months or

Table 2
Laboratory, clinical, genetic and histological characteristics of different non-progressive familial intrahepatic cholestasis.

Non-progressive familial intrahepatic cholestasis				
	BRIC	ICP	DIC	LPAC
Locus/gene/protein	18q21-22/ATP8B1/FIC1 2q24/ABCB11/BSEP 18q21.1/MyosinVB/MYO5B	18q21-22/ATP8B1/FIC1 2q24/ABCB11/BSEP 7q21/ABCB4/MDR3 9q21.11/TJP2/Z0-2 12q23.1/NR1H4/FXR	2q24/ABCB11/BSEP 7q21/ABCB4/MDR3	7q21/ABCB4/MDR3
Clinics	Intermittent severe cholestasis (intervals weeks to years); hearing loss, pancreatitis, diarrhea	Transient cholestasis + itching during pregnancy; post-natal resolution; potentially serious fetal complications	Chronic liver injury; acute hepatitis; fulminant hepatic failure Use of herbal remedies and naturopathic substances should be investigated; Onset < 1-12 months by drug adiministration	<40y cholelithiasis; intrahepatic microlithiasis; recurrence of biliary symptoms after cholecystectomy; previous episodes of ICP; familial history of gallstones
Laboratory				
BA	High during attack	High during pregnancy	Normal or mild elevation	High during obstruction
GGT	Low or normal	Normal or mild elevation	Variable	High
ALP	High	Normal or mild elevation	High	Normal to high
AST/ALT	Normal or mild elevation	Normal or mild elevation	Moderate or severe elevation	Normal or mild elevation
AFP	No data	No data	No data	No data
Histology	Centrolobular cholestasis, no alterations of liver structure, no BSEP tissue expression	Not performed	Loss of BSEP/MDR3 expression, canalicular cholestasis, hepatocellular inflammation	Not required; imaging based diagnosis

BRIC: benign recurrent intrahepatic cholestasis; ICP: intrahepatic cholestasis of pregnancy; DIC: drug induced cholestasis; LPAC: low-phospholipid-associated cholelithiasis; BSEP: biliary salt export pump; MDR-3: class III multidrug resistance P-glycoprotein; TJP-2: tight junctions protein-2; zo-2: zona occludens-2; FXR: fanesoid X receptor; MYO5B: myosinVB protein; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutammyl transferase; ALP: alkaline phosphatase; AFP: alpha-1-fetoprotein BA: bile acids.

years; (b) laboratory tests suggestive of intrahepatic cholestasis; (c) cholestasis-induced severe pruritus; (d) cholangiography showing normal intra- and extrahepatic bile ducts; (e) liver histology suggesting centrolobular cholestasis; (f) absence of other causes of cholestasis [44].

In addition to BRIC1 and BRIC2, defects in MYO5B gene have been associated with progressive, recurrent and transient forms of intrahepatic cholestasis (Fig. 2) [16]. In a Chinese study cohort, three children suffered from recurrent cholestasis similar to BRIC1 and 2 but with younger age on onset. Each patient had two episodes of cholestasis, with itching during the attacks and no symptoms between the episodes. They all had the first episode of cholestasis within the first year of life. Of note, a child presented with cholelithiasis at the age of 4 years and another had a recurrent episode of cholestasis at ten years triggered by cefixime and amoxicillin. The cholestatic bouts lasted several months and recurred after a few years. The authors postulated that the cholestatic phenotypes associated with MYO5B mutations might depend on the 'modifier' genes or possibly also on unknown environmental factors or epigenetic changes [16].

The treatment of BRIC is symptomatic and aims to reduce the frequency of the cholestatic attacks and to prevent the relapses. For instance, UDCA, cholestyramine, corticosteroids, rifampicin, antihistamines, phenobarbital, and carbamazepine, have been proposed [42]. Non-medical treatments include short-term nasobiliary drainage, which can improve the pruritus and reduce the duration of cholestatic attacks [45]. The extracorporeal albumin dialysis in a Molecular Absorbent Recycling System, can remove BS from the body [46]. The PBD can be considered in selected cases [47].

