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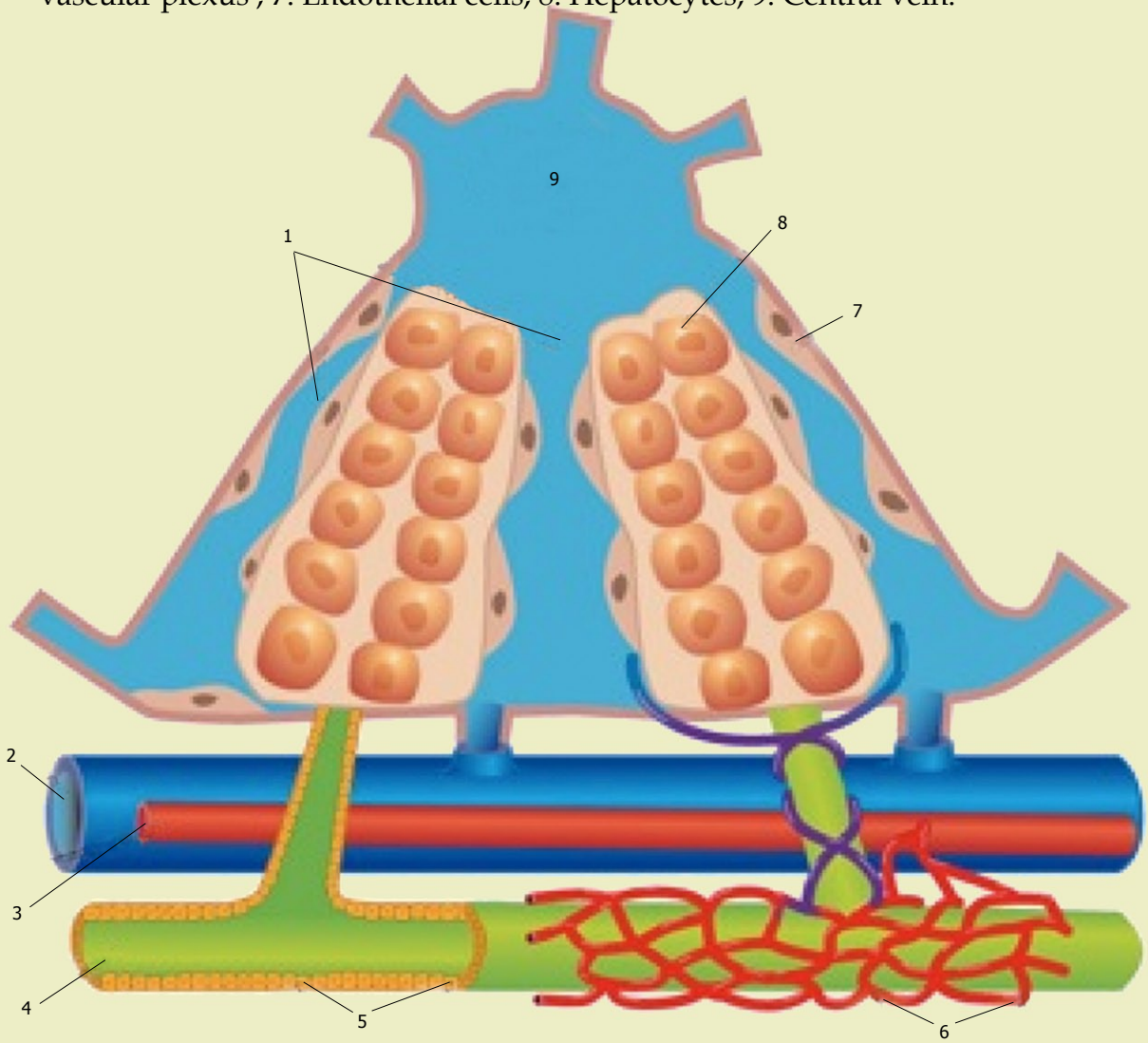
Graphic image of blood flow through the liver and the key cells involved. 1: Hepatic sinusoids; 2: Hepatic portal vein; 3: Hepatic artery; 4: Bile duct; 5: Bile duct cells (cholangiocytes); 6: Peribiliary vascular plexus ; 7: Endothelial cells; 8: Hepatocytes; 9: Central vein.



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EDITORIAL

# Aberrant DNA methylation profile in cholangiocarcinoma

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## Abstract

Cholangiocarcinoma (CCA) is a notoriously lethal epithelial cancer originating from the biliary system. As radical resection offers a poor success rate and limited effective adjuvant modalities exist in its advanced stage, the disease leads to a fairly poor prognosis. As the incidence of CCA is increasing, although the mortality rate remains stable, and few other definite etiologies have yet to be established, renewing our knowledge of its fundamental carcinogenesis is advisable. The latest advances in molecular carcinogenesis have highlighted the roles of epigenetic perturbations and cancer-related inflammation in CCA. This review focuses on the reciprocal effects between aberrant DNA methylation and inflammatory microenvironment in CCA.



**Key words:** Cholangiocarcinoma; Epigenome; DNA methylation; Cancer-related inflammation; Microenvironment

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## INTRODUCTION

Cholangiocarcinoma (CCA) is the second most common epithelial cancer originating from the biliary system, accounting for 10% to 20% of primary liver cancer[1]. As the presentation of symptoms is delayed, an R0 resection (both gross and microscope negative margin) can be achieved in less than 80% of those 10% early-stage patients in whom radical surgical intervention can be applied[2,3]. Additionally, the limited adjuvant modalities available for advanced patients have failed to show substantial benefit[3]. The aforementioned factors offer CCA a fairly poor prognosis, as overall 5-year survival rates in the resectable cases are less than 40% in both extrahepatic cholangiocarcinoma (ECC) and intrahepatic cholangiocarcinoma (ICC), and the median survival time is less than 12 mo in unresectable or metastatic cases[2,3]. Incidences of CCA, especially ICC, are increasing worldwide, although mortality has remained stable during the last four decades, with the exception of the decline in gallbladder carcinoma[1,4]. To date, though some well-documented risk factors have been identified, the majority of CCA etiology has remained

unknown[1,5,6]. Emerging advances in CCA molecular research have highlighted the roles of epigenetic perturbations and cancer-related inflammation[1,6-11]. The aberrant DNA methylation of CCA, which regarded as one of the best-characterized, mitotically heritable and reversible epigenetic modulations, has been seen to affect multiple steps of cholangiocarcinogenesis[7-12].

Epigenetic controls of gene expression orchestrate changes of chromatin architecture tissue-specifically and dynamically without affecting gene sequences, encompassing some basic mechanisms like post-translational modification of histones, displacement of nucleosomes and DNA methylations. Broadly, it may also include RNAi and non-coding RNAs. Within the above epigenetic modulations, DNA methylation is best characterized and delineated as a post-replicative addition of a methyl (-$CH_3$) group to the cytosine-5-carbon position, which is catalyzed by at least three DNA methyltransferases (DNMTs): DNMT1, DNMT3a and DNMT3b. Methylation occurs mainly at CpG dinucleotides, CpNpG and rarely at non-CpG dinucleotides like CpA, CpT and CpC[13-15] of the CpG islands or the CpG island shores[16] located in the promoter or encoding regions of genes[17]. Methylated cytosine will allow binding with methyl-CpG binding domain (MBD) proteins (MeCP1 or MeCP2) to remodel the chromatin architecture, a process that has been recognized as essential and versatile for epigenetic modification[18]. To date, global genome hypomethylation and local tumor suppressor genes hypermethylation have been noticed in tumorigenesis[19,20]. Furthermore, abundant bench and bedside evidence supports the putative association between inflammation and cancer[12], which potentially leads to dysregulated DNA methylation in CCA[7-11]. Though clinicopathological and epidemiological differences exist between ICC and ECC, emerging evidence has revealed that both of them are closely related to chronic inflammation[1,2,5-10]. This review aims to summarize current reported aberrant DNA methylation profiles of CCA and outline the involving role of cancer-related inflammation.

## RECIPROCAL EFFECTS BETWEEN INFLAMMATORY MICROENVIRONMENT AND CCA

Briefly, there are two pathways bridging inflammation to CCA: the extrinsic pathway (risk factors or related environmental exposures of CCA) and the intrinsic pathway (congenital or acquired genetic alterations, e.g. activation of oncogenes, inactivation of tumor suppressors, senescence-related perturbations, etc). The milieu of chronic inflammation may environmentally select the adaptive transformed cholangiocytes, thereby initiating cholangiocarcinogenesis. For instance, inactivation of 9p21 gene cluster ($p16^{INK4a}$/$p14^{ARF}$/$p15^{Ink4b}$) has been unraveled in liver fluke-related CCA[21] and primary sclerosing cholangitis-associated CCA[22]. Also, a high frequency of microsatellite instability (MSI) and inactivation of hMLH1 has been observed in thorotrast-related CCA[23]. Although a recent study of thyroid carcinoma has uncovered that an early genetic event is necessary and sufficient for initiating a cancerous development by promoting an inflammatory microenvironment[24], similar instances of an intrinsic pathway have yet to be addressed in CCA. Instead, a mounting body of evidence of genetic alterations in CCA has indicated these pathways indirectly, such as mutations or deletions of *K-ras*, *p53*, $p16^{INK4a}$, $p15I^{nk4b}$, $p14^{ARF}$ or *Smad4*; loss of heterozygosity (LOH) of *adenomatous polyposis coli gene* (APC) or allelic losses on 3p13-p21 and 8q22[25-30].

## INFLAMMATION-RELATED EPIGENETIC PERTURBATIONS IN CCA

Some key intrinsic factors can mediate inflammation-related gene regulation in CCA, including transcription factors [signal transducer and activator of transcription 3 (STAT3), etc], cytokines (IL-6, TNF-α, etc), growth factors, nitric oxide, reactive nitrogen oxide species (RNOS) and bile acids[1]. Among the aforementioned mediators, IL-6 plays a crucial role in many cancers, especially in epithelial cancers[31]. Upregulation of IL-6 in carcinogenesis is triggered by an autocrine[32,33] or paracrine loop[34,35], or even by an intrinsic somatic mutation of epidermal growth factor receptor (EGFR)[36]. It is also well documented that in chronic cholangiopathies or biliary infection, the level of IL-6 is increased in bile. Briefly, in CCA, the negative feedback of the IL-6 pathway is deficient and replaced by an unlimited autocrine loop owing to aberrant epigenetic silence of suppressors of cytokine signaling 3 (SOCS-3), which is mediated by the IL-6/STAT3 pathway to maintain hypermethylation of the gene promoter[33]. Aberrant activated IL-6 in CCA leads to carcinogenesis promotion, through mechanisms such as up-regulating anti-apoptotic myeloid cell leukemia-1 (Mcl-1) mediated by phosphorylated STAT3[33,37]; up-regulating EGFR expression through decreasing its promoter methylation level mediated by undefined mechanisms[38]; and activating telomerase[39,40]. In breast cancer, overloaded IL-6 also enhances expression of stem cell survival regulator Notch-3 and activates the hypoxia-resistant gene carbonic anhydrase Ⅸ (CA-Ⅸ) through the Notch-3/Jagged-1 pathway[41]. However, effect of IL-6 such as these on cancer stem/progenitor cells in CCA has yet to be clearly defined. Moreover, IL-6 had been shown to affect microRNA profiles of CCA. Overloaded IL-6 also increased let-7a expression *via* an undetermined mechanism, resulting in suppression of neurofibromatosis 2 (NF2) and a subsequent increase of phosphorylated STAT3[42]. Recently, Meng and colleagues have revealed that overload of IL-6 in CCA can up-regulate expressions of two DNA methyltransferases, DNMT1 and HASJ4442, leading to hypermethylation of the CpG island where the miR-370 encoding gene was

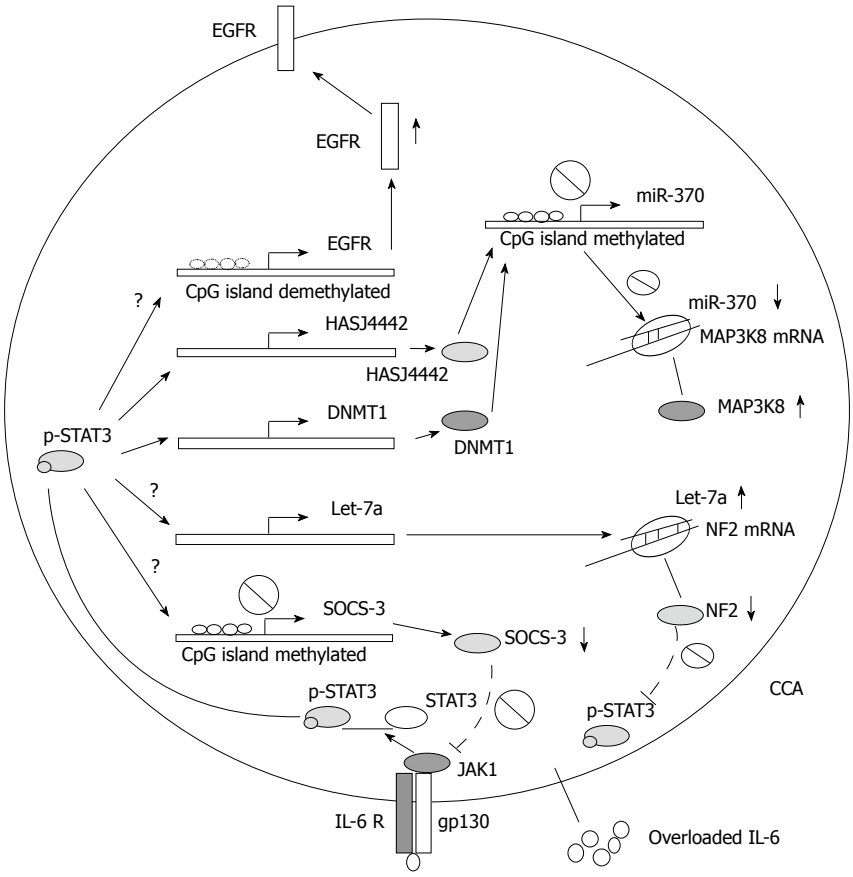


**Figure 1 The role IL-6 signaling in CCA inflammation-related epigenetic regulation.** In CCA, the negative feedback of the IL-6 pathway is deficient owing to aberrant epigenetic silence of suppressors of cytokine signaling 3 (SOCS-3), which is mediated by the IL-6/STAT3 pathway to maintain gene promoter hypermethylated. Up-regulation of EGFR expression through decreasing its promoter methylation level mediated by undefined mechanisms. Moreover, overloaded IL-6 also increased let-7a expression via an undetermined mechanism, resulting in suppression of neurofibromatosis 2 (NF2) and subsequently increasing phosphorylated STAT3. Overloaded IL-6 in CCA can up-regulate expressions of two DNA methyltransferases, DNMT1 and HASJ4442, which leading to hypermethylation of the miR-370 gene promoter and abrogating suppressing effect of miR-370 on oncogenic mitogen-activated protein kinase kinase kinase 8 (MAP3K8).

embedded. Consequently, oncogenic mitogen-activated protein kinase kinase kinase 8 (MAP3K8) suppressing effect of miR-370 was abrogated in CCA[43]. (The role of IL-6 signaling in CCA inflammation-related epigenetic regulation is summarized in Figure 1).

Infiltrated leukocytes also fuel the inflammation-related epigenetic perturbations in CCA. The mechanisms of leukocyte recruitment and homing in cholangiopathies, like primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and chronic viral hepatitis C, have long been investigated. Briefly, chemokines CCL21, CCL28, CX3CL1, CXCL9 and CXCL10 mediate the recruitment of leukocytes into the portal tract and subsequently CX3CL1, CXCL12, CXCL16 and CCL28 retain these inflammatory cells in the bile duct to serve their undetermined roles[44,45]. Little research has been conducted regarding the immunosuppressive milieu of CCA-related inflammation. Recently, the roles of PD-L1 (also termed B7-H1 or CD274, an inhibitor of activated effector T cells)[46] and Foxp3+ regulatory T cells[47] in ICC have been explored. Meanwhile, Opisthorchis viverrini infection has been shown to upregulate inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression in rodent macrophages through the Toll-like receptor 2/NF-κB pathway[48]. However, epigenetic modulations on immunity against CCA have yet to be unraveled. Actually, in the Foxp3+ regulatory T cells of the mouse colitis model, the immunosuppressive effect related to Foxp3 was mediated by its co-repressors, Eos and C-terminal binding protein-1 (CtBP1), by promoting hypermethylation of IL-2 gene promoter, trimethylation of histone 3 lysine 4 and acetylation of histone 3 and histone 4[49]. Furthermore, both of the antigen-presentation molecule major histocompatibility complex class 2 (MHC-Ⅱ) and its co-activator, class Ⅱ transactivator (CⅡTA) can be epigenetically modulated in cancer[50]. Briefly, in renal carcinoma and pancreatic carcinoma, dysregulated IL-10 and Decoy receptor 3 (DcR3) can suppress CⅡTA expression respectively in immune cells through epigenetic modifications on CⅡTA gene promoter[51-52].

Besides leukocytes, much research has also highlighted the pivotal roles of other stroma constituents in the progression of chronic cholangiopathies, encompassing fibroblasts, hepatic stellate cells (HSC), extracellular matrix (ECM), etc[53-57]. Recently stromal effect on parenchyma epithelial-to-mesenchymal transition (EMT) has also been noticed in cholangiopathies[54,58-60]. However, in CCA, less evidence involving the contribution of remodeled stroma to the epigenetic perturbations than in other cancers could be obtained. Briefly, cancer-associated fibroblasts (CAFs) have been proven to exert their epigenetic modulations on neighboring immortalized human breast epithelial cells MCF10A by direct cell-cell contact, simultaneously activating Akt1 and suppressing Akt1 repressor, inositol polyphosphate-4-phosphatase type Ⅱ (INPP4B), subsequently leading to de novo promoter hypermethylation of the tumor suppressor gene Cystatin M (CST6)[61]. Furthermore, mechanical forces of the remodeled ECM or tissue architecture

**Table 1 DNA methylation profiles of cholangiocarcinoma varying with specific risk factors**

| | Methylated gene (proportion rate) |
|---|---|
| **Definite risk factors** | |
| Primary sclerosing cholangitis | *p16* (25%)[22] |
| Liver fluke infection (opisthorchis viverrini, or less frequently, clonorchis sinensis) | *RUNX3* (49.1%)[70]; *p14* (40.2%), *p15* (48.9%), *p16* (28.3%)[21]; *hMLH1* (44.6%)[71] |
| Hepatolithiasis | *p16* (100%[72], 54.6%[73]); *TFF1* (37.5%)[74] |
| Biliary malformation (congenital choledochal cysts, caroli's disease, etc) | NA |
| Thorotrast | *hMLH1* (45.8%), *hMSH2* (25.0%)[23] |
| **Probable risk factors** | |
| Hepatitis C or hepatitis B | NA |
| Cirrhosis | NA |
| Toxins (dioxin, polyvinyl chloride, nitrosamines, etc) | NA |
| Biliary-enteric anastomosis or cholangiojejunostomy | NA |

NA: Not available; *RUNX3*: Runt-related transcription factor 3; *hMLH1*: Human mutL homolog 1; *TFF1*: Trefoil factor family 1; *hMSH2*: Human mutS homolog 2.

may also exert potential modulated effects on epigenetic perturbations of cholangiocarcinogenesis. In this context, some details about dynamic biochemical pathways and mechanotransduction, termed "mechanoreciprocity", have been explained in other cancers, like breast cancer[61-64]. Effects of ECM on the cellular epigenome are increasingly being deciphered[62-64]. Briefly, the ECM exert their cis- and trans-regulations on transcription *via* specific membrane receptors such as integrin, and various intracellular molecules like focal adhension kinase (FAK), RhoGTPases, ATP-dependent chromatin remodeling complexes (SWI/SNF, ISWI, CHD, INO80 and SWR1), which couple extracellular signals to the cytoskeleton and chromatin and mainly mediate by targeting the ECM-responsive elements (EREs) in the genome as well as transcription factors[62-65]. Moreover, it is increasingly accepted that ECM-mediated mechanotransduction can "prepare" chromatin structures to receive specific biochemical signals and can control common sets of genes in cells possessing similar morphology. Subsequently, defined biochemical signaling networks permit further tissue-specific transcription in differentiation-specific genes[62-64]. One typical example of this intricate reciprocity is nuclear lamina-associated transcriptional silencing[62]. The nuclear lamina is made of intermediate filament proteins like lamins A/C and B, which can bind smaller nesprins that belong to the inner nuclear membrane proteins. Smaller nesprins can shuttle between the outer and inner nuclear membrane, while the biggest nesprins are anchored in the outer nuclear membrane where they can extend into the cytoplasm and bind actin microfilaments and intermediate filaments[62]. Moreover, associations of negative transcription factors with components of the nuclear lamina have also been explained. For instance, the lamin B receptor can bind histone 3 methylated lysine 9 and heterochromatin component HP1[66], while lamin A/C and lamin B can bind MOK2 and Oct-1, respectively[67,68]. Recently, a genome-wide high-resolution mapping of lamin B1-associated DNA domains further implied this putative association between remodeled ECM and epigenetic control, revealing that lamin B1-associated DNA domains contain H3K27me3, the insulator protein CTCF or methylated CpG islands[69].

