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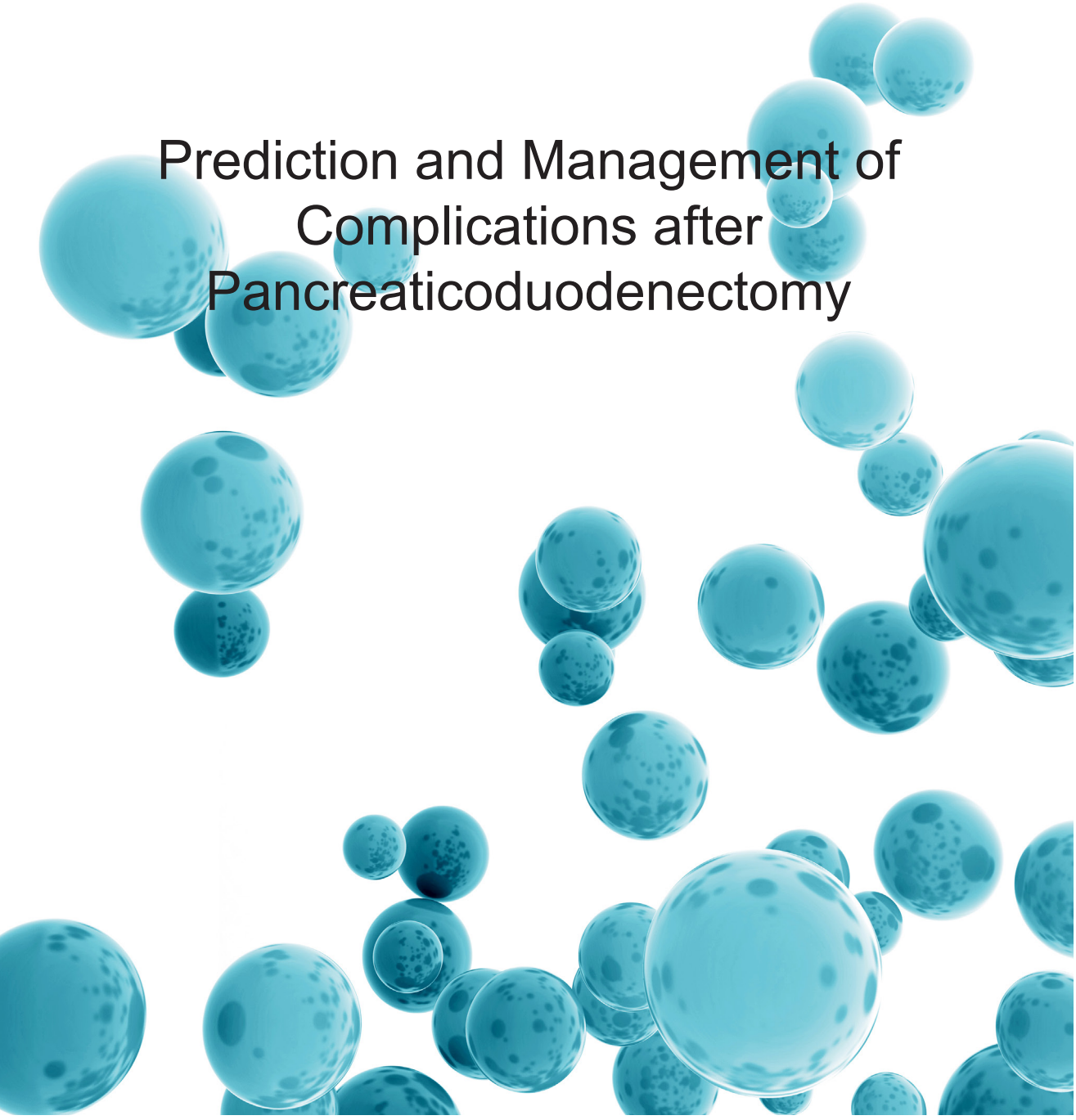
MATIAS LAANINEN

Prediction and Management of Complications after Pancreaticoduodenectomy



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Prediction and Management of Complications after Pancreaticoduodenectomy

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ACADEMIC DISSERTATION
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Fiat secundum artem.

Let it be made according to art.

ABSTRACT

Pancreaticoduodenectomy (PD) is a challenging surgical operation, in most cases performed for suspected neoplasms of the head of pancreas, distal common bile duct or periampullary region. Over the years mortality in PD has decreased to 1 – 5% but almost half of patients still suffer from clinically significant postoperative complications. Postoperative pancreatitis has attracted more interest as a possible mediator of PD related complications. Studies on experimental animal models and cell cultures have shown that pancreatic acinar and stellate cells have the capability to play a role in the inflammatory pathway, and our understanding of this pancreatic inflammation process has changed in the recent years.

The aim of this thesis was to investigate postoperative pancreatic inflammation and its possible connection to postoperative complications. Study I showed that a large number of pancreatic acinar cells correlated significantly with postoperative pancreatic inflammation and complications. A 40% acini cut-off value was noted to identify a risk patient. This cut-off value was later validated in Study IV, where patients with under 40% acinar cells had very few major complications.

The second study of this thesis aimed to investigate the differences in postoperative inflammation between patients with high-risk (acinar cell-rich) and low-risk (fibrotic) pancreas. A significant amplification in postoperative inflammation – seen as raised NF-κB and MCP-1 activity – was observed within the first 4 hours postoperatively in the high-risk pancreata, whereas in fibrotic pancreata this remained at a minimum. This supports our speculation that the postoperative inflammatory process in the normal, acinar cell-rich pancreas begins almost immediately after surgical trauma. A co-culture of pancreatic acinar and stellate cells in Study III was designed to further explore the possible interaction between these two cell types. It was shown that acinar and stellate cells do in fact have paracrine interplay: acinar cells seem to stimulate migration of stellate cells and increased release of extracellular matrix protein, and

stellate cells cause morphology deformation and loss of secretory function in acinar cells. Thus stellate cells (or fibrosis) would protect the pancreas from an acute inflammation cascade caused by surgical trauma – a phenomenon shown in the patients Study I.

Study IV was a randomized, placebo controlled trial providing anti-inflammatory treatment to reduce postoperative inflammation and complications among high-risk patients. High-risk patients (40% acini cut-off) were determined intraoperatively and the hydrocortisone treatment originally initiated preoperatively was continued for two postoperative days. Patients receiving hydrocortisone had significantly fewer major complications than those in the placebo group, 18 vs. 41%.

On the basis of these four studies it can be concluded that postoperative pancreatic inflammation is a probable mediator of complications after pancreaticoduodenectomy. These complications can be significantly reduced with perioperative hydrocortisone treatment, and this treatment can moreover be effectively targeted at high-risk patients using the 40% acini cut-off method.

TIIVISTELMÄ

Haiman pään poistoleikkaus on vaativa operaatio, joka tehdään haiman pään, yhteisen sappitiehyen tai ampullan (sapenjohtimen ja haimatiehyen yhteisen osan avartuman) alueen kasvainten vuoksi. Vuosien mittaan haiman pään poistoleikkauksen kuolleisuus on laskenut 1 – 5 prosenttiin, mutta yhä melkein puolet potilaista kärsii merkittävästä leikkauksenjälkeisestä komplikaatiosta. Leikkauksenjälkeinen haimatulehdus on noussut esiin mahdollisena altistavana tekijänä haiman pään poistoleikkauksen komplikaatioille. Eläinkokeet ovat osoittaneet, että sekä haiman asinussolut (rauhasrakkulat) että satelliittisolut voivat toimia aktiivisesti tulehdusprosesseissa ja käsitys haiman tulehduksesta on muuttunut viime vuosien aikana.

