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TOPIC HIGHLIGHT

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Paediatric cholestatic liver disease: Diagnosis, assessment of disease progression and mechanisms of fibrogenesis

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Abstract

Cholestatic liver disease causes significant morbidity and mortality in children. The diagnosis and management of these diseases can be complicated by an inability to detect early stages of fibrosis and a lack of adequate interventional therapy. There is no single gold standard test that accurately reflects the presence of liver disease, or that can be used to monitor fibrosis progression, particularly in conditions such as cystic fibrosis. This has lead to controversy over how suspected liver disease in children is detected and diagnosed. This review discusses the challenges in using commonly available methods to diagnose hepatic fibrosis and monitor disease progression in children with cholestatic liver disease. In addition, the review examines the mechanisms hypothesised to be involved in the development of hepatic fibrogenesis in paediatric cholestatic liver injury which may ultimately aid in identifying new modalities to assist in both disease detection and therapeutic intervention.

Key words: Cystic fibrosis; Biliary atresia; Liver biopsy; Ultrasound; Hepatic fibrosis; Cirrhosis; Hepatic stellate cell; Bile acid; Chemotaxis; Monocyte chemotaxis protein-1

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INTRODUCTION

Cholestatic liver disease is a significant cause of morbidity and mortality in infants and children. The inability to detect early stages of fibrosis and to monitor progressive hepatic injury hampers both the diagnosis and management of these diseases. Recent studies aimed at understanding the cellular and molecular basis of hepatic fibrogenesis in adult and paediatric liver disease

have the potential to improve diagnostic capability and may lead to improved therapeutic intervention. This review details the difficulties associated with the use of commonly available methods to detect liver injury, diagnose hepatic fibrosis and monitor progression to cirrhosis in children with cholestatic liver disease, in particular in infants with biliary atresia and children with liver disease associated with cystic fibrosis, and examines the proposed mechanisms associated with the development of hepatic fibrogenesis in these conditions.

DIAGNOSIS OF FIBROSIS AND ASSESSMENT OF DISEASE PROGRESSION

Common paediatric cholestatic liver diseases

The most common diagnosis in infants presenting with clinical or biochemical evidence of liver disease is benign Idiopathic Neonatal Hepatitis accounting for up to 40% of cases[1], with incidence rates reported between 1 in 4800 and 1 in 9000 live births[2]. Bilary Atresia is a liver disease of the newborn affecting the intra- and extra-hepatic bile ducts, with incidence rates reported to be between 1 in 8000 to 1 in 21000 live births (reviewed in[3]). Biliary atresia is the major indication for liver transplantation in children. The natural history of the disease is variable, with an unpredictable rate of progression and outcome. Diagnosis is complicated as infants have clinical symptoms which can be indistinguishable from Neonatal Hepatitis. A confirmed diagnosis of biliary atresia is made by operative cholangiogram, during which a liver biopsy is performed to assess the extent of hepatic fibrosis. If a diagnosis of biliary atresia is confirmed, then a portoenterostomy (Kasai procedure) is usually performed before 100 d of life. However, the successful establishment of bile drainage with this procedure is variable and up to 40% of children will develop significant fibrosis and progress to liver transplantation within the first few years of life[3]. The autosomal recessive disorder Alpha-1-antitrypsin deficiency affects 1 in 1800 live births and is the most common genetic cause of liver disease in children. A mutation in the ATZ protein renders the molecule incapable of correct folding resulting in the aggregation of misfolded protein in the endoplasmic reticulum, subsequently leading to liver damage[4]. However, not all patients with the ATZ mutation develop liver disease[5]. The natural history of the disease is variable suggesting that both host and genetic factors play an important part in the pathogenesis[4].

Another relatively common paediatric cholestatic condition is liver disease associated with cystic fibrosis (CF). With increasing life expectancy of children born with CF, the prevalence of liver disease is escalating and the progression of fibrosis to cirrhosis is contributing increasingly to adverse outcomes in the CF population. This review will focus on current modalities used to diagnose fibrosis and to monitor fibrosis progression to cirrhosis in these children. The diagnosis of liver disease and more importantly, fibrosing liver disease in children with CF is difficult. There are limited biochemical and clinical tests that give definitive diagnoses of disease or offer an accurate, minimally invasive method of monitoring the progression of fibrosis.

Diagnosis and monitoring CF liver disease

As the life expectancy of children and adults with CF has increased over the past decade, there has been a steady increase in the incidence of non-respiratory complications of CF such as liver disease[6]. The origin of the pathogenic lesion in CF is focal hepatic biliary fibrosis[7] which typically progresses slowly and unpredictably during childhood and adolescence. Clinical presentation with hepatomegaly and/or splenomegaly is usually around 10 years of age. Diagnosis of liver disease relies on a combination of clinical, biochemical, radiological and histological assessments; however, this is complicated by inconsistent use of definitions for what constitutes a diagnosis of liver disease[7].

It is estimated that up to 17% of children with CF will develop significant liver disease[8,9], with up to 10% developing cirrhosis, and prior to the advent of transplantation, end stage liver disease was the primary cause of death for 5% of patients with CF[10]. It has long been suspected that liver cirrhosis is also an important factor in premature death from other primary causes such as respiratory failure. However, the true prevalence of CF liver disease (CFLD) is unknown due to the poor sensitivity and specificity of available clinical tools used in diagnosis and monitoring disease progression. Based on radiological methods (ultrasound scanning), biochemical tests, clinical methods [presence or absence of hepato (± spleno) megaly] and histological assessment, the estimated prevalence of hepatic fibrosis and liver disease is proposed to be between 26%-45% in patients with CF[10-12]. However in studies undertaken at autopsy, the prevalence of significant liver disease is suggested to be as high as 10% in children, and 72% in adults[13]. Methods that are sensitive and specific enough to detect early evidence of cholestatic liver disease, and that can accurately monitor hepatic fibrosis progression are lacking[8]. This is particularly important in the setting of CF in which early detection of hepatic injury and fibrosis alerts the clinician to a more complicated future with further increased energy expenditure, impaired GI function and the need for more aggressive clinical management. It also allows for the timely commencement of ursodeoxycholic acid therapy which is proposed, though not demonstrated, to have a better efficacy earlier in the natural history of cholestatic liver diseases.

Diagnosis of liver disease using the presence of hepatomegaly and/or splenomegaly: Clinical liver disease is defined as an increase in volume and harder consistency of the liver, particularly of the right lobe with or without splenomegaly[14,15]. Studies using the pres-

ence of hepatomegaly, alone or in combination with splenomegaly, as indicative of liver disease report a prevalence rate of 4%-40%[7,12,16-18]. The use of hepato/splenomegaly as a method for the diagnosis of liver disease is inconsistent and controversial.

