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NATIVE LIVER FIBROSIS IN BILIARY ATRESIA

FROM PATHOBIOLOGY TO CLINICAL OUTCOMES

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ACADEMIC DISSERTATION

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"It always seems impossible until it's done."

Nelson Mandela

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ABSTRACT

Background. Biliary atresia (BA) is a destructive, obliterative fibroinflammatory cholangiopathy of infancy, affecting around 1 in 20 000 newborns a year. Despite successful surgical restoration of bile flow by portoenterostomy (PE), liver fibrosis progresses into liver failure and the need for liver transplantation (LTx) before adulthood in most patients. Pathogenesis underlying progressive liver fibrosis after successful PE is unclear.

Aims. The aim of this study was to investigate the molecular mechanisms underlying persistent liver fibrogenesis after successful PE and to address the predictors of outcomes.

Patients and methods. Three cross-sectional studies investigated gene and protein expression of liver biopsies and serum levels of profibrogenic growth factors, proinflammatory cytokines and extracellular matrix mediators from 25–28 BA patients treated in Helsinki University Hospital between 1991 and 2013, taken at PE and over three years of follow-up after successful operation. The change in survival rates before and after centralization and predictive values were analyzed in BA patients treated in Helsinki between 1987 and 2016 (n=61).

Results. During three years following successful PE, serum bilirubin levels remained low [median 10 (interquartile range 4–17) μmol/L]. Despite the resolution of histologic cholestasis and reduction in inflammation, liver fibrosis persisted. Ductular proliferation prevailed and periportal hepatocyte cytokeratin 7 immunopositivity, indicating hepatocyte-to-cholangiocyte metaplasia, increased. If clearance of jaundice (COJ) was not achieved, both histologic cholestasis and inflammation persisted, and progression of liver fibrosis was faster.

Of the matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) studied, MMP-7 was uniquely upregulated in BA when compared to both non-fibrotic and fibrotic control biopsies at protein, gene and serum levels. MMP-7 localized to the biliary epithelium and was related to liver fibrosis stage. Serum MMP-7 showed significant predictive value for portal fibrosis after successful PE.

Protein expression of collagen 1, alfa-smooth muscle actin (α-SMA, indicator of activated myofibroblasts), transforming growth factor-beta (TGF-β)-1, TGF-β2, decorin and connective tissue growth factor (CTGF) were all increased at PE and at follow-up compared to non-fibrotic controls. After successful PE, protein expression of TGF-β1 and CTGF, but not that of decorin or TGF-β2, decreased. Both protein and gene expression of TGF-β1 and protein expression of TGF-β2

correlated with Metavir fibrosis stage. At PE, all patients had periductal α-SMA protein expression, but it persisted in 64% at follow-up and was associated with the progression of fibrosis and ductal reactions, and serum bilirubin and bile acid levels. Gene expression of platelet-derived growth factor (*PDGF)* was elevated compared to fibrotic and non-fibrotic controls, and it correlated with α-SMA and collagen expression. Gene expression of proinflammatory cytokines (interleukins, tumor necrosis alfa and interferon gamma) was decreased or comparable when compared to fibrotic and non-fibrotic controls.

Syndromic patients (43%) showed less progressive fibrosis and ductal proliferation and decreased expression of TGF-β1, α-SMA and MMP-7 when compared with isolated patients.

After the centralization of treatment in Helsinki (2005), the median caseload increased from 1 to 3–4 per year. There was an increase in COJ rate from 42% to 80%, a five-year native liver survival from 38% to 70%, and a five-year overall survival rate from 68% to 94%. In multivariate analysis, the only predictor of COJ was high-grade portal inflammation at PE, and the normalization of bilirubin within 3 months predicted native liver survival. Cytokeratin-7 immunopositivity of periportal hepatocytes was the only predictor of follow-up liver fibrosis in multiple regression analysis.

Conclusions. After successful PE, a molecular signature of active fibrogenesis prevails, while histologic portal inflammation and expression of proinflammatory cytokines decreases. An increased hepatic expression of MMP-7 is unique to BA and offers a potential follow-up and therapeutic target to extend native liver survival after COJ. TGF-β2 and PDGF might be essentially involved with BA liver fibrogenesis after cholestasis has resolved.

ABSTRAKTI

Tausta. Sappitieatresia on vastasyntyneiden inflammatorinen, sappiteitä tuhoava ja tukkiva sairaus, joka hoitamattomana johtaa kuolemaan varhaislapsuudessa. Sappitieatresian ilmaantuvuus on 1:20 000 vastasyntynyttä vuodessa, ja se on yleisin lasten maksan siirron syy. Huolimatta varhaisesta kirurgisesta leikkaushoidosta, portoenterostomiasta, tauti etenee useimmilla maksakirroosiin ja maksan vajaatoimintaan viimeistään aikuisiässä. Maksafibroosin kehittymiseen vaikuttavat molekyylitason tekijät ovat huonosti tunnettuja. Patogeneesin ymmärtäminen on olennaista tehokkaampien hoitojen kehittämiseksi.

Tavoitteet. Tutkimuksen tavoitteena oli selvittää etenevän maksafibroosin molekyylitason mekanismeja tutkimalla maksan proteiini -ja geeniekspressiota sappitieatresiapotilailta onnistuneen portoenterostomian jälkeen, sekä arvioida hoidon tuloksiin vaikuttavia tekijöitä.

Potilaat ja metodit. Kolmessa poikkileikkaustutkimuksessa tarkasteltiin fibrogeneettisten ja proinflammatoristen solujen välittäjäainemolekyylien geeni -ja proteiiniekspressiota maksabiopsioissa sekä näiden seerumiarvoja Helsingin yliopistosairaalassa vuosien 1991 ja 2013 välillä hoidetuilla 25–28 sappitieatresiapotilaalla. Analyysit tehtiin sekä portoenterostomian aikana, että yli kolme vuotta onnistuneen kirurgisen hoidon jälkeen otetuista näytteistä. Hoidon tuloksia ja niihin vaikuttavia tekijöitä arvioitiin vertailemalla Helsingin yliopistosairaalassa hoidettuja potilaita ennen ja jälkeen vuoden 2005 keskittämispäätöksen (n=61).

Tulokset. Huolimatta histologisen ja biokemiallisen kolestaasin häviämisestä onnistuneen portoenterostomian jälkeen, maksafibroosi pysyi ennallaan tai eteni. Vaikka sappitieproliferaation määrässä ei tapahtunut muutoksia, periportaalisten hepatosyyttien sytokeratiini 7 -immunopositiivisuus, joka viittaa hepatosyytti-kolangiosyytti-metaplasiaan, lisääntyi.

Tutkituista matriksin metalloproteinaaseista (MMP) ja niiden kudosinhibiittoreista (TIMP) MMP-7:n määrä maksabiopsioissa geeni -ja proteiinitasolla sekä seerumissa oli sappitietatresiassa lisääntynyt voimakkaasti. Maksassa sen ekspressio lokalisoitui kolangiosyytteihin ja oli verrannollinen maksafibroosin asteeseen. Seerumin MMP-7 oli tilastollisesti merkitsevä ennustetekijä arvioitaessa portaalialueiden fibroosiastetta onnistuneen portoenterostomian jälkeen.

Kollageeni 1:n, alfa-sileälihasaktiinin (α-SMA, myofibroblastien aktivoitumismarkkeri), TGF-β-1 ja -2 kasvutekijöiden, dekoriinin ja CTGF -kasvutekijän proteiiniekspressio oli lisääntynyt sekä portoenterostomian että seurannassa. Onnis-

tuneen portoenterostomian jälkeen TGF-β1:n ja CTGF:n, mutta ei dekoriinin tai TGF-β2:n, proteiiniekspressio väheni. TGF-β1:n ja TGF-β2:n ekspressio korreloi maksan histologiseen fibroosiin. Portoenterostomian aikana kaikki potilaat ilmensivät α-SMA proteiinia periduktaalialueella, ja sen säilyminen seurannassa (64%) assosioitui fibroosin etenemiseen, sappitiproliferaatioon sekä seerumin bilirubiinin ja sappihappojen pitoisuuteen. PDGF -kasvutekijän geeniekspressio oli lisääntynyt sappitieatresiapotilailla, ja se korreloi α-SMA:n ja kollageenin ekspressioon. Tutkittujen proinflammatoristen sytokiinien geeniekspressio oli seurannassa matala.

Potilailla, joilla oli liitännäisanomalioita (43%), fibroosin ja sappitieproliferaation eteneminen oli lievempää, ja TGF-β1:n, α-SMA:n ja MMP-7:n ekspressio vähäisempää verrattuna isoloituihin sappitieatresiapotilaisiin.

Sen jälkeen kun sappitieatresiapotilaiden hoito keskitettiin Helsinkiin vuonna 2005, vuosittainen potilasmäärä lisääntyi yhdestä 3–4:ään vuodessa. Portoenterostomia onnistui ennen keskittämistä 42%:lla ja keskittämisen jälkeen 80%:lla. Viiden vuoden kuluttua leikkauksesta omalla maksalla eläviä oli 70% (vs 38% ennen keskittämistä) ja kaiken kaikkiaan elossa oli 94% (vs 68%). Multivarianttianalyysissä ainoa onnistunutta portoenterostomiaa ennustava tekijä oli korkea-asteinen portaalialueiden inflammaatio protenterostomian aikana, ja bilirubiiniarvon normaalistuminen kolmen kuukauden kuluttua leikkauksesta ennusti omalla maksalla selviytymistä. Sytokeratiini-7:n immunopositiivisten hepatosyyttien määrä oli ainoa seurannan aikaista maksafibroosin astetta ennustava tekijä.

Johtopäätökset. Onnistuneen portoenterostomian jälkeen fibrogeneettisten kasvutekijöiden lisääntynyt ilmentyminen hallitsee etenevää fibroosia samalla kun histologinen inflammaatio sekä proinflammatoristen sytokiinien ilmentyminen vähenee. MMP-7:n lisääntynyt ilmentyminen on ominaista sappitieatresialle ja tarjoaa mahdollisen seurantavälineen sekä anfibrogeneettisten hoitojen kohteen.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals I–IV:

I.   Kerola A, Lampela H, Lohi J, Heikkilä P, Mutanen A, Jalanko H, Pakarinen MP. Molecular signature of active fibrogenesis prevails in biliary atresia after successful portoenterostomy. Surgery 2017;162(3):548–556.

II. Kerola A, Lohi J, Heikkilä P, Mutanen A, Jalanko H, Pakarinen MP. Divergent expression of liver transforming growth factor superfamily cytokines after successful portoenterostomy in biliary atresia. Surgery 2019; doi: 10.1016/j.surg.2018.12.003. [Epub ahead of print]

III. Kerola A, Lampela H, Lohi J, Heikkilä P, Mutanen A, Hagström J, Tervahartiala T, Sorsa T, Haglund C, Jalanko H, Pakarinen MP. Increased MMP-7 expression in biliary epithelium and serum underpins native liver fibrosis after successful portoenterostomy in biliary atresia. J Pathol Clin Res 2016;12:187–898.

IV. Hukkinen M*, Kerola A*, Lohi J, Heikkilä P, Merras-Salmio L, Jahnukainen T, Koivusalo A, Jalanko H, Pakarinen MP. Treatment policy and liver histopathology predict biliary atresia outcomes – Results after national centralization and protocol biopsies. (*The first two co-authors share an equal contribution to this work.) J Am Coll Surg 2018 Jan;226(1):46–57.

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ABBREVIATIONS

In alphabetical order

α-SMA	alfa-smooth muscle actin
APRI	aspartate transferase to platelet ratio index
AUROC	area under the receiver operating characteristic curve
BA	biliary atresia
BASM	biliary atresia splenic malformation
BEC	biliary epithelial cell
CI	confidence interval
CK	cytokeratin
COJ	clearance of jaundice
CTGF	connective tissue growth factor
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-to-mesenchymal transformation
HSC	hepatic stellate cell
IQR	interquartile range
LTx	liver transplantation
MMP	matrix metalloproteinase
PDGF	platelet-derived growth factor
PE	portoenterostomy
RNA	ribonucleic acid
TGF-β	transforming growth factor-beta
TIMP	tissue inhibitor of matrix metalloproteinase
UDCA	ursodeoxycholic acid

1 INTRODUCTION

Biliary atresia (BA) is a rare disease of infancy that affects both extra- and intrahepatic bile ducts. Obliteration leads to cholestasis and liver failure by the age of two years if left untreated [1, 2]. Bile flow can be restored in over 40% of patients with a surgical operation called portoenterostomy (PE), but despite that, PE is still considered a palliative treatment since most patients will develop progressive liver fibrosis and liver failure before adulthood [1, 3]. Despite affecting only 1 in 20 000 children per year in Europe, BA is the leading indication for pediatric LTx worldwide [1]. In Europe, five-year native liver survival rates over 40% and overall survival rates over 85% are achieved [4-6]. The most important predictive factors for increased native liver survival are clearance of jaundice (COJ) after PE and younger age at the time of operation [1].

The molecular mechanisms underlying progressive fibrosis despite successful PE and resolution of cholestasis are unknown. At PE, liver histology is dominated by cholestasis and inflammation [7, 8]. Postoperative treatment with anti-inflammatory corticosteroids may enhance COJ but does not affect the native liver or overall survival [9].

In liver fibrogenesis, the extracellular matrix (ECM), e.g., collagen, accumulates in excess due to the activation of alfa-smooth muscle actin (α-SMA) expressing hepatic stellate cells and portal myofibroblasts. Transforming growth factor-beta (TGF-β) superfamily molecules and platelet-derived growth factor (PDGF) are the main profibrogenic growth factors in liver fibrogenesis [10]. Previous studies have shown that TGF-β, connective tissue growth factor (CTGF) and PDGF are upregulated in BA at the time of PE [11-13]. Of the essential molecules in ECM turnover, matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), especially MMP-7 expression, have been shown to be increased in BA at the time of PE [14, 15].

The molecular mechanisms underlying active fibrogenesis after successful PE, when biochemical cholestasis is resolved, are still unknown. If these factors were unraveled, native liver survival could possibly be pharmacologically extended, and the need for LTx would be reduced or delayed.

