Thesis for doctoral degree (PhD)

# Development, immunohistochemical analysis and diagnostic criteria of fibrosis in the normal pancreas, alcoholic chronic pancreatitis and autoimmune pancreatitis

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# List of papers

This PhD thesis is based on the four papers listed below, which are referred to in the text by Roman numerals. The studies were carried out in the period from 2004 - 2008 at the Department of Pathology, University Hospital Schleswig-Holstein, Campus Kiel, Germany, and at the Department of Gastroenterology, Mech-Sense, Aalborg Hospital, Denmark.

**I:** Sönke Detlefsen, Bence Sipos, Bernd Feyerabend and Günter Klöppel. **Pancreatic fibrosis associated with age and ductal papillary hyperplasia.** *Virchows Archiv* 2005;447:800-805.

**II:** Sönke Detlefsen, Bence Sipos, Bernd Feyerabend and Günter Klöppel. **Fibrogenesis in alcoholic chronic pancreatitis: the role of tissue necrosis, macrophages, myofibroblasts and cytokines.** *Modern Pathology* 2006;19(8):1019-1026.

**III:** Sönke Detlefsen, Bence Sipos, Jingbo Zhao, Asbjørn Mohr Drewes and Günter Klöppel. **Autoimmune pancreatitis: expression and cellular source of profibrotic cytokines and their receptors.** *The American Journal of Surgical Pathology* 2008;32(7):986-995.

**IV:** Sönke Detlefsen, Asbjørn Mohr Drewes, Mogens Vyberg and Günter Klöppel. **Diagnosis of autoimmune pancreatitis by core needle biopsy: application of six microscopic criteria.** *[Submitted]*

# Acknowledgments

This PhD thesis is based on investigations carried out from 2004 to 2008 at the Department of Pathology, University Hospital Schleswig-Holstein, Campus Kiel, Germany, and at the Department of Gastroenterology, Mech-Sense, Aalborg Hospital, Denmark, in collaboration with the Center for Sensory-Motor-Interaction (SMI), Department of Health Science and Technology, Aalborg University, Denmark.

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Aalborg, November 2008
Sönke Detlefsen

## Abbreviations

ABC method: avidin-biotin-complex method
ACP: alcoholic chronic pancreatitis
AIP: autoimmune pancreatitis
b-FGF: basic fibroblast growth factor
CD: cluster of differentiation
CP: chronic pancreatitis
CTGF: connective tissue growth factor
DAB: diaminobenzidine-tetrahydrochloride
DPH: ductal papillary hyperplasia
ECM: extracellular matrix
EGF: epidermal growth factor
EUS: endoscopic ultrasound
FNA: fine needle aspiration
GEL: granulocytic epithelial lesion
GFAP: glial fibrillary acidic protein
H&E: hematoxylin & eosin
HPF: high power field
IgG4: immunoglobulin G4
IL: interleukin
IPMN: intraductal papillary mucinous neoplasm
LAP: latency associated peptide
LTBP-1: latent TGF-β-binding protein 1
MCP-1: monocyte chemoattractant protein 1
MMP: matrix metalloproteinase
OCP: obstructive chronic pancreatitis
PanIN: pancreatic intraepithelial neoplasia
PDGF-B: platelet-derived growth factor B
PDGF-Rα and PDGF-Rβ: α and β isoforms of the PDGF receptor
PLFE: patchy lobular fibrosis in the elderly
PSC: pancreatic stellate cell
α-SMA: alpha smooth-muscle actin
TGF-α and -β: α and β isoforms of the transforming growth factor
TGF-β-RI and -RII: transforming growth factor β receptor I and II
TIMP: tissue inhibitor of metalloproteinase
TNF-α: tumor necrosis factor α

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# 1. Introduction

## 1.1 Fibrosis in the pancreas – from a clinical perspective

Fibrosis of the pancreas is the key histological feature of chronic pancreatitis (CP). It progressively replaces the pancreatic parenchyma, thereby forming different patterns. It has been shown that the pattern of fibrosis in the pancreas is related to the damage to the parenchyma. Besides, the kind and extent of the damage depend on the etiology of CP (Klöppel *et al.*, 2004). It is therefore essential that our understanding of the fibrogenesis in the pancreas be included in the discussion of the development, pathogenesis and classification of pancreatitis (Etemad and Whitcomb, 2001;Klöppel, 2007b). Since the patterns of fibrosis appear to be related to etiologic entities of CP, the recognition of the pattern of fibrosis can contribute to the diagnosis of CP.

In alcoholic chronic pancreatitis (ACP), the fibrosis is predominantly interlobular (Klöppel and Maillet, 1993), and in autoimmune pancreatitis (AIP) and hereditary CP, the fibrosis is located periductally and interlobularly (Klöppel *et al.*, 2003;Zamboni *et al.*, 2004;Klöppel *et al.*, 2005). In obstructive CP due to for example a ductal adenocarcinoma of the pancreatic head compressing the main pancreatic duct, the fibrosis is mainly diffuse, with an intra- and interlobular deposition pattern (Klöppel *et al.*, 2004). However, fibrotic changes may also be observed in the “normal pancreas,” i.e. the pancreas of persons without any known or macroscopically visible pancreatic disease. The etiology of this type of pancreatic fibrosis is not well known.

Fibrotic replacement of the normal pancreatic tissue represents a significant challenge in today’s medicine. It is commonly accompanied by a loss of the exocrine and endocrine function of the pancreas. Hence, complications arising from excessive fibrogenesis in the pancreas are, amongst others, maldigestion and diabetes mellitus. Fibrosis is generally defined as the accumulation of excessive amounts of extracellular matrix (ECM) proteins in a tissue. It is now believed that fibrosis is not an irreversible process of chronic tissue injury, but a multistage process which may be reversible at least in early stages (Apte and Wilson, 2004). Interestingly, it has been described in a few AIP patients that fibrotic lesions have resolved after steroid treatment (Saito *et al.*, 2002;Song *et al.*, 2005b). This has so far not been reported in ACP. Hence, it is tempting to speculate that there may be differences in the expression of profibrotic cytokines and their receptors in human pancreatic specimens with ACP versus AIP.

In the pancreas, ECM synthesis and production have been ascribed to stellate-shaped cells, i.e. the pancreatic stellate cells (PSCs), which can transform into myofibroblasts when activated by cytokines (Apte *et al.*, 1999;Luttenberger *et al.*, 2000;Shek *et al.*, 2002). The term “myofibroblast” was introduced in 1971, describing cells with the ultrastructural features of both smooth musle cells and fibroblasts (Majno *et al.*, 1971). In 1997, periacinar fibroblast-like cells were isolated from the pancreas. They were shown to in cell culture transform into myofibroblast-like cells expressing alpha smooth-muscle actin (α-SMA) and producing ECM components (Saotome *et al.*, 1997). Shortly thereafter, it was found that inactive PSCs from rats

store vitamin A and express desmin and glial fibrillary acidic protein (GFAP). These features made them fully comparable with the Ito cells of the liver.

## 1.2 Hepatic stellate cells

Studies on pancreatic fibrogenesis were anticipated by intense examinations of fibrogenesis in the liver (Gressner and Bachem, 1995). The equivalent of PSCs in the liver are the hepatic stellate cells, resident liver cells deriving from the perisinusoidal space of Disse, which is located between the hepatocytes and the sinusoidal lining (Blomhoff and Wake, 1991). They are characterized by long cytoplasmic processes, giving them a stellate appearance. Moreover, hepatic stellate cells store 80-90% of the total retinol in the liver (Blomhoff and Wake, 1991). Both inactive PSCs and hepatic stellate cells are characterized by intracytoplasmatic lipid droplets containing high amounts of vitamin A. In 1976, the term “vitamin A storing cell system” was established, because vitamin A storing stellate shaped cells were observed not only in the liver but also in many other kinds of tissues, i. e. the stomach, intestine, lungs and skin (Yamada and Hirosawa, 1976;Wake, 1980). The differentiation between Kupffer cells (or liver macrophages) and hepatic stellate cells (also named Ito cells) has been intensely debated, until a review published in 1980 rectified an error introduced by Kupffer in 1898 (Wake, 1980). In 1876, Kupffer had described “Sternzellen” (German term for “stellate cell”) as a separate liver cell type that was different from phagocytic cells (Kupffer, 1876). However, while reviewing his results in 1898, he suggested incorrectly that the stellated cells were mainly phagocytic – interpreting their inclusion bodies as fragments of phagocytosed erythrocytes (Blomhoff and Wake, 1991).

## 1.3 Pancreatic stellate cells

In the pancreas, vitamin A storing cells were first described in 1982 (Watari *et al.*, 1982). This study was based on examinations of pancreatic tissue from vitamin A loaded mice using fluorescence microscopy and electron microscopy. It has been proposed that these retinoid-containing fat-storing cells in the pancreas might originate from two cell lineages. One lineage might derive from fibroblasts and another from pericytes of the blood capillaries (Watari *et al.*, 1982). In 1990, similar cells were for the first time described in the human normal pancreas and in tissue from patients with ACP (Ikejiri, 1990). In 1997, periacinar fibroblast-like cells were isolated from the pancreas and, in *cell culture*, it was shown that these cells transform into myofibroblast-like cells expressing α-SMA, which actively produce ECM components (Saotome *et al.*, 1997). A year later, in *cell culture* experiments with isolated vitamin A storing cells from the pancreas, PSCs in rat tissue were shown to express desmin and GFAP and, after 48 hours of cell culture, α-SMA (Apte *et al.*, 1998).

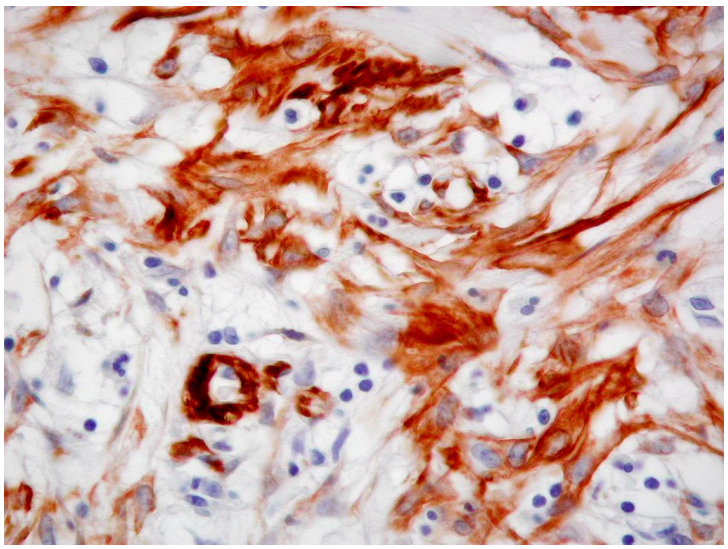


**Figure 1.1** Spindle shaped PSCs in alcoholic chronic pancreatitis, stage I. The α-SMA immunopositivity indicates that transformation into active PSCs/myofibroblasts has begun (α-SMA, x400).

During the following years, several *in vitro* studies confirmed that inactivated PSCs have a triangular morphology and are characterized by their capacity to store lipids and vitamin A (Apte *et al.*, 1998;Bachem *et al.*, 1998;Apte *et al.*, 1999;Luttenberger *et al.*, 2000). Furthermore, the transformation of inactive PSCs into activated, myofibroblast-like cells was shown to be associated with the expression of α-SMA (Fig. 1.1) and by a decrease of the retinoid content. Moreover, it was shown that activated PSCs are capable of producing fibril forming collagens, i. e. collagens type I and III, fibronectin and laminin (Bachem *et al.*, 1998;Apte *et al.*, 1999). Also, in *in vitro* studies it was shown that the transformation of quiescent PSCs into proliferating myofibroblasts/activated PSCs is furtherly stimulated by transforming growth factor β1 (TGF-β1) and platelet-derived growth factor B (PDGF-B) (Bachem *et al.*, 1998;Apte *et al.*, 1999;Luttenberger *et al.*, 2000;Shek *et al.*, 2002). Other factors also known to stimulate activation of PSCs are TGF-α, basic fibroblast growth factor (b-FGF), tumor necrosis factor α (TNF-α) (Schneider *et al.*, 2001), interleukin 1 (IL-1) and IL-6 (Mews *et al.*, 2002).

## 1.4 Study outlines

During the last decade, several studies have examined PSCs/myofibroblasts in *cell culture* and *animal* experiments. However, only little is known about the process of fibrogenesis in *human pancreatic tissue*. For this reason, we looked at human pancreatic tissues with fibrosis showing various patterns and associated with different etiologies. A total of 244 human pancreas specimens were collected. Eighty-nine of the specimens (study I) derived from persons without clinically recognized or macroscopically evident pancreatic disease. Fifty-nine specimens derived from patients who had ACP and who had undergone partial pancreatic resection because of pseudocysts, jaundice and/or pain (study II). Fifty-two specimens were obtained from autoimmune pancreatitis (AIP) patients who had undergone partial pancreatic

resection because of suspected pancreatic cancer (study III). Finally (study IV), 44 specimens were obtained by pancreatic core needle biopsy from AIP patients (29 specimens) and patients diagnosed with ACP (eight specimens) and tumor-associated, obstructive CP (OCP) (seven specimens). Studies II - III are based on a two-step approach: firstly, we described and clarified the fibrosis according to its pattern and distribution of inflammatory cells and activated PSCs/myofibroblasts. Secondly, we studied the expression and cellular distribution of profibrotic cytokines and their receptors. Finally, because of the possibility of treating AIP with steroids, we aimed at establishing criteria for its diagnosis in pancreatic core needle biopsy specimens on the basis of characteristic histopathological features including the pattern of fibrosis (study IV).

## 1.5 Special aims of the thesis

### 1.5.1 Study I

(1) To describe the distribution and pattern of fibrosis observed in the pancreas of persons without clinically or macroscopically evident pancreatic disease and to define criteria for fibrotic changes in the “normal” pancreas.

(2) To elucidate the pathomechanism of the fibrosis in the “normal” pancreas.

(3) To relate the localization of activated PSCs/myofibroblasts to the fibrotic changes in the “normal” pancreas.

### 1.5.2 Study II

(1) To develop a histopathological staging system for human alcoholic chronic pancreatitis (ACP) that allows the distinction of early and intermediate from late stages.

(2) To examine the localization and distribution of α-SMA positive activated PSCs/myofibroblasts in early, intermediate and late stages of ACP.

(3) To examine the localization and distribution of CD68 positive macrophages in early, intermediate and late stages of ACP.

(4) To examine the expression of the profibrotic cytokines TGF-β1 propeptide and PDGF-B and their receptors in early, intermediate and late stages of ACP.

### 1.5.3 Study III

(1) To test whether a recently developed histopathological grading system for autoimmune pancreatitis (AIP) can be used to examine the localization and distribution of activated PSCs/myofibroblasts in the pancreas in mild, moderate and severe grades of AIP.

(2) To examine the localization and distribution of α-SMA positive activated PSCs/myofibroblasts in mild, moderate and severe grades of AIP.

(3) To examine the localization and distribution of CD68 positive macrophages, CD4 and CD8 positive lymphocytes and CD79a and IgG4 positive plasma cells in in mild, moderate and severe grades of AIP.

(4) To examine the expression of the profibrotic cytokines TGF-β1 propeptide and PDGF-B and their receptors in mild, moderate and severe grades of AIP.

(5) To test the hypothesis that there are significant differences in the expression of the profibrotic cytokines TGF-β1 propeptide and PDGF-B and their receptors in ACP versus AIP.

(6) To test the hypothesis that there are significant differences in the distribution of activated PSCs/myofibroblasts in ACP versus AIP.

### 1.5.4 Study IV

(1) To evaluate, whether the diagnosis of AIP can be made on a pancreatic core needle biopsy specimen.

(2) To define characteristic histopathological features based on fibrotic and inflammatory changes enabling the differential diagnosis of AIP versus ACP and the margin of pancreatic neoplasms containing inflammation and fibrosis in pancreatic core needle biopsy specimens.

# 2. Materials and Methods

## 2.1 Tissue specimens

A total number of 244 human pancreatic specimens were examined. Eighty-nine of the specimens (study I) derived from persons without clinically recognized or macroscopically evident pancreatic disease. All persons had died of nonpancreatic disease, most frequently of cardiovascular, neoplastic, or respiratory disease. The specimens were removed within 4-24 hours post mortem. From each pancreatic specimen, eight paraffin-embedded sections deriving from different regions in the pancreas were examined (Fig. 2.1): two blocks were taken from the upper part of the pancreatic head including the accessory pancreatic duct (region A), and two further blocks from the lower part of the pancreatic head with the main pancreatic duct (region B). Two blocks were taken from the body (region C) and the tail (region D) of pancreas, respectively.

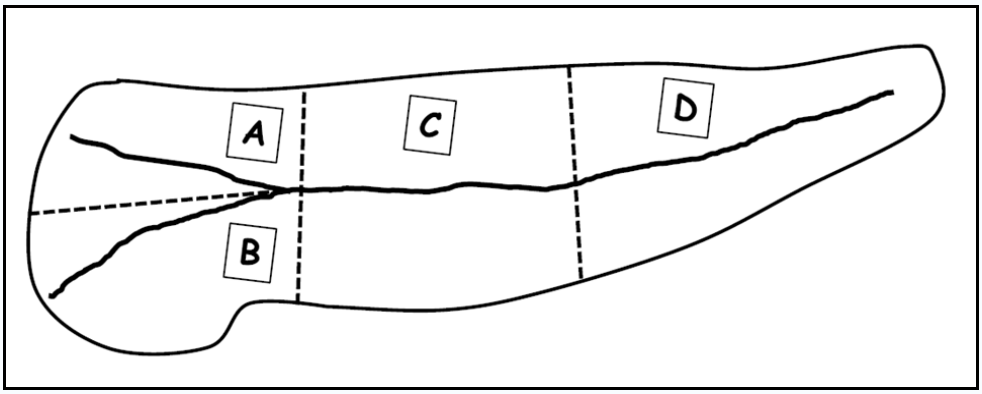



**Figure 2.1** The four different regions in the pancreas from which tissue blocks were obtained for study I. **(A)** Upper part of the pancreatic head including the accessory pancreatic duct, **(B)** lower part of the pancreatic head including the main pancreatic duct and the uncinate process, **(C)** body of the pancreas, **(D)** tail of the pancreas.

Fifty-nine of the specimens (study II) derived from patients who were diagnosed with alcoholic chronic pancreatitis (ACP) and who had undergone partial pancreatic resection (42 Whipple resections, 17 left-sided pancreatic resections) because of pseudocysts, jaundice and/ or pain. From each case there were at least two and in some cases ten tissue blocks available.

Fifty-two of the specimens (study III) were surgical specimens from autoimmune pancreatitis (AIP) patients who had undergone partial pancreatic resection (pancreaticoduodenectomy (45 patients) or left-sided pancreatic resection (seven patients)) because of suspected pancreatic cancer. At least two tissue blocks were available per case.

Fourty-four specimens (study IV) consisted of pancreatic core needle biopsy specimens obtained from AIP patients (29 specimens) and patients diagnosed with ACP (eight specimens) and tumor-associated, obstructive CP (OCP) (seven specimens). From each of these biopsies, between one and four tissue cylinders

were available. Table 2.1 summarizes the general schedule for the studies I-IV. For more detailed information, see the respective chapters in this thesis.

| Topic | Number of pancreatic specimens | Origin of pancreatic specimens | Methods of investigation |
|---|---|---|---|
| **(I)** Pancreatic fibrosis associated with age and ductal papillary hyperplasia | 89 | Postmortem specimens from persons without evident pancreatic disease | Histopathology<br>Immunohisto-chemistry (on a subgroup of the specimens) |
| **(II)** Fibrogenesis in ACP: the role of tissue necrosis, macrophages, myofibroblasts and cytokines | 59 | Surgical resection specimens from patients with ACP | Histopathology<br>Immunohisto-chemistry (on a subgroup of the specimens) |
| **(III)** AIP: expression and cellular source of profibrotic cytokines and their receptors | 52 | Surgical resection specimens from patients with AIP | Histopathology<br>Immunohisto-chemistry (on a subgroup of the specimens) |
| **(IV)** Diagnosis of AIP by core needle biopsy: application of six microscopic criteria | 44 | Pancreatic core needle biopsy specimens from patients with AIP, ACP and OCP | Histopathology<br>Immunohisto-chemistry |

**Table 2.1** Outline of the studies included in the thesis. ACP: alcoholic chronic pancreatitis, AIP: autoimmune pancreatitis, OCP: tumor-associated, obstructive chronic pancreatitis.

## 2.2 Immunohistochemical staining protocols

### 2.2.1 ABC method

In studies I-III, the immunohistochemical staining procedure was started by pipetting 150 µl of the antibody solution (primary antibody in the concentration given in the Materials and Methods section of the respective paper) onto the tissue slides. After incubation for 45 minutes, the reaction was stopped by rinsing in washing buffer three times. The next step of the staining procedure was incubation with the biotinylated secondary antibody from the kit (ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA) at a dilution of 5 µl per ml total volume of the solution for 30 minutes. This procedure was also stopped by rinsing in washing buffer.

The avidin-biotin-complex (ABC) was pipetted onto the slides for 30 minutes at

a dilution of 10 µl per ml, followed by a washing procedure in washing buffer. Then the chromogen diaminobenzidine-tetrahydrochloride (DAB) from the substrate kit for peroxidase (Vector Laboratories) was added. The solution was composed of 20 µl DAB, 20 µl buffer solution, 20 µl hydrogen peroxide and 940 µl distilled water. After rinsing in distilled water, the slides were counterstained with hemalum and washed in rinsing water, followed by dehydration using solutions with increasing ethanol concentrations and finally xylene. They were covered with pertex (Medite).

### 2.2.2 EnVision method

In study III, the immunohistochemical staining procedure for the antigens CD4, CD79a and IgG4 (also study IV) was started by pipetting 150 µl of the antibody solution (primary antibody in the concentration given in the Materials and Methods section of paper III) onto the tissue slides. After incubation for 45 minutes, the reaction was stopped by rinsing three times in washing buffer. The next step of the staining procedure was incubation with the EnVision polymer binding mouse antibodies (Dako K4001, Dako A/S, Glostrup, Denmark) for 30 minutes. This procedure was also stopped by rinsing in washing buffer. The chromogen DAB was added using the substrate kit for peroxidase (Vector Laboratories). After rinsing in distilled water, the slides were counterstained with hemalum and washed in rinsing water, followed by dehydration using solutions with increasing ethanol concentrations and finally xylene. They were covered with pertex (Medite).

## 2.3 Statistical analysis

In study I, the $\chi^2$ test (SPSS for Windows, version 10.1) was used to compare (a) the number of 20 to 59-year-old subjects with pancreatic lobular fibrosis with that of the 60 to 86-year-old subjects, and (b) the number of subjects with fibrotic foci and associated PanIN-1B lesions with the number of subjects who had PanIN-1B lesions which were not associated with fibrosis. Moreover, (c) to compare the severity of intralobular fibrosis with that of interlobular fibrosis in specimens with patchy lobular fibrosis in the elderly (PLFE), McNemar's Test was used. In study IV, statistical analysis was carried out using SigmaStat 3.0. Evaluations of results obtained in pancreatic core needle biopsy specimens from AIP patients and core biopsy specimens from patients diagnosed with non-AIP chronic pancreatitis (CP) was performed using the Fisher exact test (Table 3 in the respective paper) and the $\chi^2$ test for tabulated data (Table 4 in the respective paper). The level of significance was set at $P < 0.05$.