Biochemical markers of liver disease: In children with suspected CFLD, abnormalities in liver function tests (LFTs) are unreliable for the detection of significant liver disease and fibrosis[8], and hence are not useful to detect or measure the progression of fibrosis. Abnormal LFTs in CF are likely to be from more benign causes such as intercurrent infections, drug reactions and steatosis, and many children with advanced fibrosis have normal biochemistry. There is no consensus in the literature on a definition of "biochemically indicated liver disease" further complicating the assessment and use of biochemical markers of liver disease. The United States cystic fibrosis Foundation recommend that liver disease should be suspected if the child has any liver enzyme elevated by more than 1.5 times the upper limit of normal on two concurrent occasions and recommends more frequent testing of LFTs[8]. In comparison many clinical studies define biochemical liver disease as an elevation of LFTs for more than 2 years in patients who are > 4 years of age[10].

There is considerable evidence to suggest that children can have normal LFTs but underlying fibrogenesis[12,18,19]. When compared with fibrosis staged by liver biopsy, significant histological disease has been reported in up to 56% of patients with normal LFTs[15]. Abnormal LFTs are seen in 17%-80% of patients with CF, unrelated to the presence of neonatal cholestatsis[8,10,12,15,16,20], and in the absence of overt histological involvement. Many children who present with biochemical liver disease do not go on to develop histological liver disease[10], but abnormal biochemical markers have been associated with future development of abnormal ultrasound or the presence of clinical hepato/splenomegaly in 75% of children[20]. In patients with CF, treatment with ursodeoxycholic acid leads to improvement of biochemical markers of liver disease (ALT/AST)[21], however there is little evidence that it changes the natural history of the disease, further supporting the idea that biochemical markers of liver disease do not accurately reflect the underlying pathogenesis.

Ultrasound imaging: Hepatic ultrasound scanning is a common clinical tool used to detect and diagnose liver fibrosis in children with cholestatic liver disease, specifically in children with suspected CFLD. Although widely used, ultrasound has poor sensitivity and specificity for detecting and staging fibrosis[22]. Between 18% and 35% of children with CF will display abnormalities detected by ultrasound scanning by age 6[20,23], irrespective of evidence of biochemical or histological liver disease. Abnormal ultrasound scores do not correlate with biochemical markers of liver disease or with the presence of hepatomegaly, with abnormal echogenicity frequently found in the absence of biochemical, or clinical indicators of liver disease[24].

A diagnosis of fibrosis based only on ultrasound may be erroneous because steatosis appears sonographically similar to focal fibrosis in the liver, both lesions being common in the setting of CFLD. A recent study examined the relationship between ultrasound scores and fibrosis staged by dual pass liver biopsy in children with suspected CFLD[22]. This study found that ultrasound scanning had poor sensitivity and specificity in diagnosing the absence of fibrosis but had some utility in confirming the presence of advanced liver fibrosis and cirrhosis. In children with indeterminate ultrasound scores, liver histology ranged from normal with no evidence of fibrosis to advanced stages of fibrosis including cirrhosis.

Because of poor sensitivity for early and moderate liver fibrosis, ultrasound is a poor predictor of the future development of serious liver complications. Children with normal hepatic ultrasound scores can still develop clinically significant liver disease and display evidence of fibrosis upon liver biopsy[22]. In most paediatric cholestatic liver diseases, ultrasound is a better diagnostic tool for detecting the presence of ascites, hepatic vein dilation, gallstones and common bile duct stones[8].

Ling and colleagues demonstrated that over a 4 year follow-up period, 92% of children with CF showed some evidence of liver abnormality determined by either biochemical tests, ultrasound or the presence of hepato/splenomegaly. Biochemical and ultrasound abnormalities were often intermittent suggesting a high rate of false positivity[23]. Biochemical testing, ultrasound scanning and the presence of hepatosplenomegaly are poor diagnostic indicators of sustained hepatic fibrosis and give a poor indication of the underlying fibrogenesis.

Use of liver biopsy to detect fibrosis in CF liver disease: Given the lack of sensitivity and specificity in the use of clinical, biochemical or radiological tests, liver biopsy is considered the gold standard to detect hepatic fibrosis. However, the use of liver biopsies to detect fibrosis in CF is not routine, and mainly limited to tertiary paediatric transplant centres. Liver biopsy is not without risk. Patient discomfort, the use of a general anaesthetic in children, and the risk of rare, but serious complications including blood transfusion for bleeding, biliary peritonitis and pneumothorax are noted disadvantages. Liver biopsy in adults had an estimated morbidity of 3% and a mortality rate of 0.03%, prior to the more recent practice of ultrasound guidance. The pathogenic lesion in CFLD is the formation of focal biliary fibrosis which can ultimately progress to multilobular cirrhosis. Thus, the focal nature of CFLD can influence the reproducibility and reliability of liver biopsy in demonstrating fibrosis; cirrhosis can be difficult to diagnose given the irregularity of fibrosis distribution and the sample size associated with needle biopsies[25]. Studies have suggested that multiple biopsies

will only have a concordant diagnosis of cirrhosis in 33% of cases (reviewed in[26]). Contamination by stroma and nodularity may suggest cirrhosis, however, a complete regenerative nodule is required for an accurate diagnosis of cirrhosis. The likelihood of significant sampling error is not limited to cases of cirrhosis given that it is estimated that only 1/50 000th of the liver is sampled. This is compounded even further in cholestatic disorders such as CFLD, PSC, Alagilles where fibrogenesis is more heterogeneous compared to primarily hepatitic liver diseases such as hepatitis C virus (HCV) infection and Non-Alcoholic Steatohepatitis (NASH).

A recent study evaluated the utility of liver biopsy to diagnose liver disease and detect fibrosis in children with suspected CFLD[19]. This preliminary study illustrated that dual pass liver biopsies improved detection of liver fibrosis compared with a single pass; there was a significant level of discordance between the first and second pass with 35% of liver biopsy pairs found to be non-concordant. Additionally, a diagnosis of fibrosis would have been missed in approximately 1 in 5 cases. However, sampling error and inter-observer error can be reduced by using dual pass liver biopsies, and rejecting biopsies that have < 5 portal tracts available for analysis[27].

Given the major limitations of biochemical and radiological tests to detect fibrosis and monitor the progression of fibrosis, it must be inferred that liver biopsy is the best currently available tool to monitor fibrosis progression. However, there are no studies available examining fibrogenesis in multiple liver biopsies in children with CFLD. Preliminary results from a recent clinical study suggest that increasing stage of fibrosis may predict the development of portal hypertension[19], although further confirmatory studies are required.

Utility of additional non-invasive methodologies for fibrosis detection: With the aforementioned risks associated with liver biopsy especially in children, it is desirable to find alternative methods to accurately detect and stage liver fibrosis and to monitor fibrosis progression in children with cholestatic liver disease.