2 REVIEW OF THE LITERATURE

2.1 Biliary atresia (BA) – the fundamentals

2.1.1 Definition, epidemiology and etiology

Biliary atresia is an obliterative fibroinflammatory cholangiopathy of infancy that affects both intra- and extrahepatic bile ducts. Their destruction prevents bile flow from the liver to the bowel and causes not only cholestasis but also chronic liver disease and cirrhosis, ending in liver failure and death if left untreated [1, 2, 16-21]. BA is classified into three types depending on the level of the most proximal biliary obstruction: in type 1 (around 5% of cases), atresia is in the common bile duct, in type 2 (2%) in the common hepatic duct, and in type 3 (the most common form at over 90%), the entire extrahepatic biliary duct system is occluded to the level of portae hepatis [1, 22] (anatomy shown in Figure 1). The incidence of cholestatic jaundice is around 1 in 2500 term infants, and 25–40% are caused by BA, making it the most common cause of cholestasis in the first months of life [23, 24]. BA is a rare disorder worldwide, but its incidence varies among countries, with lowest rates reported in Croatia (1 in 23 600) and the highest in French Polynesia (from 1 in 3120). In European countries and Canada, the incidence is around 1 in 18 000– 20 000. Figure 2 shows the incidence in different countries, depicted in 10 000 live births a year [5, 6, 25-36].

The etiology of BA is largely unknown, and it is thought to be multifactorial. The generally accepted view is to see BA as a common phenotype of possibly multiple different insults, which can cause neonatal liver and bile ducts to respond in a stereotypical fibroinflammatory manner [1, 17, 18, 37-39]. This is called the "multiple hit" phenomenon [38]. The possible factors that trigger the pathologic response can be environmental, genetic, developmental and immunological [20, 37]. Environmental factors comprise viruses like reo-, rota-, human papilloma and cytomegalovirus with the latter gaining most recent evidence of forming possibly even an own entity among BA patients [29]. Different viruses have been detected in BA patients' liver tissue, and a *rhesus rotavirus* (RRV)–induced mice model has been created [2, 19, 20, 37-41]. Observations of hepatobiliary injury in newborn lambs after their dams had eaten toxic weed led to the discovery of the toxin, called biliatresone, and in following animal studies, that toxin caused selective extrahepatic biliary damage in larval zebrafish and mice [2, 16, 19]. However, no clear proof of an environmental toxin being the culprit of BA in human beings has been found, and firm proof of a causal relationship between a hepatobiliary injury and viral infection in BA is lacking [2, 16, 17, 20, 37]. Research on predisposing

genetic factors has proposed inactivation or overexpression of numerous genes (for example, laterality genes and human leukocyte antigen), but even if the evidence suggests that patients probably have a genetic susceptibility to the disease, no specific gene responsible for BA has been identified [2, 17, 19, 39]. Genetic or environmental insult might cause a disturbance in bile duct development, which ends up in the pathology of BA. That theory is supported by studies exploring BA patients' liver biopsies and finding histologic ductal plate malformations, which are part of embryogenesis, but should not appear after bile ducts have matured [2, 19, 20, 37, 41, 42]. Defects in prenatal hepatic circulation have also been noted but no clear causality observed [19, 20, 37]. Increased amounts of activated immune cells have been detected in BA livers during the time of diagnosis, and research supports the role of the immune system and inflammation in epithelial injury and bile duct obstruction. However, the exact mechanism that triggers the inflammation and its causality in the pathogenesis of BA is still unclear [17, 19-21, 43]. It might also be that inflammation is a stronger pathogenic mechanism in some cases, but not all, and that it has more impact at earlier stages of the disease [21, 44]. Basic research on etiopathogenesis in BA is based extensively on animal models (rodents with BA-mimicking disease caused by injection with *rhesus rotavirus* or bile duct ligation) and the BA patients' liver histology and genetics at the time of PE [2, 19].
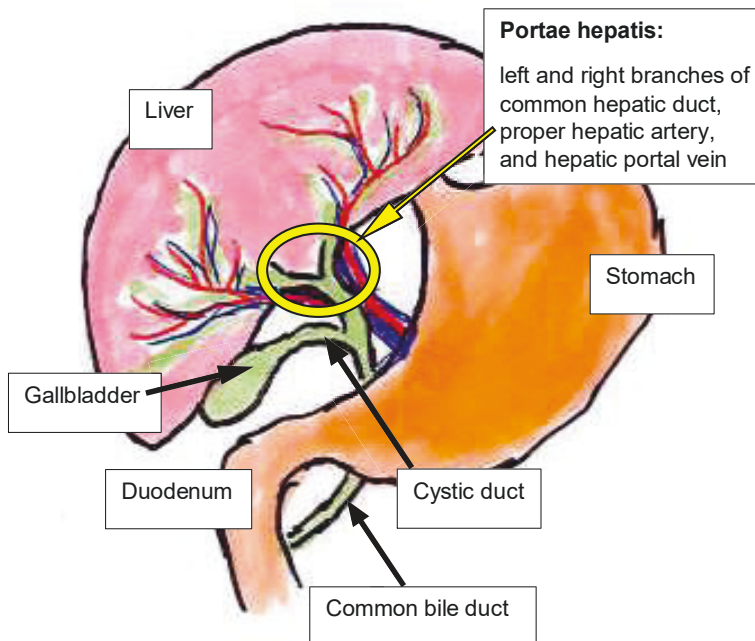

Figure 1. Liver and bile duct anatomy. In biliary atresia type 3, the entire extrahepatic biliary duct system (common bile duct, cystic duct, gallbladder, common hepatic duct, and left and right hepatic ducts) are rudimentary and occluded.
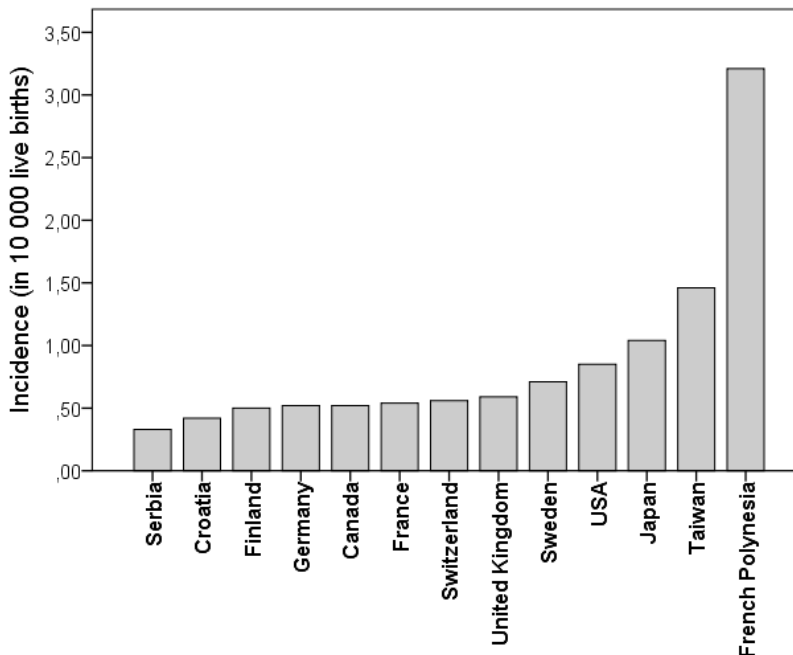
Figure 2. Epidemiology of BA. Incidence in different countries, depicted in 10 000 live births a year. Ref [5, 6, 25–36].

2.1.2 Isolated and syndromic BA

Epidemiologic studies show that 8–25% of BA patients have congenital anomalies [30, 45-51]. Some papers have reported even higher numbers, up to 36%, while the lowest comes from Taiwan (3%) [34, 52-54]. BA is classified as *isolated* (no anomalies) or *syndromic* (at least one other congenital anomaly). The nomenclature of *embryonic* or *congenital* has also been used as synonyms of syndromic form, and *perinatal* or *acquired* of isolated form, and they hint to the possibly different etiological origins of the two forms. This is based on the hypothesis that other non-hepatic congenital anomalies bespeak a defect in embryogenesis and thus a common origin, but studies are controversial. Despite the observation of possibly different gene expression profilings between those two groups, both syndromic and isolated BA have been found to be present antenatally [55-58]. In this study, the term *syndromic* is used for patients with any major anomaly/anomalies and *isolated* for patients without anomalies.

Some BA patients have a combination of typical anomalies belonging to polysplenia syndrome. These include multiple spleens, cardiovascular anomalies, intestinal malrotation, situs inversus (i.e., organ transposition through the sagittal plane), preduodenal portal vein, interrupted or absent infrahepatic inferior vena

cava, atypical hepatic artery anatomy, and organ isomerism or heterotaxy (i.e., different arrangement) [57, 59-61]. These anomalies are also referred to as *laterality sequence anomalies* and refer to a common defect in embryogenesis during determination of organ laterality and the embryonic midline (around week 5). The extrahepatic biliary system arises from the ventral foregut by the same week [46, 49, 61, 62]. However, no mutations in typical laterality sequence genes have been found in BA patients' livers [63].

The term *biliary atresia splenic malformation* (BASM) syndrome was first introduced in 1993 and referred to a specific group of BA patients with a macroscopic splenic abnormality (polysplenia, asplenia or double spleen); usually, but not necessarily, it also included another anomaly, typically from the laterality sequence group [62, 64]. Epidemiologic studies show that 4–17% of BA patients have BASM syndrome [4-6, 25, 30, 32, 45, 46, 50, 65-67]. However, the definition of BASM syndrome is not clear and varies among studies since there are BA patients with anomalies belonging to the laterality sequence group but without splenic anomaly. The term *biliary atresia structural malformation* has been proposed as a better possible representation of this patient group [46, 49, 60, 61]. In this study, the original definition is used, i.e., a BA patient has BASM syndrome if they have a macroscopic splenic abnormality with or without another congenital anomaly [62, 64].

Apart from isolated and syndromic BA, two other categories have been proposed as their own entities in BA: *cystic BA* (a cystic change in an otherwise occluded extrahepatic biliary tree) and *cytomegalovirus-associated BA* [22, 40, 68]. In some papers, cystic BA and BASM are combined to become *developmental BA* [31, 69].

2.1.3 Diagnosis

Clinical characteristics of BA are persistent jaundice, pale stools and dark urine in the first weeks of life. Patients are usually born at term with normal birth weight and an otherwise healthy appearance [1, 22]. Visible jaundice appears when the serum bilirubin level rises above 42–51 µmol/L [23]. If jaundice persists for two weeks after birth, further evaluation should be done [1, 17, 23]. Laboratory studies show elevated total and direct/conjugated bilirubin levels [23]. Preoperative diagnostic imaging methods include abdominal ultrasound, hepatobiliary scintigraphy, endoscopic retrograde cholangiopancreatography and magnetic resonance retrograde cholangiopancreatography, and they are used variably in different centers, but neither can reliably diagnose BA [1, 17, 22-24]. Percutaneous liver biopsy is the usual diagnostic method of choice in many countries, and it has the highest sensitivity (91%) in diagnosing BA, with a specificity of 93% and a diagnostic accuracy of up to 92% [70]. The complication rate in percutaneous ultrasound–guided liver biopsy in children is 0.4–1.7% [70, 71]. If the diagnosis of BA cannot

be ruled out after these diagnostic tests, the next step is laparotomy. If possible, an intraoperative cholangiography, where the discontinuity of extrahepatic biliary tree is confirmed, should be done before performing a portoenterostomy (PE) [1, 23, 37, 72].

2.1.4 Treatment

Before the 1950s, type 3 BA was called the "uncorrectable biliary atresia" because surgical attempts to relieve the biliary obstruction failed, and patients died of liver failure by the age of three years [1, 17, 37, 68]. In 1955, Japanese pediatric surgeon Morio Kasai developed a new surgical strategy where the remnants of the extrahepatic biliary tree are excised up to the level of porta hepatis, which is anastomosed to a jejunal Roux-en-Y loop (Figure 3). Portoenterostomy (PE) allows the drainage of bile via microscopic remaining ducts. Kasai published his results in 1959, and the operation became globally accepted as the first line of treatment of BA by the 1970s [73, 74]. PE is considered successful when biochemical cholestasis resolves postoperatively and serum bilirubin concentration normalizes. This is referred to as *clearance of jaundice* (COJ) [1, 4].

