GALLBLADDER AND PANCREATIC DISEASE IN PATIENTS WITH PRIMARY SCLEROSING CHOLANGITIS

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M.D.

Stockholm 2010
Tell me, and I will forget.
Show me, and I may remember.
Involve me, and I will understand. (Confucius, BC 450)
ABSTRACT

The aims of this study were to assess the occurrence of gallbladder abnormalities and dysfunction, to evaluate clinically useful biomarkers for premalignancy and malignancy in gallbladder epithelium and to assess presence of pancreatic duct changes and early pancreatic abnormalities in patients with primary sclerosing cholangitis (PSC).

In paper I and III we investigated presence of gallbladder abnormalities in 286 patients with PSC. Gallbladder abnormalities were found in 41% of the patients, gallstones in 25% and cholecystitis in 25%. Six % (18/286) of the patients had a gallbladder mass lesion with a mean size of 21(±9) mm (SD) of whom 56% (10/18) constituted adenocarcinoma. All available gallbladder specimens (n=53) were re-reviewed and immunohistochemical staining was compared on all available paraffin blocks of gallbladdercarcinoma/dysplasia (n=13) and benign tissue (n =6). A significant association was found between presence of moderate-severe lymphoplasmacytic chronic inflammation and fibrosis and dysplasia/adenoocarcinoma. Immunoreactivity for the cell-cycle-regulating proteins p53, Ki67, Cyclin D1 and pCEA were detected in significantly more cases of dysplasia and carcinoma of the gallbladder compared to non-cancerous epithelium, and the thioredoxin family proteins TrxR1-v,2,3,5 was significantly overexpressed in the dysplastic and tumors tissue whereas Grx1 was downregulated.

In paper II we studied gallbladder volumes in patients with PSC (n=20) and healthy controls (n=10) with magnetic resonance imaging (MRI). Median fasting and postprandial gallbladder volumes PSC were significantly larger than in healthy controls. There was no difference in ejection fraction or gallbladder emptying volume between PSC patients and controls. Contrast enhancement of the gallbladder wall in PSC patients was higher than in controls. No significant association was found between the gallbladder volumes and occurrence of abdominal pain in patients and controls.

Paper IV evaluated the presence of pancreatic parenchymal and duct changes using MRI and magnetic resonance cholangio pancreatography (MRCP) in 103 patients with PSC. Pancreatic duct changes were found in 24%. The pancreatic duct changes were associated with extrahepatic biliary involvement and long duration of PSC but neither associated with early radiological signs of chronic pancreatitis such as pancreas-spleen signal intensity ratio (SIR), arterial and early venous phase ratio (A/PV) nor to pancreas size, previous episodes of acute pancreatitis. Severe pancreatic duct changes were significantly associated to abdominal pain.

Conclusions: Gallbladder and pancreatic abnormalities are common in PSC and gallbladder mass lesions regardless of their size are frequently malignant in PSC. Our data support an inflammation-fibrosis-dysplasia-carcinoma sequence of the gallbladder epithelium. The overexpression of TrxR1-v2,3,5 and downregulation of Grx1 in dysplastic gallbladder epithelium may be of help for the early diagnosis of biliary malignancy in PSC but needs to be further evaluated. Pancreatic duct changes seem to be part of the spectrum of PSC and should not be defined as chronic pancreatitis. Severe pancreatic duct changes may contribute to abdominal pain in PSC, however, gallbladder size or emptying does not seem to be involved in the development of abdominal pain in PSC.
LIST OF PUBLICATIONS


III. **Said K**, Glaumann H, Björnstedt H, Bergquist A. Thioredoxin family proteins and proliferation markers in dysplastic and malignant gallbladders in patients with primary sclerosing cholangitis. Submitted for publication.

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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>AIP</td>
<td>autoimmune pancreatitis</td>
</tr>
<tr>
<td>AIH</td>
<td>autoimmune hepatitis</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CT</td>
<td>computerized tomography</td>
</tr>
<tr>
<td>ERCP</td>
<td>endoscopic retrograde cholangio pancreatography</td>
</tr>
<tr>
<td>GR</td>
<td>glutathione reductase</td>
</tr>
<tr>
<td>Grx</td>
<td>glutaredoxin</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>IAC</td>
<td>IgG4 associated cholangitis</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>IgG4</td>
<td>immunoglobulin G4</td>
</tr>
<tr>
<td>MRCP</td>
<td>magnetic resonance cholangio pancreatography</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear</td>
</tr>
<tr>
<td>PSC</td>
<td>primary sclerosing cholangitis</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SI</td>
<td>signal intensity</td>
</tr>
<tr>
<td>SIR</td>
<td>signal intensity ratio</td>
</tr>
<tr>
<td>Trx</td>
<td>thioredoxin</td>
</tr>
<tr>
<td>TrxR</td>
<td>thioredoxin reductase</td>
</tr>
<tr>
<td>UC</td>
<td>ulcerative colitis</td>
</tr>
<tr>
<td>UDCA</td>
<td>ursodeoxycholic acid</td>
</tr>
<tr>
<td>VAS</td>
<td>visual analogue scale</td>
</tr>
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</table>
1 INTRODUCTION

1.1 PRIMARY SCLEROSING CHOLANGITIS

1.1.1 Background

Primary sclerosing cholangitis (PSC) is an idiopathic chronic cholestatic inflammatory liver disease characterized by diffuse fibrosing inflammation of the intrahepatic and/or extrahepatic bile ducts, resulting in bile-duct obliteration, biliary cirrhosis, and eventually hepatic failure. Although the etiology of PSC remains unknown, findings such as a decreased number of circulating T lymphocytes, hypergammaglobulinemia, high prevalence of serum autoantibodies and the association with other autoimmune diseases suggest a strong component of autoimmunity in the pathogenesis of this disease. As high-quality epidemiologic studies are difficult to conduct, the true prevalence of PSC is unknown. The prevalence of PSC in Sweden can be estimated to be 6 per 100,000, in Norway the point prevalence is 8.5 cases per 100,000 and in Spain the prevalence of PSC was estimated to be 0.22 cases per 100,000. The prevalence of PSC in the United States has been reported to be 1-13.6 per 100,000 persons. Sixty to 70% of affected individuals are of male gender, with an average age of 42 years at diagnosis. PSC is strongly associated with inflammatory bowel disease (IBD), which is present in up to 80% of patients with PSC. The prevalence of PSC in patients with IBD has been reported widely, and ranges from 2.4 to 7.5%. No effective medical therapy is currently available, and liver transplantation is still the only life-extending treatment option for patients with end-stage PSC.

1.1.2 Clinical presentation

The clinical presentation of PSC is variable. The most common symptoms at time of presentation are shown in table 1. Asymptomatic patients are becoming increasingly common; in recent reports they make up between 15% and 55% of patients with PSC. This variation may be due to physicians’ increasing awareness of the disease, the availability and use of noninvasive imaging of the biliary tract, such as magnetic resonance cholangiography (MRC), and the increasingly common practice of screening patients with inflammatory bowel disease (IBD) for liver test abnormalities and performing cholangiography when indicated. Of the initially asymptomatic patients, 22-53% develop symptoms during a follow-up period of 6 years. Several studies show that the diagnosis of PSC is made with a mean delay of 4 years from the
first presentation of biochemical abnormalities consistent with PSC. The physical examination is usually unremarkable in early stages. If positive, it may disclose hepatomegaly (45%), splenomegaly (30%), skin hyperpigmentation (25%), excoriations (20%), or ascites (1%).

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Frequency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic</td>
<td>15-56</td>
</tr>
<tr>
<td>Fatigue</td>
<td>40-75</td>
</tr>
<tr>
<td>Pruritus</td>
<td>40-70</td>
</tr>
<tr>
<td>Jaundice</td>
<td>10-70</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>15-80</td>
</tr>
<tr>
<td>Weight loss</td>
<td>10-34</td>
</tr>
<tr>
<td>Fever and cholangitis</td>
<td>5-28</td>
</tr>
</tbody>
</table>

1.1.3 Diagnosis of PSC

The diagnosis of PSC is based on presence of a cholestatic biochemical profile, when MRC-based cholangiography or endoscopic retrograde cholangiography (ERC) shows characteristic irregularities and beading of intrahepatic and/or extrahepatic bile ducts (Figure 1), and secondary causes of sclerosing cholangitis (SSC) have been excluded (Figure 2).

Fig.1 Endoscopic retrograde cholangiography shows characteristic irregularities and beading of intrahepatic and extrahepatic bile ducts.
Patients who present with clinical, biochemical and histological features compatible with PSC, but have a normal cholangiogram, are classified as small duct PSC. The histological characteristics include the presence of portal tract inflammation, periductal fibrosis and bile duct proliferation. Periductal concentric (“onion-skin”) fibrosis is a classic histopathologic finding of PSC, but this observation is infrequent in PSC liver biopsy specimens and may also be observed in SSC. Given an abnormal cholangiogram, a liver biopsy is not required to establish a diagnosis of large duct PSC but is essential in suspected small duct PSC as well as for the assessment of possible overlap syndromes.

PSC - Autoimmune hepatitis (AIH) overlap syndrome is a disorder mainly described in children and young adults. It is characterized by the clinical, biochemical, and histological features of AIH in the presence of cholangiographic findings identical to PSC.

Secondary sclerosing cholangitis is morphologically similar to PSC but originates from a known pathological process. Its clinical and cholangiographic features may mimic PSC. Well-described causes of SSC include intraductal stone disease, surgical or blunt abdominal trauma, intra-arterial chemotherapy, and recurrent pancreatitis. Other associations have been reported recently, including autoimmune pancreatitis / IgG4 associated cholangitis, portal biliopathy, primary immune deficiency, and AIDS-related...
cholangiopathy. Differentiating between primary and secondary sclerosing cholangitis can be difficult when gallstones are present, especially in cases without concomitant inflammatory bowel disease.

Fig 3. Hematoxylin and eosin staining shows the typical lesion of fibrous cholangitis. Concentric fibrosis with inflammation surrounds the bile duct in an onion-skin pattern.

### 1.1.4 Natural history of PSC and prognosis

PSC is a progressive disease with a rate of progression that is highly variable and difficult to predict. Ultimately, however, PSC leads to cirrhosis and death from complications of end-stage liver disease; for patients with end-stage liver disease, the only effective therapeutic option is liver transplantation.

Although PSC is an uncommon disease, it is among the most common indications for liver transplantation in the Nordic Countries, Europe and the United States. The overall median survival time of patients with PSC from diagnosis to death, or liver transplantation, has been reported to range from 10 to 12 years.

There is no medical treatment that can prevent the development of fibrosis and reduce the need of liver transplantation. The most studied drug in PSC is ursodeoxycholic acid, which, despite a range of potentially valuable actions on the cholestatic liver, has not been proven to be beneficial and reduce the need of liver transplantation or the development of cirrhosis.

Cholangiocarcinoma (CCA) develops frequently in patients with PSC and is a leading cause of death in patients with this disease. The reported frequency of CCA has ranged between 6% to 11% in studies describing the natural history of PSC and 7% to 36% in PSC patients undergoing liver transplantation. The risk of CCA development appears to be unrelated to the duration of PSC and the occurrence of CCA is unpredictable and often difficult to diagnose. In contrast to PSC patients,
without CCA, the survival of PSC patients with CCA is very poor with or without liver transplantation 33, 39, 40. Lewis et al. have shown that there is a close association between gallbladder neoplasia and intrahepatic biliary neoplasia in patients with PSC 42. The mechanisms responsible for the increased risk of malignancy in primary sclerosing cholangitis are not clear, but the combined influence of exposure to chronic inflammation and hydrophobic bile acids with persistent cholestasis is most likely important 43.

Patients with small-duct PSC have a better long-term prognosis than those with large-duct PSC. Cholangiocarcinoma does not seem to occur in patients with small-duct PSC unless the disease has progressed to large-duct PSC 24.

While several prognostic models and risk scores have been constructed for patients with PSC10, 20, 34, 44, the major limitation of them all is the inability to predict the development of cholangiocarcinoma. These prognostic models seem to be useful in predicting outcome in patient cohorts but their ability to precisely predict the outcome in an individual patient is limited 27.