Transient elastography: In the search for non-invasive tools to detect fibrosis in adult liver disease, transient elastography shows significant promise. Transient elastography assesses the stiffness of the liver by measuring the elastic shear of a vibrational wave that propagates through the liver tissue. The harder the tissue, the faster the shear wave is propagated[28]. This technology offers a non-invasive, easily reproducible, bedside method of measuring liver stiffness and is increasingly used to determine and monitor liver fibrosis in diseases such as HCV and NASH[28]. Transient elastography can be performed on most patients except for those who are obese or have ascites[29]. A significant advantage of this technique is the increased proportion of the liver that is sampled and a lower intra- and inter-observer error when compared with liver biopsy. Transient elastography has a sample size of approximately 3cm^2, some 100 times greater than the sample size of liver biopsy[29]. Transient elastography has been validated for use in adults with either Hepatitis B virus (HBV), HCV, NASH, alcoholic liver disease or haemochromatosis. However, this technology has not been studied extensively in adults or children with cholestatic liver disease (reviewed in[30]). The utility of transient elastography in diagnosing liver fibrosis in most patients may lie in distinguishing cirrhotic patients from non-cirrhotic patients[30].

To date, four studies have examined the utility of transient elastography in detecting hepatic fibrosis in children, including NASH[31], CFLD[32], a mixed population of chronic liver diseases including CFLD, HBV, HCV, biliary atresia, Autoimmune Hepatitis, Wilsons disease[33], and in children with the congenital heart defect resulting in Fontan circulation[30]. The most extensive study was that conducted in NASH[31], where hepatic fibrosis was assessed using both transient elastography and liver biopsy. This study suggested that transient elastography was able to distinguish between no fibrosis, significant fibrosis and advanced fibrosis. However, while there was a significant correlation between increasing elastography scores and the Brunt histology score, there was overlap in the values determined by transient elastography between fibrosis stages 0 and 1, and between fibrosis stages 1 and 2. This suggests that transient elastography has utility in distinguishing between no fibrosis and advanced fibrosis, but has limited sensitivity for detecting mild or moderate fibrosis and thus cannot be definitively used to stage fibrosis[31] (similar results were seen in children with Fontan circulation[30]).

The study by De Ledinghen and colleagues compared transient elastography results with fibrosis staged by liver biopsy in 33 children who had chronic liver disease due to varying different aetiologies[33]. The majority of children had liver diseases as defined as 'other' by the authors (n = 18), biliary atresia (n = 9) and Autoimmune Hepatitis (n = 5). Overall, increased elastography scores correlated with increasing METAVIR fibrosis stage, however, the authors did not examine this relationship in specific disease groups and from this paper it was not possible to determine whether transient elastography has any utility in the diagnosis of fibrosis in cholestatic liver disease. Finally, Witters and colleagues used transient elastography to detect liver fibrosis in children with CFLD[32]. This study did not perform liver biopsy and instead used biochemical evidence of liver disease (LFTs) and/or the presence of hepatomegaly ± splenomegaly. Hence, given all the limitations of these clinical modalities in CFLD, as discussed above, the value of elastography in CFLD was not confirmed. The use of transient elastography to diagnose fibrosis in cholestatic liver diseases is confounded by the fact that extrahepatic cholestasis is associated with increased liver stiffness, irrespective of the stage of liver fibrosis. High elastography values, normally indicative of cirrhosis in other liver diseases (HCV, alcoholic liver disease),

were not associated with cirrhosis in adult patients with cholestatic liver disease[34]. A recent study of 49 children with biliary atresia suggested transient elastography may be useful in identifying oesophageal or gastric varices in children post-Kasai portoenterostomy[35], suggesting that while transient elastography is not useful in identifying liver fibrosis in cholestatic disease, it may help identify other significant liver associated problems.

Poor study design, inconsistent classification of liver disease (especially in the case of CFLD) and lack of comparison to fibrosis staged by liver biopsy have hindered studies attempting to validate transient elastography in children with cholestatic liver disease. Importantly there is limited data on the ability of transient elastography to predict development of serious liver complications. In other diseases (e.g. HCV) the 5 year mortality and morbidity outcome derived from transient elastography data is similar to that determined from liver biopsy data. This suggests that in diseases where transient elastography reflects liver fibrosis staged by liver biopsy, outcome data generated by transient elastography may be valid. Further investigation and validation of this technology is required, especially in paediatric cholestatic liver diseases.

Serum markers of hepatic fibrosis: In the search for an alternative diagnostic to liver biopsy, serum markers show some promise. The common pathway for cirrhosis development in CFLD is *via* hepatic fibrogenesis due to an imbalance between the synthesis and degradation of extracellular matrix by hepatic stellate cells (HSC), resulting in increased fibrillar collagen deposition[36]. Evaluating the mechanisms involved in the development of fibrogenesis may provide a method of determining fibrogenic activity in the liver and thus assist in the diagnosis of hepatic fibrosis. There is considerable evidence in adult liver diseases that serum markers of hepatic fibrogenesis provide a good indication of current underlying liver function. Serum collagen type Ⅳ (CL-Ⅳ), prolyl hydroxylase, procollagen Ⅲ polypeptide (PⅢP), and matrix metalloproteinase-1 (MMP-1) are increased in cirrhosis due to various different liver diseases[37]. In chronic HCV infection, Walsh and colleagues demonstrated elevated serum tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2[38], whereas others have shown increased levels of hyaluronic acid[39]. CL-Ⅳ and laminin have been demonstrated to be increased in alcoholic hepatitis[40]. In patients with HFE-haemochromatosis, serum TIMP-1, hyaluronic acid, CL-Ⅳ and MMP-2 are elevated[41,42], with only CL-Ⅳ and MMP-2 levels shown to be associated with fibrosis progression[42], whereas an elevated hyaluronic acid > 46.5 ng/mL has been shown to accurately diagnose patients with cirrhosis with 100% sensitivity and specificity[43]. These results suggest that certain serum markers may be both disease-specific and may better predict differing stages of fibrogenesis.

To date, the majority of serum marker analyses have been performed in adults. It is important to note that some serum markers such as MMPs and TIMPs, can be influenced by childhood growth as seen in kidney and bone[44,45]. Despite this, many serum fibrosis markers are not influenced by growth and development as reported in a study of Indian Childhood Cirrhosis, which showed elevated levels of serum CL-Ⅳ, laminin and PⅢP vs age-matched controls[46]. In CF, the multi-systemic nature of the disease makes it difficult to identify liver-specific serum fibrosis markers. Many of these markers are involved in extracellular matrix remodelling, a process which clearly occurs in the lung and pancreas associated with CF.