# 3. Fibrosis in the pancreas of the elderly

Until recently, only little was known about the frequency, type and pathogenesis of fibrotic changes that may occur in the pancreas without any clinically apparent or macroscopically visible disease. In textbooks on anatomy and histology it is mentioned that the pancreas is surrounded by a thin, fibrous capsule and that the pancreatic ducts are surrounded by thin cords of connective tissue. Furthermore, it is well known that there are small amounts of connective tissue between the lobules but almost none within them (Junqueira *et al.*, 1996). However, nothing is mentioned about pancreatic fibrosis developing in elderly persons. This is due to the fact that so far only few studies have described fibrosis in pancreatic tissues that were regarded as normal, i.e. that were not affected by any clinically obvious disease known to lead to fibrotic changes in the pancreas.

## 3.1 Association of pancreatic fibrosis with age

The first indication of an association between pancreatic fibrosis and age came from a study by Pitchumoni et al. (Pitchumoni *et al.*, 1984). The specimens examined were divided into two groups, those obtained from alcoholics and those from non-alcoholics. Small amounts of diffuse fibrosis were found in 68% (41/60 cases) of the alcoholics, which contrasted with the patchy type of fibrosis seen in the aged non-alcoholics, in whom small amounts of fibrosis were detected in 44% of the cases (18/41). Beyond the age of 65 in the non-alcoholics, the proportion of pancreatic specimens containing small amounts of fibrosis increased to 67% (14/21 cases). However, the authors did not analyze the pathomechanism of fibrosis in the aged non-alcoholics and no clear typing of the fibrotic changes was attempted.

Fibrotic changes were also described by Stamm (Stamm, 1984) and Shimizu (Shimizu *et al.*, 1989) in normal pancreatic specimens, but only Stamm reported that the fibrotic changes related to age. In Stamm´s study, 30% (34/112) of the cases contained areas of interlobular or perilobular fibrosis. Stamm also reported that papillary hyperplasia correlated with more advanced degrees of fibrosis, but did not explain this finding pathogenetically (Stamm, 1984). Shimizu and coworkers also detected “interstitial”, intralobular fibrosis in 68% (52/76) of the cases enrolled in their study. Furthermore, they concluded from their data that periductal fibrosis might be related to intralobular fibrosis, and that the periductal fibrosis might be the result of hyperplasia of the ductal epithelium. However, in this study, data were only given on the non-papillary type of ductal hyperplasia, recently classified as pancreatic intraepithelial neoplasia type 1A (PanIN-1A), formerly named mucinous hypertrophy (Shimizu *et al.*, 1989), and not on the frequency of ductal papillary hyperplasia.

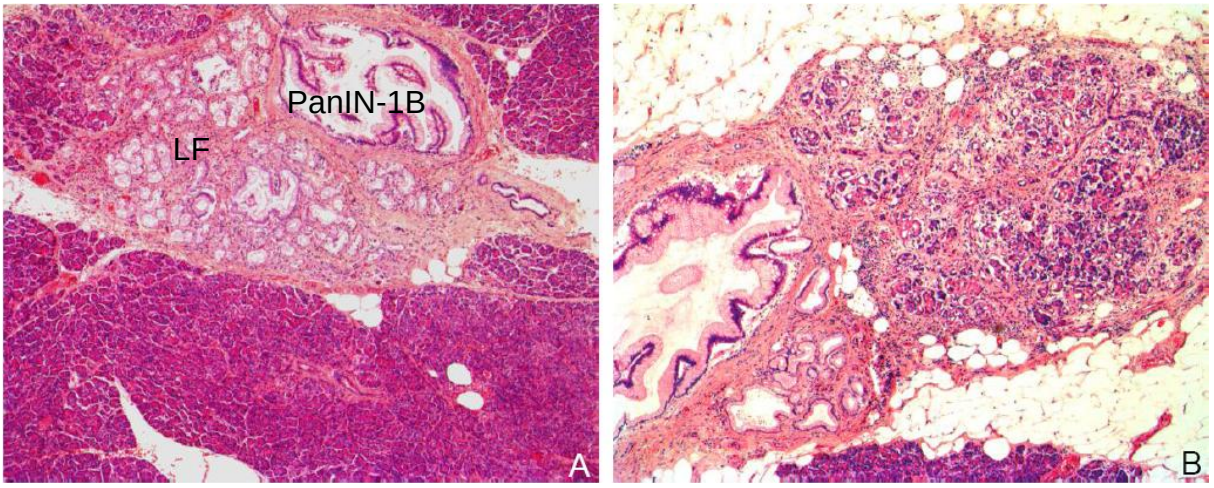



**Figure 3.1 (A-B)** PanIN-1B lesion in the pancreas obtained from an elderly person without any known pancreatic disease, resulting in various grades of lobular fibrosis (LF) (H&E, x50).

In the 1970s and 1980s, two other studies focused on hyperplastic changes in the pancreatic duct system and noticed that periductal fibrosis in these areas increased with age (Schmitz-Moormann and Hein, 1976;Allen-Mersh, 1985). In Schmitz-Moormann´s study, 88% (44/50 cases) revealed intralobular fibrosis. However, in study I, in which we aimed at gaining insights into the pathogenesis of pancreatic fibrosis by examination of the type and extent of pancreatic fibrosis in persons without any known pancreatic disease, we demonstrated that there is an association between the occurrence of hyperplastic duct changes and the development of fibrosis. Moreover, we showed that the intralobular fibrosis is not associated with the clinical symptoms of chronic pancreatitis (CP) (I).

We examined 89 postmortem specimens and determined the distribution of fibrosis, graded it and related it to two age classes (younger or older than 60 years) (I). Furthermore, we focused on changes in the ducts draining the fibrotic lobules. These duct changes have become known as “ductal papillary hyperplasia” (DPH) (Klöppel and Lüttges, 2001) and were recently classified among the precursor lesions of the pancreatic ductal adenocarcinoma, as PanIN type 1B (PanIN-1B) (Fig. 3.1 & Fig. 3.4-3.5) (Hruban *et al.*, 2001). We found an age dependent development of fibrosis in the pancreas of these persons who had no clinically apparent pancreatic disease and whose pancreas showed no macroscopic changes. Because most pancreata contained more than one fibrotic focus, we termed the pattern of multifocal intralobular fibrosis as “patchy lobular fibrosis in the elderly” (PLFE) (Fig. 3.2). PLFE was usually detected in peripheral lobules and often contained remnants of acini, small ducts, islets and small aggregates of lymphocytes (Fig. 3.1 & Fig. 3.4) (I). The distributional pattern of PLFE in the pancreata is schematically illustrated in Figure 3.2.

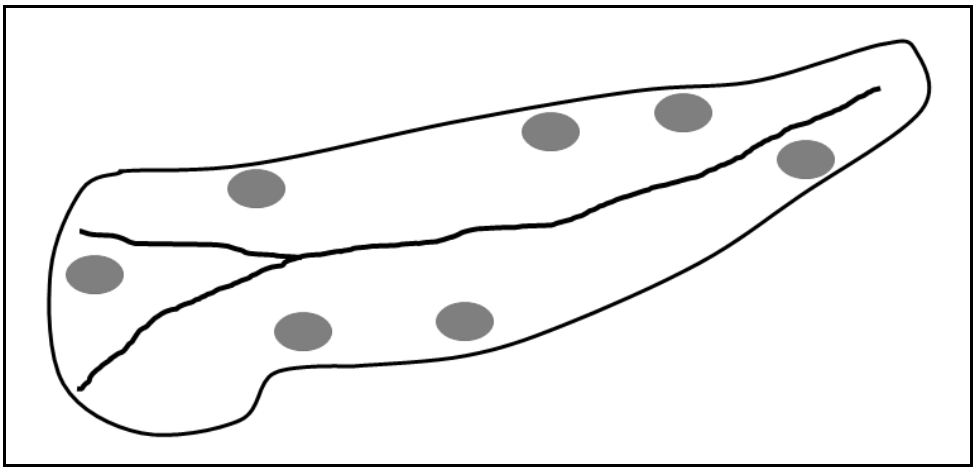


**Figure 3.2** Schematic illustration of the distribution pattern of patchy lobular fibrosis in the elderly (PLFE). The fibrosis affected mainly peripheral lobules of the pancreas and was strongly associated with PanIN-1B lesions of medium-sized interlobular ducts.

## 3.2 Pancreatic fibrosis and ductal papillary hyperplasia

The fact that PLFE was age related and usually involved single peripheral lobuli suggested a causative factor that is focally active and needs time to exert its effect (I). From tumors such as ductal adenocarcinomas affecting the head of the pancreas and obstructing the main pancreatic duct it is known that this obstruction may lead to a replacement of the acinar tissue by fibrosis, a change called obstructive CP (OCP). The experimental model of this disease is the duct ligation procedure, which results in similar alterations in the pancreas upstream of the ligation (Hultquist and Joensson, 1965;Isaksson *et al.*, 1983;Wang *et al.*, 1995). As the fibrotic changes resembled OCP as seen in experimental duct ligation, we searched for obliterating changes in the ducts draining the lobules affected by fibrosis (I). When we analyzed this duct lesion in the pancreatic specimens we found a close association between PanIN-1B lesions and lobular fibrotic foci. This suggests that PanIN-1B lesions may cause partial obstruction of the respective duct and thus lead to a decrease in the flow of pancreatic fluid, promoting apoptosis of acinar cells and followed by their replacement by fibrosis (I). This would be in accord with the hypothesis that the pattern of fibrosis in the pancreas depends on the site of the initial lesion which, in turn, depends on the respective etiology of CP (Klöppel *et al.*, 2004).

The development of PLFE, based on our findings in study I, is schematically illustrated in Figure 3.3. In addition to the described partial obstruction theory, another pathogenetic relation between PanINs and PLFE in the pancreas is possible. This has recently been outlined by Brune and coworkers, who examined eight pancreatic resection specimens from asymptomatic persons with a strong family history of pancreatic cancer. In these patients, who underwent surgery because of radiologic evidence of early pancreatic neoplasia, they found multiple lesions associated with PanIN-1B, but also with PanIN-1A, similary to those described by us (Brune *et al.*, 2006). Because mucins and aquaporins are known to be abnormally expressed in PanINs, their expression could alter the viscosity of the intraluminal secretions and thereby cause a relative reduction in their flow (Brune *et al.*, 2006). In

Brune´s study, the extent of the lobular parenchymal atrophy correlated well with the preoperative endoscopic ultrasound changes (Brune *et al.*, 2006). Hence, PLFE may be comparable with shadows casted by the PanIN-1B lesions. Interestingly, the PLFE lesions which we describe in study I represent the to date only by modern endoscopic ultrasound (EUS) detectable changes deriving from these precursor lesions of the ductal adenocarcinoma of the pancreas in the screening of patients at high familial risk of developing pancreatic cancer (Brune *et al.*, 2006;Hruban *et al.*, 2007) (I).

Intraductal papillary mucinous neoplasms (IPMNs) of the pancreas show several morphological similarities with PanIN lesions. However, they are, in contrast to PanINs, grossly visible and affect mainly the larger pancreatic ducts (Hruban *et al.*, 2008). A cut-off of 0.5 to 1.0 cm is recommended as grossly dividing line between PanINs and IPMNs. Also, the papillae in IPMNs are taller and more complex, and their epithelial lining is characterized by abundant luminal mucin. Expression of MUC2 is frequently noted in IPMNs but is lacking in most PanINs (Hruban *et al.*, 2008). IPMNs are grossly subclassified into those that primarily involve the main pancreatic duct, those that primarily involve secondary branches of the main pancreatic duct and those that involve both the main pancreatic duct and its branches (Hruban *et al.*, 2008). It is tempting to speculate that PanIN-1B lesions may give rise to branch duct type IPMNs, because both lesions are characterized by hyperplasia of the ductal epithelium in branching pancreatic ducts, and because also IPMNs are associated with the occurrence of fibrosis. However, IPMNs occur predominantly in the head of pancreas, while the PanIN-1B lesions in study I were evenly distributed throughout the periphery of the gland, with a slightly lower frequency in the inferior part of the head (unpublished data). These findings are in line with other studies on the occurrence of PanINs in pancreatic specimens without any known pancreatic disease and thus do not support this hypothesis (Lüttges *et al.*, 1999). However, further studies are needed to shed more light on the question whether some PanIN lesions give rise to branch duct type IPMNs.

## 3.3 Other profibrotic mechanisms in the pancreas of the elderly

It has been suggested that atherosclerosis of the celiac trunk and the superior mesenteric artery and its branches are associated with fibrosis (and lipomatosis) of the pancreas (Stamm, 1984), but Schmitz-Moorman and Hein were not able to establish such an association (Schmitz-Moormann and Hein, 1976). Furthermore, it must be taken into account that only the intralobular arteries are terminal vessels, while the big arteries of the pancreas are connected to each other by several communicating vessels (Hayasaka and Sasano, 1970).

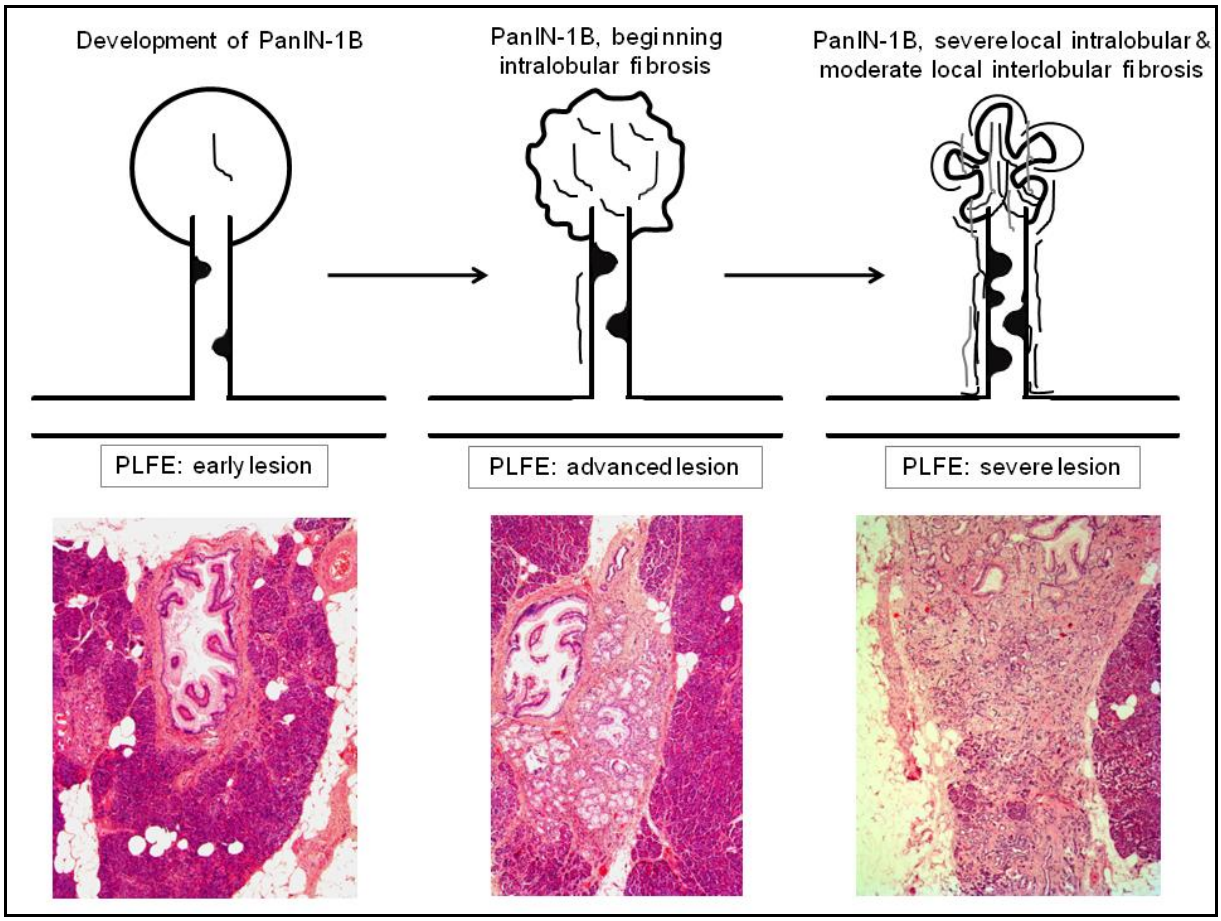



**Figure 3.3** Development of patchy lobular fibrosis (PLFE) in the pancreas of elderly persons without any known pancreatic disease. ***Left:*** Early-stage PLFE represented by two PanIN-1B lesions in a medium-sized interlobular duct. ***Center:*** Advanced PLFE, in which the persistance of the PanIN-1B lesions has resulted in impairment of the flow of pancreatic juice, promoting apoptosis of acinar cells, which was followed by their replacement by small amounts of fibrosis. ***Right:*** Severe PLFE lesion with strong lobular fibrosis.

Olsen stated a relationship between diabetes mellitus and interstitial fibrosis combined with chronic inflammation in the pancreas (Olsen, 1978), but these findings could later not be confirmed by others (Stamm, 1984;Shimizu *et al.*, 1989). However, some specimens in the elderly age group in our series showed fibrosis that was not clear-cut spatially associated with PanIN-1B lesions. Besides, there is an age-dependent decrease of pancreatic exocrine function in elderly persons without known pancreatic disease (Rothenbacher *et al.*, 2005). In a subgroup of these elderly patients who were treated with angiotensin receptor blockers for arterial hypertension, this exocrine pancreatic insufficiency was less pronounced (Rothenbacher *et al.*, 2005). The existence of a local renin-angiotensin system in the pancreas is well established (Leung, 2007). Also, angiotensin-converting enzyme inhibitor treatment has been shown to attenuate inflammation and fibrosis in male Wistar Bonn/Kobori rats (Kuno *et al.*, 2003), and angiotensin II stimulates pancreatic fibrogenesis, among other things through promotion of the proliferation of activated pancreatic stellate cells (PSCs) (Hama *et al.*, 2006). These data provide indirect evidence that the loss of exocrine function in the elderly goes in parallel with an

increase in fibrosis. Also, insulin has a trophic effect on acinar cells, and in those patients with type 1 diabetes mellitus showing residual insulin positivity, the pancreatic exocrine atrophy is less pronounced than in patients lacking residual insulin positivity (Löhr and Klöppel, 1987). Moreover, angiotensin II dose-dependently inhibited insulin release from isolated mouse islets (Lau *et al.*, 2004). Besides, impaired insulin sensitivity is a common finding in elderly persons (Broughton and Taylor, 1991;Ferrannini *et al.*, 1996). Hence, the susceptibility of pancreatic acinar cells to the trophic effect of insulin is likely impaired in elderly persons. This may result in increased apopotosis of acinar cells and their replacement by fibrosis. Hence, these findings could play a role for the development of the more diffuse type of fibrosis in the elderly which lacks association with PanIN-1B lesions.

However, because most of these conditions alone are related to advancing age, the association with fibrosis could also be merely a concomitant feature rather than causative.

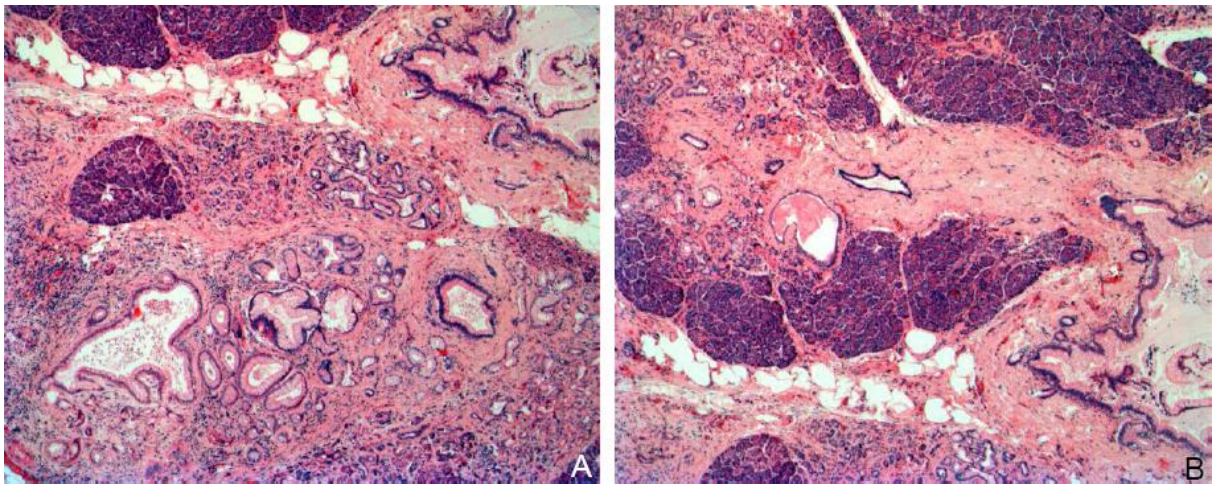



**Figure 3.4 (A-B)** PanIN-1B lesion in the pancreas obtained from an elderly person without any known pancreatic disease, resulting in various grades of lobular fibrosis and ectasia of some pancreatic ducts (H&E, x50).

## 3.4 Criteria for the diagnosis “patchy lobular fibrosis in the elderly”

On the basis of fine-needle aspiration (FNA) from a region containing PLFE, the most likely findings will be fibrosis and mild infiltration by lymphocytes and also a few plasma cells. However, as these features can also be seen in ACP (II, IV), in AIP (III, IV), in tumor-associated OCP and in the margin of pancreatic cancer containing inflammation and fibrosis (IV), FNA cytology is likely to be inadequate in the diagnosis of PLFE. However, if a pancreatic core needle biopsy or resection specimen shows ducts with a PanIN-1B lesion, accompanied by lobular fibrosis, this fibrosis may represent PLFE. PLFE should be diagnosed only by exclusion, when ACP (indicated by the presence of fat tissue necrosis, fibrosis and/or calcified protein plugs in the ducts), AIP (indicated by the occurrence of granulocytic epithelial lesions (GELs) or four or more of the six microscopic criteria which we describe in study IV), OCP and malignancy (see also Chapter 6) have been excluded.

## 3.5 Activated PSCs/myofibroblasts in the pancreas of the elderly

Myofibroblasts (i.e. activated PSCs) are known to play a pivotal role in pancreatic fibrogenesis and have been described in the normal human pancreas (Barth *et al.*, 2002) (I) and in human CP (Fukumura *et al.*, 2006) (II, III). In the pancreas from normal elderly persons, we detected α-SMA positive myofibroblasts in the PLFE foci, which were associated with PanIN-1B lesions (Fig. 3.5) (I). This implies that there is ongoing fibrogenesis in these areas, probably triggered by the hampered flow of secretions in the narrowed draining ducts. Experimental work has shown that the cytokines PDGF-B and TGF-β1 cause activation and proliferation of the resident fibroblasts/quiescent PSCs, inducing them to transform into activated PSCs/myofibroblasts, which are capable of producing extracellular matrix (ECM) (Haber *et al.*, 1999;Shek *et al.*, 2002). Considering these data, we assume that PanIN-1B lesions in the normal pancreas of elderly persons provoke damage to acinar cells due to decreased flow of pancreatic fluid in the ducts, followed by apoptosis and phagocytosis of acinar cells (I). Afterwards these cells appear to be replaced by ECM synthesized by α-SMA positive myofibroblasts/activated PSCs (Fig. 3.5).