However, despite restoring bile flow and clearing jaundice in 40–60% of cases, liver fibrosis advances into cirrhosis, so that 75–80% of patients require liver transplantation (LTx) before reaching adulthood [1, 75] (Table 1). In the 1980s, LTx was introduced as a second-line treatment option for BA, and it revolutionized the overall survival rates (reviewed in section 2.4) [68, 75]. Up to 75% of pediatric LTx procedures are performed for BA patients [1]. LTx is indicated if PE has not restored bile flow. It is also recommended for patients whose jaundice was cleared but whose chronic liver disease proceeds into end-stage liver disease with ensuing complications, including recurrent bacterial cholangitis, failure-to-thrive, portal hypertension, ascites, difficult pruritus, hepatopulmonary syndrome, portopulmonary hypertension or hepatorenal syndrome resulting in kidney failure. Rare indication for LTx is hepatic malignancy (around 1% of patients) [24, 76, 77]. COJ rates significantly fall when PE is done after the age of three months, but even one-fourth of those late-operated BA patients can survive without LTx over five years, and over 10% reach adolescence with their native livers [76-79]. It has been estimated that PE might be a reasonable option for over 95% of BA patients, and it might have a protective effect on graft survival when LTx is later performed [68, 80]. However, survival after LTx decreases with deterioration in a patient's nutritional status and cholestasis, so correct timing of LTx is crucial [76, 81].
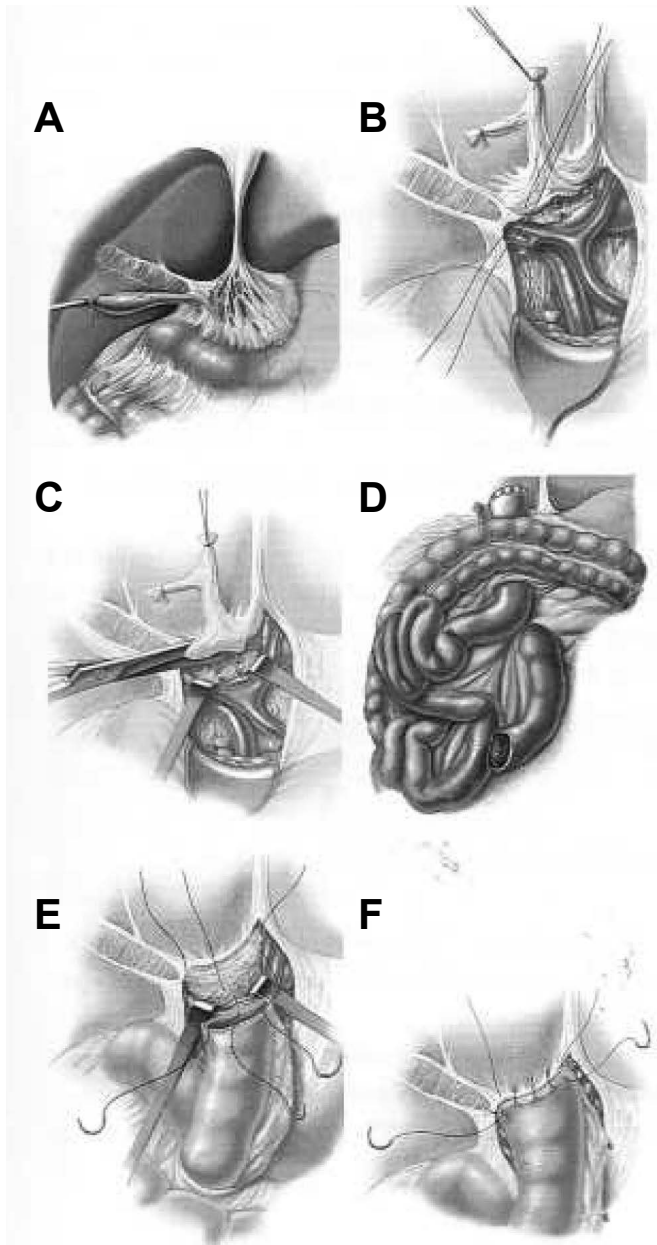
Figure 3. Operative technique of Kasai portoenterostomy. After laparotomy incision and confirmation of BA diagnosis, the liver is mobilized, the hepatoduodenal ligament is dissected, the hepatic artery and portal vein are identified and fibrous remnants of extrahepatic bile ducts and gall bladder excised (A-B). Portae hepatis is widely exposed and the Roux-en-Y-Loop mobilized by dividing jejunum approximately 10 cm from the Treitz ligament and positioning this 50 cm loop retrocolically to reach the portae hepatis. Entero-enterostomy for the ileum is done to the remaining end (C-D). Anastomosis of the jejunal loop to the tissue at the porta hepatis is done with a posterior and then an anterior row of sutures (E-F). The mesocolic window is closed and then the laparotomy is closed. From Pediatric Surgery Digest, by Zacharias Zachariou (Editor), 2009. Reprinted with kind permission from Springer Nature.

Following PE, adjuvant medical therapy includes ursodeoxycholic acid (UDCA), corticosteroids and antibiotics to prevent complications and improve prognosis [9]. Corticosteroids could theoretically reduce inflammation and therefore delay liver fibrogenesis, but even though data support the beneficial effects of high-dose steroid use on the improvement of bile drainage in the early postoperative period, no statistically significant difference is seen in native or overall survival [45, 82, 83]. Taken orally, UDCA is a hydrophilic bile acid with proven efficacy in primary biliary cirrhosis and sclerosing cholangitis. Despite having beneficial short-term effects also in BA, data advocating its beneficial long-term effects on survival are lacking [9, 66, 84]. Antibiotics are given to prevent episodes of cholangitis, but the specific drug and duration of treatment vary among countries, and their usage is based more on theory and practical experience than on scientific evidence [9, 45, 66, 85]. Growth failure after PE is associated with worse outcomes, and careful nutritional care is important postoperatively [86].

Despite achieving adequate bile drainage in as many as 60% of BA children, of which 80% are likely to survive over 10 years with good quality of life, PE is still considered a palliative treatment [2, 3]. One-fourth of BA patients will reach their 20th birthdays with their native livers [6, 87-90]. Although over half of them have good quality of life and 11–71% may have normal liver biochemistry, nearly all of them have progressive liver fibrosis and develop significant complications related to chronic liver disease or liver failure by the age of 30 [3, 88-94].

If the progressive liver fibrogenesis could be halted or at least delayed by medication, the complication rate would slow down and the need for LTx would be reduced or delayed. The driving forces and the molecular mechanisms of ongoing liver fibrogenesis despite the restoration of biliary drainage are unknown, but it is one of the top priorities in the current BA research field [2, 18, 37, 95].

Table 1. Outcome of biliary atresia. The national reports with the most recent and/or largest data available.

Ref	Country, period	Number of patients (number of PE)	Age at PE (days)[a]	Clearance of jaundice (%)	Primary LTx (%)	Native liver survival (%), years[b]	Overall survival (%), years
[4]	England & Wales 1999–2009	443 (424)	54	55%	3%	46%, 5 y 40%, 10 y	90%, 5 y 89%, 10 y
[6]	France 1986–2009	1107 (1044)	59	38%	4%	40%, 5 y 36%, 10 y 32%, 15 y 30%, 20 y	81%, 5 y 80%, 10 y 79%, 15 y 78%, 20 y
[30]	Switzerland 1994–2004	48 (43)	68	40%	10%	41%, 2 y 33%, 5 y	92%, 2 y 92%, 5 y
[45]	Netherlands 1987–2008	231 (214)	59	36%	3%	46%, 4 y[c]	73%, 4 y[c]
[5]	Finland 1987–2010	72 (64) NA (20)[d]	64 (57)[d]	42% (75%)[d]	NA	38%, 2 y (75%, 2 y)[d]	68%, 2 y (92%, 2 y)[d]
[36]	Sweden 1987–1997	85 (NA)	NA	NA	NA	41%, 2 y	NA
[33]	Germany 2001–2005	183 (159)	57 (mean)	NA	11%	20%, 2 y	83%, 2 y
[25]	Croatia 1992–2006	29 (28)	66	39%	0%	52%, 5 y[c] 39%, 10 y[c]	76%, 5 y[c] 76%, 10 y[c]
[32]	Canada 1985–2002	349 (312)	65	NA	8%	33%, 4 y 24%, 10 y	77%, 4 y 75%, 10 y
[50]	USA 2004–2011	137 (137[c])	59 (mean)	50% (at 3 months)	NA[c]	54%, 2 y	93%, 2 y
[35]	Japan 1989–1999	1381 (1181)	NA (23% 46–60 days)	57%	0.2%	60%, 5 y 53%, 10 y	75%, 5 y 67%, 10 y
[53]	Taiwan 1976–2000	185 (163)	64	60%	0%	35%, 5 y[c] 31%, 10 y[c]	42%, 5 y[c] 40%, 10 y[c]
[199]	South Korea 1995–2009	72 (59)	70	NA	18%	39%, 10 y	95%, 10 y
[208]	Brazil 1982–2008	513 (392)	79	NA	14%	68%, ? y (last follow-up not determined)	37%, 4 y
[66]	Australia 1999–2014	29 (25)	68	29%	14%	46%, 5 y[c]	NA

NA=data not available; [a] Data as median unless otherwise stated; [b] Native liver survival concerning *all* patients, and not only those who underwent PE (unless otherwise stated); [c] Only those who underwent PE were included; [d] After centralization 2005–2010

2.2 Liver injury in BA

2.2.1 Liver injury at the time of diagnosis

In cholestasis, bile pigment accumulates within bile canaliculi and hepatocytes. Accumulating bile acids are toxic to all liver cells, especially hepatocytes. A few weeks after bile flow obstruction, hepatocytes become swollen, and Kupffer cells, the hepatic macrophages, become congested with bile and assemble in cholestatic areas. A distinctive triad of portal changes develops: portal swelling and edema, accumulation of inflammatory cells (especially neutrophils), and an increased number of irregular bile ducts called ductular reactions. Apoptosis of hepatocytes and necrosis develop, which in turn activates hepatic stellate cells (HSCs) to acquire myofibroblastic phenotype and liver fibrogenesis—the abnormal accumulation of the extracellular matrix (ECM)—begins. Biliary epithelial cells (BECs) also contribute to this process by producing fibrogenic cytokines and growth factors. In cholestatic conditions, liver fibrosis accumulates first into portal areas. After fibrous expansion of portal areas, portal-to-portal bridging septae develop and widen, and eventually, strong septae surround and outline groups of hepatocytes called hepatic lobules. This last stage is called cirrhosis [7, 96].

Intrahepatic bile ductules are lined by cholangiocytes (i.e., BECs) [97]. Ductal (or ductular) reactions are reactive processes that arise at the interface of portal and parenchymal areas in acute and chronic liver disease [97, 98]. They are irregular ductules without a regular lumen [99]. Their epithelial cells can vary in origin from proliferating BECs to intermediate hepatobiliary cells, which have features of both BECs and hepatocytes. They can be identified by their expression of cytokeratin (CK): bipotent intermediate hepatobiliary cells express CK-19 and CK-7, whereas only BECs (and not mature hepatocytes) express CK-7 [100]. Their source may vary from local hepatic progenitor cells to hepatocyte-to-cholangiocyte metaplasia and bone marrow–derived circulating multipotent stem cells [42, 96-103]. In this study, the epithelial cells surrounding bile ducts and ductal reactions are called BECs, regardless of their possible origin. Ductal reactions are distinctive to biliary obstruction, and they are known to correlate with portal fibrosis; however, the cause-effect relationship is not established [98, 101, 104]. Ductal plate malformations, which are a part of normal bile ductule formation in the developing fetus, are commonly observed in BA [8, 42, 105].

Hepatocyte-to-cholangiocyte metaplasia refers to the phenomenon of fully matured hepatocytes gaining properties and characteristics usually expressed by BECs and forming ductular reactions or even fully functioning bile ductules [42, 100, 102, 103, 106]. Especially periportal hepatocytes have been shown to express CK-7 [42, 102, 103]. However, even though there is evidence of hepatocyte-to-cholangiocyte metaplasia during normal embryogenesis, some studies question

the full metaplasia of mature hepatocytes and suggest instead that these lobular CK-7 positive cells should be called periportal progenitor cells [96, 100, 107, 108].

In liver biopsies at the time of PE, there is usually a significant inflammatory component. Portal tracts are infiltrated with lymphocytes (CD4$^+$ and CD8$^+$ T-cells and natural killer cells) and Kupffer cells [7, 43, 109]. Portal areas are edematous and ductular cholestasis and ductular reactions are present, while fibrosis is concentrated around the portal instead of lobular areas. The amount of fibrosis depends on the age of the patient, reflecting the duration of obstruction and damaging cholestasis [7, 48, 110-113]. The most accurate histologic findings in differential diagnosis are bile plugs in portal bile ducts, moderate to marked ductular reaction, portal stromal edema and the absence of lobular fibrosis [110, 114]. Advanced fibrosis or cirrhosis have been found in 26–81% of hepatic biopsies during PE, and most of the rest have moderate fibrosis [48, 66, 115-119]. Ductular reactions are abundant and CK-7 positive periportal hepatocytes are present [8, 117, 120].

When liver injury has progressed to end-stage liver failure and the need for LTx, histology is dominated by cirrhosis, moderate cholestasis and ductular proliferation. However, there is usually only a mild chronic inflammatory cell infiltrate in contrast to the time of PE [8, 121].

2.2.2 Liver injury after successful PE

Few studies have examined native liver biopsies after successful PE in patients with normal or near-normal bilirubin levels [8, 74, 89, 119, 122-125]. Two papers did not compare the control biopsies to the histology at PE: *Hadžić et al.* examined liver biopsies from 16 patients at a median age of 27 months and noticed fibrosis present in 95% of them; 54% had mild to moderate fibrosis and 41% cirrhosis. Cholangitis was seen only in 19% of the biopsies [89]. *Laurent et al.* found cirrhosis in 19 of 21 patients with normal bilirubin levels at a mean age of 6.5 years [122].

Tomita et al. compared liver biopsies from 15 patients at PE and after successful PE at a mean age of 9.3 years and found fibrosis to be relieved in 7 patients (47%), persisting in 5 (33%) and progressive in 3 (20%), who also had signs of severe portal hypertension and probable requirement of LTx in the near future [119]. *Altman et al.* correlated the liver histology of 11 eight-month-old patients to the time of PE and discovered the absence of cholestasis, progressive fibrosis and distortion of bile ducts [124]. *Mustard et al.* discovered similar results with progression of fibrosis and reduction of cholestasis in the liver biopsies of four children taken 2–12 months after surgery. There was also a reduction in bile duct proliferation [123]. *Kasai et al.* had quite opposite findings in 1968 when they examined postoperative biopsies from five children and noticed nearly complete recovery of fibrosis and cholestasis. However, the follow-up time when the biopsies were taken was not

revealed [74]. In 1975, *Kasai et al.* examined 14 BA patients at the median age of 5.1 years postoperatively and noticed diminished fibrosis in 8 biopsy specimens (67%), unchanged in 3 (25%) and increased in 1 (8%). Cellular inflammation and bile ductular proliferation were diminished or resolved in 13 (93%) and 12 (86%) biopsies, respectively [125]. *Lampela et al.* examined liver biopsy specimens of 19 patients during PE (median age 63 days) and at 4.2 years after successful surgery. Fibrosis increased in 10 patients (53%) and diminished in 4 (21%). All samples showed decreased or resolved cholestasis and portal inflammation. CK-7 positive periportal hepatocytes tended to increase, while ductular proliferation remained unchanged [8].

2.3 Liver fibrosis in BA

2.3.1 Molecular mechanisms of liver fibrogenesis

The extracellular matrix (ECM) is composed of strictly organized and controlled structural and signaling molecules. In normal circumstances, their synthesis and degradation are critically balanced. In liver fibrosis, this balance is impaired, and the deposition of ECM exceeds its degradation, and ECM molecules— especially collagen I, III and IV and fibronectin—accumulate. ECM is produced by myofibroblasts of heterogenic origin [126, 127]. HSCs are quiescent hepatic cells located between hepatocytes and sinusoidal endothelial cells. Various fibrogenic cytokines and growth factors activate HSCs to create a fibrogenic and contractile phenotype [128]. Their expression of α-smooth muscle actin (α-SMA) is one of the most reliable markers of their activation, and the amount of hepatic α-SMA expression correlates with active fibrogenesis in acute and chronic liver diseases [128, 129]. In liver fibrogenesis, activated HSCs are considered the principle source of fibrotic tissue, but other sources for myofibroblasts exist [127, 130]. Especially in biliary fibrosis, these include portal fibroblasts [10, 130]. In BA at the time of PE and LTx, α-SMA has been found to be expressed, especially in periportal areas and proliferating bile ductules and ductular reactions, and it colocalizes with collagen in fibrous septae and around BECs [131-138]. Activated myofibroblasts accumulate around biliary structures in portal fibrosis, and cross-talk between BECs and myofibroblasts is a crucial part of ongoing fibrogenesis [96, 128, 132]. Other potential sources of myofibroblasts are bone marrow–derived cells, circulating fibrocytes and epithelial-to-mesenchymal transformation (EMT) [130, 139]. EMT refers to the phenomenon of epithelial cells acquiring mesenchymal properties under the appropriate regulatory microenvironment [99].

