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# PROGRESSIVE FAMILIAL INTRAHEPATIC CHOLESTASIS – CLINICAL, BIOCHEMICAL, GENETIC AND HISTOPATHOLOGICAL ASPECTS

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To the two greatest men of their time

– my father and my son

For reminding me what life is about

## ABSTRACT

In the early 1980's, most cases of neonatal cholestasis (often referred to as neonatal hepatitis) remained unexplained. By today, many of these diseases have been characterized in detail. Progressive familial intrahepatic cholestasis (PFIC) is one of these cholestatic entities with early onset, where new techniques in genetics and molecular biology have contributed substantially to our understanding.

The aims of this thesis were to further extend the knowledge of PFIC in terms of disease genetics, the effects of surgical diversion of bile, and the distribution of bile flows during cholestatic episodes and in remission. We studied a total of 18 patients.

Genetic linkage analysis excluded involvement of the known disease locus at 18q21-22, and suggested that PFIC is a genetically heterogeneous disease. After the identification of the gene *ABCB11*, which causes PFIC type 2, genetic characterization showed a homozygous missense mutation (c.890A>G) causing an amino acid shift (p.E297G) in a majority of the Swedish children studied. One compound heterozygous child carried a microdeletion that had not previously been reported, and two children were negative for mutations in the coding sequence of *ABCB11*.

A majority of the children studied presented with signs of coagulopathy, ranging in severity from bruises or nose bleeds to bleeding in the lung or brain, as a consequence of vitamin K malabsorption due to hampered hepatobiliary bile excretion. Complete relief of pruritus was observed in 7 of the 13 operated children within one month after partial external biliary diversion (PEBD). Six of the children were treated for increased stomal bile losses during the first 2-6 weeks after surgery. One patient underwent liver transplantation two months after PEBD due to end-stage liver disease, and one patient died of hepatocellular carcinoma 14 months after PEBD.

At early follow-up after 11 to 21 months the children showed improved growth, and the biochemical markers of cholestasis were significantly reduced. All operated children suffered one or more cholestatic episode(s) of varying duration during the total follow-up period of 5 to 12 years. The total duration of these episodes correlated positively with the stage of liver fibrosis at the most recent follow-up liver biopsy ($r=0.62$, $p<0.05$). A statistically significant regress in histologic cholestasis was noted 3 years and 5 years after PEBD, and in fibrosis 5 years and more than 10 years after PEBD.

A total of 13 scintigraphic examinations were performed on 9 operated children during episodes of cholestasis (n=5) and in remission (n=8). When we compared the fractions of isotopic activity lost through the stoma, in urine and remaining in the body during cholestasis and remission, we found a significantly larger fraction lost through the stoma (median 90% vs. 22%, $p<0.05$), and a smaller fraction into the urine (median 2.5% vs. 15%, $p<0.05$) in remission, than during cholestasis.

We conclude that PFIC encompasses not one, but several cholestatic diseases, all caused by different defects in the formation of bile. Most children with PFIC caused by mutations in *ABCB11* who undergo PEBD have a favorable long-term prognosis including histological improvement and long-term survival without the need for liver transplantation.



Key words: Progressive familial intrahepatic cholestasis (PFIC), genetic linkage analysis, *ABCB11*, immunohistochemistry, partial external biliary diversion (PEBD), nuclear scintigraphy, fibrosis, histological regress.

## LIST OF PUBLICATIONS

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# CONTENTS

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 4-PBA | 4-phenylbutyric acid |
| AATD | $\alpha_1$-antitrypsin deficiency |
| ABCB4 | ATP-binding cassette, subfamily B, member 4 |
| ABCB11 | ATP-binding cassette, subfamily B, member 11 |
| ASBT | Apical $Na^+$- dependent bile acid transporter |
| ATP | Adenosine triphosphate |
| ATP8B1 | ATPase, type 8B, member 1 |
| BA | Biliary atresia |
| BAAT | Bile acid co-enzyme A amino acid N-acyltransferase |
| BASD | Bile acid synthesis defect |
| BRIC | Benign recurrent intrahepatic cholestasis |
| BSEP | Bile salt export pump |
| CA | Cholic acid |
| CDCA | Chenodeoxycholic acid |
| CK19 | Cytokeratin 19 |
| cM | Centimorgan |
| CYP3A4 | Cytochrome P450, family 3, subfamily A, polypeptide 4 |
| CYP7A1 | Cholesterol 7α-hydroxylase |
| CYP27A1 | Sterol 27-hydroxylase |
| DCA | Deoxycholic acid |
| DNA | Deoxyribonucleic acid |
| EBV | Epstein-Barr virus |
| EMBL | European Molecular Biology Laboratory |
| FIC1 | Familial intrahepatic cholestasis 1 |
| FXR | Farnesoid X receptor |
| GGT | γ-Glutamyl transpeptidase |
| HCC | Hepatocellular carcinoma |
| HSC | Hepatic stellate cell |
| IB | Ileal bypass |
| IBD | Inflammatory bowel disease |
| ICP | Intrahepatic cholestasis of pregnancy |
| IDA | Iminodiacetic acid |
| IE | Ileal exclusion |
| IGF-1 | Insulin growth factor 1 |
| INR | International normalized ratio |
| LCA | Lithocholic acid |
| LCS | Lymphedema cholestasis syndrome |
| LOD | Logarithm of odds |
| LRLT | Living related liver transplantation |
| LXR | Liver X receptor |
| MARS | Molecular adsorbents recirculation system |
| MBq | Megabecquerel |
| MDR3 | Multidrug resistance protein 3 |
| MRP2, 3 and 4 | Multidrug resistance-associated proteins 2, 3 and 4 |
| NBD | Nasobiliary drainage |

| | |
|---|---|
| NTCP | $Na^+$/taurocholate co-transporting polypeptide |
| OATP | Organic anion-transporting polypeptide |
| OLT | Orthotopic liver transplantation |
| OSTα/OSTβ | Organic solute and steroid transporter α/β |
| PAC | Plasmid artificial chromosome |
| PAS | Periodic acid-Schiff |
| PBC | Primary biliary cirrhosis |
| PC | Phosphatidylcholine |
| PCR | Polymerase chain reaction |
| PEBD | Partial external biliary diversion |
| PFIC | Progressive familial intrahepatic cholestasis |
| PIBD | Partial internal biliary diversion |
| PS | Phosphatidylserine |
| PSC | Primary sclerosing cholangitis |
| PT | Prothrombin time |
| RXR | Retinoid X receptor |
| SDS | Standard deviation score |
| SNP | Single nucleotide polymorphism |
| Spgp | Sister of P-glycoprotein |
| Tc99-mbf | Technetium-99m-labeled mebrofenin |
| TJP2 | Tight junction protein 2 |
| UDCA | Ursodeoxycholic acid |
| UGT1A1 | Uridine diphosphate glucuronosyltransferase family 1, member A1 |

# 1 INTRODUCTION

The liver plays a central role in a number of vital physiological processes, including most steps in protein, carbohydrate and lipid metabolism. It is involved in the synthesis of bile constituents and of many vital proteins (*e.g.* coagulation factors, albumin, growth factors, hormones). It is also crucially involved in the storage of several important substances (*e.g.* carbohydrates, vitamins, trace elements), and in the breakdown and excretion into bile of a number of the potentially toxic waste elements in the body (*e.g.* cholesterol, ammonia, drug intermediates).

During the first months of life the demands on the liver are high while its functional maturation is still low, which renders the liver especially vulnerable. In fact, in the healthy neonate or young infant, the serum concentrations of bile acids, and the biliary bile acid pattern is similar to that of cholestasis in the adult. In other words, the newborn infant is in a state of chronic physiological cholestasis [1].

The word cholestasis is derived from the Greek words for "bile" – χολη, and "stoppage" - στασις. "Bile stoppage", or the *impairment of bile flow* remains the generally accepted definition of cholestasis. This reduced bile flow may be caused by defect hepatocytic secretion of bile acids or of any other major constituent of bile, or by obstruction of intra- or extrahepatic bile ducts. This results in retention of potentially toxic bile acids and other metabolites in the hepatocyte, which further impedes the secretion mechanisms, and exacerbates cholestasis [2].

## 1.1 A SPECTRUM OF DISORDERS AND RISK FACTORS

The reported overall incidence of neonatal cholestatic liver disease is approximately 1 in 2,500 live births, even though the true incidence may be higher, due to probable underreporting of milder disease [3], and transient disorders passing undiagnosed. Population screening programs have been proposed [4] and tested on a small scale [5]. Even if the spectrum of possible causes is wide, the etiology remains unknown in 20-25% of infants [6]. The two most common causes of neonatal cholestatic liver disease are biliary atresia (BA), a progressive obstructive disorder of the intra- and extrahepatic bile ducts, and $\alpha_1$-antitrypsin deficiency (AATD), an inherited disorder which causes intrahepatic cholestasis associated with defect protein folding and subsequent hepatocellular sequestration of $\alpha_1$-antitrypsin, an important protease inhibitor.

The incidence of BA is 1 in 12,000 to 20,000 live births [7, 8]. AATD is seen in 1 of 1,600 live births, among which approximately 10% will present with hepatobiliary disease of varying severity [9]. These figures imply, that among all infants in Sweden investigated due to prolonged jaundice and conjugated hyperbilirubinemia, 40-50 infants/year would be diagnosed with neonatal cholestatic disease, of whom 6-8 infants each would have BA and AATD-related liver disease.

The term intrahepatic cholestasis to describe a complete or incomplete cessation of bile flow "in the absence of apparent extrahepatic biliary obstruction" was first used in a

clinical review by Popper and Szanto in 1956 [10]. **Table 1** presents an overview of the different diagnoses of neonatal *intrahepatic* cholestatic disease, including progressive familial intrahepatic cholestasis (PFIC).

| | | |
|---|---|---|
| **Viral and bacterial infections** | **Genetic and metabolic disorders** | **Endocrine disorders** |
| CMV, Parvo B19, Herpes simplex, HIV | α1-antitrypsin deficiency | Isolated deficiencies |
| Sepsis | Alagille syndrome | Hypothyroidism |
| Urinary tract infections | Progressive familial intrahepatic cholestasis | Hypocortisolism |
| | Benign recurrent intrahepatic cholestasis | Panhypopituitarism |
| **Systemic causes** | Cystic fibrosis | |
| Shock, ischemia and asphyxia | Bile acid synthesis defects | **Toxic causes** |
| Heart failure | Galactosemia | Drugs |
| Neonatal lupus | | Parenteral nutrition |

**Table 1**. Common causes of neonatal intrahepatic cholestatic disease.

## 1.2 PROGRESSIVE FAMILIAL INTRAHEPATIC CHOLESTASIS

PFIC was first described in members of an extended Amish kindred by Robert Clayton, who called it Byler's disease [11, 12]. It was evidently hereditary, affecting siblings in closely related Amish families, and it was fatal in almost all cases. Reports of similar clinical and biochemical findings in non-Amish children [13] led to the use of the more inclusive term progressive familial intrahepatic cholestasis (PFIC) and uniform clinical characteristics were delineated to differentiate the disease from other similar entities [14, 15]. These diagnostic criteria are still in use: chronic unremitting cholestasis with onset during infancy (typically before 6 months of age), with biochemical markers signaling hepatobiliary disease, including conjugated hyperbilirubinemia, increased serum concentrations of alkaline phosphatase and fasting bile acids, normal or even low levels of γ-glutamyl transpeptidase (GGT), and excretion patterns of urinary bile acids ruling out bile acid synthesis defects [13, 16]. The serum levels of transaminases are usually mildly to moderately increased. Early histological findings are unspecific; there is no significant inflammation, and varying degrees of giant cell transformation. The cholestasis is both intracellular and canalicular, and neither paucity of bile ducts nor significant bile duct proliferation is a common finding in the light microscope. Transmission electron microscopy reveals coarsely granular canalicular "Byler bile" in children of Amish origin, in contrast to the densely amorphous bile plugs later described in patients of non-Amish origin [17].

Most children are born full term. Jaundice and signs of fat-soluble vitamin deficiency are often the first signs of disease, presenting during the first months after birth, whereas a progressing failure to thrive, and an unremitting cholestatic pruritus normally ensues during the second half of the child's first year. Early descriptions of PFIC or Byler's disease also frequently included findings of soft, foul-smelling stools, and hepatosplenomegaly.

According to the scarce literature on the epidemiology of PFIC, there is an approximate annual incidence of 1:50-100,000 [18, 19], which means that one or two infants with a biochemical cholestasis with increased serum levels of bile acids and normal GGT would be diagnosed with PFIC each year in Sweden [6].

# 2 BACKGROUND

## 2.1 THE PHYSIOLOGY OF BILE IN HEALTH AND DISEASE

### 2.1.1 Bile composition and function

Gallbladder bile in the healthy adult is composed of the following (with molar percentages in brackets): bile salts (12%), phospholipids (4%), cholesterol (~ 1%), plasma proteins and bilirubin (~ 1%). The rest of the bile (> 80%) consists of water and inorganic electrolytes (in concentrations closely reflecting those in plasma). Under normal non-cholestatic conditions, adults produce 600-800 mL bile/day, to be stored and concentrated in the gallbladder and excreted into the duodenum after meals, in order to facilitate the absorption of fat and fat-soluble vitamins. It has been estimated that bile salts transport up to 25 times their own weight in fat from the intestinal lumen each day [20]. Besides facilitating dietary uptake, bile serves as the main excretory pathway for degradation products (*e.g.* different xenobiotics, excess cholesterol, bilirubin, hormone intermediates). The bile acids have several functions, as comprehensively reviewed by Alan Hofmann [21]. For example, in the **liver**, they induce bile flow, promote mitosis and regulate apoptosis during hepatic regeneration and cholestasis, regulate bile acid synthesis, and regulate hepatic blood flow. In the **cholangiocyte**, they stimulate bicarbonate secretion and promote cell proliferation during cholestasis. In the **gallbladder**, they promote mucin secretion. In the **small intestinal lumen**, they enhance bile acid-dependent co-lipase, aid in tryptic hydrolysis, and exert antimicrobial effects. In the **ileal enterocyte**, they regulate the bile acid transporters, and finally, in the **large intestine**, they promote defecation and modulate fluid and electrolyte absorption.