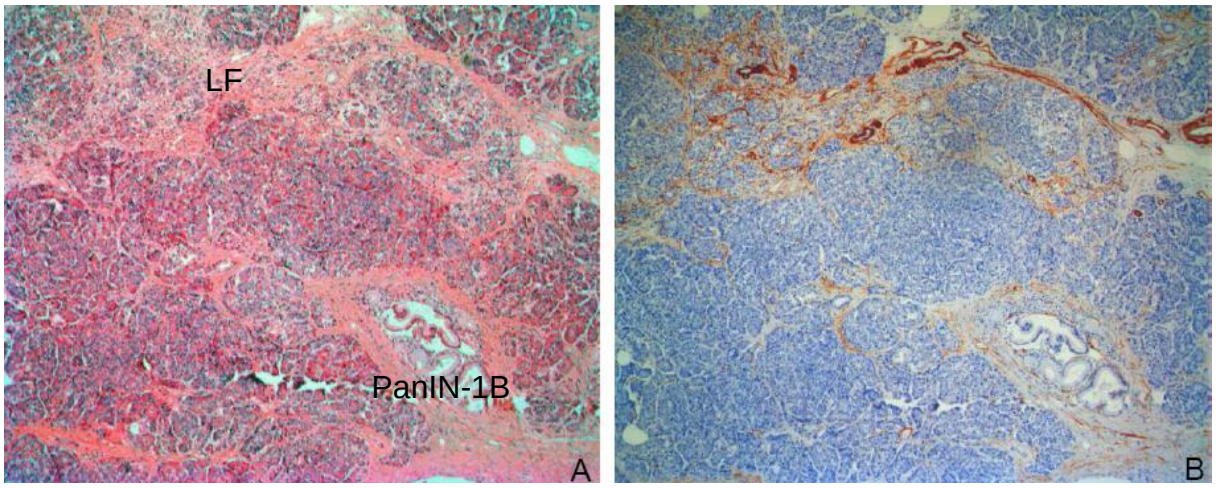



**Figure 3.5 (A)** PanIN-1B lesion in the pancreas obtained from an elderly person without any known pancreatic disease, resulting in a mild lobular fibrosis (LF) (H&E, x50). **(B)** Immunostaining of α-SMA reveals numerous activated PSCs/myofibroblasts inside the fibrotic foci (x50).

# 4. Fibrogenesis in alcoholic chronic pancreatitis

## 4.1 Pathogenesis of alcoholic chronic pancreatitis

Over the years, several hypotheses on the pathogenesis of alcoholic chronic pancreatitis (ACP) have been discussed (Wilson *et al.*, 1982;Braganza, 1983;DiMagno *et al.*, 1993;Wilson and Apte, 2003). However, two of the theories, the lithostatin hypothesis and the necrosis-fibrosis-sequence, have dominated the debate for the last decade. Therefore, these theories are described in detail below.

### 4.1.1 The lithostatin hypothesis

The lithostatin hypothesis is based on the assumption that alcohol in chronic alcoholics results in an increased secretion of proteins by a cholinergic mechanism (Sarles, 1986). The general composition of pancreatic calculi is mostly calcium carbonate in the form of calcite, explaining why most calculi in the pancreas are visible on x-ray of the abdomen (Sarles, 1986). The average protein content in pancreatic juice is 0.5%. Besides, it contains small amounts of polysaccharides (Sarles, 1986). In chronic alcoholics, however, the protein concentration in pancreatic juice is increased, resulting in increased viscosity and probably hampering of flow in the ducts (Sarles, 1986).

The lithostatin hypothesis states also, that chronic alcohol consumption impairs the synthesis and secretion of pancreatic stone protein, more recently referred to as „lithostatin“, in acinar cells. It is believed that lithostatin, in healthy subjects, protects the precipitation of insoluble calcium salts in the pancreatic ducts (Sarles, 1986). Sarles and coworkers demonstrated this experimentally, where $CaCl_2$ (calcite) was added to a mixture of $NaCO_3H$ together with different ions in concentrations similar to the physiological composition of pancreatic juice with and without lithostatin (Sarles, 1986). Lithostatin represents the main protein fraction in pancreatic protein plugs, in addition to trypsin and other enzymes. Hence, a decrease in the lithostatin concentration of pancreatic juice will result in calcification of protein precipitates, terminating in the formation of calculi. These calculi will, in late stages, provoke ulceration and erosion of the ductal epithelium followed by a circular fibrosis surrounding these ulcerations. Another consequence of the circular periductal fibrosis is prestenotic atrophy of acini (Sarles, 1986). However, the lithostatin hypothesis assumes that fibrogenesis in ACP is preceded by the formation of calculi and that calculi act as a causative factor for the formation of fibrosis (Sarles, 1986). In line with these assumptions is the finding that the relative concentration of lithostatin to the total pancreatic juice protein is constantly decreased in patients presenting with chronic calcifying pancreatitis (Sarles, 1986). In conclusion, the lithostatin hypothesis states that pancreatic lithogenesis is a two-stage process. Firstly, precipitation of proteins and insoluble calcium salts take place, leading to the formation of precipitated protein plugs in pancreatic ducts (Sarles, 1986). In late stages of this process, the precipitation of calcium increases, resulting in the formation of calcified protein plugs, „calculi“.

### 4.1.2 The necrosis-fibrosis-sequence

The lithostatin hypothesis has been criticized by many authors, in particular because also acute alcoholic pancreatitis may be caused by alcohol. Besides, acute and chronic alcoholic pancreatitis have several common clinical and morphological features. In particular histopathological hallmarks of acute alcoholic pancreatitis, such as fat tissue necrosis and pseudocysts, are also common features of early-stage ACP. Moreover, as described below, early- and mid-stage ACP lack calcifications in the pancreatic ducts (Ammann *et al.*, 1996) (II). Thus, the lithostatin-hypothesis has in recent years been challenged by the necrosis-fibrosis-sequence (Comfort *et al.*, 1946a;Comfort *et al.*, 1946b;Klöppel and Maillet, 1993), and in study II we developed a staging system which describes the histopathological findings in ACP and which is in line with this concept (II). One of the strongest points of the necrosis-fibrosis-sequence is its accordance with current concepts of the pathogenesis of acute alcoholic pancreatitis. It is widely accepted that the initial event in acute alcoholic pancreatitis is the intra-acinar activation of proteolytic enzymes, resulting in premature enzyme activation and autodigestion of the pancreas (see also Chapter 4.2). Ethanol, its metabolites and oxidant stress exert a number of toxic effects on acinar cells that predispose the gland to autodigestive injury and acute necroinflammation including fat tissue necrosis (Apte *et al.*, 2006).

The necrosis-fibrosis-sequence postulates that the lesions in the pancreas caused by autodigestion during an acute attack of pancreatitis will recover completely in many cases (Klöppel, 2004). However, in severe and prolonged cases, the normal tissue architecture may not be reobtained completely, resulting in the induction of fibrosis by myofibroblasts equipped with receptors for profibrotic cytokines (Fig. 4.7D). Our data indicate that fibrogenesis is mediated by the profibrotic cytokines TGF-β1 and PDGF-B, in part released by macrophages during their resorption of necrotic fat tissue (Fig. 4.7C) (II). Interestingly, the fibrosis is primarily seen in interlobular areas where the fat tissue and hemorrhagic necroses occur (Klöppel and Maillet, 1998b;Klöppel, 2004) (II). This interlobular fibrosis surrounds interlobular pancreatic ducts and affects them mechanically. In particular strictures of the ducts and dilated segments hamper the flow of pancreatic juice within their lumina (Klöppel, 2004). Impairment of the flow of the pancreatic secretions triggers the spontaneous precipitation of proteins (histologically represented by stage III ACP) and, subsequently, their calcification (stage IV ACP) (II). At the end of this process also intralobular fibrosis occurs, as a result of the interrupted flow of pancreatic juice, promoting apoptosis of acinar cells drained by the respective duct. Afterwards, the apoptotic acinar cells are replaced by fibrosis.

## 4.2 Tissue damage and fibrogenesis in alcoholic chronic pancreatitis

Alcoholic pancreatitis is characterized by intra-acinar activation of proteolytic enzymes (Klöppel and Maillet, 1993;Klöppel, 1999). Because the secretion of pancreatic enzymes in acute attacks of pancreatitis is reduced, zymogen granules in

acinar cells fusionate with lysosomal vacuoles containing cathepsin B, which contributes to enzyme activation and results in autodigestion of the pancreas (Liddle and Nathan, 2004). Also, ethanol sensitizes the pancreas to cholecystokinine, leading to pathological zymogen activation (Gutierrez-Ruiz *et al.*, 2002). Moreover, enzyme activation stimulates the release of inflammatory mediators, in part mediated by activation of the transcription factors nuclear factor kappaB and activator protein 1 (Gutierrez-Ruiz *et al.*, 2002). These mediators contribute to fat tissue and hemorrhagic necrosis in the pancreas. It is well established that acute pancreatic necroinflammation is associated with upregulation of mitogenic and proinflammatory chemokines in the pancreas, most importantly TNF-α, IL-1, IL-6, IL-8 and monocyte chemoattractant protein 1 (MCP-1) (Norman, 1998) (for a short review: see the respective section in (Drewes *et al.*, 2008)). Some of these inflammatory chemokines are probably involved in the upregulation of profibrotic cytokines in the pancreatic tissue.

Acute pancreatitis results from pancreatic injury that in turn leads to the recruitment of leukocytes. Firstly, leukocytes get into close contact to the wall of blood vessels through labile adhesions, where they move with the blood flow. This is mediated by adhesion molecules of the selectin family which are located in endothelial cells and takes place with low affinity (Gutierrez-Ruiz *et al.*, 2002). The process is followed by high affinity binding through chemoattractants, namely chemokines, establishing a gradient surrounding the inflammatory trigger. Interaction between leukocytes and chemokines results in upregulation of integrin receptors, leading to extravasation of leukocytes (Gutierrez-Ruiz *et al.*, 2002).

The contribution of inflammation to the development of fibrosis varies in different conditions, but, exemplified by the necrosis-fibrosis-sequence (Klöppel and Maillet, 1993), it is probably high in the progression from acute to chronic alcoholic pancreatitis. Therefore, an understanding of the interaction between the acute inflammatory processes and fibrogenesis in the pancreas is substantial. Simplified, in acute alcoholic pancreatitis, the damage to acinar cells culminates in the upregulation of inflammatory chemokines like IL-8 and MCP-1, which in turn probably contribute to the upregulation of profibrotic cytokines during the progression from acute to chronic pancreatitis.

## 4.3 Histological criteria for the staging of alcoholic chronic pancreatitis

Mild acute alcoholic pancreatitis is characterized by patchily distributed, small peripancreatic fat tissue necroses caused probably by lipase, an enzyme that does not require activation, sometimes accompanied by an interstitial pancreatic edema (Klöppel and Maillet, 1998a). Severe pancreatitis, on the other hand, reveals necrotic foci (both acinar necrosis, duct necrosis and interlobular fat tissue necrosis) also inside the pancreas. The number of interlobular fat tissue necroses depends on the intensity of intrapancreatic lipomatosis. Sometimes, there is also (mainly venous) vessel necrosis, eventually combined with intraparenchymal hemorrhage.

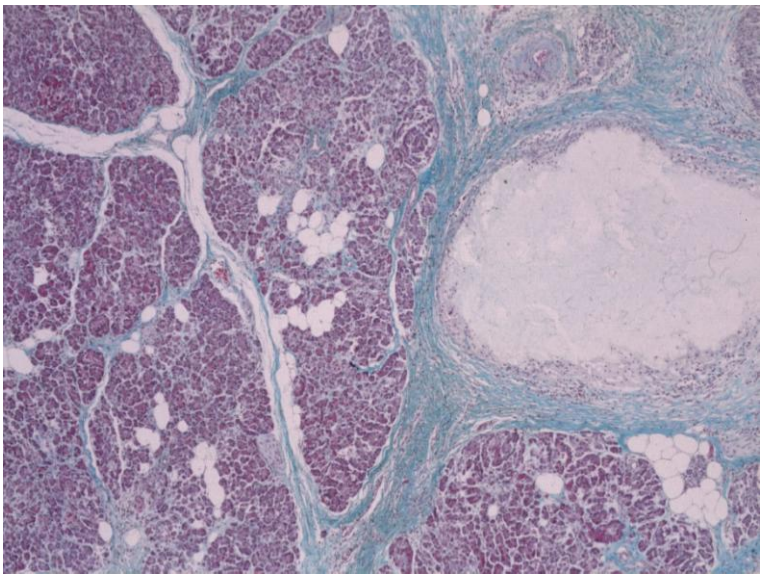


**Figure 4.1** Formation of a pseudocyst in alcoholic chronic pancreatitis, stage I (Goldner, x100).

Dependent on their size, the areas with fat tissue necrosis either resolve entirely (which is mostly the case if their diameter is below 1 cm) or first demarcate by macrophages and afterwards reabsorb (if the diameter is between 2 to 4 cm). If no spontaneous resolvement occurs (mostly seen if the diameter is larger than 5 cm), a thin layer of granulation tissue develops within 10-20 days. This is followed by a fibrotic capsule after 20-30 days and results in the formation of a pseudocyst (Fig. 4.1) (Klöppel and Maillet, 1998a). In some cases, reabsorption of interlobular fat tissue necroses may only take place slowly, in particular if inflammatory bursts occur repeatedly, resulting in interlobular fibrosis. In severe cases, characterized by recurrent episodes of the necrosis-fibrosis-sequence, acute alcoholic pancreatitis may progress to ACP (Klöppel and Maillet, 1992).

In stage I of the histological staging system of ACP which we developed in study II (Fig. 4.1 & Fig. 4.7), the pancreatic tissue shows necrotic changes as well as a mild cellular interlobular fibrosis. Early cases of stage I ACP represent an overlap stage between alcoholic acute and alcoholic chronic pancreatitis. Hallmarks are fat tissue necroses, hemorrhagic necroses and pseudocysts (Fig. 4.1). The margins of the necrotic foci are lined by numerous macrophages, some of which are transformed into foamy cells (Fig.4.7C). Also, there are a few lymphocytes, plasma cells and neutrophilic granulocytes (II). The inflammatory infiltrate is surrounded by myofibroblasts/activated PSCs which represent the main component of the cellular fibrotic tissue separating the normal pancreatic parenchyma from inflammatory necrotic areas (Fig. 4.1 & Fig. 4.7B). Acinar cell or duct necroses are rare and only observed in areas with extensive interstitial fat and/or hemorrhagic necrosis (II).

In stage II ACP (Fig. 4.2A), there is intensive cellular interlobular fibrosis that, in addition to myofibroblasts/activated PSCs (Fig. 4.2B), contains scattered macrophages, lymphocytes and occasionally neutrophilic granulocytes (II). The

inflammatory infiltrate occasionally also involves acinar tissue. The interlobular ducts are embedded in the cellular fibrosis but appear otherwise mostly unaltered (II).

In stage III ACP (Fig. 4.3A), the pancreatic tissue shows marked interlobular fibrosis associated with intralobular fibrosis that involves single lobuli or groups of lobuli and replaces acinar cells. Enlarged nerve fibers, surrounded by inflammatory infiltrates and frequently entrapped by fibrosis, are numerous (Fig. 4.3A). The fibrotic tissue shows some fibroblasts and fibrocytes, but α-SMA positive myofibroblasts/ activated PSCs are rare (Fig. 4.3B). Furthermore, there are scattered macrophages, lymphocytes and plasma cells, while granulocytes are absent. Many of the interlobular ducts embedded in fibrotic tissue show an irregular lumen and contain protein plugs (II).

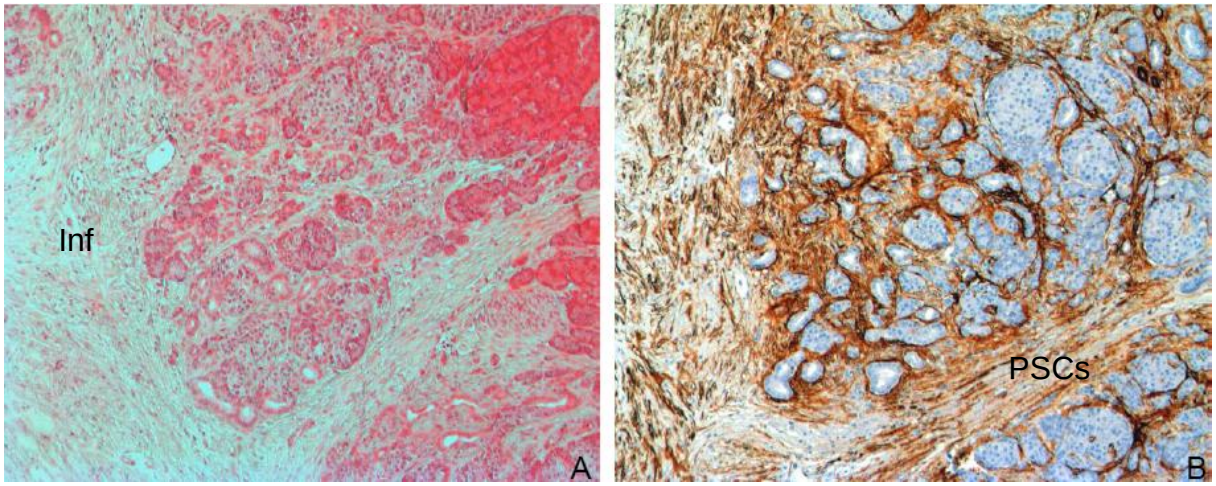



**Figure 4.2** Alcoholic chronic pancreatitis, stage II: Strong interlobular infiltration (Inf) by macrophages, lymphocytes and neutrophilic granulocytes. **(A)** (H&E, x100). **(B)** α-SMA immunostaining reveals that the infiltrate is intermingled with myofibroblasts/activated PSCs (x100).

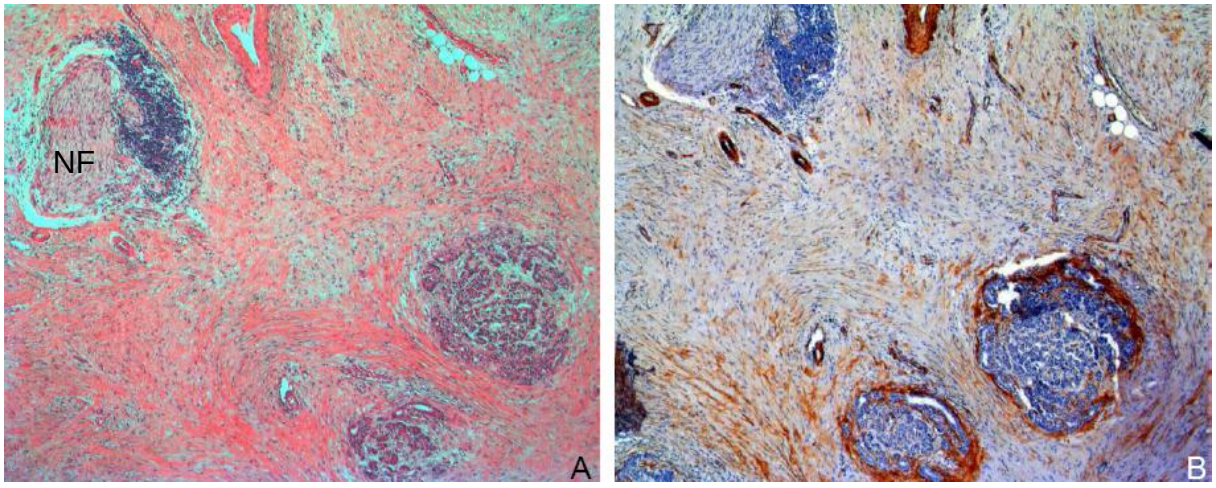



**Figure 4.3** Alcoholic chronic pancreatitis, stage III: Marked fibrosis with entrapment of interlobular ducts, remnants of acini, enlarged nerve fibers (NF) and islets. **(A)** (H&E, x50). **(B)** In this stage of ACP, only a few α-SMA positive myofibroblasts/activated PSCs are present (x50).

In stage IV ACP (Fig. 4.4A), the extent of the inter- and in particular the intralobular fibrosis is larger than in stage III. In addition, some of the interlobular ducts, which frequently are severely distorted and show irregular lumina, contain calculi (II). Sometimes, the calculi erode the epithelium. The destruction of the epithelium is accompanied by an adjacent inflammatory infiltrate, consisting of neutrophilic granulocytes, macrophages, some lymphocytes, plasma cells and also fibroblasts and numerous α-SMA positive myofibroblasts/activated PSCs (Fig. 4.4B) (II).

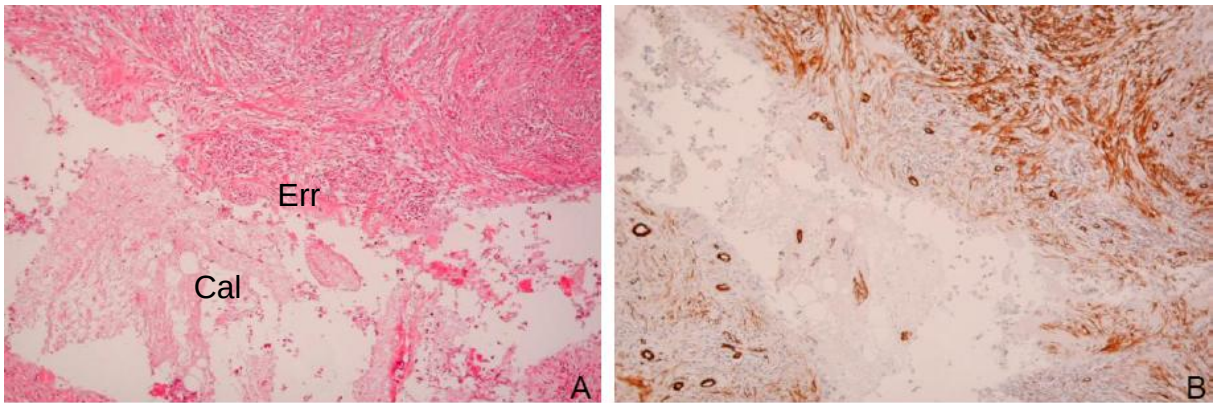



**Figure 4.4** Alcoholic chronic pancreatitis, stage IV: A calculus (Cal) eroding the epithelium (Err) of a medium-sized interlobular duct. **(A)** (H&E, x50). **(B)** Intense accumulation of α-SMA positive myofibroblasts/activated PSCs is seen only adjacent to erosion of the epithelium by calculi (x50).

The histological staging system of ACP which we defined is schematically summarized in Figure 4.5.

## 4.4 Localization of TGF-β1 and its receptors in alcoholic chronic pancreatitis

The studies on PSCs in *cell culture* and *animal models* have been valuable for the characterization of PSCs. However, cell culture experiments do not imitate the *in vivo* conditions in human pancreatitis completely, and the capability of animal models to imitate the time-course of tissue injury and fibrogenesis in different etiologies of human CP is limited. Hence, it is important to localize PSCs and the mediators playing a role in PSC activation within the diseased human tissue. Moreover, it is important to identify the cellular source of cytokines activating PSCs, and the best method to localize these peptides within the tissue is immunohistochemical studies. Those studies localized TGF-β1 (as well as its propeptides and receptors) and PDGF-B and PDGF-R to inflammatory cells and to epithelial cells in ACP tissue (Slater *et al.*, 1995;Van Laethem *et al.*, 1995;Ebert *et al.*, 1998;Ishihara *et al.*, 1998;Satoh *et al.*, 1998;di Mola *et al.*, 1999;Casini *et al.*, 2000) (II).