## ABERRANT DNA METHYLATION PROFILES VARYING WITH SPECIFIC RISK FACTORS OF CCA

As the inflammatory microenvironment may vary with diverse cholangiopathies, disease-specific DNA methylation profiles or epigenome can reasonably be expected. Some of the aberrantly methylated genes reported in currently available literature are summarized in Table 1[70-74], according to respective specific risk factors of CCA.

## CONCLUSION

From the above Table 1, more details about aberrant DNA methylation profiles in CCA remain to be unraveled. To date, although the mechanism of demethylation and the candidate enzymes exhibiting direct demethylase activity and related cofactors are not yet firmly established in mammalian cells, recent trends in research about DNA demethylation have revealed the putative role of the BER/NER pathways and the association with DNMT1[75,76]. Moreover, repair-mediated DNA demethylation of *Oct-4* gene promoter by Gadd45a has been observed[77]. Recently, Gadd45a has also been shown to connect neuronal circuit activity with DNA demethylation in mature neurons for extrinsic modulation of adult neurogenesis[78]. Further progresses in this field will help to facilitate our understanding about mechanisms of aberrant DNA methylation in CCA further.

In conclusion, chronic inflammation of cholangiopathies predisposes individuals to CCA. Since alterations of the cellular epigenome usually precede morphologic changes and genetic alterations, identification of related aberrant DNA methylation profiles according to specific inflammation milieu may serve as a reasonable early diagnostic marker and an intervention target for CCA.

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TOPIC HIGHLIGHT

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# Involvement of cholangiocyte proliferation in biliary fibrosis

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## Abstract

Cholangiocytes are the epithelial cells that line the biliary tree. In the adult liver, they are a mitotically dormant cell population, unless ductular reaction is triggered by injury. The ability of cholangiocytes to proliferate is important in many different human pathological liver conditions that target this cell type, which are termed cholangiopathies (i.e. primary biliary cirrhosis, primary sclerosing cholangitis and biliary atresia). In our article, we provide background information on the morphological and functional heterogeneity of cholangiocytes, summarize what is currently known about their proliferative processes, and briefly describe the diseases that target these cells. In addition, we address recent findings that suggest cholangiocyte involvement in epithelial-to-mesenchymal transformation and liver fibrosis, and propose directions for future studies.



**Key words:** Biliary epithelium; Cholangiopathies; Cholestasis; Integrins; Liver fibrosis; Proliferation

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## INTRODUCTION

Cholangiocytes are epithelial cells that line the biliary system and make up 3%-5% of the liver cell population[1,2]. The biliary system is a tree-like, three-dimensional network of ducts that range in size from small (< 15 μm) to large bile (> 15 μm) ducts in animal models[3,4]. These ducts are lined by cholangiocytes that also vary in size, dependent upon the size of the bile duct[5]. For example, large bile ducts are lined by large cholangiocytes and small bile duct by small cholangiocytes[3,4,6]. The major functions of the biliary system are to deliver bile from the liver to the gallbladder to the duodenum and the modification of

bile of canalicular origin[5,7,8]. Cholangiocytes modify bile[9-13] through a series of re-absorptive and secretory processes involving water, ions, and solutes, which are heavily regulated by gastrointestinal hormones, such as secretin[11,13]. Secretin receptors (SR) are present only on cholangiocytes in the liver[14]. Large but not small cholangiocytes express SR and are responsive to secretin in the normal rodent liver[3]. Small cholangiocytes *de novo* express secretin receptor during pathological conditions where large functionally active cholangiocytes are damaged[12,15]. In large cholangiocytes, secretin increases cyclic adenosine 3', 5'-monophosphate (cAMP) levels[4,16-19] and induces the opening of the Cl- channel (cystic fibrosis transmembrane conductance regulator, CFTR), which leads to the activation of the Cl-/$HCO_3$- anion exchanger 2 (AE2) and the secretion of bicarbonate in bile[3]. In addition to their normal biliary function, cholangiocytes have also been found to detoxify xenobiotics[9-12] and they also provide one of the first lines of defense against microbes in the biliary system[7,20]. Our knowledge of the factors that control cholangiocyte function has greatly increased in recent years, due to an increased interest in liver diseases, such as biliary cancer, biliary fibrosis and cholestatic liver disease[7,9-11].

Cholangiocytes are the target of many diseases, referred to as cholangiopathies, with a high impact in terms of morbidity and mortality in both children and adults[7,10,21,22]. These diseases have a diversity of etiologies, such as genetic predisposition, e.g. Alagille's syndrome, Cystic Fibrosis, fibropolysystic diseases; immune-mediated diseases like PSC, PBC[3,7,23,24], liver allograft rejection, and graft-versus-host disease[3,7,10,23,24]; a variety of infections - bacterial, fungal, parasitic and viral cholangitis; AIDS cholangiopathy; biliary atresia; idiopathic causes such as sarcoidosis; and malignant ones, such as cholangiocarcinoma[3,7,23,24]. Cholangiopathies are the leading cause of liver transplantations in pediatric patients (50%) and the third leading cause in adults (20%)[3,23,25]. Cholangiopathies are characterized by cholestasis, the loss of cholangiocytes through necrosis or apoptosis, with cholangiocyte proliferation resulting in the formation of new side branches to ducts in an effort to regain function[13,26,27], and portal/periportal inflammation[26]. Obstructive cholestasis contributes to hepatic cirrhosis and portal hypertension[28]. Portal fibroblasts and hepatic stellate cells (HSCs) are recruited to the area, and followed by parenchyma invasion and biliary fibrosis[26]. This remodeling process involves crosstalk between mesenchymal cells and cholangiocytes, the latter being able to secrete chemokines [interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), interlukin 8 (IL-8), Monocyte chemotactic protein-1 (MCP1) and profibrogenic factors (platelet derived growth factor-BB (PDGF-BB), endothelin-1 (ET1), connective tissue growth factor (CTGF), and transforming growth factor-beta 2 (TGFβ2)][26,29].

## CHOLANGIOCYTE PROLIFERATION

In animal models of cholestasis and biliary tract injury, cholangiocyte proliferation is coordinately regulated by a number of neuroendocrine factors, such as hormones, neuropeptides and neurotransmitters, which have been recently reviewed[1,3]. Proliferating cholangiocytes display neuroendocrine phenotypes, and as such, secrete and respond to a number of hormones, neuropeptides and neurotransmitters[9]. The capacity of cholangiocytes to proliferate is evident under specific experimental con ditions in animal models as well as in human pathological conditions[9,30,31]. Four types of "ductular reaction" have been described in animal models[9,30,31]. Cholangio cyte proliferation is described as "ductular reaction", a term coined by Popper to identify the expanded popula tion of epithelial cells at the interface of the biliary tree, which refers to proliferation of pre-existing ductules, progenitor cell activation, and the appearance of intermediate hepatocytes[5,9,30,31]. The ability of cholangiocytes to proliferate is important in many different human pathological conditions. Type I or "typical" cho langiocyte proliferation results in an increased number of intrahepatic bile ducts (hyperplasia), which re mains confined to portal spaces[31]. The proliferating cho langiocytes form a well-differentiated three-dimensional network of tubular structures with a well-defined lumen,which originates from pre-existing bile ducts located within portal areas[31]. In humans, this type is observed in acute obstructive cholestasis, extrahepatic biliary atresia[9,31,32], and in early phases of chronic cholestatic liver diseases (in association with "atypical" pro liferation)[9,32]. In the rat, "typical" cholangiocyte proliferation occurs after bile duct ligation (BDL), partial hepatectomy, chronic a-naphthylisothiocyanate (ANIT) fe eding, chronic L-proline treatment and prolonged oral administration of certain bile acids[31].

Type Ⅱ or "atypical" proliferation is characterized by irregular proliferation of intrahepatic bile ducts not only confined to portal areas, but also sprouting into periportal and parenchymal regions[9,31,32]. This implies that the newly formed bile ducts are functionally ineffective[31]. In humans, this type is observed in PSC, PBC, after massive hepatic necrosis, focal nodular hyperplasia, chronic cholestatic diseases, alcoholic liver diseases, and a long-standing hepatic biliary obstruction[9,31,32]. Type Ⅱ refers to ductular metaplasia of liver cell plates, predominantly observed in chronic cholestatic conditions like PBC[6,31]. It is thought that the "atypical" proliferation arises from both proliferation of pre-existing ductules and expansion of the hepatic progenitor cell compartment[31].

Type Ⅲ ductular proliferation, sometimes called "ductular hyperplasia" or "oval cell" in the past[32] consists in the massive proliferation of ductular hepatocytes (derived from oval cells) or progenitor cells in the liver, with submassive hepatic necrosis[9,31]. It involves activation and proliferation of hepatic progenitor cells,

appearing as periportal ductular structures in the case of submassive hepatocellular necrosis, and representing an alternative parenchymal regeneration when hepatocellular regeneratory capacity is insufficient, which is primarily the case in chronic liver disease[6,31].

Type IV ductular hyperplasia, now called "oval cell", occurs in early stages of carcinogenesis in rat liver and is caused by chemicals, like ethionine, 2-acetylaminofluorene, and furan[9,32,33]. This type of proliferation induces the formation of disorganized tubular structures with a poorly defined duct lumen, which randomly sprout into hepatic lobules, creating a distorted hepatic architecture[31]. "Oval cells" are cell types activated to proliferate in early stages of intoxication with carcinogens. The nature of these cells, that is, whether they are fibroblasts, endothelial cells, transformed hepatocytes, or biliary ductules, is a subject of debate[6].

One of the functional characteristics of proliferating cholangiocytes is that they acquire a neuroendocrine phenotype, especially in "atypical" ductular reaction[9,32,34,35]. Proliferating cholangiocytes, from "atypical" proliferation, show phenotypical features of neuroendocrine cells like chromogranin A, S-100 protein glycolipid A2-B4, and a neural cell adhesion molecule[9]. During cholestatic liver diseases, the cholangiocytes express neuroendocrine phenotypes and respond to a number of hormones and neuropeptides[34]. For example, these proliferating cholangiocytes have increased expression and secretion of serotonin, endogenous opioid peptides and neurothrophins (and their corresponding receptors)[25]. They also show an enhanced response to hormones and neuropeptides such as secretin, gastrin, somatostatin, acetylcholine, and adrenergic and dopaminergic agonists[9,35]. Studies suggest that the formation of a neuroendocrine compartment is crucially instrumental in the progression of liver disease[9]. Thus, understanding how we can manage the proliferation of cholangiocytes is important for the development of treatments for liver diseases.

## CHOLESTATIC LIVER DISEASES AND BILIARY FIBROSIS

In response to acute liver injury, cholangiocytes proliferate in an effort to regain proper liver function. Human chronic liver diseases are characterized by repetitive liver injury due to chronic infection by viral agents (hepatitis B and C viruses), toxin/drug exposure (alcohol consumption), and autoimmune attack (PBC/PSC)[26]. Chronic liver diseases cause a continuous activation of the wound-healing response that results in an accumulation of extracellular matrix, eventually leading to liver cirrhosis and hepatic failure[26]. Thus, cirrhosis can be defined as an advanced stage of fibrosis involving the formation of abnormal cell clusters surrounded by excessive extra- cellular matrix, which also results in significant changes in vascular framework[26,36-38]. As originally described, liver fibrosis during acute and chronic cholestasis involves the stepwise process that includes "ductular reaction", accompanied by polymorphonuclear leukocytes and an increase in matrix deposition, leading to periportal fibrosis and eventually biliary cirrhosis[39].

## APOPTOSIS IN CHOLESTATIC LIVER DISEASES

Apoptosis is thought to play a major role in cholestatic liver diseases such as PBC, PSC and biliary atresia[40,41]. In immune-mediated liver diseases, such as PBC, PSC and autoimmune hepatitis, recent studies have indicated that programmed cell death ligands and circulating apoptotic markers might serve as diagnostic markers for these diseases[40,42]. Apoptosis of cholangiocytes has been observed in a number of animal models of cholestasis and biliary injury[12,15,43-45]. A recent study has shown that anti- death receptor 5 (DR5) monoclonal antibody induced cholangitis that exhibited the typical histological appearance of PSC and PBC[46]. These findings led them to believe the death signal mediated by TNF-related apoptosis-inducing ligand (TRAIL) receptor 2/DR5 may be a key regulator of cholestatic liver injury[46].

## SECRETION OF PROFIBROGENIC FACTORS BY CHOLANGIOCYTES

Sedlaczek *et al*[47] demonstrated that during the progression of biliary fibrosis, proliferating bile duct epithelial cells are the predominant source of the profibrogenic factor CTGF. Newly formed bile ducts also express the message for alpha 1 (IV) procollagen, indicating that proliferating cholangiocytes are a potential source of hepatic collagen during fibrosis[48]. TGFb2 expression has been shown to be a specific property of proliferating bile duct epithelial cells and it has been postulated that its expression was related to the formation of specialized periductular connective tissue during bile duct proliferation[49]. In addition, platelet-derived growth factor is expressed in proliferating cholangiocytes during experimental biliary fibrosis in rats[50]. Administration of pentoxifylline exerts an antifibrogenic effect by reducing the PDGF-induced ERK-dependent signaling and proliferation of extracellular matrix- producing cells[51]. Other studies have shown that during biliary injury and fibrosis, the hedgehog pathway activation induces cholangiocyte production of chemokines that recruit natural killer T cells to portal tracts[52]. Hedgehog ligands regulate tissue-remodeling responses during embryogenesis and adult tissue repair[52,53]. Cholangiocytes produce and respond to hedgehog ligands[38,52]. Hedgehog pathway activation promotes proliferation and enhances viability of these cells, which unrestrained, could cause progressive fi

brosis and hepatic architectural distortion[52]. The targeting of the profibrogenic program that is activated in proliferating cholangiocytes and the profibrogenic factors they secrete, might provide an unique opportunity for the development of treatments for biliary fibrosis.

## EPITHELIAL-TO-MESENCHYMAL TRANSITION (EMT)

Cholangiocytes normally exist in an highly differentiated state that allows them to modify bile of canalicular origins by a coordinated series of hormone- regulated secretory and absorptive processes[54]. Cholangiocytes proliferate in response cholestasis induced by bile duct ligation and during other pathological conditions such as, partial hepatectomy and $CCl_4$-induced liver damage[54]. Evidence suggests that proliferating cholangiocytes have a role in the induction of fibrosis, either directly through epithelial-mesenchymal transition[55], or indirectly through the activation of hepatic stellate cells[38]. EMT is a complex process that involves cross talk among several signaling pathways that collaborate to affect global, but gradual, changes in cell structure and function[56]. In this dynamic process cells eventually lose their typical epithelial characteristics (proteins that mediate cell-cell, cell-matrix contacts and cytoskeletal organization)[57,58]. These changes cause epithelial cells to disassociate from their neighbors, gradually acquire a motile phenotype, and eventually migrate out of epithelial sheets and into adjacent mesenchyme[21,56]. There are three biological subtypes of EMT, which have been previously reviewed[57,59]. Type 1 is present during implantation, embryogenesis and organ development. Type 1 also generates mesodermal and endodermal mesenchyme that can then undergo mesenchymal-to-epithelial transition (MET) to generate a secondary epithelia that can undergo additional rounds of EMT and MET to form various organs[57,59]. Although these concepts remain to be proven, it is possible that the balance between EMT and MET controls the outcome of chronic liver injury. Type 2 is associated with inflammation[57,59]. When there is injury with inflammation, this type generates fibroblastic cells that eventually cause organ destruction[57,59]. Type 3 is the result of genetic and epigenetic changes in cancer cells with invasion and spread of tumor cells that eventually form metastatic tumors apart from the primary tumor[57].

EMT is involved in tissues that are being developed or remodeled[57]. The presence of EMT in embryonic development[55,57] and cancer invasion and metastasis[60] is well established, and there is some evidence for EMT in the liver[55]. As a highly regenerative organ, the liver has the ability to restore its mass even in the face of extensive functional cell loss. However in situations of prolonged injury, this method of repair can lead to fibrosis[61]. Recent data suggest the classification at cellular, molecular and tissue level, multiple mechanisms for fibrosis as follows: (1) chronic activation of the wound-healing reaction; (2) oxidative stress and related reactive intermediates; and (3) profibrogenic derivatives of EMT[26]. Liver fibrosis develops from a heterogeneous population of profibrogenic hepatic myofibroblasts (MFs) that may originate from activated hepatic stellate cells and portal fibroblasts, bone marrow derived cells, or possibly cholangiocytes and hepatocytes that have undergone EMT[26]. These myofibroblasts are characterized by increased proliferation, migration, and contractility, and a relative resistance to apoptosis[37]. TGF(s) play a major role in the induction of EMT in development, carcinogenesis, and fibrosis, with different isoforms mediating various effects depending the cell type and setting[62]. EMT in response to TGFβ-1 and in fibrosis is mediated predominantly *via* Smad-dependent (mainly Smad3) pathways[63]. TGFβ-1 has previously been shown to play a critical role in the progression of liver fibrosis[64]. In fact, a recent study demonstrated that blockage of TGFβ-1 in proliferating biliary epithelia retards biliary fibrosis in an animal model of liver fibrosis[65]. Interestingly, a recent study demonstrated that EMT contributes to portal tract fibrogenesis during human chronic liver disease, which is characterized by chronic inflammation[65]. In fact, inflammation plays a key role as the convergence point between EMT and the progression of fibrosis in many organ systems, and has been previously reviewed[66].

EMT has been implicated as a key mechanism in the pathogenesis of liver fibrosis. In study of human samples from several types of liver diseases, Diaz and colleagues present convincing histological data revealing that EMT occurs in human liver fibrosis, particularly in disease associated with prominent bile ductular pro liferation, such as biliary atresia and PBC[55]. They observed significant colocalization between cytokeratin (CK-19, a cholangiocyte- specific epithelial marker) and other markers of EMT (i.e. vimentin, Snail, and fibroblast-specific protein 1) in biliary atresia and PBC. Robertson *et al*[67] have also demonstrated that biliary EMT occurs during post-transplantation recurrence of PBC. The study found that in livers affected by early recurrent PBC, there were indications that biliary EMT was occurring which was associated by cholangiocyte expression of S100A4 (a key marker of early fibroblast lineage), vimentin and pSMAD 2/3 with the data indicating that this process was driven by TGF-β[67]. S100A4 expression appears to occur prior to the onset of the appearance of other features of recurrent PBC, which suggests that EMT may be an initiating event, and may potentially explain the loss of bile duct epithelia during the course of the disease[67]. Rygiel and colleagues have also clearly demonstrated that EMT occurs during portal tract fibrosis[24]. Their work shows that cholangiocytes forming the small and medium sized bile ducts and responding with ductular reaction undergo

EMT during chronic liver disease, which results in the formation of invasive fibroblasts[24]. Similar findings have been demonstrated in liver cells from rodents, and humans can undergo EMT[68]. This study found that both hepatic stellate cells and hepatic epithelial progenitor cells coexpress epithelial and mesenchymal markers indicating that EMT occurs in adult livers[68]. This recent evidence indicates that EMT probably plays a critical role in the process of portal fibrosis during chronic liver diseases.