1.1.5 Associated diseases

PSC is strongly associated with IBD. In most series of patients from Northern Europe and North America, the prevalence of IBD in PSC has been in the range 60%-80% 10, 21, 45; a lower prevalence was observed in Japan23 and in Southern Europe7. The most frequent type of IBD in PSC is ulcerative colitis. The characteristics of IBD associated with PSC are shown in table 2 27. PSC patients with UC are at an increased risk of colorectal dysplasia/cancer compared with patients with UC alone46, 47; this continues to be the case even after the PSC patients with UC have undergone orthotopic liver transplantation48, 49.

<table>
<thead>
<tr>
<th>Table 2. Characteristics of inflammatory bowel disease associated with primary sclerosing cholangitis</th>
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<tbody>
<tr>
<td>Extensive colitis (with right-sided predominance of inflammatory activity)</td>
</tr>
<tr>
<td>Rectal sparing</td>
</tr>
<tr>
<td>Backwash ileitis</td>
</tr>
<tr>
<td>Mild or quiescent course</td>
</tr>
<tr>
<td>Increased risk of colorectal neoplasia</td>
</tr>
<tr>
<td>Increased risk of pouchitis in patients undergoing proctocolectomy with IPAA</td>
</tr>
<tr>
<td>Increased risk of peristomal varices in patients undergoing proctocolectomy with ileostomy</td>
</tr>
</tbody>
</table>

IPAA, ileal pouch-anal anastomosis
Autoimmune disorders (diabetes mellitus and thyroid diseases) are more frequent among PSC patients compared to IBD patients without liver disease. However, these associated autoimmune diseases do not influence the outcome or clinical presentation of PSC. The association between PSC and pancreatic disease is discussed separately below.

### 1.1.6 Liver fibrosis

Hepatic fibrosis is a major histological finding associated with the progression of chronic liver disease to cirrhosis; it is characterized by increased deposition of components of the extracellular matrix (ECM), in particular fibrillar collagens types I and III. The excess deposition of ECM disrupts the normal architecture of the liver, resulting in pathophysiological damage to the organ. Typically, hepatic injury leads to initiation of fibrogenesis. Stimuli may include hepatocyte necrosis, oxidative stress apoptosis, inflammatory cell infiltration, and ECM alterations. Both parenchymal and nonparenchymal cells participate in the response to injury. The hepatic stellate cell (HSC), commonly considered to be the perisinusoidal cell of the liver, has many unique characteristics. It is well established that the activated HSC is the primary cell type in the liver responsible for the excess synthesis and deposition of ECM following a fibrotic stimulus. Following liver injury, the HSC undergoes a complex transformation or activation process whereby it changes from a quiescent, vitamin A-storing cell to an activated, myofibroblast-like cell. Two major cellular changes are associated with HSC activation. First, HSCs change their pattern of gene expression, which results in an enormous increase in the synthesis and deposition of ECM. Secondly, the proliferation rate of HSCs increases. The activation of HSC is initiated by several mediators and pathways that can act simultaneously. These activation factors including cytokines such as platelet-derived growth factor (PDGF), fibroblast growth factor, TGF-β1, and endothelin-1(ET-1).

There are other sources of ECM-producing fibroblast cells in injured liver besides those derived from activated HSCs, including: Kupffer cells, hepatocytes, sinusoidal endothelial, and bile duct epithelial cells. Biliary epithelial cells play particularly important roles in fibrogenesis associated with cholestatic liver diseases, for example, primary biliary cirrhosis and sclerosing cholangitis. These cells contribute to biliary fibrosis by several mechanisms; both directly by synthesis of matrix components and indirectly by regulating matrix degradation, transdifferentiation into matrix producing cells. 
myofibroblasts, and cross communication with other cell types. They produce profibrogenic cytokines, such as TGF-β1, PDGF-BB, tumor necrosis factor-alpha (TNFα), and connective tissue growth factor, that stimulate myofibroblast activation in cholestatic models of these diseases.

1.2 GALLBLADDER DISEASE IN PATIENTS WITH PSC

Gallbladder involvement in PSC has been recognised and described since the first report of the disease was published by Schwartz and Dale in 1958. However, only a few studies have evaluated gallbladder abnormalities in PSC. In one, gallbladder abnormalities were frequently observed in PSC patients and the prevalence of overall gallbladder abnormalities, including gallstones (26%), thickening of the gallbladder wall (15%) and malignancy (4%), was reported to be 41%. Fasting gallbladder volume has been reported to be increased in patients with PSC.

PSC-associated inflammation of the gallbladder epithelium and cholangiographic abnormalities of the cystic duct have been reported in patients with PSC. One of the most common symptoms at the time of presentation of PSC is mild to severe abdominal pain localized in the right upper quadrant. This kind of abdominal pain may be misdiagnosed as related to cholelithiasis and the patient undergoes cholecystectomy. In these cases, the PSC diagnosis was established during the operation with perioperative cholangiography. The cause of abdominal pain is unclear but seems to be unrelated to the degree of bile duct strictures. Moreover, cholecystectomy seldom improves abdominal pain in these patients. A possible association between enlarged fasting gallbladder volume, ejection fraction and abdominal pain has never been investigated in patients with PSC.

1.2.1 Pathophysiology of gallstone formation

The majority (80–90%) of gallstones formed within the gallbladder consist mainly of cholesterol (70%) in a matrix of bile pigments, calcium salts and glycoproteins. Gallstones can be pure or mixed cholesterol gallstones as well as pure pigment stones. Pigment gallstones are divided into two categories: black-pigment stones composed primarily of bilirubin polymers, and brown-pigment stones composed predominantly of calcium bilirubinate. Important factors in the process of the formation of brown-pigment stone are bacterial infection and biliary stases. Black-pigmented stone formation in the gallbladder is not associated with bile infection, but rather with chronic
haemolysis, leading to overproduction of bilirubin and in patients with cystic fibrosis. Mucin, a glycoprotein mixture originating from the bile ducts and gallbladder, has consistently been defined as a crystallization promoting protein in gallbladder sludge, although the role of mucin in gallstone formation is still unclear.

Conditions associated with bile salt malabsorption, such as active ileal Crohn’s disease or after ileal resection, lead to biliary cholesterol supersaturation and promote cholesterol gallstones.

Three mechanisms are of major importance for the formation of cholesterol gallbladder stones: (i) cholesterol supersaturation of bile; (ii) gallbladder hypomotility; and (iii) kinetic, pro-nucleating protein factors (Figure 4).

![Fig.4 Pathophysiology of cholesterol gallstone formation. Cholesterol crystals aggregate in bile supersaturated with cholesterol, are nucleated in the presence of pro-nucleating factors such as mucin, and grow to stones in an enlarged gallbladder with hypomotility. Figure provided by Professor Marschall HU, reprinted with permission from John Wiley and Sons.](image)

### 1.2.2 Gallstones in patients with PSC

Previous studies regarding gallbladder disease in patients with PSC have shown that one-fourth of the patients had gallstones; this seems to be part of the spectrum of PSC. Gallstones have also been shown to be associated with symptoms such as abdominal pain, pruritus and bacterial cholangitis. The mechanisms underlying the formation of gallstones in PSC are not fully understood. However, as mentioned previously, chronic cholestasis and infections predispose for the development of pigment gallstones and a high frequency of pigment stones has been reported in PSC.
1.2.3 Gallbladder motility in general

Neural control of gallbladder emptying is mediated by both parasympathetic and sympathetic innervation; the former increases gallbladder contractility, the latter causes relaxation. Numerous neurotransmitters and hormones are capable of causing gallbladder contraction. The three compounds that clearly participate in gallbladder contractile events are cholecystokinin (CCK), acetylcholine, and tachykinins. CCK is the most potent physiologic stimulator of gallbladder contraction. Postprandial gallbladder contraction is triggered by gastric emptying, leading to release of CCK from enterochromaffin cells in the epithelial lining of the duodenum.

The mucosa of the gallbladder has a high ability to absorb water. The volume of hepatic bile residing in the gallbladder decreases by 80% to 90% as a result of active sodium transport coupled with passive water absorption.

1.2.4 Gallbladder motility in PSC

Functional impairment of the gallbladder in PSC has rarely been studied. Van de Meeberg et al. showed enlarged fasting gallbladder volumes and increased postprandial volumes in patients with PSC compared with patients with primary biliary cirrhosis and healthy controls. Conversely, the pathophysiological mechanisms responsible for gallbladder enlargement in their study could not be explained. Other studies in patients with PSC have not found gallbladder enlargement. Increased gallbladder volume or gallbladder retention and altered gallbladder motility are known to occur in conditions other than PSC, such as truncal vagotomy, chronic pancreatitis, octreotide therapy, obesity, diabetes mellitus, pregnancy, and distal biliary obstruction.

1.2.5 Histopathological features of gallbladder in PSC

Several studies in recent years have investigated histopathological changes in the gallbladder in patients with PSC. All of them confirmed that patients with PSC have a diffuse lymphoplasmacytic cholecystitis. Jessurun et al. considered this to be characteristic of PSC, while Abraham et al. concluded that diffuse lymphoplasmacytic chronic cholecystitis is highly specific for extrahepatic biliary tract disease but does not distinguish between primary and secondary sclerosing cholangiopathy. Abraham et al. studied histopathological changes in patients with PSC.
and in control groups consisting of patients with malignancy-associated obstructive jaundice and a group with chronic cholelithiasis.

1.2.6 Gallbladder carcinoma

Gallbladder carcinoma is the fifth most common malignancy of the gastrointestinal tract, characterized by female preponderance, late diagnosis, ineffective treatment and poor outcome. Carcinoma of the gallbladder generally occurs in elderly patients, with a mean age of 65 yr. In most cases, the diagnosis is not made preoperatively and gallbladder cancer is found incidentally during surgery performed for other indications. This tumour is traditionally regarded as a highly lethal disease with an overall 5-year survival of less than 5%. Multiple risk factors appear to be or have been claimed to be important for the development of gallbladder carcinoma in general populations, including genetic characteristics, chronic cholecystitis and gallstone, bile composition, calcification of the gallbladder wall, anomalous junction of the biliary and pancreatic ducts, some infections, environmental carcinogens, and drugs. It is well established that gallstones are associated with cancer of the gallbladder; however, the pathogenic mechanism underlying this association is unknown.

1.2.7 Gallbladder mass lesions in PSC

The prevalence of gallbladder malignancy in patients with PSC is unclear but a study of 102 PSC patients undergoing cholecystectomy found a high risk of cancer associated with a gallbladder mass lesion. Fourteen of these patients (13.7%) had a gallbladder mass lesion, eight (57%) of which were adenocarcinomas. Furthermore, another investigation of 72 gallbladders from patients with end-stage PSC who underwent cholecystectomy (6 obtained prior to and 66 removed at liver transplantation) revealed low-grade or high-grade dysplasia in 27 (37%) and adenocarcinoma in 10 (14%). Moreover, a high frequency of pyloric metaplasia, intestinal metaplasia, dysplasia and invasive adenocarcinoma in gallbladder has been shown in patients with end-stage PSC, which supports the presence of a metaplasia-dysplasia-carcinoma sequence in PSC. Most polypoid lesions of the gallbladder smaller than 1 cm are benign in non-PSC populations, whereas adenocarcinoma was reported in gallbladder lesions less than 1 cm in patients with PSC. The frequency of malignancy in polypoid lesions of the gallbladder in patients with PSC, as well in lesions less than 1 cm, is much higher than the reported prevalence of malignancy among patients with gallbladder mass lesions in a non-PSC population, which ranges from 0.2% to 20%.
1.2.8 Markers for gallbladder neoplasia

1.2.8.1 Serological biomarkers in carcinoma of the gallbladder
Carcinoma of the gallbladder is often detected in advanced tumour stages, has a poor prognosis and reliable markers of early malignancy are lacking. Several biomarkers have been evaluated, including carbohydrate antigen 19-9 (CA 19-9), carcinoembryonic antigen (CEA), and carbohydrate antigen 125 (CA 125); of these, serum estimation of CA 19-9 holds the most promise and is most widely used, as elevated values have been found in several studies of gallbladder cancers. However, this marker has a low specificity as it is also elevated in carcinoma of the pancreas, extrahepatic bile duct cancers and even in benign diseases of the gallbladder and bile duct. CA19-9 and CA 125 seem to be clinically useful adjuncts to imaging for distinguishing carcinoma of the gallbladder from cholelithiasis.\textsuperscript{109,110}

1.2.8.2 Immunohistochemical staining of the gallbladder neoplasma
Various molecular biomarkers have also been evaluated in gallbladder cancer without PSC, including cyclin D1 and p53. These are genes involved in control of G1 to S transition in the cell cycle, which is one of the important steps abrogated in the development of many malignant human neoplasms. Alterations of these genes result in unregulated cell growth and an increased risk of tumor formation. P53 is mutated in more than 50% of all human malignancies, including PSC associated cholangiocarcinoma. P53 mutation can be detected as an immunohistochemical overexpression\textsuperscript{111}. Cyclin D1 overexpression has been shown to be associated with poor histological differentiation, high cellular proliferative activity, and a poor prognosis in patients with intrahepatic cholangiocarcinoma\textsuperscript{112}. The expression of CEA in gallbladder cancer in patients without PSC has been examined in just a few studies and its expression in patients with PSC has not been studied previously. The studies that have been conducted suggest that the expression of CEA plays an important role in the development of cancer cells and in the metastasization of human gallbladder cancer\textsuperscript{113}. None of the previous studies of the gallbladder in PSC has included immunohistochemical analysis to study the expression of biomarkers.