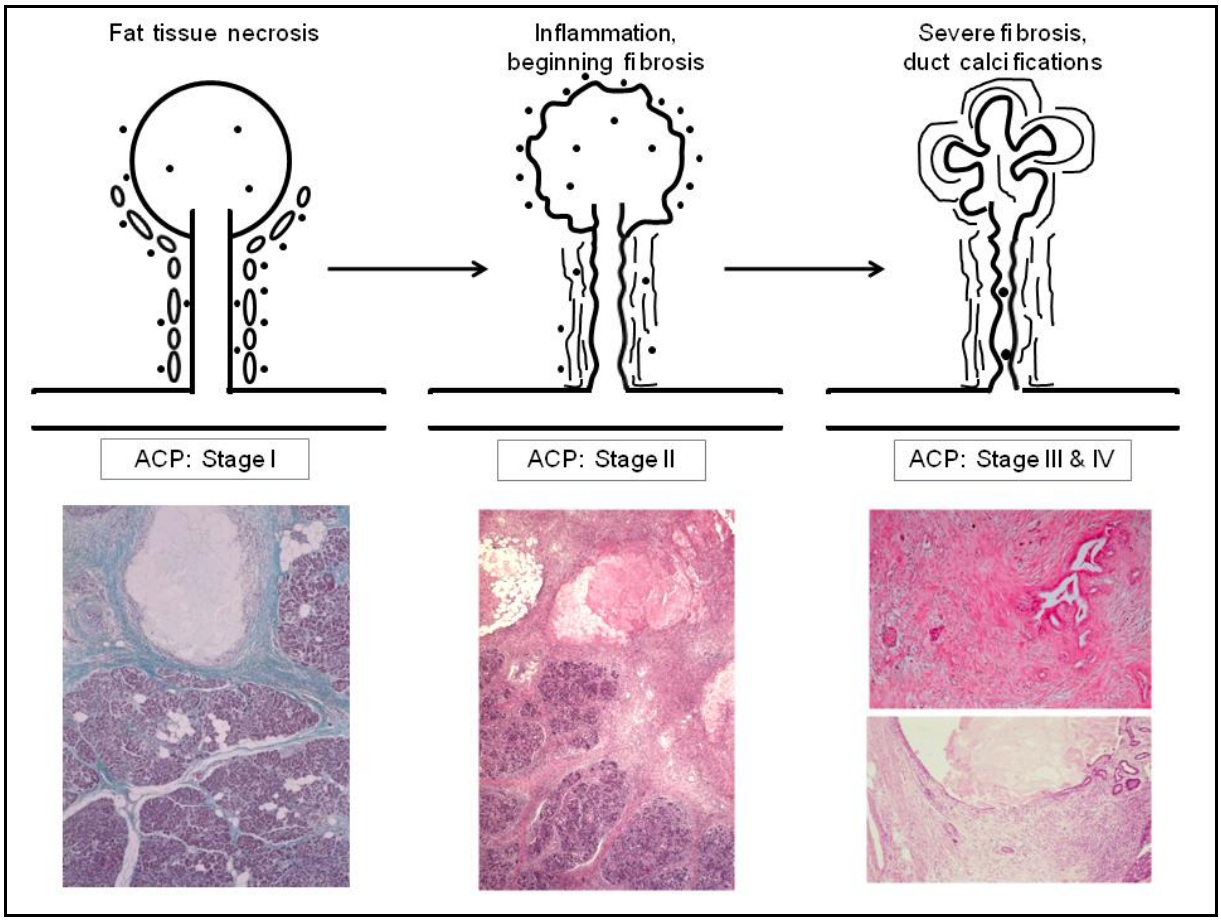



**Figure 4.5** Schematic illustration of the development of fibrosis in alcoholic chronic pancreatitis (ACP), which is based on the four histological ACP stages which we defined in study II (see also Chapter 4.3). ***Left:*** In stage I ACP, interlobular fat tissue necroses are present, some of which transform into pseudocysts while reabsorbed by macrophages. Fibrosis is mainly seen in the wall of pseudocysts and, mildly, in interlobular areas. ***Center:*** In stage II ACP, the necroinflammation extends to interlobular and intralobular areas. There is a prominent interlobular cellular fibrosis. ***Right:*** In stage III & IV ACP, when the branching ducts are severely narrowed and wide areas of the parenchyma have been replaced by fibrosis, there is only little inflammation. However, in stage IV, some medium- and large-sized ducts contain calculi (large dots), eroding the epithelium. This process is accompanied by focal inflammation and focal fibrogenesis. (Figure showing stage III ACP is reprinted from (II), with permission).

In tissue from ACP patients, the immunoreactivity of the TGF-β1 propeptide latency associated peptide (LAP) has been observed particularly in macrophages by ourselves and others (Van Laethem *et al.*, 1995) (II). TGF-β1 is secreted in a latent form connected to LAP and latent TGF-β-binding protein 1 (LTBP-1). Together, these proteins form a high molecular complex, the “latent TGF-β-complex” (Kanzaki *et al.*, 1990), and this complex is necessary for the secretion of TGF-β1 (Miyazono *et al.*, 1988;Saharinen *et al.*, 1999). The TGF-β1 propeptide (LAP) immunoreactivity may therefore be employed as tool for identification of those cells that actively release TGF-β1. We noted that macrophages represent the major source of TGF-β1 also in AIP (see Chapter 5.4) (III), and these findings are in accord with observations from Fukumura and coworkers in cancer-associated, obstructive CP (OCP) (Fukumura *et*

*al.*, 2006). These data taken together indicate that mainly macrophages, but also tubuloacinar cells and myofibroblasts/activated PSCs, release TGF-β1 and activate nearby resident fibroblasts and quiescent PSCs, to transform into myofibroblasts/ activated PSCs. These findings, reflecting the *in vivo* localization of LAP/TGF-β1 in human pancreatic tissue, are in line with cell culture studies on interactions between macrophages and PSCs, showing that macrophages also *in vitro* are an important source of TGF-β1 (Schmid-Kotsas *et al.*, 1999). Myofibroblasts, on the other hand, express LAP and/or TGF-β1 less intensely, but are strongly positive for transforming growth factor β receptor II (TGF-β-RII) (Satoh *et al.*, 1998;di Mola *et al.*, 1999) (II, III) and TGF-β-RI (di Mola *et al.*, 1999). They are therefore probably susceptible to TGF-β1.

Interestingly, in cancer-associated OCP, macrophages were shown to be strongly TGF-β1 and weakly TGF-β3 positive, but TGF-β2 negative (Fukumura *et al.*, 2006). TGF-β1 might at least in part reveal its profibrogenic effects through induction of connective tissue growth factor (CTGF). This is a cysteine-rich peptide originally identified in human endothelial cells (Ryseck *et al.*, 1991). CTGF exhibits chemotactic and mitogenic effects for mesenchymal cells and is upregulated in cultured human foreskin fibroblasts after stimulation with TGF-β1, but not other profibrogenic cytokines like PDGF, epidermal growth factor (EGF) or b-FGF (Grotendorst *et al.*, 1996). CTGF has been shown to be upregulated in acinar cells and fibroblasts in human ACP tissue samples (di Mola *et al.*, 1999).

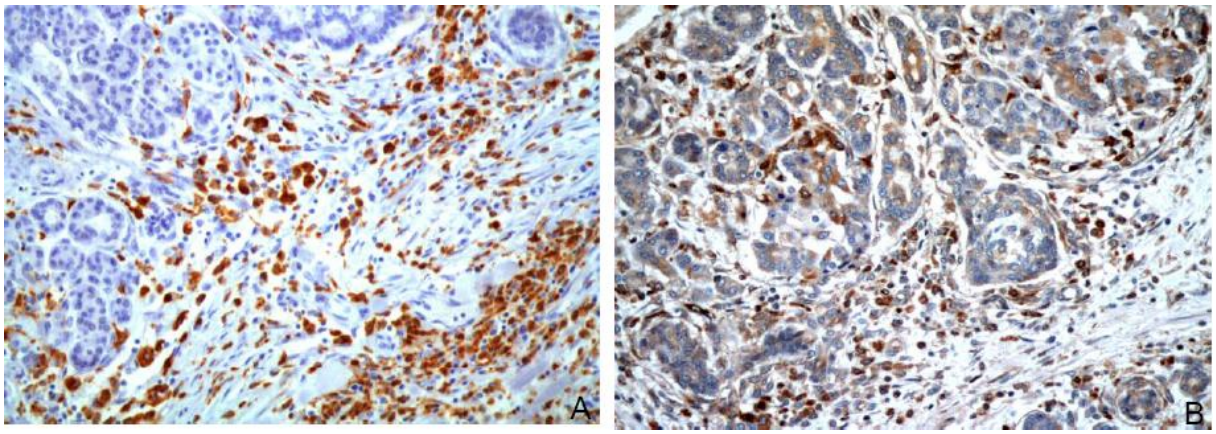



**Figure 4.6** Alcoholic chronic pancreatitis, stage II: **(A)** CD68 positive macrophages are intermingled in the cellular interlobular fibrosis and invade also into the lobuli (x200). **(B)** Many of the macrophages are TGF-β1 propeptide positive (x200).

## 4.5 Localization of PDGF-B and its receptors in alcoholic chronic pancreatitis

Only few studies elucidated the *in vivo* tissue distribution of PDGF-B and corresponding receptors in human CP (Ebert *et al.*, 1998). Moreover, to the best of our knowledge, only study II and III of this thesis explore the expression pattern of this cytokine and its receptors in relation to the natural course of the disease in human ACP (II) and human AIP (III). Ductal cells and tubuloacinar cells next to

inflammatory infiltrates were found to be the most important source of PDGF-B (II). This indicates that epithelial cells in the pancreas, particularly the ductal cells, are involved in the production of PDGF-B and thus stimulate myofibroblasts/activated PSCs that are equipped with PDGF receptor α (PDGF-Rα) (Fig. 4.7D) (II) and PDGF receptor β (PDGF-Rβ). This might result in proliferation of the resident myofibroblasts/activated PSCs in the immediate vicinity of the tissue damage, inducing them to accumulate in ACP (II).

The expression of PDGF-B in ductal cells indicates the existence of an autocrine loop, in which ductal cells produce PDGF-B which, in turn, stimulates neighbouring ductal cells to increase their synthesis of PDGF-B. This is in line with the strong expression of PDGF-Rα in ductal and tubuloacinar cells in ACP (II). Interestingly, ductal cells are probably able to synthesize several major ECM components, and laminin and hyaluronic acid have been demonstrated to be actively

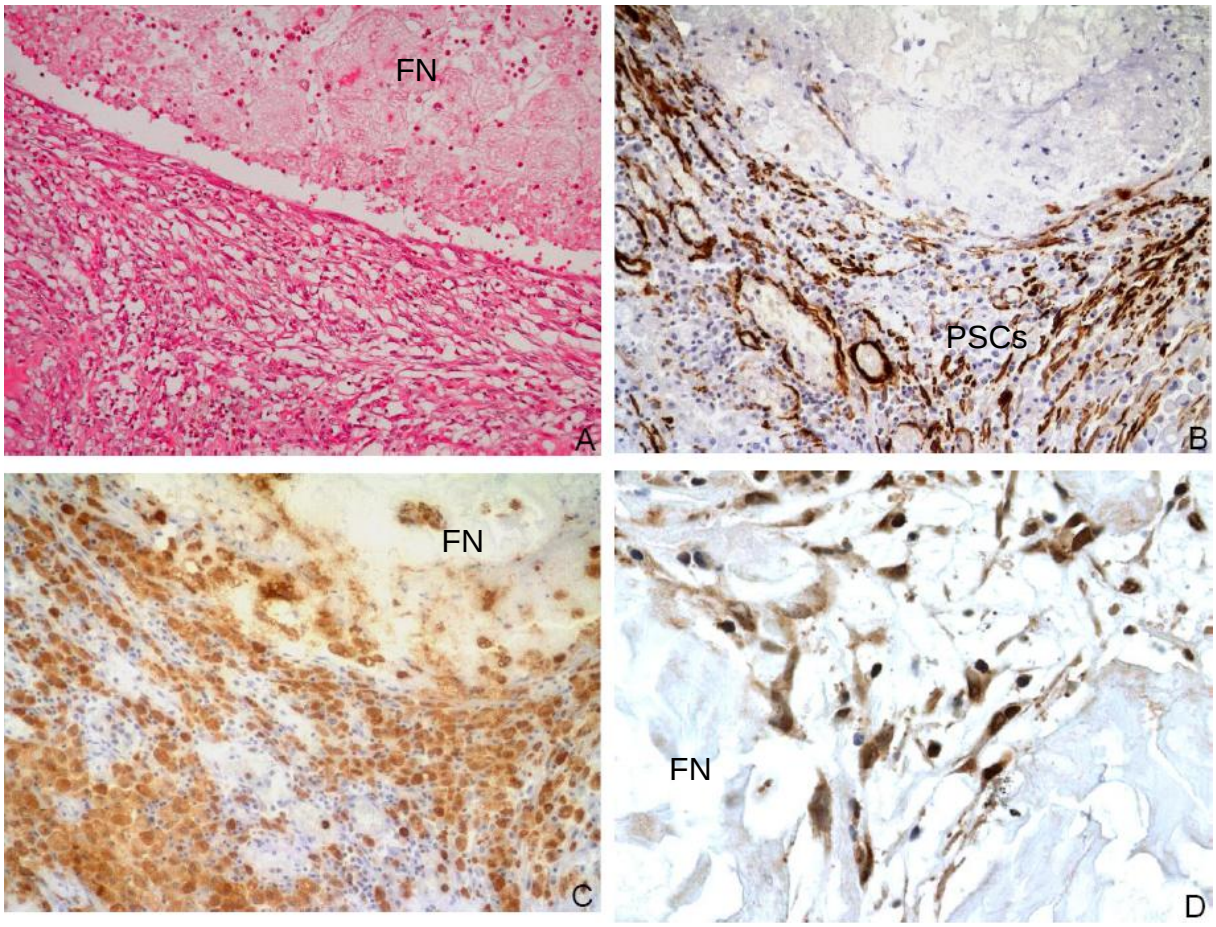



**Figure 4.7** Alcoholic chronic pancreatitis, stage I: **(A)** Fat tissue necrosis (FN) undergoing transformation into a pseudocyst (H&E, x100). **(B)** The myofibroblasts/activated PSCs stain for α-SMA (x200). **(C)** Macrophages in the same area staining for CD68 (x200). **(D)** PDGF-Rα positive myofibroblasts/activated PSCs adjacent to fat tissue necrosis (x400). (Figures 4.7B-D are reprinted from (II), with permission).

secreted into the pancreatic juice (Löhr *et al.*, 1999). Also, pancreatic ductal adenocarcinoma cells express several ECM components, as demonstrated in cell

culture and on nude mice (Löhr *et al.*, 1994). Moreover, epithelial-mesenchymal transition is a well-known phenomenon in pancreatic cancer and plays a significant role for tumor invasion and metastasis. Besides, it is known that ductal cells differentiate to endocrine cells in experiments based on the duct-ligation procedure (Wang *et al.*, 1995). In these experiments, transitional cytodifferentiation forms between ductal cells expressing cytokeratin 20 and beta cells expressing insulin, or alpha cells expressing glucagon, were demonstrated (Wang *et al.*, 1995). Hence, the expression of PDGF-B and PDGF-Rα in ductal cells is an indirect indicator of epithelial-mesenchymal transition (II). Ductal cells are likely stimulated by para- and autocrine mechanisms to produce ECM components and synthesize profibrotic cytokines, which in part could explain why in some patients fibrogenic activity is maintained, even though the patients have cessated their alcohol abuse. Also, these findings emphasize the important role of tubular complexes in the pathogenesis of fibrosis in CP.

## 4.6 Tissue degradation and implications for matrix turnover

Matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes which have been classified into at least 25 types according to substrate specificity (Arthur, 2000). There has been reported an increased secretion of matrix-degrading enzymes (MMP-2 and MMP-9) by PSCs in cell culture when exposed to ethanol, TGF-β1, TNF-α or IL-6, indicating that PSCs participate not only in ECM synthesis but also in its degradation (Phillips *et al.*, 2003). However, in another cell culture study, TGF-β1 resulted in unaltered MMP-2 and even decreasing MMP-3 and MMP-9 synthesis in PSCs, pointing towards another profibrogenic effect of TGF-β1 (Shek *et al.*, 2002). Also tissue inhibitors of MMP-1 and -2 (TIMP-1 and TIMP-2) are expressed by cultured rat PSCs (Phillips *et al.*, 2003). TIMPs have been shown to inhibit MMP activity by binding to their active site, and this binding is reversible and non-covalent. Furthermore, both MMP-2 and its inhibitor TIMP-2 are upregulated in ductal cells and, less intensely, mononuclear cells, in CP (Ishihara *et al.*, 1998). MMP-2 degrades basement membrane collagen (collagen type IV) and it has been hypothesized that the increased degradation of basement membrane collagen results in deposition of fibrillar (abnormal) collagen. Besides, also MMP-9 degrades collagen type IV (Ishihara *et al.*, 1998). This abnormal collagen might then promote the development of fibrosis (Apte and Wilson, 2003).

## 4.7 Model of fibrogenesis in alcoholic chronic pancreatitis

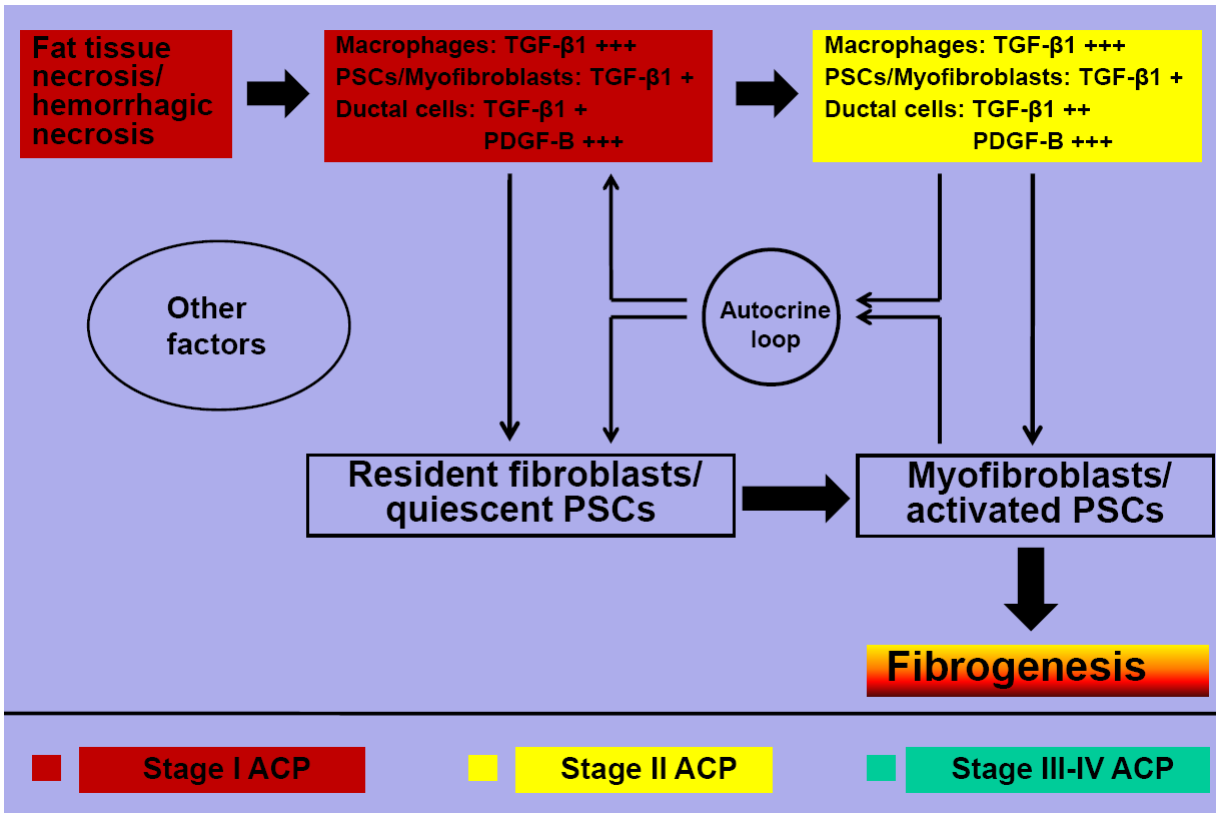
The interaction between myofibroblasts/activated PSCs, macrophages, epithelial cells and profibrotic cytokines during fibrogenesis and thus the progression from acute to chronic alcoholic pancreatitis is initiated by damage to acinar cells and ductal cells. It is illustrated schematically in Figure 4.8. The damage leads to pancreatic necroinflammation, which in turn is characterized by upregulation of mitogenic and proinflammatory chemokines in the pancreas, most importantly TNF-α,

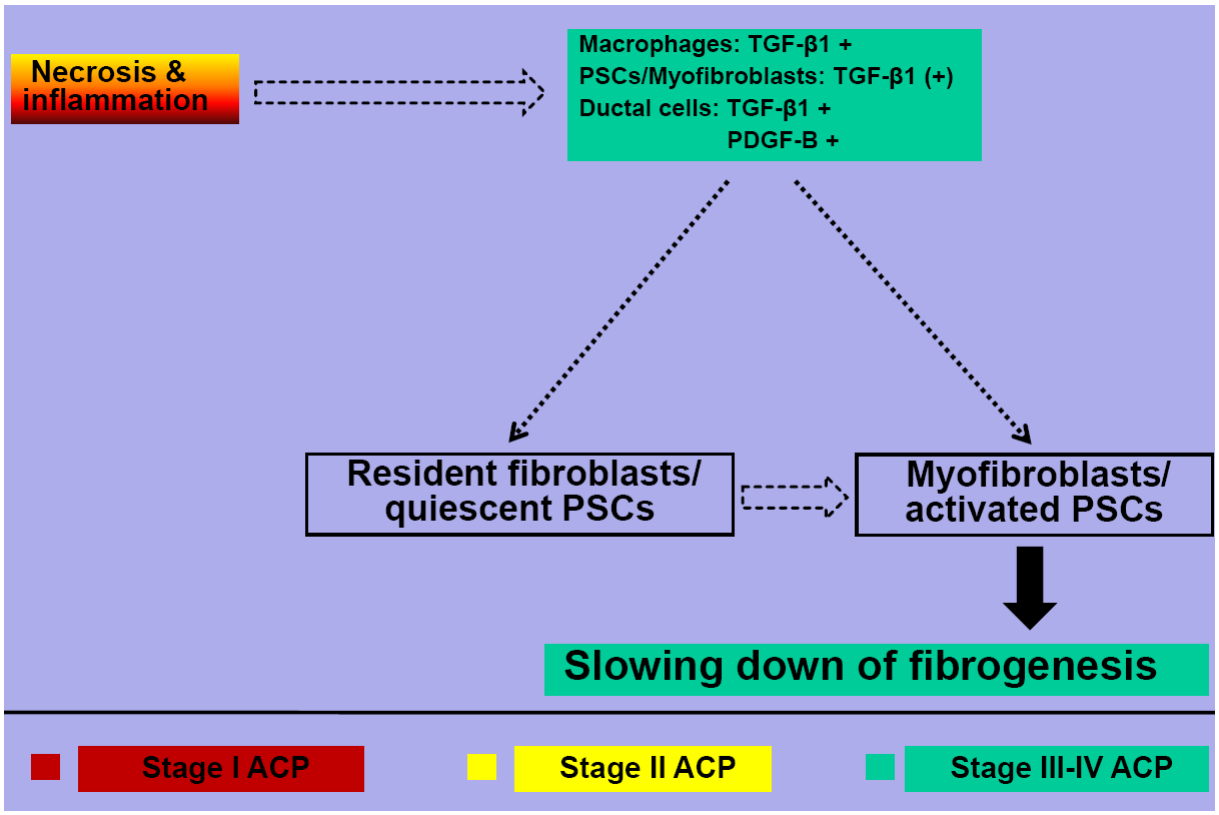
IL-1, IL-6, IL-8 and MCP-1 (Norman, 1998). Some of these inflammatory cytokines are probably also involved in the upregulation of TGF-β1 in macrophages and PDGF-B in ductal cells, and the subsequent activation of quiescent PSCs and resident fibroblasts (II). PDGF-B derives mainly from ductal cells and stimulates proliferation of myofibroblasts/activated PSCs (Apte *et al.*, 1999;Luttenberger *et al.*, 2000) (II). TGF-β1, synthesized in mainly macrophages but also epithelial cells, stimulates myofibroblasts/activated PSCs to increase their synthesis of extracellular matrix (Apte *et al.*, 1999;Luttenberger *et al.*, 2000) (II). As shown in study II, the expression and cellular distribution of the profibrotic cytokines TGF-β1 propeptide and PDGF-B is intimately related to the severity of fat tissue necrosis in ACP.