## INTEGRINS AND BILIARY FIBROSIS

Integrins are a family of heterodimeric transmembrane glycoproteins composed of α and β chain protein subunits that act as cell surface receptors[69-71]. Integrins are a large family of 24 heterodimers formed from eight β subunits and α subunits that have been identified. Integrins play a role in communicating messages between the cell and the environment *via* extracellular matrix interactions[69-71]. The binding to extracellular matrix to integrins results cytoplasmic signals in the integrin-expressing cell contributing to cell growth, differentiation, invasion, metastasis, and survival[65,72-74]. Integrins also play a key role in how cells sense to mechanical stimuli in the environment[65,72,73]. Integrins have been shown to interact with cell surface ligands, growth factors, pathogens, soluble proteases and transmembrane proteins[75,76]. The loss of integrin-mediated contacts, usually leads to apoptosis, a process called *anoikis*[74,77].

Two recent studies have demonstrated that targeting αvβ6 integrin expressed by proliferating biliary epithelia might provide a novel antifibrotic therapy[65,78]. Patsenker *et al* demonstrated that αvβ6 integrin is strongly upregulated in proliferating biliary epithelium in rodent (BDL, thioacetamide, Mdr2 (Abcb4)$^{-/-}$ mice) and human models (chronic hepatitis C[65,78]) and that it drives fibrogenesis *via* adhesion to fibronectin and stimulates auto/paracrine TGF-β1 activation[65]. Most importantly, they demonstrated in vivo that a single dose of a small molecule αvβ6 integrin inhibitor induced antifibrogenic and profibrinolytic genes, reduced activated cholangiocyte proliferation, and adhesion to fibronectin[65,78]. In addition, increased vascularization has a key role in the development of biliary fibrosis, as supported by fibrosis that has been limited in animal models where angiogenesis has been inhibited[14].

## REVERSAL OF BILIARY FIBROSIS

Although we have made impressive progress in our understanding of the pathogenesis of liver fibrosis in the past two decades, translation of this knowledge into antifibrotic therapies has ground to a halt short of clinical trials[37]. The reduction of fibrosis within cirrhotic liver tissue would lead to a reduction of portal hypertension and consequent clinical complications, thus improving overall liver function, which would extend the complication-free patient survival time and reduce the need for liver transplants[26]. Studies have suggested the reversibility of liver fibrosis, but the mechanisms for such a reversal are poorly understood[79]. In BDL rats, Popov and colleagues introduced macrophages to damaged biliary epithelium *via* Roux-en-Y bilio-jejunal anastomosis. After engulfing apoptotic cholangiocytes, macrophages upregulate matrix metalloproteinases and become fibrolytic effector cells[79]. This suggests a link between apoptotic epithelial cells, macrophages, and the reversal of fibrosis.

## FUTURE DIRECTIONS

In order to develop clinical treatments, we need to learn more about how cholangiocytes interact with other cell types, and the role that EMT contributes to biliary fibrosis. The studies presented in this review raise the important question of the relationship between cholangiocytes and myofibroblasts as to whether cholangiocytes may be an additional source of fibroblasts during chronic liver injury. Also, important will be to determine how cholangiocytes contribute to soluble factors and to the activation of myofibroblasts and to the deposition of extracellular matrix. Understanding the interactions and contributions of these cell types to the process of biliary fibrosis will be essential for determining whether different mechanisms of fibrosis occur in the various cholangiopathies, which, in turn, will aid in designing disease-specific therapies.

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TOPIC HIGHLIGHT

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# Histamine regulation of hyperplastic and neoplastic cell growth in cholangiocytes

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## Abstract

Histamine has long been known to be involved in inflammatory events. The discovery of antihistamines dates back to the first half of the 20th century when a Swiss-Italian pharmacologist, Daniel Bovet began his work. In 1957 he was awarded a Nobel Prize for his production of antihistamines for allergy relief. Since that time, histamine has been found to play a role in other events besides allergic reaction. Possibly unbelievable to Bovet and his peers, histamine has now been marked as playing a role in liver pathologies including hepatobiliary diseases.



**Key words:** Histamine; Receptors; Cholangiocytes; Cholestasis; Cholangiocarcinoma

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## INTRODUCTION

The liver is a dynamic organ that is able to repair and regenerate itself after injury. Numerous resident liver cell types are involved in maintaining a homeostatic state of the liver as well as playing a role during liver injury and disease. Cholangiocytes play a critical role in the regulation and overall function of the liver. These cells are hormone-responsive and much effort has been put

forth to delineate the mechanisms behind cholangiocyte function in liver disease. An emerging role for histamine and liver diseases has begun to be investigated.

In this review we will begin with a brief overview of the intrahepatic biliary tree, cholangiocyte function, hepatic vasculature, cholangiocarcinoma and histamine. After, we will discuss the latest findings regarding histaminergic activation in the liver as well as the most recent findings in regards to cholangiocyte regulation by histamine and histamine receptors. Finally, we will conclude with a section on histamine and cholangiocarcinoma and our speculation for the future of histamine in the progression of liver diseases.

## INTRAHEPATIC BILIARY TREE

The biliary tree is the common term used for the pathway where bile is secreted from the liver that begins with initiation of bile from the hepatocytes[1]. It starts with numerous small branches that terminate in the common bile duct, also known as the trunk of the biliary tree[2]. It is here that the common duct comes together with branches of the hepatic artery and portal vein forming the central axis of the portal triad[2]. Lining the intrahepatic biliary tree are epithelial cells known as cholangiocytes[1,3,4]. Once defined as "simple" epithelia, cholangiocytes are now regarded as key players in liver pathophysiology[3-7]. Cholangiocytes are responsible for the modification and release of bile from the liver and the transport of bile acids[6,8-12]. These cells are hormone-responsive, and express a multitude of hormone receptor binding sites that enable them to interact with mediators to induce varying effects on liver pathology[1,6,13-18]. These cells, that are cuboidal by nature, line the three-dimensional network of interconnecting ducts[1]. Cholangiocytes are a heterogeneous population of cells[1-4,16,17,19,20] that are derived from small intrahepatic bile ducts (lined by small cholangiocytes)[3,20,21] and large intrahepatic bile ducts (lined by large cholangiocytes)[3,20,21].

### *Cholangiocyte proliferation*

Heterogeneous cholangiocytes have been identified as the target cells for a number of liver diseases (cholangiopathies) that include primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC)[1]. In these diseases there is a destruction of the bile ducts that leads to hyperplastic transformation, termed "typical" or "atypical" proliferation[7,14,22]. "Typical" cholangiocyte proliferation is characterized by an increase in cholangiocyte proliferation that is limited to the portal area, whereas "atypical"[23] is seen in patients with prolonged cholangiopathies (i.e. PBC and PSC) and is noted by irregular proliferation of cholangiocytes (with a well structured lumen) that extend into periportal and parenchymal regions with occasional anastomosing cords with adjacent hepatocytes[7,14,22]. "Oval" cell proliferation is a pre-malignant stage that is seen in early carcinogenesis and characterized by the presence of a disorganized tubular structure without any well-defined lumen that can transform into a full-blown neoplastic condition known as cholangiocarcinoma (CCA)[7,14,22,24].

### *Models of proliferation*

Several animal models of "typical" proliferation have rapidly become an excellent tool for the dissection of the mechanisms behind this event. Bile duct ligation (BDL) is commonly used to mimic "typical" proliferation[19,25-27]. Ligation of the bile duct induces a build-up of bile and increased biliary pressure[8,28] that induce an increase in cholangiocyte proliferation (mostly of large cholangiocytes)[19] and resembles biliary hyperplasia seen in patients with diseases like PBC[7,19,29]. Other models used to mimic "typical" proliferation include 70% partial hepatectomy (PH), acute carbon tetrachloride ($CCl_4$) treatment and chronic feeding of $\alpha$-naphthylisothiocyanate (ANIT) or bile salts[7,12,14,16,17,30]. All of these models are found to be associated with an upregulation of secretin receptor (SR) gene expression and secretin-stimulated cAMP levels and ductal secretory activity[7,13,14,16,17,30,31]. Chronic treatment of normal rats with forskolin has also been shown to increase biliary hyperplasia (similar to BDL) and secretin-induced responses in cAMP levels as well as downstream signaling components[32]. While BDL targets mainly the large cholangiocytes[3,29], after $CCl_4$ treatment, only small cholangiocytes respond and de novo proliferate[16,17]. This occurs with a parallel loss of large ductal mass[16,17]. After 70% PH, small and large cholangiocytes proliferate during liver regeneration[14,24]. These tools are useful in evaluating the mechanisms of cholangiocyte proliferation during liver cholestasis and disease progression.

## CHOLANGIOCYTE FUNCTION

Using the tools described above has enabled researchers to uncover important information regarding cholangiocyte function in relation to secretion, proliferation and apoptosis during liver disease[1,4,6,7,13,18].

### *Secretory function*

In BDL rats, there is increased cholangiocyte proliferation coupled with increased bile flow and bicarbonate secretion after stimulation with secretin[3,8,13,19,25,27,33]. This is an excellent tool to use to evaluate functionality of cholangiocytes in normal and diseased states as well as in response to stimulation by numerous factors. Over the years it has been shown that, in addition to secretin, vasoactive intestinal peptide increases bicarbonate secretion, whereas the hormones somatostatin, insulin and gastrin all decrease bile flow and bicarbonate secretion[33-36]. Other factors have been shown to inhibit the secretin-induced increased cholangiocyte response. These include the alpha 2 adrenergic receptor agonist UK,14304[37] and the phenolic compound, caffeic phenethyl ester (CAPE)[38]. The majority of these responses occur through a cAMP-dependent pathway, however other studies have shown

that $Ca^{2+}$ can also play a role in cholangiocyte secretion. Le Sage, *et al*[15] have demonstrated that the alpha-1 adrenergic receptor agonist, phenylephrine, increases secretin-stimulated choleresis through activation of $Ca^{2+}$/protein kinase c (PKC) mechanisms, whereas the hormone gastrin was found to decrease secretin-stimulated ductal secretion in BDL rats *via* increased expression of $Ca^{2+}$-dependent PKC isoforms[27].

### Proliferative and apoptotic functions

Cholangiocyte proliferation and apoptosis is affected by many hormones and peptides[13]. Recently, Mancinelli *et al*[31] have shown that the sex hormone, follicle-stimulating hormone (FSH) increases cholangiocyte proliferation through cAMP/PKA-dependent phosphorylation of extracellular signal-regulated kinase (ERK1/2) and Elk-1, a member of the ETS oncogene family. The forkhead box proteins A1 and A1 (Foxa1 and Foxa2) have been descried as "terminators" of bile duct expansion by their ability to inhibit interleukin-6 (IL-6) expression[39]. In BDL Foxl1 (-/-) knockout mice it was found that loss of the winged helix transcription factor Foxl1 induced a decrease in cholangiocyte proliferation and loss of bile ductular mass by activation of the canonical Wnt/beta-catenin pathway, suggesting that this transcription factor plays a critical role in cholangiocyte proliferation during cholestasis[40]. After treating BDL rats with CAPE, Mancinelli *et al*[38] demonstrated a large decrease in cholangiocyte proliferation coupled with increased cholangiocyte apoptosis that was reversed after chronic feeding with the bile acid taurocholic acid (TC). TC feeding also led to recuperation in the expression of VEGF proteins and receptors (R2 and R3), implicating bile acids and angiogenic factors in the course of cholangiocyte cholestasis[38]. The endocannabinoid, anandamide (AEA) was found to inhibit cholangiocyte proliferation and increase apoptosis through activation of thioredoxin 1/redox factor 1 and activator protein-1 (AP-1), demonstrating the potential role for endocannabinoids in cholestasis[41]. Genetic knockdown of the transcription factors c-Fos and c-Jun ablated the AEA-induced cholangiocyte death[41]. Tumor necrosis factor-alpha (TNF-$\alpha$) has been shown to induce cholangiocyte apoptosis and decrease proliferation when coupled with a single injection of actinomycin D[42]. Chronic feeding with TC has been found to reverse this ductopenic effect *via* a phosphatidylinositol-3-kinase (PI3K)-dependent pathway[43]. These studies have provided strong evidence that cholangiocyte function is susceptible to inhibition and stimulation by numerous factors.

### Hepatic vasculature

It is pertinent to discuss hepatic vasculature within this article as the microanatomy (including cholangiocytes) of the liver is significantly influenced by blood flow. There is a constant flow of large amounts of blood to and from the liver that is controlled through two separate

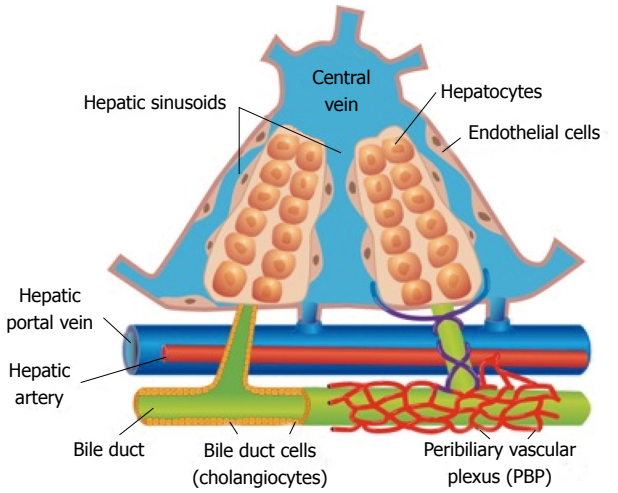


**Figure 1 Graphic image of blood flow through the liver and the key cells involved.**

blood supplies: the portal vein (PV) and the hepatic artery (HA)[44,45]. There are conflicting views regarding HA distribution and where this artery terminates in the liver parenchyma[46-48]. Recently, a detailed study using scanning electron microscopy of vascular corrosion casts demonstrated that the terminal HA branches do not end directly in the sinusoidal beds[49,50]. The terminal HA give rise to capillaries that form the peribiliary plexus, periportal plexus and single capillaries of the portal space; this implies that only venous blood at a lowered pressure reaches the sinusoids and vena portal branches[49,50].

Structurally, the sinusoids are vascular channels lined with fenestrated endothelial cells (sinusoidal endothelial cells or SEC[51]). It is this fenestrated structure that makes the sinusoids very "leaky", allowing fluid passage to occur[52]. Hepatocytes are found around the sinusoids and are responsible for the initiation, formation and secretion of bile[53]. After secretion of bile by the hepatocytes, bile is modified by cholangiocytes[53]. The hepatic artery is the main supplier of blood to the biliary tract and cholangiocytes within the structure termed peribiliary vascular plexus (PBP)[26,50]. This system has been found to be a source rich in vascular growth factors and other vasoactive substances[50]. Recent work has shown that the PBP has a great influence on cholangiocyte proliferation and/or loss that is mediated by VEGF expression[26]. The bile acid TC has also been shown to influence cholangiocyte growth/loss by regulating VEGF[38]. Please refer to Figure 1 for a graphic representation and diagram of the microcirculation in the liver with regards to blood and bile flow.

### Effects of blood flow on liver microanatomy

Significant findings have shown that blood flow in the liver directly influences its microanatomy and how it responds to stress and/or disease. In rats with BDL-induced cholestasis, interruption of the hepatic artery blood supply *via* hepatic artery ligation (HAL) causes a

significant loss of the PBP and increases cholangiocyte apoptosis[26]. Ischemic bile duct injury can occur in association with hepatic artery thrombosis during liver transplantation and can induce the development of biliary casts, bile duct death or chronic disease that mimics PSC[54].

## CHOLANGIOCARCINOMA

### Description of progression and treatment options

Cholangiocyte hyperplasia that becomes uncontrollable or differentiates into a further neoplastic state can lead to cholangiocarcinoma. Cholangiocarcinoma is defined as a tumor from either intrahepatic or extrahepatic origin[55]. Prominent risk factors of CCA are chronic inflammation, congenital abnormalities of the biliary tree and genetic predisposition[56]. Tumors of CCA progress in a slow and undramatic manner with biliary sepsis, malnutrition and liver failure being typical causes of death associated with this disease[57]. CCA is the second most common type of cancer in the liver after hepatocellular carcinoma and the number of incidences of this disease is on the rise worldwide[55]. Early discovery of cholangiocarcinoma is difficult, resulting in limited treatment options. Long-term survival is only reachable by complete surgical resection of the tumor, which is not feasible for some patients and can be highly unsuccessful. Conventional chemotherapy and radiation have not proven to be successful at prolonging survival rates[55,57]. Photodynamic therapy has recently appeared as a possible treatment option to relieve pain and increase survival, however further studies are needed[56]. A most recent review of the latest advances in CCA diagnosis, treatment and patient care can be found in the reference by Aljiffry, *et al*[56]. Given the current lack of satisfactory treatments, understanding the cellular mechanisms behind the development of CCA will be critical in the development of future curative therapies.

### Recent findings

Research to investigate CCA development and mechanism of action has increased in the last decade. A glimpse into the literature reveals thousands of entries including both reviews and original articles. Highlights of some of the latest findings from 2009 are shown here. Most recently, Blechacz *et al*[58] have shown that Sorafenib, an approved treatment for primary renal cancer, inhibits CCA growth in vitro and *in vivo* by sensitizing tumor cells to apoptosis. Blocking the production and secretion of dopamine, over-produced in CCA, has also been shown to decrease CCA growth in both cultured cells and an *in vivo* model of CCA[59]. In liver fluke-associated CCA, inhibition of galectin-3 was found to stimulate apoptosis that was increased by 10 fold by treatment with cisplatin or 5-fluorouracil[60]. Using CCA cell lines, Okada *et al*[61] demonstrated that rapamycin decreased cell proliferation that was synergistic with treatment with gemcitabine. Suppression of the nuclear factor kappa beta (NF-κB) pathway by treatment with CAPE was found to decrease cholangiocarcinoma growth both in culture and *in vivo* by increasing apoptosis[62]. Endothelin (ET-1) has been shown in both *in vitro* and *in vivo* models to decrease CCA growth by inhibiting VEGF and its receptors[63]. Coupled with decreased angiogenic factors, ET-1 also increased apoptosis and collagen tissue deposition[63]. Tamoxifen, the estrogen receptor antagonist, is showing promise as a possible therapeutic agent in CCA through calmodulin targets AKT [protein kinase B (PKB)] and c-FLIP (cellular-FLICE inhibitory protein)[64]. These studies are just the pinnacle of the multitude of studies that have been performed to investigate potential therapies for this devastating tumor.

## HISTAMINE

In 1910 the British scientist, Sir Henry Dale identified histamine as a substance that is released and acts as a "mediator" during an allergic responses. Today we know that histamine is involved in many bodily processes in addition to allergic reaction.