1.2.8.3 Redox proteins
In primary sclerosing cholangitis, the epithelium of the biliary tract is exposed to long-
standing chronic inflammation, which may result in the production of harmful reactive oxygen species (ROS), inducing DNA damage and chronic stimulation of biliary cell proliferation 114-116. It is therefore possible that dysfunction of the intracellular reduction-oxidation regulatory system may be involved in biliary tract carcinogenesis. Recently, several reports have been published concerning the role of the thioredoxin system in tumour growth and drug resistance 117.

Reactive oxygen species and reactive nitrogen species (RNS) play important roles in the regulation of cell survival. In general, moderate levels of ROS/RNS may function as signals to promote cell proliferation and survival, whereas a severe increase in ROS/RNS can induce cell death. Normally, cellular reduction/oxidation (or redox) homeostasis ensures that cells respond properly to endogenous and exogenous stimuli. However, an increase in ROS production or a decrease in ROS-scavenging capacity due to exogenous stimuli or endogenous metabolic alterations can disrupt redox homeostasis, leading to an overall increase in intracellular ROS levels, or oxidative stress118-120. This may lead to aberrant cell death and contribute to the development of diseases such as cancer and degenerative disorders.

Trxs (thioredoxins) and Grxs (glutaredoxins) are small (9–16 kDa) thiol-disulphide oxidoreductases, first identified in Escherichia coli as a hydrogen donor for ribonucleotide reductase, an essential enzyme for DNA synthesis 121. The thioredoxin and glutaredoxin systems are two multifunctional redox active protein disulphide reductase systems which play an important role for tumour growth and drug resistance 117. Knowledge of the thioredoxin and glutaredoxin families has grown in recent years; numerous isoforms have been identified in different organisms with quite different structures and catalytic activities.

The thioredoxin system consists of thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH. The core function is as a protein disulfide reductase system 122. The system is of great importance since it maintains multiple redox processes essential for cell function, such as involvement in antioxidant defence, cell proliferation and redox regulated cell signaling122, 123. The glutaredoxin system consists of NADPH, glutathione reductase (GR), glutathione (GSH) and glutaredoxin(Grx). Glutaredoxin also plays an important role in redox regulatory mechanisms through catalysis of glutathionylation reactions 124. The glutaredoxin system utilizes the cellular abundance
of glutathione to catalyze protein disulfide reductions in the presence of NADPH and glutathione reductase. Grxs and Trxs can compensate for each other’s functions to a large extent; at the same time, each system has unique functions.

All redox proteins exist as isoenzymes, where Trx1, TrxR1 and Grx1 are mainly cytosolic and Trx2, TrxR2 and Grx2 predominantly localized to the mitochondria. The expression of the cytosolic TrxR1 is particularly complex, with multiple alternative transcripts leading to the translation of five different isoforms, denoted TrxR1v.1-v.5, where the less abundant forms are TrxR1v.2, TrxR1v.3 and TrxR1v.5. The relative expression of these isoforms in tumours and normal tissues has been studied to a limited extent; however, clear upregulation in lung cancer and leukaemia has previously been reported for the less abundant forms as compared to total TrxR, indicating the importance of isoform expression in cancer. Both the thioredoxin and the glutaredoxin system have been implicated in a variety of processes, including regulation of cell proliferation, apoptosis and defence against oxidative stress. Many studies have investigated the role of the thioredoxin system in various tumors. Fernandes et al. showed that the thioredoxin family of proteins is important for the growth and differentiation of lung cancer cells. In a study by Cunnea et al., redox proteins exhibited elevated expression levels in tumor tissue compared to internal control, with the endoplasmic reticulum member of the thioredoxin super family, ERdj5, showing a remarkable threefold increase in expression. Furthermore, in brain tumors, Trx expression has been associated with tumor grading and poor prognosis, and levels of Trx correlated to differentiation and proliferation in gastric cancer.

Most recently, Yoon and coworkers presented evidence supporting a substantial role for Trx also in the malignant transformation of biliary epithelium in hamster as well as in humans. The potential of the thiroredoxin system as a drug target in cancer therapy has been extensively studied in several kinds of malignancy.

1.3 PANCREATIC DISEASE IN PSC

The association between pancreatic changes and PSC is well established; reported frequencies range from 0% to 77%. However, most of these studies are small and suffer from a lack of clinical data. A study by Lindström et al of 17 cases showed that pancreatic changes are present in a proportion of patients with PSC despite no clinical suspicion of pancreatitis. Resnick et al found that 10 of 29 (35%) patients
with PSC had prepancreatic and pancreatic abnormalities, including dilated pancreatic ducts in three patients \(^{138}\). In a retrospective study by Ito et al. of 24 cases of PSC, the most common MRI finding was increased signal intensity of the pancreas on T2-weighted images (73%), followed by decreased signal intensity on T1-weighted images (55%) and decreased enhancement on arterial-phase contrast-enhanced images (50%); the last two findings reflect parenchymal pathology of the pancreas, including chronic pancreatitis\(^{139}\). In another study reviewing MR/MRCP images of 29 PSC patients, Ozkavukcu et al. found that the most common pancreatic changes in PSC patients were decreased T1-signal intensity (44%) and dilatation of the pancreatic duct (13.8%). The enhancement pattern of the pancreas was not taken into consideration in this study \(^{140}\). It is unclear whether the morphological pancreatic changes seen in PSC represent a separate diagnostic entity or are part of the disease’s spectrum; information is lacking on whether the pancreatic duct changes are of clinical relevance.

In a previous study by Bergquist et al., the risk of pancreatic carcinoma in patients with PSC was found to be 10-14 times higher than in the general population \(^{41}\). The mechanism underlying the increased risk of pancreatic carcinoma in PSC is not clear.

1.4 IMMUNOGLOBULIN G4-ASSOCIATED CHOLANGITIS (IAC)

IgG4-associated cholangitis (IAC) is a recently defined disease entity which shares a number of clinical, biochemical, and radiological features with PSC\(^{141-143}\). IAC is regarded as one variant of IgG4-related systemic disease, of which autoimmune pancreatitis (AIP) is the best studied organ manifestation \(^{144}\). IAC is associated with AIP in up to 92% of cases \(^{32, 145}\) and is characterized by an immune reaction predominantly mediated by Th2 cells and regulatory T cells (Tregs) and infiltration of immunoglobulin G4 -bearing plasma cells in bile ducts and other affected tissues \(^{141, 142}\).

Two sets of criteria for the diagnosis of AIP have been proposed recently and independently: the HISORt criteria (Histology, Imaging, Serology, Other organ involvement, and Response to steroid therapy) \(^{146}\) and the Kim criteria \(^{147}\). Both sets include histological, imaging and serological findings as well as responsiveness to corticosteroid treatment.

The cholangiographic feature of AIC is characterized by segmental and distal bile duct strictures, whereas pancreatic mass or diffuse enlargement is a diagnostic criteria for AIP, in association with diffuse pancreatic duct abnormalities \(^{146}\).
More than 80% of AIP/IAC patients seem to have high IgG4 levels in the course of the disease. Serum IgG4 levels are liable to fluctuate over time and sensitivity is higher when repeated analysis is performed. In a large cohort of patients with various pancreatic diseases, including 45 patients with AIP, Ghazale et al. reported a 93% specificity of elevated serum IgG4 (>140 mg/dl), increasing to 99% when a cut-off value of 280 mg/dl was used, thereby reaching a positive predictive value of 75%. In the largest cohort of 53 patients so far reported, the most frequent clinical signs and symptoms at presentation included jaundice (77%), weight loss (51%), mild to moderate abdominal pain (26%). The cholangiographic appearance of IgG4-associated cholangitis can be difficult to differentiate from primary sclerosing cholangitis. Moreover, raised levels of serum IgG4 have recently been found in 9% of patients with primary sclerosing cholangitis. Differences between PSC and IAC are shown in Table 3. In contrast to PSC, IAC responds to immunosuppressive treatment; however, relapses are common after steroid withdrawal, especially with proximal strictures of the bile ducts.

Table 3. Differences between PSC och IAC

<table>
<thead>
<tr>
<th></th>
<th>PSC</th>
<th>IAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, years</td>
<td>38-42</td>
<td>60-65</td>
</tr>
<tr>
<td>Gender, male</td>
<td>65%</td>
<td>80%</td>
</tr>
<tr>
<td>Association with AIP</td>
<td>-</td>
<td>90%</td>
</tr>
<tr>
<td>Association with IBD</td>
<td>80%</td>
<td>+/-</td>
</tr>
<tr>
<td>Elevated serum IgG4</td>
<td>9%</td>
<td>80%</td>
</tr>
<tr>
<td>Histological findings</td>
<td>periductal fibrosis and bile duct proliferation, cirrhosis.</td>
<td>infiltration of immunoglobulin G4-bearing plasma cells.</td>
</tr>
<tr>
<td>Cholangiographic findings</td>
<td>irregularities and beading of bile ducts</td>
<td>segmental and distal bile duct strictures, pancreatic mass</td>
</tr>
<tr>
<td>Response to steroids</td>
<td>-</td>
<td>+++</td>
</tr>
</tbody>
</table>

1.5 CHRONIC PANCREATITIS

Chronic pancreatitis (CP) is a progressive inflammatory condition that leads to irreversible loss of pancreatic structure and function. The annual incidence of CP in several retrospective studies ranged from 3 to 9 cases per 100,000 inhabitants.
This variation may partly be due to differences in alcohol consumption in different populations.

1.5.1  Etiology and natural history

In western countries, alcohol is the cause of 70% to 80% of all cases of chronic pancreatitis. However, only 10–20% of alcoholics develop the disease. The risk of alcoholic chronic pancreatitis increases logarithmically with increasing alcohol use but there is no threshold value below which the disease does not occur. Prolonged alcohol intake (6-12 years) is required to develop symptomatic chronic pancreatitis and potential cofactors that have been proposed include a diet high in fat and protein, and smoking. The etiology of chronic pancreatitis is unclear in about 10-20 % of all cases (Table 4).