Tämän väitöskirjan tavoitteena oli tutkia haiman leikkauksenjälkeistä tulehdusta ja sen mahdollista yhteyttä komplikaatioihin. Ensimmäinen osatyö osoitti, että asinus-solujen suuri määrä on yhteydessä lisääntyneeseen haiman leikkauksenjälkeiseen tulehdukseen sekä komplikaatioihin. Riskipotilaan rajaksi tunnistettiin 40% asinussolupitoisuus haiman katkaisupinnan pinta-alasta. Löydös vahvistui neljännessä osatyössä: Alle 40% asinussolupinta-alan potilaat välttyivät käytännössä kokonaan merkittäviltä komplikaatioilta.

Toinen osatyö keskittyi asinussolurikkaan riskihaiman ja sidekudosrikkaan, matalan riskin, haiman leikkaustenjälkeisten tulehdusreaktioiden eroihin. Riskihaimassa leikkauksenjälkeinen tulehdusreaktio (Nf-κB ja MCP-1 aktivaatio) voimistui huomattavasti ensimmäisen 4 tunnin aikana haiman katkaisun jälkeen, kun taas sidekudosrikkaassa haimassa tulehdusreaktio pysyi ennallaan. Löydös tukee ajatusta, että leikkauksenjälkeinen tulehdusprosessi alkaa välittömästi kirurgisen vaurion jälkeen. Asinus- ja satelliittisolujen yhdistelmäviljelmä luotiin kolmannessa osatyössä solujen parakriinisten vuorovaikutusten tutkimista varten. Tulokset paljastivat, että asinus- ja satelliittisolut stimuloivat toisiaan: asinussolut menettivät

normaalin erityskykynsä, ja satelliittisolut aktivoituivat, mikä näkyi solunulkoisten proteiinien määrän ja solujen liikkumisen lisääntymisenä. Siten satelliittisolut (tai niiden muodostama sidekudos) suojaisi haimaa akuutilta vaurion aiheuttamalta tulehduskaskadilta – ilmiö joka osoitettiin potilailla osatyössä I.

Neljäs väitöskirjatutkimus oli satunnaistettu, lumelääkekontrolloitu tutkimus, jossa pyrittiin vähentämään leikkauksenjälkeistä tulehdusreaktiota ja komplikaatioita riskipotilailla tulehdusta hillitsevällä hydrokortisonihoidolla. Korkean riskin potilaat (yli 40% asinussolupinta-ala) määritettiin leikkauksen aikana ja juuri ennen leikkausta aloitettua lääkehoitoa jatkettiin kahden leikkauksenjälkeisen päivän ajan. Hydrokortisoniryhmän potilaat saivat huomattavasti vähemmän merkittäviä komplikaatiota kuin lumelääkettä saaneet, 18 vs. 41%.

Väitöskirjan neljän tutkimuksen perusteella voidaan todeta, että leikkauksenjälkeinen haimatulehdus on todennäköinen välittäjä haiman pään poistoleikkauksen komplikaatioissa. Komplikaatioita voidaan vähentää merkittävästi hydrokortisonihoidolla, joka kohdistetaan riskipotilaisiin. Riskipotilaat tunnistetaan helposti määrittämällä asinussolujen pinta-ala haiman katkaisupinnasta.

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LIST OF ORIGINAL COMMUNICATIONS

1. The risk for immediate postoperative complications after pancreaticoduodenectomy is increased by high frequency of acinar cells and decreased by prevalent fibrosis of the cut edge of pancreas. Laaninen M, Bläuer M, Vasama K, Jin H, Räty S, Sand J, Nordback I, Laukkarinen J. Pancreas. 2012; 41:957-61.

2. Difference in early activation of NF-κB and MCP-1 in acinar-cell-rich versus fibrotic human pancreas exposed to surgical trauma and hypoxia. Laaninen M, Bläuer M, Sand J, Nordback I, Laukkarinen J. Gastroenterology Research and Practice. 2014; 2014:460363.

3. Reciprocal stimulation of pancreatic acinar and stellate cells in a novel long term in vitro co-culture model. Bläuer M, Laaninen M, Sand J, Laukkarinen J. Pancreatology. In press.

4. Perioperative hydrocortisone treatment reduces complications after pancreaticoduodenectomy. A randomised controlled trial. Laaninen M, Sand J, Nordback I, Vasama K, Laukkarinen J. Submitted.

ABBREVIATIONS

ASA	American Society of Anesthesiologists
CT	computed tomography
DGE	delayed gastric emptying
ERCP	endoscopic retrograde cholangiopancreatography
EUS	endoscopic ultrasonography
FCS	fetal calf serum
H(2)S	hydrogen sulfide
ICAM-1	intracellular adhesion molecule 1
IL-1β	interleukin 1 beta
IL-6	interleukin 6
IL-8	interleukin 8
IPMN	intraductal papillary mucinous neoplasm
ISGPF	International Study Group of Pancreatic Fistula

ISGPS	International Study Group of Pancreatic Surgery
MCP-1	monocyte chemoattractant protein 1
MDCT	multidetector computed tomography
MRCP	magnetic resonance cholangiopancreatography
NaHS	sodium hydrosulfate
NF-κB	nuclear factor kappa B
PD	pancreaticoduodenectomy
PET	positron emission tomography
PG	pancreaticogastrostomy
PJ	pancreaticojejunostomy
POPF	postoperative pancreatic fistula
PPH	postpancreatectomy hemorrhage
PSC	pancreatic stellate cell
TGFβ1	transforming growth factor beta 1
TNF-α	tumor necrosis factor α
VIP	vasoactive intestinal peptide

INTRODUCTION

Pancreaticoduodenectomy (PD) is a challenging surgical operation with high postoperative morbidity and mortality. The operation is in most cases performed due to suspected neoplasms in the head of the pancreas, distal common bile duct or in the periampullary region (Bond-Smith et al., 2012; Schulick & Cameron, 2007).

The first PDs were performed over 100 years ago. Initially both mortality and morbidity were intolerably high. Over the years, mortality has diminished in high volume centers (1 - 5%), but morbidity remains high at 18-58% (Braga et al., 2011; DeOliveira et al., 2006; Halloran et al., 2002). Several small modifications to the operation and especially to the pancreaticoenteric anastomoses have been developed, but no significant improvement in terms of complication rates has been achieved in recent years (Diener et al., 2011; Yang et al., 2011).

The postoperative complications of PD operation include pancreatic fistula (POPF), postpancreatectomy hemorrhage (PPH) and delayed gastric emptying (DGE). POPF is often considered to be the clinically most challenging complication. The incidence, 12 – 27% on average, varies widely across the literature, partly due to different definitions of complications. (Cameron et al., 2006; Reid-Lombardo et al., 2007). Today, international definitions of the most common complications of PD make it easier to compare studies (Bassi et al., 2005a; Wente et al., 2007a; Wente et al., 2007c). POPF may lead to or be combined with PPH and DGE, or abscesses and sepsis, possibly leading to subsequent shock, multiorgan failure and mortality (Callery et al., 2009; Gouillat & Gigot, 2001). The previously reported risk factors for POPF include soft pancreatic texture and normal exocrine function, narrow pancreatic duct, severe intraoperative blood loss and ischemia (Callery et al., 2009; Kleespies et al., 2008; Raty et al., 2006) but the exact pathophysiology for POPF has remained obscure.