One of the most profibrogenic cytokines is transforming growth factor beta (TGF-β) [10, 140-142]. The TGF-β superfamily consists of over 30 proteins involved

in normal tissue homeostasis by regulating cellular differentiation, proliferation, apoptosis, motility and adhesion [141, 143, 144]. Three isoforms of TGF-β exist: TGF-β1, TGF-β2 and TGF-β3. Despite having highly similar biological activities *in vitro*, their *in vivo* expression patterns differ [141, 144]. TGF-β1 isoform is usually considered the most important cytokine in liver fibrogenesis [128, 139]. TGF-β2 expression is peculiar to BECs and periportal regions, emphasizing its possible relevance in biliary fibrosis [63, 127, 145, 146].

Figure 4 shows the relation of TGF-β and other growth factors and functions in liver fibrogenesis. TGF-β is the most potent activator of HSCs, turning them into collagen-producing myofibroblasts. It upregulates the collagen genes (*COL1A1* and *COL1A2*) of HSCs and promotes net deposition of ECM proteins also by altering HSC expression of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) [10, 126, 128, 140, 141, 147]. In addition to being profibrogenic, TGF-β can also modulate immune response and has anti-inflammatory and immunosuppressive effects [141, 147, 148].
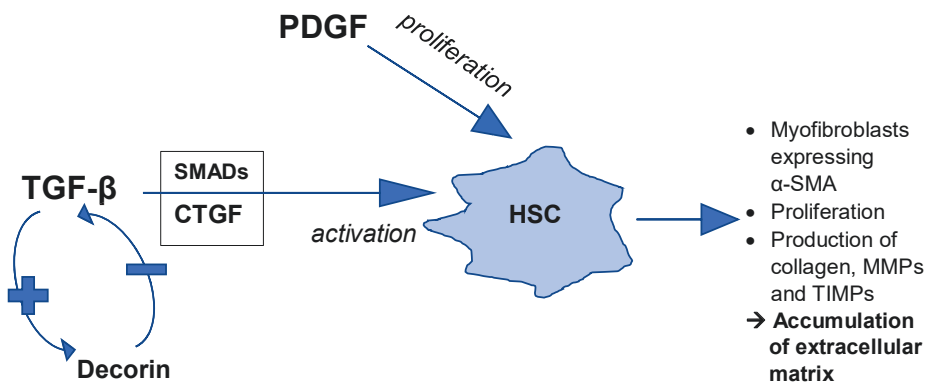

Figure 4. The relation of transforming growth factor-beta (TGF-β) with other growth factors in liver fibrogenesis. TGF-β is a potent activator of hepatic stellate cells (HSCs), which transform into activated myofibroblasts; it performs its actions through down-stream mediators like SMAD proteins and connective tissue growth factor (CTGF). TGF-β promotes decorin expression, which negatively affects TGF-β activation. Platelet-derived growth factor (PDGF) is the most potent mitogen of HSCs. MMP=matrix metalloproteinase; TIMP=tissue inhibitor of matrix metalloproteinase.

TGF-β has many downstream signaling pathways, e.g., SMAD proteins, but it can also execute its actions through other growth factors, like connective tissue growth factor (CTGF), which is greatly induced by TGF-β [130, 140-144, 149]. CTGF regulates proliferation, adhesion, migration and differentiation of many cell types, including HSCs in liver fibrosis [139, 142, 150]. CTGF can be produced by multiple mesenchymal and epithelial cell types, including myofibroblasts, HSCs, hepatocytes and BECs [142].

Decorin is a proteoglycan present in low quantities in a normal liver, but during liver fibrogenesis, TGF-β can upregulate its expression. Studies support its antifibrotic effects by preventing the activation of TGF-β [128, 141, 151-153].

The most potent mitogen for HSC is platelet-derived growth factor (PDGF), which also promotes their activation into myofibroblasts [10, 126-128, 130, 139, 147]. PDGF is upregulated and overexpressed during acute and chronic liver injury and correlates with the degree of fibrosis and inflammation [10, 139, 147]. In addition to platelets, during liver fibrosis, HSCs and BECs are able to produce PDGF, and it is also found to be a major activator of HSC proliferation in cholestatic liver diseases and biliary fibrosis [147].

Matrix metalloproteinases (MMPs) are a group of enzymes that are crucial for balanced ECM homeostasis together with their specific tissue inhibitors (TIMPs). They are involved in many normal biological processes, including embryonic development (and intrahepatic bile duct formation), organ morphogenesis, bone remodeling, angiogenesis and wound healing. However, they are also upregulated during pathological processes, e.g., arthritis, cardiovascular disease, gastric ulcer, and fibrosis of different tissues [154-158]. They can promote tumor progression by enhancing cancer cell invasion and proliferation and hindering apoptosis, and they modulate immune system responses in various inflammatory and repair responses by mediating immune cell migration, leukocyte activation, chemokine processing and antimicrobial defense [155, 157, 159, 160]. Despite acting mostly by degrading collagen and other ECM molecules, MMPs can also mobilize and activate growth factors through the cleavage of carrier proteins [155]. The main sources of MMPs and TIMPs during liver fibrogenesis are activated myofibroblasts, hepatocytes and inflammatory cells [139, 154].

An imbalance of MMPs and TIMPs leads to accumulation of ECM [154, 157, 161]. TGF-β affects their expression, and activated HSCs are known to secrete MMPs and TIMPs [128, 140, 154, 161]. In addition to inhibiting MMPs to degrade ECM molecules, thus enhancing accumulation in liver fibrosis, TIMPs can also inhibit HSC apoptosis [128].

TIMPs are generally considered to be profibrogenic and MMPs antifibrogenic. However, the metalloproteinases can also activate other cytokines and even promote HSC activation and proliferation by altering cell-matrix interactions; thus, their exact role in fibrogenesis is probably multifactorial and not determined by simple matrix breakdown. More significant is the imbalance between MMP and TIMP expression, not the exact amount of different proteinases or their inhibitors [126, 157, 162]. In cholestatic liver injury, TIMP-1, MMP-13 and MMP-7 have been found to be upregulated and coupled with fibrosis [15, 163-168].

MMP-7, also called matrilysin, is normally expressed by exocrine and mucosal epithelial cells throughout the body [160, 169, 170]. MMP-7 is able to degrade fibronectin, gelatinases, elastin and collagen IV, and during inflammatory

processes, it can control neutrophil activation [139, 160, 171]. In normal adult livers, MMP-7 is expressed by BECs but not by fully matured hepatocytes, which can express MMP-7 during early embryonic stages [156, 170]. In ductal cells, MMP-7 is normally expressed in the luminal cell parts, probably to maintain the glandular lumen [170, 171]. MMP-7 may activate latent forms of other MMPs and be more resistant to inhibition by TIMPs than other MMPs [171]. Upregulation of MMP-7 correlates with worsened outcomes in cholangiocellular and hepatocellular carcinomas [172, 173]. Its overexpression is established in hepatic, pulmonary and renal fibrosis [139, 157, 174-177].

2.3.2 Liver fibrogenesis in BA

Georgiev et al. showed that in bile duct–ligated rats, hepatic TGF-β1 gene expression increased rapidly after injury and then decreased when the liver fibrosis stabilized [177]. Liver gene and protein expressions of TGF-β1 and TGF-β2 in human BA patients have been studied at PE and at LTx, when they have been increased compared to healthy controls or patients with neonatal hepatitis–related liver fibrosis or congenital biliary dilatation [11, 13, 178-182]. In BA, TGF-β1 is expressed by Kupffer cells, HSCs and BECs of ductular reactions and hepatocytes adjacent to fibrotic areas. The expression pattern by periportal hepatocytes and BECs is peculiar to BA, highlighting their crucial role in BA liver fibrogenesis [11, 12, 132, 133, 135, 179, 181, 183]. In BA, there is a correlation between TGF-β1 expression and the degree of liver fibrosis and ductular reaction [11, 12, 179]. However, the relation with outcome is different: *Lee et al.* and *de Oliveira et al.* compared hepatic TGF-β1 expression at PE and at LTx and found either no change or decreased expression during the course of the disease, and *Kobayashi et al.* found decreased TGF-β1 expression in patients with persistent jaundice after PE compared to patients after successful PE [181-183]. Studies exploring plasma TGF-β1 expression have found decreased levels at the time of LTx compared to PE, and a relation of increased levels to better outcomes (COJ vs. persistent jaundice) [181, 183-185]. However, in healthy young children, the age is negatively correlated with plasma TGF-β1 expression, and *Rosensweig et al.* did not find a correlation between plasma and hepatic protein expression, indicating that plasma levels might not be a reliable marker of hepatic TGF-β1 expression [11]. Furthermore, serum levels may be grossly contaminated not only by the activation of platelets as a source of TGF-β1 but also by the possible production by other organs and cells, making the interpretation of hepatic activity unreliable [141, 186].

Three papers have studied gene and protein expression of TGF-β2 isoform in BA during PE and LTx. It localized to myofibroblasts, hepatocytes and BECs. Differences in expression at the time of PE compared to LTx and their relationship to fibrosis are controversial [13, 134, 182]. *Lee et al.* studied plasma TGF-β2 expression

and did not find any difference between BA patients and healthy controls at the time of LTx and PE [182].

The hepatic upregulation of CTGF in BA at the time of PE and LTx resembles TGF-β1. CTGF is produced by activated myofibroblasts, inflammatory cells, hepatocytes and BECs, the latter being peculiar to BA compared to healthy controls and those with other chronic liver disease [12, 187, 188]. Plasma CTGF levels after PE have also been higher compared to healthy controls [149, 189]. How CTGF liver expression and plasma values relate to BA liver fibrosis and outcomes is controversial [12, 149, 185, 187-190].

Four studies have explored the hepatic protein and gene expression of PDGF in BA during PE and LTx. Its expression is increased compared to healthy controls, and it localizes to inflammatory cells, myofibroblasts, hepatocytes and BECs, the latter being peculiar to BA [13, 134, 179, 191]. *Faiz Kabir Uddin Ahmed et al.* found increased expression at the time of PE compared to LTx, while *Malizia et al.* found a positive correlation to stage of fibrosis and ductular reactions [13, 179].

In studies with experimental BA animal models, a steady increase in hepatic expression of MMP-7 and TIMP-1 has been found [162, 166, 177, 192-194]. In BA patients at the time of PE and LTx, TIMP-1 expression is upregulated compared to healthy controls with controversial results of correlation to the stage of fibrosis [165, 178, 195, 196]. MMP-2 is increased and MMP-9 decreased at the time of LTx compared to healthy controls, but no statistically significant correlation to histologic fibrosis has been found [15, 134, 163-165, 195, 196].

Increased hepatic protein and gene expression of MMP-7 has been repeatedly associated with BA liver fibrosis [15, 163, 165]. Its expression is also increased compared to neonatal intrahepatic cholestasis and parenteral-nutrition–related biliary cirrhosis [164, 197]. *Huang et al.* explored the localization of MMP-7 protein and gene expression at the time of PE and LTx and found that it is expressed in Kupffer cells, hepatocytes and BECs in both healthy controls and BA patients. The expression was significantly increased in BA compared to controls, and it correlated with the stage of fibrosis [15]. *Iordanskaia et al.* compared 47 BA patients with fibrotic and inflammatory gene signature at the time of PE and found a twofold increase in MMP-7 gene expression in the former group [163]. *Lertudomphonwanit et al.* noted an increased MMP-7 protein and gene expression in BA at the time of PE compared to healthy controls and those with other neonatal liver diseases, and expression was localized mostly to BECs [14].

MMP-7 serum levels were upregulated in two different studies in BA at the time of PE compared to healthy controls and those with age-matched neonatal intrahepatic cholestasis; diagnostic sensitivity was 98% and specificity 95%. MMP-7 was suggested as a potential diagnostic biomarker for BA. At the time of PE, serum levels of MMP-7 did not correlate with histologic fibrosis [14, 198].

2.4 Outcome

2.4.1 Clearance of jaundice, overall survival and native liver survival

Several measures are used to evaluate treatment outcomes: median age at PE, COJ, native liver survival and overall survival. Median age at PE reflects the primary care practitioners' awareness to diagnose BA early and the effectiveness of diagnostic procedures to make early operation possible. COJ measures the efficacy of surgery. Native liver survival reflects not only the success rate of PE but also the long-term postoperative (medical) treatment and regular follow-up of the patients. Additionally, overall survival depicts the availability and outcomes of LTx [22].

Table 1 shows the national reports with the most recent and/or largest data available (age at PE, primary LTx, COJ, native liver and overall survival rates) from 13 countries worldwide. Median age at PE is 54–79 days, and in most European countries, it has been around 60 days in recent years. Of operated children, 29–75% achieve COJ, with the usual rate around 40%. The percentage of primary LTx varies among countries from nearly 0% (Taiwan, Japan and Croatia) to 18% (South Korea). In Japan, deceased donor LTx was legalized only in 1999, even though living donor LTx was available 10 years earlier [90]. Five- and ten-year native liver survival rates vary from 33% (Switzerland) to 60% (Japan) and from 24% (Canada) to 53% (Japan), respectively. Overall survival rates 5 and 10 years after birth are from 42% (Taiwan) to 92% (Switzerland) and from 40% (Taiwan) to 95% (South Korea), respectively. In most European countries, COJ rates over 40%, 5-year native liver survival over 40% and 5-year overall survival over 85% are achieved (Table 1).

2.4.2 Predictive factors

The most confirmed predictive factor for better native liver survival is the rate of COJ after PE; i.e., when the PE is successful, the likelihood of surviving longer with a native liver is higher [30, 33, 45, 50, 53, 54, 111, 113, 118, 199-203].