### 2.1.2 Bile acid synthesis and its regulation

The daily hepatic excretion of bile acids is 20-40 g, or 50-60 µmol/kg body weight. More than 97% is recycled, and less than 3% is lost into feces and urine [22]. Thus, the *de-novo* synthesis only needs to balance these losses, in order to keep a stable total bile acid pool. The bile acids are produced from cholesterol along two different metabolic pathways, consisting of a number of enzymatic steps, most of which take place in the hepatocyte [23]. The neutral (or classical) pathway is regarded as the major pathway in adults under normal conditions. It mainly synthesizes cholic acid (CA), starting via hydroxylation of cholesterol at the 7α-position by the rate limiting enzyme **CYP7A1** (cholesterol 7α-hydroxylase). The other metabolic route is called the acidic (or alternative) pathway, which mainly, but not exclusively, synthesizes chenodeoxycholic acid (CDCA), starting with the hydroxylation of cholesterol at 27α-position by the rate limiting enzyme **CYP27A1** (sterol 27-hydroxylase). This pathway, which produces a minor fraction of the total bile acids in adults under non-cholestatic conditions, has been suggested to be more important in neonates and during the first year of life [24]. The rate limiting enzyme CYP7A1 is under strict control by several different regulatory feedback mechanisms, including an intricate system of small polypeptides acting together directly on transcription of the enzyme. These polypeptides are in their turn controlled by the nuclear receptors **FXR** (farnesoid x-receptor), **LXR** (liver x-receptor)

and **RXR** (retinoid x-receptor) [25]. The rate limiting enzyme of the acidic pathway, CYP27A1, does not seem to be under the same thorough feed-back control [26], *i.e.* its activity is more stable, relatively uninfluenced by bile acid concentration.

Under non-cholestatic conditions, the two major human primary bile acids and their metabolites constitute the bile acid pool, of which ~ 60% is CA and its main metabolite deoxycholic acid (DCA), whereas ~ 40% is CDCA and its main metabolite, lithocholic acid (LCA) [27]. This is somewhat different from the newborn baby, where CA is the predominating bile acid, and where almost no secondary bile acids are detectable until 2 to 3 months of age [28]. Also, small amounts of "atypical bile acids" can be found in bile during the first weeks of infancy, similar to the bile acid pattern in adult cholestasis [28]. During cholestasis in adults, the total bile acid pool is decreased, mainly as a consequence of a smaller CA pool, whereas there are only minor differences in CDCA kinetics [29, 30].

### 2.1.3 The enterohepatic circulation and the transporters of bile

More than 97% of secreted bile acids are recycled to the liver via active and passive intestinal uptake, and resecreted into the canaliculus. Under normal conditions, each bile acid molecule would be secreted and recycled up to 10 times daily [22]. Mauritius van Reverhorst (see Thesis cover) postulated the existence of this enterohepatic circulation (EHC) already by the end of the 17$^{th}$ century [31], and it was outlined in greater detail 200 years later by Mauritz Schiff [32]. The different main transporters in the cells involved in the EHC (*i.e.* the hepatocyte, the cholangiocyte, and the ileal enterocyte) have since then been identified and characterized, and are briefly presented in the text below and in **Figure 1**.

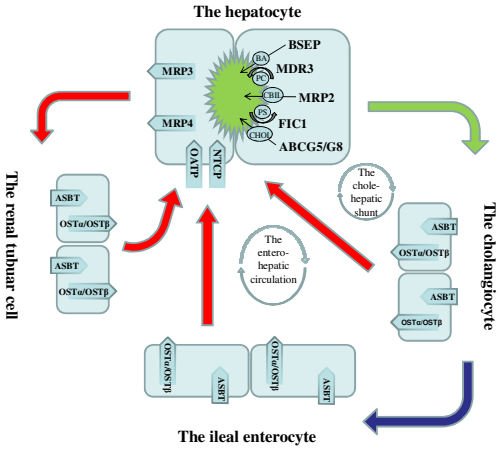


**Figure 1**. The most important transporters of bile acids and bile constituents in the hepatocyte, the cholangiocyte, the ileal enterocyte, and the renal tubular cell. Red arrows depict bile acids in the blood circulation, the green arrow those in the bile and the blue arrow those in the intestinal fluid. BA, bile acids; CBIL, conjugated bilirubin; CHOL, cholesterol (for further abbreviations see text or List of Abbreviations).

After uptake into the hepatocyte from the sinusoidal space, the recycled bile acids together with the small fraction of *de-novo* synthesized primary bile acids are conjugated with one of the amino acids taurine and glycine; glycine conjugation predominates in the adult hepatocyte, whereas taurine is more abundant and thus more commonly used during infancy [1]. The conjugated bile acids are then actively secreted against an up to 1,000-fold concentration gradient mainly by **BSEP** (bile salt export pump), the main bile acid transporter in humans. A smaller amount is exported by

**MRP2** (multidrug resistance-associated protein 2), which primarily transports conjugated bilirubin and other organic anions into the canaliculus.

Once leaving the hepatocyte, the bile acids are mainly ionized at physiological pH, and hence will be called bile salts. During passage down the biliary tract, bile salts form micelles with the phospholipids released from the outer canalicular membrane leaflet, translocated from the inner leaflet by **MDR3** (multidrug resistance protein 3), a so-called floppase with high affinity for phosphatidylcholine (PC). The third constituent necessary for formation of mixed micelles in bile is cholesterol, which is transported into the canaliculus by the canalicular cholesterol transporter **ABCG5/G8**. Upon reaching the gallbladder, the bile is concentrated and stored until gallbladder contraction releases it into the duodenal lumen.

A minor fraction of non-ionized bile acids (mainly CDCA-derivates) passively diffuse through the enterocytes of jejunum and proximal ileum, from whence they enter the portal blood. The larger fraction of ionized, conjugated bile salts undergo active uptake when reaching the distal ileum, through the action of **ASBT** (apical $Na^+$- dependent bile acid transporter) located in the apical membrane of the ileal enterocyte. They are thereafter effluxed by the basolaterally located heterodimer **OSTα/OSTβ** (organic solute and steroid transporter α and β) [33], and enter the mesenterial or portal blood. The bile salts that remain in the intestinal lumen undergo deconjugation and dehydroxylation in the distal ileum and proximal colon through the action of intestinal bacteria, which convert the primary conjugated bile salts into the secondary deconjugated bile acids DCA (deoxycholic acid) and LCA (lithocholic acid). These highly hydrophobic non-polar molecules then readily diffuse through the intestinal enterocyte of the colon and are recycled to the liver via the venous blood flow. On reaching the liver, the bile salts are absorbed into the hepatocyte from the sinusoidal space mainly by the action of **NTCP** ($Na^+$/taurocholate co-transporting polypeptide), and to a lesser extent through the **OATPs** ($Na^+$- independent organic anion-transporting polypeptides) [34]. Apart from these influx transporters at the basolateral membrane of the hepatocyte, there is also a basolateral set of "salvage proteins" including the bile acid transporters **MRP3** and **MRP4** (multidrug resistance-associated proteins 3 and 4 ). These salvage proteins aid in the export of organic anions, including the hepatotoxic bile acids retained in the hepatocyte during cholestatic conditions, when the expression of these proteins increases dramatically [22].

Although not directly involved in the transport of bile acids, the phospholipid transporter **FIC1** (familial intrahepatic cholestasis 1) is an important factor for maintaining the phospholipid asymmetry of the canalicular membrane, by translocating phosphatidylserine (PS) from the outer to the inner membrane leaflet (hence it is called a flippase). This asymmetry provides the canalicular membrane with the mechanical stability that has been proven crucial for proper function of biological membranes [35]. FIC1 is also an important inducer of FXR, a central regulator of bile acid synthesis, excretion and uptake (see 2.1.3.2) [36].

#### *2.1.3.1 The cholehepatic shunt*

In addition to the enterohepatic recycling of bile acids, there is also a cholehepatic shunt pathway, which mainly recycles unconjugated bile acids. These are actively absorbed by ASBT in the cholangiocyte, effluxed through OSTα/OSTβ on the basolateral membrane, and recycled back via the peribiliary venous plexus into the hepatocyte through NTCP [37]. The exact importance of the cholehepatic shunt remains to be clarified, but it has been suggested to be important for a functional bile acid dependent bile flow [38], and perhaps also as a part of the cholangiocyte's salvage system during cholestasis.

#### *2.1.3.2 The role of FXR in bile acid synthesis, secretion and uptake*

The expression of BSEP is controlled by FXR (farnesoid X- receptor), a nuclear transcription factor for bile acid synthesis and secretion. Intracellular CDCA and CA phosphorylate and thus activate FXR, which forms a complex with RXR (retinoid X-receptor). This heterodimer then translocates to the nucleus, and binds directly to the promoter site of the gene *ABCB11* (encoding BSEP), thereby inducing transcription and increasing the secretion of bile acids. Activated FXR downregulates the *de-novo* synthesis of bile acids by indirect inhibition on the transcription of CYP7A1, the rate limiting enzyme of bile acid synthesis. It also inhibits the transcription of ASBT, and thus decreases the active uptake of bile acids in the cholangiocyte, in the ileal enterocyte, and in the renal tubular cell, thereby preventing the re-uptake of excreted bile acids from the urine [36].

### 2.1.4 Bile and bile acids during infancy

After birth, bile acids accumulate in serum, soon reaching levels comparable with those of adults with cholestatic liver disease. This occurs despite the infant's small total circulating bile acid pool [28] (weight for weight corresponding to only about 30% of the adult pool), and the relatively underdeveloped active intestinal reabsorption of bile acids in the newborn [39]. This state of "physiological cholestasis" remains during at least the first 2 months, and thereafter a gradual decline of serum bile acids ensues to attain non-cholestatic, adult levels during the second half of the infants' first year [1]. This physiological cholestasis may have clinical implications under conditions that place extra demands on liver function, and also for "extrahepatic causes", for instance during metabolic stress due to a bacterial infection, after surgery, during parenteral nutrition, or in starvation.

### 2.1.5 Cholestasis and its pathophysiologic effects

Cholestasis is defined as a reduced canalicular bile flow, and its causes may be subdivided according to the primary defect into precanalicular, canalicular and postcanalicular [40, 41]. The precanalicular causes of cholestasis include $\alpha_1$-antitrypsin deficiency, parenteral nutrition-induced cholestasis, infectious hepatitis and bile acid synthesis defects. Canalicular causes of cholestasis include the bile acid secretion defects, *e.g.* defects in BSEP (as in PFIC type 2), or in MRP2 (as in Dubin-Johnson disease). Biliary atresia and CF-induced cholestasis are examples of postcanalicular causes of reduced canalicular bile flow.

Regardless of etiology, the reduced canalicular bile flow of cholestasis leads to hepatocytic retention of potentially harmful compounds, for instance bile acids. This further aggravates the hepatocellular damage, which decreases the bile acid secretion, resulting in further reduction of the bile acid-dependent bile flow, and decreased secretion of organic anions including bilirubin. This adds another brick to the burden of cholestasis by further reducing the bile acid-independent bile flow. The retained bile acids escape into the blood circulation, where it has been thought to elicit cholestatic pruritus. On the other hand, there is now growing consensus that the cholestatic pruritus is associated with increased opioid neurotransmission, and not directly with the retention of bile acids [42]. This vicious circle quickly leads to decreased micellar formation and thus to malabsorption of fat and fat-soluble vitamins with potentially life-threatening consequences, as well as to hepatocytic accumulation of neurotoxic substances such as ammonia, which may have deleterious effects on the central nervous system. The ongoing hepatocytic damage increases cell turn-over and necrosis, eventually leading to fibrosis and cirrhosis, with liver failure as a consequence. The mechanisms and physiologic effects of cholestasis have been outlined further detail in a number of recent reviews [41].

## 2.2 HISTORICAL BACKGROUND OF PFIC

### 2.2.1 The early history

In the late 17$^{th}$ century ideological differences led to a schism within the Anabaptist-Mennonite church, and a large minority group under leadership of the Elder Jakob Ammann of Erlenbach from the canton of Bern in Switzerland formed the Amish-Mennonite religious movement, later referred to as the Amish [43]. Part of this congregation emigrated to North America due to religious persecution during the first half of the 18$^{th}$ century, and settled down in the "Paradise of Pennsylvania". In 1737 the families of Beiler and Kauffmann arrived in a second wave of emigrants [44]. The families settled along the border between Berks and Lancaster counties in south-eastern Pennsylvania, which now harbor the second largest Amish community in the world [45]. Jacob (now Byler) and Nancy Kaufmann married and had children. The Amish' religious and cultural isolation led to genetic isolation, which increased the likelihood of otherwise rare recessive diseases [46, 47]. In time, this population presented glutaric aciduria type I [48], familial hypercholanemia [49], Ellis-van Creveld syndrome [50] – and progressive familial intrahepatic cholestasis or Byler's disease [11].

### 2.2.2 The first clinical descriptions

The first detailed report of patients with episodic cholestasis, with jaundice, pruritus, conjugated hyperbilirubinemia and prolonged prothrombin time was published by Summerskill and Walshe in 1959 [51]. The two patients presented their first symptoms during adolescence, responded poorly to different pharmacological treatments, but their pruritus disappeared after surgical drainage of the common bile duct, despite the lack of mechanical biliary obstruction. Since both patients recovered completely between their attacks, the authors proposed the descriptive term benign recurrent cholestasis, and suggested that the underlying mechanism could involve defects in bile secretion. A

similar clinical picture was described in a number of publications in the years to follow [52-55]. Niels Tygstrup described two boys from the isolated Faeroe Islands, with early childhood disease presentation, and very similar disease features. Although they were not known to be closely related to each other, the author suggested that a genetic factor caused the disease [52].

Less than one decade later Clayton described six young children from four distantly related Amish sibships, all named Byler, with symptoms and biochemical findings of conjugated hyperbilirubinemia and with hypoprothrombinemia that responded to parenteral vitamin K, but with normal levels of cholesterol [11]. These children lacked demonstrable obstruction of the extrahepatic biliary ducts, and there was an apparent familial component. In addition to this, and in contrast to most of the earlier descriptions, the cholestasis was not episodic; it progressed to liver failure and death in a majority of the patients already during early childhood [12].