### Histamine activation and function

This biogenic amine interacts with four G-protein coupled receptors (GPCRs), H1HR, H2HR, H3HR and H4HR[65-68] that exert their actions on various G-proteins[69]. It has been shown that stimulation of the H1 histamine receptor (HR) activates Gαq, inducing a $Ca^{2+}$-dependent effect in various cell systems[66,70,71]. In contrast, the H2HR appears to signal mainly through Gαs, stimulating a cAMP- dependent action in a variety of cell types[65,71,72]. Coupling of H3 and H4 HR has been linked to Gαi, inducing a negative regulation of cAMP-dependent signaling[65,73] as well as Gαo, mediating cellular regulation through a phospholipase C/$Ca^{2+}$-dependent pathway[25,73]. Histamine receptors are also able to induce both inhibitory and stimulatory effects in different cell types. In Leydig cells, the H1HR induces an inhibitory effect, whereas the H2HR has a stimulatory effect on steroidogenesis[74]. In cholangiocytes and cholangiopathies, it has been shown that the H1HR stimulates growth, whereas the H3HR inhibits hyperplastic proliferation[65,66]. Histamine and the histamine receptors have been shown to induce a multitude of effects on various cellular pathologies including the H3HR in Alzheimer's Disease[75]; histamine and the H1HR in vascular disease[76]; the H4HR in treatment of chronic pruritus disease[77] and many more. There is not yet a proven direct link between histamine and cancer, however growing evidence suggests that histamine and the histamine receptors may be involved in tumor growth and/or depletion[25,78]. Further, dysregulation of the enzymes that are responsible for histamine synthesis, histidine decarboxylase (HDC) and monoamine oxidase-B (MAO-B), have been implicated in certain cancers[79]. The interaction between cells and the four histamine receptors also complicates our understanding of the regulation of liver function in both normal and diseased (including cancer) states.

### *Histamine and liver disease*

Histamine and the histamine receptors play a role in numerous processes including disease progression in the liver. Histamine, *via* H2HR activation, has been shown to play a protective role in alcohol-induced liver injury by lowering liver enzymes [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] and decreasing inflammation and necrosis in ethanol treated rats[80]. In patients with cirrhotic-related hepatic encephalopathy, evaluation of brain tissue showed an upregulation of histamine and H1 receptors and decreased H3 receptor density, highlighting the importance of histamine regulation in liver pathologies[81,82]. An H2 receptor antagonist, rantidine[83] and an H3/H4 antagonist, thioperamide[84] have both been shown to suppress ischemic reperfusion liver injury[83-85]. Stimulation with dimaprit, an H2 and H4 agonist showed similar results by inducing a protective effect against ischemic reperfusion injury by decreasing cytokine release[85]. The density of H2 histamine receptors is decreased in hepatic tissues from cirrhotic patients with portal hypertension[86]. Furthermore, in H1HR and H2HR knockout mice it was found that the protective effects of histamine on lipopolysaccharide (LPS)-induced liver injury was partially or completely blocked, suggesting that histamine and its receptors have a protective role in endotoxin-induced hepatic injury[87]. It has also been found that histamine levels in plasma from patients with both PSC and PBC are increased compared to healthy controls[88]. Histamine, *via* the H2HR, has been shown to protect against fulminant hepatitis[89].

### *Histamine and liver cancer*

There have been numerous studies involving histamine and histamine receptors in liver cancers. In a study using hepatocellular carcinoma cell lines, histamine stimulation was found to induce a differential effect by decreasing the growth of one line while increasing the growth of another[90]. Histamine-induced effects were attenuated by inhibition of either H1 or H2 HRs[90]. The H2HR antagonist, cimetidine is a common drug used for treatment of gastric ulcers and has also been shown to reduce liver metastasis *via* activation of selectins in liver sinusoids[91]. Both gastrin and histamine H2 receptor antagonists may play a synergistic role in helicobacter-induced gastric cancer[92].

## HISTAMINE AND THE BILIARY TREE

The above studies have alluded to the role of histamine in a broad range of liver pathologies from hepatitis to hepatocellular carcinoma. Here we will reflect on the recent studies involving histamine and the histamine receptors in specific cholangiocyte pathologies ranging from cholestasis to cholangiocarcinoma.

### *Biliary hyperplasia and histamine*

To date, only a handful of articles have addressed the direct role of histamine in biliary diseases. Though limited, these studies have provided evidence that there is a strong link between histamine and its receptors in the regulation of cholangiocyte function. Using a BDL-induced model of cholestasis, we have shown that chronic treatment of BDL rats with an H3HR agonist, (R)-(α)-(-)-methylhistamine dihydrobromide (RAMH), results in a significant decrease in biliary hyperplasia[65]. Numerous techniques revealed that liver tissue and RNA from both normal rats and BDL rats express all four of the histamine receptors[65]. Figure 2 depicts expression of the H3 receptor in proliferating cholangiocytes (induced by BDL) by immunohistochemical analysis. This finding is in accordance with other studies showing that cholangiocytes express a wide array of hormone receptors[13]. Biliary hyperplasia was reduced *in vivo* and also *in vitro* in freshly isolated cholangiocytes stimulated with RAMH *via* cAMP/PKA/ERK/Elk1 signaling. By downregulating this signaling pathway, the H3HR is able to decrease and manage biliary hyperplasia that could be a useful therapeutic tool in cholestatic liver diseases like PBC where cholangiocyte proliferation is prevalent.

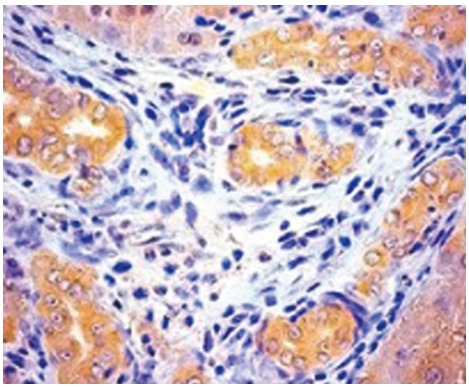

**Figure 2 Immunohistochemistry for the H3 histamine receptor in proliferating cholangiocytes (induced by bile duct ligation).** Original magnification × 40.

In contrast, the H1HR agonist, HTMT dimaleate, increases the growth of small murine mouse cholangiocytes, but not large[66]. This study has great implication on the effects of histamine and histamine receptors in the heterogeneous population of cholangiocytes where cholangiopathies are likely to occur. These findings show that the H1HR elicits its effects by activation of Gαq, mobilization of calcium and increased intracellular inositol triphosphate receptor ($IP_3$) levels. This was coupled with increased (1) phosphorylation of calmodulin-dependent protein kinase I (CaMKI) and ERK and (2) activation of the transcription factor CREB (cAMP response element binding protein). It was further shown that knockdown of CaMKI resulted in the loss of CREB activation and H1HR-induced increased proliferation in small cholangiocytes[66]. The importance of calcium-dependent signaling in cholangiocyte function and regulation is demonstrated by this study and is in accordance with other studies[93-95]. Taken together, these studies implicate histamine and the histamine receptors in cholangiocyte regulation.

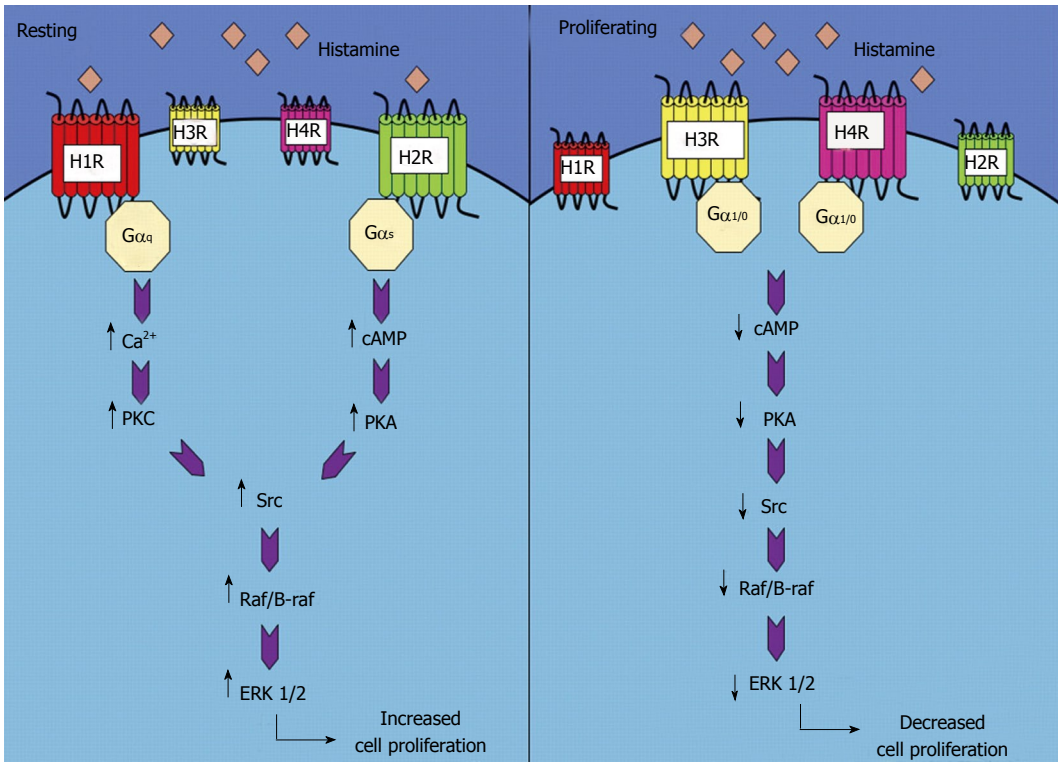


**Figure 3 Differential signaling of histamine receptors in resting and proliferating cholangiocytes.** Reprinted with permission from Demorrow S, Francis H, Alpini G, Biogenic Amine Actions on Cholangiocyte Function. *Exp Biol Med (Maywood)* 2007; **232**: 1005-1013.

Other recent studies have demonstrated that treatment with histamine; the H1HR agonist or the H2HR agonist (but not the H3HR agonist) stimulates the proliferation of cholangiocytes in normal rats[96]. Preliminary data suggests that there is a differential effect induced by the H1 and H2 HRs where the H1HR appears to signal predominantly through calcium-dependent mechanisms, whereas the H2HR is more prone to work through a cAMP/PKA-dependent signaling pathway[96]. Histamine is able to utilize both signaling pathways, but may have a preference for the H1HR-mediated path[96]. Figure 3 illustrates the differential signaling induced by histamine and histamine receptors in both normal and proliferating cholangiocytes[5]. It appears that histamine receptors may also mediate the VEGF-regulated cholangiocyte response during liver regeneration after 70% partial hepatectomy[96]. After 3 d post PH, treatment with the H1HR agonist, HTMT, induced a 50x fold increase in cholangiocyte proliferation that was coupled with increased VEGF expression[96]. This study has huge potential to lend a therapeutic "hand" to physicians treating patients after liver transplantation by aiding in the acceleration of liver regeneration and healing. Overall, it is clearly apparent that this biogenic amine and its receptors play numerous, critical roles in liver cholangiopathies and regeneration. Further investigations of these events are currently underway.

### *Biliary neoplasia and histamine*

Little is currently known about the possible interaction between cholangiocarcinoma and histamine. Following our study with the cholestatic rat model and the H3HR agonist, RAMH, we evaluated the role and mechanisms of action by which RAMH regulates CCA growth[25]. Using numerous CCA cell lines and normal cholangiocytes, it was found that all four of the histamine receptors were expressed with an upregulation of H3HR expression compared to normal tissue. Treatment with RAMH induced a decrease in growth in all CCA cell lines, but had no effect in normal cholangiocytes. Interestingly, in this study we demonstrated that RAMH was potentially signaling *via* Gαo by inducing an increase in intracellular $IP_3$ levels, but having no effect on cAMP signaling, thus ruling out any interaction with this pathway. Inhibitors for $[Ca^{2+}]_i$, PKC and PLC all blocked the RAMH-induced inhibitory action in CCA cells. Because PKC signaling has previously been shown to be important in cholangiocarcinoma[11,97,98], the role of this protein was investigated further. RAMH induced phosphorylation of PKC alpha (PKCα) and also caused translocation of PKCα from the cytosolic region into the membrane of CCA cells. Knockdown of PKCα ablated the suppressive effects induced by RAMH as well as reversing ERK phosphorylation. In final studies, *in vivo* examination was performed in a nude mouse model[59,62,63], implanted with CCA cells and treated with RAMH over the course of several weeks. Tumor growth was significantly inhibited by RAMH treatment compared to tumor growth from NaCl-treated mice. This was coupled with decreased

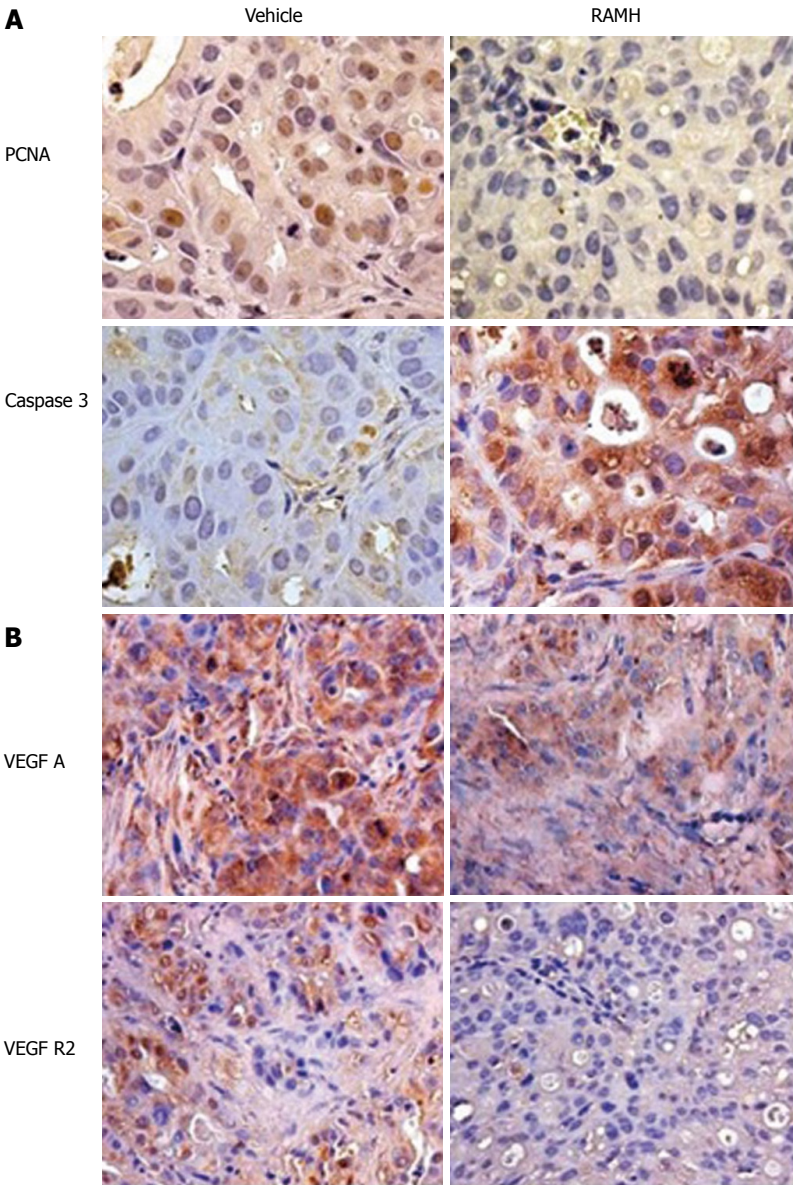


**Figure 4 Immunohistochemistry images of tumors excised from vehicle- and RAMH-treated mice (× 40).** A: Proliferating cholangiocytes (PCNA) and apoptotic cholangiocytes (Caspase 3). RAMH induced a decrease in PCNA-positive cholangiocytes coupled with an increase in apoptotic cholangiocytes compared to vehicle-treated mice; B: VEGF-A and VEGF-R2. RAMH treatment caused a decrease in the expression of both VEGF-A and VEGF-R2.

proliferation and increased apoptosis. Figure 4A shows a representative image for immunohistochemical staining for both PCNA and caspase 3. Total PKCα expression was also increased in RAMH-treated tumors while VEGF and its receptors were decreased as seen by immunohistochemistry images provided in Figure 4B[25]. This is an important finding related to the regulation of PKCα in cholangiocarcinoma growth and also the ability of histamine to mediate VEGF expression, that plays a critical role in tumor development[63,99-102]. In work that is currently underway, we have found a role for histamine secretion and for other histamine receptor agonists and antagonists. Like other cancers, we have found that HDC and MAO-B are dysregulated in CCA and that CCA cell lines secrete an increased amount of histamine compared to normal cholangiocytes[103]. Further investigation has shown that acute histamine treatment has no significant impact on CCA growth, but that long-term stimulation (up to 2 wk) can increase growth that is coupled with increased VEGF gene expression[103]. *In vivo* treatment with histamine in our nude mouse model also increased tumor growth that was blocked by treatment with an HDC inhibitor[103]. These mechanisms are still being worked out, but it is clear that histamine has a trophic effect in CCA growth. We have also found that the histamine receptors are upregulated in CCA cell lines and have differential effects with regards to changes in proliferation[103]. As stated above, the H3HR agonist decreases CCA growth. However, we have seen that blocking the H2HR with cimetidine has no effect in either the CCA cell lines or *in vivo* (unpublished observations, Alpini and Francis). Stimulation with an H1HR antagonist also decreases CCA growth and increases apoptosis *in vitro* (unpublished observations, Alpini and Francis, 2007). Most recently we have found that clobenpropit, a highly potent H4HR agonist and selective H3HR antagonist can decrease CCA growth by integrin-mediated events including decreasing the invasive capacity of CCA cells[104]. These findings are complex and intricately woven together. Please refer to

**Table 1 Effects of histamine and histamine receptors on cholangiocarcinomagrowth**

| | Expression level | Agonist effect | Antagonist effect | *In vitro*/*in vivo* model | Known or potential signaling mechanism |
|---|---|---|---|---|---|
| Histidine decarboxylase (HDC) | ↑↑ | | ↓↓ CCA growth | Both | Inhibits histamine synthesis |
| Monoamine oxidase-B (MAOB) | ↓↓ | Unknown | Unknown | *In vitro* | Unknown |
| Histamine | ↑↑ secretion by CCA | ↑↑ CCA growth | ↓↓ CCA growth | Both | ↑↓ VEGF expression |
| H1HR | ↑↑ | ↑ CCA growth | ↓↓ CCA growth | *In vitro* | $Ca^{2+}$-mediated signaling |
| H2HR | ↑↑ | ↔ | ↔ | Both | Unknown |
| H3HR | ↑↑ | ↓↓ CCA growth | | Both | PKCa-dependent signaling |
| H4HR | ↑↑ | ↓↓ CCA growth | | *In vitro* | Integrin-dependent decreased invasion |

CCA: Cholangiocarcinoma; HDC: Histidine decarboxylase; MAOB: Monoamine oxidase-B; HR: Histamine receptor; VEGF: Vascular endothelial growth factor.

Table 1 for a summary of the effects of histamine and histamine receptors in CCA regulation.

## FUTURE PERSPECTIVES

While the studies reviewed above are relevant and important, it is also critical to remember that there are numerous other factors that may be involved in histamine regulation of liver disease. *In vivo*, the role of mast cells must be addressed as information regarding the interplay between hepatic mast cells and liver pathologies is increasing. The main source for histamine release is mast cells[105-107] and therefore these cells are likely to play some role in histamine regulation of cholangiocyte function. Bile acids have also been shown to play a major role in cholangiocyte proliferation, secretion and function[9,12,108-113]. We know that bile acids are also able to act on mast cells and can influence the type and amount of a mediator (like histamine) that is being released[114-117]. Not all bile acids induce the same effect. Lipophilic bile acids have been shown to activate mast cells and induce a large amount of histamine release, whereas ursodeoxycholate has almost no effect on histamine secretion from mast cells[118]. Bile acids are currently used as a treatment option for patients with etiologies ranging from gastro-esophageal reflux disease (GERD) to cholestatic liver diseases[119,120]. Histamine receptor agonists and antagonists are also used to treat various digestive diseases. The H2 antagonists, cimetidine and rantidine have both shown promise in treating GERD[121]. Because of the involvement of mast cells in irritable bowel syndrome (IBS), blocking mast cell interaction through either second generation antihistamines or proteinase-activated receptor antagonists could relieve pain in suffering patients[122]. How bile acids interact with hepatic mast cells and possibly cholangiocytes is unknown. However, understanding the interconnecting relationship between *bile acids* ← *mast cells* → *cholangiocytes* could allow us to take our current understanding of histamine-mediated regulation of cholangiocyte function to a new level and thereby allow us to turn translational science into bedside practice.