Besides, paracrine and autocrine loops seem to contribute to fibrogenesis in ACP, incorporating both TGF-β1 and PDGF-B signaling and leading to excessive stimulation of epithelial cells and their subsequent degeneration, even though there is no relapse of autodigestive necrosis. A likely explanation for the expression of TGF-β1 propeptide in activated PSCs/myofibroblasts is the autocrine stimulation of neigbouring PSCs, which are equipped with TGF-β-RII (II). Similarly, PDGF-B produced in ductal cells may stimulate neighboring ductal cells bearing the PDGF-Rα. However, in late stages, the only damage to pancreatic parenchyma that maintains active fibrogenesis is the duct ulceration that is caused by calculus formation in the ducts. These foci of active fibrogenesis, however, are very small and contribute only minimally to the development of fibrosis in late-stage ACP (II). Hence, in stage III and stage IV ACP, the fibrogenic activity is decreasing (II).

In study III we wanted to test our hypothesis that there are significant differences in the expression and localization of these profibrotic cytokines and their receptors and in the distribution of activated PSCs/myofibroblasts in ACP versus AIP (see Chapter 5).

**Figure 4.8 (next page)** Schematic, simplified illustration of fibrogenesis and thus the progression from acute to chronic alcoholic pancreatitis (ACP). ***Top:*** In stage I and II ACP, fat tissue and hemorrhagic necrosis result in accumulation of macrophages, platelets and neutrophilic granulocytes. Mainly macrophages are stimulated to synthesize TGF-β1, and mainly ductal and tubuloacinar cells are stimulated to synthesize PDGF-B. These profibrotic cytokines stimulate resident fibroblasts and quiescent PSCs to transform into myofibroblasts/ activated PSCs, resulting in strong fibrogenic activity during stage I and II ACP. ***Bottom:*** Decreasing expression of TGF-β1 and PDGF-B results in a reduction of myofibroblast activation and a slowing down of the fibrogenic activity in late stages of ACP. *Abbreviations:* PDGF-B: platelet-derived growth factor B, PSCs: pancreatic stellate cells, TGF-β1: transforming growth factor β1.


Fat tissue necrosis/ hemorrhagic necrosis
Macrophages: TGF-β1 +++
PSCs/Myofibroblasts: TGF-β1 +
Ductal cells: TGF-β1 +
PDGF-B +++
Macrophages: TGF-β1 +++
PSCs/Myofibroblasts: TGF-β1 +
Ductal cells: TGF-β1 ++
PDGF-B +++
Other factors
Autocrine loop
Resident fibroblasts/ quiescent PSCs
Myofibroblasts/ activated PSCs
Fibrogenesis
Stage I ACP
Stage II ACP
Stage III-IV ACP


Necrosis & inflammation
Macrophages: TGF-β1 +
PSCs/Myofibroblasts: TGF-β1 (+)
Ductal cells: TGF-β1 +
PDGF-B +
Resident fibroblasts/ quiescent PSCs
Myofibroblasts/ activated PSCs
Slowing down of fibrogenesis
Stage I ACP
Stage II ACP
Stage III-IV ACP

# 5. Fibrogenesis in autoimmune pancreatitis

## 5.1 Background

Autoimmune pancreatitis (AIP) has been established as a special entity of CP that is responsive to steroid treatment. However, its clinical distinction from pancreatic cancer and other entities of CP is difficult, because of the similarity of these diseases and the lack of a reliable marker of AIP (Japan Pancreas Society, 2002;Chari *et al.*, 2006;Finkelberg *et al.*, 2006;Kim *et al.*, 2006;Löhr, 2007;Okazaki *et al.*, 2007). Hallmarks of the disease are narrowing of the pancreatic duct system and the bile duct by periductal lymphoplasmacytic inflammation and sclerosis, in many cases resulting in obstructive jaundice (Ectors *et al.*, 1997;Hamano *et al.*, 2001;Klöppel *et al.*, 2003;Weber *et al.*, 2003;Klimstra and Adsay, 2004;Zamboni *et al.*, 2004). In severe stages, the pancreas is affected by large amounts of periductal, inter- and intralobular fibrosis (Ectors *et al.*, 1997;Klöppel *et al.*, 2003;Weber *et al.*, 2003;Klimstra and Adsay, 2004;Zamboni *et al.*, 2004) (III).

The probably first reports of AIP came from Ball and coworkers (Ball *et al.*, 1950) in 1950 and from Sarles et al. in 1961 (Sarles *et al.*, 1961). The term “autoimmune pancreatitis” was introduced in a paper by Yoshida and coworkers in 1995 (Yoshida *et al.*, 1995). Named “lymphoplasmacytic sclerosing pancreatitis with cholangitis”, the disease has been proposed as a special variant of primary sclerosing cholangitis by Kawaguchi and coworkers in 1991 (Kawaguchi *et al.*, 1991). Several names have been used, ranging from “lymphoplasmacytic sclerosing pancreatitis” (Notohara *et al.*, 2003), “lobulocentric AIP” (Deshpande *et al.*, 2006) “ductocentric AIP” (Deshpande *et al.*, 2006) to “idiopathic duct-destructive pancreatitis” (Notohara *et al.*, 2003). However, most recently, the term “autoimmune pancreatitis” is mainly used, and an autoimmune pathogenesis is assumed and indicated by several clinicopathological and immunological findings (see below).

Only little is known about the incidence and prevalence of AIP. Data on the prevalence derive firstly from retrospective analysis of resection specimens from different countries. In a review on Japanese pancreatic specimens, 21/451 cases (4.7%) with CP were classified as AIP. In a similar setting at the Mayo Clinic, Rochester (USA), AIP was established as the final diagnosis in 27/254 (10.6%) pancreatic resection specimens with CP. Secondly, in an Italian multicenter study that enrolled 383 patients suffering from CP during the years 2001 and 2002, 23 patients (6%) were classified with “autoimmunity as an associated factor” (Pearson *et al.*, 2003). Mean age of patients suffering from AIP is 56 (range 40 – 60) (Ectors *et al.*, 1997;Weber *et al.*, 2003;Abraham *et al.*, 2003;Zamboni *et al.*, 2004). Some patients are younger, below the age of 20, and a few have been reported to be older than 70. In our own series which we examined in study III, the mean age was 54 years. The youngest patient was 13 and the oldest patient was 78 years old (III), while in our series based on core needle biopsy specimens, the age range was ten to 79 years (mean age: 51 years) (IV). To the best of our knowledge, no studies exist clarifying the incidence and prevalence of AIP in the Scandinavian population.

## 5.2 Pathogenesis of autoimmune pancreatitis

The pathogenesis of AIP is still not well known. Several findings suggest that an autoimmune mechanism is involved. For example, similar to Sjögren´s disease and primary sclerosing cholangitis, there is a predominance of CD4+ Th1 cells over Th2 cells in some cases (Okazaki *et al.*, 2000). AIP patients in Japan show a particular human leukocyte antigen two-loci haplotype, DRB1*0405-DQB1*0401 (Kawa *et al.*, 2002). Autoimmune antibodies like antinuclear antibody, anti-lactoferrin and anti-carbonic anhydrase II (Okazaki *et al.*, 2000) as well as elevated serum-IgG and -IgG4 levels (Hamano *et al.*, 2001) and the common association of AIP with other autoimmune diseases, namely Sjögren´s syndrome (Montefusco *et al.*, 1984;Yoshida *et al.*, 1995), are further indicators of an autoimmune pathomechanism.

In a recent study it was suggested that AIP might be a systemic immune complex mediated disease (Deshpande *et al.*, 2006). It was shown in renal biopsy specimens from AIP patients, that tubular basement membrane granular deposits contained IgG4 (Deshpande *et al.*, 2006). Kamisawa and coworkers noted that extensive IgG4 positive plasma cell and T lymphocyte infiltration not only is present in the pancreas but also in other organs in a number of AIP patients (Kamisawa *et al.*, 2003;Kamisawa *et al.*, 2006). Hence, he proposed a new clinicopathological entity of IgG4-related sclerosing disease, including AIP as one of the main manifestations together with retroperitoneal fibrosis, sclerosing sialadenitis and sclerosing cholangitis (Kamisawa and Okamoto, 2006).

In peripheral blood from AIP patients, increased numbers of T cells bearing HLA-DR were found (Okazaki *et al.*, 2000). Interestingly, HLA-DR antigens have also been detected on pancreatic duct cells (Ectors *et al.*, 1997;Uchida *et al.*, 2000). In study III, we detected α-SMA, TGF-β-RII and PDGF-R positive activated PSCs/myofibroblasts which accumulated around small intralobular ducts (III, se below). Moreover, they sometimes formed a thin layer directly beneath the epithelium of interlobular ducts in addition to the capsule-like lesions which surrounded the periductal lymphoplasmacytic infiltrates (see Chapter 5.3) (III). It remains to be elucidated, whether this attraction of activated PSCs/myofibroblasts to epithelial structures of intralobular and interlobular ducts in AIP is related to a potential antigen located in the ductal epithelium and why this immune process is mostly focal and not diffuse.

## 5.3 Distribution pattern of fibrosis, lymphocytes, plasma cells, macrophages and activated PSCs/myofibroblasts in autoimmune pancreatitis

Several authors have contributed to the main histopathological findings in AIP (Klöppel *et al.*, 2003;Weber *et al.*, 2003;Klöppel *et al.*, 2005;Klöppel *et al.*, 2007) (III). Zamboni and coworkers established a grading system (Zamboni *et al.*, 2004) which, together with our experience with 52 AIP specimens in study III, forms the basis of the following description of the histopathological findings in AIP. Our observations in pancreatic core needle biopsies from AIP patients are described in Chapter 6 (IV).

Severe grades of AIP are characterized by increasing amounts of fibrosis in the pancreas. Our findings on the pattern and development of fibrosis in AIP are schematically illustrated in Figure 5.1 (III).

Grade 1 AIP is characterized by scattered periductal lymphoplasmacytic infiltrates resulting in mild narrowing of medium- and large-sized ducts (Zamboni *et al.*, 2004). The infiltrates consist of CD4 and CD8 positive lymophocytes and CD79a and IgG4 positive plasma cells (III). Moreover, as shown by CD68 immunostaining, the infiltrates are intermingled with macrophages (III). Intralobular areas are almost unaffected.

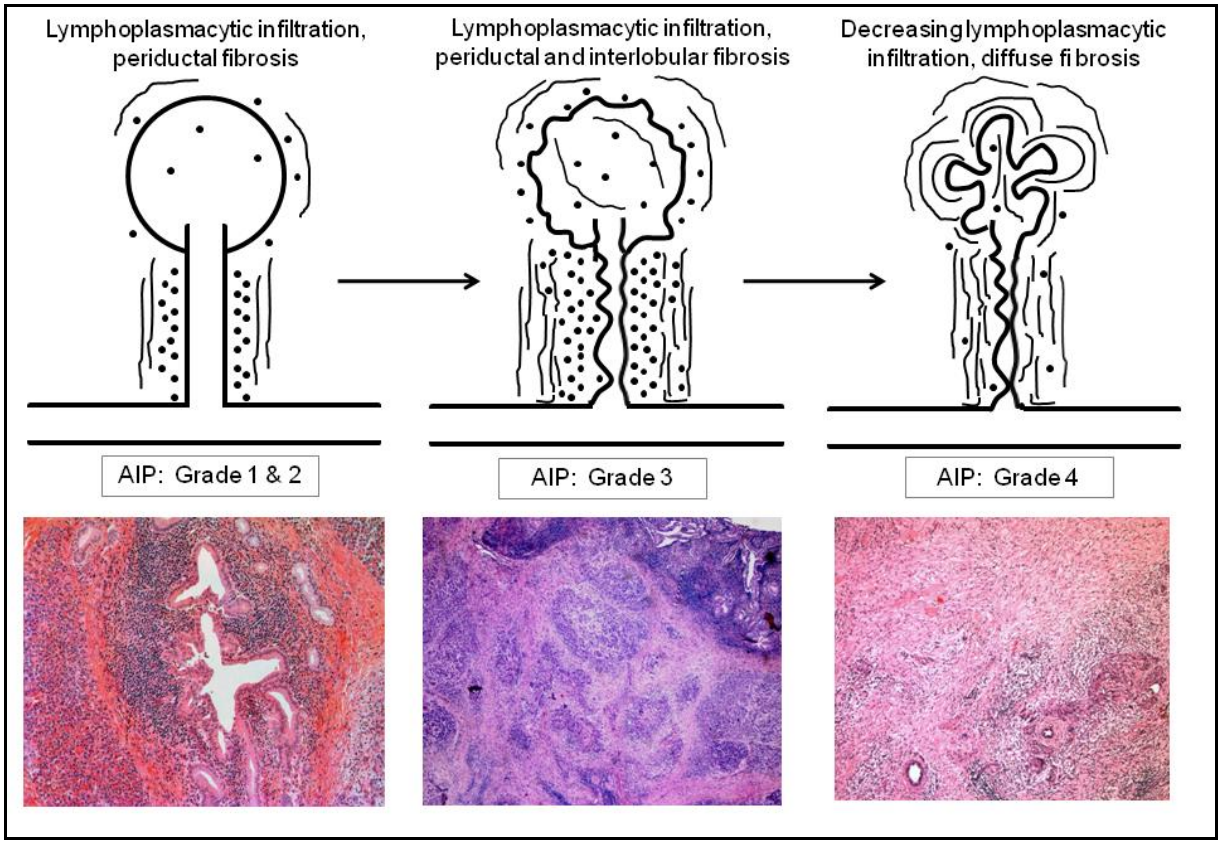



**Figure 5.1** Schematic illustration of the development of fibrosis in autoimmune pancreatitis (AIP). ***Left:*** In grade 1&2 AIP, fibrosis is mainly seen in the capsule-like lesions surrounding the periductal lymphoplasmacytic infiltrates. ***Center:*** In grade 3 AIP, the fibrosis extends to interlobular and intralobular areas, accompanied by inflammatory infiltrates consisting of lymphocytes, plasma cells and macrophages. ***Right:*** In grade 4 AIP, when the ducts are severely narrowed and most acinar tissue has been replaced by fibrosis, fibrosis is diffuse, but still most prominent in periductal areas. Lymphoplasmacytic infiltration is less intense than in grade 2&3 AIP. (Parts of this figure are reprinted from (III), with permission).

In grade 2 AIP, there are numerous periductal lymphoplasmacytic infiltrates, mild periductal fibrosis, and mild interlobular and acinar inflammation (Fig. 5.2A) (Zamboni *et al.*, 2004). Sometimes there are areas with focal inflammatory cellular fibrosis replacing acinar tissue and mild venulitis. The lymphoplasmacytic infiltrates are intermingled with CD68 positive macrophages (Fig. 5.2B-C) (III). Myofibroblasts form capsule-like lesions surrounding the periductal lymphoplasmacytic infiltrates of

medium- and large-sized interlobular ducts (Fig. 5.2D) (III).

Grade 3 AIP is characterized by diffuse periductal lymphoplasmacytic infiltrates and marked periductal fibrosis causing partial duct obstruction (Fig. 5.3A) (Zamboni *et al.*, 2004). The partial duct destruction gives the lumen a star-like structure, and fibrosis intermingled with activated PSCs/myofibroblasts encompasses the ducts almost completely (III). Furthermore, there is strong lymphoplasmacytic infiltration in interlobular and acinar spaces. We noted that myofibroblasts/activated PSCs are frequently arranged in a thin layer directly beneath the epithelium of small intralobular and medium-sized interlobular ducts (Fig. 5.3B) (III). Focally, there are lesions with moderate cellular fibrosis replacing the acinar tissue (Fig. 5.4A) (III). These lesions are composed of plasma cells (Fig. 5.4B), lymphocytes (Fig. 5.4C) and macrophages (III). The inflammatory cells are intermingled with myofibroblasts/ activated PSCs, which are arranged in a storiform, whirling pattern (Fig. 5.4D) (III). Venulitis is frequently noted, and some lymphoid follicles are distributed throughout the tissue (III).

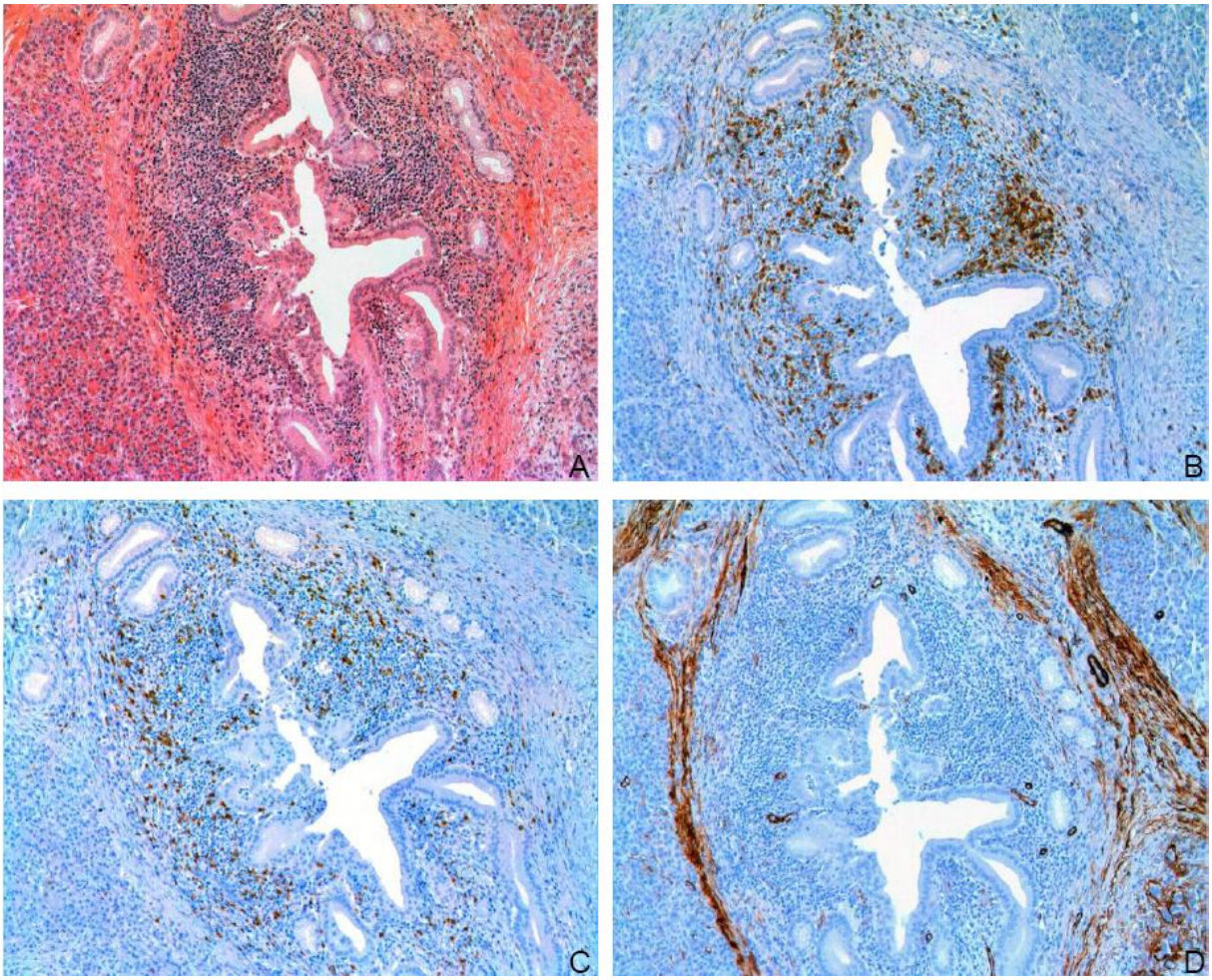



**Figure 5.2** Periductal lymphoplasmacytic infiltration in autoimmune pancreatitis, grade 2. **(A)** (H&E, x100). **(B)** CD79a positive plasma cells (x100). **(C)** CD8 positive lymphocytes (x100). **(D)** α-SMA positive myofibroblasts/activated PSCs forming a capsule-like lesion surrounding the lymphoplasmacytic infiltrate (x100). (Figures 5.2A-B & 5.2D are reprinted from (III), with permission).

In grade 4 AIP, there still are diffuse periductal lymphoplasmacytic infiltrates, causing sometimes total duct obstruction. There is severe periductal, interlobular and intralobular fibrosis (Fig. 5.10) (III). The lymphoplasmacytic inflammation surrounding medium- and large-sized ducts, which is also seen interlobularly and intralobularly, is stepwise replaced by fibrotic tissue (III). The cellular fibrosis replacing acinar tissue is severe and accompanied by a diffuse sclerosis. Venulitis is sometimes accompanied by arteritis. Moreover, there are scattered and occasionally prominent lymphoid follicles. As illustrated schematically in Figure 5.1, grade 4 AIP is characterized by increasing amounts of fibrosis in the pancreas, while lymphoplasmacytic inflammation is decreasing (III).

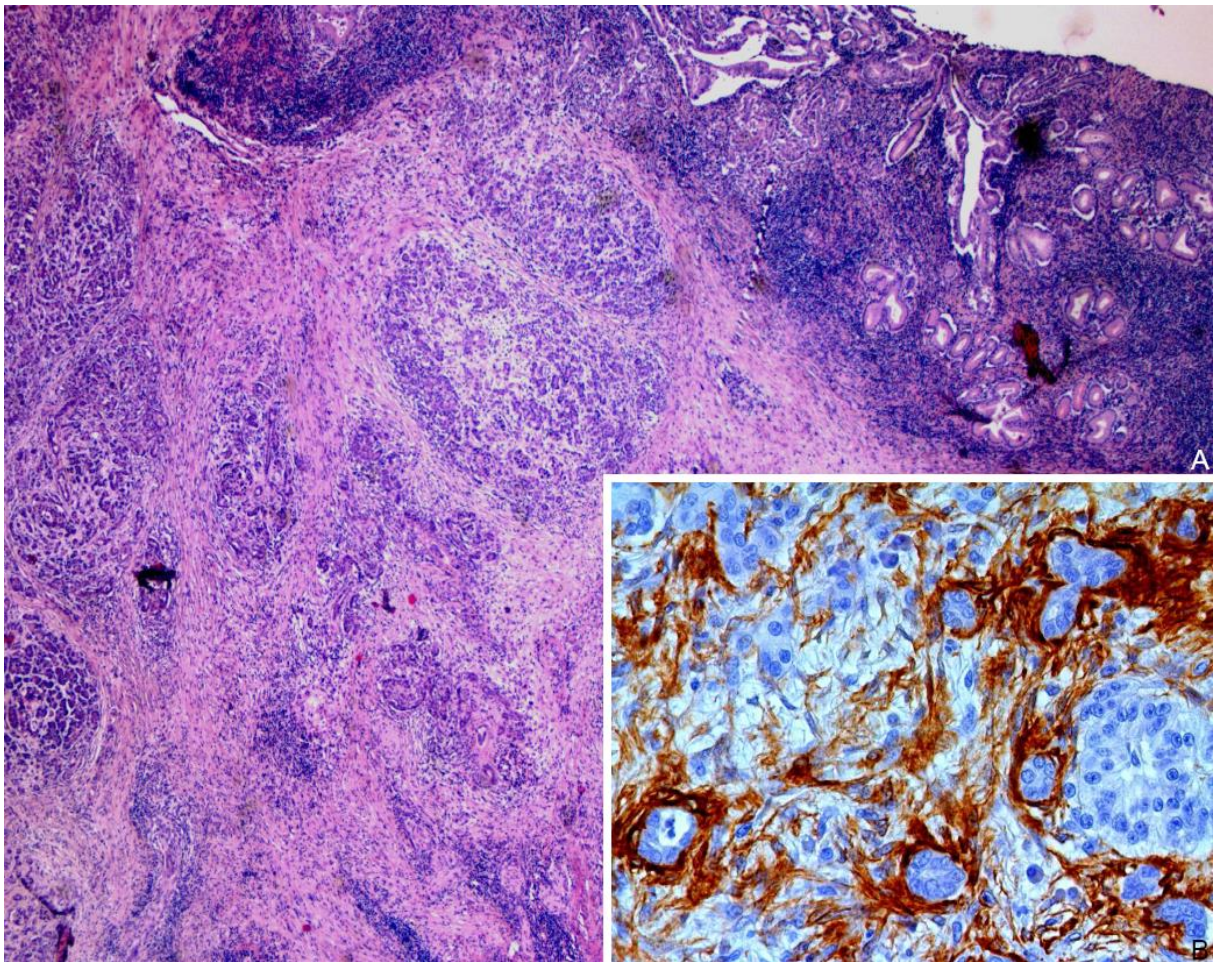



**Figure 5.3** Autoimmune pancreatitis, grade 3: Periductal lymphoplasmacytic infiltrates extend to interlobular and intralobular areas. **(A)** (H&E, x50). **(B)** Inset: α-SMA positive myofibroblasts/activated PSCs surrounding intralobular tubular complexes (x400).