## 2.3 THE CLINICAL SPECTRUM AND THE GENETICS OF PFIC

### 2.3.1 The early descriptions of familial intrahepatic cholestasis

During the late 60s and the 70s, several case reports of related patients with progressive familial intrahepatic cholestasis similar to that in the children with Byler's disease were published. Common findings included early clinical presentation, most often but not always during the first year of life, with jaundice, poor growth, signs of fat-soluble vitamin deficiency and pruritus, and a progressing biochemical cholestasis, often with normal or almost normal cholesterol levels [56-69]. There were also noticeable differences between the cases reported, for instance responses to pharmacological treatment [57, 58, 62]. Liver histology also differed: most patients had liver fibrosis to some degree; some had a paucity of bile ducts [58, 60, 62], others had proliferating bile ducts [61, 63, 66], or normal liver histology [57, 59, 63, 68]. Children with concomitant developmental delays [56, 59], or slowly progressing neurological abnormalities, with low levels of vitamin E [64] were also reported, later shown to improve after treatment with α-tocopherol [65]. The disease spectrum and the clinical characteristics of PFIC were reviewed by Caroline Riely in 1987 [13]. The next ten years of genetic linkage studies demonstrated that PFIC was not one, but in fact several diseases, as outlined in the text and summarized in **Table 2**, page 12.

### 2.3.2 PFIC type 1

The search for a disease-causing gene in a small number of distantly related members of a geographically isolated kindred in The Netherlands led to a first important breakthrough. These patients had a disease similar to that described by Summerskill and Walshe [51], *i.e.* benign recurrent intrahepatic cholestasis (BRIC). Houwen *et al.* assumed an increased statistical likelihood that the affected patients would have inherited not only an ancestral, thus identical genetic mutation, but also the identical genetic sequence surrounding the disease locus, *i.e.* that they had inherited a shared haplotype, identical by descent. On this assumption, three affected patients and their healthy parents were genotyped for 250 highly polymorphic genetic markers, each with known chromosomal location, evenly distributed along the genome. A region on

chromosome 18 was found, where 5 out of 6 tested chromosomes carried an identical haplotype in a number of consecutive highly polymorphic microsatellite markers, spanning a genetic distance of 19 cM. Based on this finding the region was strongly suspected of carrying a common disease-causing genetic mutation [70].

Selectively studying this locus in two distantly related patients from the Amish kindred originally described by Clayton [12], Carlton *et al.* showed shared haplotypes in all four chromosomes (*i.e.* linkage) to the BRIC-locus at 18q21-q22, and proposed the diseases to be allelic, *i.e.* different disease phenotypes caused by mutations in the same gene [16]. The region of interest was further refined to 11-12 cM in linkage studies of another seven patients from the original kindred [17]. Additional mapping based on a total of 97 families with both BRIC and PFIC, using the assumption of allelic diseases, narrowed the region of interest to less than 1 cM [71]. Using various physical mapping strategies, including database search of previously mapped expressed sequence tags (ESTs), sequence scanning, and the successful hybridization of a genetically mapped PAC (plasmid artificial chromosome) to a liver tissue cDNA-library, Bull *et al.* identified a putative gene, *FIC1* (familial intrahepatic cholestasis 1) [72], belonging to a group of ATPases, later named *ATP8B1* (ATPase subfamily 8B, member 1). Several mutations in *FIC1* were characterized in patients with as well BRIC as PFIC, indicating that the two different diseases in fact were caused by mutations in the same gene. Since a second chromosomal locus for PFIC had been established at this time, the intrahepatic cholestasis linked to 18q21-q22 was assigned the name PFIC type 1 (or PFIC1), and later also BRIC type 1 (or BRIC1).

Searching for mutations in *ATP8B1* in patients with similar phenotypes, Tygstrup *et al.* found a common homozygous genetic mutation in the patients from the Faeroe Islands, previously described as suffering from BRIC with variable clinical phenotype [52, 73]. In a number of indigenous Inuit families with a fatal form of intrahepatic cholestasis called GFC (Greenlandic familial cholestasis) [67], where indications of linkage to 18q previously had been established [74, 75], Klomp *et al.* reported a common probably disease-causing mutation [76]. In 2004, the same group presented > 50 different *ATP8B1* mutations in 180 families with PFIC or BRIC, and proposed a genotype-phenotype correlation, with a higher proportion of missense mutations in BRIC than in PFIC [77]. *ATP8B1* belongs to a large gene family encoding transporter proteins that – when mutated – cause several human diseases, for example the copper transport disorders Menke's and Wilson's diseases, with mutations found in *ATP7A* and *ATP7B*, respectively [78].

In functional studies, Eppens *et al.* showed that the human FIC1-protein was located in the canalicular membrane [79]. Ujhazy *et al.* studied the localization and function of *Fic1* in rat liver, and suggested that the protein is crucial in maintaining the proper distribution of phospholipids between the outer and inner leaflets of the epithelial plasma membrane, proposing it to be a flippase located in the hepatobiliary canalicular membrane [80]. When Paulusma *et al.* studied the effects on membrane stability, they found that canalicular membranes of *Atb8b1* mutant mice were less resistant to hydrophobic bile acids, and caused cholestasis. They suggested that this was due to loss of phospholipid asymmetry caused by the decreased *Atp8b1*-flippase activity [81], and

showed recently that *ATP8B1* indeed is involved in the internalization of phosphatidylserine (PS), *i.e.* a PS-flippase [82]. In a recent publication, Stapelbroek *et al.* elegantly showed that mutations in *Atp8b1* in mice cause progressive degeneration of cochlear hair cells and result in hearing loss, and that patients with BRIC type 1 have severe hearing loss, unlike other patients with cholestasis (primary sclerosing cholangitis and BRIC type 2) [83]. This suggests the first plausible explanation for the earlier sporadic reports of hearing loss in patients with PFIC [84].

An explanation for the difference in severity of PFIC type 1 *versus* BRIC type 1 was provided by Folmer *et al.* [85], after studying the interaction between *ATP8B1* and *CDC50A* (shown earlier by the same group to be required for proper function of the endoplasmatic reticulum [82]) in cell cultures. The different disease-causing mutations not only led to differences in protein stability and canalicular expression, but it was also observed that mutations causing PFIC type 1 led to decreased interaction between *ATP8B1* and *CDC50A*, in contrast to mutations causing milder disease, such as BRIC type 1 or ICP (intrahepatic cholestasis of pregnancy).

In studies of possible transcriptional interaction between transporters of bile and the main bile acid regulating factor FXR, a link between mutations in *ATP8B1* and the decreased expression of BSEP has evolved, that perhaps could further explain the cholestasis seen in PFIC type 1. It is known that activated FXR stimulates the expression of BSEP by direct action on the gene *ABCB11* and inhibits the expression of ASBT [86], leading to increased secretion and reduced recycling of bile acids. It has now been convincingly shown by different research groups using different methods, that mutations in *ATP8B1* decrease FXR-activity, with inhibited bile acid secretion and increased ileal uptake and recycling of bile acids as a consequence, both mechanisms supposedly leading to cholestasis [87, 88].

### 2.3.3 PFIC type 2

Strautnieks *et al.* [89] genotyped unaffected and affected members from a large multiplex family previously described by Kagalwalla *et al.* [68], along with four additional unrelated consanguineous families, in search of shared haplotypes using the same set of polymorphic markers mapped to 18q21-22 as had been used in previous linkage studies [16, 70]. Evidence against linkage to this locus was found, suggesting genetic heterogeneity in PFIC. This was further substantiated when siblings from two unrelated non-Amish families with similar clinical features were studied [17]. Employing a genome-wide mapping approach, including members from six consanguineous families with no known relation to each other, and typing them for polymorphic microsatellite markers evenly distributed over the genome, Strautnieks *et al.* found a second disease locus at chromosomal region 2q24, spanning a region corresponding to 2 cM, *i.e.* approx. 2 Mb (2 million base pairs) of coding sequence [90].

Based on this finding, on the isolation by Gerloff *et al.* of a gene encoding a bile salt transporter *Spgp* (Sister of P-glycoprotein) predominantly expressed in the hepatobiliary canalicular membrane in mammals (pig and rat) [91], and on the isolation

and genetic mapping to 2q21 of a PAC containing the human orthologue of the rat *Spgp* by Childs *et al.* [92], a putative human gene was isolated and the first disease-causing mutations of the gene *ABCB11* were characterized [93]. We contributed to this work by providing patient data and genomic DNA from the same Swedish patients with Byler-like intrahepatic cholestasis as described in **Paper I**. Examination of conserved regions of homozygosity in a number of closely linked polymorphic microsatellite markers in three consanguineous families revealed a small number of critical recombination events leading to loss of homozygosity. This made it possible to further refine the putative gene-harboring region to less than 1 cM. A yeast artificial chromosome (YAC), with a size of 870 kb, was found to encompass three of these markers, flanking the putative disease locus. Genomic sequence from the recently isolated and mapped PAC, containing the human *Spgp*-orthologue, was used for creating suitable PCR-primers. With the help of these primers, identical DNA-sequences could be amplified using the YAC (containing the putative disease locus) and the PAC (containing the *Spgp*-orthologue), a finding strongly suggesting that both the PFIC type 2 and the human *Spgp* (called BSEP) were localized on the same 870 kb YAC, thus identifying a possible candidate gene. cDNA sequences from BSEP were generated, and gene expression limited to human liver was demonstrated by northern blot analysis. In patients linked to the disease locus, a number of different sequence variations were found, that were not found in matched controls. One of these probable mutations was a nucleotide change in exon 9 at position 890 (c.890A>G), leading to the substitution of glutamic acid with glycine at amino acid position 297 (p.E297G). This mutation was found in a majority of the Swedish patients in **paper I** (page 26).

Janssen *et al.* generated polyclonal pig *Bsep* antibodies, and studied the BSEP expression in 28 patients and *ABCB11* mutations in 19 patients with clinical low-GGT PFIC [94]. They found mutations in 10 patients, none of them expressing BSEP, proposing a close correlation between mutations in *ABCB11* and negative BSEP staining. In 2008, Strautnieks *et al.* described a large number of mutations causing PFIC type 2 (or BSEP disease) [95], adding up to well over 100 known genetic aberrations in *ABCB11* [96-105], including recent findings by Liu *et al.* of 12 novel mutations found in the mainland Chinese population [106]. van Mil *et al.* sequenced *ABCB11* in *ATP8B1*-negative patients from 20 different families with benign recurrent cholestasis (BRIC) and detected 1 splice site- and 7 missense *ABCB11* mutations in 11 patients from 8 different families; they proposed that this new entity be named BRIC type 2 [107]. Two patients with BRIC type 2 and slowly progressing hepatic fibrosis was found to be homozygous for the previously described *ABCB11* mutation c.889A>G (p.E297G).

Functional studies of BSEP and of common mutations in *ABCB11* were performed, using different *in vitro* systems [98, 108-112]: In studies of substrate specificities, Byrne *et al.* showed high affinities to BSEP for the major bile acids, and found a strong inhibition of the transport of bile acids by different therapeutic drugs, suggesting a mechanism for drug-induced cholestasis [109]. Noe *et al.* studied the *BSEP* expression with the gene cloned into a virus vector [110], and the function of four human *ABCB11* mutations *in vitro* was studied using cells infected with the a virus vector after site directed mutagenesis. They could demonstrate normal expression, but reduced bile

acid transport ability in one patient with clinical BRIC type 2 and no expression in the patient with clinical PFIC type 2 [98]. Wang *et al.* introduced human *ABCB11* mutations into rat *Abcb11* to investigate the canalicular expression and bile acid transport capabilities, and showed a wide range of effects on the trafficking to the apical membrane, on the varying ability to transport bile acids, and on the degradation patterns in the different tested PFIC type 2-mutants. They recently suggested the endoplasmatic reticulum as one of possible therapeutic targets [111, 112]. Hayashi *et al.* studied the common European *ABCB11* mutations E297G and D482G in cell systems infected with viral vectors carrying the mutations and found close to normal bile acid transport function, but decreased trafficking to the canalicular membrane [99]. They then investigated the effect *in vitro* and *in vivo* (on rats) of 4-phenylbutyric acid (4-PBA), a drug that had been shown to decrease the degradation of mutant protein in cystic fibrosis [113], and found that 4-PBA in fact increased the expression of mutant BSEP at the apical membrane, probably by prolonging its half-life, thus stabilizing the protein. The same finding was shown in rats treated with 4-PBA.

| Name | **PFIC type 1** | **PFIC type 2** | **PFIC type 3** |
|---|---|---|---|
| Gene | *ATP8B1* | *ABCB11* | *ABCB4* |
| Locus | 18q21-22 | 2q24 | 7q21 |
| Protein | FIC1 | BSEP | MDR3 |
| Function | Phospholipid flippase; transporting PS from outer to inner membrane; maintaining asymmetric distribution of PS, improving membrane mechanical stability and chemical resistance. | The main transporter of bile acids into the biliary canaliculus. | Phospholipid floppase; transporting PC from inner to outer membrane; maintaining integrity of cell membrane transport and stability, protecting it from chemical damge. |
| S-GGT | Low | Low | High |
| S-Bile acids | High | High | High |
| Pruritus | Intermediate-intense | Intense | Intermediate |
| Extrahepatic symptoms | Diarrhea, pancreatitis, deafness | Gallstones (rare) | Gallstones (frequent) |
| Biochemical consequence in bile | Increased biliary levels of phosphatidylserine and cholesterol, decreased levels of bile salts. | Decreased levels of bile salts. | Decreased biliary levels of phosphatidyl-choline, increased fractions of non-micellar bound bile salts. |
| Histology | Byler bile | Giant cell transformation | Bile duct proliferation |
| References | [16, 72, 80-83] | [90, 93, 108-111] | [114-118] |

**Table 2**. Genetic, biochemical and histopathological characteristics in PFIC types 1-3.

### 2.3.4 PFIC type 3

Smit *et al.* studied *Mdr2* knock-out mice developing severe cholestasis with low biliary levels of phosphatidylcholine. They could show a functionally defect protein and suggested this to be a phospholipid transport protein [115]. Deleuze *et al.* found absent MDR3 expression (the human homolog to mouse Mdr2) in liver tissue from a patient suffering from PFIC with high serum GGT and low biliary levels of phospholipids, and suggested that the liver damage may be caused by toxic effects of bile acids on the biliary epithelium in the absence of biliary phospholipids to protect the membrane [116]. de Vree studied the *ABCB4* gene sequence and MDR3 expression using anti-MDR3 antibodies in two patients with high-GGT PFIC from two consanguineous, unrelated families. The findings of homozygous mutations in the gene resulting in a truncated MDR3-protein, and absent MDR3 staining in liver tissue, suggested that a mutant protein could explain the lack of demonstrable immunohistochemical staining, *i.e.* causing PFIC type 3 [117]. Jacquemin *et al.* studied genetic sequence variations in

*ABCB4* in 31 patients with PFIC type 3, and found homozygous mutations in more than a third. They also found a varying age at onset of symptoms, from infancy (rarely) to early adulthood, and normal concentrations of biliary bile acids and phospholipids, in contrast to the findings in bile from patients with low-GGT PFIC types 1 and 2 [118].