## CONCLUSION

Taking the evidence discussed in this review we can glimpse into the future and see that histamine and histamine receptor regulation can have a major impact on liver diseases. However this information is only useful if we are able to dissect the mechanisms of histaminergic action that occur during disease progression.

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TOPIC HIGHLIGHT

**Sharon DeMorrow, PhD,** *Series Editor*

# Role of sex hormones in the modulation of cholangiocyte function

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## Abstract

Over the last years, cholangiocytes, the cells that line the biliary tree, have been considered an important object of study for their biological properties which involves bile formation, proliferation, injury repair, fibrosis and angiogenesis. Cholangiocyte proliferation occurs in all pathologic conditions of liver injury where it is associated with inflammation and regeneration. During these processes, biliary cells start to secrete different cytokines, growth factors, neuropeptides and hormones which represent potential mechanisms for cross talk with other liver cells. Several studies suggest that hormones, and in particular, sex hormones, play a fundamental role in the modulation of the growth of this compartment in the injured liver which functionally conditions the progression of liver disease. Understanding the mechanisms of action and the intracellular pathways of these compounds on cholangiocyte pathophysiology will provide new potential strategies for the management of chronic liver diseases. The purpose of this review is to summarize the recent findings on the role of sex hormones in cholangiocyte proliferation and biology.



**Key words:** Biliary epithelium; Sex hormones; Cholestatic diseases

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## INTRODUCTION

The intrahepatic biliary tree is a complex three-dime-

nsional network of interconnected ducts which starts at the level of canals of Hering, continues into intrahepatic ducts of increasing diameter and ends at the level of the extrahepatic bile ducts[1]. The intrahepatic biliary tree plays a critical role in many liver functions including bile formation, regeneration, injury repair, fibrosis, angiogenesis and regulation of blood flow. Most of these events are regulated by several neuropeptides, hormones, cytokines and growth factors which target cholangiocytes[2], the epithelial cells lining the biliary tree. Cholangiocytes are the preferential target of damage in a group of chronic cholestatic liver diseases called cholangiopathies, with a high social and economic impact due to their high prevalence and morbidity such as primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), polycystic liver disease (PCLD) and cholangiocarcinoma (CCA)[3-7]. In laboratory animals, "typical" cholangiocyte proliferation is achieved by a number of experimental models including bile duct ligation (BDL), partial hepatectomy, acute $CCl_4$ feeding and chronic feeding of a-naphthylisothiocyanate (ANIT) or bile salts[8-11]. In these hyperplastic models, cholangiocyte proliferation is closely associated with increased secretin receptor gene expression and secretin-stimulated cAMP levels[12-18]. A variety of clinical and epidemiological observations have shown the involvement of sex hormones as inducers of growth and differentiation of target cells expressing their receptors[3]. The molecular mechanism of these complicated events, especially sex hormone-dependent growth enhancement, has been studied extensively. In MCF-7 cells, an estrogen-induced autocrine loop has been demonstrated to play an important role for estrogen-dependent growth. Androgen has been also found to promote the growth of SC-3 cell through the induction of several autocrine growth factors[4].

Sex hormones such as estrogens and androgens have been well known to regulate the growth of normal as well as transformed target cells[19-24]. Generally, they have been proposed to promote cell growth whereas so-called anti-hormones inhibit the hormone-dependent growth. For example, anti-androgens such as cyproterone acetate have also been known to accelerate the growth of prostate cancer in some circumstances[5]. Conversely, administration of a large amount of estrogens frequently causes the regression of estrogen-receptor-positive breast cancer[6]. Tamoxifen, the most widely used therapeutic agent of estrogen-dependent breast cancer, exhibits organ- and species-dependent differences in cell growth regulation[7]. Even in the same cells, growth response to tamoxifen has been observed to differ in a dose-dependent manner. One plausible explanation is that two pathways exist for estrogen-dependent growth in target cells, one for the stimulatory and another for the inhibitory signal[4] (Figure 1).

In particular, estrogens exert a trophic action in several target organs such as liver[8] where they modulate growth and repair, intervening in neonatal liver growth and regeneration after injury in adults[3]. Moreover, chronic administration of estrogens for pharmacological purposes induces an enlargement of liver mass[25,26] and after partial hepatectomy, ERs expression in hepatocytes increases with subsequent transcription of genes involved in proliferation to restore a normal liver mass[25,27]. With this review, we aim to summarize the latest findings about the role of sex hormones on biliary epithelium function, their effects and alterations during cholestasis. The role and mechanism by which sex hormones modulate cholangiocyte functions have been explored only over the last few years at both experimental and clinical levels[28-50].

## SEX HORMONES

Hormones are the chemical messengers of the body since they are involved in transmission of information from one tissue to another and from cell to cell. Hormones circulate in the bloodstream and interact with target cells that possess receptors that can only be activated by a specific type of hormone. Several kinds of hormones exist for their structure or activity in the cell. Usually they are known as steroids and peptides (Figure 2). In general, steroids are sex hormones, chemical substances made from cholesterol and produced by a sex gland or other organ that has an effect on the sexual features of an organism[9]. Like many other kinds of hormones, they may also be artificially synthesized. On the other hand, peptides are made from long strings of amino acids to regulate other functions and are sometimes referred to as "protein" hormones.

Sex hormones are divided into 3 groups: (1) female sex hormones or estrogens; (2) male sex hormones or androgens; and (3) pregnancy hormones or progestins.

### *Estrogens*

Estrogen is a generic term for estrus-producing compounds; the female sex hormones including estradiol, estriol and estrone (Figure 3). In humans, the estrogens are formed in the ovary, adrenal cortex, testis and fetoplacental unit and are responsible for female secondary sex characteristic development and, during the menstrual cycle, act on the female genitalia to produce an environment suitable for fertilization, implantation and nutrition of the early embryo[10]. Uses for estrogens include oral contraceptives, hormone replacement therapy, advanced prostate or postmenopausal breast carcinoma treatment and osteoporosis prophylaxis[51-55]. They also antagonize the effects of the parathyroid hormone minimizing the loss of calcium from bones and thus helping to keep bones strong[11]. Estradiol (E2) is the main female sex hormone. Its actions are mediated by two members of the nuclear receptor superfamily, estrogen receptor ERα and ERβ, and a recently discovered G protein-coupled membrane receptor, GPR30[56,57]. Mechanisms by which ERα and ERβ bind ligand, dimerize, associate with coactivators or corepressors and regulate gene transcription are typically

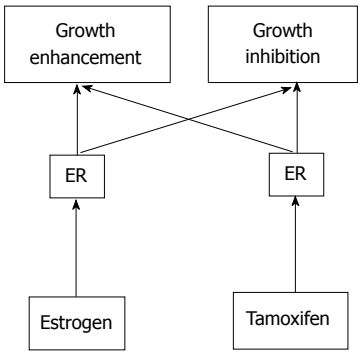


**Figure 1 Two-pathway theory of estrogen-receptor-dependent growth.** Many estrogen target cells contain growth-stimulatory and -inhibitory pathways. Both pathways are mediated through ERs. Tamoxifen may possess relatively high affinity for the growth-inhibitory pathway whereas estrogen can mainly activate the growth-stimulatory pathway. However, any ligand for ER can stimulate both pathways.

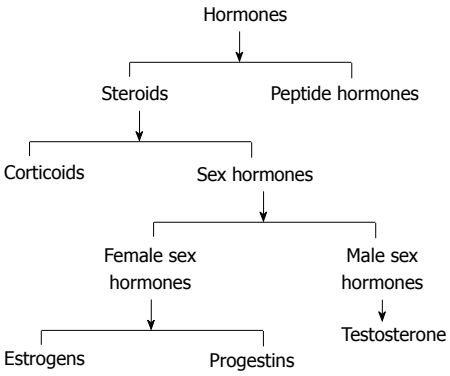


**Figure 2 Scheme of the two classes of hormones, steroids and peptide hormones that successively can be divided in corticoids and sex hormones.**

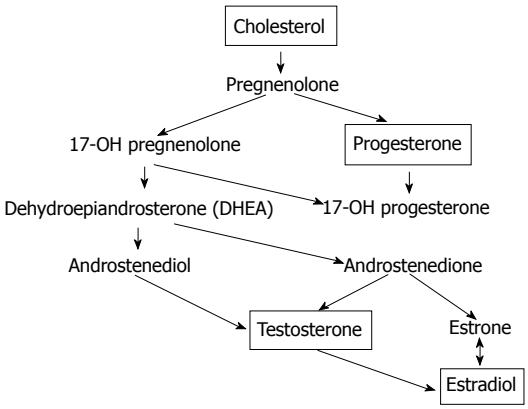


**Figure 3 The biosynthesis of the sex hormones starts with the oxidation of the side chain of cholesterol, which is catalyzed by the enzyme cytochrome P450$_{scc}$ to form pregnenolone.** The next steps in the biosynthesis of testosterone can proceed via two different routes. Pregnenolone can be oxidised first by cytochrome P450$_{17a}$ to 17a-hydroxypregnenolone. The enzyme 3β-HSD also can convert pregnenolone first into progesterone. Both pregnenolone and progesterone are accepted as substrate by the enzyme cytochrome P450$_{17a}$. In this way, after 3β-hydroxy-5-androstene-17-one (DHEA) synthesis, there is the testosterone and successively the estradiol formation.

referred to as "genomic" actions[12], which ultimately regulate both cell proliferation and survival[58-61]. Estrogens play biological activities in several organs[13] including the cardiovascular system, nervous system, digestive system and "male" organs such as the prostate. In target tissues, estrogens may exert opposite actions and heterogeneous effects[62-66]. In detail, overexpression of ERα has been associated with cancer development and progression in several organs[14]. The functions of ERβ are linked to a protective effect against uncontrolled or neoplastic cell proliferation[64,67]. In different types of cancer, estrogens synergize the effects of growth factors by acting at both receptor and post-receptor levels favouring the growth and spreading of tumour mass[68-73]. The liver is a hormone-sensitive organ. Both normal liver and hepatocellular carcinoma (HCC) tissues from male and female mammals have been shown to express specific ERs, stimulating both *in vivo* and *in vitro* hepatocyte proliferation[15]. Moreover, anti-estrogens like tamoxifen have been shown to reduce levels of ERs and to inhibit hepatocyte proliferation following partial hepatectomy[16]. Long-term use of oral contraceptives (OCs) and anabolic androgenic steroids (AASs) can induce both benign and hepatocellular tumors[17]. Other experimental findings suggest that estrogens have numerous neuroprotective actions. This responsiveness can diminish with age, reducing neuroprotective actions of estrogen[18]. Hormonal treatment plays an established role in several solid tumors, first of all in breast cancer where, for the last decades, the antiestrogen tamoxifen has been the most commonly used treatment for patients with estrogen receptor alpha (ER)-positive breast cancer[19]. Tamoxifen is characterized by a favourable toxicity profile which, together with the easy oral administration, makes this drug an interesting candidate for treatment of other solid tumors potentially responding to hormonal manipulation[74-77]. In addition, there is increasing evidence showing that adipose tissue is a site of steroid metabolism, including the interconversion of estrone (E1) and E2. The presence of both estrogen receptors (ERα and ERβ) in preadipocytes and mature adipocytes strongly suggest a role for active estrogen in these cells. For that reason, adipose tissue can be considered a significant source of estrogenic compounds.

### *Androgens*

Androgens are a special kind of fat molecule with a four-ringed, carbon atom backbone or core[20]. A series of chemical reactions transform cholesterol first into the steroid pregnenolone and then into testosterone and other androgens (Figure 3). Like all steroid hormones, androgens produce effects by docking with receptors on the cell's membrane surface or inside the cell in the liquid cytoplasm[20]. The steroid hormone/receptor unit moves into the nucleus activating specific genes. These genes drive the cell changes guiding androgen-controlled growth and development[21]. Scientists have studied androgens since the 18th century. John Hunter initially described androgenic actions in 1771. Almost

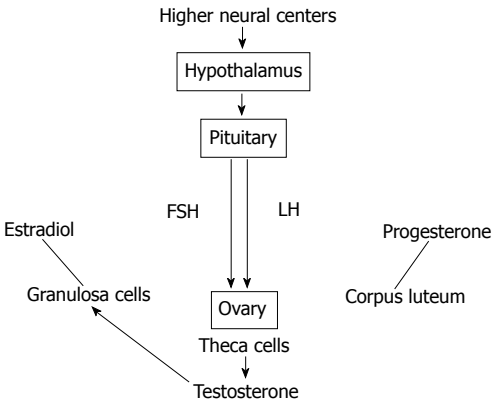


**Figure 4 Scheme of hypothalamic-pituitary-gonadal (HPG) axis control exerted by both circulating and in situ locally produced estradiol in men.** This axis controls development, reproduction, and aging in animals. The hypothalamus produces gonadotropin-releasing hormone (GnRH). The pituitary gland produces luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and the gonads produce estrogen, progesterone and testosterone from different kinds of cells.

two century later in 1935, Leopold Ruzicka worked out the chemical structure of the "androgenic principle" from the testes, calling it testosterone[22]. Testosterone and dihydrotestosterone (17-beta-hydroxy-5-alpha-androstan-3-one) are the most potent androgens in humans and four-legged vertebrates[20]. The weaker androgens androstenedione and dehydroepiandrosterone (DHEA) occur in small amounts in all vertebrates[23]. Testosterone is essential for the production of sperm and is manufactured by the interstitial Leydig cells of the testes. Its secretion increases sharply at puberty and is responsible for the development of the so-called secondary sexual characteristics of men during puberty[20]. Synthetic testosterone analogs are used in medicine to promote muscle and tissue growth in patients with muscular atrophy[24]. Testosterone therapy is indicated in adult men for the treatment of hypogonadism[25]. Over the last three decades it has become apparent that testosterone plays a significant role in the maintenance of bone and muscle mass, in erythropoiesis and in mental functions. Androgens are also key players in glucose homeostasis and lipid metabolism and exert an important role in liver. In fact, it has been observed that androgen receptors (ARs) are present in normal liver tissue from both males and females and that their expression is increased in tumor tissue[16]. Moreover, cross-sectional epidemiological studies have reported a direct correlation between plasma testosterone and insulin sensitivity and low testosterone levels are associated with an increased risk of type 2 diabetes mellitus, dramatically illustrated by androgen deprivation in men with prostate carcinoma[42,43]. Prostate cancer is one of the most common cancers among men and androgens are involved in controlling the growth of androgen-sensitive malignant prostatic cells[26]. The model of LNCaP prostate cancer cell line was used to study androgen and estrogen metabolism during the transformation process. It was discovered that substantial changes in androgen and estrogen metabolism occur in the cells during the process[45-47]. Recent evidence indicates androgen actions in protecting the brain against neurodegenerative diseases and their positive effects on age-related testosterone loss in men and increased risk for Alzheimer's disease (AD)[27]. The successful use of hormone therapies in aging men and women to delay, prevent and/or treat Alzheimer's disease will require additional research to optimize key parameters of hormone therapy[28].

### *Progestins*

The term progestins is defined as the natural or synthetic progestational substances that mimics some or all of the actions of progesterone, a crude hormone of the corpus luteum from which progesterone can be isolated in pure form[29]. Progesterone is responsible for preparing the uterus for implementation of the fertilized egg. It also has an important role as a birth control agent[30]. It is a steroid hormone produced in the ovary under the control of the pituitary gonadotropins[78-80] (Figure 4). It has also been recently demonstrated that the synthetic progestogen, levonorgestrel, increases progesterone accumulation in cultured, stable porcine granulosa cells, the JC-410[81-93]. Results of those studies have been interpreted to suggest that progestins may affect progesterone synthesis by the regulation of steroidogenic enzymes, the cytochrome P450 side-chain cleavage ($P450_{scc}$) and 3β-hydroxysteroid dehydrogenase (3β-HSD)[31]. The genomic action of progesterone is mediated by two progesterone receptor (PR) isoforms, A and B[84,85]. PR-B is a strong activator of gene transcription, whereas PR-A can act as a ligand-dependent trans-repressor of PR-B[32]. The large majority of PR target genes have been identified in breast cancer cells[87,88]. Different evidence indicates that this hormone also exerts neuroprotective effects on the central nervous system (CNS). Its neuroprotective actions make it a particularly promising therapeutic agent for neuroinjury and neurodegenerative diseases. Progesterone appears to exert its protective effects by protecting or rebuilding the blood-brain barrier, decreasing the development of cerebral edema, down-regulating the inflammatory cascade and limiting cellular necrosis and apoptosis[33]. The family of anti-progestins, i.e. mifepristone, includes pure agonists such as progesterone itself or progestins and, at the other end of the biological spectrum, pure progesterone receptor antagonists (PA). Selective progesterone receptor modulators (SPRM) have mixed agonist-antagonist properties and occupy an intermediate position of the spectrum. Mifepristone is used to terminate pregnancy[34]. Many PA and SPRM display direct antiproliferative effects in the endometrium although with variable actions which seem product- and dose-dependent. This property justifies their use in the treatment of myomas and endometriosis. Interestingly, clinical data show that treatment with these compounds is not associated with hypo-estrogenism and bone

loss. The potential clinical applications of these compounds cover a broad field and are very promising in major public health areas. Further developments might also include hormone replacement therapy in post-menopausal women as well as the treatment of hormone-dependent tumors[35].

### *Other sex hormones*

Another group of substances secreted by the pituitary gland can be defined sex hormones. They include the follicle-stimulating hormone (FSH), the luteinizing hormone (LH) and the prolactin (Prl). The synthesis and secretion of estrogens is stimulated by FSH which is in turn controlled by the hypothalamic gonadotropin releasing hormone (GnRH) (Figure 4). High levels of estrogens suppress the release of GnRH providing a negative-feedback control of hormone levels[36]. Progesterone production is stimulated by LH which is also stimulated by GnRH[36]. Elevated levels of progesterone control themselves by the same negative feedback loop used by estrogen[37]. The two gonadotropins, FSH and LH, are key regulators of ovarian cell functions and the potential role of gonadotropins in the pathogenesis of ovarian cancer is suggested. The presence of gonadotropins in ovarian tumor fluid and their receptors expression suggests the importance of these factors in the transformation and progression of ovarian cancers as well as being prognostic indicators[94-97]. The recent cDNA microarray analyses and characterization in the molecular mechanisms of gonadotropin signaling have indicated the effects of gonadotropins on the regulation of some ovarian cancer cell growth, survival and metastasis that may involve other growth factors[38]. Prl is another hormone released by the pituitary gland that stimulates breast development and milk production in women[39]. It is secreted by so-called lactotrophs in the anterior pituitary as a prohormone. Although the pleiotropic actions of Prl are recognized, its role in regulating growth and differentiation of mammary tissues is better understood[98,99]. Several lines of evidence have also indicated that Prl acts as an autocrine, paracrine and endocrine progression factor for mammary carcinoma *in vitro* and *in vivo* in rodents and humans[100]. These data include recent epidemiologic studies indicating that postmenopausal women with 'high-normal' levels of Prl are at increased risk of breast cancer[101-105]. Elevated prolactin (hyperprolactinemia) may be due to a benign tumor in the pituitary gland called a prolactinoma. Abnormally low prolactin (hypoprolactinemia) can cause menstrual disorders and lead to inadequate lactation[31]. It is concluded that the rat is a predictive model for human mammary carcinogenesis and that rat mammary carcinogenesis induced by hyperprolactinaemic drugs may have greater importance in human toxicological risk than previously thought[40].