One possible predisposing factor of PD related complications is pancreatic inflammation or postoperative pancreatitis. Postoperative pancreatitis has been shown to predispose to POPF and DGE (Raty et al., 2006; Uemura et al., 2012). Earlier it was thought that during acute pancreatic inflammation activation of pancreatic enzymes such as trypsinogen was the most important pathogenic event. Recent experimental animal studies of acute pancreatitis, however, have suggested that inflammation cascade may also occur parallel to trypsinogen activation, and acinar cells may even behave as inflammatory cells (Sah & Saluja, 2011). Systemic and local inflammation cascade is the same without trypsinogen activation (Dawra et al., 2011), and future therapies for acute pancreatitis may be targeted at the inflammation itself rather than at blocking pancreatic enzymes.

The main hypothesis of this dissertation was that surgical trauma during or immediately after PD operation may trigger an inflammation cascade leading to other complications such as POPF, DGE and PPH. Factors predisposing to this postoperative pancreatic inflammation, the molecular and cellular level events of the inflammation cascade, and ultimately prevention of postoperative complications with anti-inflammatory treatment were studied.

REVIEW OF THE LITERATURE

1.1 Anatomy and physiology of the pancreas

The pancreas is a retroperitoneal organ specialized in both endocrine and exocrine secretory functions. Anatomically the pancreas is divided into five parts: the uncinate process, head, neck, body and tail. The uncinate process originates from the lower part of the head and passes posterior to the superior mesenteric vessels. The head is located alongside the C-shaped concavity of the duodenum. The neck lies anterior to the superior mesenteric vessels and the body continues from it towards the spleen. Altogether the pancreas is approximately 20 cm long. The anatomical border of the body and the tail has not been clearly defined. The tail ends between layers of the splenorenal ligament. (Drake et al., 2005; Netter, 2006).

Exocrine secretions of the pancreas (pancreatic juice) eventually end up inside the duodenum. The orientation of small pancreatic ducts has wide individual variation, but usually a few main structures can be found. Pancreatic juice is gathered from smaller ducts and eventually flows through the main pancreatic duct (duct of Wirsung). In most people, the main pancreatic duct has a common distal end with the bile duct. This structure is called the hepatopancreatic ampulla or the ampulla of Vater. It descends to the duodenum via the major duodenal papilla. It is surrounded by a sphincter consisting of smooth muscle (sphincter of Oddi). Some people also have an accessory pancreatic duct which descends into the minor duodenal papilla located just above the major duodenal papilla. (Drake et al., 2005; Hruban & Wilenz, 2005).

Microscopically the pancreas is mainly composed of exocrine and endocrine components. Fat cells and fibrosis may also be present, the quantity of these is related to diseases, aging, genetics, and other individual factors. The islets of Langerhans constitute only 1 – 2% of the organ but are responsible for its endocrine function.

These islets of Langerhans include four major cell types, β, α, δ and PP that secrete insulin, glucagon, somatostatin, and pancreatic polypeptide. Insulin and glucagon regulate fatty acid, amino acid, and especially blood glucose levels and therefore have a major systemic impact on the human body. Somatostatin suppresses insulin, glucagon, and pancreatic polypeptide release, and has complex systemic effects such as inhibitory consequences on the gastrointestinal tract. Somatostatin also inhibits the exocrine function of the pancreas. Pancreatic polypeptide inhibits gastrointestinal motility and pancreatic exocrine secretion. All these pancreatic hormones and other neuropeptides (such as ghrelin, amylin, pancreastatin, VIP, and substance P) seem to regulate each other's secretion and pancreatic exocrine function. Despite the wide range of studies, the exact mechanism of this complex system remains unknown. (Barreto et al., 2010; Barrett et al., 2010; Hruban & Wilenz, 2005).

Pancreatic acinar cells are responsible for the exocrine part of the organ. An acinus is constructed from a group of acinar cells, pyramid shaped cells pointing towards a lumen. Pancreatic juice is secreted from the acini into the lumen, where it runs into tiny ductules and eventually into the main pancreatic duct. The walls of ductules and ducts are of cuboidal epithelium and a few mucus secreting goblet cells. (Hruban & Wilenz, 2005).

An adult secretes approximately 1.5 - 2 liters of alkaline pancreatic juice per day. It contains electrolytes (especially bicarbonate), albumin, immunoglobulins, and several different digestive enzymes. These include trypsinogen, chymotrypsinogen, amylase, elastase, carboxypeptidases, phospholipase A_2, colipase, and other lipases. The enzymes digest food, more specifically proteins, nucleic acids, triglycerides, and starch as they reach the duodenum. These molecules are also components of normal human structures. It is clear that the enzymes should not be activated before the duodenum. Most of the enzymes are therefore secreted as inactive molecules. Activation takes place in the duodenum due to the effects of colipase, enterokinase, and trypsin. Villi of the small intestines activate enterokinase that in turn converts inactive trypsinogen into trypsin. (Barrett et al., 2010; Hruban & Wilenz, 2005; Koike et al., 1982; Steer et al., 1984; Steer & Meldolesi, 1987). Trypsin is known to launch the activation of nearly every pancreatic enzyme. Pancreas also has other protective

measures against self-digestion. These include *inter alia* autolysis of trypsin, enzyme compartmentalization, and different types of trypsin inhibitors and low intracellular concentrations of ionized Ca^{2+}. (Frossard et al., 2008).

1.2 Pancreaticoduodenectomy

1.2.1 Neoplasms leading to pancreaticoduodenectomy

The decidedly most common reason for pancreaticoduodenectomy (PD) is a mass seen in the head of the pancreas or in the periampullary region. The type of the tumor is confirmed after the surgery, when histopathological specimens have been analyzed. Etiology may vary widely, but the most commonly found diseases are pancreatic ductal adenocarcinoma, other pancreatic exocrine and endocrine tumors, periampullary tumors, duodenal adenocarcinoma, cholangiocarcinoma, and chronic pancreatitis with associated mass lesion. (Bond-Smith et al., 2012; Schulick & Cameron, 2007).

According to the Finnish Cancer Registry, pancreatic cancer was the ninth most common cancer in Finland in 2012 among both women (3.6%) and men (3.3%). Its age-adjusted incidence was among women was 7.0 / 100 000 and among men 9.2 / 100 000 in 2012. The probability of pancreatic cancer is clearly associated with old age. The incidence begins to rise significantly after the age of 60 among both women and men reaching 134.7 and 135.5 at the age of 85 respectively. Pancreatic cancer has extremely low survival rates. Relative survival was 27% / 25% after one year and 7% / 5% after five years among women and men respectively. (Finnish Cancer Registry 2015).