A number of groups have demonstrated increased levels of serum collagen type-Ⅵ[47], hyaluronic acid[48], as well as PⅢP and prolyl hydroxylase[49] in children with CFLD. Additionally, serum TIMP-1, prolyl hydroxylase and CL-Ⅳ levels have been shown to be significantly elevated in children with CFLD compared with children with CF and no liver disease (CFnoLD) and age-matched controls, suggesting that these serum markers may have relative specificity for liver injury in CF[50]. Serum hyaluronic acid levels have been reported to be significantly increased in CFLD compared with controls, but not when compared with CFnoLD[50], suggesting that the extra-hepatic complications associated with CF may have a confounding influence over the use of certain serum markers. Serum TIMP-1, prolyl hydroxylase[50] and monocyte chemotaxis protein-1 (MCP-1)[51] were significantly higher in children with CFLD who had minimal or no histological evidence of fibrosis, suggesting a potential role for these markers in the early detection of liver injury, and potential utility in distinguishing between serious liver fibrosis and no fibrosis. These few studies suggest that further investigation and development of panels of serum markers may provide an excellent surrogate to assess fibrosis progression and predict the future development of serious liver complications.

In lieu of a viable, minimally invasive alternative me thod to detect and monitor fibrosis in paediatric cholestatic liver disease, liver biopsy remains the gold standard. Biochemical and clinical markers of disease are inadequate in detecting fibrosis, monitoring fibrosis progression, and predicting future development of serious liver complications such as portal hypertension. It remains to be seen whether transient elastography will be useful in the cholestatic setting, given the presence of increased liver stiffness in the absence of overt liver disease and fibrosis. However, serum markers detecting the underlying processes of hepatic fibrogenesis show significant promise and warrant further investigation.

MECHANISMS OF HEPATIC FIBROGENESIS AND DEVELOPMENT OF CIRRHOSIS

Despite the diverse aetiologies of paediatric cholestatic liver diseases, bile acid accumulation, resulting in hepatotoxicity, is common to all conditions. Bile acid toxicity impacts all liver cells, and thus can have either

direct or indirect effects on the phenotype of HSC, the principal source of fibrotic tissue in the liver. This section of the review will discuss the potential mechanisms associated with the cholestasis-induced transformation of HSC into a myofibroblastic phenotype. New and emerging concepts including the heterogeneity of HSC, the role of HSC in eliciting portal hypertension and the interplay between HSC and cells of the ductular reaction and immune system will also be discussed.

Bile acid synthesis in the liver

Bile acids are generated from cholesterol metabolism within hepatocytes, secreted into bile canaliculi with bile subsequently hydrated by cholangiocytes as it drains into the common bile duct and gall bladder. Bile from the gall bladder is released into the duodenum where bile acids aid in the solubilisation and absorption of fats and fat soluble vitamins. Chenodeoxycholic acid (CDCA) and cholic acid (CA) are primary bile acids produced in the liver and subsequently modified by gut bacteria in the small intestine to produce the secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA). All bile acids are reabsorbed in the gut and recycled back to the liver in the portal venous system, i.e. *via* the enterohepatic circulation. These bile acids are conjugated with either taurine or glycine and rarely exist in an unconjugated form in the normal human bile acid pool.

The polar nature of these bile salts, which is essential for their function in fat digestion, makes them toxic to cell membranes of liver and gut cells. Bile toxicity is determined by bile acid polarity, with hydrophobic bile acids being more toxic that hydrophilic bile acids. Ursodeoxycholic acid (UDCA) is a hydrophilic bile salt that plays a role in hepatoprotection when the proportion of UDCA in the bile is elevated relative to the proportion of hydrophobic bile salts. Bile acid toxicity is ranked as follows: LCA < DCA < CDCA < CA < UDCA[52]. In the normal liver toxicity is moderated by the formation of mixed micelles (with bilirubin, cholesterol, phospholipids proteins), bile hydration, conjugation, alkalinisation, the presence of mucin and the bile flow out of the liver. If any of these factors are perturbed, cholestasis ensues.

In patients with CFLD, there is a correlation between serum cholic acid levels and the stage of hepatic fibrosis, inflammation score, and limiting plate disruption[53]. A similar correlation has also been demonstrated when using the cholic acid/chenodeoxycholic acid ratio. This same study demonstrated that endogenous biliary levels of UDCA are increased in CFnoLD patients when compared to patients with CFLD and controls, suggesting a potential mechanism may exist to protect against liver disease in a cohort of patients with CF[53]. In a more recent study the hydrophobic bile acid taurine-conjugated cholic acid (or taurocholate), was increased in the bile of patients with CFLD and also in an animal model of cholestatic liver injury, the bile duct ligated (BDL) rat[51]. Serum taurocholate was also correlated with the stage of hepatic fibrosis in both CFLD and in the animal model, suggesting a potential causal association.

Control of bile acid metabolism

Cytochrome P7A1 (CYP7A1) or cholesterol 7a-hydroxylase is the rate limiting step in the conversion of cholesterol to bile acids in hepatocytes[54]. In the normal liver this enzyme is controlled at the level of transcription by a short heterodimer partner (SHP) which is in turn regulated by interaction of the farnezoid-X receptor (FxR) with bile acids[55,56]. In patients with cholestatic liver disease, the presence of excess bile acids results in the concomitant upregulation of FxR. However, there is no change in the level of SHP which suggests a different pathway of regulation is at play. Recent work has suggested this may involve fibroblast growth factor 19 (FGF19)[57,58]. FGF19 is an endocrine growth factor that is produced by enterocytes of the terminal ileum in response to uptake of bile salts from the small intestine[57]. While FGF19 mRNA is not expressed in normal liver, it is markedly increased in both liver and serum in early cholestasis[59]. In the presence of excess bile acids, FxR stimulates the production of FGF19 which along with its signalling cofactor, β-Klotho, binds to fibroblast growth factor receptor 4 (FGFR4) to downregulate CYP7A1 and decrease de-novo bile acid synthesis[57].

Since FGFs are crucial hormones in bile acid synthesis, they are important disease-specific genes to be considered when attempting to understand the mechanisms associated with the development of cholestatic liver disease. While basic FGF (bFGF) has been shown to impact on HSC activation[60], the potential role of FGF19 and FGFR4 in HSC activation associated with cholestatic injury remains to be investigated. Given the early induction of FGF19 in cholestasis, this molecule along with other protein family members may be viable targets to investigate further in the detection of early liver injury and fibrogenesis.