Finally, BRIC can be considered a relatively benign condition with favorable prognosis but also the cases with an intermediate phenotype representing a clinical *continuum* between BRIC and FIC or with progression from BRIC to FIC have been described [48–50]. Therefore, the patients with BRIC require a regular clinical follow-up.

3.2. Intrahepatic cholestasis of pregnancy

The heterozygous mutations in ATP8B1, ABCB11 and ABCB4 genes in ICP have been reported (Fig. 2) [51]. The ICP is the most common liver disease of the pregnancy. It is characterized by an onset of transient cholestasis and itching during the pregnancy with post-natal resolution [52]. Notably, serious fetal complications especially in ICP patients with high levels of BS (>40 μmol/L) can be registered [53] while the risk of stillbirth is increased when serum BS concentrations are ≥100 μmol/L [54]. A link between PFIC genes and ICP has been established in families with children with MDR3 deficiency, where each child has nonsense or missense homozygous ABCB4 mutation while a heterozygous mother has experienced recurrent episodes of ICP [36]. The reproductive hormones are central in the pathogenesis of this illness. In fact, the ICP is more common in twin pregnancies and in women with history of the fertility treatments or of the oral contraceptive-induced pruritus [55]. In fact, the disease usually starts in the third trimester when the hormonal concentrations are higher and able to reduce the activity of the BSEP [56,57]. The recurrence in subsequent pregnancies is likely.

The Incidence of ICP ranges from 0.1% to 15.6% worldwide [58,59]. The role of the environmental factors is evidenced by a more relevant prevalence during the winter among pregnant women in Sweden, Finland and Chile. High levels of BS, AST and ALT can be present while GGT levels are often modestly increased or within normal range (Table 2) while they are high in MDR3 deficiency [60]. Mutations in ABCB4 were the first to be described in ICP [37] and are implicated in up to 20% of ICP cases [61].

When it comes to the ABCB11 gene, polymorphism c.1331 T > C (p.V444A) in exon 13, has been consistently observed in pregnant women since the patients with at least one c.1331 T allele tended to have lower levels of BSEP expression as demonstrated by higher levels of BS in the carriers of alanine allele versus the carriers of valine allele [60,62]. It was reported in 83% of Caucasians with ICP [63] and the susceptibility to this disorder was increased if com-

bined with other mutations in the same gene or in the ABCB11 [64].

The heterozygous mutations have also been identified in ATP8B1 gene during ICP but a small number of cases is described [65,66].

The NR1H4 gene, associated with a new form of progressive intrahepatic familial cholestasis (PFIC5), has been previously associated with the ICP. Van Mil et al. [67] identified four novel heterozygous FXR variants (c.-1G > T, p.M1V, p.W80R, and p.M173T110) in 92 British women with ICP, hypothesizing that genetic variation in FXR confers susceptibility to this cholestatic form. The variant c.-1G > T in NR1H4 is implicated in the ICP when combined with the mutations of ABCB11 in V444 A and of ABCB4 in p.S320 F, respectively [68].

Recently, in an ICP cohort of 147 women, three patients showed variants of potential interest in TJP2, suggesting a correlation between ICP and this gene: c.185C > T in two unrelated cases and c.1877C > G in one mother [69].

Differential diagnosis should include other causes of jaundice such as HELLP syndrome (a pregnancy-associated disease characterized by Hemolysis, Elevated Liver enzymes, and Low Platelets in the mother), acute fatty liver of pregnancy, viral hepatitis, Budd–Chiari syndrome and Primary Biliary Cholangitis (PBC) [23]. The following drugs have been used to improve the symptoms in ICP: cholestyramine, dexamethasone, S-Adenosyl-L-methionine, rifampicin and UDCA [70,71]. The UDCA represents the first-line therapy being able to reduce maternal and fetal complications of ICP [36] and to improve maternal symptoms and biochemistry in the majority of cases [52]. Pregnant women with previous ICP and fetal mortality should start UDCA therapy early in subsequent pregnancies. The mechanisms of action of UDCA are not fully understood but include improved bile acid transport, anti-apoptotic effect and detoxification of pruritogens [72].