### 2.3.5 PFIC-genes contributing to other cholestatic diseases

#### *2.3.5.1 Intrahepatic cholestasis of pregnancy (ICP)*

Although most cases of ICP are sporadic, families with both dominant and recessive inheritance patterns have been reported, suggesting a genetic predisposition [119, 120]. Genetic sequence variations in *ABCB4* may contribute to ICP; a number of studies have linked both sporadic and familial cases of high-GGT ICP to heterozygous sequence variations (mainly missense or nonsense mutations) in *ABCB4* [121-124], or to significant haplotype frequency differences compared to healthy controls [125].

The link between the genes *ATP8B1* and *ABCB11* and ICP is clearly weaker, and most studies on genetic sequence variations and haplotype frequencies have been negative [125-127]. However, Eloranta *et al.* concluded that genetic sequence variations in *ABCB11* may contribute to ICP [128], and Mullenbach *et al.* suggested a possible genetic contribution of *ATP8B1* in a minority of the British patients studied [129]. Lastly, Keitel *et al.* presented one case of severe early-onset high-GGT ICP with reduced MDR3 expression and homozygous genetic variants in *ABCB11* and *ABCB4*, proposing that this combination may have caused the cholestasis [130].

#### *2.3.5.2 Primary biliary cirrhosis and primary sclerosing cholangitis*

Pauli-Magnus *et al.* found no frequency differences of disease haplotypes in *ABCB11* and *ABCB4* in patients with primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC), compared to healthy controls [131]. Lucena *et al.* presented a case involving a patient heterozygous for a missense mutation in *ABCB4*, with recurrent attacks of juvenile cholelithiasis and ICP, who later developed PBC. They suggested that mutations in *ABCB4* may be involved in the etiology of several distinct cholestatic diseases [132], in congruence with proposals by Jacquemin *et al.* [118, 133].

#### *2.3.5.3 Transient neonatal cholestasis*

A few cases of neonatal transient cholestasis (TNC) involving heterozygous mutations in *ABCB4* have been reported [133], and also a heterozygous deletion of the entire *ABCB11* gene in a young infant with transient low-GGT cholestasis [19]. These findings suggest that mutations in genes encoding important bile and bile acid transporters may be a predisposing factor for TNC, in addition to the other known risk factors, including prematurity, asphyxia, peri- or postnatal infections, delayed enteral feeding, and surgery. Still, these are only occasional observations, and systematic studies are lacking so far.

### 2.3.6 Other cholestatic diseases with low GGT

In up to 25% of patients with clinical and biochemical features in full accordance with PFIC types 1 and 2, no mutations have been detected in *ATP8B1* or *ABCB11*. This

suggests that undiscovered disease-causing genes or other mechanisms in known disease-causing genes remain to be uncovered [107, 134]. Carlton *et al.* described mutations in two different genes (encoding the bile acid co-enzyme A amino acid N-acyltransferase, BAAT; and the tight junction protein 2, *TJP2*) in patients suffering from failure to thrive, rickets, coagulopathy, pruritus and increased serum bile acids with low or normal GGT [49, 135]. The suggested mechanisms of cholestasis are described as an impeded conjugation of bile acids (in *BAAT*-mutants) and leakage of bile acids through defect tight junctions of the biliary canaliculi (in *TJP2*-mutants). In addition to these rare defects, there are a number of cholestatic diseases or groups of diseases with onset during infancy, closely resembling low-GGT PFIC.

#### *2.3.6.1 Bile acid synthesis defects (BASD)*

Most, but not all [136], patients with BASD or inborn errors of bile acid metabolism present early during infancy, as outlined by Clayton [137]. BASD is similar to PFIC in many respects, and has even been proposed to be a "PFIC type 4" [138]. Pruritus is uncommon, as it is in PFIC during the first months of disease. In contrast to PFIC, most infants with BASD show normal or low serum levels of bile acids, when measured with standard hospital methods. Early recognition and diagnosis of BASD is important, since most defects can be treated with supplementation of oral bile acids other than UDCA [139].

#### *2.3.6.2 Aagenæs syndrome*

Neonatal cholestasis with jaundice and pale stools presenting during the first week of life was described for the first time in 1968 by Øystein Aagenæs in a group of Norwegian children all belonging to the same large kindred [140]. Apart from the apparent clinical cholestasis, these children show moderately increased serum levels of bile acids, and often, but not always increased levels of GGT. Pruritus is common after 6 months of age, but the lymphedema typical of this disease (also called lymphedema-cholestasis syndrome, LCS) is usually not a prominent feature until the early school years. In children presenting with normal GGT-levels and without the typical lymphedema, the diagnosis of Aagenæs syndrome may not be easily distinguished from the low-GGT cholestasis of PFIC types 1 and 2 [141]. The first disease locus has been genetically mapped to 15q [142], but the disease-causing gene still remains to be found.

#### *2.3.6.3 Arthrogryposis, renal dysfunction and cholestasis (ARC syndrome)*

The clinical diagnosis of ARC syndrome is based on the findings of arthrogryposis, renal tubular dysfunction and cholestasis with low serum GGT [143]. Children present early with severe failure to thrive, and with a spectrum of clinical manifestations signaling multiorgan disorder, such as cardiac defect, diarrhea, deafness, ichthyosis, neural tube defect, pancreatic insufficiency, and platelet dysfunction. The disease is almost invariably fatal during the first 2 years of life. Mutations in the disease-causing gene VPS33B, located at 15q26.1 [144], lead to varying phenotypic severity, with no clear genotype-phenotype correlation [143].

## 2.4 NON-SURGICAL TREATMENT OF PFIC

The first attempts at non-surgical treatment were intended to compensate for the apparent dietary malabsorption and fat-soluble vitamin deficiency. Formulas rich in medium-chain triglycerides [145], and supplementation with extra vitamins A, D, E and K [12, 51] were used. In order to relieve the handicapping pruritus, different pharmacological modalities and a few non-pharmacological alternatives have been tried with various rates of success, often only short-term.

### 2.4.1 Pharmacological treatment

Numerous treatment attempts have been reported, including substances that target the smooth muscle, such as papaverine, amyl nitrite, magnesium sulfate and atropine [55], and steroid derivatives [51-53, 146], mostly with little or no success. Antihistamines have shown no significant effects on cholestatic pruritus [147], this despite reports of increased serum levels of histamine in hepatobiliary disease in animals and humans [148, 149]. The dietary supplement S-adenosylmethionine has been used in patients with chronic intrahepatic cholestasis of different etiologies including intrahepatic cholestasis of pregnancy [150]. However, it did not ameliorate the pruritus or affect biochemical markers of cholestasis in four children with BRIC [151].

Bile acid binding resins such as cholestyramine, which interrupt the enterohepatic circulation of bile acids by reducing their uptake in the small intestine, have been used since the early 1960's in cholestatic liver disease [152-155]. Cholestyramine has also been useful in selected cases of PFIC [12, 62], but the use of the drug in children with cholestatic liver disease is limited. The unpalatability of cholestyramine and its gastrointestinal side-effects (diarrhea and constipation) often leads to poor compliance [156].

Phenobarbital, a potent inducer of hepatic enzymes, has several proposed mechanisms of action, including the induction of bile acid-independent bile flow and increased biliary secretion of possible pruritogens [20, 157-159]. Use of this drug has resulted in decreased pruritus and reduced levels of bile acids in children with cholestasis [160, 161], but the neurological side-effects (including drowsiness, behavioral changes and depression) have limited its usefulness substantially [156].

UDCA has been shown to decrease both biochemical cholestasis and cholestatic pruritus in several studies of pediatric patient groups, including a group of 27 children with PFIC, of whom 23 experienced relief from pruritus on UDCA 15 mg/kg/day [162]. Jacquemin *et al.* showed similar results in a non-controlled, non-randomized study of 39 children with PFIC. Significant changes in the degree of pruritus before and after treatment with UDCA in doses of 20-30 mg/kg/day were seen, accompanied by a complete biochemical resolution in up to 46% of the children [163]. It has been questioned whether the demonstrated reductions in biochemical markers of cholestasis imply a true effect on the disease progression. Narkewicz *et al*. described significant reductions in transaminases and pruritus, but no differences in dynamic liver function

tests (*e.g.* galactose elimination tests) in 13 patients with intrahepatic cholestasis (including 2 with PFIC) after UDCA 15-20 mg/kg per day for 12 months [164].

The main mechanisms of action of UDCA have been summarized as a) protecting injured cholangiocytes against cytotoxic hydrophobic bile acids, b) upregulating important transporting proteins such as BSEP and MDR3, thereby enhancing impaired hepatobiliary secretion, c) stimulating detoxification of hydrophobic bile acids, and d) protecting hepatocytes from apoptosis by inhibiting mitochondrial membrane permeability transition [165]. These suggested mechanisms of action have mainly been demonstrated in experimental animal studies (mainly rats or mice), whereas yet other experimental studies on knock-out mice have shown detrimental effects on the biliary epithelium after treatment with UDCA [166]. Recent *in vivo* studies by Marschall *et al.* on healthy human patients with gallstones have shown that UDCA acts through a number of mechanisms mainly involving protein upregulation, including the induction of expression BSEP and MDR3, and also of the alternative bile transporting protein MRP4, part of the salvage system during cholestasis, located in the basolateral membrane of the hepatocyte. Treatment with UDCA led to a marked increase in the serum concentrations of bile acids due to the increased UDCA concentration, but no significant changes of primary or secondary bile acids were found in gall bladder bile, compared to controls [167].

Studies of rifampicin in pediatric patients with cholestasis are scarce, and even fewer children with PFIC have been described. No significant biochemical changes or reported side-effects were seen in any of these studies; Cynamon *et al.* showed significant amelioration of cholestatic pruritus on rifampicin 10 mg/kg/d in 5 children with chronic cholestatic liver disease in a prospective, double-blinded, placebo-controlled, cross-over study [168]. Gregorio *et al.* observed a response in 3 out of 8 children with PFIC, and an overall response rate of 52% on pruritus on 5 mg/kg/day in the 33 retrospectively reviewed children with cholestasis [169]. Cancado *et al.* reported that 10 mg/kg/day yielded improvement in 3 children with low-GGT intrahepatic cholestasis (BRIC and PFIC) with severe pruritus, who had been unresponsive to all pharmacological treatments tested until then [170]. Yerushalmi *et al.* presented data on 24 children with chronic cholestasis treated with 10 mg/kg/day. The overall response rate was 90%, with a better response in children with biliary atresia than in children with intrahepatic cholestasis including 3 children with PFIC [171].

The mechanisms explaining the actions of rifampicin on cholestatic pruritus are complex. Miguet *et al.* demonstrated its enzyme-inducing effects in a small group of healthy adults in 1977 [172]. Galeazzi *et al.* studied fasting and postprandial serum bile acid levels before and after rifampicin, showed increased serum levels after rifampicin, and proposed that rifampicin prevented active uptake of bile acids into the hepatocyte [173]. Wietholz *et al.* studied urinary bile acid excretion patterns in healthy individuals before and after rifampicin, and found a shift in hepatocytic bile acid accumulation towards less cytotoxic and more hydrophilic bile acids. They suggested that this was caused by on of rifampicin on intestinal bacteria and on hepatic enzyme induction [174]. This was further supported by the same group, studying the expression of important hepatocytic transporters after rifampicin treatment in otherwise healthy

patients with gallstones [167]. The drug increased the conjugation and excretion of bilirubin by inducing expression of UGT1A1 and MRP2 [175]. It also enhanced bile acid detoxification, and *de-novo* synthesis of primary bile acids, by inducing the expression of and CYP3A4 and CYP7A1. In contrast to UDCA, there were no changes in expression of any of the bile acid or phospholipid transporters. The net effects in serum were unchanged bile acid concentrations, and lower levels of conjugated bilirubin. The biliary concentrations of primary bile acids (CA and CDCA) increased, whereas the secondary bile acids (DCA and LCA) decreased, which resulted in a shift of the total bile salt pool in favor of less hydrophobic, less toxic bile salts [167].

### 2.4.2 Alternative treatments

Plasmapheresis, used to remove pruritogens from the circulation in patients with cholestasis, has provided symptom relief for weeks to months in patients with PBC [176, 177]. Anecdotal reports showing its efficacy in intrahepatic cholestasis of pregnancy, including one pregnant woman with PFIC have been published [178, 179].

Molecular adsorbents recirculating system (MARS) is a liver support system that have been studied in patients with advanced liver disease as a bridge to liver transplantation [180]. Until now, there have been only a few reports on the effectiveness of this procedure to relieve the pruritus of BRIC and PFIC [181-183].