Pancreatic ductal adenocarcinoma accounts approximately 80% of pancreatic carcinomas and in clinical practice these terms are often used synonymously. Ductal adenocarcinoma has especially very poor prognosis: Only 3 – 5% of patients are alive 5 years after diagnosis. (Abraham et al., 2003; Michalski et al., 2007; Winter et al.,

2006; Yeo et al., 2002). Patients are often symptomatic when the cancer has already spread: about 80% of pancreatic ductal adenocarcinomas are metastatic at the time of diagnosis and curative resection is not achievable. Painless jaundice is a typical advanced state symptom but earlier symptoms are non-specific: weight loss, abdominal pain and malaise. Patients who undergo surgery with curative intent have a 5-year survival of 10 – 41%. The best prognostic features are small tumor size, negative lymph node and resection margins, and good to moderate histopathological differentiation. (Bond-Smith et al., 2012; Cameron et al., 2006; Michalski et al., 2007; Winter et al., 2006). Risk factors for pancreatic cancer include age, smoking, chronic pancreatitis, obesity, diabetes and heavy alcohol consumption. New onset diabetes may also be an indicator of pancreatic cancer. Many genetic mutations such as BRCA2, CDKN2A, STK11/LKB1 and PRSS1 are also strongly associated with the development of pancreatic cancer. (Poruk et al., 2012). In a study by Yachida and colleagues, genomic sequencing was performed on pancreatic cancer cells. On the basis of mutations and primary and metastatic lesions, the authors estimated that at least 15 years is needed for pancreatic cancer to develop from tumor initiation to metastatic stage (Yachida et al., 2010). This finding has significantly increased the urge to develop proper screening methods. In a review by Poruk and colleagues, it was considered that the biomarkers for pancreatic cancer such as CA19-9 do not have high enough sensitivity or specificity for systematic screening. They can only be used with high risk groups, e.g. patients with genetic mutations. In future certain panels of serum biomarkers may be adapted for screening purposes. The cancer suspect patients could be then directed further to confirmatory imaging. (Poruk et al., 2012).

Pancreatic ductal adenocarcinoma is the most common pancreatic tumor but several other types of pancreatic neoplasms with or without malignant potential have been reported. There are numerous different cystic tumors in the pancreas. The most common are intraductal papillary mucinous neoplasms (IPMNs), mucinous cystic neoplasm and serous cystadenoma. These cystic neoplasms may sometimes prove hard to differentiate from each other leading to problems when considering treatment. Serous cystadenomas are always benign and may not require resection. Mucinous cystic neoplasms appear almost exclusively in women and are benign, borderline, or malignant. Treatment is almost always resection. (Pitman et al., 2010). IPMNs have

different malignant potential regarding size, morphology, and location (main pancreatic duct vs. branch duct) (Turner & Brugge, 2010). The indications for surgical resection of IPMNs have been a subject of intense debate in recent years. An international guideline for IPMNs was created in 2006 and later in greater detail in 2012. Main-duct IPMNs, and branch-duct IPMNs with worrisome features (e.g. rapid growth, >30 mm size, symptomatic, high-grade atypia), should be surgically resected. It was also proposed that asymptomatic branch duct IPMNs of even >30 mm size without mural nodules or high grade atypia in cyst fluid could be observed without immediate resection, particularly in elderly patients. (Tanaka et al., 2006; Tanaka et al., 2012). Analysis of DNA mutations and protein expression from the cyst fluid may be useful in differentiating benign cystic tumors from those with malignant potential in the future (Kwon, 2012).

Neuroendocrine tumors account for only 1 – 2% of pancreatic neoplasms. Tumor biology, clinical symptoms, and prognosis vary widely between different neuroendocrine tumors. The primary treatment is still surgical resection but new, systemic therapies for example with thyrosin kinase inhibitors for unresectable disease have shown promising results. (Milan & Yeo, 2012). Acinar cell carcinoma accounts for 1% of pancreatic neoplasms and small, localized tumors are resectable (Matos et al., 2009). Metastatic cancer, usually from renal cell carcinoma, to the pancreas accounts for 2 – 3% of pancreatic solid tumors. Other rare pancreatic tumors are solid pseudopapillary tumor and primary pancreatic lymphoma. (Mortenson et al., 2008).

1.2.1.1 Preoperative imaging

The first phase imaging study for patients with jaundice or suspicious abdominal pain is usually abdominal ultrasound. This is useful, especially when further investigating the etiology of obstructive jaundice. Both bile and pancreatic duct dilation (double duct sign) is an indirect sign of a pancreatic tumor. Liver metastases and ascites may also be seen. Small pancreatic tumors, however, cannot be reliably detected due to the retroperitoneal position of the pancreas. (Bond-Smith et al., 2012; Poruk et al., 2012). Endoscopic retrograde cholangiopancreatography (ERCP) with biopsies is reliable in

the diagnosis of pancreatic ductal adenocarcinoma (sensitivity 90 – 95%) but has a significant risk for postoperative pancreatitis (3 – 7%) and is therefore reserved only for cases requiring endoscopic procedures. These include biliary obstruction or diagnostic need for unusual pancreatic neoplasm. (Bond-Smith et al., 2012; Enestvedt & Ahmad, 2012).

Modern, multidetector-row computed tomography (MDCT) achieves 86% sensitivity in diagnosing pancreatic masses. The problem with MDCT is low sensitivity (55%) for tumors less than 3 cm (Sahani et al., 2008) but it reaches up to 91% positive predicting value when assessing the resectability of a tumor and 99% negative predictive value when assessing vascular invasion (Manak et al., 2009). CT exposes to radiation, is unsafe for patients with renal failure and should only be used when malignancy is strongly suspected. Magnetic resonance cholangiopancreatography (MRCP) may provide complementary information about a pancreatic tumor when considering resection: It offers excellent visualization of the biliary and pancreatic duct and may also reveal small metastases that do not appear in CT. Endoscopic ultrasound is a good choice when further evaluating small pancreatic tumors under 3 cm. Fine needle aspirations also can be obtained during the examination. Endoscopic ultrasound needs an expert to produce reliable results and is therefore only available in specialized centers (Sahani et al., 2008).

1.2.2 Technique

In the early twentieth century, Kausch was the first to perform PD (Kausch, 1912). The operation was later popularized by Whipple in the 1930s (Whipple et al., 1935). The first part of the operation is to determine whether the tumor is resectable. Peritoneal surfaces, organs, lymph nodes, and adjacent tissues, such as vessels, are carefully inspected. Cholecystectomy is then performed and the pancreas is transected from the neck. (Michalski et al., 2007; Schulick & Cameron, 2007). The classic Whipple procedure involves resection of the proximal pancreas, distal stomach, duodenum, distal bile duct, and gallbladder (Whipple et al., 1935). The pylorus-preserving method spares the stomach, thus intuitively reducing DGE, but clinical

trials have produced modest results. No difference in cancer survival has been found between these two techniques (Chapter 1.6.1).

The Whipple procedure has been modified several times over the years. Laparoscopic technique for PD has recently aroused interest. Gumbs and colleagues showed that laparoscopic PD has similar mortality and morbidity rates than open PD. (Gumbs et al., 2011). In an attempt to improve the outcomes of pancreatic cancer surgery, more extensive procedures including total pancreatectomy, regional pancreatectomy and extended lymphadenectomy have been introduced (Strasberg et al., 1997). A recent consensus statement of extended PD from the International Study Group of Pancreatic Surgery (ISGPS) was published in 2014. PD is considered to be extended if more than the stomach antrum, colon with relevant vascular structures, small bowel beyond the first segment of the jejunum, portal or mesenteric veins, hepatic artery or celiac trunk / superior mesenteric artery, inferior vena cava, right adrenal gland, right kidney, liver or diaphragmatic crura is resected. Postoperative morbidity seems to increase with extended PD (particularly if the above-mentioned arteries are involved), but extended PD for locally advanced pancreatic cancer seems to produce superior results in 5-year survival than palliative by-pass surgery or radiochemotherapy. (Hartwig et al., 2014).