The rifampicin, a potent pregnane-X receptor agonist, is useful to treat the pruritus in many forms of intrahepatic cholestasis [73]. Only a retrospective study with few cases reported the efficacy of rifampicin in one third of pregnant women not responsive to UDCA alone [71]. According to the available data, the use of rifampicin in the third trimester of pregnancy is feasible as a second line therapy [52]. There is little information about the use of cholestyramine in ICP [74]. The efficacy of S-Adenosyl-L-methionine in improving the itching and the biochemistry while dexamethasone is useful only to promote the fetal lung maturity. In the clinical practice, physicians induce the birth between 37 and 38 weeks of gestation with the aim of reducing the incidence of intrauterine death [51].

Women having ICP history present an increased risk of hepatic impairment when taking the combined oral contraceptives [52].

Although the blood tests are usually normalized within 2–8 weeks, a follow-up is necessary for ICP patients because they may develop cirrhosis, non-alcoholic pancreatitis and cholelithiasis over time [75].

3.3. Drug induced cholestasis

Drug induced Liver Injury (DILI) represents major public health issue, accounting for about 5% of hospitalizations for jaundice and more of 40% of hepatitis in adults over 50 years [60]. DILI is the most common cause of acute fulminant hepatic failure (more than 50% of cases) [76]. Herbal remedies have been reported to cause different liver disorders (Table 2). Approximately 30% of DILI are cholestatic [Drug induced Cholestasis (DIC)] [77].

The main limit during the systemic clearance of lipophilic drugs and their metabolites by the liver is their excretion into the bile. This process is regulated by several ATP-dependent canalicular transporters including BSEP protein (ABCB11), the multidrug resistance protein-2 (MRP2, ABCC2) able to cause BS independent flow by excretion of glutathione, the multidrug resistance-1 protein (MDR1, ABCB1) which transports organic cations and the MDR3 protein (ABCB4).

The list of the drugs that can induce cholestasis includes the non-steroidal anti-inflammatory drugs, antihypertensives, antidiabetic, anticonvulsants, lipid-lowering agents, and psychotropic drugs [60].

Many drugs cause cholestasis through the interaction with hepatic transporters. To date, a relatively strong association between DIC and attenuated BSEP activity has been proposed [76,78]. Inhibition of BSEP due to a drug activity, should lead to reduced BS secretion and their retention within hepatocytes, leading to cholestasis.

The drugs such as rifampicin, cyclosporine A, rifamycin, bosentan, troglitazone, erythromycin, glibenclamide inhibit the BSEP protein with a dose-dependent action [76].

Although not a drug transporter, the MDR3 protein plays a key role in the biliary secretion of phosphatidylcholine. Several mutations in ABCB4 result in exposure of the biliary epithelium to the toxic detergent effects of BS, thus increasing susceptibility to DIC [76].

Chlorpromazine, imipramine, itraconazole, haloperidol, ketoconazole, sequinavir, clotrimazole, ritonavir and troglitazone restrain the MDR3 activity *in vitro*, and can lead to DIC or to the vanishing bile duct syndrome.

Interestingly, 27% of women with ICP history complain of itching when taking oral contraceptives or during the second half of the menstrual cycle [79].

Acute or chronic hepatitis can be clinical manifestations of DIC. The cholestasis is often asymptomatic while laboratory tests are only modestly increased [76]. Sometimes the itching, the xantomas and/or the melanoderma are present [80].

The diagnosis of DIC can be difficult, due to the presence of the concomitant medications or of other chronic liver diseases [60]. The most studied genes are ABCB11 and ABCB4; the clinical role of genetic variants for the development of DIC is currently being researched. In addition to the presence of the disease-causing mutations, several polymorphisms have been associated with DIC. Considering the ABCB11, the most cited example is V444A that is significantly more common in patients with ICP, DIC and genetic cholestasis than in the general population [60,81,82]. In addition, FXR is critical in the transcriptional activation of MDR3 and BSEP, as the mutations in this gene are a risk factor for the onset of liver injury [60].