## 2.5 SURGICAL TREATMENT OF PFIC

### 2.5.1 Biliary diversion

#### *2.5.1.1 Biliary diversion in non-obstructive cholestasis*

Biliary drainage has been used in clinical settings for more than 75 years to treat patients with non-obstructive cholestatic liver disease [184, 185]. The underlying theory supporting the use of biliary drainage was presented by Foster *et al.* in 1919 [186]. He suggested that an interruption of the enterohepatic recirculation of bile would decrease the total bile acid pool, reduce the hepatic “pre-load” of bile acids, and thereby alleviate cholestasis. Since then, several authors have shown good response for up to two or three weeks in patients with non-obstructive cholestasis of various etiologies, including BRIC [187] using surgical methods [188, 189], nasobiliary drainage [187, 190], or both [191].

#### *2.5.1.2 The effects of biliary diversion in PFIC*

In 1988, Whitington *et al*. presented the first results on children with progressive familial intrahepatic cholestasis with decreasing serum levels of bile acids and relief from pruritus after partial diversion of the bile flow through an external stoma (**Figure 2**, page 18), *i.e.* partial external biliary diversion (PEBD) or cholecystojejunocutaneostomy. Two patients were investigated with scintigraphy after PEBD to study the fractions of bile diverted, and approximately 50% of the bile was diverted through the stoma during remission in both patients (one with AS and one with PFIC) [192]. The method was quickly adopted by centers worldwide as an alternative surgical treatment to orthotopic liver transplantation (OLT), to treat children with PFIC, and other

diseases of intrahepatic cholestasis, such as Alagille syndrome, and non-syndromic paucity of bile ducts [193-196] (**Table 3**).

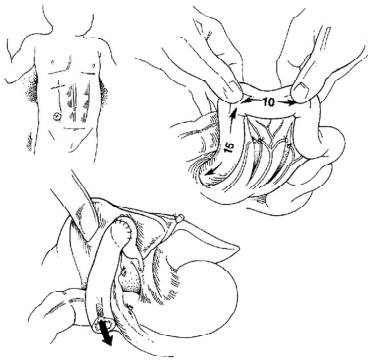


**Figure 2**. Partial external biliary diversion. Division of the proximal jejunum 15 cm distal to the ligament of Treitz permits the creation of a 10-cm jejunal conduit to divert gallbladder bile to the exterior. Reprinted from Emond *et al.,* J Ped Surg (1995) with permission from Elsevier.

2.5.1.2.1 Bile and bile acids after PEBD

To date, the exact mechanism by which biliary diversion decreases hepatocytic damage is still poorly understood, and the idea put forward 90 years ago by Foster *et al.* [186] suggesting that the operation reduced the hepatic "pre-load" of bile acids still prevails.

The first qualitative studies of bile acids in children with PFIC was presented by Linarelli *et al.* 1972, who demonstrated a defect secretion of conjugated bile acids into bile and high plasma levels of LCA in one boy presumably with PFIC type 1 [60]. Subsequent studies have consistently shown a pattern where the bile acid concentrations in biliary bile of patients with PFIC type 1 (or at least low-GGT PFIC) are low, and that CA is the predominant biliary bile acid [17, 94, 196-199], suggesting a selective defect in CDCA synthesis or secretion. This was also found by Kurbegov *et al.*, who added that the CDCA/CA-ratio had normalized in one patient with low-GGT PFIC five years after PEBD [199].

Emerick *et al.* presented results on biliary lipid analyses before and after PEBD in genetically characterized patients with Alagille syndrome (AS), and PFIC types 1 and 2. In 4 patients with PFIC type 1, there was a significant increase in phospholipid content after PEBD, but no changes in the CDCA/CA–ratio, and no measurable biliary CDCA after PEBD in 3 patients with PFIC type 2. The children responding to PEBD tended to show increased CDCA/CA-ratio postoperatively, compared to non-responders. The patients with AS showed normal concentrations of bile acids, cholesterol and phospholipids both pre- and postoperatively, in contrast to the patients with PFIC [196].

2.5.1.2.2 Growth after PEBD

Positive effects of PEBD on growth in PFIC were demonstrated in individual patients in a number of studies [15, 199-202], with suggested mechanisms of action including an improved nutrient absorption due to decreasing cholestasis, improved hepatic metabolism, and increased secretion of IGF-1. The first systematic review was presented by Melter *et al.,* who showed a substantial growth improvement one year after PEBD in all six patients studied [203].

2.5.1.2.3 Effects on the progression of histological changes

Although several outcome studies on PEBD in patients with PFIC have appeared since the first paper in 1988, so far, regression of histological abnormalities in the liver after PEBD has been reported in only a handful of cases [192, 199, 200], and no long-term outcome analyses of histological findings before and after PEBD in children with genetically characterized PFIC type 2 have been published so far (**Table 3**).

| **Investigators** | | **Patients** | | | | **Follow-up after diversion** | |
|---|---|---|---|---|---|---|---|
| First author | Ref | n | Category* | Age at operation | Duration | Biopsies | Result |
| Whitington | [192] | 4 | γ-GT | 3-9 yrs | 3-8 yrs | Pre-/postop | Clinical remission in all, histological improvement in 2. |
| Whitington | [15] | 14 | γ-GT | ns | ns | ns | Complete or partial clinical remission in 10. 3 to OLT. |
| Emond | [200] | 8 | γ-GT | 10.5 yrs (mean) | 2.6-5 yrs | Pre-/postop | Complete or partial clinical remission in 6. 2 to OLT. |
| Rebhandl, Felberberbauer | [204-5] | 1 | | 13 yrs | 2.5 yrs | Pre-/postop | Appendicostomy. Clinical improvement, chronic cholestasis. |
| Ng | [193] | 4 | γ-GT | 2-10 yrs | 1-7 yrs | Preop | Clinical remission in all. |
| Melter | [203] | 6 | γ-GT | 20 mo-12.5 yrs | 2 yrs | Preop | Clinical remission in all, accelerated growth. |
| Kalicinski | [202] | 20 | ns | 9 mo-11 yrs | 1-4 yrs | Preop | Clinical remission in 15, partial remission in 3. |
| Kurbegov | [199] | 3 | γ-GT | 1-5.5 yrs | 1-5 yrs | Pre-/postop | Improved histologies. |
| Wachters-Hagedoorn | [206] | 2 | *BSEP* | 4 - 8 yrs | 2 yrs | Preop | Clinical improvement, cholestatic episodes. |
| Metzelder | [207] | 4 | γ-GT | 0.5-3 years | 1.5-2.5 mo | Preop | Laparoscopy. Clinially and biochemically reduced cholestasis. |
| Mattei | [195] | 1 | ns | 11 mo | 9 mo | ns | Clinical remission. |
| Englert | [208] | 13<br>4 | *BSEP*<br>*MDR3* | ns | ns | Preop | OLT performed in 10, and 3 waiting for OLT. |
| Bustorff-Silva | [209] | 2 | γ-GT | 15-17 yrs | ns | Preop | Partial internal biliary diversion. Clinical remission. |
| Ekinci | [210] | 2 | γ-GT | 4-7 yrs | 7-13 mo | Preop | Clinical improvement in both. |
| Emerick | [196] | 4<br>3 | *FIC1*<br>*BSEP* | 0.5-7.3 yrs<br>0.4-4.5 yrs | ns | Preop | Clinical remission in 3.<br>Clinical remission in 1. |
| Yang | [211] | 11 | *BSEP*** | 5.3 yrs (mean) | 2.0-5.7 yrs | Preop | Clinical relief in 75%, chronic pruritus in 30%. |

* ns = not stated, γ-GT = patients with normal serum concentrations of γ-glutamyltranferase, *FIC1* = patients with mutations in *ATP8B1* , *BSEP* = patients with mutations in *ABCB11*, *MDR3* = patients with mutations in *ABCB4*

** all 11 showed low-GGT, and 8 of 11 patients had mutations in *ABCB11 (* BSEP*)*

**Table 3**: Overview of outcome studies in patients with PFIC after biliary diversion.

*2.5.1.3 Alternative surgical methods*

Rebhandl *et al.* and Gauderer *et al.* presented results in two children who underwent successful PEBD **using the appendix** instead of a part of jejunum (*i.e.* cholecysto-appendicostomy). Both children, one with PFIC type 2 and the other with Alagille syndrome, were well at follow-up, 2 and 3 years after PEBD [204, 212].

Successful **laparoscopic PEBD** was first described by Metzelder *et al.*, who used the method in 4 patients with low-GGT PFIC, operated at 0.5-3 years of age, without immediate or early problems at follow-up 2.5-3 months later [207].

**Ileal exclusion** (or ileal bypass, IB) was described by Whitington *et al.* [15] in two children who had undergone cholecystectomy prior to PEBD, and thus were not suitable for PEBD [15]. Instead, they were operated excluding the last 15% (10–100 cm) of the small intestine, to circumvent the active ileal uptake of bile in the distal parts the small intestine, and thereby reducing the enterohepatic recirculation of bile acids. The children experienced relief from pruritus, but instead suffered from disabling choleretic diarrhea. Subsequently, Hollands *et al.* described successful IB in five children (15 months – 17 years of age) [213]. One of the patients suffered postoperative bleeding from the anastomosis, necessitating re-operation. At follow-up 6-22 months after IB, four of the five patients experienced clinical relief, and one of them had a liver biopsy taken six months after surgery which showed significant improvement. None of them suffered from diarrhea. Ismail *et al.* presented data from 5 patients (9 months–11 years; mean 6.5 years) who underwent IB, including one child who had already

undergone PEBD, but was converted to IB due to large losses of bile through the stoma. Three of the initial 4 responders relapsed within a year after surgery, and one of them was successfully converted to PEBD [201].

Bustorff-Silva *et al.* recently described a third surgical method of diverting the bile, using a segment of the jejunum, partially diverting the bile directly into the ascending colon, thereby avoiding a cutaneous stoma, *i.e.* **partial internal biliary diversion** (PIBD). Two patients underwent PIBD, one boy 17 years of age with a slowly progressing PFIC, and one girl 15 years of age with a PFIC, who prior to this surgery had already been operated with PEBD at 2 years of age. At short-term follow-up, both patients were clinically well and had no pruritus [209].

### 2.5.2 Liver transplantation

The only other surgical alternative for patients with PFIC is orthotopic liver transplantation (OLT). Although graft survival rates have improved tremendously since the first liver transplantation in a child almost 50 years ago [214], OLT in children is still associated with substantial morbidity and mortality [200, 201, 215-218].

There have been several reports of follow-up after OLT in patients with Byler's disease or PFIC type 1, stating that these children continue to suffer from extrahepatic manifestations such as diarrhea [216, 219], and that they develop hepatic steatosis after OLT [219, 220], with a high risk of progressing into steatohepatitis and cirrhosis as recently reported in a number of patients [221].

The results of OLT in patients with proven PFIC type 2 have been more reassuring; outcome is as good as in other common pediatric diseases requiring OLT. However, only a limited number of patients have been reported so far. Two groups recently reported post-transplantation *de-novo* development of anti-BSEP antibodies in patients with PFIC type 2. Keitel *et al.* described the development of anti-BSEP antibodies after OLT leading to graft failure and necessitating retransplantation, in a patient with PFIC type 2, operated with PEBD prior to transplantation [222]. Jara *et al.* described similar findings in three children with PFIC type 2 [223], who suffered sudden episodes of cholestasis up to 12 years after OLT. Anti-BSEP antibodies were detected in all three, and were shown to act directly on the hepatobiliary canalicular membrane in tested rat liver. The authors propose that children undergoing liver transplantation for this reason should be checked for circulating anti-BSEP antibodies.

The results of living-related liver transplantation (LRLT) in a number of patients from Japan [224], and Saudi Arabia [225] have been published. In a study where the outcome after LRLT in seven cases of PFIC was compared with seven parent-child pairs with biliary atresia undergoing LRLT, the authors concluded that LRLT was safe even when the donor presumably is heterozygous for a PFIC-gene mutation [226].

# 3 AIMS OF THE THESIS

The general purpose of this thesis was to study the group of Swedish patients with progressive familial intrahepatic cholestasis with focus on clinical, biochemical, genetic and histopathological aspects of the disease, in order to learn how surgical diversion of bile affected the outcome in the patients.

To this end, we aimed to answer the following questions:

Genetics and immunohistochemistry

- Is PFIC a genetically homogenous disease? (**Paper I**)
- Which are the genetic mutations in the Swedish patients? (**Papers II-III**)
- Is there a consistent genotype-phenotype correlation with regard to disease presentation, effects of treatment and long-term outcome? (**Paper III**)
- Do the results of BSEP staining correspond to that of genetic mutation analyses? (**Paper III**)

Clinical and biochemical outcome

- What are the short- and long-term effects of PEBD? (**Papers II-III**)
- What proportions of bile flow are found in the stomal bag, urine and stools after PEBD? (**Paper IV**)
- Are there any significant differences in the relative distribution of bile flow between these compartments in remission compared to periods of cholestasis? (**Paper IV**)

Histopathological outcome

- Can PEBD stop or even reverse the development of fibrosis in PFIC? (**Paper III**)
- How do recurrent cholestatic episodes after PEBD affect the development of fibrosis? (**Paper III**)

# 4 PATIENTS AND METHODS

## 4.1 SETTING AND SAMPLES

The generally accepted criteria for progressive familial intrahepatic cholestasis include early onset (typically before 6 months of age) clinical signs of chronic cholestasis with jaundice and an unremitting cholestatic pruritus, and biochemical signs including conjugated hyperbilirubinemia with increased fasting serum levels of bile acids, normal serum levels of GGT, mildly to moderately increased serum levels of transaminases. Finally, other causes of early-onset chronic cholestasis, including bile acid synthesis defects, must be ruled out [13, 15].

For more than 25 years, children with hepatobiliary diseases from most parts of Sweden have been referred to the tertiary center for pediatric hepatology at Karolinska University Hospital, Huddinge. Among these, a total of 18 children (11 girls and 7 boys), fulfilled the disease criteria, and 16 were included in one or more of the four studies of this thesis. Two children were excluded; one child was referred to us at 12 years of age due to her unbearable cholestatic pruritus, and one child did not undergo PEBD until after the completion of the studies (**Table 4**, page 25).