## ESTROGENS AND BILIARY EPITHELIUM

Estrogens and their metabolites have been hypothesized to have a pathogenic role in the diseases which preferentially affect the female sex[106-109]. Furthermore, marked alterations of estrogen hepatic metabolism occurs in cholestasis which is one of the hallmarks of cholangiopathies, including the decreased hepatic levels of P450-dependent microsomal enzymes with a consequent enhanced estradiol serum level[41]. Over the last years, we have discovered that rat cholangiocytes express both ERα and ERβ subtypes while hepatocytes only express ERα[28]. In addition to that, cholangiocyte proliferation after BDL is associated with a marked increase in the expression of ER and especially the ERβ while hepatocytes which do not proliferate after BDL display a decrease of ERα protein expression[42] (Figure 5). This important role of estrogens in modulating cholangiocyte proliferation during BDL is associated with enlarged bile duct mass and enhanced estradiol serum levels[29].

The role of estrogens in modulating cholangiocyte proliferation has been confirmed by experiments showing that when BDL rats were treated with tamoxifen or the pure ER antagonist, ICI 182,780, the intrahepatic bile duct mass was markedly decreased in comparison with the control rats by impaired proliferation and enhanced cholangiocyte apoptosis. In breast cancer and hepatocellular carcinoma, tamoxifen induces cell death by multiple mechanisms including the blocking of the mitogenic effect of estrogens and induction of apoptosis-related genes[43]. In fact, the Fas receptor/Fas ligand pathway plays a crucial role in tamoxifen-induced apoptosis in hepatocellular carcinoma and cholangiocarcinoma cell lines[110-112]. To support the positive modulatory effect of estrogens on cholangiocyte proliferation, *in vitro* experiments show that proliferation of isolated rat cholangiocytes were significantly increased by 17β-estradiol and that these effects were individually blocked by ER antagonists[28]. Regarding the role of endogenous estrogens on modulating cholangiocyte proliferation during experimental cholestasis, we also evaluated the effects of ovariectomy (OVX) and estrogen replacement treatment in BDL rats[44]. OVX rats were submitted to BDL and the bile duct mass was compared with control BDL rats submitted to sham-OVX and with BDL-OVX rats treated with exogenous administration of 17β estradiol. OVX induced a significant reduction of bile duct mass in BDL rats that was associated with a decreased expression of ERβ. Administration of 17β estradiol induced a normalization of bile duct mass, ER expression and cholangiocyte proliferation in comparison with untreated BDL rats. A probable cross-talk between estrogens and growth factors including IGF1 (insulin like growth factor) has been proposed and later demonstrated that result in a synergistic growth stimulation[4,112]. This signaling cascade, typically activated by growth factors acting through tyrosine kinase receptors, involves the recruitment of the steroid receptor-coactivator (Src) and adapter protein Shc (Src-homology/collagen protein) which act upstream to the mitogen-activated protein (MAP) kinase isoforms

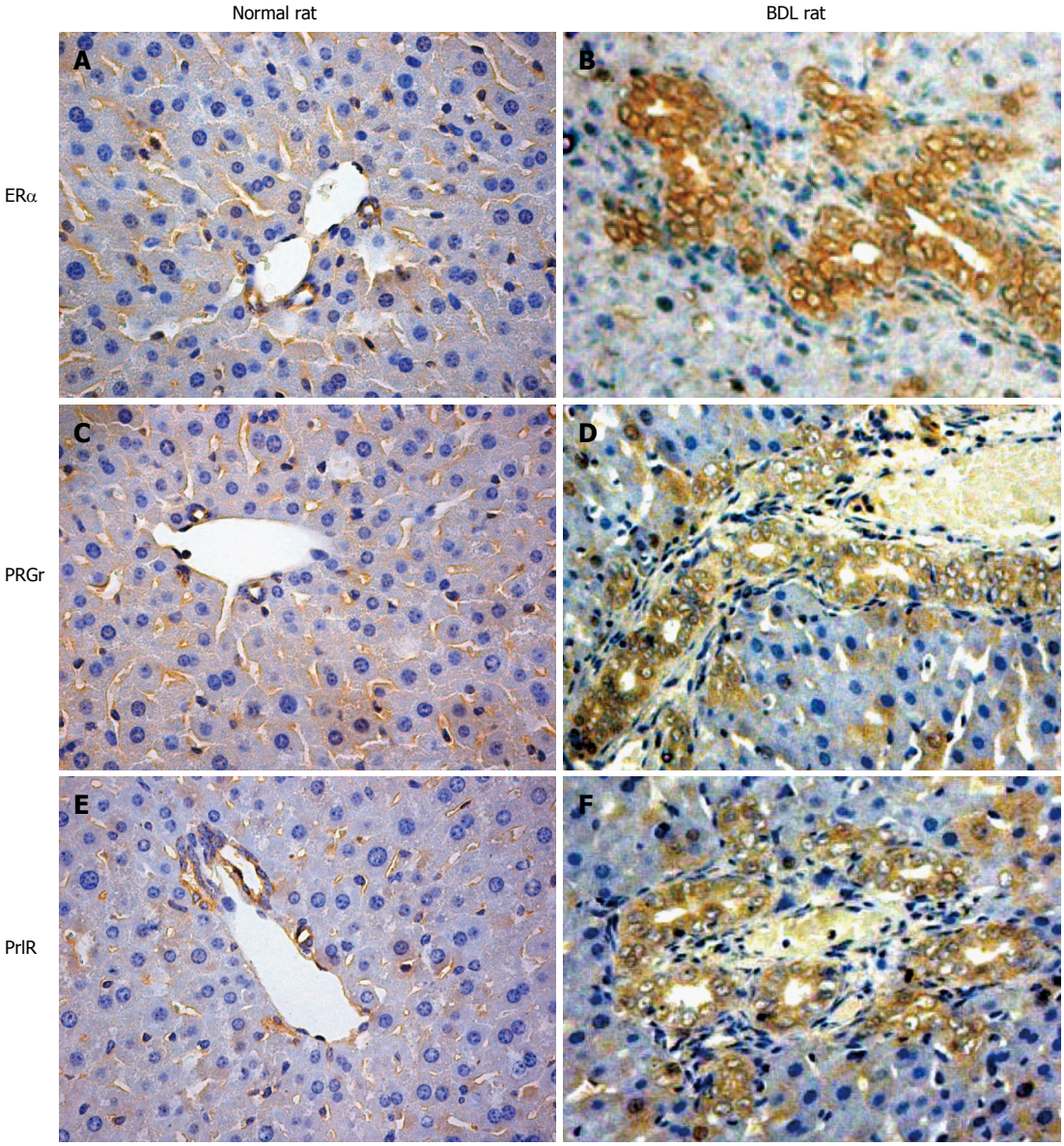


**Figure 5 Some representative immunohistochemistry for Erα, progesterone and prolactin receptors in normal and bile duct ligation (BDL) rats.** A, B: Erα receptors; C, D: progesterone receptors; E, F: prolactin receptors. The expression of all these receptors is highly increased after BDL compared with that in the biliary epithelium of the normal animal. Original magnification × 40.

ERK1/2 (extracellular signal-regulated kinase)[113,114]. We found that cholangiocyte proliferation induced by BDL involves the activation of the Src/Shc/ERK signalling cascade blocked through administration of ER antagonists[45].

Normally, human cholangiocytes do not express ERs but they stain positive for ERα and β in different pathological conditions such as primary biliary cirrhosis (PBC)[31], polycystic liver disease[115] and cholangiocarcinoma[116-121]. All these conditions are characterized by reactive or neoplastic cholangiocyte proliferation, suggesting that estrogens and their receptors may play a role in modulating the proliferative activities of cholangiocytes and therefore the course of these diseases.

PBC is one of the chronic cholestatic liver diseases which represent the most frequent acquired cholangiopathy. This is an autoimmune liver disease in that the key pathology involves the attack upon the small, microscopic bile ducts by immune system inflammatory cells. The result is a chronic granulomatous inflammatory infiltrate invading and progressively destroying the small bile ducts within the portal tracts of the liver[46]. The disease predominantly affects females with a typical clinical presentation occurring during the peri- and postmenopausal period[47]. Recent findings suggest that estrogens may influence the course of PBC by directly modulating the pathophysiology of cholangiocytes[31]. In fact, in PBC, such as in other chronic cholestatic conditions, estrogen serum levels are increased as a consequence of impaired hepatic metabolism and biliary excretion of estrogens and their metabolites[48]. However, estrogen replacement therapy as osteoporosis

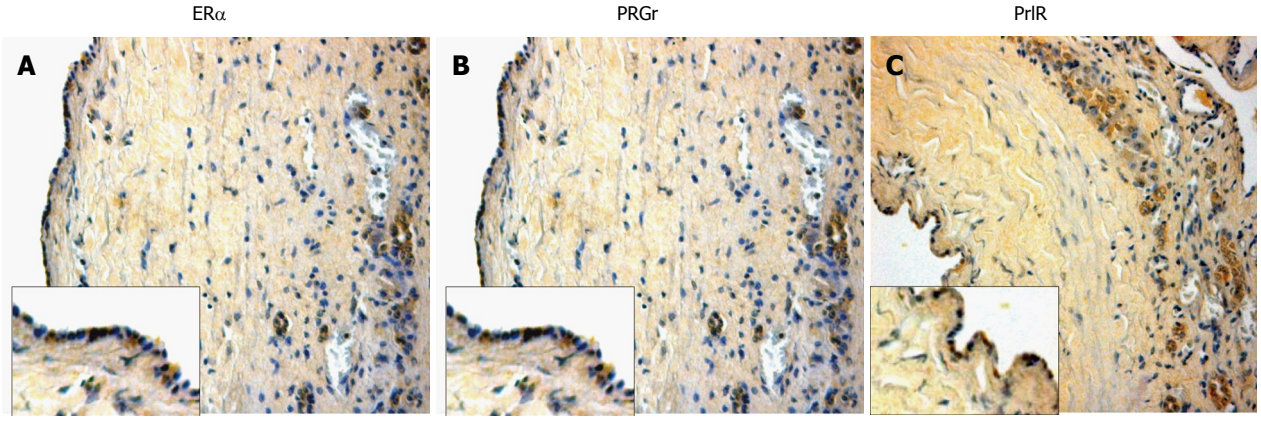


**Figure 6 Immunohistochemistry for ERα, progesterone and prolactin receptors in liver sections from patients affected with polycystic liver disease.** A: Erα receptors; B: progesterone recepors; C: prolactin receptors. Also in course of human cholangiopathies, these three considered receptors seem to play an important role in cholangiocyte physiology. Original magnification × 20.

treatment has been shown to be safe in PBC patients[49]. These clinical studies allowed summarizing the concept that administration of estrogens in PBC patients exerts deleterious effects on the liver but they can improve liver function. During PBC, the ER expression varies according to different stages and correlates with markers of proliferation and apoptosis. ERα expression increases from 1% of cholangiocytes in PBC stage Ⅰ to 12% in stage Ⅲ while ERβ is stably high in all histological stages. Interestingly, in stages Ⅰ-Ⅲ, ERα expression co-localizes with PCNA indicating that the expression of this receptor subtype is a typical feature of proliferating cholangiocytes. Furthermore, in stage Ⅳ of PBC where there is the maximal degree of ductopenia, cholangiocytes are negative for ERα and express the lowest proliferation/apoptosis ratio[31]. We can speculate that a relative proliferative deficiency of cholangiocytes in the terminal ductopenic stages of PBC is associated with the disappearance of ERα. These findings could have important therapeutic implication by the modulation of ERs. To this latter regard, preliminary clinical observations indicate that tamoxifen improves biochemical parameters of cholestatis in PBC patients. Interestingly, through the ERα, estrogens can positively modulate the GH/ IGF-1 axis[14].

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most prevalent human genetic diseases[50]. Hepatic cysts are the most common extrarenal clinical manifestation of ADPKD[51]. Estrogens have a role in the development and progression of hepatic cysts in ADPKD patients. The probability of developing hepatic cysts is higher in women than in men. Many studies and the clinical observations show a strict estrogen sensitivity of cyst formation and progression in ADPKD patients[122,123]. First of all, the epithelial layer of hepatic cysts presents the expression of ERβ and this occurs in all cysts examined, whereas the staining for ERα was less evident (Figure 6). Estrogens act not only directly but also by promoting the synthesis and release of growth factors from the cyst epithelium[115]. These findings show how the formation and progression of hepatic cysts is highly sensitive to changes in the estrogen status in the body[4,115].

Cholangiocarcinoma is a malignant tumor arising from cholangiocytes and characterized by a poor prognosis and scarce response to current therapies[124-132]. Human intrahepatic cholangiocarcinoma and the human intrahepatic cholangiocarcinoma cell line HuH-28 express ERs. The use of 17β-estradiol stimulates proliferation and inhibits apoptosis of HuH-28 cell lines, findings comparable with the proliferative response of MCF7, a breast cancer cell line. Proliferation of these cells induced by 17β-estradiol is associated with enhanced protein expression of ERα, p-ERK1/2 and pAKT but with decreased protein expression of ERβ[32,116]. This further supports the role of ERα in the estrogen-dependent modulation of neoplastic cell growth. Estrogens appear to act in several critical points of the IGF signal transduction pathway. ERα and IGF-1R have been shown to co-precipitate and their state of activation as well as the related signaling pathways have been shown to be potentiated by their coupling[52]. Finally, this mechanism may converge at different common transduction pathways modulating proliferation including ERK and phosphatidylinositol-3 kinase/Akt pathways[53]. Thus, the role played by estrogens and their receptors in the growth of ER-positive neoplasms represents the basis for the pharmacological treatment and/or prevention of different cancers with ER antagonists.

## ANDROGENS AND BILIARY EPITHELIUM

The role of androgens on biliary epithelium has been poorly investigated. In fact, we have only preliminary data on the castration effects in normal and experimental rat model of BDL in which there is a decrease in androgen receptors expression and impairment in cholangiocyte growth especially after bile duct ligation to support the hypothesis that testosterone, as estrogens, may play a key role in biliary epithelium proliferation[54]. In human

conditions, several studies exist on the use of anabolic androgenic steroid (AAS). They are frequently utilized at high doses by bodybuilders to achieve a rapid increase in muscle mass although they are associated with a number of side effects. Several liver disorders have been reported to be associated with AAS consumption such as cholestasis, peliosis hepatis and liver tumors[55]. In recent times, this use has also been proved to be involved in the development of hepatic adenomas (HA)[56]. Although more than 750 cases of oral contraceptive-induced HA have been reported, apparently androgen-induced HA are relatively rare. HA are not malignant tumors but surgical intervention may be required if sudden massive bleeding or liver failure occurs; rupture of HA with haemoperitoneum can be a life threatening complication[57]. A non-surgical approach should be considered for androgen-induced HA given that some tumors have regressed after AAS administration was stopped[58]. In any case, after a diagnosis of liver tumors the administration of AAS should cease[133-135].

## PROGESTINS AND BILIARY EPITHELIUM

As previously summarized, a number of studies have shown that, not only estrogens and androgens but also progestins strongly regulate cholangiocyte functions. Glaser *et al* have been found that female and male rat cholangiocytes express nuclear and membrane receptors that bind progesterone (PR, PGRMC1, PGRMC2, and mPR$\alpha$). Following chronic administration of progesterone to normal female and male rats, there is an increase in biliary growth which can be partly prevented by the simultaneous administration of the nuclear progesterone receptor antagonist RU-486[59] or with administration of a neutralizing anti-progesterone antibody[60]. Finally, this study also demonstrated for the first time that the biliary epithelium possesses the enzymatic pathway for the steroidogenesis of progesterone and secrete progesterone, indicating that, in addition to a paracrine pathway, cholangiocytes regulate their growth in an autocrine mechanism[136-142] (Figure 6). In humans, the concentrations in serum of sulfated metabolites of progesterone are known to be elevated in patients with intrahepatic cholestasis of pregnancy (ICP)[61]. Some studies propose that patients with ICP have a selective defect in this secretion into bile probably for a genetic polymorphism of canalicular transporters for steroid sulphates or their regulation. Interaction with estrogen metabolites may further enhance the process triggering ICP in genetically predisposed individuals[62]. Ursodeoxycholic acid, an important bile acid, stimulates the biliary excretion of these metabolites, particularly those with a 3alpha-hydroxy-5alpha (H) configuration and disulphates. The effect appears to be independent of the stimulation of bile acid secretion. An effect of ursodeoxycholic acid on the reductive metabolism of progesterone cannot be excluded[63].

## OTHER SEX HORMONES AND BILIARY EPITHELIUM

Information on the role of FSH in liver pathophysiology is limited[64]. A study has demonstrated that liver cirrhosis is associated with endocrine dysfunction, notably in the gonadal axis[65]. In males it has been recognized that cirrhotic liver disease is associated with hypogonadism and feminization parallel with impairments in the serum level of sex hormones[66]. The derangement of hypothalamic-pituitary function may play a role in the sexual dysfunction and changes in sex hormones in male patients with cirrhosis[64]. For the first time, we have shown that the biliary epithelium expresses FSHR and that FSH is a trophic factor for the biliary epithelium since chronic administration of FSH to normal rats increased cholangiocyte proliferation and intrahepatic ductal mass by cAMP-dependent phosphorylation of ERK1/2 and Elk-1[12-13]. In support of the findings that FSH treatment increases cholangiocyte FSH receptor expression, it has been demonstrated that it induces follicular growth and ovulation together with an increase in FSH binding and mRNA levels in ovaries[143,144]. In addition, another study has demonstrated that treatment of these cells with FSH increases the levels of two FSH receptor mRNA transcripts[67]. Although FSH may modulate cholangiocyte growth by a paracrine mechanism, our studies support the novel concept that FSH is a key player in the autocrine loop regulating the balance between cholangiocyte proliferation and loss. These findings have important pathological implications since modulation of cholangiocyte expression and secretion of the trophic factor FSH may be important in the management of chronic cholestatic liver diseases[145].

Regarding the other gonadotropin, it has been observed that with a reduction in the plasma level of testosterone there is an elevation of the LH level in BDL rats demonstrating a primary defect in testosterone production by testes[146].

If the BDL rats were treated with L-NAME, a NO inhibitor, to reduce its over production during bile duct ligation, there is an interesting effect on the LH levels[68]. Prolonged L-NAME treatment could not decrease the elevated level of LH in BDL rats while it could increase the level of testosterone in those rats. These data suggest that the primary effect of bile duct ligation is at the level of Leydig cells and the increase of LH is secondary to the decrease in circulating testosterone. One interpretation is that L-NAME has only a partial effect on the NO inhibited Leydig cells which can produce normal levels of testosterone after being stimulated by an increased level of LH. The other interpretation is based on the complex effect of NO on gonadotropin secretion. In fact, it has previously been clearly demonstrated that NO stimulates LHRH secretion by activating guanylate cyclase and supports a potential role of NO as a neuroactive agent involved in the control of LHRH secretion and, thereby, reproductive functions[69]. It has also been suggested

that the endogenous level of NO may determine the sensitivity of GnRH-stimulated gonadotropin released by the anterior pituitary[146-151].

In addition, Prl participates in the regulation of liver function. Their receptors (PrlR) are expressed by rat hepatocytes in the sinusoidal domain of cellular membranes and in perinuclear areas[70]. They are also expressed by human hepatocytes of patients with obstructive jaundice of different etiology but prolactin receptor expression is lower in hepatocytes compared to human cholangiocytes[71]. The expression pattern and regulation of PrlR isoforms is totally different in cholangiocytes compared to hepatocytes. In fact, mature rat cholangiocytes express low levels of PrlR while it is very high in hepatocytes; only the long isoform is detected in cholangiocytes while the short isoform predominates in hepatocytes; and PrlR levels in cholangiocytes are induced by obstructive cholestasis while it is the opposite in hepatocytes. From these data, the actions of prolactin on liver are anticipated to exhibit strong cell-type specificity in both normal and pathological conditions[72] (Figure 6).