One of the most studied possible factors concerning the outcome in BA is the age at PE. Theoretically, the younger the patient is during the corrective surgery, histologic changes in the liver would also be milder and therefore the outcome would be better. Some studies support the positive correlation between age and hepatic fibrosis at the time of PE [48, 110-113]. Although many studies support the protective role of younger age at PE, there is controversy concerning the critical cut-off age, and 30, 45, 60 or even 90 days have been suggested [6, 29, 30, 32, 35, 45, 78, 79, 199, 203-208]. In Taiwan, a screening system with a stool color card was implemented nationally, and the rate of performing PE before 60 days of age raised from 49% to 66%, leading to an improved COJ rate from 35% to 61%, a

five-year jaundice-free native liver survival rate from 27% to 64%, and an overall survival rate from 56% to 89% [209].

BASM has often been found to have a negative effect on outcomes including COJ, native liver and overall survival [6, 62, 90, 205, 210-212]. However, not all studies have found a difference in survival rates between isolated and BASM patients [5, 30, 45, 47, 59, 65, 67, 213]. One explanation might be the different definitions of BASM among the studies. Some studies have shown a decreased age at PE in BASM [4, 69] while others have not [49, 62, 67]. The degree of histologic liver fibrosis at the time of PE appears comparable to isolated BA [64, 67]. Two studies have found decreased COJ and overall survival rates among syndromic patients (i.e., patients with any kind of congenital anomaly) [4, 50], while there was no difference in outcomes between these two groups in other studies [36, 49, 51, 67].

Concerning how the amount of histologic fibrosis or ductular reaction at the time of PE affects outcomes, the available data are controversial, which may be a result of variable analytical methods and patient age [8, 48, 66, 111-113, 115-118, 120, 124, 213, 214]. A positive correlation between hepatic collagen and α-SMA expression during PE and outcome have been found in multiple studies [111, 112, 131, 138, 181]. Concerning inflammation, *Lampela et al.* did not find a correlation between histologic inflammation at PE and native liver survival by two years [8]. However, *Azarow et al.* did find a correlation with the amount of lobular inflammation and failure of PE, and *Davenport et al.* noticed that a decreased amount of macrophages correlated with better outcomes postoperatively [109, 215].

2.4.3 Concentration of care

Considering the rarity of BA, it would be logical to achieve improvements in surgical expertise and multi-professional care and follow-up if the care of BA patients were centralized to selected expert centers. The first paper supporting this was published in 1985 in the United Kingdom; 114 patients were treated in 16 different centers during 1980–1982. There was a statistically significant improvement in COJ rates in those centers, which treated over five cases a year (43%) compared to those with only one case a year (11%) [216]. Two further studies have affirmed these results: in 1993–1995, slightly fewer than 100 BA patients were treated in the UK in 15 different centers, with only two centers treating over five cases a year. Those centers had significantly better results in 5- and 13-year native liver survival rates (61% and 54%) compared to those with less than five cases a year (14% and 27%). Overall survival was significantly better after 5 years but not after 13 years, which might reflect the already established centralization of LTx [47, 200]. After these findings, the British government centralized the treatment of BA patients to three centers in 1999, which all treated over five cases (7–20) a year during 1999–2009 with comparable results [4, 210].

In France, the population and incidence of BA are roughly the same as in the UK. In 1999, a French study group published results of national BA treatment during 1986–1996. At that time, there were 32 different centers caring for BA patients, with 29 treating less than two cases a year and only one center treating over 20 cases (the remaining two treated 3–5 cases a year). Both native liver survival and overall survival were significantly improved with a growing center caseload [212]. Subsequently, the French Observatory of BA was created, and all centers were encouraged to actively collaborate with more experienced units. Since then, the native liver survival rates have improved in centers with lower caseloads, and no effect of center size on outcomes was observed anymore in cohorts 1997–2002 and 2003–2009. However, the 10-year native liver survival rate did improve only in centers that treated 3–5 patients a year (from 28% to 42%) [6, 211].

In Germany, 183 BA patients were treated in 29 clinics in 2001–2005, with the majority of hospitals (n=22) treating less than five cases during the study period. Two-year native liver survival improved from 8% to 26% and overall survival from 74% to 84% when compared to centers that treated over five cases during that time [33].

In Finland, the treatment of BA patients was centralized from five university hospitals to one in 2005. *Lampela et al.* studied cohorts 1987–2005 and 2005–2010 and found that COJ, two-year native liver and overall survival improved significantly (from 42% to 75%, 38% to 75%, and 68% to 92%, respectively) after centralization [5]. In a multicenter retrospective observational study of six centers in the Nordic countries, COJ rate was 64%, and cumulative 5-year native and overall survival rates were 53% and 88%, respectively. The annual caseload of over three patients a year predicted both early performance of PE and long-term native liver survival [203].

In Canada, 230 BA patients were treated in 12 university hospitals in 1992–2002. There was no difference in native liver and overall survival rates when the one center treating over five cases a year was compared to other centers with less than five patients (six treated less than one patient a year). It was concluded that the treatment is already centralized to academic hospitals, and because of the low population density, centralization to a lesser number of hospitals could cause delays and logistic problems [217].

In Switzerland, where the population is less than 15% of that in France and the UK, five surgical centers treated 43 BA patients in 1994–2004 (two treated 1 case each, one 6 cases, one 14 cases, and one treated 21 cases). No statistically significant difference was found between these five centers concerning the rate of four-year native liver survival [30].

Collectively, the available data suggest that the concentration of BA care results in improved outcomes, which are reachable with a minimum of 3–5 patients per year.

2.4.4 Future improvements in outcome

BA is the most common indication for pediatric LTx worldwide. Even after the successful restoration of bile flow after PE, the progression of liver fibrosis leads to ensuing complications and ultimately liver failure usually before adulthood. The development of antifibrogenic therapy to postpone or reduce the need for LTx would further improve native liver and overall survival rates and reduce morbidity related to transplantation. The invention of such therapy requires knowledge of the underlying molecular pathobiology of progressive liver fibrosis after successful PE and recognition of subgroups of possibly different etiopathogenesis.

3   AIMS OF THE STUDY

The general aim of this thesis was to investigate the pathobiology underlying liver fibrogenesis after successful PE and to address predictors of survival.

The specific aims were:

1) To explore mediators of fibrogenesis after successful PE (I,II).

2) To investigate the hepatic expression of MMP-7 and other matrix metallo-proteinases after successful PE, their correlations to fibrosis, and their possible roles as serum markers of liver fibrosis after successful PE (III).

3) To address predictors of COJ, native liver survival and overall survival in BA, and investigate predictors of outcome (IV).

4) To examine possibly different outcomes and hepatic expression of fibrogenic cytokines in syndromic and isolated BA patients.

4   PATIENTS AND METHODS

4.1 Patients and clinical data

Study subjects included BA patients treated at Helsinki University Hospital in Finland during 1991–2013 (studies I–II), 1991–2011 (study III), and 1987–2016 (study IV). For studies I–III, only those who cleared their jaundice (serum bilirubin fell below 20 μmol/L after PE) were included. Tables 2 and 3 show the study design outline and patient characteristics. Treatment of BA patients in Finland was centralized to Helsinki University Hospital in 2005, and management and follow-up protocol were standardized, as depicted in detail in study IV. Hospital records were reviewed for laboratory tests, imaging studies, operative findings, and outcomes (Table 4). The AST to platelet ratio index (APRI) was calculated as depicted in Table 4. Syndromic BA was defined as the presence of any congenital structural malformation, and BASM as the presence of polysplenia or asplenia [62, 64]. Anomalies were recorded retrospectively from all available imaging studies (x-ray, ultrasound, magnetic resonance imaging) and intraoperative findings. Splenomegaly was defined as spleen length over two standard deviations above the reference value of age- and gender-matched healthy children [218]. Portal hypertension was defined by endoscopic verification of esophageal varices or the presence of splenomegaly and thrombocytopenia (platelet count below 150 x 10^9/L) [219].

4.2 Controls

For studies I–III, as depicted in Table 2, 10 children [median age 11.4 (interquartile range IQR 7.8–14.8) years] undergoing operation for complicated cholelithiasis with (n=3) or without liver disease were used as non-fibrotic controls for ribonucleic acid (RNA) expression analyses. 19 (studies I–II) or 14 (studies III) donor liver biopsies were used as controls for immunohistochemical studies, with a median age of 15 (8–16) or 14.2 (8.0–16.2) years, respectively. As fibrotic controls, intestinal failure patients with associated liver disease were used for ribonucleic acid (RNA) (studies I–III), immunohistochemical (studies I,III) and serum analyses (study III) [11 for studies I–II, age 4.7 (3.5–9.7) years and 10 for study III, age 4.6 (3.0–8.4) years]. Blood samples from healthy controls were obtained from 47 [study II, age 6.5 (4.2–12.6) years] or 78 [study III, age 8.5 (4.5–14) years] day-surgery patients without any evidence of hepatobiliary, metabolic or endocrinological diseases.

Table 2. Study design outline.

	Study I	Study II	Study III	Study IV
Main issue	Expression of profibrotic and inflammatory mediators after successful PE	Expression of TGF-β superfamily cytokines after successful PE	Expression of MMPs and TIMPs after successful PE	Effect of centralization on BA outcomes and predictive factors of COJ and survival
Study design	Cross-sectional	Cross-sectional	Cross-sectional	Prospective / retrospective, observational
Inclusion criteria	Of the BA patients (n=51) treated in Helsinki University Hospital between 1991 and 2013, those who cleared their jaundice after PE were included	Of the BA patients (n=51) treated in Helsinki University Hospital between 1991 and 2013, those who cleared their jaundice after PE were included	Of the BA patients (n=46) treated in Helsinki University Hospital between 1991 and 2011, those who cleared their jaundice after PE were included	BA patients treated in Helsinki University Hospital between 1987 and 2016 with at least 4-month follow-up
BA patients	28	28	25	61
Controls	10 non-fibrotic liver biopsies (RNA) 19 non-fibrotic donor liver biopsies (immuno-histology) 11 fibrotic controls (RNA, immunohistology)	10 non-fibrotic liver biopsies (RNA) 19 non-fibrotic donor liver biopsies (immuno-histology) 11 fibrotic controls (RNA) 47 non-fibrotic serum controls	10 non-fibrotic liver biopsies (RNA) 14 non-fibrotic donor liver biopsies (immuno-histology) 10 fibrotic controls (RNA, immunohistology, serum) 78 non-fibrotic serum controls	-

BA=biliary atresia; PE=portoenterostomy; TGF-β=transforming growth factor-beta; MMP=matrix metalloproteinase; TIMP=tissue inhibitor of matrix metalloproteinase, RNA=ribonucleic acid;

Table 3. Patient characteristics.

	Study I-II	Study III	Study IV
Patients (n)	28	25	61
Age at PE	61 (40–84) days	2.0 (1.3–2.9) months	64 (39–85) days
Age at follow-up	3.0 (2.1–6.7) years	3.3 (2.1–7.4) years	-
Male (n,%)	14 (50%)	13 (52%)	28 (46%)
Anomalies (n,%)	12 (43%)	9 (36%)	22 (36%)
BASM (n,%)	8 (29%)	-	10 (16%)
Splenomegaly (n,%)	9 (32%)	9 (36%)	-
Portal hypertension (n,%)	14 (50%)	13 (52%)	-

PE=portoenterostomy; BASM=biliary atresia splenic malformation syndrome

4.3 Liver biopsies and hepatic protein expression analyses

Liver biopsies obtained at PE and at follow-up for studies I–III are depicted in Table 4. All follow-up liver biopsies were taken percutaneously with ultrasound guidance under general anesthesia for endoscopic variceal surveillance and were part of the routine follow-up liver biopsies for BA patients initiated in 2005 and performed 1, 5, and 10 years after PE and at transition or when clinically indicated. Biopsies were fixed in formalin, embedded in paraffin, sliced and stained with H&E and other conventional stains, CK-7 (analyzed with SP52 monoclonal antibody and ultraView Universal DAB Detection Kit, Ventana, Tucson, AZ) and specific immunohistologic stains: MMP-7, TIMP-1, Collagen 1, α-SMA, TGF-β1, TGF-β2, CTGF and decorin (Tables 5 and 6, and specific instructions for MMP-7 and TIMP-1 analyses in study III). Two experienced pediatric pathologists blinded to clinical data analyzed conventional histology and CK-7 immunoreactivity semiquantitatively until consensus was reached (Table 5). Antibodies and dilutions used for specific immunohistologic stains are depicted in Table 6. Anna Kerola performed semiquantitative grading for those with a Leica DM RXA Microscope (Leica Microsystems GmbH, Wetzal, Germany), as depicted in Table 7 In studies I–II, for CK-7, α-SMA and Collagen 1, the area fraction (proportion of the antibody-positive area to the entire biopsy area) was calculated with ImageJ Image Analysis Software (SciJava Common open source software; Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA; http://imagej.nih.gov.ij/; 1997–2014).

Table 4. Laboratory values and liver histology at portoenterostomy (PE) and at follow-up.

	Scale/unit	Study I-II (n=28)		Studies III (n=25)	
		at PE	at follow-up	at PE	at follow-up
Liver biochemistry					
Bilirubin total	µmol/L	159 (116–204)[d]	10 (4–17)	159 (116–204)[d]	10 (4–17)
Bilirubin direct/conjugated	µmol/L	115 (83–159)[d]	4 (2–8)	115 (83–159)[d]	4 (2–8)
Gamma-glutamyl transferase (GGT)	U/L	-	-	-	-
Bile acids	µmol/L	143 (73–197)[d]	32 (16–92)	143 (73–197)[d]	32 (16–92)
Prealbumin	mg/L	-	-	-	-
Alanine aminotransferase (ALT)	U/L	87 (42–164)[d]	45 (24–94)	87 (42–164)[d]	45 (24–94)
APRI[a]		0.83 (0.42–1.41)	1.18 (0.45–1.95)	0.83 (0.42–1.41)	1.18 (0.45–1.95)
Liver histology (n)		24	28	24	28
Metavir fibrosis	0–4	2 (2–3)	2 (1–4)	2 (2–3)	2 (1–4)
Portal fibrosis	0–4	-	-	-	-
Intracanalicular cholestasis	0–3	2 (1–3)[d]	0 (0–0)	2 (1–3)[d]	0 (0–0)
Bile ductular cholestasis	0–3	-	-	-	-
Intracellular cholestasis	0–3	-	-	-	-
Ductal proliferation[b]	0–2	-	-	-	-
Cytokeratin-7 expression of periportal hepatocytes	0–4	-	-	-	-
Ductal reaction[c]	%	5.0 (3.3–7.0)	2.4 (1.8–4.9)	5.0 (3.3–7.0)	2.4 (1.8–4.9)
Portal inflammatory cell infiltrate	0–3	2 (2–3)[d]	1 (0–1)	2 (2–3)[d]	1 (0–1)

[a] APRI=[Aspartate aminotransferase (AST, U/L)/50/Platelet count (E9/L)]; [b] Ductal proliferation analyzed using cytokeratin-7 immunostaining; [c] Cytokeratin-7 expression in proliferative bile ductules, adjacent periportal hepatocytes, and bile ducts; [d] P <0.05 in Wilcoxon signed-rank test between PE and follow-up

Table 5. Grading and staging of histologic findings in liver biopsies.