Toxic effects of bile acids

The toxic effects of bile acids are varied, but include hepatocellular apoptosis, which in turn may play a role in the activation of HSC into myofibroblasts (Figure 1). Apoptosis (or programmed cell death) is a process that occurs in the normal liver to remove unwanted, senescent or damaged cells, but in cholestasis apoptosis is increased and dysregulated. Apoptosis is histologically characterised by cell shrinking and nuclear fragmentation and is regulated by either extrinsic factors such as death receptors, including tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)[61,62], or by intrinsic pathways such as mitochondrial release of pro-apoptotic factors. Both result in the release of effector caspases (intracellular proteases and endonucleases) that result in the degradation of cellular components into apoptotic bodies which are phagocytosed by Kupffer cells, macrophages and HSC. Electron transport mechanisms are impaired in hepatic mitochondria resulting in the production of lipid peroxidation metabolites which are the main reactive oxygen species (ROS) in cholestasis.

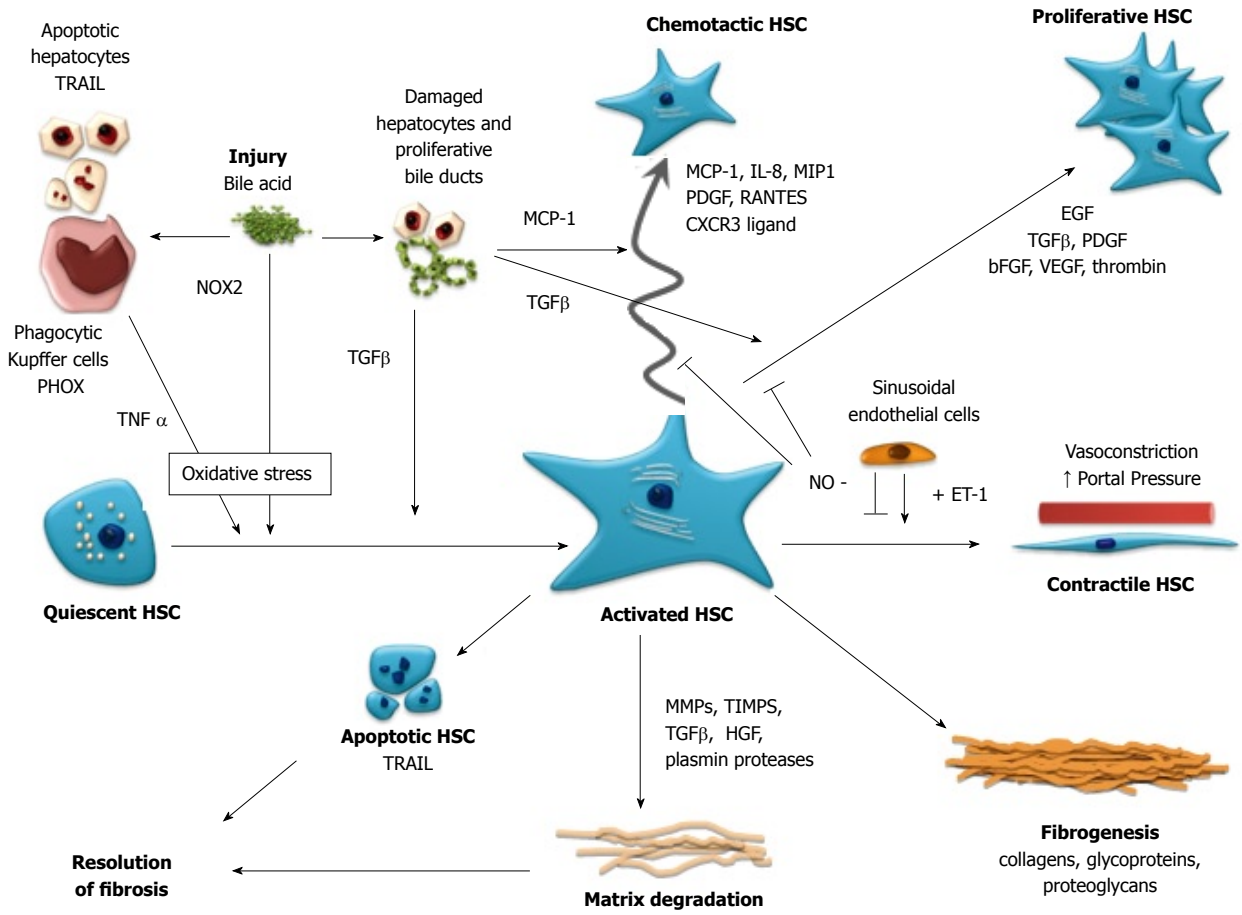

Figure 1 Schematic representation of the activation, function and interaction of Hepatic Stellate Cells (HSC) with other cells of the liver in cholestatic liver injury. Bile acid mediated injury is proposed to impact on HSC directly and indirectly *via* oxidative stress mediated pathways, resulting in the transformation of quiescent HSC to an activated phenotype, i.e. myofibroblast. Activated HSC are proliferative and fibrogenic and are responsible for increased production and deposition of fibrillar collagens and extracellular matrix, leading to fibrosis and cirrhosis. In response to hepatocyte and cholangiocyte-derived chemokines, motile HSC are recruited to the site of injury along the growing margin of scar tissue, with HSC and portal myofibroblasts also demonstrated surrounding bile ducts. HSC assume a vasoconstrictive phenotype resulting in increased portal pressure. Hepatic fibrosis is ultimately resolvable with disease treatment or cessation of injury, as HSC produce fibrinolytic enzymes and are themselves subject to apoptosis as part of the process of fibrosis resolution. TRAIL: tumor necrosis factor-related apoptosis-inducing ligand; PHOX: phagocytic NADPH oxidase; NOX2: non-phagocytic NADPH oxidase; TNFα: tumor necrosis factor α; TGFβ: transforming growth factor β; MCP-1: monocyte chemotaxis protein-1; IL-8: interleukin-8; MIP1: macrophage inflammatory protein 1; PDGF: platelet derived growth factor; RANTES: regulated upon activation, normal T cell expressed and secreted; CXCR3 ligand: chemokine (C-X-C) receptor 3 ligand; EGF: epidermal growth factor; bFGF: basic fibroblast growth factor; VEGF: vascular endothelial growth factor; NO: nitric oxide; ET-1: endothelin 1; MMP-1: matrix metalloproteinase-1; TIMPS: tissue inhibitors of metalloproteinase; HGF: hepatocyte growth factor.

Phagocytic NADPH oxidase (PHOX) is an enzyme which catalyses the production of further ROS in Kupffer cells. $CD68^+$ Kupffer cells have been identified in the perisinusoidal space in close proximity to scar tissue in the liver of patients with biliary atresia[63]. These cells produce tumor necrosis factor α (TNFα)[64] which can activate HSC. Bile acids also induced oxidative stress directly in HSC and this is mediated by the non-phagocytic NADPH oxidase (NOX2)[65].