Granulocytic epithelial lesions (GELs) represent a specific histopathological feature in approximately 45% of AIP patients (Fig. 5.6A) (Zamboni *et al.*, 2004) (III, IV). This lesion is characterized by focal disruption and destruction of the duct epithelium resulting from invasion of mainly neutrophilic granulocytes (Ectors *et al.*, 1997). GELs are recognized in the subgroup of younger AIP patients with an equal gender distribution which sometimes is associated with chronic inflammatory bowel disease (Zamboni *et al.*, 2004). GELs do not only consist of neutrophilic granulocytes

but occasionally also of eosinophilic granulocytes and, as shown by CD68 immunostaining, macrophages (Fig. 5.6C) (III).

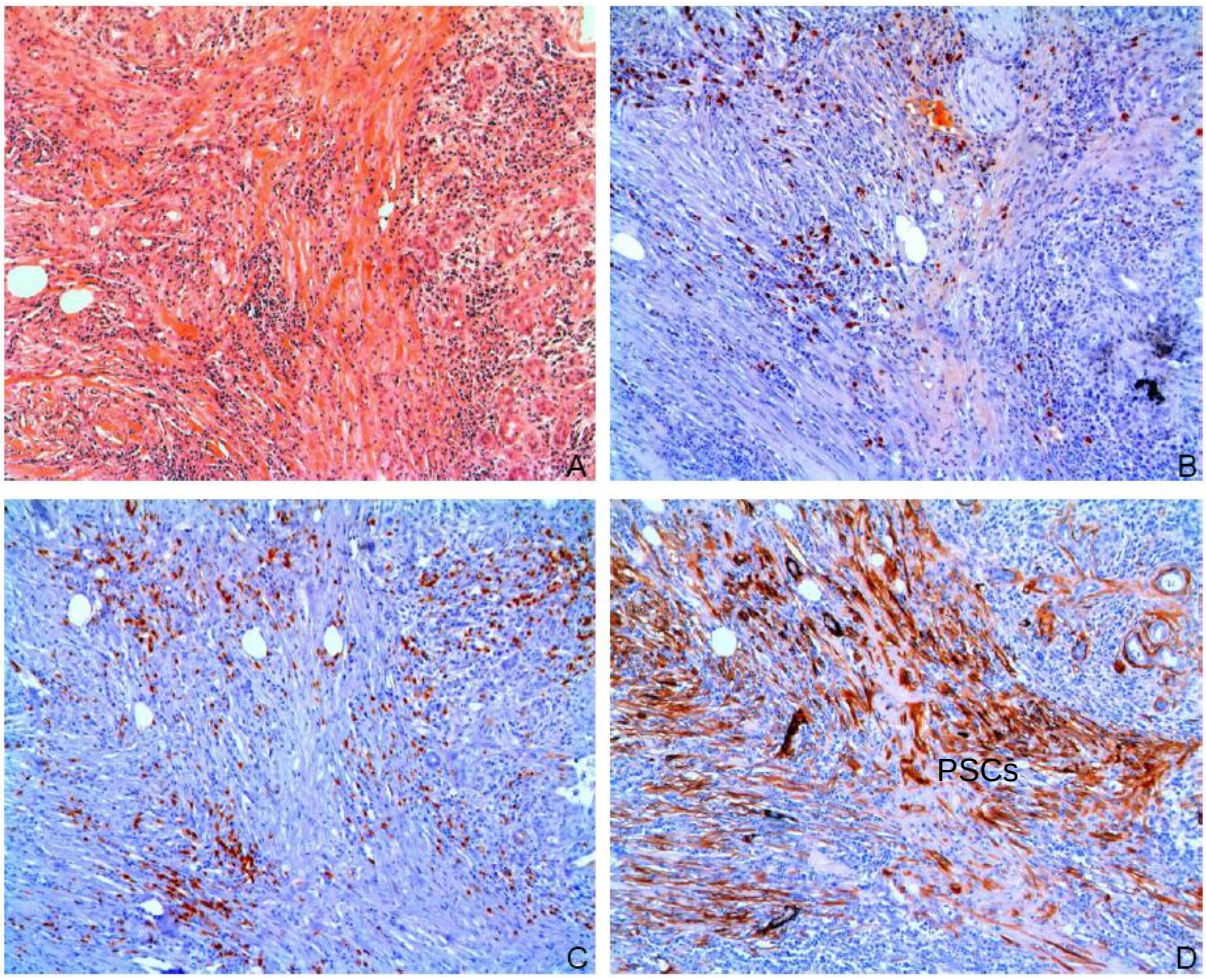



**Figure 5.4** Cellular fibrosis with inflammation in autoimmune pancreatitis, grade 3. **(A)** (H&E, x100). **(B)** IgG4 positive plasma cells (x100). **(C)** CD8 positive lymphocytes (x100). **(D)** α-SMA positive myofibroblasts/activated PSCs (x100).

## 5.4 Localization of TGF-β1, PDGF-B and their receptors in autoimmune pancreatitis

As mentioned earlier, it is important to elucidate the cellular source of the profibrotic cytokines that stimulate PSCs/myofibroblasts to produce ECM, and the best method to localize these peptides within the tissue is immunohistochemical studies. Moreover, as it has been described in a few AIP patients that fibrotic lesions have resolved after steroid treatment (Saito *et al.*, 2002;Song *et al.*, 2005b), it is tempting to speculate that there may be differences in the expression of profibrotic cytokines and their receptors in human pancreatic specimens with ACP (see Chapter 4) versus AIP. Immunopositivity for the TGF-β1 propeptide LAP has in ACP been observed particularly in macrophages by ourselves and others (Van Laethem *et al.*, 1995) (II), and Fukumura and coworkers noted a similar expression profile in cancer-associated, obstructive CP (Fukumura *et al.*, 2006). We were able to show that this is

also the case in tissue specimens with AIP (III). Together with activated PSCs/myofibroblasts, macrophages expressed TGF-β1 propeptide, and this expression was strongest in grade III AIP (Fig. 5.5D & Fig. 5.6.D). These data indicate strongly that mainly macrophages, but also tubuloacinar cells, release TGF-β1 and activate nearby resident fibroblasts and quiescent PSCs to transform into myofibroblasts/ activated PSCs also in AIP (III). Activated PSCs/myofibroblasts express TGF-β1 less intensely (III), but are strongly positive for TGF-β-RII (Satoh *et al.*, 1998;di Mola *et al.*, 1999) (II) and TGF-β-RI (di Mola *et al.*, 1999) not only in ACP but also in AIP (Fig. 5.5F) (III). They are therefore probably susceptible to TGF-β1. However, in grade 4 AIP the expression of TGF-β1 propeptide and TGF-β-RII was decreasing (III).

We found ductal cells and tubuloacinar cells next to lymphoplasmacytic inflammatory infiltrates together with myofibroblasts/activated PSCs to be the most important source of PDGF-B in AIP (Fig. 5.5E & Fig. 5.7) (III). This indicates that epithelial cells in AIP, particularly the ductal cells, are involved in the production of PDGF-B and stimulate quiescent PSCs/resident fibroblasts to transform into activated PSCs/ myofibroblasts. Besides, they probably stimulate activated PSCs/myofibroblasts that are equipped with PDGF-Rα and -Rβ (Fig. 5.8B & Fig. 5.9B). This results in proliferation of the myofibroblasts/activated PSCs in the immediate vicinity of the pancreatic ducts and induces them to accumulate (III). The expression of PDGF-B and its receptors was strongest in grade 2 and 3 and decreasing in grade 4 AIP (Fig. 5.10B) (III).

## 5.5 Model of fibrogenesis in autoimmune pancreatitis

We noted that the severity of the lymphoplasmacytic infiltration in AIP correlates well with the distribution and number of myofibroblasts/activated PSCs. Moreover, we show that the expression of the two most important profibrotic cytokines, the TGF-β1 propeptide LAP and PDGF-B, by macrophages and ductal cells and the expression of TGF-β-RII and PDGF-Rα/PDGF-Rβ in myofibroblasts/activated PSCs, are strongest in grade 2 and 3 of AIP (III). In grade 4, the inflammatory activity was decreasing, which resulted in a slowing down of fibrogenesis, as emphasized by decreasing expression of the profibrotic cytokines and their receptors (see also Figure 5.11). Paracrine and autocrine loops seem to contribute to fibrogenesis also in AIP, incorporating both TGF-β1 and PDGF-B signaling. Similarly to our findings in ACP (II), the expression of TGF-β1 propeptide in activated PSCs/myofibroblasts in AIP probably leads to autocrine stimulation of neigbouring PSCs, which are equipped with TGF-β-RII (III). Also, PDGF-B produced in ductal cells and activated PSCs/myofibroblasts in AIP may stimulate neighboring ductal cells bearing the PDGF-Rα and myofibroblasts expressing the PDGF-Rα and -Rβ (III).

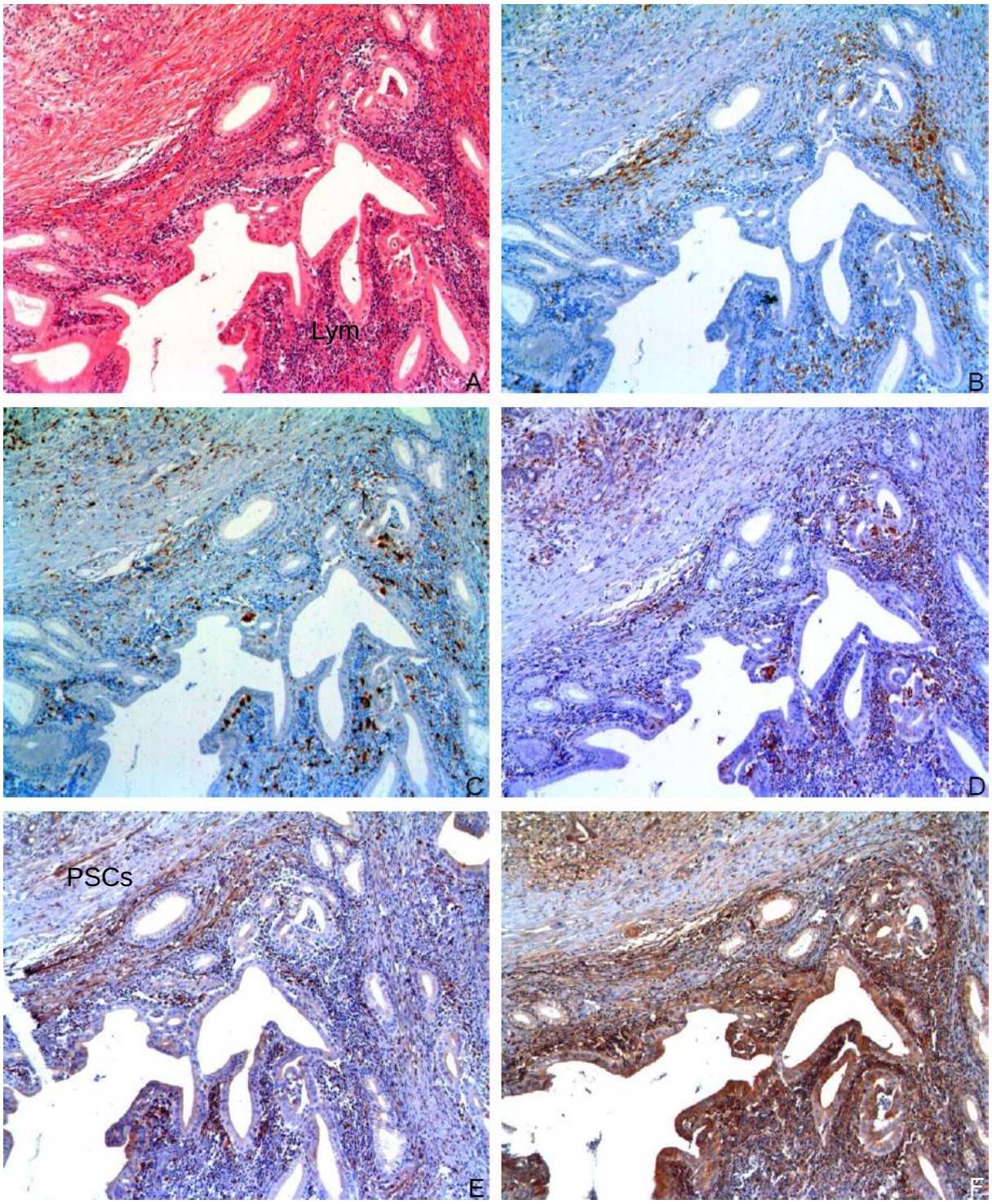



**Figure 5.5** Periductal lymphoplasmacytic infiltration in autoimmune pancreatitis, grade 3. **(A)** The lymphoplasmacytic infiltrate (Lym) is surrounded by a collar of fibrotic tissue admixed with myofibroblasts/activated PSCs (H&E, x100). **(B)** CD79a positive plasma cells (x100). **(C)** CD68 positive macrophages. **(D)** TGF-β1 propeptide positive macrophages (x100). **(E)** PDGF-B positive myofibroblasts/activated PSCs, macrophages and ductal cells (x100). **(F)** TGF-β-RII positive myofibroblasts/activated PSCs, ductal cells and macrophages (x100).

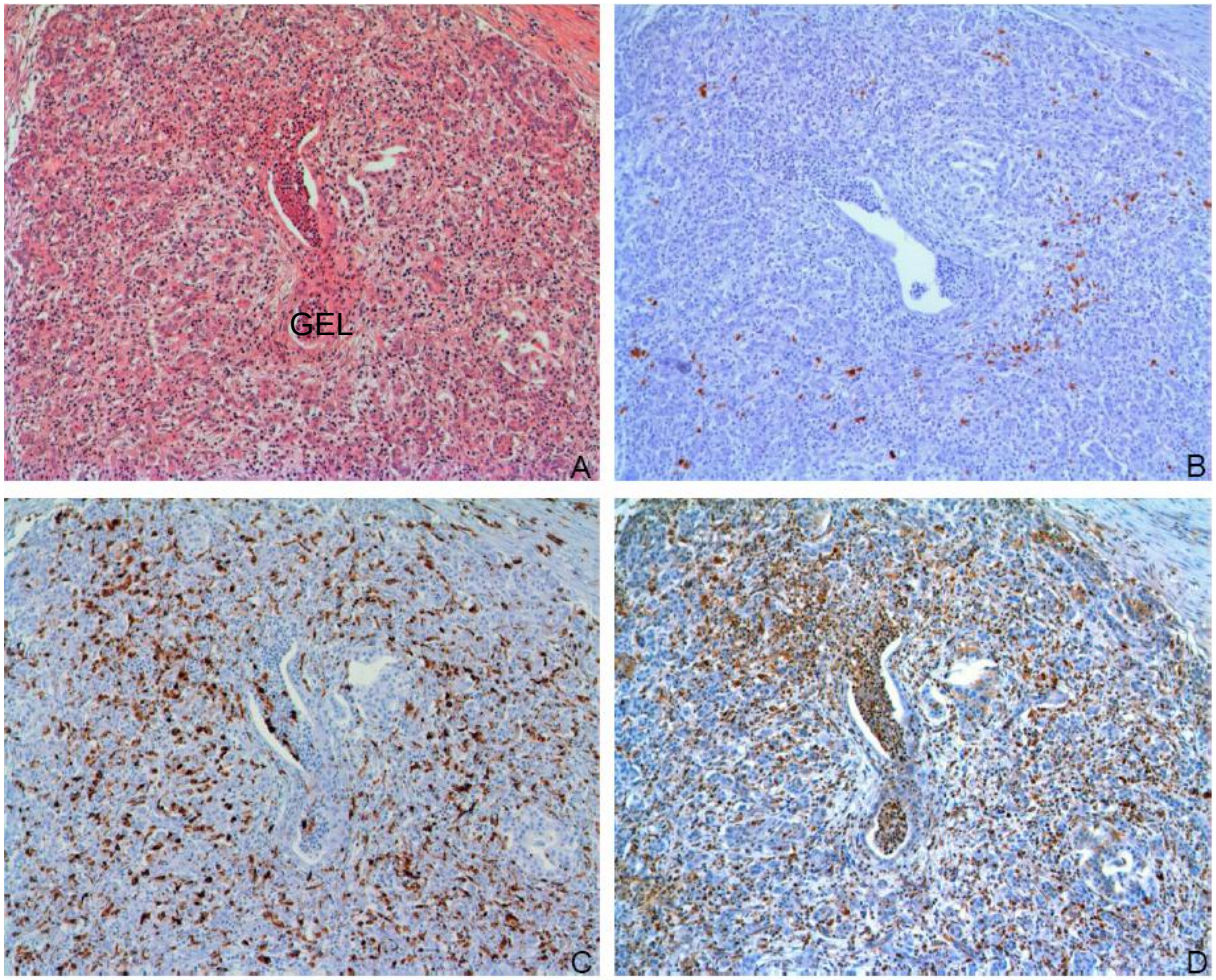



**Figure 5.6** Granulocytic epithelial lesion (GEL) in autoimmune pancreatitis, grade 3. **(A)** The GEL is surrounded by strong intralobular lymphoplasmacytic inflammation (H&E, x100). **(B)** CD79a positive plasma cells (x100). **(C)** CD68 positive macrophages (x100). **(D)** TGF-β1 propeptide positive macrophages (x100). (This figure is reprinted from (III), with permission).

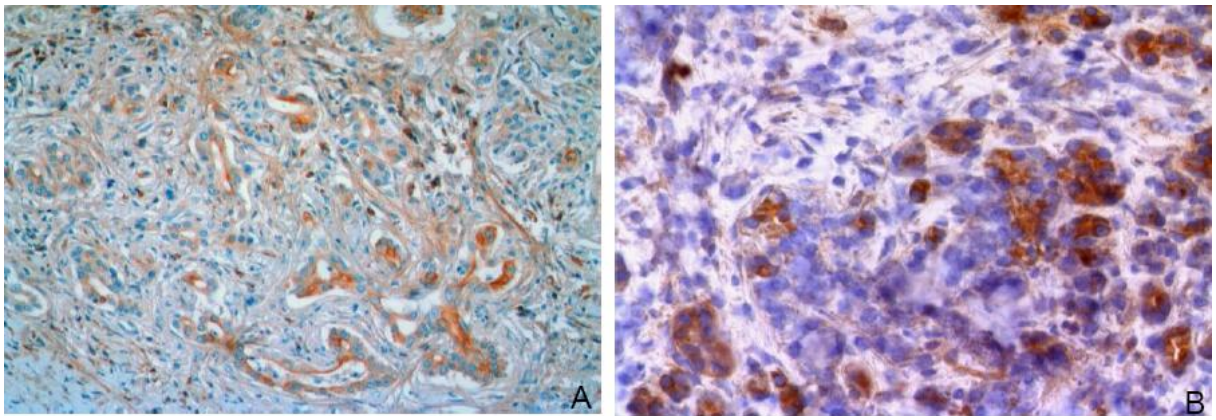



**Figure 5.7** Autoimmune pancreatitis, grade 3. PDGF-B positivity in ductal cells, tubular complexes and myofibroblasts/activated PSCs. **(A)** (x200). **(B)** (x400).

Hence, even though it has been described in a few patients that fibrotic lesions in AIP have resolved after steroid treatment (Saito *et al.*, 2002;Song *et al.*, 2005b), our data did not reveal fundamental differences in expression of profibrotic cytokines and their receptors in ACP versus AIP (II, III). A possible alternative explanation

came from a study demonstrating that there are remarkable differences in the deposition of collagen type IV between AIP and ACP (Song *et al.*, 2005a). In this study, the staining of collagen type IV was absent or sparse in ACP but almost normal in AIP. With intact framework of the basement membrane, it has been shown several decades ago that the initial structure of the pancreas can regenerate (Fitzgerald *et al.*, 1968). Because collagen type IV serves as scaffold of the basement membrane, it was hypothesized that the maintained expression of collagen type IV in AIP would result in the persistence of the potential of acinar cells to regenerate and in the resolution of fibrosis (Song *et al.*, 2005a).

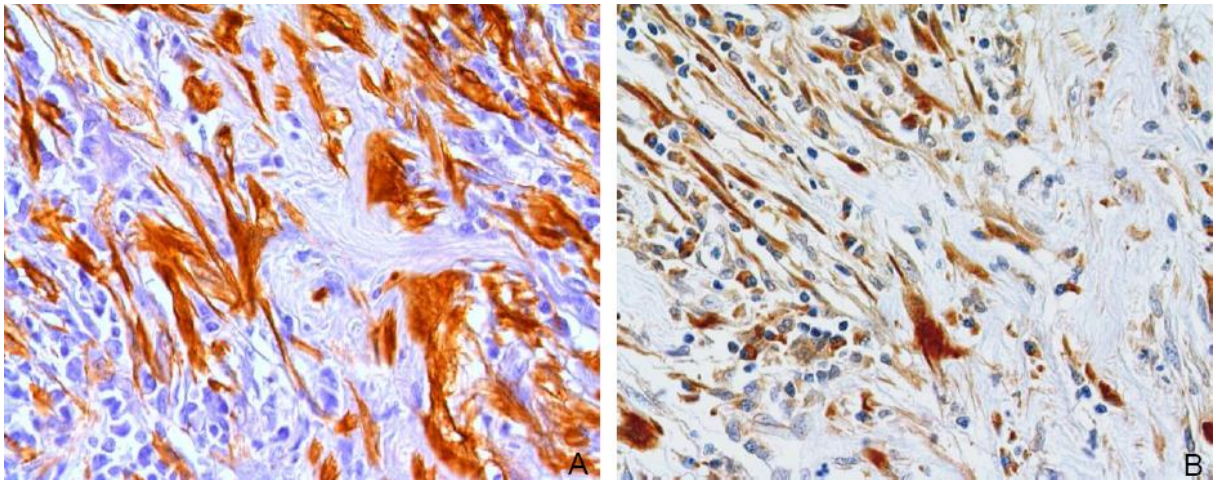



**Figure 5.8** Cellular fibrosis replacing acinar tissue in autoimmune pancreatitis, grade 3. **(A)** α-SMA positive myofibroblasts/activated PSCs are intermingled with lymphocytes, macrophages and plasma cells in a storiform pattern (x400). **(B)** Many myofibroblasts/ activated PSCs show PDGF-Rα positivity (x400).