Table 4. Etiology of chronic pancreatitis

<table>
<thead>
<tr>
<th>Etiology of chronic pancreatitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol 70-80%</td>
</tr>
<tr>
<td>Idiopathic 10-20%</td>
</tr>
<tr>
<td>Other 10-15%</td>
</tr>
<tr>
<td>-Hereditary</td>
</tr>
<tr>
<td>-Metabolic</td>
</tr>
<tr>
<td>-Hypercalcemia</td>
</tr>
<tr>
<td>-Hypertriglyceridemia</td>
</tr>
<tr>
<td>-Obstructive</td>
</tr>
<tr>
<td>-Traumatic stricture</td>
</tr>
<tr>
<td>-Stenosis of sphincter Oddi</td>
</tr>
<tr>
<td>-Pancreas divisum</td>
</tr>
<tr>
<td>-Malignant pancreatic duct obstruction</td>
</tr>
<tr>
<td>-Autoimmune pancreatitis</td>
</tr>
</tbody>
</table>

During the last two decades, several epidemiological and clinical studies have suggested a possible genetic factor in the development of chronic pancreatitis and several gene mutations have been reported, including the cationic trypsinogen gene (PRSS1), and the serine protease inhibitor, Kazal type 1 (SPINK1), which provides a defence mechanism against premature activation of trypsinogen. Other candidates include cystic fibrosis transmembrane conductance regulator (CFTR), the gene responsible for cystic fibrosis and anionic trypsinogen (PRSS2), which may have a protective effect against CP.
The median time to development of pancreatic insufficiency after disease onset depends on the type of pancreatitis. Several studies have demonstrated a long median time (6-13 years) to develop exocrine insufficiency in patients with alcoholic chronic pancreatitis and a median time to endocrine insufficiency of 8-20 years. The median time to develop endocrine insufficiency is much longer (27 years) in patients with early onset idiopathic pancreatitis. Patients with CP have a markedly increased risk of pancreatic cancer compared with the general population.

1.5.2 Pancreatic fibrosis

Traditionally, alcoholic pancreatitis has been considered to be a form of CP from the start, interrupted during its course by acute exacerbations. The current concept favours the necrosis-fibrosis hypothesis that alcoholic pancreatitis begins as an acute process that progresses to chronic irreversible damage as a result of repeated acute attacks. Interestingly, in a long-term population-based study, Lankisch et al. have recently shown that acute pancreatitis may progress to CP, but how frequently and under what circumstances this happens is unknown. The necrosis-fibrosis concept seems to be applicable not only to alcoholic CP but also to non-alcohol-related pancreatitis (Figure 4).

The pathogenesis of pancreatic fibrosis, a characteristic feature of chronic pancreatitis, has received increasing attention over the past few years, largely due to the identification and characterization of stellate cells in the pancreas. With regard to the putative role of pancreatic stellate cells in the production of pathologic fibrosis in the
pancreas, it is hypothesized that these cells are activated during pancreatic injury (in an approach parallel to hepatic stellate cells) 171, 172. The pancreas may be predisposed to autodigestive injury, either because of abnormal trypsin activation/inactivation mechanisms or because of the effects of toxins such as ethanol on acinar cells. An appropriate trigger factor (environmental or genetic) then stimulates overt pancreatic necrosis. Repeated episodes of acute necroinflammation (regardless of etiology) and the release of proinflammatory cytokines lead to the activation of pancreatic stellate cells 157.

Most recently, it has been reported that pancreatic stellate cells themselves are capable of synthesizing cytokines. Endogenous production of TGFβ, IL1, and IL6 has been identified using real-time PCR techniques 173, 174. Furthermore, factors such as ethanol and acetaldehyde and oxidant stress have been reported to stimulate endogenous cytokine production in pancreatic stellate cells 174. Taken together, these observations suggest a possible pathway for persistent activation of pancreatic stellate cells. Such activation may potentiate extracellular matrix production, eventually causing pancreatic fibrosis 171, 172. It is now generally accepted that fibrosis is not a mere end-product of chronic injury, but an active, dynamic process that may be reversible in the early stages 172.

1.5.3 Clinical features

The major clinical features of CP are abdominal pain, maldigestion, weight loss and pancreatic diabetes. While abdominal pain is considered to be the hallmark of CP, a subgroup of patients may have no pain at all. The pathomechanisms of pain in CP are unclear but several mechanisms have been suggested, such as inflammation of the pancreas, increased intrapancreatic pressure, alteration in pancreatic nerves and mechanical obstruction of the common bile duct or duodenum 175, 176. Maldigestion with steatorrhea occurs in advanced disease and only after the capacity for pancreatic lipase secretion is reduced by more than 90% 177.

1.5.4 Diagnosis

1.5.4.1 Diagnostic systems

The diagnosis of CP relies on relevant symptoms, imaging modalities to assess pancreatic structure, and assessment of pancreatic function. The most widely used diagnostic classification systems include the Marseille classification of 1963 with
revisions in 1984 and 1988, and the Cambridge classification of 1984. There are also widely used scoring systems such as the Mayo Clinic (Table 5) or the Lüneburg Clinic scoring system. The best method for diagnosis of CP at an early stage is an adequate surgical biopsy from the pancreas, which is seldom available. The histological characteristics include irregular fibrosis with destruction and loss of exocrine parenchyma in tissue specimens. The sensitivities of serum pancreatic enzymes (trypsinogen, lipase, or amylase) are less than 60%, so none of them per se are helpful in diagnosing CP.

Table 5. Mayo Clinic diagnostic scoring system for chronic pancreatitis

<table>
<thead>
<tr>
<th>Diagnosis is based on a total score of 4 or more derived from morphological and functional criteria (scores are in brackets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Pancreatic calcification: definite (4) or probable (2)</td>
</tr>
<tr>
<td>• Histology: definite (4) or probable (2)</td>
</tr>
<tr>
<td>• Steatorrhoea or lipase output less than 2 standard deviations below mean normal value—determined for each laboratory (2)</td>
</tr>
<tr>
<td>• Pancreatic duct abnormalities at endoscopic retrograde cholangiopancreatography (ERCP), CT, magnetic resonance cholangiopancreatography (MRCP), Cambridge classification I to III (3)</td>
</tr>
<tr>
<td>• Major clinical criteria—upper abdominal pain or weight loss over 10 kg in 12 months (2)</td>
</tr>
<tr>
<td>• Diabetes (fasting glucose &gt;140 mg/dL) (1).</td>
</tr>
</tbody>
</table>

1.5.4.2 Functional testing

The secretin-cerulein test, which is an invasive function test, is regarded as the gold standard for the detection of exocrine pancreatic insufficiency. This test, which measures stimulated secretory capacity, is available at just a few referral centres and is time consuming and uncomfortable for the patient. Non-invasive function tests have also been developed but are of limited diagnostic value, particularly in early-stage CP. In mild or moderate pancreatic insufficiency, the sensitivity of these tests is inadequate. It is only in severe disease that pancreatic function tests show a high sensitivity because these tests do not differentiate CP from pancreatic insufficiency without pancreatitis.

1.5.4.3 Imaging procedures

CP is easily diagnosed when the disease is severe and is characterized by extensive
calcifications and ductal dilatation and associated with distinct symptoms. The challenge in diagnosis arises in patients with early and mild CP. Endoscopic ultrasonography (EUS) is claimed to be a sensitive modality for diagnosing CP and evaluating its severity. The "Rosemont criteria" for EUS diagnosis of CP were established recently but the role of EUS in a diagnostic algorithm for diagnosis of CP remains unclear.  

The gold standard for the diagnosis of CP has until recently been ERCP. The most commonly used classification of ERCP changes in chronic pancreatitis is the Cambridge classification, developed nearly three decades ago and based on abnormalities seen in the main pancreatic duct and in the side branches which are secondary to chronic parenchymal changes (Table 6).

Table 6. Cambridge classification of chronic pancreatitis by ERCP

<table>
<thead>
<tr>
<th>Grade</th>
<th>Main pancreatic duct</th>
<th>Side branches</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>II-Equivocal</td>
<td>Normal</td>
<td>$&lt;3$ abnormal</td>
</tr>
<tr>
<td>III-Mild</td>
<td>Normal</td>
<td>$\geq 3$ abnormal</td>
</tr>
<tr>
<td>IV-Moderate</td>
<td>Abnormal</td>
<td>$\geq 3$ abnormal</td>
</tr>
<tr>
<td>V-Marked</td>
<td>Abnormal, plus at least one of the following: Large cavity. Duct obstruction. Dilation or Irregularity. Intraductal filling defects</td>
<td></td>
</tr>
</tbody>
</table>

The ERCP procedure is invasive, requires sedation, and is associated with a risk of cholangitis and pancreatitis. This has led to an increased use of MRCP and magnetic resonance imaging (MRI). MRCP correlates well with ERCP for diagnostic purposes in CP. In early phases of CP, the degree of pancreatic dysfunction and structural changes is subtle. The arterial blood flow to the pancreatic gland is impaired both in acute inflammatory attacks and secondary to fibrosis in CP. The enhancement pattern and signal intensity of the pancreatic gland using dynamic MRI in early diagnosis of CP has therefore been investigated in several recent studies. Delayed pancreatic parenchymal enhancement is reported to be a sensitive parameter for early diagnosis of CP prior to development of pancreatic duct changes.

The pancreatic signal on T1-weighted fat-saturated images represents a normal pancreatic parenchyma. A high pancreatic parenchymal signal indicates a rich proteinous content of the acinar cells. A segmental or diffusely decreased parenchymal
signal reflects parenchymal pathology, including chronic pancreatitis \(^{194, 200}\). It has been shown that in the early stages of chronic pancreatitis the parenchymal signal on T1 weighted fat-saturated images may not decrease compared to the control group as early as arterial peak enhancement of the gland \(^{197}\). The delayed peak enhancement in early chronic pancreatitis may be related to a combination of decreased pancreatic blood flow and delayed washout, because fibrosis has an enlarged extracellular space \(^{201}\). Arterial enhancement and parenchymal signal are both correlated with the concentration of the indirect pancreatic function test, fecal elastase-1\(^{195}\).
2 AIMS OF THE STUDY

- To assess the occurrence of gallbladder abnormalities, including gallstones, cholecystitis and gallbladder mass, in a large cohort of PSC patients, and study the impact of gallbladder abnormalities on the prognosis of PSC (Paper I).

- To evaluate fasting and postprandial gallbladder volumes and assess whether or not gallbladder emptying is associated with abdominal pain in patients with PSC (Paper II).

- To evaluate if the presence of imaging signs of chronic cholecystitis is correlated to gallbladder volume, the emptying process or abdominal pain (Paper II).

- To evaluate the possibility of an inflammation-dysplasia-carcinoma sequence in the gallbladder in PSC and clinically useful biomarkers for premalignancy and malignancy using immunohistochemical analysis of the cell-cycle-regulating proteins p53, Ki-67 and CyclinD1 and the redox proteins TrxR1, isoform-TrxR1-v.2.3.5, Trx1 and Grx1 (Paper III).

- To describe the presence of pancreatic duct changes and early pancreatic abnormalities by evaluating the pancreatic parenchymal enhancement pattern using MRCP/MRI in a large cohort of PSC patients, and to identify possible risk factors for the development of such changes in PSC and their clinical importance (Paper IV).
3 MATERIAL AND METHODS

3.1 SUBJECTS
The baseline cohort of this study consisted of 286 patients with well-defined PSC. All these patients were included in study I. Of these patients, 20 were included in study II, 53 in study III and 103 in study IV. Several patients were included in more than one study. However, all patients in study III had undergone previous cholecystectomy and none of them was included in study II (Figure 5).

Fig. 5. Patients with primary sclerosing cholangitis included in the thesis.

3.1.1 Papers I and III
All patients with a well-defined PSC treated at the Liver Unit, Karolinska University Hospital, Huddinge, between January 1970 and June 2005 were included in the study; 286 patients were identified and included in paper I. All available gallbladder specimens (n=53) taken at cholecystectomies in patients with PSC or liver transplantation performed for PSC at Karolinska University Hospital between 1985 and 2005 were re-reviewed histologically and clinical data were obtained by review of the patients’ charts.

3.1.2 Paper II
Twenty PSC patients (14 men and 6 women) were included in the study between January 2005 and July 2006. Ten healthy subjects (5 men and 5 women) without any
history of gastrointestinal disease or previous abdominal surgery served as controls. Informed consent for study participation was received from all patients and controls.

3.1.3 Paper IV

All patients who had been referred to the Department of Radiology at Karolinska University Hospital, Huddinge, for a liver/pancreas MRI using a dynamic contrast media protocol in a 1.5 T scanner during the period from January 2001 to August 2005 were identified. Among them, 112 PSC patients were identified by matching all referrals with our local PSC registry. Nine patients were excluded (5 were liver transplanted and 4 were incompletely visualized). The remaining 103 PSC patients were included in the study.