Bile acid-induced hepatocellular injury, whether due to liver cell apoptosis, the generation of ROS or the release of soluble factors such as cytokines, results in the activation of HSC from a quiescent to a myofibroblastic phenotype. In the normal liver HSC are responsible for maintaining the basement membrane and are a store for vitamin A. HSC are quiescent and are located in the perisinusoidal Space of Dissé, with projections that come into close contact with hepatocytes. Upon injury to the liver, HSC are transformed into myofibroblasts[66] which are proliferative, fibrogenic (as well as fibrolytic), contractile and motile. Activated HSC have been demonstrated to be present in CFLD liver biopsies prior to histological evidence of fibrosis or procollagen I mRNA expression[36].

It is envisaged that both necrosis and apoptosis contribute to HSC activation in cholestatic liver disease[67]. At high concentrations (> 100 μmol/L), hydrophobic bile acids can have a detergent action and cell necrosis may predominate. Necrosis is characterized by cell swelling, disruption of intracellular and plasma membrane, ATP depletion, ion dysregulation, mitochondrial swelling, activation of degradative enzymes and cell lysis. However, the exact mechanisms linking necrosis to HSC activation are not yet well characterised.

Activation of HSC to a myofibroblastic phenotype

Transforming growth factor β (TGF-β) is a key profibrogenic cytokine present in various tissues, including the lungs, kidneys, skin (reviewed[68]), and the liver[69]. TGF-β1 is elevated in the liver of children with biliary atresia[63] and CFLD[36]. In these studies, TGF-β1 was expressed predominantly in bile duct epithelial cells, but also in HSCs and hepatocytes at the interface between normal liver and scar tissue. TGF-β protein is produced as a large latent form which is bound to liver extracellular matrix and is activated by proteases, ROS and integrins[70]. The active TGF-β signals through (serine/threonine kinase) type Ⅰ and type Ⅱ receptors which in turn complex with mothers against decapentaplegic homolog (Smad) 2, 3 and 4 proteins to translocate to the cell nucleus and interact with DNA binding proteins to modulate several cellular processes[71]. Smad 6 and 7 proteins are inhibitory molecules[72]. TGF-β increases the expression of extracellular matrix components[73] by modulating the expression of MMPs and enzymes such as plasminogen activator inhibitor 1 (PAI-1)[74] and TIMP[75] in HSC. In patients with CFLD, TGF-β expression correlates with the stage of hepatic fibrosis[36].

Factors which stimulate the proliferation of HSC

PDGF is the major driver of HSC proliferation in cholestatic liver disease[76]. HSC produce PDGF and also express receptors for PDGF[77]. Four isoforms of PDGF have been identified (A, B, C and D) and of these PDGF-D is thought to be the most potent HSC mitogen[78]. The downstream effectors of PDGF-mediated HSC proliferation include the phosphatidylinositol 3-kinase (PI3K)[79,80] and extracellular signal-related protein kinase 5 (ERK5)[81] signalling pathways. PI3K also controls other aspects of HSC function such as collagen synthesis[80] and potentially plays a role in upregulation of proinflammatory mediators of fibrosis such as ICAM-1, RANTES and IL-1β[82]. Other HSC mitogens include epidermal growth factor (EGF), bFGF[60], VEGF[83] and thrombin[84] (Figure 1). EGF[85] and thrombin[86] receptors have also been identified on HSC.

Fibrogenesis and fibrolysis mediated by HSC

HSC are the principle source of fibrotic tissue including collagens Ⅰ, Ⅲ and Ⅳ[87,88], glycoproteins (laminins, SPARC, undulin, elastin, hyaluronan, tenascin)[89,90], and proteoglycans (biglycan, decorin, BIGH3, fibronectin and vesican)[91-93] (Figure 1). The composition of the fibrotic matrix is thought to be similar in all forms of liver fibrosis irrespective of aetiology[94] which suggests myofibroblasts, regardless of their origin, produce the same components. However, this hypothesis needs to be validated in light of growing evidence for the heterogeneity of both HSC[95], and the myofibroblastic population[96], discussed in subsequent sections.

HSC are also responsible for the remodeling of fibrotic tissue *via* the production of collagenases, MMPs[97] and their inhibitors, TIMPs[98]. Many of the components of the fibrolysis system including MMPs, TGFβ and Hepatocyte Growth Factor (HGF) are secreted in an inactive form with the plasmin protease system essential for their activation. Plasmin is itself activated from inactive plasminogen by tissue plasminogen activator (tPA) and uroplasminogen activator (uPA) which are in turn activated by IGFBP-5 and inhibited by PAI-1. Many components of the plasmin protease system are produced in the liver[99]. PAI-1 is produced by HSC[100] and its expression is decreased in cirrhotic livers[101]. PAI-1 is a key mediator of cholestatic liver disease in bile duct-ligated, PAI-1 knockout mice[102,103]. Insulin-like growth factor binding protein-5 (IGFBP-5) binds to PAI-1 in the extracellular matrix[104] and in the absence of PAI-1, IGFBP-5 has been shown to enhance the effect of tPA on plasminogen[105], suggesting that IGFBP-5 plays a role in MMP modulation. Thus, it is postulated that decreased PAI-1 and increased IGFBP-5 could increase MMP activity.

Toll-like receptor expression in HSC

HSC express toll like receptors (TLR) which are usually involved in recognising unmethylated bacterial DNA, naturally rich in cytidine-phosphate-guanosine (CpG) sequences[106]. When mammalian hepatic cells undergo apoptosis they are subject to severe modifications which may include the enrichment of CpG sequences[107]. DNA from apoptotic hepatocytes induces the differentiation and chemotaxis of human and mouse HSC *via* TLR9 and PDGF[108]. Bile duct-ligated TLR9$^{-/-}$ mice have been demonstrated to exhibit reduced fibrosis, HSC activation and MCP-1 expression compared to control wild type mice, suggesting a role in cholestasis-induced injury[109]. A single nucleotide polymorphism (SNP) in the TLR4 gene at c.1196C > T (rs4986791, p.T399I) is shown to confer protection from fibrosis in patients with HCV infection[110]. This SNP along with another at c.896A > G (rs4986790, p.D299G), was functionally linked to a lower apoptosis threshold in the cultured HSC LX2 cell line[111]. The role of TLRs in cholestasis-induced liver disease is deserving of further investigation.