In **Paper I**, a total of 11 children with PFIC (7 girls, 4 boys) from nine Swedish families were included. In **Papers II** and **III**, we described the initial presentation, and the short- and long-term biochemical and histological results after partial external biliary diversion (PEBD) in the 13 patients (7 girls, 6 boys) who had undergone PEBD at the time of the study. In **Paper IV**, the distribution of bile during cholestatic and non-cholestatic conditions in nine (5 girls, 4 boys) of the operated children were presented.

## 4.2 METHODS

### 4.2.1 Genetic analyses

For the linkage analysis in **Paper I**, pedigrees from nine Swedish non-consanguineous, two-generation families were constructed. They were all consistent with autosomally recessive pattern of disease inheritance, an assumption used for data input into the computerized linkage programs. The geographic distribution of the patients made us suspect that several families could have a common ancestry. In seven of the families, one (n=1) or both (n=6) parents originated from a geographically limited area along the Northern coast of the Baltic sea.

Venous blood samples for DNA extraction were collected from all but one of the affected children, their parents, and their unaffected siblings (total n=37), to yield as much information as possible for the linkage analysis. From one child (patient P, **table 4**, page 25) who had died due to an EBV-associated acute liver failure, there was no blood available for genotyping. DNA extraction from leukocytes was manually performed using standard methods [227]. PCR-amplification of radioisotope-labeled highly polymorphic markers (*i.e.* dinucleotide repeat microsatellite markers), evenly

distributed along the coding sequence of the genome [228], was performed. Markers flanking both sides of the region of interest, *i.e.* the region already genetically mapped to the *FIC1*-locus on 18q21-22 [16, 70] were used. The PCR fragments obtained were separated according to length (*i.e.,* number of dinucleotide repeats) on denaturing polyacrylamide gels using electrophoresis. They were then visualized as dark bands (or dots) on autoradiography after overnight exposure on film. Haplotypes encompassing the region of interest for each individual were constructed on the basis of these results (**Paper I**, Figure 1). These data were used for the computerized analyses, which included two-point and multipoint analyses, using the LINUX-adopted software developed based on calculations by Lathrop *et al.* [229, 230]. The laboratory and computer work including microsatellite typing, physical YAC mapping, cDNA library screening, designing of suitable primers from rat cDNA, northern blot analyses and mutation detection necessary for cloning of *ABCB11* and characterizing the first mutations [93] are briefly described in Background (page 11).

The genetic mutation analyses of the disease-causing gene *ABCB11* presented in **Papers II** and **III** were performed using previously published exon flanking primer pairs [107], except for the primer pairs flanking exons 9 and 14, which we redesigned using computer software on the basis of human genomic sequence EMBL: AF190696. We used public databases containing known genetic polymorphisms to distinguish possible disease-causing mutations from single nucleotide polymorphisms. A potential deletion in exon 8 of *ABCB11* in one of the patients was characterized by constructing plasmids using a commercially available vector system into which the deletion-bearing exon 8 was cloned. Thereafter the plasmids were transformed into cell cultures, from which DNA was prepared and sequences obtained using plasmid-specific primers. In children where no mutations in *ABCB11* were identified, *ATP8B1*, the disease-causing gene in PFIC type 1 was sequenced by JN Painter, Helsinki, Finland (**Paper III**).

### 4.2.2 BSEP immunohistochemistry

Immunohistochemical staining using anti-BSEP antibodies as previously described [110], with antibodies against MRP2 and MDR3 as controls, was performed and judged by AS Kinsley, London, UK, without knowledge of clinical outcome or genetic status. The findings on biopsy sections from the 18 children, including the 13 children who underwent PEBD, were scored as absent or near-absent; weak; and near-normal or normal (**Table 4**, page 25 and **Paper III**, Table 1).

### 4.2.3 Clinical data

Clinical data, including description of symptoms, changes in bile output and growth were documented in each child's individual patient charts, together with results from routine biochemistry and radiographic results. Hospital standard laboratory analyses were used for biochemical liver function tests, *i.e.* serum concentrations of conjugated and total bilirubin, transaminases (AST and ALT), GGT, albumin, and measurement of the prothrombin time (expressed as INR and/or PT%). Standard methods were also used for plasma concentration analyses of fat-soluble vitamins, cholesterol and triglycerides. Fasting serum analyses of bile acid concentrations were performed using colorimetric methods measuring 3-OH bile acids, as outlined by Talalay [231]. Urine

was investigated to rule out bile acid synthesis defects; to this end, bile acids were extracted, concentrated and analyzed using electrospray ionization or fast atom bombardment mass spectrometry as described elsewhere [136, 232]. To assess possible improved growth after PEBD (**Paper II**), Δ–standard deviation scores (Δ-SDS or z-scores) in each patient were calculated, *i.e.* the change in SDS for weight and height at PEBD and at follow-up. The Swedish standard growth charts were used for mean values and standard deviations [233].

### 4.2.4 Hepatobiliary scintigraphy after PEBD

For the scintigraphic examinations (**Paper IV**) we used radioactively labeled tracer mebrofenin (99Tc-mbf) and the quantified the tracer with a gamma camera (Millennium$^{TM}$). The examinations were performed after an overnight fast in children who had been instructed to discharge bile from their draining bags, and (when possible) to void urine, and pass stools. After this, 2.8 MBq/kg body weight of 99Tc-mbf was administered intravenously. During the following 24 hours, the stomal bile excreted into the draining bag, and all urine and feces were collected and transferred into different standardized specimen cans, which were analyzed using the gamma camera. A whole body scan was made using the same gamma camera. The proportions of activity in the different compartments could be calculated, and related to the activity administered to the patient.

### 4.2.5 Histopathological scoring

Hospital standard staining methods of liver biopsy material included hematoxylin and eosin, Sirius red, periodic acid-Schiff staining with and without diastase pre-digestion, iron stain, and immunohistochemical staining applying cytokeratin 19 (CK19). For the analysis of histological changes at presentation and at follow-up after PEBD (**Papers II** and **III**), we analyzed biopsy specimens taken from patients at initial investigation, if available (n=17 patients), at PEBD (n=13), and at one year (n=12), at three years (n=10), at five years (n=11) and at > 10 years after PEBD (n=8). The assessments were performed by two investigators, blinded for patient identity and for clinical and biochemical status of the patient. Seven histological features were scored: inflammation, fibrosis, hepatocyte giant cell transformation, steatosis, cholestasis, ductular reaction and paucity of bile ducts. The Batts and Ludwig scale [234] was used for scoring fibrosis and inflammation (for more details on the scoring see **Paper III**).

## 4.3 STATISTICAL METHODS

The two-point and multipoint linkage analyses in **Paper I** were integrated in the computer programs used, as described elsewhere [229, 230]. Paired Student's *t*-test was used to analyze parametric data under the assumption of normal distribution, including the levels of biochemical markers before and after PEBD in **Paper II**. For the analysis of non-parametric data in **Papers III** and **IV**, including the comparisons of degrees of cholestasis and stages of fibrosis before and after PEBD, correlating the effects of the total duration of cholestatic episodes after PEBD on the progression of fibrosis, and comparing two different groups of scintigraphic examinations, the Wilcoxon signed rank test, the Spearman's rank correlation and the Mann-Whitney *U*-test were used.

LOD-scores above 3.0 and below -2.0 (**Paper I**), and p-values below 0.05 (**Papers II-IV**) were considered significant. The STATISTICA package (Stata Soft, Inc. Tulsa, OK, USA) was used for creating box plots and pie charts in **Papers II-IV**.

## 4.4 ETHICAL CONSIDERATIONS

The studies included in this thesis were approved by the local ethics committee. Ethical permit numbers were: dnr 71/01 (**Papers II** and **III**), 464/03 (**Papers II** and **III**) and 565/03 (**Paper IV**).

| | At presentation and inclusion in studies | | | | | Age at surgery | | Fibrosis stage [1] | | | Most recent follow-up | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | Paper | Sex[2] | Age | ABCB11[3] | BSEP[4] | PEBD | OLT | At PEBD | > 5 yrs | > 10 yrs | Age | Duration |
| A | I-IV | M | 6 mo | a/a | neg | 154 mo | - | 2 | 0 | 0 | 25 yrs | 12 yrs |
| B | I-IV | M | 3 mo | a/a | weak | 121 mo | - | 3 | 0 | 0 | 20 yrs | 10 yrs |
| C[5] | I-III | F | 1 mo | a/a | weak | 38 mo | - | 3 | 1 | 1 | 14 yrs | 10 yrs |
| D | I-IV | F | 1 mo | a/a | neg | 35 mo | - | 3 | 2 | 2 | 14 yrs | 11 yrs |
| E | I-IV | F | 3 mo | a/a | weak | 18 mo | - | 3 | 1 | 0 | 13 yrs | 12 yrs |
| F | I-IV | F | 6 mo | a/a | weak | 13 mo | - | 3 | 0 | 0 | 12 yrs | 11 yrs |
| G | I-IV | F | 1 mo | a/a | weak | 16 mo | - | 2 | 0 | 0 | 12 yrs | 11 yrs |
| H | II-IV | F | 6 mo | a/b | neg | 19 mo | - | 2 | 1 | 2 | 12 yrs | 10 yrs |
| I | II-IV | M | 6 mo | n/n | pos | 11 mo | - | 1 | 0 | - | 7 yrs | 6 yrs |
| J | II-IV | M | 5 mo | a/a | weak | 13 mo | - | 3 | 2 | - | 6 yrs | 5 yrs |
| K | II-III | M | 1 mo | n/n | pos | 6 mo | - | 2 | 3 | - | 6 yrs | 5 yrs |
| L[6] | I-III | M | 1 mo | a/a | neg | 11 mo | 25 mo | 3 | - | - | 2 yrs | 1 yr |
| M[7] | II-III | F | 5 mo | a/c | neg | 9 mo | 11 mo | 4 | - | - | - | - |
| N[8] | - | F | 12 yrs[8] | d/d | weak | - | - | - | - | - | | - |
| O | I | M | 2 mo | a/e | neg | - | 45 mo | - | - | - | - | - |
| P[9] | I | F | 1 mo | - | neg | - | - | - | - | - | - | - |
| Q | I | F | 1 mo | n/n | neg | - | 34 mo | - | - | - | - | - |
| R[10] | - | F | 2 mo | c/n | neg | 22 mo[10] | - | - | - | - | - | - |

[1] Stage of fibrosis determined according to Batts and Ludwig [233]; 0: no fibrosis, 1: portal fibrosis, 2: periportal fibrosis, 3: bridging fibrosis, 4: cirrhosis

[2] Sex: M indicates male; F female

[3] *ABCB11*-genotype: a-e indicate different mutations; *i.e.* a/a indicates homozygosity for mutation a; a: c.890A>G, b: c.3268C>T, c: c.3382C>T, d: c.1708G>A, e: c.541-546delAAAATC, n: no mutations found, -: not genotyped

[4] BSEP: immunohistochemical findings using BSEP antibodies: neg: absent or near-absent BSEP expression; weak: weak BSEP expression; pos: near-normal or normal BSEP expression

[5] Underwent a total external biliary diversion at 8½ years of age

[6] Died 14 months after PEBD, 1 week after orthotopic liver transplantation (OLT) due to hepatocellular carcinoma

[7] Underwent living related liver transplantation 2 months after PEBD

[8] Presented to us at 12 years of age; jaundice presented before 6 months of age

[9] Not genotyped. It may be presumed that she carries he same mutation (a/a) as her sibling (L).

[10] Recently underwent PEBD; after the finalization of Papers II and III

**Table 4**. Characteristics of the 18 patients included in the thesis, including the paper(s) in which they appear. Age at presentation, genotype, BSEP staining and stages of fibrosis at PEBD and follow-up.

# 5 RESULTS

## 5.1 EXCLUSION OF GENETIC LINKAGE TO CHROMOSOME 18

In **Paper I** we could demonstrate that the disease and the markers did not co-segregate and thus excluded linkage in three of the families (**Paper I**, Figure 1). In addition to this, the affected children of two closely related families did not share the same haplotypes, as would have been expected if this chromosomal region in fact harbored the disease causing gene. Linkage to the region in the remaining families could not be excluded, as the affected and unaffected siblings did not share the same haplotypes in the region. When all the allele information of the four microsatellite markers D18S69, D18S64, D18S55 and D18S68 tested in all members of the eight families with > one child was added together, the multipoint linkage analysis showed a maximum LOD-score (Logarithm of odds) of -5.8, thus providing a statistically significant evidence against linkage to this chromosomal region (**Paper I**, Figure 2).

## 5.2 SPECTRUM OF MUTATIONS IN THE PATIENTS

When sequencing the 28 exons of *ABCB11* on available genomic DNA from 17 children (all except for patient P, **Table 4**, page 25), we found probable disease causing alterations (mutations or deletions) in 14 of them, on 27 of 34 chromosomes. The most common finding was the missense mutation c.890A>G (corresponding to p.E297G) on exon 9, as described in most of the children studied in **Paper I** (see Strautnieks *et al.* [93], Table 1). Nine of the children carried the mutation in homozygous form, whereas 3 children were heterozygous carriers for c890A>G. Of these 3, one boy demonstrated a microdeletion on his other allele, inherited from his healthy father, encompassing six nucleotides in exon 3 (c.541-546delAAAATC), with a corresponding loss of two amino acid-residues (p.K208-I209del). The two other compound heterozygous children carried previously documented mutations in exon 25 [94, 95] on their other alleles: one nonsense mutation, c.3268C>T (corresponding to p.R1090X), and one missense mutation 3382C>T (corresponding to p.R1128C). The same mutation was found in another patient, where no other changes were found on the other allele. One girl was homozygous for the missense mutation c.1708G>A at exon 15 (corresponding to p.A570T). This was previously described in a patient with milder, non-fibrosing "BRIC-like" disease [107]. Three children had no demonstrable mutations in coding sequence of either *ABCB11* or *ATP8B1*.