3.2 METHODOLOGY

3.2.1 Clinical assessment (Papers I, II, III, IV)

A structured protocol was used to assess the clinical history and clinical data were obtained by review of the complete medical history collected from patient files. The diagnosis of PSC was based on typical cholangiographic findings in combination with clinical, biochemical, and histological data. Secondary causes of sclerosing cholangitis were excluded before establishing the diagnosis of PSC. The following data were collected and recorded in a protocol: (1) demographic characteristics, substance use, onset, extension, stage; (2) characteristics of PSC, duration and treatment; (3) symptoms and signs attributable to PSC, including abdominal pain, jaundice, pruritus, fever, weight loss, ascites, bleeding from esophageal varices, and hepatobiliary malignancy; (4) information about IBD, type, onset, extension, duration, treatment, surgery, presence of colon dysplasia/colon cancer; (5) gallbladder abnormalities, including cholecystitis, stones, polyps and malignancy; gallstones were confirmed by one or more radiological methods, including abdominal ultrasonography, computerized tomography, and/or MRC; (6) presence of clinical sign of CP with enzyme supplementation; (7) previous investigation with ERCP and post ERCP complications; (8) laboratory data; (9) history of liver transplantation; (10) indications for cholecystectomy; (11) body mass index.
3.2.2 Laboratory data (Papers I, II, IV)
All biochemical variables were obtained and analyzed using standard procedures at the Karolinska University Hospital, Stockholm. The IgG4 subclasses were measured using sandwich type ELISA (Invitrogen, Sweden). Briefly, coated microtitre plates capture IgG4 subclass from the serum samples. The captured IgG4 is then labelled with a horseradish peroxidase anti-human IgG. The signal generated is then proportional to the amount of human IgG4. The absorbance was then measured at the correct wavelength in an ELISA reader and results were calculated using a 4-parameter curve. Patients with a serum IgG4 concentration >140 mg/dl were considered to have elevated IgG4\textsuperscript{32,150}.

3.2.3 Histologic evaluation (Papers I, II)
Routine haematoxylin and eosin stained gallbladder sections (n=53) were re-reviewed. Evaluations of the following histological features were noted: active inflammation in the gallbladder epithelium ( 0=absent, 1=mild, 2=moderate , 3=severe) ; inflammatory cell infiltrate [predominantly plasmacytic, lymphocytic or polymorphonuclear (PMN)]; fibrosis ( 0=absent, 1=mild, 2=moderate , 3=severe); site of the inflammation and fibrosis (superficial = confined to the lamina propria, deep = in or beyond muscularis propria); smooth muscle hypertrophy ( 0=absent, 1=mild, 2=moderate , 3=severe); dysplasia (low-grade or high-grade) and carcinoma. Cellular dysplasia was diagnosed when the following were present: loss of polarity, cellular enlargement, nucleus enlarged, stippled and hyperchromatic, varying shape and outline of nuclei, increased nuclear/cytoplasm ratio and mitotic figures and/or nuclear pleomorphism. High-grade dysplasia was considered when the nuclear abnormalities were more pronounced, with more marked nuclear enlargement and more irregular nuclear membranes. The extent of inflammation and fibrosis was divided into two subgroups: absence-to-mild and moderate-to-severe.

3.2.4 Immunohistochemistry (Paper III)
In this study, immunohistochemical staining was performed on all available paraffin blocks of gallbladder (n =19), carcinoma (n=6), dysplasia without presence of gallbladder carcinoma (n=7) and non-cancerous gallbladder epithelium (n=6). The formalin-fixed, paraffin-embedded 4-μm sections from these cases were immunostained with antibodies for cell-cycle-regulating proteins p53, Ki-67, Cyclin D1, epithelial marker Ber-PE4, CK7 and tumour marker CA19-9, polyclonal
carcinoembryonic antigen (pCEA) according to routine practice. The antigens were retrieved with Dako target retrieval solution. The tissue sections were incubated with primary antibodies for 30 minutes at room temperature. The proportion of positive cells was expressed as a percentage of the total number of epithelial cells examined, and divided into three categories: negative 0-5%, slightly positive between >5% and <30% and positive ≥ 30%. For statistical reasons, we collapsed these categories into two main categories: negative <30% and positive ≥30%. All markers were analyzed for expression in 6 cases of carcinoma, including neighbouring areas of high-grade mucosal dysplasia (n=6), 5 cases of low-grade dysplasia, 2 cases of high-grade dysplasia and 6 cases of non-cancerous gallbladder epithelium.

All available paraffin blocks of gallbladder were also evaluated for expression of redox enzymes. For this purpose, 3-μm tissue sections were deparaffinized in xylol and rehydrated in decreasing concentrations of ethanol. The tissue sections were heated in a microwave oven for 10 min in 0.01 M citrate buffer, pH 6.0, for antigen retrieval. The automatic Dako TechMate 500 was used for staining (Dako, Glostrup, Danmark). Tissue slides were stained with primary antibodies against TrxR1 (Upstate, Billerica, MA, USA), 1:1000; Grx1 (IMCO, Stockholm, Sweden), 1:50; the isoforms TrxR1-v,2,3,5 (Agrisera, Sweden) 127, 1:250; and Trx1 (IMCO), 1:100 diluted in ChemMate antibody diluent (Deko), for 25 min at room temperature. The cytoplasmic and nuclear saturation of the hue was analysed and classified into four categories (0=negative, 1=weak positivity, 2=moderate positivity, 3=strong positivity). These categories were collapsed into two main categories, negative (0-1) and positive (2-3) for simplicity. The analyses were performed by two liver pathologists and one hepatologist.

3.2.5 Questionnaire for assessment of abdominal pain (Paper II)

Every subject filled in a questionnaire for the assessment of abdominal pain localized in the right upper quadrant, abdominal discomfort and nausea, before the first MRI, just before and one and three hours after meal ingestion. The questionnaire consisted of visual analogue scales (VAS) on which the patient marked the degree of symptoms, including abdominal pain, nausea and abdominal discomfort.

3.2.6 Gallbladder volume measurements by MRI (Paper II)

3.2.6.1 Procedure

Patients with PSC (n=20) and healthy controls (n=10) underwent, after overnight fasting, an MRI investigation using a 1.5 T magnetic resonance system [Magnetom
Symphony \( (n = 1 \text{ PSC}) \), Vision \( (n = 7 \text{ PSC}) \) or Avanto \( (n = 12 \text{ PSC} \text{ and } 10 \text{ controls}) \), Siemens, Erlangen, Germany. The fasting gallbladder volume was analysed by MRI prior to injection of the contrast agent, time = 0 min. One hour later (time = 1 h) a test meal was ingested consisting of 200 g “Swedish hash” (fried diced meat, onions and potatoes served with beetroot), 250 mL milk (3% fat) and an apple, totalling 2064 kJ including 21 g fat. Postprandial gallbladder volume and ejection fraction were obtained at 2.5 h (time = 2.5 h), that is, an hour and a half after ingestion of the fat-meal, at which point gallbladder contraction is supposed to be maximal.

Gd-BOPTA (MultiHance® 0.5 mmol/mL, Bracco, Milan, Italy) at a dosage of 0.1 mmol/kg was injected. Axial breath-hold 3D-T1-weighted scans (VIBE, slice thickness 1.7-2.5 mm) were performed natively and dynamically in arterial, portal-venous and delayed 5-min phase for clinical diagnosis. Postprandially, in the hepatobiliary phase, the hepatobiliary system was rescanned (VIBE). Each patient was examined using the same unit before and after the meal.

3.2.6.2 Gallbladder volume measurements

The volume of the gallbladder was measured fasting and in the postprandial phase (Figure 6). In the latter hepatobiliary phase, contrast filling of the gallbladder was also noted. The 3D-T1-weighted scans were analysed using a Voxar® 3D workstation (Barco NV, Kortrijk, Belgium) (Figure 7). The analyses were made in consensus by two radiologists. The ejection volume was measured in microliters using the formula:

\[
\text{Ejection volume} = \text{volume (fasting)} - \text{volume (postprandial)}
\]

**Fig. 6.** Gallbladder volumes using magnetic resonance imaging; fasting 1, postprandial 2.
The ejection fraction or gallbladder emptying was measured in per cent using the formula:

\[
Ejection \ fraction = \left( \frac{volume(fasting) - volume(postprandial)}{volume(fasting)} \right) \times 100
\]

Gallbladder fasting volume, ejection fraction and postprandial gallbladder volume of patients with PSC were compared with healthy controls.

Fig. 7. Measurement of gallbladder volume was performed by using the 3D-T1-weighted image at sagittal, coronal, and transverse planes. The images were analysed using a Voxar® 3D workstation.

3.2.7 Gallbladder wall thickness and contrast enhancement (Paper II)

Increased gallbladder wall thickness and contrast enhancement indicate the presence of inflammation of the gallbladder wall. To investigate the presence of inflammatory changes in the gallbladder wall in patients with PSC we measured thickness and contrast enhancement. Thickness was measured on the axial T2 Haste slices at three different areas of the gallbladder (Figure 8).

Fig. 8. Measurement of gallbladder wall thickness (a) and contrast enhancement (b) using MRI at three different areas of the gallbladder.
The mean values of the measurements were calculated for each patient. Contrast enhancement of the gallbladder wall was analyzed in per cent using the formula:

\[
\text{Contrast enhancement} = \frac{\text{SI (portal venous phase) - SI (native)}}{\text{SI (native)}} \times 100.
\]

In each patient, the signal intensity (SI) of the wall was measured in a single voxel in three different areas, trying to avoid vessels and adjacent intestinal loops or the liver parenchyma. The same areas were measured natively and in the portal venous phase and the enhancement was calculated for each part. The mean of the measurements was calculated for each patient.

### 3.2.8 Radiologic assessment of pancreatic abnormalities (Paper IV)

#### 3.2.8.1 MRI and MRCP procedures

All patients underwent MRI of the upper abdomen, including MRCP. The examinations were performed early in the morning after at least 4 hours of fasting, using a 1.5 T magnetic resonance system (Magnetom Vision, Siemens, Erlangen, Germany) and combining the spine and the flexible body array coil. All patients were scanned using the clinical comprehensive protocol, including a dynamic contrast media protocol using 0.1 mmol/kg Gd-BOPTA (MultiHance® 0.5 mmol/ml, Bracco, Milan, Italy). Axial breath-hold 3D-T1-weighted scans (VIBE, TE 1.9 ms, TR 4.5 ms, FOV 40 cm and 120 1.7 mm thick slices) of the liver were performed before any contrast media was administered (before test bolus), during the arterial and the early and late venous phases (48 seconds and 5 minutes after the arterial phase). The MRI examinations were transferred to the clinical picture archiving and communicating system (PACS) and analysed using a workstation (Sectra, Linköping, Sweden). The analyses were made in consensus by two radiologists, who were blinded to the clinical and laboratory results.

#### 3.2.8.2 Signal intensity (SI) of the pancreas

The signal intensity (SI) of the pancreatic head, corpus and tail was measured in the three phases with the placement of a circular region of interest (ROI) of approximately 0.7 cm². The ROIs were placed by two senior radiologists in consensus. The signal intensity (SI) of each pancreatic segment and the spleen was measured on T1-weighted fat-saturated images. The signal intensity ratio (SIR) between each segment of the pancreas and the spleen was determined separately. SIR >1 was defined as normal. The average SIR of the pancreatic head, body, and tail was taken to represent the SIR of the whole pancreas.
3.2.8.3 Enhancement of the pancreatic gland
The enhancement of the pancreatic gland was measured in arterio-portal (A), and late portal venous (PV) phases. The ratio between arterial phase and portal venous phase enhancement (A/PV ratio) was calculated for each segment of the pancreas. The A/PV values were separately scored as normal or abnormal for A/PV >1 or A/PV<1, respectively. The mean A/PV ratio of the three segments was calculated and is referred to in the results section as the A/PV ratio.