Intestinal continuity is reconstructed with gastrojejunostomy, choledochojejunostomy and pancreaticojejunostomy, PJ (or pancreaticogastrostomy, PG). Pancreatic and biliary anastomoses are reconstructed 45 – 60 cm proximal to the gastrojejunostomy. (Strasberg et al., 1997). An enteroenterostomy is often constructed between the afferent and efferent jejunal limbs so that bile and pancreatic juice do not flow through the gastrojejunal anastomosis. This may reduce alkaline reflux gastritis, bile vomiting and postoperative DGE. (Hochwald et al., 2010). Different methods for pancreaticojejunal anastomosis have been created in an attempt to reduce mortality and morbidity (Chapter 1.6.1).

For resected pancreatic cancers, adjuvant therapy with gemsitabine or fluorouracil is indicated for improved long-term survival. The use of neo- and adjuvant radiation therapy remains controversial. Multiagent chemotherapy with fluorouracil,

irinotecan, oxaliplatin, and leucovorin (Folfirinox) and gemcitabine plus albumin-bound paclitaxel particles (nab-paclitaxel) for both neo- and adjuvant use have shown promising initial results (Ryan et al., 2014).

1.2.3 Morbidity and mortality

For decades PD was only performed in rare cases due to substantial mortality 20 – 40% and even higher morbidity (Gudjonsson, 1987; Shapiro, 1975). Since the 1980s the mortality has decreased significantly, especially in high volume centers and has led to increased use of the procedure (Cameron et al., 2006; Strasberg et al., 1997). Centralization of the operation to high volume hospitals is extremely important since it is known to significantly reduce PD associated mortality and morbidity (de Wilde et al., 2012; Nordback et al., 2002). Nowadays mortality is as low as 1 – 5% but morbidity is still considered intolerably high 18 – 58%, even in high volume centers. Complication rates between studies vary widely because some centers only report major complications as morbidity. Leading causes of mortality include sepsis, cardiovascular events and hemorrhage. POPF, biliary fistula, PPH, intra-abdominal abscess, postoperative pancreatitis and DGE are the most common reasons for morbidity. (Braga et al., 2011; DeOliveira et al., 2006; Halloran et al., 2002).

Complications are life threatening, prolong hospital stay and delay oncological adjuvant treatment, may require radiological intervention or re-operation and, naturally, increase costs (Halloran et al., 2002; Michalski et al., 2007). Enestvedt and colleagues recently estimated that a major complication nearly doubles the cost of a single PD (Enestvedt et al., 2012). In an earlier study by Daskalaki and colleagues, POPF was found to significantly increase costs after PD (Daskalaki et al., 2011).

PD also has significant readmission rates. In a study by Ahmad and colleagues, medical records of PDs in six high volume centers were reviewed. 30 and 90-day readmission rates were 15% and 19% respectively, the most common causes being infectious complications, DGE, and failure to thrive. Chronic pancreatitis, high transfusion requirements, and postoperative complications such as POPF and abscesses increased the probability of readmission. (Ahmad et al., 2012).

Lack of international definitions for complications and different types of surgical techniques have previously made it difficult to compare studies. Today efforts to achieve international consensus and exact definitions for DGE, POPF, and PPH have been made (Bassi et al., 2005a; Wente et al., 2007a; Wente et al., 2007c). These studies are still under debate but as the definitions begin to converge, better meta-analyses and statistical comparisons are to be expected.

1.3 Postoperative complications of pancreaticoduodenectomy

1.3.1 Postoperative pancreatic fistula

POPF has been the number one complication of pancreatic surgery. Soft consistency of the pancreas has made it more difficult to suture, thus creating a labile anastomosis. If the anastomosis leaks, pancreatic digestive enzymes cause inflammation and necrosis in the peripancreatic region and the abdominal cavity. POPF may lead to hemorrhage, abscesses, retroperitoneal sepsis and DGE. Possible subsequent mortality is caused by hemorrhage, shock and multiorgan failure. (Callery et al., 2009; Gouillat & Gigot, 2001). Many risk factors have been identified, such as pancreatic texture and exocrine function, duct size, operative technique and intraoperative blood loss (Callery et al., 2009; Kleespies et al., 2008) but the exact pathophysiology of POPF is still unknown. Several different surgical techniques have been created in an attempt to prevent POPF, but the results have been modest (Chapter 1.6.1). Nowadays postoperative pancreatitis and its role in the origin of POPF have attracted more attention (Chapter 1.4).

According to the International Study Group of Pancreatic Fistula (ISGPF), the best term for the leakage of pancreaticojejunal anastomosis is postoperative pancreatic fistula, POPF. Bassi and colleagues defined POPF using the amylase content of operatively (or subsequently placed percutaneous) drain on postoperative day 3. POPF is diagnosed if the amylase content is three times higher than the normal upper

serum value. The volume of the drain fluid has no impact on the grading. (Bassi et al., 2005a).

The original ISGPF definition divides POPF into three grades: A, B, and C. Grade A has no clinical impact related to the leak. Grade C is life threatening and a major change in the postoperative management is required; e.g. relaparotomy or ICU stay is needed. Grade B in turn means non-A and non-C: Enteral nutrition is delayed, signs of infection may occur, peripancreatic drains are kept in place (usually for over 3 weeks) and readmission may be necessary. If invasive procedures are needed, POPF shifts to grade C. (Bassi et al., 2005a). There has been discussion as to whether the postoperative placement of a percutaneous drain should be categorized as grade B or C (Reid-Lombardo et al., 2007).

In a study by the Pancreatic Anastomotic Leak Study Group (Reid-Lombardo et al., 2007) The definition of ISGPF was evaluated using postoperative data from 1507 patients undergoing PD. It was found that drain fluid analysis is an appropriate way to diagnose POPF, but some cases of intra-abdominal abscess were not detected in the analysis. This was confirmed in a study by Moskovic and colleagues, where postoperative drain amylase analysis did not predict all clinically significant leakages (Moskovic et al., 2010). This may be explained by malfunction of the drain, and the operating surgeon must keep in mind that few anastomotic leakages are missed with sole drain data analysis (Reid-Lombardo et al., 2007). ISGPF's definition of POPF has also been criticized as it increases the rate of leakages and 49-67% of these are considered clinically insignificant (Grade A) (Moskovic et al., 2010; Reid-Lombardo et al., 2007). Most study groups have thus reported only the clinically significant B & C fistulas. It has been shown that ISGPF defined morbidity correlates with ICU stay and postoperative stay (Tan et al., 2011).

1.3.2 Delayed gastric emptying

The exact mechanism of DGE is still poorly understood. It is often a result of other intra-abdominal complications such as POPF and abscesses, but the operation itself is one probable reason. The resection of the duodenum and subsequent decrease in

plasma motilin levels are considered a factor behind gastroparesis. This is supported by the fact that distal pancreatectomy patients rarely experience DGE. (Kleespies et al., 2008; Kunstman et al., 2012; Michalski et al., 2007; Wente et al., 2007a). Postoperative pancreatic inflammation has also been found to increase DGE (Raty et al., 2006).

A definition for DGE was proposed by the ISGPS in 2007. DGE was also divided into grades A, B, and C, depending on the tolerance of solid oral intake, requirement of nasogastric tube, vomiting, and the need for prokinetics. Grade A does not usually have significant clinical consequences, whereas grade B prolongs the length of hospital stay and impairs the quality of life. Grade C patients require nutritional support, prolonged hospital stay, suffer from substantial discomfort, and are associated with other complications. (Wente et al., 2007a). Several studies indicate that the ISGPS definition of DGE is a useful tool when assessing clinical outcomes (Akizuki et al., 2009; Malleo et al., 2010; Park et al., 2009; Welsch et al., 2010).