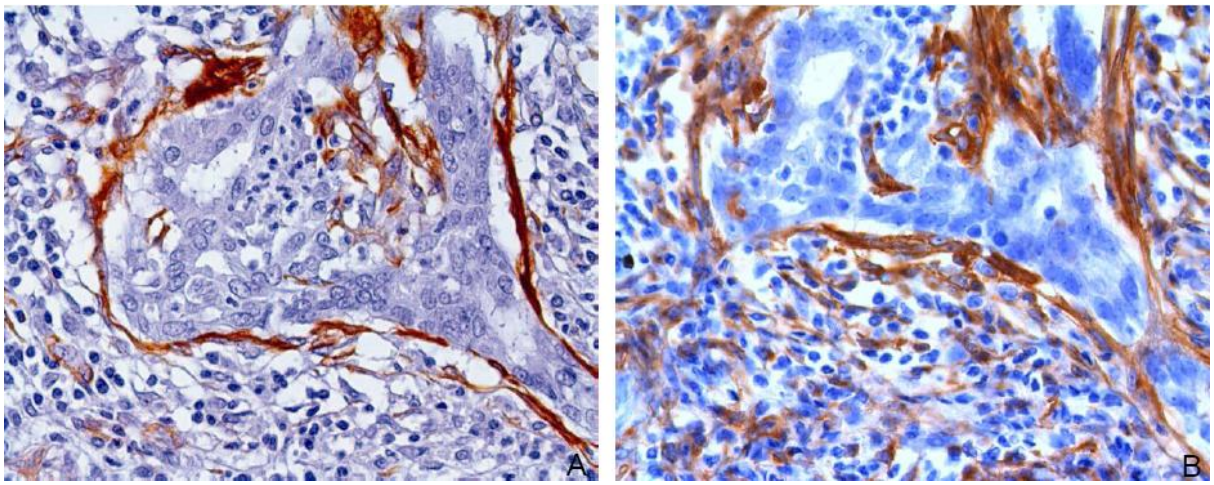



**Figure 5.9** Periductal lymphoplasmacytic infiltration in autoimmune pancreatitis, grade 3. **(A)** α-SMA positive myofibroblasts/activated PSCs directly beneath the epithelium of a pancreatic duct showing a granulocytic epithelial lesion (GEL) (x400). **(B)** Almost all myofibroblasts/activated PSCs show PDGF-Rβ positivity (x400).

We found, however, fundamental differences in the distribution pattern of activated PSCs/myofibroblasts in AIP versus ACP. In stage I ACP, activated PSCs/ myofibroblasts were concentrated in the wall of pseudocysts, while in AIP, they for-

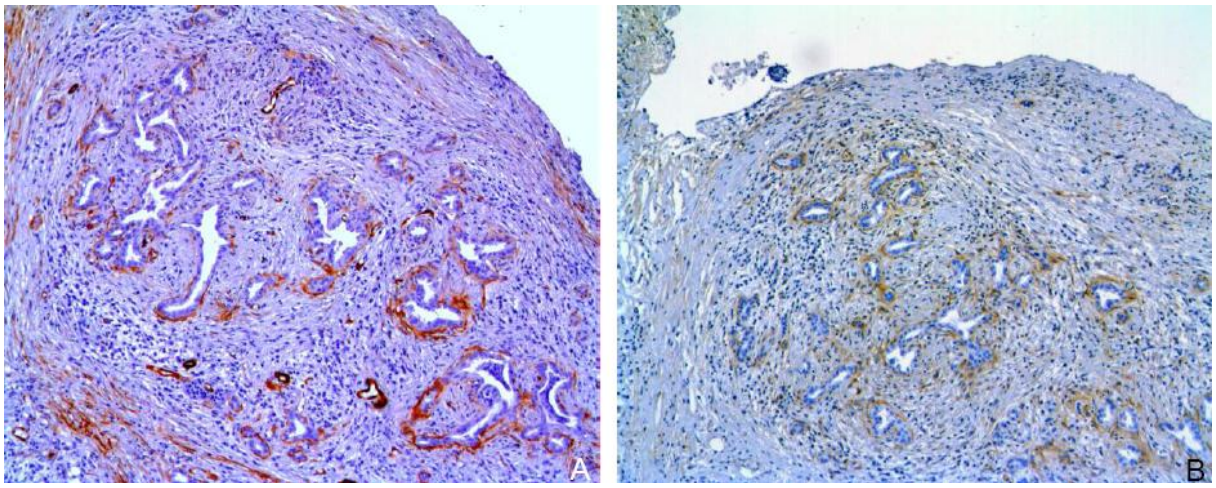
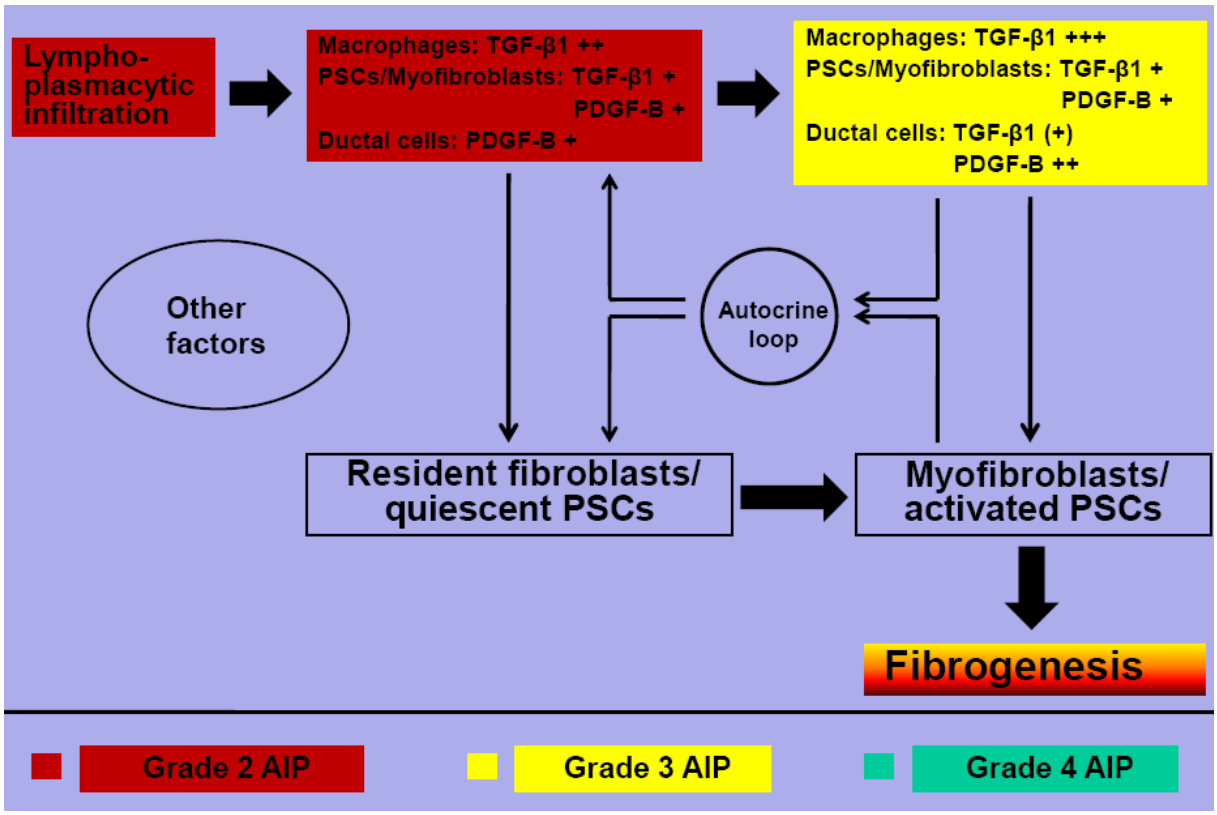
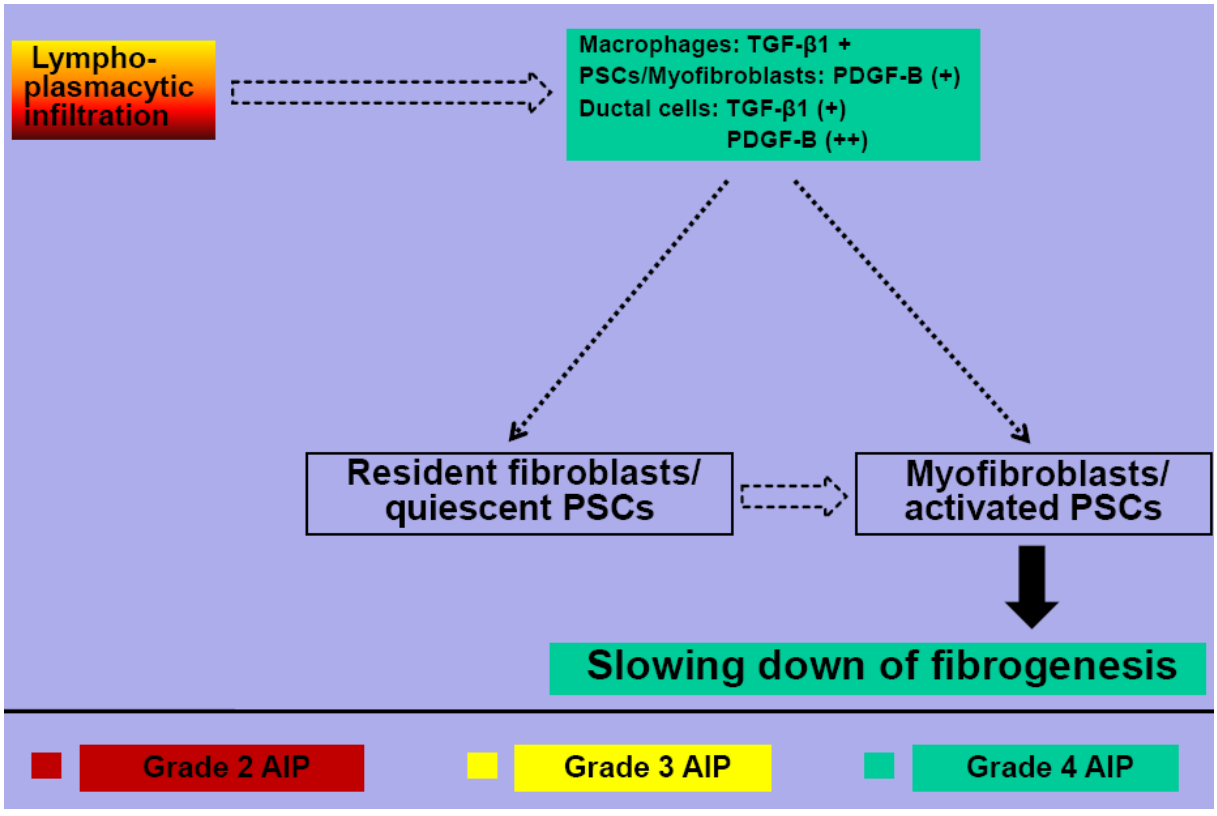



**Figure 5.10** Fibrosis in autoimmune pancreatitis, grade 4. **(A)** α-SMA positive myofibroblasts/activated PSCs are sparse and mainly located directly beneath the epithelium of pancreatic ducts (x100). **(B)** Scattered PDGF-Rβ positive myofibroblasts/ activated PSCs (x100). (Reprinted from (III), with permission).

med capsule-like lesions surrounding the periductal lymphoplasmacytic infiltrates and accumulated directly beneath the epithelium of small and medium-sized pancreatic ducts (II, III). In late stages of the two diseases, however, scattered myofibroblasts/ activated PSCs were noted also interlobularly and intralobularly (II, III). To elucidate if and to what extent steroid treatment really can resolve fibrosis in AIP, it would be interesting to obtain pancreatic core needle biopsies from a larger number of AIP patients before and after treatment. However, even though the performance of EUS guided trucut biopsy is associated with only very rarely occuring complications including pancreatitis, bleeding and tumor seeding (Levy *et al.*, 2006), the decision to perform another biopsy after treatment would of course have to be otherwise clinically justified in the respective patients. Besides, we wondered if the recognition of inflammatory and fibrotic changes could help to improve the utility of pancreatic core needle biopsy for the differential diagnosis AIP versus ACP and the margin of pancreatic cancer showing inflammation and fibrosis. Therefore, in study IV we focused on the usefulness of pancreatic core needle biopsy for the diagnosis of AIP (see Chapter 6).

**Figure 5.11 (next page)** Schematic, simplified illustration of fibrogenesis in autoimmune pancreatitis (AIP). ***Top:*** In grade 2 and 3 AIP, lymphoplasmacytic infiltration results in accumulation of lymphocytes, plasma cells, macrophages and activated PSCs/ myofibroblasts. Mainly macrophages are stimulated to synthesize TGF-β1, and mainly ductal cells/tubuloacinar cells are stimulated to synthesize PDGF-B. These profibrotic cytokines stimulate resident fibroblasts and quiescent PSCs to transform into myofibroblasts/activated PSCs, resulting in strong fibrogenic activity during grade 2 and 3 AIP. ***Bottom:*** Decreasing expression of TGF-β1 and PDGF-B results in a reduction of myofibroblast activation and a slowing down of the fibrogenic activity in grade 4 AIP. *Abbreviations:* PDGF-B: platelet-derived growth factor B, PSCs: pancreatic stellate cells, TGF-β1: transforming growth factor β1.


Lympho-plasmacytic infiltration
Macrophages: TGF-β1 ++
PSCs/Myofibroblasts: TGF-β1 +
PDGF-B +
Ductal cells: PDGF-B +
Macrophages: TGF-β1 +++
PSCs/Myofibroblasts: TGF-β1 +
PDGF-B +
Ductal cells: TGF-β1 (+)
PDGF-B ++
Other factors
Autocrine loop
Resident fibroblasts/ quiescent PSCs
Myofibroblasts/ activated PSCs
Fibrogenesis
Grade 2 AIP
Grade 3 AIP
Grade 4 AIP


Lympho-plasmacytic infiltration
Macrophages: TGF-β1 +
PSCs/Myofibroblasts: PDGF-B (+)
Ductal cells: TGF-β1 (+)
PDGF-B (++)
Resident fibroblasts/ quiescent PSCs
Myofibroblasts/ activated PSCs
Slowing down of fibrogenesis
Grade 2 AIP
Grade 3 AIP
Grade 4 AIP

# 6. Diagnosis of autoimmune pancreatitis by core needle biopsy

## 6.1 Background

According to the criteria of the Japan Pancreas Society, AIP can be diagnosed on the basis of imaging findings together with *either* laboratory findings (elevated levels of serum-gammaglobulin and/or -IgG or presence of autoantibodies) *or* histo-pathological findings (marked lymphoplasmacytic infiltration and fibrosis) (Japan Pancreas Society, 2002;Okazaki *et al.*, 2007). The criteria of the Korean working group include imaging, laboratory findings, histological findings and response to steroid treatment. For the diagnosis of AIP, the imaging criterion is required along with any one of the other three features (Kim *et al.*, 2006). The criteria of the Mayo Clinic (HISORt criteria) are histopathology (marked lymphoplasmacytic infiltration and occurrence of more than 10 IgG4 positive plasma cells per high power field (HPF)), imaging, serology, presence of other organ involvement and response to steroid treatment (Chari *et al.*, 2006). Interestingly, also a two-week steroid trial can be useful in differentiating AIP from pancreatic cancer in some cases (Chari *et al.*, 2006;Moon *et al.*, 2008). In contrast to the other working groups, the Mayo Clinic group already diagnoses AIP if only the histology is positive. The fact that with the HISORt criteria AIP can be diagnosed on the basis of typical histopathological features alone, highlights the special significance of pancreatic core needle biopsy. However, the role of pancreatic biopsy in the diagnosis of AIP is still controversial, because the available data are sparse and its usefulness is not generally accepted. In a previous study, diagnostically relevant lesions were noted in three of four needle biopsy specimens (Zamboni *et al.*, 2004). In other studies, the effectiveness of core needle biopsy in diagnosing AIP was less significant (Weber *et al.*, 2003;Deshpande *et al.*, 2005a;Bang *et al.*, 2008). This also held for fine-needle aspiration cytology (FNA), which was thought to be able to exclude carcinoma, but which lacks specific features enabling the diagnosis of AIP in most patients (Levy *et al.*, 2005;Deshpande *et al.*, 2005b;Levy *et al.*, 2006).

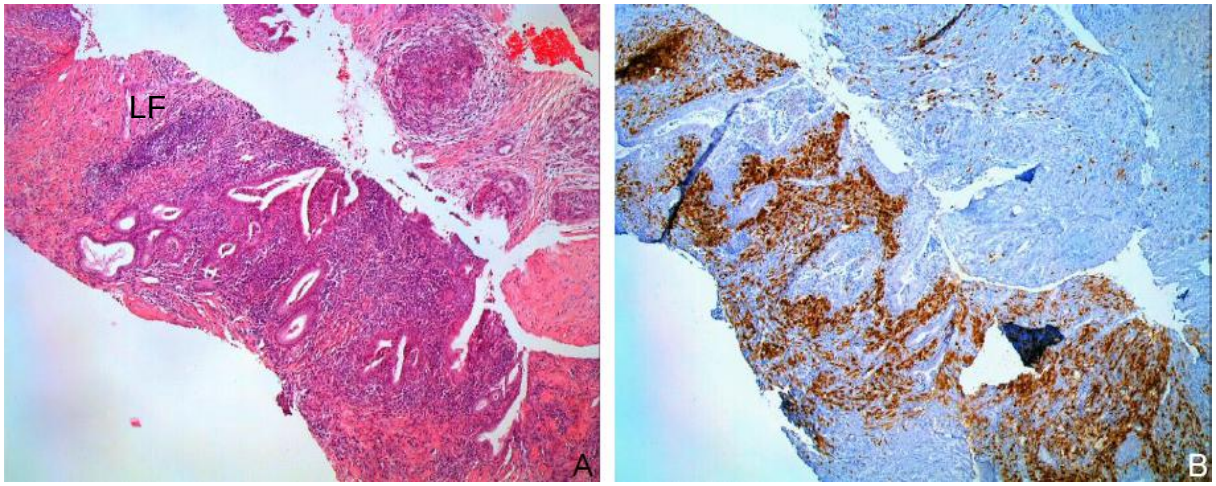



**Figure 6.1** Core needle biopsy showing autoimmune pancreatitis, grade 3: Periductal lymphoplasmacytic infiltration accompanied by a lymphoid follicle-like lesion (LF). **(A)** (H&E, x50). **(B)** Strong infiltration with CD79a positive plasma cells (x50).

## 6.2 Criteria for the diagnosis of autoimmune pancreatitis by core needle biopsy

AIP shows distinct histopathological features that allow it to be diagnosed in pancreatic resection specimens and that distinguish it clearly from other types of CP and also from ductal adenocarcinoma of the pancreas (Ectors *et al.*, 1997;Klöppel *et al.*, 2003;Zamboni *et al.*, 2004;Finkelberg *et al.*, 2006) (III). However, in pancreatic core needle biopsy specimens AIP seems to be difficult to recognize (Levy *et al.*, 2005;Deshpande *et al.*, 2005b;Chari *et al.*, 2006;Levy *et al.*, 2006;Bang *et al.*, 2008). In particular when the core needle biopsy shows the margin of a pancreatic cancer and contains inflammation and fibrosis but lacks malignant cell infiltration, the differential diagnosis AIP versus pancreatic cancer is difficult. Hence, the usefulness of pancreatic core needle biopsy for the diagnosis of AIP has been debated (Löhr and Klöppel, 2005;Kwon *et al.*, 2007).

In a series of five core needle biopsy specimens, Deshpande found only one to be diagnostic, because the two histological hallmarks of AIP, periductal collar of inflammation and venulitis, were only present in this specimen (Deshpande *et al.*, 2005a). Levy et al. reported on three AIP cases for which trucut biopsy specimens were available. They established the diagnosis of AIP in two of the three cases (Levy *et al.*, 2005). In another study including 16 pancreatic core needle biopsy specimens from AIP patients, seven showed the “full spectrum” of characteristic histological changes (Chari *et al.*, 2006). The largest survey of core biopsy specimens so far was based on 22 Japanese AIP patients (Bang *et al.*, 2008). In this study, which relied on the presence of periductal lymphoplasmacytic infiltrates, cellular fibrosis and venulitis as well as the demonstration of more than 10 IgG4 positive plasma cells per HPF as a diagnostic criterion of AIP, the diagnosis was only made in 6/22 (27%) cases.

In our series of 44 core needle biopsy specimens from the pancreas, 22 showed four or more of the six features we had chosen (Tab. 6.1) (IV). All of these 22 biopsy specimens were obtained from patients whose clinical diagnosis and follow-up were consistent with AIP. In contrast, the specimens obtained from patients with non-AIP CP never displayed more than three of the six features and 7/15 revealed none or only one of them. This suggests that the presence of four or more of the microscopic features that we chose is diagnostic of AIP in a pancreatic core needle biopsy specimen (IV). The criteria we applied included the following microscopic features: (1) granulocytic epithelial lesions (GELs), (2) >10 IgG4 positive plasma cells/HPF, (3) >10 eosinophilic granulocytes/HPF, (4) cellular fibrosis with inflammation, (5) lymphoplasmacytic infiltration (periductal or diffuse), and (6) venulitis (Tab. 6.2). Based on this list of purely morphological criteria and a cut-off level of four criteria, our evaluation reached a diagnostic sensitivity of 76% for AIP. Besides, 86% of the AIP core needle biopsy specimens were detected, when a cut-off level of three features was applied in those cases that contained GELs (IV).

| No. of features | During follow-up diagnosed with AIP | During follow-up diagnosed with non-AIP CP |
|---|---|---|
| **0** | 0% (0/29) | 33.3% (5/15) |
| **1** | 0% (0/29) | 13.3% (2/15) |
| **2** | 0% (0/29) | 26.7% (4/15) |
| **3** | 24.1% (7/29) | 26.7% (4/15) |
| **4** | 48.3% (13/29) | 0% (0/15) |
| **5** | 24.1% (8/29) | 0% (0/15) |
| **6** | 3.5% (1/29) | 0% (0/15) |

**Table 6.1** Number of microscopic features per core needle biopsy specimen observed in 29 specimens from patients whose clinical diagnosis and follow-up were consistent with autoimmune pancreatitis (AIP) and in 15 core needle biopsies from patients presenting with non-AIP CP (alcoholic CP or tumor-associated OCP) (IV).

Among the applied features, most crucial and discriminative were the presence of GELs and the demonstration of more than 10 IgG4 positive plasma cells per HPF (Tab. 6.2). GELs were not noted in non-AIP CP cases, suggesting that GELs can be regarded as diagnostic of AIP. More than 10 IgG4 positive plasma cells were, apart from the AIP cases, also seen in 2/15 non-AIP CP cases, implying that the abundance of IgG4 cells is suggestive of, but not specific to AIP (IV). All the biopsies with three features in which IgG4 positivity or GELs represented one criterion were obtained from AIP patients. Moreover, we did not note more than 10 IgG4 positive plasma cells/HPF in the non-AIP CP cases in which three of the six features were present (IV). However, it must be anticipated that, in larger series of specimens, some non-AIP CP specimens will contain three features including IgG4 positivity. Hence, even though the sensitivity of our features increases to 93% using a cut-off level of three features in those cases that contain GELs *or* more than 10 IgG4 positive plasma cells/HPF, the specificity would likely decrease simultaneously (IV). However, the diagnosis of AIP is unlikely, if three (or less) of the six features are present and no GELs and no IgG4 positivity are noted. Core needle biopsy appears to be superior to FNA. Although only few studies on the usefulness of FNA for the diagnosis of AIP are available, their data suggest that it might help to exclude carcinoma, thus preventing pancreatic resection, but that it is not diagnostic and barely suggestive of AIP (Weber *et al.*, 2003;Farrell *et al.*, 2004;Levy *et al.*, 2005;Deshpande *et al.*, 2005b;Levy *et al.*, 2006;Pace *et al.*, 2007;Salla *et al.*, 2007).