3.2.8.4 Measurement of pancreas size
The anteroposterior (AP) diameter of the pancreas was measured according to the method described by Heuck et al. Pancreatic segment size was considered to be decreased if its AP diameter was below the lower limit of its age-related size (mean-2SD). For each segment, a normal pancreatic AP diameter was scored 1 and a decreased pancreatic diameter was scored 0. The mean AP diameter of the head, body, and tail was then calculated. A total score of three was considered to represent normal size.
3.3 STATISTICAL ANALYSIS
Statistical comparisons to test differences between two independent groups were made by use of Student’s t-test for uncorrelated means. In order to evaluate hypotheses of variables in contingency tables, the chi-square test was used or, in the case of small expected frequencies, Fisher’s Exact Test. In addition, descriptive statistics was used to characterize the data. Values were expressed as mean and standard deviation or as median (range). Survival analysis was done with Kaplan–Meier estimates, using the log rank test. All analyses were carried out by using the SAS system for Windows, version 9.1, and Statistica release 7. The probability value (p-value) is given for statistically significant results; a p-value <0.05 was considered significant.

3.4 ETHICAL APPROVALS
The study (Papers I, II, III, IV) was approved by the local Ethics Committee at Karolinska Institutet, Karolinska University Hospital, Huddinge, Stockholm, Sweden.
4 RESULTS

4.1 PAPER I

4.1.1 Clinical data

286 patients were included in this study. Their clinical characteristics are shown in Table 7. Of these patients, 73 (26%) had a biopsy-verified diagnosis of liver cirrhosis. The mean follow-up time was 9 (±7) years. Twenty-six patients had either colon carcinoma (n = 9) or high-grade colonic dysplasia (n = 17). The frequency of hepatobiliary malignancy was 16% (46/286); 33 (11.5%) had cholangiocarcinoma, 10 (3.5%) had gallbladder carcinoma and 3 (1%) had hepatocellular carcinoma.

Table 7. The clinical characteristics of 286 patients with PSC

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender:</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>66% (189/286)</td>
</tr>
<tr>
<td>Female</td>
<td>34% (97/286)</td>
</tr>
<tr>
<td>Mean age at diagnosis in years (±SD)</td>
<td>36 (±14)</td>
</tr>
<tr>
<td>Liver transplantation</td>
<td>30% (87/286)</td>
</tr>
<tr>
<td>Associated IBD:</td>
<td></td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>68% (196/286)</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>10% (28/286)</td>
</tr>
<tr>
<td>Indeterminate colitis</td>
<td>1% (3/286)</td>
</tr>
<tr>
<td>Colectomy</td>
<td>20% (56/286)</td>
</tr>
<tr>
<td>Treatment with UDCA</td>
<td>61% (161/263)</td>
</tr>
<tr>
<td>Mean follow up time (years to end point) (±SD)</td>
<td>9 (±7)</td>
</tr>
<tr>
<td>Cholangiographic distribution of PSC:</td>
<td></td>
</tr>
<tr>
<td>Extra- and intra-hepatic involvement</td>
<td>74% (213/286)</td>
</tr>
<tr>
<td>Extrahepatic changes</td>
<td>1% (3/286)</td>
</tr>
<tr>
<td>Intrahepatic changes</td>
<td>19% (54/286)</td>
</tr>
<tr>
<td>Small bile duct PSC</td>
<td>6% (16/286)</td>
</tr>
<tr>
<td>Alive at the end of follow-up</td>
<td>73% (210/286)</td>
</tr>
<tr>
<td>Any symptom</td>
<td>61% (175/286)</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>39% (110/286)</td>
</tr>
<tr>
<td>Jaundice</td>
<td>26% (76/286)</td>
</tr>
<tr>
<td>Pruritus</td>
<td>25% (72/286)</td>
</tr>
</tbody>
</table>

4.1.2 Gallbladder abnormalities

Gallbladder abnormalities, including cholecystitis, gallstones, gallbladder mass and gallbladder malignancy, are presented in Table 8. The prevalence of gallstones among
patients with extrahepatic PSC (28% (60/213)) tended to be higher than among those with intrahepatic PSC only (17% (12/70)) \((P = 0.058)\). There was concomitant

Table 8. Gallbladder (GB) abnormalities in 286 patients with PSC

<table>
<thead>
<tr>
<th>GB abnormality</th>
<th>Frequency in all patients</th>
<th>Frequency in males/females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallstone</td>
<td>25% (72/283)</td>
<td>22% (41/187)/32% (31/96)</td>
</tr>
<tr>
<td>Cholecystitis</td>
<td>25% (71/284)</td>
<td>28% (53/188)/9% (18/96)</td>
</tr>
<tr>
<td>GB mass lesion</td>
<td>6% (18/286)</td>
<td>7% (13/189)/5% (5/97)</td>
</tr>
<tr>
<td>GB malignancy</td>
<td>3.5% (10/286)</td>
<td>3% (6/189)/4% (4/97)</td>
</tr>
<tr>
<td>One or more abnormalities</td>
<td>41% (116/286)</td>
<td>40% (75/189)/42% (41/97)</td>
</tr>
</tbody>
</table>

cholecystitis in 31% (22/72) of the patients with gallstones but no significant association between the presence of cholecystitis and gallstones. The frequency of gallstones did not differ significantly either between UDCA treated and untreated patients or between patients with or without IBD. Previous studies on gallstones showed a significant association between occurrence of cholesterol gallstone and presence of Crohn’s disease; however, no such association was found in this study. The frequency of cholecystitis in patients with extrahepatic involvement of PSC (30% (65/214)) was significantly higher than in those with intrahepatic involvement only (9% (6/70)) \((P < 0.0001)\). Adenocarcinoma was found in 56% (10/18) of patients with gallbladder mass lesion. The presence of a gallbladder mass lesion was not correlated to the presence of gallstones, gender or liver cirrhosis.

The reassessment of all available gallbladder specimens \((n = 53)\) showed presence of high-grade dysplasia in two cases and low-grade dysplasia in seven cases. All these cases of dysplasia were previously unknown and no gallbladder mass lesions were detected on preoperative imaging. Moreover, the histological review showed a significant association between the severity of inflammation in the gallbladder wall and extrahepatic involvement of PSC; of the patients with an extrahepatic spread of PSC, 51% (25/49) had moderate or severe inflammation as against none of those with an intrahepatic distribution, a difference that is statistically significant \((P < 0.05)\). The mean size of the gallbladder mass lesions was 21 mm \(\pm 9\).

4.1.3 Prognosis

Gallbladder stones and cholecystitis had no impact on the prognosis. Seven out of eight
patients with benign gallbladder masses were still alive at follow up as against only one patient with gallbladder cancer.

4.2 PAPER II

4.2.1 Gallbladder volumes

The mean age of patients with PSC (n=20) and healthy subjects (n=10) was 39 ± 10 yrs and 47 ± 13 yrs, respectively (NS). There was no significant difference between the two groups regarding age and body mass index (BMI). PSC patients had significantly higher serum alkaline phosphatase and alanine aminotransferase than the control group. The median fasting gallbladder volume and postprandial gallbladder volume were significantly higher in patients with PSC compared with the healthy controls, while there was no significant difference between the two groups in the ejection fraction and ejection volume (Figure 9).

Fig. 9. Fasting gallbladder volume (A), postprandial gallbladder volume (B), ejection volume (C), ejection volume as percentage of fasting gallbladder volume and ejection fraction (D) measured with magnetic resonance imaging (MRI) in patients with primary sclerosing cholangitis (PSC) (n = 20) and in healthy controls (n = 10).
Abnormalities of cystic ducts were visualised in 13 (60%) of the PSC patients but in none in the control group. No significant correlation was found between increased gallbladder fasting volume, decreased ejection fraction or lack of postprandial gallbladder refilling and presence of abnormalities of cystic ducts.

4.2.2 Gallbladder wall enhancement
There was a significantly increased contrast enhancement of the gallbladder wall (mean ± SD) in PSC patients compared to controls, 69% (± 32%) and 43% (± 21%) respectively (p<0.05). In all subjects there was a significant correlation between high contrast enhancement of the gallbladder wall and large gallbladder volume at fasting (p<0.05) (r = 0.39). Postprandial gallbladder volume and ejection fraction were not significantly correlated to contrast enhancement of the gallbladder wall.

4.2.3 Abdominal pain
No significant association was found between gallbladder volumes or contrast enhancement and occurrence of abdominal pain, abdominal discomfort and nausea in either PSC patients or controls. Before the fatty meal, altogether 25% of PSC patients experienced abdominal pain, the VAS ranging from 1-4; 25% of PSC patients experienced nausea, the VAS ranging from 1-2; and 20% of PSC patients experienced abdominal discomfort, the VAS ranging from 1-4. There was no significant increase in symptoms in the PSC group at one or three hours after meal ingestion. None of the healthy controls experienced symptoms pre- or post-prandially.

4.3 PAPER III

4.3.1 Histology
30% (16/53) of cholecystectomized and end-stage liver disease patients with PSC had gallbladder dysplasia and adenocarcinoma. 17% (9/53) of the patients had gallbladder dysplasia without carcinoma and 13% (7/53) had gallbladder carcinoma. Gallbladders with high-grade dysplasia harbored separate areas of low-grade dysplasia and had severe diffuse lymphoplasmacytic chronic inflammation of the gallbladder wall. All gallbladders with adenocarcinoma (one adenocarcinoma in situ, one well and five poorly differentiated adenocarcinoma) had separate areas of low-grade and high-grade mucosal dysplasia (Figure 10) and all had moderate to severe diffuse
lymphoplasmacytic chronic inflammation of the gallbladder wall. All patients with dysplasia and adenocarcinoma of the gallbladder had extrahepatic involvement of PSC.

Gallbladder dysplasia and adenocarcinoma were more common in gallbladder tissue with moderate-severe diffuse lymphoplasmacytic chronic inflammation [48% (12/25)] than in gallbladder specimen with absent-mild inflammation [12% (4/28)] (p<0.01). Moderate to severe fibrosis was significantly more frequent among patients with moderate to severe inflammation in the gallbladder epithelium, 64% (16/25), than among patients with mild inflammation 18% (5/28) (p<0.001).

Fig.10. Hematoxylin and eosin staining of gallbladder in primary sclerosing cholangitis showing adenocarcinoma and had high-grade dysplasia in the surrounding gallbladder epithelium.

There was no statistical association between the occurrence of gallstones and presence of moderate to severe lymphoplasmacytic chronic inflammation, dysplasia or carcinoma of the gallbladder. However, gallbladder dysplasia/adenocarcinoma was more frequent in gallbladders with moderate to severe fibrosis than in those with absent to mild fibrosis, 62% (13/21) and 9% (3/32) respectively (p<0.0001).

4.3.2 Immunohistochemistry
Immunoreactivity for p53, Ki67, Cyclin D1 and pCEA was detected in significantly more cases of dysplasia and carcinoma of the gallbladder compared to non-cancerous epithelium (Table 9). A similar expression pattern was noted when we analyzed the immunoreactivity for these markers in non-neoplastic epithelium compared to dysplasia alone. Carcinoma cases separately were not compared statistically with non-neoplastic epithelium due to the limited number of cases.
Table 9. Comparison of frequencies of positive (≥ 30% positive cells) samples in normal gallbladder epithelium and in gallbladder dysplasia/carcinoma with immunohistochemistry.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Non-neoplastic epithelium (n=19)</th>
<th>Dysplasia (n=13)</th>
<th>Carcinoma (n=6)</th>
<th>Dysplasia and carcinoma (n=19)</th>
<th>P-value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>0(0%)</td>
<td>5(39%)</td>
<td>2(33%)</td>
<td>7(37%)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Ki-67</td>
<td>1(5%)</td>
<td>4(31%)</td>
<td>3(50%)</td>
<td>7(37%)</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>0(0%)</td>
<td>6(46%)</td>
<td>3(50%)</td>
<td>9(47%)</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>pCEA</td>
<td>1(5%)</td>
<td>9(69%)</td>
<td>4(67%)</td>
<td>13(68%)</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>CA 19-9</td>
<td>14(74%)</td>
<td>12(92%)</td>
<td>6(100%)</td>
<td>18(95%)</td>
<td>NS</td>
</tr>
<tr>
<td>Ber-EP4</td>
<td>13(68%)</td>
<td>8(67%)</td>
<td>4(67%)</td>
<td>12(63%)</td>
<td>NS</td>
</tr>
<tr>
<td>CK7</td>
<td>19(100%)</td>
<td>13(100%)</td>
<td>6(100%)</td>
<td>19(100%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Non-neoplastic vs dysplasia and carcinoma

The expression pattern of members of the thioredoxin superfamily (Trx1, TrxR1, TrxR1-v2,3,5 and Grx1) are shown in table 10. Areas of low grade dysplasia were not identified in the new sections in two cases of low grade dysplasia and they were therefore excluded. TrxR1-v,2,3,5 was significantly overexpressed in the dysplastic and tumor tissue, whereas Grx1 was downregulated (Table 10). Hematoxylin and eosin, and immunohistochemistry staining are shown in figure 11.