1.3.3 Postpancreatectomy hemorrhage

Early PPHs are usually related to coagulopathy or technical failures of appropriate hemostasis at anastomotic sites (Rumstadt et al., 1998; Welsch et al., 2011). These include bleeding from the PJ anastomosis, ulceration in the gastroenteric anastomosis or leakage of venous anastomosis after portal vein resection. Late PPHs are mostly severe and usually result from a POPF or a local septic focus, and the most important bleeding site is the area of peripancreatic vessels. (Roulin et al., 2011).

The ISGPS also presented criteria for PPH with grades A, B, and C. Grades are defined by the time of onset (early < or late > 24 hrs), severity, site of bleeding (intra- or extraluminal) and clinical impact (especially the amount of packed blood cells required). Grade A patients have only mild early blood loss, their clinical condition is well, and they are not in need of invasive treatment or other therapeutic interventions than volume resuscitation or blood transfusions (max 3 units of blood). Grade B patients have severe early or mild late blood loss and are often in need of invasive treatment, but their clinical condition is rarely life threatening. Grade C patients have

serious late blood loss and their clinical condition is severely impaired. (Wente et al., 2007c). Later studies have concluded that ISGPS defined PPH is suitable for clinical and scientific use as it accurately predicts mortality, morbidity, and length of hospital stay (Grutzmann et al., 2012; Welsch et al., 2011).

1.3.4 The Clavien-Dindo classification

Registration of POPF, PPH and DGE is important, but each of these only measures one specific PD-related complication. The Clavien-Dindo classification was created in 2004 for general surgery to evaluate overall complications (Dindo et al., 2004). The 5-step grading system is based on the therapeutic consequences of a complication. Grade I complications lead to only minor deviation from the normal postoperative course, e.g. bedside wound revisions, analgetics, electrolytes and antiemetics. Grade II patients require pharmacological intervention such as antibiotics, blood transfusions or total parenteral nutrition. Grade III patients require surgical, radiological or endoscopic intervention and grade IV patients suffer from a life-threatening complication that requires intermediate care (IC) or intensive care unit (ICU) treatment. Grade V means mortality. This classification has been found to be applicable for pancreatic surgery (DeOliveira et al., 2006). The scoring system is easy to use and thus provides an excellent method to compare overall complication rates between different studies (DeOliveira et al., 2006).

1.4 Postoperative pancreatic inflammation

1.4.1 Cellular and molecular mechanisms of pancreatic inflammation

Acute pancreatitis is a disease with occasional severe manifestations leading to substantial morbidity and mortality. The mechanisms of pancreatic inflammation have therefore been studied intensively, and its pathogenesis has begun to emerge in recent decades. Typical triggers of acute pancreatitis are pancreatic hyperstimulation, gallstones or alcohol. (Frossard et al., 2008). Classically it has been thought that

unregulated activation of trypsin in the acinar cells leads to uncontrolled autodigestion of the pancreas and a local inflammation process. According to the co-localization hypothesis, the digestive zymogens of the pancreas become co-localized with lysosomal hydrolases in acinar cell cytoplasmic vacuoles and then lysosomal hydrolases such as cathepsin B, activate trypsinogen. Trypsin then activates the other digestive enzyme zymogens and the activated digestive enzymes gain access to the cell interior leading to cell necrosis (Koike et al., 1982; Steer et al., 1984; Steer & Meldolesi, 1987). Kinin and complement pathways are stimulated followed by the secretion of inflammatory mediators by local leukocytes. As the inflammation proceeds, more leukocytes migrate into the pancreas from the bloodstream exacerbating the injury. Hypoxia and oxygen-derived free radicals have also been known to play an exacerbating role in this setting. (Frossard et al., 2008). Recent findings suggest that disturbed calcium signaling may play a pivotal role in mediating acinar cell necrosis (Frick, 2012).

New experimental models of acute pancreatitis have increased the knowledge about acute pancreatitis. Experimental models for biliary acute pancreatitis have been developed only recently. The ability to induce experimental biliary acute pancreatitis offers new opportunities to compare the pathogenesis of acute pancreatitis caused by different etiologies (Laukkarinen et al., 2007; Laukkarinen et al., 2008; Perides et al., 2010a; Perides et al., 2010b; Perides et al., 2011), for example, it was found that G-protein-coupled, cell surface bile acid receptor (Gpbar1) might play a significant role in bile-acid-induced pancreatitis (Perides et al., 2010a). The pivotal role of trypsinogen activation on the other hand was attenuated in several studies (Sah & Saluja, 2011). Singh and colleagues *inter alia* showed in their experimental animal model that suppressing trypsin with protease inhibitors reduced pancreatic injury and acinar cell death but did not decrease inflammation (Singh et al., 2009). Genetically engineered mice without the pathologic early trypsinogen activation were recently developed by Dawra and colleagues in an attempt to learn more about this setting. Surprisingly, in cerulein induced acute pancreatitis, progression of local and systemic inflammation was similar between normal and genetically engineered mice. Trypsinogen activation did, however, affect acinar cell necrosis as it accounted for 50% of pancreatic damage. These results seem to confirm that trypsinogen activation

induces acinar cell damage but does not affect the inflammatory response. (Dawra et al., 2011).

Acinar cells play a key role in emergence of acute pancreatitis. Research suggests that it can act as an inflammatory cell. Inflammatory mediator nuclear factor kappa B (NF-κB) was first found to activate in acinar cells during acute pancreatitis in 1998 (Gukovsky et al., 1998) and later it was shown to occur parallel to trypsin activation (Hietaranta et al., 2001). Subsequent studies implied that NF-κB activation plays an important role in the pathogenesis of acute pancreatitis. In the animal models of Chen et al. and Baumann et al., activation of NF-κB induced pancreatitis and systemic inflammatory response (Baumann et al., 2007; Chen et al., 2002).

Acinar cells have been shown also to exhibit other inflammatory mediators such as TNF-α, MCP-1, IL-6, and IL-1β, and adhesion molecule ICAM-1, after noxious stimuli in animal models (Dios, 2010; Vonlaufen et al., 2007). Acinar cells express MCP-1 as early as 60 minutes after supramaximal cerulein infusion (Grady et al., 1997). These signaling molecules, chemokines, and cytokines, are known to attract leukocytes from the bloodstream into the pancreas and disperse the inflammation systemically. The leukocytes infiltrate the pancreas from three hours after the stimulus. This promotes local and systemic complications and is therefore a vital event for clinical manifestations of acute pancreatitis. (Dios, 2010; Vonlaufen et al., 2007). Other pancreatic parenchymal cells that attract leukocytes are resident macrophages and stellate cells. Pancreatic stellate cells were found in 1998 by Apte and colleagues (Apte et al., 1998), and subsequently these cells have been seen to express early chemokines such as IL-8, MCP-1, and cytokines TGFβ1 and IL-1, after noxious stimuli such as alcohol (Apte et al., 2010; Apte et al., 2012). Interestingly, stellate cells have also been found to phagocytose necrotic debris and aged polymorphonuclear cells (Shimizu et al., 2005) thus implying that stellate cells may behave like macrophages.