Heterogeneity of myofibroblasts

It is now recognised that fibrotic tissue is produced in the liver by a heterogeneous population of activated myofibroblastic cells[75]. In addition to the perisinusoidal HSC, portal (myo) fibroblasts which are located around bile ducts in the portal tract are thought to be important contributors to biliary fibrosis. However, it is unclear whether they are an extension of the differentiation lineage of activated HSC or if they are from a different embryological origin. In mouse embryos both HSCs and portal myofibroblasts originate from mesenchymal cells which express the p75 neurotrophin receptor (p75NTR)[112]. Both HSC and portal myofibroblasts express alpha-smooth muscle actin (αSMA) and both perisinusoidal and periductal αSMA expression has been demonstrated in the liver of children with CFLD and

biliary atresia[36,63]. Efforts to identify markers unique to each cell type have produced conflicting data[113-116]. However, recent studies suggest that Fibulin-2, Thy-1[117] and gremlin[118] are unique to portal myofibroblasts while laminin is expressed only by HSC[119]. An important question which needs to be addressed is whether the two cell types produce fibrotic tissue of different compositions. The temporal activation of the two cell types may also differ. In cholestatic liver diseases, HSC may drive the initial fibrotic response, with portal myofibroblasts assuming a greater role later in fibrosis development; in addition, there may also be differences in acute versus chronic cholestatic injury[120]. These may be important questions to take into consideration when designing therapeutic targets for cholestatic liver diseases.

Bone marrow-derived cells are also recruited to the liver and contribute to hepatic fibrosis. Mice transplanted with green fluorescent protein (GFP)-expressing bone marrow cells showed GFP-positive HSC in the liver[121-122]. Human patients who underwent gender-mismatched bone marrow or liver transplants have provided further evidence for the bone marrow as a source of HSC, or myofibroblasts in the liver[123]. However, a different study suggested these cells are not mature HSC; rather, that these bone marrow-derived cells are mesenchymal precursors (or fibrocytes) which differentiate into myofibroblasts after taking residence in the liver[124].

Further sources of myofibroblastic cells have been identified. These include the myofibroblasts derived from the transformation of hepatocytes[125], and/or cholangiocytes[126], *via* the process of epithelial-mesenchymal transition (EMT). Fibroblasts of the Glisson's capsule[127] and smooth muscle cells (termed second layer cells) around the central vein[128] also contribute to fibrotic tissue in the liver.

Ductular reaction

In the normal liver regeneration occurs *via* hepatocyte replication. If this process is impaired or overwhelmed, a secondary pathway involving hepatic progenitor cells is activated. These progenitor cells give rise to small reactive bile ducts as well as intermediate hepatocytes. The term ductular reaction was coined by Popper and colleagues in 1957[129] to describe a lesion they observed which was characterised by the swelling and proliferation of cholangiocytes. A strong correlation exists between the ductular reaction and hepatic fibrosis, not only in cholestatic liver disease such as seen in biliary atresia[130], but also in hepatocellular injury associated with HCV[131] and NASH[132]. Conversely, patients with Alagille Syndrome, who have no reactive bile ductules[133], are slow to develop fibrosis despite severe cholestasis and puritis[134]. The cells of the ductular reaction appear to play a role in the development of hepatic fibrosis but the nature of this interaction is yet to be adequately defined. It has been suggested that the ductular reaction drives fibrosis; indeed a recent study has shown that both hepatic progenitor cells and HSC express epithelial and mesenchymal markers[135], which suggest direct mesenchymal-epithelial transition is possible between progenitor cells and HSC. Alternatively, HSC activation may be mediated by proinflammatory or profibrogenic factors released by hepatic progenitor cells, or cells of the ductular reaction[136] although this hypothesis remains to be evaluated. Other theories suggest that the ductular reaction and hepatic fibrosis are not interdependent but rather, either occur in parallel in response to a common stimulus (reviewed in[137]), or even that progenitor cell expansion occurs after fibrosis is initiated by HSC[138]. Clearly, controversial, this field of research warrants further extensive investigation.

A recent study demonstrated the potential for direct progenitor cell and HSC interaction driving chemotaxis-associated inflammation associated with wound healing and hepatic regeneration in a murine model of portal fibrosis[139]. This proinflammatory pathway was initiated *via* lymphotoxin-β (LT-β), a cell surface-bound ligand expressed on progenitor cells interacting with the LT-β receptor expressed on adjacent HSC. This interaction induced an NFκB-regulated signalling pathway which upregulated the expression of chemotaxis-associated factors RANTES and ICAM-1, which was proposed to cause the recruitment of $CCR5^+$ inflammatory cells, HSC and progenitor cells to the site of hepatic injury aiding in wound healing and fibrogenesis[139].

HSC chemotaxis in response to MCP-1

HSC are responsive to a variety of different chemokines and chemoattractants including PDGF[140,141], CXCR3 ligands[142], macrophage inflammatory protein 1 (MIP-1)[143], CCL5/RANTES[144] and IL-8[145] (Figure 1).

One of the most potent HSC chemokines is MCP-1[146,147]. Elevated MCP-1 expression has been demonstrated in cholangiocytes in adult patients with PBC[148], and elevated serum MCP-1 has been observed in children with Bilary Atresia[149]. These findings were confirmed in a well characterised cohort of children with cholestatic liver disease (CFLD and biliary atresia) and also in the BDL rat model of cholestatic liver injury[51]. In the liver, MCP-1 protein was expressed predominantly by hepatocytes at the scar margin and also by cholangiocytes of reactive bile ductules in close proximity to activated HSC and myofibroblasts, respectively. Using in situ hybridisation, MCP-1 mRNA was also seen in perisinusoidal cells[51], suggesting HSC themselves produce MCP-1[150] as demonstrated *in vitro*. MCP-1 was localized to the apical membrane of cholangiocytes and the pericanalicular membrane of hepatocytes suggesting it may be actively secreted into bile. Elevated MCP-1 was also detected in the bile in both CFLD and in the animal model. Importantly, MCP-1 expression was elevated in CFLD patients and cholestatic rats with stage 0 fibrosis, i.e., prior to the histological evidence of fibrosis, suggesting that MCP-1 plays a crucial role in the early events associated with hepatic fibrogenesis[51,136].

In this same study, hepatocytes isolated from BDL

rats produced increased levels of MCP-1 which caused HSC chemotaxis, *in vitro*[51]. This effect was inhibited in a dose-dependent manner by up to 80% using a neutralizing antibody to MCP-1. The bile acid taurocholate was demonstrated to induce MCP-1 expression in normal control hepatocytes suggesting its potential as an initiating stimulus in cholestasis, which was verified in both CFLD and cholestatic rats showing a correlation between taurocholate and MCP-1 in serum and bile. The primary receptor for MCP-1 on monocytes, chemokine (C-C) receptor 2 (CCR2), has not been demonstrated on human HSC[146] or rat portal myofibroblasts[151], although a recent study has identified CCR2 on mouse HSC[152]. Other receptors may play a role in eliciting the chemotactic effects on rat and human HSC, and portal myofibroblasts, although these remain to be identified.

Role of HSC contractility in portal hypertension associated with fibrosis

Portal hypertension is a common complication of hepatic fibrosis. It is seen in biliary atresia patients even after successful Kasai portoenterostomy[153], as well as children with CFLD with varying degrees of liver disease. Portal hypertension is defined as a portal pressure gradient between the portal vein and the hepatic vein of greater than 5 mm Hg. HSC are proposed to contribute to portal hypertension by several mechanisms including increased contractility, the deposition of collagen, sinusoidal remodelling and angiogenesis[94,154].