Table 10. Comparison of the positive cytoplasmatic and nuclear expression frequencies of redox proteins in normal gallbladder epithelium and in gallbladder dysplasia/carcinoma with immunohistochemistry.

<table>
<thead>
<tr>
<th>Redox proteins</th>
<th>Non-neoplastic epithelium (n=19)</th>
<th>Dysplasia (n=11)</th>
<th>Carcinoma (n=6)</th>
<th>Dysplasia and carcinoma (n=17)</th>
<th>P-value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trx1/n</td>
<td>10(53%)</td>
<td>8(73%)</td>
<td>3(50%)</td>
<td>11(65%)</td>
<td>ns</td>
</tr>
<tr>
<td>Trx1/c</td>
<td>13(68%)</td>
<td>9(82%)</td>
<td>4(67%)</td>
<td>13(76%)</td>
<td>ns</td>
</tr>
<tr>
<td>Grx1/n</td>
<td>9(47%)</td>
<td>3(27%)</td>
<td>2(33%)</td>
<td>5(29%)</td>
<td>ns</td>
</tr>
<tr>
<td>Grx1/c</td>
<td>15(79%)</td>
<td>6(55%)</td>
<td>1(17%)</td>
<td>7(41%)</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>TrxR1/n</td>
<td>6(32%)</td>
<td>5(46%)</td>
<td>3(50%)</td>
<td>8(47%)</td>
<td>ns</td>
</tr>
<tr>
<td>TrxR1/c</td>
<td>15(79%)</td>
<td>10(91%)</td>
<td>3(50%)</td>
<td>13(76%)</td>
<td>ns</td>
</tr>
<tr>
<td>TrxR1-v2,3,5/n</td>
<td>0(0%)</td>
<td>1(9%)</td>
<td>0(0%)</td>
<td>1(6%)</td>
<td>ns</td>
</tr>
<tr>
<td>TrxR1-v2,3,5/c</td>
<td>2(11%)</td>
<td>5(46%)</td>
<td>2(33%)</td>
<td>7(41%)</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>

* Non-neoplastic vs dysplasia and carcinoma
Fig. 11. Hematoxylin and eosin (H&E) and immunohistochemistry staining of gallbladder in primary sclerosing cholangitis showing: a; H&E-normal epithelium, b; H&E-dysplasia, c; Htx-carcinoma, d; Ki67-normal epithelium (negative expression), e; Ki67-dysplasia (expression ≥ 30%), f; Ki67-carcinoma (expression ≥ 30%), g; TrxR1v2,3,5-normal epithelium (negative cytoplasmic saturation), h; TrxR1v2,3,5-dysplasia (positive cytoplasmic saturation), i; TrxR1v2,3,5-carcinoma (positive cytoplasmic saturation), j; Grx1-normal epithelium (strong positive cytoplasmic saturation), k; Grx1-dysplasia (weak positive cytoplasmic saturation), l; Grx1-carcinoma (negative cytoplasmic saturation).

4.4 PAPER IV

4.4.1 General features
The clinical characteristics of the 103 patients included in this study are similar to those of the baseline cohort. Only one patient with PSC had typical clinical and laboratory features of CP and was treated with enzyme supplementation therapy. No PSC patient showed classical radiological signs of autoimmune pancreatitis.
4.4.2 MRI and MRCP findings of pancreas

A summary of pancreatic changes in patients with PSC is presented in Table 11.

Table 11. MRI/MRCP findings in 103 patients with PSC

<table>
<thead>
<tr>
<th>Radiological features</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic ductal changes</td>
<td></td>
</tr>
<tr>
<td>Grade 0 (normal)</td>
<td>76% (78/103)</td>
</tr>
<tr>
<td>Grade 1</td>
<td>11% (11/103)</td>
</tr>
<tr>
<td>Grade 2</td>
<td>14% (14/103)</td>
</tr>
<tr>
<td>Decreased pancreas size</td>
<td>23% (24/103)</td>
</tr>
<tr>
<td>Abnormal contrast enhancement of pancreas (A/PV&lt;1)</td>
<td>26% (27/103)</td>
</tr>
<tr>
<td>Abnormal pancreas-spleen SIR (SIR&lt;1)</td>
<td>7% (7/103)</td>
</tr>
</tbody>
</table>

Pancreatic duct changes (grade 1-2 or only grade 2) were not associated with small pancreas size, abnormal pancreas/spleen SIR or delayed parenchymal enhancement of the pancreatic gland (A/PV <1) (Figure 12).

![Fig.12.Dynamic T1 weighted images of the pancreatic head (arrow) in a 51-year-old man with primary sclerosing cholangitis and ulcerative colitis before intravenous contrast (a), arterial phase (b), and venous phase (c). Dilated side branches (grade 1 changes) are indicated with multiple arrows on the T2 weighted image (d). The window levels are the same in a-c to show that the enhancement of the pancreas is normal, with higher signal intensity during the arterial phase (b) compared to during the venous phase (c). L, liver; K, left kidney.](image)

We found that the frequency of pancreatic duct changes among PSC patients with extrahepatic involvement of PSC was significantly higher than among those with only
intrahepatic PSC and the mean duration of PSC was longer in patients with pancreatic duct changes than those without. No association was found between previous acute or ERCP-related pancreatitis and pancreatic duct changes. The frequency of patients with A/PV enhancement ratio <1 was higher among patients with severe pancreatic duct changes 43% (6/14) than among those with ductal change grade 1 [9% (1/11)] and those with normal pancreatic duct (grade 0) [26% (20/78)]; however, this difference did not reach statistical significance.

4.4.3 Immunoglobulin G4

Clinical features and pancreatic duct changes with regard to IgG4 levels are shown in Table 12. Thirteen PSC patients (20%) had IgG4 levels above 140 mg/dL. Only 3% (2/66) of the patients had an IgG4 level above 280 mg/dL; these two patients had IBD, only intrahepatic distribution of PSC and no history of jaundice. There was no association between pancreatic duct changes and IgG4 levels.

Table 12. Clinical features and pancreatic duct changes observed using MRCP with regard to IgG4 levels in 66 patients with PSC.

<table>
<thead>
<tr>
<th></th>
<th>IgG4 &lt;140 mg/dl (n=53)</th>
<th>IgG4&gt;140 mg/dl (n=13)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (year) (±SD)</td>
<td>42(±15)</td>
<td>36(±12)</td>
<td>NS</td>
</tr>
<tr>
<td>PSC distribution:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intrahepatic only</td>
<td>19%(10/53)</td>
<td>54%(7/13)</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Intra- and extra-hepatic</td>
<td>81%(43/53)</td>
<td>46%(6/13)</td>
<td></td>
</tr>
<tr>
<td>IBD</td>
<td>76%(40/53)</td>
<td>69%(9/13)</td>
<td>NS</td>
</tr>
<tr>
<td>pancreatic duct changes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>grade 1-2</td>
<td>19%(10/53)</td>
<td>8%(1/13)</td>
<td>NS</td>
</tr>
<tr>
<td>Jaundice at PSC diagnosis</td>
<td>13%(7/53)</td>
<td>0%(0/13)</td>
<td>NS</td>
</tr>
</tbody>
</table>
5 GENERAL DISCUSSION

Our studies of gallbladder disease in patients with PSC showed gallbladder abnormalities in 41% and gallstones in 25%. PSC patients were also shown to have increased fasting gallbladder volumes and a high frequency of chronic cholecystitis. The 25% frequency of gallstones in PSC is high but similar to the figure of 26% previously reported by Brandt et al, who studied 121 PSC patients. In the general Swedish population the prevalence of gallstones varies with gender and increases with age. The mean age of our patients at PSC diagnosis was 36 years and the frequency of gallstones was 22% in men and 31% in women. These figures are higher than the reported frequencies of gallstones in 40-year-old men and women in Sweden: 4% and 11%, respectively.

The pathophysiology behind the increased prevalence of gallstones in patients with PSC remains unclear. Several mechanisms need to be considered. A high frequency of pigment gallstones in PSC has previously been reported. The bile duct changes seen in patients with PSC heighten the risk of bacterial colonisation and bacterial cholangitis is common, which adds to the risk of gallstone formation. Inflammatory changes in gallbladder epithelium may affect its capacity to secrete and absorb, thereby inducing changes in bile composition. This could, in turn, lead to an imbalance between stimulatory and inhibitory factors for the development of gallstones. The fact that patients with PSC have an increased postprandial volume – three times the values seen in healthy controls – may in itself raise the risk of developing gallstones. Thus, there are multiple factors that together may explain the increased incidence of gallstones in patients with PSC.

We have also shown that 6% of patients with PSC develop polypoid lesions of the gallbladder and 3.5% develop gallbladder carcinoma. Of the polypoid lesions, 56% were malignant. This figure is similar to that previously reported by Buckles et al, who found gallbladder mass lesions in 14% of 102 patients who underwent cholecystectomy; 57% of the gallbladder mass lesions represented adenocarcinoma. Malignant gallbladder polyps are found in 0.2% to 20% in a non-PSC population. Gallbladder polyps not more than 1.0 cm in diameter are commonly malignant in patients with PSC, whereas those of this size in the non-PSC population are
seldom malignant 107, 108. Our findings have contributed to recently published guidelines for the management of gallbladder polyps in patients with PSC from the European Association for the Study of the Liver (ESAL) and the American Association for the Study of Liver Diseases (AASLD) 27, 206. These guidelines recommend annual surveillance ultrasound to detect gallbladder mass lesions and cholecystectomy when such a lesion is identified, regardless of its size.

The reason for the higher incidence of malignant polyps in PSC compared with the general population is unknown. Gallstones are known to increase the risk of gallbladder cancer in patients without PSC. We did not, however, find any significant association between the presence of gallstones and either polypoid lesions or cancer of the gallbladder, which is in line with the study by Buckles et al 104. We have shown that half of the patients with PSC had moderate to severe diffuse chronic lymphoplasmocytic cholecystitis, and that these changes were significantly associated with the extrahepatic distribution of PSC and the presence of dysplasia and malignancy in the gallbladder mucosa, but not with gallstones. Dysplastic epithelial changes without visible polypoid lesions on preoperative radiological images were found in nine patients. This shows that dysplasia and malignancy may arise in a flat mucosa and that carcinogenesis may differ from that in the non-PSC setting. There is an obvious parallel with ulcerative colitis, where colorectal dysplasia in patients with IBD is known to develop in a flat mucosa and not necessarily from adenomas, which is the case in the non-ulcerative colitis setting 207. We have shown a high frequency of dysplasia and adenocarcinoma (30%) of gallbladder among PSC patients. This is in line with the data previously published by Lewis et al where dysplasia and carcinoma was found in 37% and 14% respectively in 72 gallbladder specimens from patients with end-stage liver disease secondary to PSC42. Moreover, gallbladders with high-grade dysplasia harboured separate areas of low-grade dysplasia and all gallbladders with adenocarcinoma (n=7) also had separate areas of low- grade to high-grade epithelial dysplasia and these changes were associated with chronic moderate to severe inflammation and fibrosis of the gallbladder wall. The role of fibrosis for development of gallbladder cancer is unclear. In breast, pancreatic and liver cancer, a strong correlation has been shown between cancer development and presence of fibrosis 208-210. It has been hypothesized that the increased cancer risk seen in fibrosis is caused by a stimulated angiogenesis driven by inflammation 211. All these observations support the theory that the gallbladder epithelium is involved in the chronic inflammatory
process affecting the biliary tree in PSC and support an inflammation-fibrosis-dysplasia-carcinoma sequence in PSC.