## 5.3 CLINICAL AND BIOCHEMICAL ASPECTS

### 5.3.1 Initial features

All 17 children who were followed from young age presented one or several symptoms of cholestasis during the first six months of life. Eleven of these (65%) presented with variable signs of vitamin K-dependent coagulopathy, some of them as early as at 4-5 weeks of age. The most common signs noted were a tendency to easy bruising and recurrent nosebleeds (epistaxis). Other manifestations of coagulopathy included bleedings in the anterior chamber of the eye (hyphema), of umbilicus, and one case of pulmonary hemorrhage with hemoptysis. One child presented at 2.5 months with a

life-threatening intracerebral hemorrhage, necessitating urgent neurosurgery and pediatric intensive care. One boy presented with hypocalcemic seizures with low serum levels of vitamin D, yet another feature of fat-soluble vitamin deficiency. No patients demonstrated any overt signs of neuromuscular abnormalities of the type reported in conjunction with severe vitamin E deficiency. Pruritus from 5-12 months was a uniform finding in all children, and one child showed signs of pruritus already at three months of age. None of the features associated with PFIC type 1 such as diarrhea, cholelithiasis, pancreatitis, nephrolithiasis or hearing loss were seen at presentation.

### 5.3.2 Clinical follow-up after PEBD

Partial external biliary diversion (PEBD; **Figure 2**, page 18) was performed in 13 patients at 6 to 157 months of age (mean, 36; median, 16), as described in **Paper II**. At surgery, all children suffered from severe pruritus, and showed signs of chronic compensated cholestatic liver disease, with increased serum levels of bile acids and conjugated hyperbilirubinemia. The surgery was free from immediate adverse events in all patients. They were discharged after 7-13 days. Seven children were apruritic one month after PEBD. Five children were readmitted due to sudden episodes of dehydration and electrolyte disturbances caused by increasing stomal losses of bile at 2-6 weeks after surgery. One patient experienced repeated episodes of stomal prolapse during the first weeks, requiring surgery. She eventually developed end-stage liver disease, and underwent living related liver transplantation (LRLT) 2 months after PEBD with no serious adverse events (**Figure 3**, page 28).

At follow-up 11-21 (median 14) months after PEBD, 11 of the 12 children showed partial or complete response to the surgery, including improved growth. Except for the child who underwent LRLT, no serious adverse events had been recorded, such as bleedings, cholangitis, gallstones or pancreatitis. The children showed significantly decreased serum markers of cholestasis (**Paper II**, Figures 2 a-f). One of the operated children with poor growth and continued cholestasis after PEBD, demonstrated high serum levels of alpha-fetoprotein. Ultrasound revealed a suspected liver malignancy, and a hepatocellular carcinoma was diagnosed on liver biopsy one year after PEBD. He died two months later.

All children experienced longer or shorter cholestatic episodes with pruritus and increased serum levels of bile acids. Six children had had their first post-operative cholestatic episode or suffered from chronic cholestasis during the first year after PEBD. Two patients remained free from cholestatic episodes for 4 years or more after PEBD before they had their first recurrence of cholestasis (**Paper III**, Figure 1). These episodes were often preceded by an upper respiratory tract infection with fever. In most cases, there was a concomitant drastic decrease in the volumes of bile discharged into the stomal bag. Pharmacological treatment during these episodes aimed at relieving the cholestatic pruritus and preventing possible effects of decreased absorption of fat and fat-soluble vitamins. First-line treatment consisted of UDCA in doses up to 100 mg/kg/day. In most cases, this had only limited effect on the pruritus, and rifampicin was added in doses of 10 mg/kg/day with variable effect. One boy with chronic

cholestatic pruritus was repeatedly treated with MARS, which usually provided good relief lasting for one or two weeks.

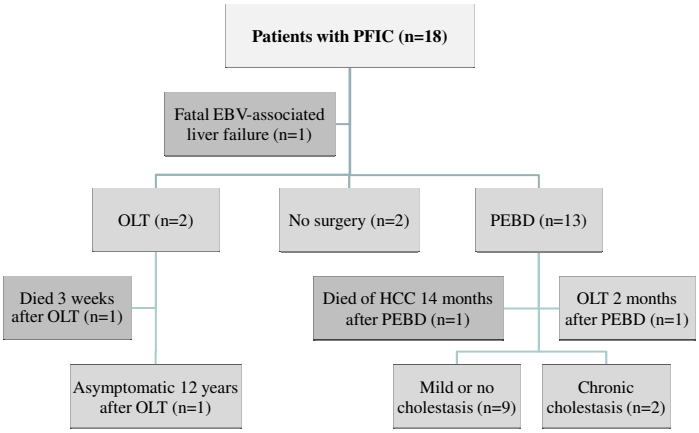


**Figure 3**. Clinical outcome in 18 patients with progressive familial intrahepatic cholestasis (PFIC) of whom 13 have undergone partial external biliary diversion (PEBD).

### 5.3.3 Growth and pubertal development after PEBD

Preoperatively, nine of 13 patients showed evident features of failure to thrive, defined as a standard deviation score (SDS) below - 2 standard deviations for length and/or weight. At follow-up 11-21 months after PEBD, we found substantial growth increment (mean change in SDS for weight was 1.5, and mean change in SDS for length was 0.84) in all but one of the patients (**Paper II**, Figures 3 a-b). At long-term follow-up 5 to 12 years after PEBD, a majority of the children showed normal growth. Two children demonstrated suboptimal growth after the initial catch-up: both suffered chronic cholestasis. One of them was diagnosed with Crohn's disease, and had delayed pubertal development (**Paper III**). To date, 7 out of 8 patients who underwent PEBD and have reached the age of expected puberty have shown normal pubertal development.

## 5.4 HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL ASPECTS

Common hepatic histological features in the infants investigated at presentation (n=17) included disturbed lobular architecture with swollen hepatocytes and mild to moderate hepatocellular and/or canalicular cholestasis. Mild to moderate (stage 1-2) fibrosis was present in 10 of 17 biopsy specimens, whereas severe fibrosis (stage 3) was found in one patient. This girl demonstrated established cirrhosis in the perioperative biopsies, and shortly thereafter developed end-stage liver disease, requiring liver transplantation (**Paper II**). On immunohistochemical assessment, the CK19 stained biopsy specimens that were informative enough to be assessed (n=11) demonstrated mild to moderate ductular reactions in 9 cases, while paucity of bile ducts was found in 3. BSEP staining

showed absent or near-absent BSEP in 9 cases, weak in 7 and near-normal or normal in 2 of the specimens (**Table 4**, page 25).

At follow-up after PEBD, there was a statistically significant decrease in histologic cholestasis after 1 and 3 years, and in fibrosis after 5 and > 10 years, compared to the histological findings at PEBD (**Paper III**, Figures 2 a-b). We found a significant, positive correlation between the stage of fibrosis at most recent follow-up, and the total duration of the cholestatic episodes in the children after PEBD. In other words, frequent and long cholestatic episodes after PEBD correlated positively to increased hepatic fibrosis (**Paper III**, Figure 3).

### 5.4.1 The genotype and histological outcome after PEBD

Nine of 13 children who underwent PEBD were homozygous for c.890A>G, *i.e.* the most common mutation. Liver specimens acquired at long-term follow-up demonstrated mild or no fibrosis in six children, and moderate fibrosis in two. The ninth child homozygous for c.890A>G died of HCC 14 months after PEBD. The outcome in the other four children with no mutations found in either *ABCB11* or *ATP8B1* (n=2), or with other mutations of *ABCB11* (n=2) was more variable; one girl underwent LRLT early after PEBD, two children suffered chronic cholestasis with moderate to severe fibrosis, and one boy showed no fibrosis at long-term follow-up (**Figure 4**).

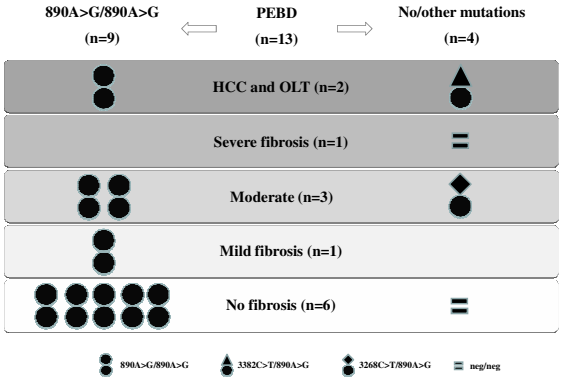


**Figure 4**. Overview of the genetic mutations and histologic fibrosis in 13 patients with PFIC operated with PEBD. At long-term follow-up after PEBD, 6 of 9 patients homozygous for c.890A>G showed mild or no fibrosis.

## 5.5 SCINTIGRAPHIC FOLLOW-UP AFTER PEBD

We analyzed a total of 13 examinations of 9 patients studied at 13 (range 5-24) years of age, 10 (range 4-14) years after PEBD. Of these examinations, 8 were performed during non-cholestatic remission (n=7 patients), and 5 during cholestasis (n=3). The patients studied during remission discharged a significantly larger fraction of isotope through the stoma (median 90% vs. 22%), and a significantly lower fraction in the urine (median 2.5% vs. 15%), compared to the patients studied during cholestasis (**Paper IV**, Figure 2). More isotopic activity appeared to be retained in the body and not excreted during cholestasis than in remission (median 54% vs. 6.5%), although this difference did not reach statistical significance.

### 5.6 OUTCOME IN NON-DIVERTED CHILDREN

Two of the five regularly followed children who did not undergo PEBD underwent OLT; one of them is healthy and free of symptoms almost 17 years after OLT, and one of them died of multiorgan failure 3 weeks after transplant surgery. Both demonstrated established liver cirrhosis in their biopsy specimens from the explanted liver. One child died of a fulminant Epstein-Barr virus (EBV) infection at 21 months of age, with established cirrhosis found in post-mortem examinations of the liver.
A fourth child, who did not undergo PEBD for non-medical reasons, presented to us at 12 years of age with pruritus, biochemical cholestasis and a stage 3 liver fibrosis. At follow-up 8 years later, she still suffers daily pruritus, and the liver histology is unchanged. Recently, after the finalization of **Papers II** and **III**, the fifth so far non-diverted child underwent PEBD with no immediate complications. The follow-up time is still too short to draw any conclusions on the outcome.

# 6 GENERAL DISCUSSION AND CONCLUSIONS

## 6.1 METHODOLOGICAL CONSIDERATIONS

The methods used in 1997 for genetic linkage analysis and in 2007 for sequencing of 28 exons of genomic DNA from 18 patients with possible mutations in *ABCB11* differ substantially. In 1997, most of the work was done manually, *i.e.* DNA-extraction from blood, preparation of PCR reactions with radioactive nucleotide labeling, preparation of polyacrylamide gels, setting up and running the gel electrophoresis, drying the gels, overnight exposure on photographic films, preparation of chemicals for developing the film, and finally reading the films and preparing the input data files. Since then, most of these manual procedures have been abandoned in favor of large-scale fully-automated and non-radioactive methods. Most of the work described in **Paper I** would nowadays have been done at an academic core facility, or by a specialized commercial center. Nevertheless, the underlying theories used to calculate the genetic probabilities are the same.

Most results in **Papers II** and **III** are based on thoroughly documented findings from routine clinical and biochemical follow-up investigations, including the routine staining of biopsy specimens. The BSEP immunostaining presented in **Paper III** is not yet a standard method; these stainings were performed on biopsy specimens prepared at the Department of Pathology, Karolinska University Hospital, Huddinge, and then sent for immunostaining to the Institute of Liver Studies, King's College Hospital in London, UK (Alex Knisely). The scintigraphic examinations presented in **Paper IV** were all performed at the Department of Radiology, Karolinska University Hospital, Solna. To our knowledge, scintigraphic methods have never been deployed more than occasionally in children operated with PEBD [192, 235]. However, the method *per se* is based on well known and reliable theory, and the technique is widely used in the pediatric population [236].

## 6.2 LIMITATIONS

As in many clinical studies in general, and in pediatric studies of uncommon diseases in particular, there are obvious methodological limitations related to the small numbers of patients. Since the children included in this study live all over Sweden, it has not been possible for practical reasons to perform investigations such as scintigraphy at the most appropriate moments, for instance at the peak of a cholestatic episode. The lack of a control group in the studies of treatment outcome such as PEBD is another evident limitation. This is at least in part due to the low number of available patients.

## 6.3 STRENGTHS

The total number of patients should still be considered high, especially when compared to previously published outcome studies (**Table 3**, page 19). In addition to this, the patients have been thoroughly characterized genetically and immunohistochemically, and systematically followed during a long period of time, before and after various

therapeutic efforts, *e.g.* PEBD, OLT, MARS, and different pharmacological treatment attempts.

### 6.4 FINDINGS, IMPLICATIONS AND CONCLUSIONS

Progressive familial intrahepatic cholestasis is not a genetically homogenous disease. The exclusion of linkage to 18q21-22 in the group of Swedish patients, and in two other groups of patients [17, 89] all proved locus heterogeneity. Following the identification of a gene encoding the main bile salt export pump [93], we found disease-causing *ABCB11* mutations in > 80% of the Swedish patients genotyped. Apart from the most common mutation c.890A>G, a few previously published missense and nonsense mutations (**Table 4**, page 25), and a number of single nucleotide polymorphisms (SNPs), we also characterized a heterozygous small deletion of 6 nucleotides in one patient. This microdeletion could probably explain this patients' rapid disease progression which led to early OLT. This dramatic clinical course contrasts sharply to the slow clinical course in his second cousin, homozygous for c.890A>G. The latter represents the opposite end of the phenotypic spectrum in PFIC type 2, with unremitting cholestatic pruritus and biochemical cholestasis, but with slowly progressing disease. Three of the investigated children (**Table 4**; patients A, B and N) showed this slow disease progression, two of which are homozygous for c.890A>G, and operated at 10 and 13 years of age, respectively. The third patient has still not undergone operation. All of them carry homozygous *ABCB11* mutations previously reported also in occasional patients with BRIC [107], and they demonstrate the broad phenotypic continuum in cholestatic disease caused by mutations in *ABCB11*.

When we compared the genotype to the effects of PEBD on long-term outcome, we found an overall favorable response to PEBD in patients with c.890A>G, and a more variable response in patients with other or no mutations in *ABCB11*, although these differences were not significant. In contrast to this, we found no consistent genotype-phenotype correlations that could explain differences in disease presentation (*e.g.* age at presenting symptoms or signs of early coagulopathy), or effect on pharmacological treatment of cholestatic pruritus. Others have also previously attempted to find consistent genotype-phenotype correlations in PFIC, but with limited success. This could be due to a number of possibly disease modifying factors, including intrinsic post-transcriptional or post-translational modifications (*e.g.* DNA methylation, mRNA splicing, or protein structural changes), or exogenous modulators (*e.g.* viruses or drugs). The small number of patients studied may also lead to lack of power to detect existing genotype-phenotype correlations.