The diagnosis of DIC is dependent on the accurate clinical history: the use of drugs, herbal remedies and naturopathic substances should be always investigated. Temporal relationships between taking the drug and development of liver injury can be different, ranging from a few days, to several weeks, or longer (1–12 months). Furthermore, the polipharmacotherapy increases the risk of drug-drug interactions. Transaminases may be in normal range or minimally increased while the AP elevation is the most common laboratory alteration [76].

The Council for International Organizations of Medical Sciences/Roussel Uclaf Causality Assessment Method (CIOMS/RUCAM) scale has been proposed to establish a causal relationship between the offending drug and the liver disease. The "Cholestatic injury" in patients with AP > 2 × ULN or Alanine transaminase (ALT)/AP ratio ≤ 2, the "Mixed Hepatocellular/cholestatic injury" in patients with an ALT/AP ratio ≤ 2–5 or the "Hepatocellular injury" if ALT > 2 × ULN or ALT/AP ratio ≥ 5. The categories are "definite or highly probable" (score > 8), "probable" (score 6–8), "possible" (score 3–5), "unlikely" (score 1–2) and "excluded" (score ≤ 0) (Table 3) [83]. The differential diagnosis should be made versus PBC, sepsis, autoimmune hepatitis and obstructive biliary diseases. Liver biopsy may be helpful when

Table 3
CIOM/RUCAM scale[a].

Time of onset	Type of liver injury				Score
	Hepatocellular		Cholestatic/mixed		
	1st treatment	2nd treatment	1st treatment	2nd treatment	
From drug intake until onset reaction	5–90 days	1–15 days	5–90 days	1–90 days	+2
From drug withdrawal until onset reaction	<5 or >90 days	>15 days	<5 or >90 days	>90 days	+1
	≤15 days	≤15 days	≤30 days	≤30 days	+1
	ALT > 2 × ULN or ALT/AP ratio ≥ 5		AP > 2 × ULN or ALT/AP ratio ≤ 2 or ALT/AP ratio ≤2–5		
	>50% improvement at 8 days			+3	
Course of reaction	>50% improvement at 30 days		>50% improvement at 180 days		+2
		<50% improvement at 8 days		+1	
	Lack of information or not improvement			0	
	Worsen or < 50% improvement at 30 days			−1	
		Alcohol or pregnancy		+1	
Risk factors	Alcohol		Age ≥55-years old		+1
	Age ≥55-years old		None		0
	None		Drug with suggesting time		−1
Concomitant therapy	Drug with suggesting time		Known liver toxicity with suggestive timing		−2
	Known liver toxicity with suggestive timing		Drugs with other evidence for a role		−3
	Drugs with other evidence for a role				
Exclusion of non drug-related causes	Virus infection, biliary obstruction, hypotensive shock		Virus infection, biliary obstruction, hypotensive shock		−3 to +2
Previous information on hepatotoxicity	Reaction in product label or published		Reaction in product label or published		+2
	No label		No label		+1
	Unknown reaction		Unknown reaction		0
	Positive		Positive		+3
Rechallenge	Compatible		Compatible		+1
	Negative		Negative		−2
	Non done or not interpretable		Non done or not interpretable		0

Results: >8 points, definitive; 6–8 points, probable; 3–5 points, possible; 1–2 points, unlikely; <0 points, excluded.
ALT: alanine aminotransferase; AP: alkaline phosphatase; ULN: upper limit of normal.

[a] Scale proposed by the Council for International Organizations of Medical Sciences/Roussel Uclaf Causality Assessment Method (CIOMS/RUCAM) to establish a causal relationship between the offending drug and the liver disease [83].

the diagnosis is difficult; a loss of BSEP/MDR3 expression at IHC increases the chance for DIC diagnosis. In DIC, the canalicular cholestasis and minimal hepatocellular inflammation are present [60].