1.4.2 Association to postoperative morbidity

Postoperative pancreatitis has usually been an underdiagnosed postoperative complication of PD. The main focus has been on POPF, PPH, DGE, and systemic or local infections. This may be because of the difficulty of the diagnosis. Postoperative pancreatitis was traditionally accurately diagnosed the same way as acute pancreatitis: by CT scan (Raty et al., 2006).

A rapid urinary tryspinogen-2 dipstick was developed by Kemppainen and colleagues as a screening test for acute pancreatitis with sensitivity of 94% and specificity of 95% (Kemppainen et al., 1997). A recent meta-analysis stated that the dipstick method is reliable in the diagnosis of acute pancreatitis with a sensitivity of 82.3% and specificity of 93.5% (Chang et al., 2012). The idea was refined in 2007, when CT-confirmed postoperative pancreatitis was successfully diagnosed with urine trypsinogen strip test by our group. The sensitivity, specificity, positive and negative predictive values were 100, 92, 81, and 100 % respectively (Raty et al., 2007).

Since an easy method for diagnosing postoperative pancreatitis has only recently been created, only few studies have been conducted on postoperative pancreatitis and its association with postoperative morbidity. CT-confirmed postoperative pancreatitis was earlier found to be a risk factor for DGE and POPF (Raty et al., 2006). This finding was supported in a study by Uemura and colleagues, where elevation of urine trypsinogen-2 was an independent risk factor for POPF. Postoperative pancreatitis was diagnosed in 14 out of 19 patients with POPF. (Uemura et al., 2012). In an earlier study, the rate of postoperative pancreatitis and levels of drain amylase after PD were significantly reduced by administering ulinastatin, an intrinsic trypsin inhibitor (Uemura et al., 2008). Although the evidence is so far scarce, postoperative pancreatic inflammation appears as a logical and an interesting potential mediator of complications related to the Whipple operation.

1.5 Predicting complications

1.5.1 Texture of the pancreas

The consistency of the pancreas has been shown to be associated with risk of POPF. Several studies have shown that intraoperative assessment of the gland to soft increases the risk for POPF (Ansorge et al., 2012b; Pratt et al., 2008; Shimoda et al., 2012). Patients with soft pancreas are three times more likely to develop POPF than are patients with firm parenchyma (Pratt et al., 2008). Soft, healthy pancreas is generally considered to be "non-firm" pancreas, meaning that the parenchyma is dominated by acinar or fat cells and has a small main pancreatic duct. Firm pancreas consists mainly of fibrosis and the pancreatic duct is dilated. This is usually a result of chronic pancreatitis or obstructive pancreatitis caused by a tumor. (Ansorge et al., 2012b).

"Fatty pancreas", fat cell dominated pancreatic parenchyma, has been investigated as a risk factor for POPF. In 2007, Mathur and colleagues reported that POPF patients had higher fat content (50%) in the pancreas than other patients (13%) (Mathur et al., 2007). This “fat score” consisted of grading specimens from the pancreatic neck (0–4) for the presence of intra- and interlobular fat. Rosso and colleagues subsequently stated that patients with over 10% fat infiltration of the pancreas were more prone to suffer POPF (sensitivity 100% and specificity 53.5%). It was also found that the intraoperatively assessed texture of the pancreas correlated with the amount of fibrosis but not with that of fat. Unfortunately, the exact method for this quantitative analysis is not described in the article as it only states that the analysis was done blindly by two pathologists in a “simple and reproducible way” (Rosso et al., 2009). To the best of our knowledge, no study has so far investigated the association of number of acinar cells and postoperative complications.

1.5.2 Body mass index

Pancreatic fat infiltration correlates with body mass index (BMI), which of itself also an independent risk factor for POPF. One hypothesis is that high BMI increases fat

infiltration into the pancreas, which in turn may impair healing of the pancreas and create a labile pancreaticojejunal anastomosis. (Mathur et al., 2007; Rosso et al., 2009; Shimoda et al., 2012). Tranchart and colleagues found that preoperative evaluation of body fat distribution by CT helps to predict POPF. Visceral fat area greater than 84 cm^2 was an independent risk factor for POPF grades B and C and was associated with fat infiltration of the pancreas. 45 % of patients with visceral fat area more than 84 cm^2 developed POPF. They also found some correlation with fatty pancreas and POPF. Half of the patients with fatty pancreas developed POPF. (Tranchart et al., 2012). A study with contradictory results was presented by Balentine and colleagues, whose results stated that neither BMI nor the amount of visceral fat (evaluated by CT) significantly predicted the risk for complications (Balentine et al., 2011).

1.5.3 Pancreatic duct diameter

The normal, mean diameter in the body of pancreas (where transection in PD is done) is 2 mm. In many pathologies of the pancreas, the duct diameter is widened (Mortele et al., 2006). One considered risk factor for POPF is small main pancreatic duct diameter. Duct diameter smaller than 3 mm is associated with POPF (Muscari et al., 2006; Pratt et al., 2008). In one study, patients with diameter < 3 mm had 38% incidence of POPF (Yang et al., 2005). In a recent study by Ansorge and colleagues, 30% of patients with < 3 mm diameter developed grade B or C POPF (Ansorge et al., 2012b).

1.5.4 Ischemia

Ischemia has been found to be associated with the probability of POPF. Earlier it has been shown by our group that patients with postoperative pancreatitis (30%) had underlying coronary heart disease more often than did patients without postoperative pancreatitis (3%) (Raty et al., 2006). Cardiovascular diseases and low cardiopulmonary reserves have been identified as significant independent risk factors for POPF, overall morbidity, and severity of complications (Ausania et al., 2012; DeOliveira et al., 2006). These findings were recently supported by a study where

evidence of ischemia – high intraperitoneal glycerol concentrations, elevated lactate/pyruvate ratios and low glucose levels – were postoperatively monitored near the pancreatojejunal anastomosis in patients who later developed POPF (Ansorge et al., 2012a).

1.5.5 Risk scores

Patients at high risk for complications would be good to identify prior to surgery. The CT-estimated volume of the pancreatic remnant has been found to correlate with POPF. In a study by Kanda and colleagues, pancreatic remnant volume of >25.5 cm3 had a sensitivity of 0.81 and a specificity of 0.82 for clinically relevant POPF (Kanda et al., 2014). These results still need to be confirmed in a larger, prospective cohort study.

Since some predisposing factors for POPF have been identified, different risk prediction scoring systems have been created. Ansorge and colleagues created their intraoperative risk scoring system on the basis of the consistency of the pancreas and the diameter of the pancreatic duct. Three different risk groups were identified. High risk patients, who had both soft pancreas and small pancreatic duct, had a 37% chance for grade B or C POPF. The risk for the same complication in the intermediate risk group (one risk factor, soft pancreas or small duct diameter) was 21%, and patients with no risk factors had no symptomatic POPFs. (Ansorge et al., 2012b). Braga and colleagues divided patients into four risk groups using ASA score (indicator of patient's overall status by the American Society of Anesthesiologists), intraoperative blood loss, pancreatic duct diameter and the consistency of the pancreas. Soft pancreas, < 3 mm pancreatic duct diameter, > 700 ml blood loss and ASA score of II or III were calculated as risk points, and the total points determined a patient's risk group. Patients in the lowest risk group had a 7% chance of a major complication, whereas patients in the highest risk group had a 36% probability of a major complication. (Braga et al., 2011). Neither one of these risk scores (Ansorge et al., 2012b; Braga et al., 2011) identifies all risk patients completely and a more specific identification method is needed.