Histologic / immunohistologic finding	Grade/ scale	Definition
Metavir fibrosis reference [225]	0	No fibrosis
	1	Portal fibrosis without fibrous septae
	2	Porto-portal fibrous septae
	3	Porto-portal and porto-central fibrous septae
	4	Cirrhosis
Portal fibrosis	0	Absent or fibrous expansions of some portal areas
	1	Fibrous expansions of most portal areas
	2	Focal portal-to-portal bridging
	3	Marked portal-to-portal bridging
	4	Cirrhosis
Intracanalicular, bile ductular and intracellular cholestasis	0	Absent
	1	Minimal
	2	Marked
	3	Prominent
Portal inflammatory cell infiltrate	0	Absent or minimal
	1	Mild
	2	Moderate
	3	Marked
Cytokeratin-7 positive ductal proliferation	0	Absent
	1	Present
	2	Prominent
Cytokeratin-7 expression in periportal hepatocytes	0	Absent
	1	Rare
	2	Present
	3	Prominent
	4	Extensive

Table 6. Antibodies for immunohistological analyses in studies I-III.

Molecule	Dilution	Antibody (clone)	Manufacturer
MMP-7	1:1500	141-72	Merck Millipore, Merck KGaA, Damstradt, Germany
TIMP-1	1:50	63515	R&D Systems, Minneapolis, MN
Collagen 1	1:1000	1-8H5	Abnova corporation, Taipei City, Taiwan
α-SMA	1:200	1A4	Dako-Cytomation, Glostrup, Denmark
TGF-β1	1:50	TGFB17	Leica Novocastra, Leica Biosystems, Nussloch, Germany
TGF-β2	1:1000	MGC116892	Abnova corporation, Taipei City, Taiwan
CTGF	1:400	L-20	Santa Cruz Biotechnology Inc., Dallas, Texas, USA
Decorin	1:750	NBP1-84970	Novus Biologicals, LLC, Littleton, CO, USA

MMP=matrix metalloproteinase; TIMP=tissue inhibitor of matrix metalloproteinase;
α-SMA=alfa smooth muscle actin, TGF-β=transforming growth factore beta;
CTGF=connective tissue growth factor

Table 7. Grading of immunohistologic findings in liver biopsies.

Immunohistologic finding	Grade	Definition
MMP-7 staining in biliary epithelial cells *Also staining of periportal hepatocytes [a]*	0 1 2 3 4	No staining Staining only at apical/luminal side Completely stained with weak intensity Completely stained with moderate intensity Completely stained with strong intensity
TIMP-1 *Also staining of periportal hepatocytes [a]*	0 1 2 3	No staining Staining of individual spindle-shaped stromal cells at portal or parenchymal areas Staining in less than 30% of hepatocytes Staining in over 30% of hepatocytes
Collagen 1	0 1 2 3 4	Slight expression at portal areas Expanded expression at portal areas With portal-to-portal septaes With portal-to-portal and portal-to-central septaes Extended nodular expression (cirrhosis)
α-SMA (modified from reference [226]) *Also periductal staining surrounding bile ducts and ductal proliferation [a]*	0 1 2 3 4	Staining in smooth muscle cells within portal vessel walls only Mild or moderate periportal expression With mild or moderate bridging between portal tracts With strong bridging between portal tracts Marked portal expression in the enire portal area with portal bridging
TGF-β1 *Also staining of periportal hepatocytes [a] and localization [b]*	0 1 2 3	No staining Mild staining at fibrotic and lobular areas Moderate staining at fibrotic and lobular areas Strong staining in fibrotic and lobular areas
TGF-β2 *Also staining of bile duct epithelial cells and periportal hepatocytes [a]*	0 1 2	No staining Mild staining Moderate staining
CTGF *Also staining of periportal hepatocytes [a] and localization [b]*	0 1 2 3	No staining Mild staining at fibrotic and lobular areas Moderate staining at fibrotic and lobular areas Strong staining in fibrotic and lobular areas
Decorin	0 1 2 3 4	Slight expression at portal areas Expanded expression at portal areas With portal-to-portal septaes With portal-to-portal and portal-to-central septaes Extended nodular expression

[a] Dichotomus grading: 0=no staining, 1=staining; [b] Most staining in 0=fibrotic, 1=parenchymal, or 2=both areas. MMP=matrix metalloproteinase; TIMP=tissue inhibitor of matrix metalloproteinase; α-SMA=alfa smooth muscle actin; TGF-β=transforming growth factor beta; CTGF=connective tissue growth factor.

4.4 Gene expression analyses

For studies I–III, gene expression analyses were performed by embedding liver tissue specimens in RNAlater solution (Ambion, Life Technologies, Thermo Fisher Scientific Inc, Waltham, MA) and freezing them until analyzed. RNA was extracted with the RNeasy Mini Kit (QIAGEN, Frederick, Maryland, USA). Integrity of RNA was assessed spectrophotometrically. Human Fibrosis RT² Profiler PCR Array (QIAGEN SABiosciences, Frederick, MD) on an ABI 7700 Sequence Detection System (Perkin-Elmer Life Sciences, Boston, MA) was used to analyze gene expression in triplicate by a quantitative real-time polymerase chain reaction, according to the manufacturer's directions. Quantification of target gene mRNA expression was performed using the ΔΔCt Method [220] and expressed after normalization to housekeeping genes (*B2M, HPRT1, RPL13A, GAPDH, ACTB*) and relative to control subjects. Genes analyzed for study I were *COL1A2, ACTA2, PDGFA, IL1A, IL1B, IFNG, TNF, IL4, IL5, IL10*, for study II *TGFB1, TGFB2, TGFB3, TGFBR1, TGFBR2, SMAD2, SMAD6, CTGF, DCN* and for study III *MMP-1, -2, -3, -7, -8, -9, -13, -14, TIMP-1, -2, -3, -4.*

4.5 Serum analyses

For studies I and III, the serum concentrations of TGF-β2, decorin, MMP-7, MMP-9 and TIMP-1 were determined with commercially available enzyme-linked immunosorbent assay (ELISA) kits, and analyses were carried out according to the manufacturer's protocol as follows: Biovendor R&D (Bratislava, Slovakia) for TGF-β2 and decorin, The Quantine® kit (R&D Systems, Inc., Minneapolis, MN) for MMP-7 and Buotrak ELISA systems (Amerisham Biosciences UK, Ltd., Buckinghamshire, UK) for MMP-9 and TIMP-1. Time-resolved immunofluorometric assay (Medix Biochemica, Kauniainen, Finland) was used for MMP-8 analyses. The inter assay coefficient of variations (CV%) and detection limits are depicted in detail in studies I (for MMP-7, -8, -9 and TIMP-1) and III (for TGFβ2 and decorin).

4.6 Statistical analyses

All data are expressed as median and interquartile range (IQR) unless otherwise stated. In study IV, categorical data are expressed as frequencies. Nonparametric tests were used for comparison. Pairwise comparisons of continuous variables were performed using the Mann-Whitney *U* test (for independent variables) or the Wilcoxon signed-rank test (for repeated measurements). Independent multiple comparisons were analyzed using the Kruskall-Wallis test. Dichotomous variables

were compared using the McNemar test (for repeated measurements) and Fisher's exact test (for independent variables), the latter also being used to compare frequencies between groups. Correlations were calculated using Spearman's rank correlation. For study I, predictive values were perceived as the area under the receiver operating characteristics curves (AUROC). For study IV, linear regression was used to evaluate predictors of bilirubin levels at 3 months after PE, the follow-up Metavir score, and fibrosis progression, and binary logistic regression was used for COJ odds ratios (OR) with 95% confidence intervals (CIs). Those predictors, which showed significant association with outcomes variables in simple regression were included in multiple regression analyses. Kaplan-Meier curves were used to analyze cumulative native liver and overall survival rates, and univariate predictors of survival were evaluated with the log-rank test. Multivariate survival models were performed by generating hazard ratios with 95% CIs with the Cox proportional hazards regression model, adjusted for statistically significant variables from univariate models and for age at PE for native liver survival. In all studies, a P value below 0.05 was considered statistically significant. Statistical analyses were performed with SPSS software (IBM Corp., Armonk, NY, USA).

4.7 Ethical considerations

The study protocol was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa (number 345/13/03/03/2008) and by the Finnish National Authority of Medicolegal Affairs and Health. In all studies, ethical guidelines of the 1975 Declaration of Helsinki were obeyed, and informed consent was obtained from all participating adults and children's legal guardians before any procedures.

5   RESULTS

5.1   Evolution of liver histology after successful PE (I,II,III,IV)

During three years after successful PE, serum bilirubin remained low (Table 4). Despite the resolution of biochemical and histologic cholestasis, histologic liver fibrosis and ductal proliferation persisted, while inflammation decreased (Figure 5, Table 4, studies I–IV). CK-7 expression of periportal hepatocytes was enhanced after PE, and the amount of CK-7 positive ductal reactions correlated with Metavir fibrosis stage (Table 4). The progression of fibrosis was more pronounced when COJ was not achieved (IV). Age did not correlate with the liver fibrosis stage at follow-up.

At the time of follow-up, BA patients with congenital anomalies (n=12) had significantly lower Metavir fibrosis stage and portal inflammation grade than isolated patients (I–II). Despite a similar trend, the difference in Metavir fibrosis stage was not statistically significant in patients with BASM compared to those without (I). Metavir fibrosis stage increased after successful PE only in isolated patients but not in syndromic ones (II). Similarly, the magnitude of ductal reaction decreased only in syndromic patients but not in isolated BA patients (II).
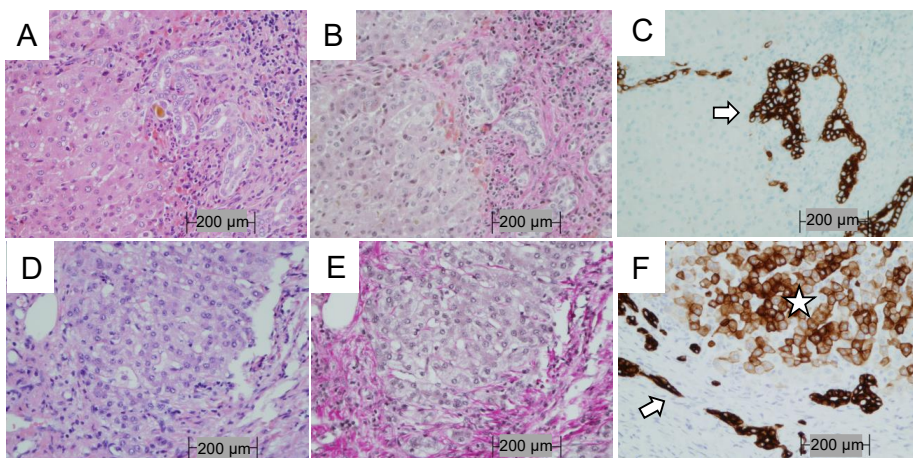
Figure 5. After portoenterostomy (PE), the degree of fibrosis and periportal hepatocyte-cholangiocyte metaplasia increased and ductal reaction persisted in all patients, while portal inflammation decreased only after successful PE. Representative native liver histology from the same patient (**A**, **B**, **C**) at PE (17 days old) and (**D**, **E**, **F**) 2.4 years after successful PE (200 x magnification). (**A**, **D**) Portal inflammation (0–3) reduced from grade 3 to 1 (hematoxylin and eosin). (**B**, **E**) Metavir fibrosis stage (0–4) increased from 2 to 4 (Herovici stain). (**C**, **F**) Ductal reaction (0–2, arrow, dark brown) increased from grade 1 to 2, and hepatocyte-cholangiocyte metaplasia in periportal hepatocytes (0–4, asterisk, light brown) from 0 to 4 (cytokeratin-7 stain). Pictures reprinted from study IV with kind permission from the publisher.