Taffetani *et al* have demonstrated that Prl regulates the growth of female cholangiocytes, presumably by an autocrine mechanism. In fact, cholangiocytes from normal and BDL female and male rats express prolactin receptors. Furthermore, Prl has a trophic effect on the growth of normal female cholangiocytes by phosphorylation of PKCβ- I and dephosphorylation of PKCα. In addition, cholangiocytes express the protein for and secrete prolactin, suggesting that prolactin participates by an autocrine mechanism in the modulation of cholangiocyte proliferation and that it may be an important therapeutic approach for the management of cholangiopathies[152-159].

## CONCLUSION

A large body of evidence supports the therapeutic potential of sex hormones in animal models and human clinical conditions in the modulation of cholangiocyte growth/loss. Mechanisms of action for most of them have been studied and others are in the course of study. Further investigations are needed to elucidate the precise mechanism of androgens, progestins and their receptors in regulating normal liver physiology and pathophysiology of cholestatic diseases. All of this interestingly suggests that sex hormones represent novel and important treatment options that could beneficially affect the pathophysiology of the biliary epithelium. Sex hormones clearly function as more than reproductive compounds by exhibiting a myriad of roles that are also essential to protect liver and biliary functions. In particular, the main concept is that estrogens and probably other hormones act by synergizing the effects of growth factors. This interaction may have more clinical implications for diseases involving the biliary epithelium in which cholangiocyte proliferation is a typical hallmark influencing disease progression and may also be relevant in the course of the neoplastic transformation. In conclusion, sex hormones are regulators of cholangiocyte proliferation in cholestasis and their modulation could represent a future therapeutic strategy for the management of cholangiopathies.

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TOPIC HIGHLIGHT

**Sharon DeMorrow, PhD,** *Series Editor*

# Biogenic amines serotonin and dopamine regulate cholangiocyte hyperplastic and neoplastic growth

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## Abstract

Biogenic amines, such as serotonin and dopamine, regulate a multitude of cellular responses. A great deal of effort has been invested into understanding the effects of these molecules and their corresponding receptor systems on cholangiocyte and cholangiocarcinoma secretion, apoptosis and growth. This review summarizes the results of these efforts and highlights the importance of these regulatory molecules on the physiology and pathophysiology of cholangiocytes. Specifically we have focused on the recent findings into the effects of serotonin and dopamine on cholangiocyte hyperplasia and neoplastic growth.



**Key words:** Ductal secretion; Cholangiocarcinoma; Monoamine oxidase A; Tryptophan hydroxylase 1; Tyrosine hydroxylase

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## INTRODUCTION

The intrahepatic biliary tree is network of interconnected ducts, which play a key role in determining the final composition of the bile reaching the duodenum by a series of secretive and absorptive events[1,2]. Cholangiocytes are the epithelial cells that line the biliary tract and regulate these secretive and absorptive events. These cells also possess marked proliferative capacity, which is evidenced during experimental conditions such as cholestasis induced by bile duct ligation (BDL), as well as in human cholangiopathies such as primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and extrahepatic biliary obstruction and biliary atresia[1,2]. These disorders are responsible for 20% of the liver transplantations among adults and close to 50% in pediatrics in the United States[3]. The ductal reactions during these diseases range from hyperplastic cholangiocyte proliferation to severe ductopenia[1,4].

What regulates cholangiocyte proliferation and death and how these mechanisms fail is still undefined.

## BIOGENIC AMINES

Serotonin, norepinephrine, epinephrine, dopamine, and histamine are often collectively referred to as "biogenic amines"[5-7]. These agents play key roles in neurotransmission and other signaling functions[5-7]. They are relatively small in size, can act as neurotransmitters to elicit various physiological responses and all have various other sites of action throughout the body[5-7]. Generally, they can be synthesized at various sites throughout the body and are released from intracellular vesicles into the surrounding tissue where they can then bind to cell membrane-located receptors on the neighboring cells to elicit their responses[8]. These molecules are capable of affecting mental functions such as mood and appetite as well as regulating blood pressure, body temperature and other bodily processes[8]. This review will focus on the effects of serotonin and dopamine on the hyperplastic and neoplastic proliferation of cholangiocytes.

## SEROTONIN

Serotonin, or 5-hydroxytryptamine (5-HT), is a neuroendocrine hormone that is synthesized in serotonergic neurons in the central nervous system[9] and in enterochromaffin cells throughout the gastrointestinal tract[10]. It is synthesized by the systematic hydroxylation and decarboxylation of the amino acid tryptophan by the enzymes tryptophan hydroxylase (TPH1) and amino acid decarboxylase, respectively[9]. There are 16 serotonin receptors through which serotonin exerts its multiple effects. With the exception of the 5-HT3 receptor, a ligand-gated ion channel, all other 5-HT receptors are G protein-coupled, seven transmembrane receptors that activate intracellular secondary messenger systems[11]. Once serotonin has activated the receptor it is cleared from the extracellular space by specific re-uptake transporters whereupon it undergoes catabolism[12]. Degradation of serotonin is carried out primarily by the enzyme monoamine oxidase (MAO), which occurs as two molecular subtypes called MAO A and MAO B, which have some differences in their tissue and cellular distributions[13]. MAO A is more selective for serotonin oxidation (and has a higher affinity for the substrate) than MAO B, as it is able to metabolize serotonin with a much lower Km value[14].

### *Serotonin in liver function*

Serotonergic nerve fibers are part of the autonomic nervous system and nerve endings have been found on the branches of the hepatic artery, portal vein, bile ducts and connective tissue of the interlobular septa in humans[15], as well as in portal tracts and the fibrous septa within rat hepatic lobules[16]. The function of serotonergic fibers in liver function appears to be related to the regulation of blood flow through the portal vein as well as sinusoidal blood flow[17].

### *Serotonin in cell proliferation*

In the liver, inhibition of the 5-HT2 receptors by ketanserin arrested liver regeneration only when administered late (16 h) after partial hepatectomy, suggesting that serotonin may have a role in the G1/S transition check point through 5-HT2 receptors[18]. Studies have shown that liver regeneration after partial hepatectomy was completely dependent upon platelet-derived serotonin, as a mouse model of thrombocytopenia inhibited normal liver regeneration in a 5-HT2 receptor-dependent manner[19].

Serotonin is involved in the pathogenesis of certain clinical features of cholangiopathies: pruritus and fatigue in particular[20,21]. In animal models of chronic cholestasis, this may be due to an enhanced release of serotonin in the central nervous system and its subsequent interactions with subtype 1 serotonin receptors[21].

Recently, it was demonstrated that cholangiocytes have the capacity to synthesize and secrete serotonin, both of which are increased in proliferating rat cholangiocytes after BDL[22]. In addition, the 5-HT 1A and 1B receptors are found predominantly on the basolateral membrane of cholangiocytes in the liver[22]. It was postulated that this autocrine loop is integral in limiting the growth of the biliary tree as a result of chronic cholestasis. This was based on the observation that chronic treatment of rats with the 5-HT 1A and 1B receptor agonists inhibited cholangiocyte proliferation in BDL rats[22]. Furthermore, this effect is more than likely due to a direct effect of the receptor agonists on cholangiocytes, as the treatment of cholangiocytes with serotonin had a similar inhibitory effect. By immunoneutralizing the endogenous serotonin secreted from cholangiocytes as a result of BDL, using an anti-serotonin antibody, we were able to enhance the growth of the biliary tree in the course of chronic cholestasis, suggesting that the autocrine secretion of serotonin does, indeed, play an important role in the control of cholangiocyte growth[22].

Certain physiological aspects of cholangiocyte function were also inhibited by 5-HT 1A and 1B receptor agonists in proliferating cholangiocytes after BDL, but not in mitotically dormant cholangiocytes[22]. Both secretin-stimulated bile and bicarbonate secretion were inhibited by chronic *in vivo* administration of the serotonin receptor agonists[22]. In freshly isolated cultures of cholangiocytes, serotonin receptor agonists inhibited both secretin-stimulated cAMP synthesis and protein kinase A (PKA) activity[22]. This suggests that activation of both 5-HT 1A and 1B receptors can modulate not only cholangiocyte proliferation and survival, but also physiological functions of cholangiocytes.

The intracellular signaling pathways that may be responsible for the antiproliferative effects of serotonin are associated with enhanced $IP_3$ levels, increased $Ca^{2+}$-

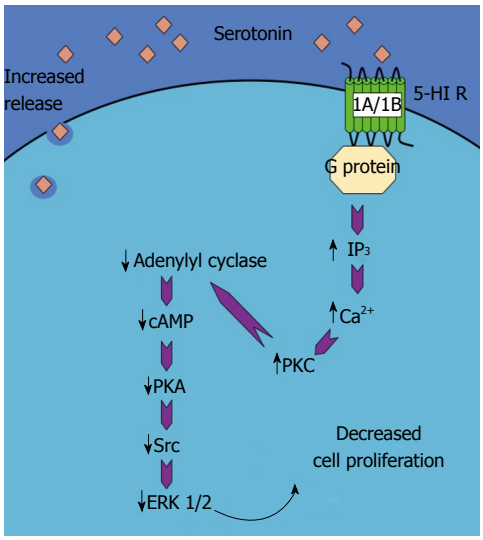


**Figure 1 Schematic representation of the mechanism of the serotonin-induced decrease in cholangiocyte proliferation.** Activation of 5-HT 1A/1B receptors results in an increase in $IP_3/Ca^{2+}$/PKC pathway, which in turn decreases the adenylyl cyclase/cAMP/PKA/ERK1/2 pathway. This ultimately leads to a decrease in cholangiocyte proliferation. (This figure was adapted from DeMorrow *et al*[3] and reproduced with permission from the Society for Experimental Biology and Medicine).

dependent PKC activity and a reduction in the cAMP/PKA pathway[22]. Downstream of these events is a reduction in the activation of the Src/ERK1/2 cascade, which directly effects cholangiocyte proliferation[22]. A schematic representation of this pathway can be seen in Figure 1 (adapted from[3]).

A role for serotonin in biliary choleresis is also supported by the observation that selective serotonin reuptake inhibitors such as citalopram and paroxetine have been linked with cholestasis as well as severe acute and chronic hepatitis in humans[23-25]. However, the mechanisms by which selective serotonin reuptake inhibitors contribute to cholestasis are unknown[17].

### *Serotonin control of neoplastic growth in the biliary epithelium*

Several opposing effects of serotonin on tumor growth have been reported[26]. On one hand, serotonin is known as a growth factor for several types of non-tumoral cells[27,28], and it has been proposed to take part in the autocrine loops of growth factors contributing to cell proliferation in aggressive tumors such as small cell lung carcinoma[29], prostate cancer[30], breast cancer[31] and bladder cancer[32]. In contrast, several studies have reported that serotonin can inhibit tumor growth, mainly via the specific vasoconstrictive effects of serotonin on the vessels irrigating the tumors[26,32-34]. In addition, the synthesis and secretion of serotonin have previously been shown to be dysregulated in neuroendocrine tumors, with these cells possessing a higher biogenic amine content than normal cells[35-37].

We have recently shown that serotonin is overproduced in cholangiocarcinoma. It can be detected in the supernatant from cholangiocarcinoma cell lines as well as in the bile taken from cholangiocarcinoma patients at higher levels, compared to those with non-malignant biliary diseases[38]. Further analysis revealed disequilibrium between the synthesis and degradation pathways of serotonin[38]. Specifically, the rate-limiting synthesis enzyme TPH1 is overexpressed in cholangiocarcinoma cell lines and tumor tissue, whereas the expression of MAO A is dramatically downregulated[38]. This increase in serotonin production has growth-promoting effects on cholangiocarcinoma and blocking serotonin synthesis by treating animals with a specific TPH1 inhibitor effectively reduces tumor growth[38].

Precisely why serotonin goes from having growth-suppressing activities in non-malignant cholangiocytes to promoting growth in neoplastic cholangiocarcinoma cells is unknown and is a topic of ongoing research in our laboratory.

## DOPAMINE

Dopamine is synthesized mainly by nervous tissue and adrenal glands, first by the enzymatic conversion of tyrosine to DOPA (3,4-dihydroxyphenilalanine) by tyrosine hydroxylase and then by the decarboxylation of DOPA by aromatic-L-amino-acid decarboxylase. As a member of the catecholamine family, dopamine is also a precursor to epinephrine and norepinephrine. The dopamine receptors are a class of metabotropic G protein-coupled receptors and, to date, there are 5 types: D1-D5[39]. Activation of these receptors has differing effects on signal transduction pathways. For example, the D1 receptor interacts with the Gs complex to activate adenylyl cyclase, whereas the D2 receptor interacts with Gi to inhibit cAMP production[39]. As with serotonin, dopamine is rapidly cleared from the extracellular space by a dopamine-specific re-uptake transporter whereupon it is degraded, predominantly by MAO A[40].

In the brain dopamine acts as a neurotransmitter where it activates dopamine receptors, but it can also act as a neurohormone which is released by the hypothalamus and exerts various effects on the pituitary[41]. Dopamine has been implicated in the etiology of Parkinson's disease[42] and schizophrenia[43] and plays a major role in the reward system of behavior[44].

### *Dopamine in hyperplastic cell proliferation in the liver*

The studies into the effects of dopaminergic innervation on cholangiocytes have focused on the D2 dopamine receptor[45]. Expression of the other dopamine receptors was absent from cholangiocytes under all conditions studied (mitotically dormant and proliferating cholangiocytes), whereas the D2 dopamine receptor was expressed in normal cholangiocytes and markedly upregulated after BDL[45]. In similar experiments to those described above for serotonin, the effects of D2 dopamine receptor activation on other aspects of cholangiocyte physiology have been determined[45].

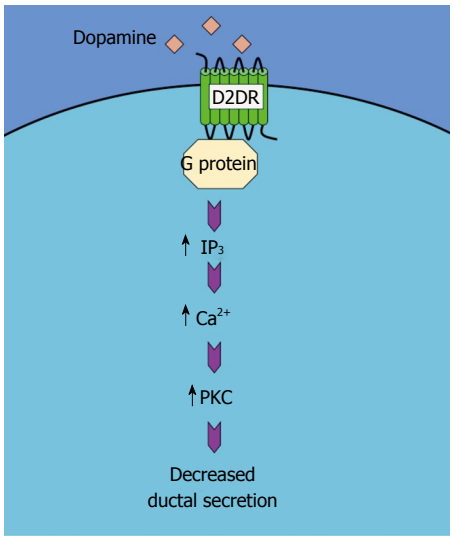


**Figure 2 Schematic representation of the mechanism of the dopamine-induced decrease in ductal secretion.** Activation of D2DR results in an increase in $IP_3/Ca^{2+}$/PKC pathway, which in turn decreases the ductal secretion.

Infusion of quinelorane had no effect on basal bile flow and bicarbonate concentration and secretion. However, co-infusion of quinelorane with secretin resulted in a decrease in secretin-stimulated bile flow and bicarbonate secretion, an effect that could be abolished with the D2 receptor antagonist eticlopride[45]. It has been repeatedly demonstrated that agents that inhibit secretin-stimulated bile flow also exhibit growth-suppressive actions on cholangiocytes[22,46-48]. This further supports a tentative role for D2 dopamine receptor activation in the suppression of cholangiocyte proliferation after BDL.

The mechanism by which quinelorane inhibits secretin-induced ductal secretion and, by extension, cholangiocyte growth, is similar to that observed after serotonin receptor activation. That is, quinelorane activates the $Ca^{2+}$-dependent Protein kinase C (PKC)-γ but not any other PKC isoform. Once again, blocking PKC-γ activity effectively inhibits the effects of D2 dopamine receptor activation on ductal secretion[45]. This pathway is summarized in Figure 2.

Information regarding the ability of cholangiocytes to synthesize and secrete dopamine is lacking, so it is not possible to say whether these dopamine-induced effects on cholangiocytes are through an autocrine mechanism and/or are a direct result of dopaminergic innervation of the liver.

### *Dopamine control of neoplastic growth in the biliary epithelium*

Similar to serotonin, several opposing effects of dopamine on tumor growth have been reported. There is an increase in the circulating level of dopamine in lung tumors, which seems to play a protective role by inhibiting cytotoxic *t*-cell proliferation, thereby preventing their ability to mount an adequate attack on the tumor cells[49,50]. Furthermore, dopamine secretion is increased in some cases of the rare malignancy pheochromocytoma[51,52] and in carcinoid tumors[53], although the consequences of this secretion on tumor growth or progression are unclear. However, agents such as dexamethasone that increase pheochromocytoma dopamine content also increase cell proliferation[54], suggesting that there may be a causal link between increased dopamine content and cell proliferation in these tumor cells. Conversely, in malignant colon tissue[55] and gastric cancer tissue[56] where dopamine levels are depleted, dopamine treatment slows tumor growth, presumably by decreasing the expression of vascular epithelial growth factor and subsequent angiogenesis[56,57]. In addition, modulation of dopamine receptors is being proposed as a possible treatment for pituitary tumors due to the suppressive effects of dopamine on prolactin secretion[58]. It may also have a role in the treatment of neuroblastoma cells, where D1DR agonists have a toxic effect on cell proliferation, which appears to be neuronal specific.

We have recently shown that dopamine, similar to serotonin, is overproduced in cholangiocarcinoma and can be detected in both the supernatant from cholangiocarcinoma cell lines and in the bile from cholangiocarcinoma patients[59]. The two enzymes responsible for dopamine synthesis, tyrosine hydroxylase and dopa decarboxylase are both overexpressed in cholangiocarcinoma cell lines and in cholangiocarcinoma tumor tissue[59]. Once again, the increased dopamine production increased cell proliferation and tumor growth, and inhibiting dopamine synthesis by using specific inhibitors of dopa decarboxylase suppresses tumor growth *in vitro* and *in vivo*[59].

As is the case with serotonin, the explanation of why dopamine has growth-inhibitory actions in cholangiocytes but has growth-promoting effects in cholangiocarcinoma is unclear and is the topic of ongoing research in our laboratory.

## CONCLUSIONS AND FUTURE DIRECTIONS

Biogenic amines such as serotonin and dopamine regulate a plethora of biological responses. We, and others, have strived to dissect the precise effects of these important biological molecules on cholangiocyte growth and physiology as well as on the malignant growth and progression of cholangiocarcinoma. Specifically, we suggest that both serotonin and dopamine have a buffering role in limiting the cholangiocyte hyperplastic proliferation seen after bile duct ligation. Conversely, somewhere during the course of the malignant transformation of cholangiocytes, serotonin and dopamine effects become growth promoting rather than growth suppressive and may contribute to tumor growth and the progression of cholangiocarcinoma. Studies have highlighted the potential importance of these molecules and their receptors in the pathological processes associated with chronic cholestatic liver diseases and cholangiocarcinoma. Further research into the molecular events

associated with the actions of the various biogenic amines on hyperplastic and neoplastic cholangiocyte proliferation is ongoing in our laboratory. Regulation of cholangiocyte growth and cell death by therapeutic agents aimed at activating or blocking these receptor systems may prove beneficial in the treatment of various cholangiopathies such as primary biliary cirrhosis and sclerosing cholangitis, as well as for blocking or slowing the progression of cholangiocarcinoma.

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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.