HSC contractility is maintained in the normal liver *via* a balance between vasodilators and vasoconstrictors. HSC dilators include nitric oxide (NO), carbon monoxide, H2S and prostaglandin, while Endothelin-1 is a potent HSC constrictor. In the normal liver NO is produced constitutively in sinusoidal endothelial cells by endothelial nitric oxide synthase (eNOS)[155] or by inducible nitric oxide synthase (iNOS) in HSC[156]. In cholestasis, and the resultant oxidative stress, eNOS-derived nitric oxide synthesis is impaired and the negative regulation of HSC contractility is lifted (Figure 1). Endothelin-1 expression is also increased. Serum endothelin-1 levels are elevated in patients with biliary atresia with portal hypertension[157]. Endothelin in produced by sinusoidal endothelial cells[158] with HSC expressing endothelin receptors[159], thus HSC are proposed to control sinusoidal blood flow by constricting the perisinusoidal space surrounding endothelial cells[154].

In addition to its vasodilatory role, nitric oxide inhibits HSC proliferation and migration. NO can elicit HSC apoptosis through mitochondrial membrane depolarisation in a mechanism which is caspase-independent[160]. Thus, the nitric oxide depletion seen in cholestasis also results in a lifting of the negative regulation of HSC apoptosis. Instead, HSC proliferate and collagen deposition is increased, further contributing to portal hypertension.

Liver immunity in cholestatic liver disease

A marked inflammatory infiltrate has been documented in the liver of children with biliary atresia, Idiopathic Neonatal Hepatitis, choledochal cysts, total parenteral nutrition[161-164] and paediatric-onset PBC[165]. These studies have shown increased levels of $CD4^+$, $CD3^+$ and $CD8^+$ T cells, $CD56^+$ Natural Killer cells and $CD68^+$ macrophages around the bile ducts. The $CD4^+$ T cells express the Th-1 cytokines interferon-γ and IL-2[162,163] as well as Th-2 cytokines, IL-4 and IL-10[166], while $CD68^+$ macrophages express TNF-α[163] and IL-18[166]. The mechanisms by which these immune cells and cytokines interact with HSC and contribute to fibrosis are not yet clear. As discussed earlier, the role of chemokines and cytokines (such as MCP-1, RANTES and TGF-β) in stimulating HSC in cholestatic livers is well established. However the role of these factors in recruiting lymphocytes to the cholestatic liver also requires further characterisation.

While $CD4^+/CD25^+$ T cell numbers have been shown to be increased in biliary atresia[161] , these cells are depleted in the liver of patients with PBC[167,168], an autoimmune disease. CD25 (which is also the IL-2 receptor alpha) is an important marker of regulatory T cells and plays a key role in maintaining self tolerance[169]. HSC are believed to contribute to the liver's immune-tolerance through T cell suppression[170], an effect which may be enhanced in PBC, although the role of these T cells in other paediatric cholestatic liver diseases is unknown.

Therapeutics to reverse hepatic fibrosis

The hepatic fibrosis which accompanies cholestatic liver disease is reversible[171,172] and HSC are crucial in this process[173]. In patients with HBV[174] and HCV[175] infections, even advanced fibrosis is reversible and patient outcomes can be improved. Several studies have attempted to reverse fibrosis by targeting various aspects of HSC activation or function (review[176]). More recently, in a BDL rat model of cholestatic injury, Rapamycin was shown to target HSC function on several levels including HSC activation and proliferation, EMT and liver progenitor cell proliferation[177]. Sorafenib[178] has been shown to reduce portal hypertension in BDL rats by reducing HSC-mediated sinusoidal constriction. Nevertheless the shortcoming of all these studies is the lack of liver specificity as these factors target collagen deposition or fibrosis in all organs. Therapeutic agents with a further level of specificity in targeting HSC, but sparing other liver cells, would be even more valuable. Some of the most promising agents being investigated are those which selectively induce apoptosis of HSC, but not hepatocytes. These include gliotoxin[179], proteosome inhibitors[180] and TRAIL[181]. These may be used alone or in conjunction with agents that block hepatocyte apoptosis[182].

As discussed earlier, UDCA is an endogenous hydrophilic (and therefore protective) bile acid which normally makes up approximately 3% of the human[183] bile acid pool. It is commonly used as a therapeutic agent in various cholestatic liver diseases[184], since it

is well tolerated and has few side effects. The exact mechanisms by which it modulates HSC function are now being elucidated. The effects of UDCA are proposed to include hepatoprotection against oxidative stress, inhibition of apoptosis, stimulation of bile flow, as well as immunomodulatory effects on cytokine suppression (reviewed in[185]). In PBC, there is some evidence to suggest that long-term use of UDCA delays fibrosis progression (reviewed in[186]). However, there is little evidence to suggest a direct influence of UDCA on the regression of hepatic fibrosis in CF[21,187], although more comprehensive long-term prospective follow-up studies are required.

CONCLUSION

Detecting hepatic fibrosis and monitoring disease progression in paediatric cholestatic liver disease remains a challenge. The development of significant liver disease in children with CF is increasingly recognised but it is difficult to identify those likely to progress to cirrhosis and at risk of greater morbidity and mortality. Neonatal Hepatitis and biliary atresia are conditions with similar clinical presentation and thus difficult to differentially diagnose without an invasive operative cholangiogram. Commonly used clinical methods have poor sensitivity and poor specificity for detecting and staging fibrosis. While liver biopsy is the gold standard to detect fibrosis, it is not without limitations, particularly in focal diseases such as CFLD. New non-invasive serum marker panels or imaging technologies may provide a minimally invasive method to stage and monitor fibrosis progression. However, given the congestive nature of cholestatic liver diseases, transient elastography may not be a clinically useful alternative in children with suspected cholestasis. Significant advances have been made in understanding the biology of HSC and the interaction between HSC and cholangicytes, hepatocytes, Kupffer cells, inflammatory cells and progenitor cells. Understanding the cellular and molecular mechanisms associated with cholestasis-induced hepatocellular injury and fibrogenesis may provide novel markers to aid in better diagnosis of liver disease, detection of fibrosis and prediction of outcome. The role of the endocrine growth factor intestinal FGF19 in regulating bile acid synthesis and the taurocholate-induced HSC chemokine MCP-1 in wound healing and fibrogenesis, have helped to identify previously unrecognised regulatory pathways of disease progression in paediatric cholestatic liver disease. Further investigation into the processes associated with wound healing will greatly assist in more accurate diagnosis and better management of infants and children with paediatric cholestatic liver disease, and ultimately aid in the development of more targeted therapeutic modalities.

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