Although the two most specific features, the presence of GELs and the abundance of IgG4 positive plasma cells, play a crucial role in the diagnosis of AIP, they also have their limitations in view of the two types of AIP that can be currently distinguished. As we and others have shown, GELs and IgG4 positivity are features that distinguish two different subgroups of AIP patients (Notohara *et al.*, 2003;Zamboni *et al.*, 2004;Zhang *et al.*, 2007). One of these two subgroups is characterized by the occurrence of GELs and seems to lack increased numbers of

IgG4 positive cells in the pancreas and probably also elevated serum IgG4 levels. This AIP subtype has also been called “ductocentric AIP” (Deshpande *et al.*, 2006) or “idiopathic duct destructive pancreatitis” (Notohara *et al.*, 2003). In European series of resection specimens it accounted for almost 50% of the AIP cases (Zamboni *et al.*, 2004) (III, IV). In our pancreatic core needle biopsy series, the second subtype, which was found to be GEL negative (Zamboni *et al.*, 2004), appears to correspond to the AIP subtype that has been called “lymphoplasmacytic sclerosing pancreatitis” (Notohara *et al.*, 2003) or “lobulocentric AIP” (Deshpande *et al.*, 2006) (IV). This subtype obviously presents with abundant IgG4 positive plasma cells within the lymphoplasmacytic infiltrates (Zhang *et al.*, 2007). These data provide an explanation why in our current study almost half of the specimens were IgG4 negative, but at the same time GEL positive, or vice versa (IV). The features IgG4 positivity and GEL are therefore complementary to each other, as they characterize two different types of AIP. It is interesting to note that the relative frequency of the two AIP subtypes in Europe (Pearson *et al.*, 2003;Aparisi *et al.*, 2005;Kleeff *et al.*, 2006;Kojima *et al.*, 2007;Löhr, 2007) and the US (Chari *et al.*, 2006) seems to differ from that in East Asia (Hamano *et al.*, 2001;Choi *et al.*, 2007). While in Europe each subtype can be expected in about 50% of the cases, IgG4 positive AIP patients predominate in East Asia.

| Feature | During follow-up diagnosed with AIP | During follow-up diagnosed with non-AIP CP |
|---|---|---|
| **Granulocytic epithelial lesion (GEL)** | 48.3% (14/29) | 0% (0/15) |
| **>10 IgG4 positive plasma cells/HPF** | 41.4% (12/29) | 13.3% (2/15) |
| **>10 eosinophilic granulocytes/HPF** | 62.1% (19/29) | 33.3% (4/15) |
| **Cellular fibrosis with inflammation** | 96.6% (29/29) | 40.0% (5/15) |
| **Lymphoplasmacytic infiltration** | 93.1% (27/29) | 33.3% (5/15) |
| **Venulitis** | 65.5% (20/29) | 26.7% (3/15) |

**Table 6.2** Frequency of the individual features in pancreatic core needle biopsy specimens. Twenty-nine specimens derived from patients whose clinical diagnosis and follow-up were consistent with autoimmune pancreatitis (AIP). The remaining 15 specimens were from patients presenting with non-AIP CP (alcoholic CP or tumor-associated OCP) (IV).

Because the presence of GELs is specific to one subtype of AIP, a diagnosis of AIP can be made if they are recognized in a biopsy specimen, regardless of the number of other features found. This increased the diagnostic sensitivity of biopsy for the GEL positive AIP subtype in study IV to 86%. Among the microscopic features that were less discriminative were the demonstration of a lymphoplasmacytic

infiltrate, cellular fibrosis with inflammation, venulitis and increased numbers of eosinophilic granulocytes, because each of these changes was also identified in a small number of non-AIP CP cases (IV). Therefore it was necessary to set a cut-off level for the number of criteria that are required for the diagnosis of AIP in a pancreatic core needle biopsy specimen.

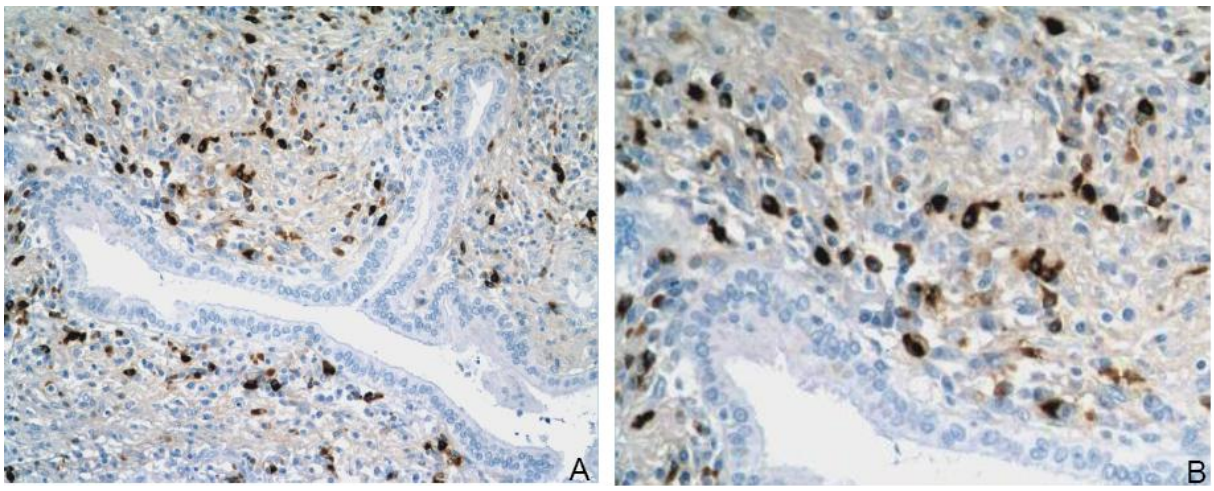



**Figure 6.2** Autoimmune pancreatitis, grade 3: Periductal infiltration with IgG4 positive plasma cells. **(A)** (x200). **(B)** (x400).

## 6.3 The role of core needle biopsy for the diagnosis of autoimmune pancreatitis

Together with imaging and serological markers, pancreatic core needle biopsy seems to be helpful in establishing the diagnosis of AIP (IV). However, histological confirmation of the diagnosis is not imperative for the initiation of steroid therapy (Japan Pancreas Society, 2002). Particularly diffuse pancreatic enlargement together with increased serum-IgG4 levels in patients with a negative initial work-up for pancreatic malignancy suspected of suffering from AIP allows the initiation of a steroid trial (Moon *et al.*, 2008). In patients presenting with a mass-forming lesion in the pancreas, pancreatic cancer represents the main differential diagnosis (Zamboni *et al.*, 2000). However, in those of these patients lacking the classic imaging criteria for pancreatic cancer, AIP may be suggested. Also in this subgroup of patients, a steroid-trial can be helpful, for both treatment and diagnosis of these patients (Moon *et al.*, 2008). If the pancreatic mass after a few weeks does not resolve almost completely, surgical biopsy should be considered (Finkelberg *et al.*, 2006). One has to bear in mind that a mass-forming lesion due to cancer in the pancreas can also be weakly or moderately decreased after steroid treatment (Finkelberg *et al.*, 2006). However, not all patients with a massforming lesion in the pancreas show imaging features typical of either pancreatic cancer or AIP. Hence, FNA is performed during the preoperative workup in many of these patients. The final role of pancreatic core needle biopsy in this setting has to be considered on the basis of larger studies testing our criteria. However, as indicated by our data from study IV, pancreatic core needle biopsy seems to be superior to FNA in distinguishing AIP from pancreatic

cancer.

In conclusion, our data indicate that the six characteristic microscopic features we used were able to recognize AIP in 76% of AIP core needle biopsy specimens when a cut-off level of four features was applied. Moreover, they were able to recognize 86% of AIP core needle biopsy specimens when, in addition to the cut-off level of four features, all specimens that contained GELs were considered, regardless of the total number of features encountered (IV). GELs or four or more of our six microscopic features were present in 25/29 AIP core needle biopsies but in none of the non-AIP CP biopsies (IV). Core needle biopsy is therefore a useful adjunct for recognizing AIP and excluding other diseases such as pancreatic cancer, even if the biopsy lacks malignant cell infiltration and shows inflammation and fibrosis deriving from the margin of the tumor. It has likely the potential to contribute to decrease the number of unnecessary pancreatic resections in AIP patients. However, our criteria for the diagnosis of AIP in pancreatic core needle biopsies have to be tested on a larger number of patients before the utility of our findings can be fully appreciated.

# 7. Conclusions

## 7.1 Study I

Eighty-nine human pancreatic specimens from persons without clinically recognized or macroscopically evident pancreatic disease were examined.

(1) The “normal” pancreas develops a specific type of focally accentuated fibrosis that is highly age related. This type of pancreatic fibrosis is patchily distributed in the peripheral lobules of the pancreas and is accompanied by atrophy of the acinar tissue.

(2) This patchy lobular fibrosis in the elderly (PLFE) is closely associated with pancreatic intraepithelial neoplasia 1B (PanIN-1B) lesions in the ducts, suggesting that the narrowing of a duct due to papillary hyperplasia of the epithelium may hamper secretion and cause fibrosis of the drained lobule.

(3) α-SMA positive myofibroblasts/activated PSCs occur in close association with the PLFE lesions, indicating ongoing fibrogenic activity.

## 7.2 Study II

Fifty-nine pancreatic specimens deriving from patients who were diagnosed with alcoholic chronic pancreatitis (ACP) were examined. In a subgroup of these patients, the expression of the profibrotic cytokines latency-associated peptide (LAP), a transforming growth factor beta 1 (TGF-β1) propeptide, and platelet-derived growth factor B (PDGF-B) was studied. Moreover, we examined the expression of their receptors (TGF-β receptor II (TGF-β-RII) and PDGF receptor α) and their cellular sources.

(1) A histopathological staging system of ACP was developed, based on the pancreatic changes that can be observed according to the presence and abundance of necrotic, inflammatory and fibrotic alterations. Four histological stages were distinguished and defined, and it turned out that this staging system is in accord with the necrosis-fibrosis-sequence for the progression of acute to chronic alcoholic pancreatitis.

(2) In stage I ACP, the stage with overt tissue injury, activated PSCs/myofibroblasts are numerous and especially associated with macrophages around areas of fat tissue necrosis. In stage II, the stage with cellular fibrosis, activated PSCs/myofibroblasts represent the main cellular component of the interlobular tissue. In stage III, the stage with dense fibrosis, activated PSCs/myofibroblasts are rare, and in stage IV, when calculi are present, they are only detected adjacent to duct ulcerations caused by calculi.

(3) In stage I ACP, CD68 positive macrophages are mainly noted in the inner layer of the wall of pseudocysts and around areas of fat tissue necrosis. In

these lesions, some macrophages transform into lipophages. In stage II ACP, macrophages are numerously found and infiltrate interlobular and also intralobular spaces. In stage III & IV ACP, where fibrosis prevails, the number of macrophages is decreasing.

(4) The expression and cellular distribution of TGF-β1 propeptide and PDGF-B is closely related to the severity of fat tissue necrosis in stage I ACP and to the severity of interlobular and intralobular inflammation in stage II ACP. TGF-β1 propeptide is mainly expressed by macrophages while PDGF-B is mainly located in ductal cells. Activated PSCs/myofibroblasts express receptors for these profibrotic cytokines, most intensely during stage I & II ACP. In stage III & IV ACP, the expression of the profibrotic cytokines and their receptors is decreasing.

## 7.3 Study III

Fifty-two specimens from autoimmune pancreatitis (AIP) patients were examined. In a subgroup of these patients, we studied the expression of the profibrotic cytokines TGF-β1 propeptide and PDGF-B and their receptors TGF-β-RII, PDGF-Rα and -Rβ as well as their cellular sources.

(1) We found that a recently developed histopathological grading system of AIP can be used to examine the localization and distribution of activated PSCs/myofibroblasts in mild, moderate and severe grades of AIP.

(2) In grade 1 and 2 AIP, activated PSCs/myofibroblasts form capsule-like lesions surrounding periductal lymphoplasmacytic infiltrates. In grade 3 AIP, there are numerous activated PSCs/myofibroblasts not only in periductal, but also in interlobular and intralobular spaces. In grade 4 AIP, which is characterized by advanced fibrosis, the number of activated PSCs/myofibroblasts is markedly decreasing.

(3) In grade 1 and 2 AIP, CD68 positive macrophages are intermingled with CD4 and CD8 positive lymphocytes and CD79a positive plasma cells in the periductal areas. Grade 3 AIP is the grade showing the strongest inflammatory activity. Macrophages are noted in close association with lymphocytes and plasma cells, infiltrating not only the periductal areas, but also the interlobular and intralobular spaces. In grade 4 AIP, when the lymphoplasmacytic inflammation in the pancreas is decreasing, only few macrophages are found.

(4) The expression and cellular distribution of TGF-β1 propeptide and PDGF-B is closely related to the severity of lymphoplasmacytic infiltration and the accumulation of macrophages in grade II & III AIP. TGF-β1 propeptide is mainly expressed by macrophages and activated PSCs/myofibroblasts, while PDGF-B is mainly located in ductal cells and activated PSCs/myofibroblasts. Myofibroblasts express receptors for these profibrotic

cytokines, most intensely during grade 3 AIP. In grade IV AIP, the expression of the profibrotic cytokines and their receptors is decreasing.

(5) The expression and cellular distribution of the profibrotic cytokines TGF-β1 propeptide and PDGF-B and their receptors are related to the severity of lymphoplasmacytic infiltration in AIP and the severity of fat tissue necrosis in ACP. Macrophages and adjacent epithelial cells are the main sources of these profibrotic cytokines, while myofibroblasts/activated PSCs express their receptors. In both AIP and ACP, the expression of TGF-β1 propeptide, PDGF-B and their receptors is decreasing in late stages. Hence, there are no fundamental differences in the expression of these profibrotic cytokines and their receptors in ACP versus AIP.

(6) There are fundamental differences in the distribution pattern of activated PSCs/myofibroblasts in ACP versus AIP. In stage I ACP, activated PSCs are concentrated in the wall of pseudocysts. In contrast, in grade 1 and 2 AIP, they form capsule-like lesions surrounding the periductal lymphoplasmacytic infiltrates and accumulate directly beneath the epithelium of small and medium-sized pancreatic ducts. However, in late stages of both AIP and ACP, the number of activated PSCs/myofibroblasts is decreasing, and they are distributed more diffusely in the pancreatic tissue.

## 7.4 Study IV

Fourty-four pancreatic core needle biopsy specimens were examined. Twenty-nine specimens were obtained from AIP patients. The remaining 15 biopsy specimens derived from patients diagnosed with ACP (eight specimens) and tumor-associated, obstructive CP (OCP) (seven specimens).

(1) The diagnosis of AIP can be made on a pancreatic core needle biopsy.

(2) We defined six characteristic microscopic AIP features for the differential diagnosis AIP versus ACP and the margin of pancreatic neoplasms showing inflammation and fibrosis in pancreatic core needle biopsy specimens. The sensitivity of our six features for AIP was 86%.

## 7.5 Future perspectives

During the last decade, our understanding of fibrogenesis in CP has improved significantly. However, several issues are unsolved. Future researchers should aim at a detailed description of the histological features of those entities of CP which yet not have been examined systematically, such as tropic pancreatitis and pancreas-divisum pancreatitis (Klöppel, 2007a). It is also important to increase our understanding of whether different subtypes of the respective entities of CP exist. In AIP, we believe there are two subtypes which can be distinguished on the basis of

IgG4 positivity and the occurrence of GELs. Besides, it has been hypothesized that AIP might be an immune complex mediated disease (Deshpande *et al.*, 2006). Data on these two topics from us are under way. Moreover, it is important to develop treatment options to prevent and/or suppress fibrogenesis in the pancreas. Suppression of the profibrotic processes which are stimulated and initiated by the profibrotic cytokines TGF-β1 and PDGF-B might at least partially be able to inhibit fibrogenesis in the pancreas. Another and maybe a better therapeutic attempt would be the prevention and/or antagonism of inflammation and tissue necrosis in early-stage CP. Hence, it is necessary to improve our ability to establish the diagnosis of CP earlier. However, the search for a trustful marker of early-stage CP is still ongoing (Löhr, 2007).

The recognition of AIP as a special entity of CP together with the more widely use of endoscopic ultrasound (EUS) guided core needle biopsy of the pancreas will probably improve our ability to differentiate pancreatic cancer from CP, in particular from AIP, preoperatively. In approximately 10% of pancreatic resections performed because of suspected pancreatic cancer, histological examination reveals that no malignancy is present. Instead, CP and/or pancreatic neoplasms other than ductal adenocarcinoma are found (Abraham *et al.*, 2003). Therefore, it is important that future research on fibrogenesis in the pancreas will include the examination of pancreatic core needle biopsy specimens.

As described above, our data from study II and III did not reveal fundamental differences in expression of profibrotic cytokines and their receptors in ACP versus AIP. However, as it has been described in a few patients that fibrotic lesions in AIP have resolved after steroid treatment (Saito *et al.*, 2002;Song *et al.*, 2005b), future researchers should aim at obtaining core needle biopsies from a larger number of AIP patients before and after steroid treatment. Also, we look forward to test our microscopic criteria for the diagnosis of AIP on a larger number of pancreatic core needle biopsy specimens. Finally, future research should concentrate on improvement of our ability to determine, which precursor lesions of the ductal adenocarcinoma of the pancreas are most likely to progress and thus require resection or close follow-up.

# 8. Abstracts

## 8.1 Abstract in English

Fibrosis of the pancreas is the key histological feature of chronic pancreatitis (CP). It progressively replaces the pancreatic parenchyma, thereby forming different patterns. Since the patterns of fibrosis appear to be related to the etiologic entities of CP, their recognition can contribute to the diagnosis of CP. Also, fibrotic changes may be observed in the “normal pancreas,” i.e. the pancreas of persons without any known or macroscopically visible pancreatic disease, but the etiology of this type of pancreatic fibrosis is not well known. Clinically, fibrotic replacement of the normal pancreatic tissue is accompanied by an impairment of the exocrine and endocrine pancreatic function. In the pancreas, the development of fibrosis has been ascribed to the pancreatic stellate cells (PSCs), which can transform into myofibroblasts when activated by cytokines. This PhD thesis is based on the examination of 244 human pancreatic tissues with fibrosis showing various patterns and associated with different entities.

Eighty-nine of the specimens (study I) derived from persons without diagnosed or macroscopically evident pancreatic disease. We found that this type of fibrosis increases with age and is significantly associated with hyperplastic lesions of the pancreatic duct epithelium. Fifty-nine specimens derived from patients who had alcoholic chronic pancreatitis (ACP) (study II) and 52 specimens were obtained from patients diagnosed with autoimmune pancreatitis (AIP) (study III). We defined a histopathological staging system of ACP and examined the distribution pattern of activated PSCs/myofibroblasts and fibrosis in ACP and AIP. We found that the expression and cellular distribution of the profibrotic cytokines transforming growth factor beta 1 propeptide (TGF-β1 propeptide) and platelet-derived growth factor B (PDGF-B) and the occurrence of activated PSCs/myofibroblasts correlate with the severity of fat tissue necrosis in ACP and the intensity of lymphoplasmacytic infiltration in AIP. We showed that macrophages are the main source of TGF-β1 and ductal cells of PDGF-B in both ACP and AIP, and that the activated PSCs/myofibroblasts are equipped with receptors for these profibrotic cytokines.

Approximately 3% of all patients undergoing pancreatic resection because of suspected pancreatic cancer are finally diagnosed with AIP, which responds to steroid treatment. Fourty-four pancreatic specimens (study IV) were obtained by pancreatic core needle biopsy from patients suspected of suffering from AIP, ACP or tumor-associated, obstructive CP (OCP). We defined histopathological criteria based on the occurrence of inflammatory and fibrotic lesions for the differential diagnosis of AIP versus ACP and the margin of pancreatic neoplasms showing inflammation and fibrosis. Their sensitivity for AIP was higher than 85%. Hence, our criteria will in the future likely contribute to reduce the number of unnecessary pancreatic resections on patients diagnosed with AIP. They will now be tested on a larger number of pancreatic core needle biopsies. Moreover, future research will concentrate on suppression of the profibrotic mechanisms which are stimulated by the profibrotic cytokines we examined.

## 8.2 Abstract in Danish

Fibrose (arvæv) i pancreas (bugspytkirtlen) er det dominerende histologiske kendetegn af kronisk pancreatitis (CP, kronisk bugspytkirtelbetændelse). Fibrosen erstatter det normale pancreasvæv hvorved der opstår forskellige fibrosemønstre. Da fibrosemønstret er relateret til entiteterne of CP, kan beskrivelsen af fibrosemønstret bidrage til at diagnosticere den pågældende entitet af CP. Derudover forekommer fibrose også i det "normale pancreas", dvs. i pancreas fra personer uden diagnosticeret eller makroskopisk synlig pancreassygdom. Ætiologien til denne type af pancreasfibrose er ukendt. Klinisk medfører fibrose der erstatter det normale pancreasvæv et fremskridende tab af den eksokrine og endokrine pancreasfunktion. Fibroseudviklingen i pancreas bliver tilskrevet de pancreatiske stjerneceller (PSC), som kan udvikle sig til myofibroblaster når de bliver aktiveret af cytokiner. Denne PhD-afhandling baserer på undersøgelsen af 244 humane pancreaspræparater med fibrose anordnet i forskellige mønstre og associeret med forskellige entiteter.

Niogfirs af disse præparater (studie I) stammer fra personer uden diagnosticeret eller makroskopisk synlig pancreassygdom. Vi påviste, at denne fibrosetype tiltager med alderen og er signifikant associeret med hyperplastiske læsioner of epitelet i udførselsgangene. Nioghalvtreds præparater stammede fra patienter med alkoholisk kronisk pancreatitis (ACP) (studie II) og 52 præparater var fra patienter med autoimmun pancreatitis (AIP) (studie III). Vi definerede en histologisk stadieinddeling af ACP og undersøgte fordelingsmønstret af aktiverede PSC/myofibroblaster og fibrose i ACP og AIP. Vi fandt at ekspressionen og den cellulære fordeling af de profibrotiske cytokiner transforming growth factor beta 1 propeptide (TGF-β1 propeptide) og platelet-derived growth factor B (PDGF-B) samt forekomsten af aktiverede PSC/myofibroblaster er tæt relateret til sværhedsgraden af fedtnekrose i ACP og til intensiteten af lymfoplasmacytisk infiltration i AIP. Vi påviste, at makrofager er hovedkilden til TGF-β1 og gangepitelceller hovedkilden til PDGF-B i både ACP og AIP, og at de aktiverede PSC/myofibroblaster er udstyret med receptorer for disse profibrotiske cytokiner.

Omtrent 3% af alle patienter der bliver pancreasreseceret på mistanke om pancreascancer viser sig efterfølgende at have AIP, der responderer på steroidbehandling. Fireogfyrre pancreasprøver (studie IV) blev udtaget som grovnålspiopsier fra patienter med mistanke om AIP, ACP eller tumor-associeret, obstruktiv CP (OCP). Vi definerede patoanatomiske kriterier baserende på forekomsten af betændelse og arvæv i grovnålene for at kunne stille differentialdiagnosen AIP versus ACP og randzonen fra pancreastumorer der indeholder betændelse og fibrose. Deres sensitivitet for AIP lå på over 85%. Vores kriterier vil derfor i fremtiden formentlig kunne bidrage til at reducere antallet af unødvendige pancreasoperationer på patienter med autoimmun pancreatitis. Vores kriterier vil nu blive testet på et større antal patienter. Fremtidige undersøgelser vil derudover fokusere på at hæmme de af de beskrevne cytokiner aktiverede arvævsfremmende processer.

# 9. References

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