Not all children with PFIC type 2-like disease demonstrated mutations in the coding sequence of *ABCB11*. In one child with rapidly progressing PFIC disease, there was total absence of BSEP, and yet we found no mutations in *ABCB11* or *ATP8B1*. This could imply that a third genetic locus for low-GGT PFIC is still to be found, or alternatively, that we should look for genetic variations in *non-coding regions* of the genome (*e.g.* downstream promoter or upstream regulator regions). This is supported by the recent report by Liu *et al.,* who found a surprisingly large number of possibly

disease-causing splice site mutations in intron sequence of *ABCB11* in patients with low-GGT PFIC [106].

The BSEP expression varied to a limited extent among children with the same *ABCB11* mutations, even among siblings (**Table 4**; patients D and G). However, there was good overall agreement between the BSEP expression and mutations in *ABCB11*. We believe that immunohistochemical BSEP staining indeed is an important and useful diagnostic tool, especially when genetic characterization is not available, or cannot be performed, or where the mutation screening has been negative.

PEBD should be regarded a safe procedure in experienced hands. A rapid response with early relief of pruritus was seen in 7 of 13 patients, although this was not a completely reliable indicator on the long-term outcome, since some of these early responders later developed longer and/or repeated periods of cholestasis. One group of children presented 2-6 weeks after PEBD with sudden transient episodes of dehydration with hyponatremia, a finding that might indicate a less favorable long-term outcome. This sudden "choleresis" has been briefly mentioned earlier in cirrhotic patients operated with PEBD [200, 202], but its cause remains unexplained. Adrenal insufficiency as a cause for this was ruled out. We speculate that the mechanism behind these rapid changes in output could be found in the cholangiocyte, rather than in the hepatocyte. The interrupted enterohepatic circulation reduces intracellular levels of bile acids in the hepatocyte and the cholangiocyte, and thus inhibits FXR [36]. The downregulation of FXR leads to a number of events, including the induction of ASBT expression in the apical membrane of the cholangiocyte, with an increased reabsorption of bile acids from ductular bile and a concomitant efflux of bicarbonate into the bile ducts as a consequence. Increased cholehepatic shunting of bile acids thus induces a bicarbonate rich hypercholeresis, a mechanism which has been suggested to be an important driving force of bile flow [237].

When studying the fractions of bile diverted into the stomal bag during remission we found that > 90% of the total bile was lost into the stomal bag, and less than 10% reached the intestine. In spite of this finding, none of the investigated children had any clinical or biochemical signs of fat-soluble vitamin deficiency. We speculate that this could be explained by alternative mechanisms facilitating the duodenal uptake of dietary fats and vitamins in the relative absence of bile. For instance, the gastric co-lipase has previously been shown to act as a bile acid facilitator, and could perhaps be such a candidate [238].

There was a drastic shift in the distribution of bile during cholestatic episodes, with a lower fraction of bile diverted through the stoma, and a larger fraction retained in the body and eliminated through the urine. The mechanism for this, as supported by the findings of Keitel *et al.*, could be an increased expression of MRP4 in children with PFIC type 2 during cholestasis [239]. This upregulation of one of the salvage proteins of the hepatocyte (page 5) aims to protect the liver from further damage by increasing the efflux of cytotoxic bile acids into the blood circulation, with increased elimination of bile acids into urine as a consequence.

PEBD not only improved clinical and biochemical markers, but also improved the long-term outcome by reversing the hepatic fibrosis. The significant reductions in biochemical and histologic cholestasis heralded significant reductions in the histologic fibrosis seen at long-term follow-up. This finding indirectly supports earlier suggestions that the hepatocanalicular cholestasis probably not only precedes, but also causes the fibrosis in PFIC [240]. Cholestatic episodes after PEBD were eventually seen in all patients, and the total duration of these episodes correlated with the development of hepatic fibrosis. From this we conclude, that the cholestatic episodes after PEBD should be kept as short as possible. It is suggested that by combining UDCA and rifampicin during these episodes, one can achieve additive effects by promoting several important bile and bile acid transporters in the apical and basolateral membranes of the hepatocyte [167].

We believe that there are several reasons to be cautious about OLT in children with PFIC, apart from the risks of complications associated with the surgical procedure and the life-long immunosuppression after OLT. The children with PFIC type 1 are not cured from the extrahepatic disease manifestations after OLT, and the reports of unexplained steatosis and steatohepatitis after OLT give cause to concern. In patients with PFIC type 2, OLT may be an alternative to PEBD in rapidly progressing disease, or at late diagnosis, with cirrhosis. In those patients, there seems to be a higher risk for post-operative complications after PEBD, and an increased risk for hepatic malignancy in patients with manifest hepatic cirrhosis [240-243]. However, the recent findings of *de-novo* BSEP antibodies after OLT should be taken into account when deciding upon method of operation.

The main finding of this thesis is that PEBD reversed hepatic fibrosis in patients with PFIC type 2, and we propose that PEBD should be regarded as the first line of treatment for patients in whom non-surgical treatments have failed or are insufficient. The operation needs to be done at an early stage of the disease, to prevent operating children with advanced hepatic fibrosis or cirrhosis.

The exact mechanisms explaining the good effect of PEBD on the clinical and histological course remain unknown. In fact, there is still no better explanation than that proposed 90 years ago, that the interruption of the enterohepatic circulation decreases the “preload” of bile acids to the liver. Whether the operation also leads to qualitative changes in bile and in the secretion patterns of bile acids, remains to be further investigated.

# 7 FUTURE CHALLENGES AND DIRECTIONS

There are several cholestatic diseases in which the clinical course and the prognosis are highly variable and where possible disease modifiers remain unknown. One such example is liver disease caused by $\alpha_1$-antitrypsin deficiency, in which the disease severity ranges from slight elevations of transaminases to early and rapid progress into end-stage liver disease. Cystic fibrosis-related liver disease and biliary atresia are other disorders of variable and unpredictable outcome. One possible candidate as disease modifier would be the bile salt export pump. Therefore, studies of genetic variations in *ABCB11* and functional studies of BSEP in other cholestatic diseases would be important.

Different animal models have shown to be of limited value when studying the kinetics of bile acid metabolism and function of the different main bile acid transporters, due to large interspecies differences. The new possibilities to use primary hepatocyte cultures for functional *in vitro* studies will hopefully circumvent the problems posed by these important differences between animals and humans [88, 244].

The mechanism behind the effect of PEBD is only partly understood. Whether there are any qualitative changes in bile acid synthesis and secretion before and after PEBD remains unknown. We aim to study these mechanisms by analyzing the urinary bile acids before and after PEBD using gas chromatography-mass spectrometry, and by following the patients with serum markers for bile acid synthesis and intestinal bile salt reabsorption [245, 246].

More than half of the patients described in this thesis have passed into adolescence, an age normally characterized by increasing self awareness, a wish to be “like everybody else”, and with rapid fluctuations in self-esteem. These patients have all more or less accepted their bile ostomy, and they all appreciate that PEBD is a life-saving procedure. Nonetheless, all of them would probably rather be without their stomal bags. To learn more about this, we aim to undertake studies of tolerability and quality of life in patients with PFIC after PEBD.

# 8 SVENSK SAMMANFATTNING

I Sverige föds varje år drygt 100.000 barn, och ett antal av dessa nyfödda kommer att utredas för oklara symptom som alla skulle kunna vara tecken på tidigt debuterande leversjukdom (t ex långdragen gulsot, dålig tillväxt och blåmärken eller näsblod). Årligen visar det sig att 40-50 av dessa lider av en leversjukdom. Trots att våra kunskaper kring dessa sjukdomar ständigt ökar, förblir upptill 25% av de sjuka spädbarnen utan diagnos. Vi försöker behandla dem, men vi kan trots alla upptänkliga diagnostiska metoder inte säga vad som orsakar sjukdomen.

De vanligaste lever- och gallvägssjukdomarna hos nyfödda är gallvägsatresi som karaktäriseras av skrumpnande gallgångar, och alfa-1 antitrypsinbrist, som orsakas av en onormal upplagring i levern av proteinet alfa-1 antitrypsin. De viktigaste orsakerna till s.k. intrahepatisk gallstas är samlade i **tabell 1** på sidan 2.

Den här avhandlingen handlar om olika aspekter på en grupp av sjukdomar som med ett samlingsnamn kallas progressiv (dvs. fortskridande, med risk för försämring) ärftlig intrahepatisk gallstas. Det engelska namnet är progressive familial intrahepatic cholestasis, och förkortas därför PFIC. I Sverige insjuknar i genomsnitt ett barn årligen i PFIC. Utan behandling skulle det barnet sannolikt inte överleva, men tack vare läkemedel och kirurgi kan numera så gott som alla barn med PFIC räddas till livet. I avhandlingens fyra delarbeten har vi undersökt olika aspekter av sjukdomen hos 16 av de totalt 18 barn från stora delar av Sverige som vi följt den senaste 25-årsperioden vid enheten för barns leversjukdomar vid Karolinska universitetssjukhuset i Huddinge. Nedan följer en sammanfattning av vad vi kommit fram till. Jag har valt att förklara på ett sådant sätt att även en icke-medicinskt kunnig person ska kunna tillgodogöra sig de stora dragen.

I **delarbete 1** undersökte vi om de svenska barnens sjukdom kunde vara orsakad av samma ärftliga anlag som det då nyligen påträffade anlag som orsakar Bylers sjukdom, en variant av PFIC. Vi kunde med s.k. genetisk kopplingsanalys konstatera att de svenska barnens sjukdom *inte* kunde ha orsakats av detta anlag. Det innebar alltså att sjukdomen PFIC kunde orsakas av flera olika ärftliga anlag, dvs. att den egentligen inte var en, utan flera olika sjukdomar som liknande varandra.

Undersökning av arvsmassa (DNA) från våra patienter bidrog till att ytterligare ett anlag kunde hittas och därefter studeras i detalj. Det visade sig att detta nyupptäckta anlag gav upphov till ett transportprotein som förflyttar gallans viktigaste beståndsdel, gallsyrorna, från levercellen ut i gallgången. Denna ”gallsyrepump” döptes på engelska till bile salt export pump (BSEP). När pumpen inte fungerar eller inte alls bildas, så blir gallsyrorna kvar i levercellen i en koncentration som är skadlig, och därför så småningom leder till ärrbildning och slutligen skrumplever.

I **delarbetena 2** och **3** undersökte och beskrev vi effekterna av den operation som 13 av de 18 svenska barnen har genomgått. Operationen kallas partiell (ofullständig) gallavledning, eller partial external biliary diversion (PEBD): en bit av tunntarmen

kopplas från gallblåsan och ut genom huden, så att en ”gallstomi” bildas på magen (se **figur 2** på sidan 18). Till stomiöppningen på magen kan en påse kopplas, där den avledda gallan samlas upp. På detta sätt kan den skadliga upplagringen av gallsyror minskas. Tack vare operationen har de flesta svenska barnen med PFIC sluppit levertransplantation, vilket annars skulle varit det enda återstående alternativet.

Vi kunde visa att de flesta barn som genomgick partiell gallavledning förbättrades efter operationen. Dels minskade de besvärliga symptomen med svår klåda och dålig tillväxt, dels minskade nivåerna av gallsyror i levern och i blodet, men framför allt bromsade ärrbildningen i levern upp. I de flesta fall till och med minskade den. Alla opererade barn besvärades även efter operationen då och då av gallstas med en mycket besvärlig klåda, men det varierade hur ofta besvären kom och hur mycket besvär barnen då hade. Vi undersökte om de olika påträffade avvikelserna i det ärftliga anlaget kunde förklara skillnader mellan barnen, t ex deras symtom vid insjuknandet, hur mycket klåda de hade efter operationen och graden av ärromvandling i levern. Vi kunde konstatera att den vanligaste anlagsförändringen hos svenska barn i de flesta fall gav en bra långtidsprognos förutsatt att barnen opererades tidigt, innan levern hunnit bli alltför ärromvandlad. Resultaten av operationen beskrivs i **tabell 4** på sidan 25, och i **figurerna 3** och **4** på sidorna 28-29.

I **delarbete 4** undersökte vi hur stor del av gallan som leddes ut via gallstomin och hur stor del som blev kvar i kroppen hos 9 av de gallvägsopererade barnen. Vi använde oss av en undersökningsmetod som kallas för nuklearskintigrafi (undersökning med gammakamera), och undersökte barnen både under perioder av pågående gallstas, och under perioder då de mådde bra. Vi upptäckte att när barnen inte hade gallstas, så hamnade mer än 90% av gallan i stomipåsen, medan bara knappt 10% hamnade i tarmen där den utförde sitt viktiga jobb med att underlätta upptaget av fett och de livsviktiga fettlösliga vitaminerna (se figur 2 i **delarbete 4**, på bokens sista sida). Trots denna lilla andel galla i tarmen uppvisade barnen inga symtom på vitamin- eller fettbrist, som blåmärken eller dålig tillväxt. Vi kunde också konstatera att en mycket mindre andel galla hamnade i gallstomin, och en större andel av gallan blev kvar i kroppen eller kissades ut, under de perioder då barnen led av gallstas.

Sammanfattningsvis har vi kunnat konstatera att den undersökta sjukdomen PFIC inte är en, utan flera olika sjukdomar, som orsakas av olika ärftliga anlag. Sannolikt finns det flera ännu oupptäckta anlag som kan förklara en del av de återstående fallen av oklar intrahepatisk gallstas. De flesta barn med den variant som orsakar sjukdomen PFIC typ 2 uppvisar en god prognos efter partiell gallavledning, där även ärrbildningen i levern kan minska eller försvinna, förutsatt att operationen sker innan denna ärrbildning hunnit gå för långt och lett till skrumplever. På så sätt kan de flesta barn slippa genomgå levertransplantation med de många risker det medför på kort och på lång sikt.

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