5.2 Expression of profibrogenic and inflammatory mediators (I,II)

After successful PE, protein expressions of collagen 1 and α-SMA were increased compared to fibrotic and non-fibrotic controls (Figure 6). In BA, collagen staining was seen at fibrotic portal areas and septae, while α-SMA concentrated to portal and periductal areas. There was no difference in the expression of collagen 1 and α-SMA between PE and follow-up (Table 8, I). The intensity of CK-7 positive ductal reactions correlated with both collagen and α-SMA expression.
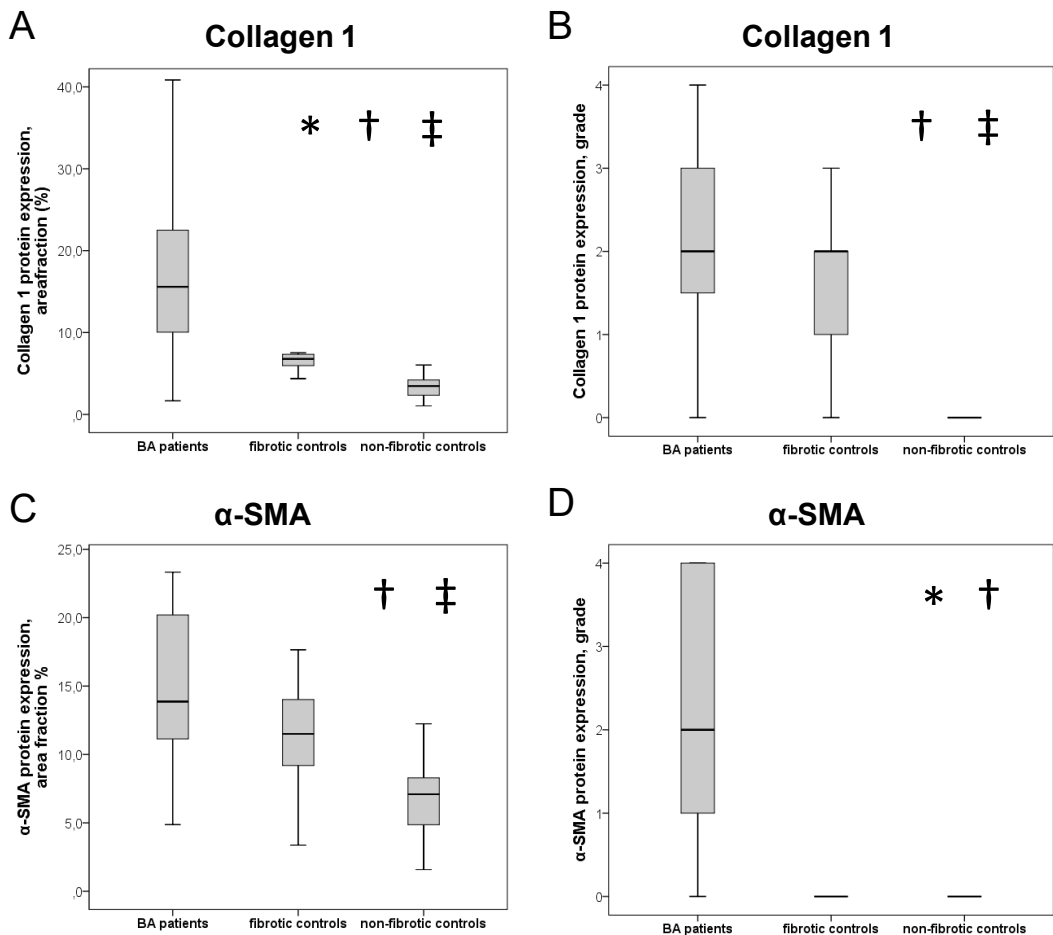
Figure 6. Hepatic protein and gene expression of collagen 1 (A–B) and alfa-smooth muscle actin (α-SMA) (C–D) 3.0 years after successful portoenterostomy in biliary atresia (n=28) compared to fibrotic (n=11) and non-fibrotic (protein n=19, gene n=10) pediatric controls. Box plots display median (bold transverse line), interquartile range (rectangle) and range. Significance evaluated by the Mann-Whitney U test for each group. P<0.05: * BA vs. fibrotic controls, † BA vs. non-fibrotic controls, ‡ fibrotic vs. non-fibrotic controls. n=28.

Table 8. Evolution of liver fibrosis and ductal reaction in relation to collagen expression, myofibroblast activation (α-SMA) and profibrotic cytokine expression (studies I and II).

	At PE n=24	At follow-up n=28	P-value
Metavir, stage (0–4)	2 (2–3)	2 (1–4)	0.170
Ductal reaction, area fraction (%)	5.0 (3.3–7.0)	2.4 (1.8–4.9)	0.227
Collagen, grade (0–4)	3 (2–3)	2 (1–3)	0.210
Collagen, area fraction (%)	17.4 (12.6–22.4)	15.6 (10.0–22.5)	0.484
α-SMA, grade (0–4)	3 (2–3)	2 (1–4)	0.833
α-SMA, area fraction (%)	16.8 (13.7–21.3)	13.9 (11.1–20.5)	0.783
TGF-β1, grade (0–3)	2 (2–3)	1 (0–1)	**<0.001**
TGF-β2, grade (0–2)	1 (1–1)	1 (0–1)	0.09
CTGF, grade (0–3)	2 (2–3)	1 (0–1)	**<0.001**
Decorin, grade (0–4)	2 (2–3)	2 (1.5–2.5)	0.409

Data are median (interquartile range). Significance between groups evaluated by Wilcon Signed rank test.

TGF-β=transforming growth factor beta; CTGF=connective tissue growth factor

Periductal α-SMA staining surrounding bile ducts and ductal proliferations was unique to BA compared to fibrotic and non-fibrotic controls. All histologic liver samples at PE stained positively with periductal α-SMA at the time of PE, but it persisted in 64% of follow-up samples and correlated positively with immunohistologic staining of collagen, α-SMA, the amount of ductal reaction, and plasma levels of total and direct bilirubin and bile acids.

In BA, gene expression of *COL1A2* (encoding for collagen 1), *ACTA* (encoding for α-SMA) and *PDGFA* (encoding for PDGF subunit A) were upregulated compared to non-fibrotic controls, as well as those of *COL1A2* and *PDGFA* when compared to fibrotic controls (Figure 7). The upregulation of *PDGFA* was positively correlated to collagen area fraction, α-SMA grade and presence of periductal α-SMA expression. RNA expression of several inflammatory cytokines (*IL1A, IL1B, IFNG, TNF, IL4, IL5, IL10*) was comparable or lower when compared to either fibrotic or non-fibrotic controls.

After successful PE, hepatic protein expression of TGF-β1, TGF-β2, CTGF and decorin were all increased compared to non-fibrotic controls (II). Compared to PE, follow-up expression of TGF-β1 and CTGF, but not that of TGF-β2 and decorin, decreased significantly (Table 8, II). Lobular hepatocytes expressed both TGF-β1 and TGF-β2, and the latter was also expressed by periportal hepatocytes, especially at follow-up. BECs did not express TGF-β2 at PE, while one-fourth expressed it during follow-up (P=0.125). CTGF localized to spindle-shaped periportal cells and lobular hepatocytes, especially periportal ones at PE compared to follow-up. Decorin expression localized to portal areas and fibrotic septae.
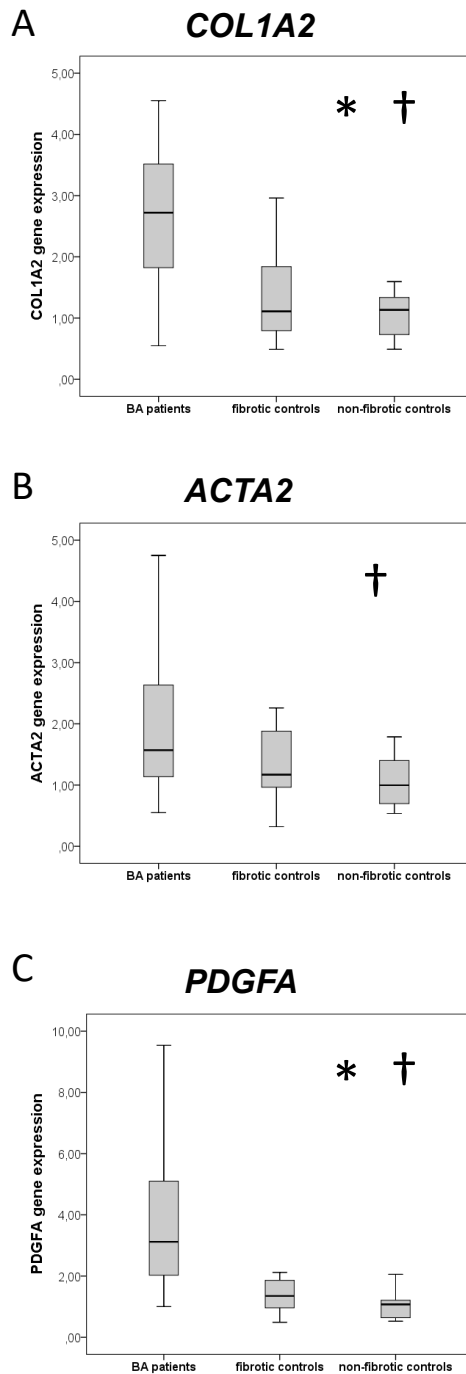
A **COL1A2**

B **ACTA2**

C **PDGFA**

Figure 7. Hepatic gene expression of COL1A2 for collagen 1 (A), ACTA for alfa-smooth muscle actin (B) and PDGFA for platelet-derived growth factor (C) 3.0 years after successful portoenterostomy in biliary atresia compared to fibrotic (n=11) and non-fibrotic (n=10) pediatric controls. Box plots display median (bold transverse line), interquartile range (rectangle) and range. Significance evaluated by the Mann-Whitney U test for each group. P<0.05: * BA vs. fibrotic controls, † BA vs. non-fibrotic controls, ‡ fibrotic vs. non-fibrotic controls. n=28.

At follow-up, the RNA expression of TGF-β2 and TGF-β3, but not that of TGF-β1, was upregulated in BA compared to both non-fibrotic and fibrotic controls (II). The expression of CTGF and decorin was upregulated compared to fibrotic controls, as well as decorin in relation to non-fibrotic controls. TGF-β receptor 1 coding *TGFBR1* and *SMAD2* was also upregulated compared to both non-fibrotic and fibrotic controls.

At follow-up, both protein and RNA expression of TGF-β1 correlated with Metavir fibrosis stage (II), which also correlated with the immunoreactivity of TGF-β2, CTGF and decorin. The magnitude of CK-7 positive ductal reaction correlated positively with the protein expression of decorin and CTGF.

The histologic marker of activated HSCs, α-SMA protein expression depicted as an area fraction correlated with gene expression of TGF-β1, protein expression of decorin, and the hepatic gene and protein expressions of TGF-β2 and CTGF (II). The gene expression of α-SMA (*ACTA*) correlated with TGF-β2, CTGF and decorin gene expression (II). PDGFA gene expression correlated positively with α-SMA protein and gene expression (r=0.542, P=0.007 and r=0.618, P=0.001, respectively).

At follow-up, serum TGF-β2 levels were increased in BA compared to non-fibrotic controls, but they did not correlate with Metavir fibrosis stage (II). There was no difference in serum decorin levels between BA patients and non-fibrotic controls. Syndromic BA patients had significantly lower protein expressions of collagen, α-SMA and TGF-β1 compared to isolated BA patients, but no difference in respective RNA expression was observed (II).

5.3 Expression of MMPs and TIMPs (III)

Of the various MMPs and TIMPs studied, RNA expression of MMP-7 (29.2-fold), MMP-2 (3.12-fold), and TIMP-1 (1.80-fold) showed the strongest upregulation after successful PE. Increased protein expression localized to the BECs of bile ducts and ductal reactions and periportal hepatocytes (Table 6). Similarly to CK-7, MMP-7 expression localized to periportal hepatocytes and BECs of ductal proliferations and bile ducts. Biliary epithelial expression of MMP-7 correlated positively with histologic fibrosis and portal inflammatory cell infiltrate. Protein expression of MMP-7 also correlated positively with plasma-conjugated bilirubin and bile acids. TIMP-1 expression was seen in hepatocytes and individual spindle-shaped stromal cells at portal or parenchymal areas, but its intensity did not differ between BA patients and controls.

Serum MMP-7 levels were increased compared to non-fibrotic controls, and they correlated positively with its protein and gene expression. Serum MMP-7 levels also correlated positively with plasma conjugated bilirubin and bile acids.

The patients without (n=4) portal fibrosis had significantly lower serum MMP-7 levels, having a significant predictive value for portal fibrosis with an AUROC of 0.925 (Figure 8).
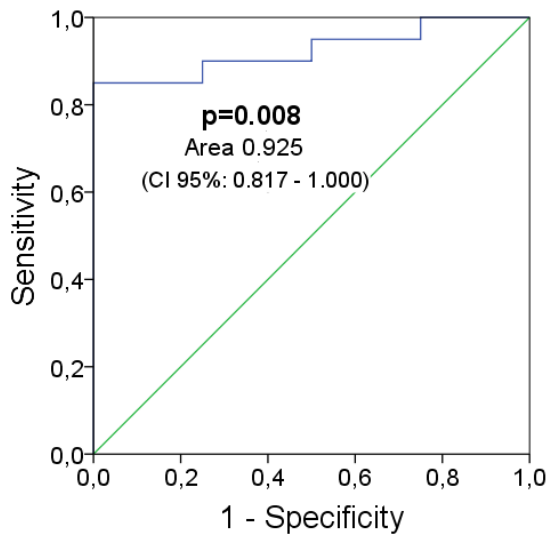

Figure 8. Area under the receiver operating characteristics curve (AUROC) showing the predictive value of serum MMP-7 for histological portal fibrosis after successful portoenterostomy (PE). Area of 1.0 represents the ideal test, whereas the area under 0.5 represents no diagnostic value. *Figure reprinted from study I with kind permission from the publisher.*

When MMP-7 expression in BA patients was compared with intestinal failure patients with comparable Metavir fibrosis stage, the BA patients showed significantly higher hepatic RNA expression, immunohistologic expression and serum levels. No correlation between Metavir fibrosis stage and liver or serum MMP-7 expression was seen among fibrotic controls.

At the time of follow-up, syndromic patients had significantly lower protein expression of MMP-7 compared to isolated ones [grade 2 (0.5–2.5) vs. 3 (2–3), P=0.015]. Despite a similar trend, there was no statistically significant difference in RNA expression [14.5 (7.67–43.0) vs. 44.2 (7.89–80.10), P=0.493] or serum concentrations [12.8 (6.22–32.3) vs. 16.0 (6.91–27.4) ng/mL, P=0.929].

5.4 Predictors of outcome (IV)

Incidence of BA in Finland is 1 in 18 600. Before centralization in 2005, the caseload in Helsinki University Hospital was 1 (1–2) per year, and after centralization, it was 3.5 (2–4.3) per year. After centralization, patients were younger at PE (54 vs. 73 days, P=0.016), and the COJ rate was higher (80% vs. 42%). In logistic regression analyses, COJ predictors were operation after centralization, high-grade portal inflammation in liver biopsy at the time of PE, and young age at operation, but in multiple regression, only the effect of high-grade portal inflammation at the time of PE remained significant.

The cumulative native liver survival is depicted in Figure 9. Two-year native liver survival before and after centralization was 37.5% (±SE 9.6%) and 77.6% (±7.5); five-year survival was 37.5% (±9.9) and 70.2% (±8.4); and ten-year survival was 33.3% (±9.6) and 65.2% (±9.2). In univariate analyses, significant predictors of native liver survival were a bilirubin level below 20 μmol/L at 3 months and at 6 months, portal inflammation grade ≥ 2 at PE, and operation after centralization. In the multivariate regression model, only a bilirubin level below 20 μmol/L at 3 months significantly predicted native liver survival.