Not recognizing a drug responsible of the cholestatic injury prolongs the exposure, which may worsen DIC and lead to unnecessary diagnostic and therapeutic proceedings. Furthermore, the drug-drug interactions, the cytokines able to down-regulate the cytochrome P450 and the genetic determinants, may influence the severity of DIC [76]. Given the different presentation patterns of DIC, its management should not be limited to the discontinuation of a specific drug, but should include genetic analysis to determine which bile transporters variants might be involved, in order to avoid relapses of DIC.

3.4. Low-phospholipid-associated cholelithiasis

Mutations in FIC genes are involved also in LPAC. The ABCB4 deficiency can cause gallstone disease in young people without other known risk factors for cholelithiasis. The prevalence of LPAC is unknown while the male to female ratio is approximately 1:3. However, up to 10% of the European and American population have gallstones, 25% of cases having symptoms [84]. The LPAC is associated with the intrahepatic biliary lithiasis, is often symptomatic after cholecystectomy and presents a good response to UDCA [36].

A reduced secretion of phospholipids in the bile decreases the solubility of cholesterol promoting its crystallization and the gallstone formation. At least two of the following criteria are necessary for the diagnosis of LPAC [84]:

- symptomatic cholelithiasis prior to 40 years;
- intrahepatic echogenic *foci* or microlithiasis at ultrasound;
- recurrence of biliary symptoms after cholecystectomy.

The diagnosis should also be suspected when previous episodes of intrahepatic cholestasis of pregnancy and a family history of gallstones in first-degree relatives are present [85].

The onset of the symptoms has been reported at the end of or soon after pregnancy. In fact, 56% of symptomatic women who are carriers of ABCB4 variants also presented a history of ICP [86]. The cholestasis results in an increase of GGT while abdominal ultrasonography, computing tomography scan and magnetic resonance cholangiopancreatography, help to evidence the intrahepatic gallstones (Table 2).

In a large cohort of 156 patients, Poupon et al. [87] discovered the ABCB4 variants in 79 cases (63 mono-allelic): similar features between subjects with and those without mutations were found and the authors hypothesized the possible involvement of unexplored genes. For example, the FXR regulates ABCB4 and ABCB11 expression and a down-regulation of this gene, due to the mutations or to the high levels of the progesterone metabolites, could promote defective BS and phospholipid secretion, leading to LPAC and ICP. While the homozygous variants cause PFIC3, the heterozygous variants lead to LPAC/ICP disorders.

The differential diagnosis should be made versus inflammatory cholestatic disorders which may cause biliary symptoms and/or intrahepatic gallstones such as Caroli disease and primary sclerosing cholangitis.

A definitive diagnosis is obtained by ABCB4 gene analysis. A careful management is recommended and an indication to cholecystectomy should take into account high rates of recurrence after surgery [88]. Moreover, a long-term therapy with UDCA could be useful to prevent complications. The up-regulation of ABCB4 by FXR agonist could become a treatment option in the future. In the setting of concomitant hypercholesterolemia statins should be preferred to fibrates as the latter increase the bile lithogenicity [84].

Furthermore, a potential association with cholangiocarcinoma has been reported [89].

4. Liver cancer

Hepatocellular carcinoma is a complication of liver cirrhosis being rare in children [9]. Knisely et al. [9] reported 11 cases of pediatric HCC in subjects affected by BSEP deficiency. However, all patients had cirrhosis when the tumor was diagnosed and 8 patients presented elevated serum levels of AFP [9]. Patients with BSEP deficiency may develop HCC or CCA in 15% of cases [10] and also pancreatic adenocarcinoma has been described [90]. The mechanisms of hepatocarcinogenesis are unknown but some authors hypothesized a mutagenic role of increased intracellular concentrations of BS [91]. Interestingly, reports of CCA in 2 young patients with PFIC and ABCB11 mutation have been described [92].

An association between HCC and the ABCB4 gene was well described in a large-scale whole-genome sequencing of the Icelandic population. Among 303 cases of liver cirrhosis and 681 cancer cases, the variants p.Gly622Glu, p.Leu445GlyfsX22, p.Asn510Ser and c.711 A > T were associated with liver cirrhosis, gallbladder cancer, HCC and CCA. Remarkably, the four ABCB4 variants were also associated with elevated ALT and GGT, secondary to phosphatidylcholine deficiency [93].