It is known that patients with PSC are at increased risk of developing cholangiocarcinoma. The mechanisms responsible for this increased risk are not clear, but the combined influence of chronic inflammation and exposure to hydrophobic bile acids seen in prolonged cholestasis is most likely important. When the accumulation of hydrophobic bile acids occurs in chronic or subchronic cholestasis, this triggers cholangiocyte proliferation. This mechanism may also play an important role in the development of gallbladder dysplasia and carcinoma. The gallbladder epithelium is a part of the extrahepatic bile duct epithelium and seems to be involved in the chronic inflammatory process affecting the bile ducts in PSC. The long-standing inflammation may result in increased oxidative stress and production of harmful reactive oxygen species which induce DNA damage and stimulation of biliary cell proliferation.

Ki-67, p53, and Cyclin D1 were found in our study to be overexpressed in dysplastic tissue, and a previous study of gallbladder tumours in patients without PSC found that overexpression of Cyclin D1 was present in 67% of precancerous adenomas. This demonstrates that dysregulation of these key proteins may be important already in early carcinogenesis (dysplasia) and that cyclin D1 can serve as a marker of both malignancy and premalignancy in gallbladders from patients with PSC. The expression pattern of the thioredoxin protein family show a clear overexpression of the TrxR1-v,2,3,5 isoform in dysplastic tissue in particular (46%) but also in tumour tissue (33%) versus non-tumorous tissue (11%). This pattern is similar to previously published data on preneoplastic and neoplastic cholangiocarcinoma lesions in a hamster model where the strongest expression was seen in the preneoplastic lesions and the TrxR1 seemed to be downregulated when cancer had developed. Expression of TrxR1 may therefore be an important event, especially in early stages of cholangiocarcinogenesis as well as in gallbladder cancer development in PSC. Grx1 expression in the present study was decreased in dysplastic tissue and even more decreased in the tumours as compared to non-neoplastic tissue. The downregulation of Grx1 is previously described in lung cancer tissue where an inverse correlation with the proliferation of the tumours was found. TrxR1 may be an additional immunohistologic marker for dysplasia in PSC. Such a marker is needed, given the high prevalence of hepatobiliary malignancy in PSC and the difficulties in differentiating benign inflammatory bile ducts, especially from
low-grade dysplastic lesions. Of particular interest is the different expression patterns of the less abundant isoforms TrxR1v2,3,5 compared to total TrxR1. This difference indicates a potential to use isoforms and alternative transcripts as tools in cancer diagnostics.

A large gallbladder volume in PSC has previously been published by Van de Meeberg et al 67, who performed ultrasound investigation of the gallbladder in patients with PSC. Our MRI study of gallbladder size and emptying confirm that patients with PSC have significantly larger fasting and postprandial gallbladder volumes and also shows a normal ejection fraction and ejection volume. The reason for the increased fasting volume is not known but several mechanisms can be considered. The large volume may be caused by a significant stricture in the cystic duct or in common bile duct distal to the cystic duct, reduced gallbladder motility or gallbladder mucosal dysfunction. Our findings weigh against cystic strictures and gallbladder motility dysfunction since we excluded patients with significant extrahepatic strictures, seventy-five percent of the PSC patients had no radiological signs of dominant stricture in the cystic duct and the patients had normal ejection fraction and volume. Presence of gallbladder mucosal dysfunction is a more likely explanation. Our histological findings show chronic inflammation of the gallbladder mucosa and a significantly increased contrast enhancement in the gallbladder wall, which is an indicator of inflammatory activity. In experimental cholecystitis, the process of fluid absorption in the gallbladder epithelium changes to fluid secretion215, 216. Furthermore, the secretory dysfunction of the gallbladder in PSC has been described previously in a case report of one PSC patient with concomitant cholecystitis who showed a high secretory capacity217. Inflammation in the gallbladder wall may, therefore, result in a dysfunctional gallbladder mucosa, impairing the ability of the gallbladder to absorb fluid and to concentrate bile. It could also lead to increased secretory activity, which would result in a markedly increased fasting volume.

UDCA has been widely used for treatment of PSC despite insufficient evidence of its efficacy 35, 36. UDCA treatment may affect gallbladder motility and several studies have shown that it results in increased fasting and postprandial gallbladder volumes, whereas gallbladder emptying has not been shown to be reduced or modified 218, 219. The PSC patients in the study by Van de Meeberg et al 67, which obtained similar results to ours, discontinued their UDCA medication for four weeks before commencement of the
study. In order to study the patients’ symptoms in a true clinical setting, we decided not to discontinue therapy with UDCA. However, our results showed no significant difference in fasting gallbladder volume and gallbladder emptying between UDCA treated and untreated patients.

One third of patients with PSC suffer from recurrent abdominal pain, although the pathogenesis of this is not fully understood. It has been suggested that the pain is partly related to gallbladder size or to impaired gallbladder motility. We did not find any correlation between gallbladder volume or ejection fraction and the patient’s reported symptoms of abdominal pain, abdominal discomfort or nausea. On the other hand, we demonstrated that pronounced pancreatic duct changes were associated with abdominal pain, which indicates that these changes may contribute to the abdominal pain in PSC. The underlying mechanisms by which severe changes in the pancreatic ducts may contribute to abdominal pain in PSC patients are unclear but several can be hypothesized. Pancreatic duct changes may cause increased intra-pancreatic pressure, either in the pancreatic ducts or in the pancreatic parenchyma, leading to pain.

Pancreatic changes have previously been reported in 0-77% of patients with PSC. Our study is the largest evaluation of pancreatic duct changes in PSC and we found such changes in 24% on MRCP. Dynamic MRI provides the opportunity to evaluate parenchymal contrast enhancement and several studies (in a non-PSC setting) have shown that decreased parenchymal enhancement and a reduced blood flow to the pancreatic parenchyma reflect CP. A delayed pancreatic enhancement and abnormal pancreas/spleen SIR on MRI may be sensitive parameters for early diagnosis of CP.

In our study, the occurrence of pancreatic duct changes was not associated with decreased enhancement of the pancreatic parenchyma, reduced pancreas size, a pathological pancreas-spleen SIR or increased IgG4 levels. Pancreatic duct changes were significantly less common in PSC with only an intrahepatic distribution, and the PSC patients with changes in the pancreatic ducts were shown to have a longer duration of PSC. This indicates that the changes in the pancreatic ducts are a primary event and appear to develop prior to CP in PSC. Pancreatic duct changes therefore seem to be part of the spectrum of PSC and the formation of changes in the bile and pancreatic ducts may have a shared pathogenesis.
The pathogenesis of pancreatic fibrosis, a characteristic feature of chronic pancreatitis, has received increasing attention in recent years, largely due to the identification and characterization of pancreatic stellate cells. Pancreatic ductal cells share features in common with cholangiocytes, in terms of embryologic origin, morphology, functional activity, and response to injury. Furthermore, the underlying pathophysiology of pancreatic fibrosis has many properties in common with the liver fibrosis that develops following hepatocyte or cholangiocyte damage. The chronic inflammatory process in the pancreatic ducts resembles that seen in the bile ducts in PSC, and damaged pancreatic duct cells are probably responsible for the release of mediators that initiate the development of pancreatic fibrosis in a process similar to that seen in PSC-associated liver fibrosis. This fibrotic process develops long after the primary insult to the pancreatic duct epithelium. We have shown that patients with A/PV < 1 tend to have a longer duration of PSC and to have severe changes in the pancreatic ducts. These findings suggest that this subgroup of PSC patients will later develop pancreatic fibrosis in a process similar to the inflammatory process seen in the bile ducts. Pancreatic duct changes in PSC do therefore not represent CP but may rather represent primary sclerosing pancreatitis. Whether the immunological attack on the pancreatic ducts shares pathogenetic mechanisms with the attack on the bile ducts, remains to be studied.
6 CONCLUSIONS

- Gallbladder disease is common in patients with PSC, with a frequency of 41%. The high frequency of carcinoma in gallbladder mass lesions regardless of their size suggests that regular examination of the gallbladder in PSC patients could be of value for early detection of a gallbladder malignancy. Cholecystectomy is recommended when such a lesion is detected, regardless of its size.

- The association between cholecystitis and extrahepatic distribution of PSC supports the theory that the gallbladder epithelium is involved in the chronic inflammatory process affecting the bile ducts in PSC.

- PSC has high frequencies of moderate to severe superficial and lymphoplasmacytic inflammation, fibrosis, dysplasia and carcinoma of the gallbladder. Our data support an inflammation-fibrosis-dysplasia-carcinoma sequence in PSC.

- The significant overexpression of TrxR1-v2.3.5 and downregulation of Grx1 in dysplastic gallbladder epithelium in patients with PSC suggest that these proteins may be future immunohistological markers in the early diagnosis of gallbladder malignancy in PSC but this needs to be further evaluated.

- Patients with PSC have increased fasting and residual gallbladder volumes, whereas gallbladder emptying is normal. Gallbladder mucosal dysfunction secondary to chronic inflammation of the gallbladder is a possible mechanism for the increased gallbladder fasting volume.

- Gallbladder size and emptying do not seem to be involved in the development of abdominal pain in patients with PSC.

- Patients with PSC have a 24% prevalence of pancreatic duct changes at MRCP which is associated with extrahepatic bile duct involvement but not with increased IgG4 levels or early MRI signs of chronic pancreatitis.

- Pancreatic duct changes are part of the spectrum in PSC and should not be referred to as chronic pancreatitis. Severe pancreatic duct changes may contribute to the abdominal pain in PSC.
7 POPULÄRVETENSKAPLIG SAMMANFATTNING


I första arbetet har vi evaluerat förekomsten av gallblåseförändringar, såsom gallblåseinflammation, gallsten samt polypösa förändringar hos en stor grupp av PSC patienter (n=286). Vi har visat att 41 % hade någon typ av gallblåseförändring, 25 % hade gallsten och 6% hade makroskopisk gallblåseförändring som hos 56% representerade gallblåsecancer (10/18). Arbete III är en uppföljning av första arbetet och syftade till att studera den carcinogenetiska processen morologiskt genom att evaluerar histologiskt inflammation och celltillväxt och genom studier av immunhistokemiska markörer av potentiell betydelse för celltillväxt, och cancerutveckling i gallblåseepitelet hos patienter med PSC. Ett annat syfte var att undersöka om det finns klinisk användbara diagnostiska markörer för tidig cancerutveckling, sk dysplasi vid PSC. Vi har hittat ett signifikant överuttryck av en av thioredoxinsystemets selenberöende redox proteiner, nämligen TrxR1-v2,3,5, i
gallblåseeptelet med cellförändringar och cancer. Cellförändringar och cancer återfinns i gallblåseeptelet hos ungefär 30% patienter med PSC och talar för att regelbundet undersöka gallblåsan hos dessa patienter för att kunna erbjuda gallblåseoperation redan vid förekomst av små polypösa förändringar.

I arbete II studerade vi gallbläsestorlek vid fasta och gallblåsetömning efter provmåltid med användning av magnetkamera hos 20 patienter med PSC och 10 kontroller för att studera relationen mellan faste gallbläsestorlek, gallblåsetömning och symptom i form av buksmärta och illamående. Gallblåsens median faste- och postprandiellvolym var större hos PSC patienterna jämfört med kontrollerna. Vi spekulerar i att detta kan orsakas av en oförmåga i gallblåseeptelet att resorbera vatten och/eller ökad vätskebildning från gallblåseeptelet till följd av en kronisk inflammation orsakad av PSC sjukdomen. Gallblåsetömningen var intakt och ingen association mellan buksmärta och gallblåsevolym kunde påvisas.

I arbete IV beskrev vi pankreasförändringar hos 103 patienter med PSC som har genomgått magnetkameraundersökning. Förändringar i pankreasgångar hittades hos 24% av patienterna. Dessa förändringar var associerade med extrahepatitisk distribution och duration av PSC men inte associerade med andra röntgenologiska förändringar i pankreas parenkymet som talade för kronisk pankreatit. Pankreasgångsförändringar hos patienter med PSC verkar vara en del av PSC spektrum och uttalade förändringar i pankreasgångarna var signifikant associerade med buksmärta, något som delvis kan förklara varför en tredjedel av patienter med PSC klagar över buksmärta.
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