Before and after centralization, cumulative 2-, 5- and 10-year overall survival rates were 68.0% (±9.3) and 93.7% (±4.3), respectively. In univariate analyses, bilirubin levels below 20 μmol/L at 3 months [100% vs. 66.7% (±8.6), P=0.001] and operation after centralization [93.7% (±4.3) vs. 68.0% (±9.3), P=0.007] predicted overall survival, but in multivariate analyses, neither remained significant.

At follow-up, in simple regression model a slower Metavir fibrosis stage progression rate was associated with a higher portal inflammation grade at PE and COJ, but in the multiple regression model, only the latter remained significant. Follow-up fibrosis stage associated with CK-7 positive periportal hepatocytes in multiple regression.
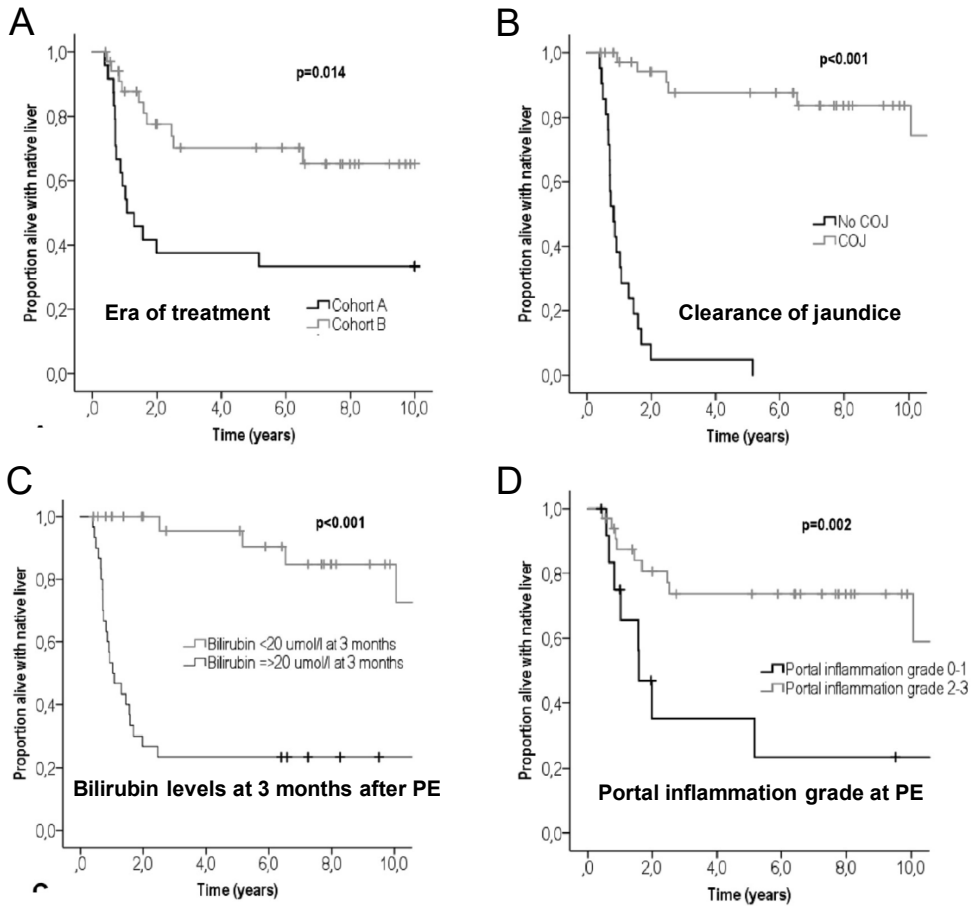
Figure 9. Cumulative native liver survival rates according to (A) era of treatment, (B) clearance of jaundice, (C) plasma bilirubin levels at 3 months after portoenterostomy (PE), and (D) portal inflammation grade at PE. Subgroups compared with the log-rank test. Cohort A=1987–2004; cohort B=2005–2016. *Figure reprinted from study IV with kind permission from the publisher.*

6 DISCUSSION

Previous studies have shown that fibrosis persists even after successful PE, despite the resolution of histologic inflammation and cholestasis [8, 89, 122, 124, 221]. Findings in studies I–IV show that after successful PE, liver fibrosis progresses or at least persists in all BA patients despite their COJ status, but inflammation diminishes, and cholestasis resolves only in those who undergo successful PE. Table 9 aggregates the observed changes in selected fibrosis and inflammatory mediators among BA patients after successful PE (I–III).

In cholestatic liver diseases, histologic cholestasis is often accompanied by inflammation, which is thought to have a crucial role in transforming BECs into reactive cells promoting fibrogenesis [7, 96]. However, in study IV, a higher portal inflammatory grade at PE predicted a slower fibrosis progression rate and better native liver survival, and inflammation decreased only after successful PE. In study I, RNA expression of proinflammatory cytokines remained relatively low after successful PE. These findings may indicate that inflammation is mainly secondary to cholestasis, while those with a predominantly inflammatory reaction at PE might represent a modifiable disease form before proceeding to a predominantly fibrotic form. This is supported by *Moyer et al.*, who found different molecular profilings in the fibrotic and inflammatory groups at the time of PE and decreased native liver survival among the fibrotic group [44]. Anti-inflammatory corticosteroids seem to improve bile drainage in the early postoperative period but have no effect on native or overall survival [9, 45, 82, 83]. Findings in studies I–IV also suggest that after successful PE, anti-inflammatory therapy might not be an effective means to reduce the progression of fibrosis, but instead studies with antifibrogenic therapies affecting profibrogenic cytokines and reactive BECs producing these molecules should be considered.

Ductular reaction is a distinctive histologic feature characteristic of biliary obstruction, and it correlates with portal fibrosis in BA [98, 101, 104, 117]. Findings in studies I–IV strengthen the role of ductal reactions in liver fibrogenesis after successful PE. MMP-7 was expressed by BECs in ductal reactions and periportal hepatocytes, and persistence of periductal α-SMA expression after PE associated with fibrosis and the degree of ductal reaction. This supports the crucial role of reactive BECs in ductal reactions sustaining and promoting fibrogenesis after successful PE. Previous studies have found CK-7 positive periportal hepatocytes in BA, possibly reflecting the role of hepatocyte-to-cholangiocyte metaplasia in the progression of the disease [8, 117, 120]. In the present study (III), the magnitude of CK-7 positive periportal hepatocyte immunoreactivity associated with MMP-7 expression. As these CK-7 positive periportal hepatocytes associated with fibrosis at

follow-up, this supports the importance of hepatocyte-to-cholangiocyte metaplasia in liver fibrogenesis after PE. More studies are needed to further investigate the function and origin of CK-7 positive periportal hepatocytes in BA-associated liver injury [102, 103, 106].

Table 9. Schematic overview of the observed changes in selected fibrosis mediators among BA patients following successful portoenterostomy.

Variable	Liver protein expression	Liver RNA expression	Serum level	Correlation to histologic liver fibrosis
Collagen	↑ ↑	↑ ↑		protein
α-SMA	↑ ↑	~ ↑		protein
TGF-β1	↑	~ ~		protein, RNA
TGF-β2	↑	↑ ↑	↑	protein
Decorin	~ ↑	↑ ↑	~	protein
CTGF	~ ↑	↑ ~		protein
PDGFA		↑ ↑		RNA
MMP-7	↑ ↑	↑ ↑	↑ ↑	protein, serum
TIMP-1	~	↑	↑	RNA
IL1A		↓ ~		
IL10		~ ↓		
TNF		~ ~		

Changes presented in relation to control patients with (blue) and without (red) liver fibrosis. Data found in studies I-III, except for liver protein expression BA vs fibrotic controls: CTGF [1 (0–1) vs 1 (0.75–2), P=0.115] and Decorin [2 (1.5–2.5) vs 1.5 (1–2), P=0.137].

α-SMA=alfa smooth muscle actin; TGF-β=transforming growth factore beta; CTGF=connective tissue growth factor; PDGFA=platelet-derived growth factor alfa; MMP=matrix metalloproteinase; TIMP=tissue inhibitor of matrix metalloproteinase; IL=interleukin; TNF=tumor necrosis factor

Study II showed that after successful PE, hepatic expression of TGF-β1 and CTGF selectively decreased, and both protein and RNA expressions of TGF-β1 correlated with fibrosis. In previous studies, TGF-β1 expression has been found to decrease from PE to LTx [181, 183]. *Kobayashi et al.* found that the expression of TGF-β1 was lower in patients with persistent jaundice compared to those with COJ and suggested that it might be due to the incapability of HSCs to produce

TGF-β1 in patients with end-stage liver fibrosis since the expression of α-SMA, the indicator of HSC activation, was also low in this group [181]. However, our findings challenge this interpretation since, in our patients with COJ, there was an abundance of α-SMA compared to healthy controls. Selective downregulation of TGF-β1 and CTGF together with persistent overexpression of antifibrogenic decorin might have a protective role in liver fibrogenesis, while persistent upregulation of TGF-β2 or PDGF may overpower their antifibrogenic effects. Profibrogenic PDGF has been shown to be overexpressed in BA and correlate with fibrosis [13, 179, 191]. Findings in study I suggest that this is an important cytokine sustaining fibrogenesis also after successful PE, and further research should concentrate on this growth factor. The coupling of hepatic protein and gene expression of TGF-β1, TGF-β2, CTGF, decorin and PDGFA with expression of α-SMA, a marker of activated myofibroblasts, strengthens their role in persisting fibrogenesis after the resolution of cholestasis in BA (study II).

In previous studies, hepatic MMP-7 expression has been shown to be upregulated in BA at the time of PE and LTx with an association to liver fibrosis [14, 15, 163, 165]. Study III strengthened the important role of MMP-7 in BA fibrosis and was the first study to show that serum and liver tissue expression of MMP-7 also increases after successful PE, is specific to BA, and associates with histologic fibrosis and ductal reactions at follow-up. Recently, serum MMP-7 has also been shown to be an accurate biomarker for BA at the time of diagnosis [14, 198]. However, MMP-7 did not reflect the degree of liver fibrosis at the time of PE [14, 198]. Our findings indicate that MMP-7 may be essentially involved with the progression of liver injury and fibrosis among patients who normalize their bilirubin by PE. Collectively, these findings encourage further investigation into the role of MMP-7 in BA fibrogenesis. It also raises the possibility for antifibrogenic therapy targeted against MMP-7. MMP-7 inhibitors *nobiletin* and *isofraxidin* have shown promising initial results in cancer cells, and *marimastat* reduced MMP hyperactivity in cystic cholangiocytes in polycystic liver diseases [222-224]. In the *rhesus rotavirus*–injected rat model of BA, the MMP-7 inhibitor *batimastat* prevented BEC injury and bile duct obstruction [14].

Overall, 36–43% of patients had at least one congenital anomaly. Anomalies included cardiac defects (at least a septal defect), polysplenia, vascular anomalies, intestinal malrotation, situs inversus and pancreatic anomalies. Just two patients had only polysplenia without other anomalies. A similarly high proportion (over 30%) of syndromic patients have been found in the USA and Canada [34, 52, 54, 115]. One explanation for the high percentage of syndromic patients could be the thorough assessment for associated malformations. In studies I–II, syndromic patients were found to have milder fibrosis compared to isolated ones, and their progressions of fibrosis and ductal proliferation were less prominent than in isolated patients after successful PE. The slower progression of fibrosis in syndromic

patients associated with decreased liver expression of collagen, α-SMA, TGF-β1 and MMP-7. This might reflect the different etiology and pathogenesis between syndromic and isolated patients. This raises the question of possible need for stratifying patients into isolated and syndromic disease forms in future studies instead of merely based on splenic anomaly in accordance with the concept of *biliary atresia structural malformation* as previously suggested [46, 49, 60, 61].

Study IV showed an improved five-year native liver survival from 38% to 70% and overall survival from 68% to 78% after the treatment of BA was centralized from five university hospitals into one in 2005, with a caseload of three to four children a year. These results demonstrated that even with the relatively low caseload, high-quality outcomes can be achieved when interdisciplinary treatment is standardized in a center with experience on complex hepatobiliary surgery. The potential benefits of centralization of BA care are well established [4-6, 33, 203, 210-212, 216]. In this respect, the crucial issue is the concentration of experience, which is also achievable by active cooperation with expert centers [6, 211].

The studies' limitations include a relatively small sample size and age range among patients and controls. Control material was obtained from several different sources due to the limited availability of liver specimens for obvious reasons. The cross-sectional study design provides only associations than proof of causality. Localization of the studied proteins relied on immunohistochemistry, which is prone to methodological inaccuracies. However, immunohistochemistry was supplemented with RNA expression to increase reliability. The other strengths of these studies include age-matched "healthy" and cholestatic controls, and the thorough evaluation of liver histology and potential fibrogenic mechanisms underlying the progressive liver fibrosis in BA after successful PE.

Sixty-four years after Morio Kasai's development of an effective surgical procedure to restore bile flow, BA is still the leading indication for pediatric LTx worldwide [1]. Molecular mechanisms underpinning ongoing liver fibrogenesis after successful PE are still mostly a mystery that needs to be resolved for the development of effective antifibrogenic therapies [2]. Our findings suggest directing future research to reactive BECs in ductal reactions and metaplastic periportal hepatocytes maintaining liver fibrosis, possibly through actions of MMP-7, TGF-β, PDGF and CTGF after successful PE in children with BA.

7   CONCLUSIONS

Based on the results, the main conclusions of these studies are the following:

1) After successful PE, a molecular signature of active fibrogenesis with increased expression of collagen, α-SMA and PDGFA prevails, and the expression of proinflammatory cytokines is low. There is a decline in the expression of TGF-β1 and CTGF, and no change in TGF-β2 and antifibrogenic decorin, which might indicate that TGF-β2 together with PDGF are important in mediating progressive liver fibrogenesis after cholestasis has resolved by successful PE.

2) After successful PE, there is an increased hepatic expression of MMP-7 specific to BA, which correlates with the degree of liver fibrosis. MMP-7 could serve as a therapeutic target to extend native liver survival and serum MMP-7 could be used as a postoperative follow-up tool in BA after successful PE.

3) High-grade inflammation in liver histology at the time of PE predicted COJ and the normalization of serum bilirubin levels following PE native liver survival. High-quality outcomes are achievable in a small country when standardized BA care is concentrated to an assigned interdisciplinary team.

4) A subgroup of syndromic patients showed less pronounced progressions of histologic fibrosis, ductal reaction, and expression of MMP-7, TGF-β1 and α-SMA compared to isolated patients. This might indicate a different etiopathogenesis between these two subgroups.

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