Zhou et al. [94] described two cases of HCC, caused by homozygous mutations in TJP2 (PFIC4). One was compound heterozygous for the TJP2 mutation, the other was homozygous for a frameshift mutation. The patients were six and twenty-six years old and the cirrhotic stage of the disease was found in both cases. The clinical and histological futures were similar to the BSEP deficiency, presenting low GGT values and chronic cholestasis. The down-regulation of TJP2 in certain tumors suggests a TJP2 induced direct tumorigenesis, but the mechanism is still unclear [94].

The lack of reports of HCC cases in the FXR-related PFIC may be due to the fact that only 2 families have been described to date, with the neonatal onset and a rapid progression to the end-stage liver disease. For instance, two patients died at 8 months and at 5 weeks while LT was performed at 22 months and at 4.4 months in the other two cases, respectively [15].

The key role of the FXR in repressing BS synthesis has been reported in the FXR null mice, which exhibit the increased BS levels and expression of pro-inflammatory cytokines, the resistance to apoptosis and the cell hyperproliferation leading to development of HCC [95]. Conversely, a long-term activation of FXR in ABCB4$^{-/-}$ mice by administration of INT-767, a more potent derivate of obeticholic acid seems to be able to reduce BS pool, re-program the BS metabolism and prevent the HCC [96]. These results could promote the potential therapeutic role of the FXR agonists in the prevention of the HCC.

Colon-rectal, bladder, gastric but not liver cancers have been associated with a MYO5B down-regulation [97–99]. Finally, patients with ABCB11/TJP2 intrahepatic cholestasis should be subjected to the screening for HCC.

5. Conclusions

Genetic variants of the ATP8B1, BSEP and MDR3 biliary transporters can lead to serious features of PFIC in the childhood, representing the indications to the PBD and to the LT at this age. Non-surgical therapy includes drugs, predominantly UDCA and rifampicin, and naso-biliary drainage, used in the patients with an intractable itching during the long-lasting cholestatic episodes.

The bile acid derivates, such as obeticholic acid and norursodeoxycholic acid, fibroblast growth factor 19 analogues and other mutation-targeted drugs represent promising therapeutic

strategies and could be used to increase the expression of functional proteins in the future.

The real incidence of PFIC is difficult to establish [1]. The NGS of targeted genes associated with cholestasis allowed to discover new gene mutations and a broad spectrum of cholestatic liver diseases with different phenotypes and severity [100]. Recently, we described multiple variants in different FIC genes in a cohort of adult patients with specific risk factors for and clinical manifestations of cholestasis [82]. It has been hypothesized a synergistic effect between various cholestatic phenotypes and/or specific interactions with environmental factors, in particular certain drugs. Therefore, the presence of the personal or familial history of juvenile cholelithiasis, drug liver injury, ICP or cryptogenic cholestasis should sensitize towards possible diagnosis of ATP8B1, ABCB11, ABCB4 and TJP2-related cholestasis.

Moreover, HCC is a potential severe complication, especially in ABCB11 and TJP2 related diseases. Recently, the WES analyses identified new genes responsible for PFIC, such as TJP2 [14] and NR1H4 gene [15], involved in PFIC 4 e PFIC5, respectively. Finally, the mutations of the gene MYO5B, which are responsible for PFIC-like pictures without microvillus inclusion disease, have been reported.

Several other genes have been implicated in the diagnosis of the inherited cholestasis in Western countries, e.g. JAG1 (Alagille syndrome), CYP27A1 (cerebrotendinous xanthomatosis), SLC25A13 (citrin deficiency), ABCC2 (Dubin-Johnson syndrome) and SERPINA1 (alpha-1 antitrypsin deficiency) [101].

Today, the existence of a relevant number of unsolved cholestatic cases in children and in adults suggests that new genetic pathophysiologic pathways should be explored, encouraging the use of WES technology as a routine diagnostic tool.

Conflict of interest

None declared.

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