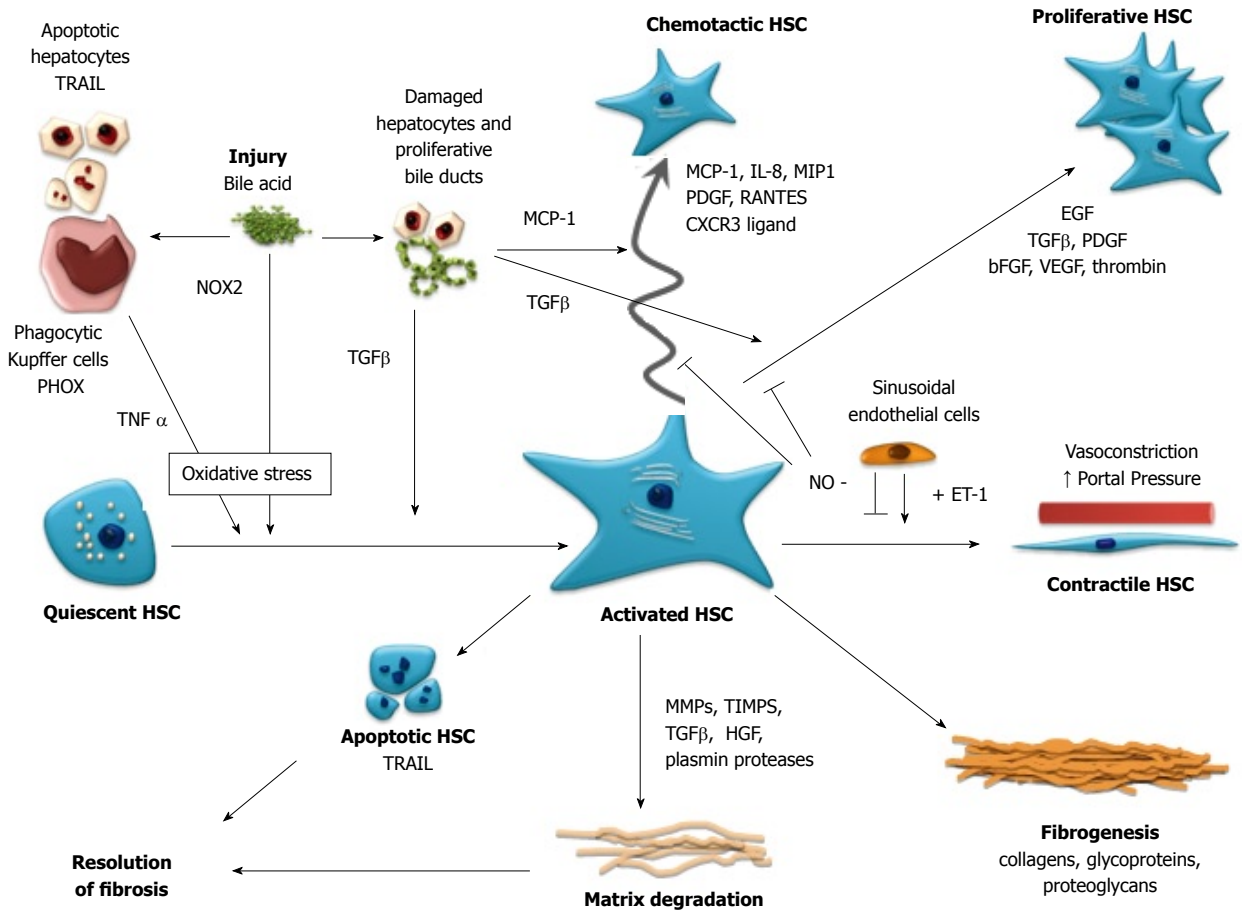


**Figure 1 Schematic representation of the activation, function and interaction of Hepatic Stellate Cells (HSC) with other cells of the liver in cholestatic liver injury.** Bile acid mediated injury is proposed to impact on HSC directly and indirectly *via* oxidative stress mediated pathways, resulting 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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REVIEW

# Pancreatic secretory trypsin inhibitor: More than a trypsin inhibitor

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## Abstract

Kazal-type serine protease inhibitor is one of the most important and widely distributed protease inhibitor families. Pancreatic secretory trypsin inhibitor (PSTI), also known as serine protease inhibitor Kazal type Ⅰ (SPINK1), binds rapidly to trypsin, inhibits its activity and is likely to protect the pancreas from prematurely activated trypsinogen. Therefore, it is an important factor in the onset of pancreatitis. Recent studies found that PSTI/SPINK1 is also involved in self-regulation of acinar cell phagocytosis, proliferation and growth of a variety of cell lines. In addition, it takes part in the response to inflammatory factor or injury and is highly related to adult type Ⅱ citrullinemia.



**Key words:** Pancreatic secretory trypsin inhibitor/serine protease inhibitor Kazal type Ⅰ; Pancreatitis; Autophagy; Cell proliferation; Inflammatory factor; Adult-Ⅱ citrullinemia.

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## INTRODUCTION

Human pancreatic secretory trypsin inhibitor (PSTI) is also known as serine protease inhibitor Kazal type Ⅰ (SPINK1). It was originally isolated from the pancreas[1] and subsequently identified in mucus producing cells of the gastrointestinal tract and in a range of other tissues including lung, liver, kidney, ovary, breast and in the collecting tubules and transitional epithelium of the renal pelvis[2]. There are homologous PSTI/SPINK1 genes in mouse and rat. Serine protease inhibitor, Kazal type 3 (*Spink3*), the homologous gene in mouse, was recently discovered in the forebrain/midbrain junction region, mesonephric tubules and other tissues during mouse embryonic development[3]. In recent years, more and more attention has been devoted to PSTI/SPINK1 with identification of more unexpected functions for PSTI/SPINK1. In this review, we summarize the diverse roles of PSTI/SPINK1 in pancreatitis, embryonic development, cancer occurrence and development, acute phase response and adult-Ⅱ citrullinemia.

## STRUCTURE OF PSTI/SPINK1 AND ITS HOMOLOGS

Kazal-type serine protease inhibitor, usually with a

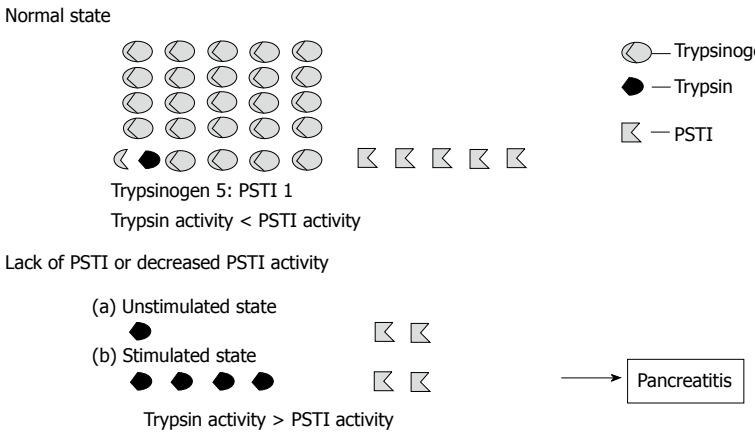


**Figure 1 The importance of PSTI/SPINK1 in onset of pancreatitis (cited from Hirota *et al*[14] and revised).**

signal peptide of about 20 amino acids at *N*-terminal, is normally composed of one or several Kazal domains which typically consist of 50-60 amino acid residues. Human PSTI gene, containing about 7.5 kb and four exons, is located on 5q32. Analysis of the PSTI gene reveals that a 40 bp DNA fragment located between kb -3.84 and -3.80 carries the element responsible for both transcriptional activity and IL-6-induced gene expression[4]. The protein of 79 amino acids encoded by human PSTI gene includes two parts. The first part of 56 amino acid residues contains three disulfide bonds and a trypsin-specific binding site formed by Lys-Ile; the second part is a 23 amino acid signal peptide. The mature PSTI is produced after being directed by signal peptide into the cavity of endoplasmic reticulum. In mouse, the homologous gene is designated *Spink3*. There are two types of cDNA, PSTI-I or SPINK3 and PSTI-Ⅱ or SPINK1, which code for the two types of PSTIs in rat. The nucleotide sequences are 91% homologous between the two cDNAs, but 68% and 65% homologous respectively when compared with human PSTI cDNA. Both amino acid sequences consist of 79 amino acids with the secretion signal peptide consisting of 18 and 23 amino acids for PSTI-Ⅰ and PSTI-Ⅱ respectively. Therefore, mature PSTI-Ⅰ consists of 61 (PSTI-61) and PSTI-Ⅱ of 56 (PSTI-56)[5,6]. There may be no difference between two PSTIs in inhibitory properties. However, PSTI-Ⅰ, also known as monitor peptide (MP), can sense the content of proteins in the intestine, promote peptide cholecystokinin (CCK) release and thereby contributes to pancreatic exocrine function, while PSTI-Ⅱ has no effect on either CCK release or pancreatic secretion function[7-9].

## PSTI/SPINK1 IN THE ONSET OF PANCREATITIS

PSTI/SPINK1 is synthesized in acinar cells of the pancreas, packaged with digestive enzymes into the secretory granule and binds covalently with erroneously activated trypsin to form an inactive, stable complex[10]. Therefore, PSTI/SPINK1's likely major role is to prevent premature activation of trypsinogen thereby ensuring the integrity of acinar cells to prevent autodigestion of the pancreas[11]. Because the SPINK1 and trypsinogen ratio is 1:5, SPINK1 can only inhibit up to 20% of the trypsin activity in the pancreas. Thus a lack of SPINK1 or decreased activity of SPINK1 may result in the premature conversion of trypsinogen into active trypsin leading to activation of various proteases that damage acinar cells and then ultimately the development of pancreatitis[12-14] (Figure 1).

There have been many reports of mutations of PS TI/SPINK1 gene in patients with pancreatitis and several hypothesized roles of these mutant proteins in pancreatitis. Pfutzer *et al*[12] compared the SPINK1 gene sequences in 112 pancreatitis patients with family history and 95 control persons and found SPINK1 mutations were very common (2%) in the population and closely related to pancreatitis. Drenth *et al*[15] showed SPINK1 gene mutations occurred in 12.2% of adult patients with alcohol-induced chronic pancreatitis and idiopathic chronic pancreatitis, indicating SPINK1 was a susceptible gene for chronic pancreatitis. In South India, SPINK1 gene mutations and environmental factors were found to be possible reasons that lead to tropical pancreatitis[16]. Furthermore, Witt *et al*[17] identified N34S missense mutation and four other kinds of mutations in SPINK1 gene in 18 of 96 (23%) patients with chronic pancreatitis. Bernardino pointed out the -253C allele for SPINK1 gene might represent a risk factor for the occurrence of pancreatitis in Brazilian population[18].

Interestingly, many previous studies have confirmed that the N34S and other SPINK1 mutations are more common in the general population. However, the incidence of pancreatitis is not very high. Lee *et al* revealed SPINK1 gene mutations only slightly increased the risk of alcohol-induced chronic pancreatitis (ACP) while the new genetic, environmental and triggering factors must be worth exploring the relationship with ACPs[19-21]. Therefore, the pathogenesis of pancreatitis may be more complex[12] and it is hypothesized that only the mutations that affect PSTI/SPINK1 binding to trypsin will contribute to the onset of pancreatitis. Recent research suggested that trypsinogen activation and trypsin activity were regulated by calcium and the

combination mutations of calcium-sensing receptor (CASR) and SPINK1 gene may further increase the risk of pancreatitis[22]. In summary, SPINK1 gene mutations may be a key factor to determine the occurrence of pancreatitis while more attention should be paid to other genetic and external environmental factors.

To better understand the physiological roles of PSTI/SPINK1 in pancreatitis, Ohmuraya *et al*[23] carried out a study by means of *Spink3* knockout mice and found that they died after birth due to excessive autophagy and impaired regeneration in pancreatic acinar cells, suggesting that *Spink3* is involved in the regulation of autophagy and is essential for maintaining exocrine integrity of the pancreas. Subsequently, Ohmuraya *et al*[24] further demonstrated that autophagy is specific to pancreatic acinar cells and involved in trypsinogen activation in experimentally induced pancreatitis. These results suggest that *Spink3* has protective roles in pancreatitis by dual mechanisms, one as a trypsin inhibitor and a second as a suppressor of autophagy.

## PSTI/SPINK1 IN CELL PROLIFERATION AND GROWTH

### *PSTI/SPINK1 and embryonic development*

In 1986, Fukayama *et al*[25] first detected SPINK1 in developing buds of the pancreas during the 8th gestational week and found that pancreatic proteinases appeared in acinar cells during the 14th wk of gestation before trypsinogen started to be produced. Thus, the development of the pancreas may be related to the earlier appearance of SPINK1 which perhaps works as a growth factor. Wang *et al*[3] detected the expression of *Spink3* during development of fetal mouse and observed *Spink3* in the foregut, midgut, hindgut and the forebrain/midbrain junction region at 9.5 d post coitus (dpc), in the pancreas, large intestine, mesonephric tubules and urogenital ridge at 11.5 dpc, in the acinar cell, small intestine and genital swelling at 13.5 dpc, in the the ductus epididymis at 17.5 dpc and in the seminal vesicle at 8 wk. These data suggest that PSTI/SPINK1 may play important roles in proliferation and/or differentiation of various cell types during development.

### *PSTI/SPINK1 and cancers*

Many previous studies have shown PSTI/SPINK1 were highly expressed in the liver and serum of hepatocellular carcinoma (HCC) patients. Ohmachi *et al*[26] early discovered the blood level of PSTI in 27 patients with HCC was significantly increased and positively correlated with tumor size, suggesting that elevated blood level of PSTI without inflammation indicates the presence of HCC. Human PSTI is also known as tumor-associated trypsin inhibitor (TATI). The results of Lee *et al*[27] concluded that TATI overexpression contributed to cell growth advantage and enhanced the metastatic potential of tumors and suggested using TATI, AFP and osteopontin as combined markers for molecular staging, the detection of HCC and for the prediction of early tumor recurrence. PSTI is highly expressed not only in HCC but also in many other tumors[28]. By means of DNA microarray analysis, quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry, PSTI was identified in the cytoplasm of intrahepatic cholangiocarcinoma (ICC) cells and had been suggested as a potential marker for identifying ICC patients with an increased risk of early recurrence after surgical resection[29]. Moreover, high TATI expression was associated with liver metastasis and was an independent predictor of poor prognosis in cancer of the colon and the breast[30,31]. Paju *et al*[32] demonstrated for the first time that TATI was expressed in the benign and malignant prostate and androgens can regulate TATI protein expression in high-grade tumors.

High expression of PSTI/SPINK1 in cancers may be highly related to its roles in cell proliferation and migration[33]. In addition, there are structural similarities between SPINK1 and epidermal growth factor (EGF) in terms of the number of amino acid residues and the presence of 3 intrachain disulfide bridges. Hence, SPINK1 may bind to EGF receptor (EGFR) to activate its downstream signaling. Recently, Ozaki first showed that SPINK1 induced proliferation and growth of NIH 3T3 cells and pancreatic cancer cell lines and this effect of EGF or SPINK1 can be completely inhibited by EGFR and MAPK/ERKK inhibitor. Taken together, these results suggest SPINK1 stimulates the proliferation of pancreatic cancer cells through the EGFR/MAPK cascade[34]. Rat PSTI-61 in vitro induced the growth and proliferation of rat intestinal epithelial cells IEC-6 in a dose-dependent manner and can effectively enhance the activity of ornithine decarboxylase, indicating polyamine metabolism may be involved in the mechanism of growth induction[35].

## PSTI/SPINK1 IN RESPONSE TO INFLAMMATORY FACTOR OR INJURY

The early stages of the host response to infection, inflammation, tissue damage and other stressors include a number of physiologic changes collectively known as the acute phase response. The acute phase response is comprised of many acute-phase reactants which rapidly increase in the serum. PSTI has been suggested to be an acute-phase reactant in humans in previous studies. Yasuda reported LPS-stimulated macrophage conditioned medium and IL-6 markedly stimulated the secretion of PSTI by cultured hepatoblastoma cells and suggested IL-6 induced PSTI secretion is mediated by cAMP dependent protein kinase A[36]. Jönsson also found the median plasma level of PSTI increased significantly at the fourth day following subtotal pancreatoduodenectomy with no increase in pancreatic juice or trypsinogen levels in pancreatic juice and plasma. Furthermore, he detected the levels of PSTI in culture medium from endotoxin-stimulated hepatocellular carcinoma cells and

pancreatic cancer cells and demonstrated that the liver is the probable source of extrapancreatic origin of plasma PSTI during the acute-phase reaction[37]. There are two PSTIs (PSTI-61 and -56) purified from rat pancreatic juice. However, only the serum PSTI-61 had increased approximately 17-fold over the initial level at 48 h after the injection in the turpentine-induced acute inflammation model and this immunoreactive PSTI-61 was detected in the liver after induction of inflammation[38]. Thus, PSTI is highly expressed mainly in the liver after stimulation by inflammatory cytokines or other stressors and thereby leads to elevated levels of plasma PSTI.

In addition, SPINK3 was found highly expressed in the pancreas and induced after pancreatic injury in mouse. Because SPINK3 may be an important serine protease inhibitor, its up-regulation may reflect an important endogenous cytoprotective mechanism in preventing further injury[39]. Marchbank *et al*[40] demonstrated that PSTI can reduce the release of cytokine in lipopolysaccharide-stimulated dendritic cells and therefore transgenic mice overexpressing human PSTI within the jejunum reduced indomethacin-induced injury by 42% and systemic recombinant hPSTI truncated injurious effects in rat models of dextran-sodium-sulfate-induced colitis. Human milk containing high concentration of PSTI stimulated migration and proliferation of intestinal HT29 cells about three fold, reduced indomethacin-induced apoptosis by about 70%-80% and gastric damage in rats by about 75%[41]. Thus, we conclude that PSTI may be involved in stabilizing intestinal mucosa against noxious agents and stimulating repair after injury. We used Rat Genome 230 2.0 array to detect the expression of rat PSTI-61 mRNA in isolated hepatocytes, stellate cells, dendritic cells, sinusoidal endothelial cells, Kupffer cells and pit cells from regenerating livers and found its expressions were significantly up-regulated in some kinds of hepatic cells, with the highest 449-fold over the control at 2 h after partial hepatectomy (PH) in isolated stellate cells and 132-fold at 2 h after PH in hepatocytes, which implies PSTI-61 may be highly related to inflammation or injury during liver regeneration.

## PSTI/SPINK1 IN ADULT-Ⅱ CITRULLINEMIA

Citrin deficiency caused by SLC25A13 gene mutations usually develops into adult-onset type Ⅱ citrullinemia (CTLN2). Previous studies have shown that the expression of hPSTI mRNA increased significantly and the concentration of hPSTI protein was higher in the liver of type Ⅱ patients than controls. Furthermore, a significant increase in serum hPSTI level with no change in the other serum markers was found, suggesting serum hPSTI is useful as a diagnostic marker for adult-onset type Ⅱ citrullinemia[42,43]. Further study indicated that CTLN2 patients may also have weight loss, hepatic steatosis, steatohepatitis and a history of pancreatitis and serum concentration of PSTI was higher when compared with non-alcoholic fatty liver disease (NAFLD) (non-SLC25A13 gene mutations). Therefore, serum PSTI may be a useful indicator for distinguishing CTLN2 patients from conventional NAFLD[44]. However, the regulatory mechanism and physiological role of high expression of PSTI in the liver remain to be elucidated.

## CONCLUSION

The roles of PSTI/SPINK1, especially its relationship to the onset of pancreatitis, have been particularly described and we have shown that activation of trypsinogen and PSTI/SPINK1 regulates the onset of pancreatitis while other genetic and external environmental factors should be considered more in future. We need to further explore possible signal pathways for PSTI/SPINK1 involved in cell proliferation, growth and migration which may contribute to a better understanding of its roles in embryonic development or types of cancers. Furthermore, we do not clearly know the mechanism involved in high expression of PSTI/SPINK1 in livers when stimulated by inflammatory factors or partial hepatectomy. In addition, the relationship between PSTI/SPINK1 and adult type Ⅱ citrullinemia remains unclear. In short, much further work is required in order to comprehensively understand the functions of PSTI/SPINK1.

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# Meetings

## Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
San Francisco, CA, United States
The Medical Management of HIV/AIDS

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# Instructions to authors

## GENERAL INFORMATION

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

*Organization as author*

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

*Both personal authors and an organization as author*

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

*No author given*

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

**Books**

*Personal author(s)*

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

**Electronic journal** (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

**Patent** (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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