1.6 Preventing complications

1.6.1 Pylorus-preserving pancreaticoduodenectomy

One attempt to reduce complications with shorter operating time and reduced blood loss is pylorus-preserving PD. A prospective randomized clinical trial comparing the pylorus-preserving method with the classic Whipple procedure was published in 2005. Operating time and blood loss were significantly reduced in the pylorus-preserving group, but the morbidity rate was only slightly, not significantly, reduced. Long-term survival, tumor recurrence and quality of life did not differ between the groups. (Seiler et al., 2005). Meta-analyses seem to confirm that pylorus-preserving PD is faster to perform with less blood loss, but no significant difference in perioperative morbidity, mortality or long term results can be seen (Diener et al., 2011; Karanicolas et al., 2007).

1.6.2 Pancreatic anastomoses

After resection of the pancreas during PD, an anastomosis is created between the pancreatic stump and the gastrointestinal tract. The anastomosis can be done with the jejunum (PJ) or with the stomach (PG). PG was introduced in clinical use in 1946 as a way to reduce postoperative morbidity. The stomach is thick-walled, easy to suture and well vascularized. The enterokinase-free, acidic environment may prevent the activation of proteolytic enzymes and negative pressure may be maintained with a nasogastric tube. (Yeo et al., 1995). Several non-randomized singe-institution reports suggest that PG has fewer complications than PJ, but prospective, randomized trials comparing these two anastomoses have found no difference in morbidity or mortality (Bassi et al., 2005b; Yeo et al., 1995). Earlier meta-analyses supported these findings with no difference between PG and PJ (Wente et al., 2007b; Yang et al., 2011), but a recent one (Menahem et al. 2015) shows slightly less POPF when using PG.

Pancreaticojejunal anastomosis can be done by end-to-end anastomosis or with end-to-side anastomosis. The latter may be done with or without duct-to-mucosa suturing. Several modifications to PJ anastomosis have been made but no anastomosis

has proven to be superior. The lack of randomized, controlled trials of different techniques hinders unbiased reviews and meta-analyses. (Lai et al., 2009; Yang et al., 2011). Some authors prefer invagination anastomosis for soft pancreas and small pancreatic duct, whereas duct-to-mucosa is used with fibrotic pancreas and dilated pancreatic duct (Lai et al., 2009).

Some new PJ anastomoses appear promising. Our group has developed an anastomosis with minimum damage to the pancreas in an attempt to protect it from postoperative pancreatitis. In this model the pancreatic stump is sunk into the jejunum and the anastomosis is tightened with a purse string in the bowel serosa, so no stiches go through the pancreatic tissue. (Nordback et al., 2008). A small clinical trial with this method had promising results with 0% mortality and only 3% POPF rate (Nordback et al., 2012). This model was also used successfully as an end-to-side anastomosis by Hashimoto and colleagues in 2012 (Hashimoto et al., 2012). Anastomoses with on the same principle were developed at that time and two different binding pancreaticojejunostomy techniques were published in 2003 and 2008 with 0% POPF rate (Chen et al., 2008; Peng et al., 2003). However, these results must be treated with caution as no other study group has conducted trials on these techniques.

1.6.3 Pancreatic duct stents

Different kinds of stents can be used inside the pancreatic duct. Internal stents are left inside the pancreaticojejunal anastomosis, where the idea is that they facilitate the removal of the pancreatic juice away from the labile anastomosis area and may thus prevent POPF. The first results with this method were promising (Yoshimi et al., 1996). Later studies, however, found no difference in morbidity or mortality when using an intraductal stent (Moriya et al., 2012; Xiong et al., 2012). Theoretically, external stents may be useful in preventing POPF by draining the pancreatic juice completely away from the pancreaticojejunal anastomosis and preventing bile from activating pancreatic enzymes. A randomized, controlled trial by Poon and colleagues showed that external stents reduce POPF when compared with non-stented patients (6.7 vs. 20 %) (Poon et al., 2007). A subsequent prospective patient study showed significant reduction in clinically relevant POPF (6 vs. 22 %), but surprisingly overall

morbidity and mortality were the same whether the patient had a stent or not (Motoi et al., 2012). In both studies, the groups were similar regarding the number of patients with soft pancreas and diameter of the pancreatic duct. No definition of "risk patient" was proposed. Both groups reported a correlation of small pancreatic duct diameter with increased POPF. More studies with larger patient data are needed to clarify the use of external pancreatic duct drainage.

1.6.4 Pharmaceutical trials

Pharmacological therapy has been noted as an option to reduce PD complications in recent years. These drug molecules may be variously targeted such as at activation of pancreatic enzymes. Intrinsic trypsin inhibitor ulinastatin has been shown to slightly reduce endoscopic retrograde cholangiopancreatography (ERCP) induced pancreatitis (Seta & Noguchi, 2011) as well as postoperative pancreatitis related to PD (Chapter 1.4.2). Corticosteroids have failed to reduce post-ERCP pancreatitis (Zheng et al. 2008), but on the other hand, hydrocortisone has been found useful among patients with shock caused by acute pancreatitis (Eklund et al. 2005). A recent study revealed that relative adrenal insufficiency (and associated low cortisol levels) are strongly associated with the most severe types of acute pancreatitis and mortality (De Waele, 2007).

Somatostatin and its analogs inhibit the exocrine function of the pancreas so its prophylactic administration during PD could in theory inhibit POPFs. Somatostatin and its analog octreotide appeared effective earlier in the treatment of POPF for they reduced the time required for the healing of the anastomosis (Hesse et al., 2001). Later meta-analyses did not find solid evidence in favor of the use of somatostatin analogs routinely. Gurusamy and colleagues found that somatostatin analogs did not decrease mortality or reoperation rates, and most of the studies conducted were at high risk of bias. However, treatment was found to decrease the rate of POPF and increased the number of asymptomatic patients. (Gurusamy et al., 2012). Gans and colleagues also found severe flaws in the methodologies of earlier studies. They concluded that solid evidence of the benefits of somatostatin analogs has not been presented, and this therapy should not be used routinely (Gans et al., 2012). A recent randomized

controlled trial (Allen et al., 2014) described a promising 10% rate of clinically significant POPF with pasireotide, a somatostatin analog, compared with an over 20% rate of the placebo group after PD. There were several drop-outs during the trial due to exclusion criteria "a history of clinically significant cardiac disease, including a corrected QT (QTc) interval longer than 450 msec." This criterion is met with in several patients scheduled for PD and thus restricts the use of this medicine.

PD is a major operation with risks for ischemia and oxidative stress (Chapter 1.5). This increases local and systemic inflammation, which may lead to complications and sometimes even to multiple organ failure (Motoyama et al., 2003). Antioxidants are hence an interesting way to try to reduce complications by alleviating oxidative stress. A pilot, placebo-controlled, randomized clinical trial was conducted using an oral nutrition supplement with antioxidants. Patients who received antioxidant therapy preoperatively had higher total endogenous antioxidant capacity values postoperatively than placebo patients, but no significant changes in oxidative stress, inflammatory response or complication rates were observed. (Braga et al., 2012). In an attempt to reduce postoperative complications, acute normovolemic hemodilution was used in one clinical trial with PD patients. Patients in the intervention group still required allogeneic transfusions and were actually more prone to suffer anastomotic complications, likely due to an increased need for intraoperative fluid administration. The authors suggested restrictive intravenous (i.v.) fluid management for PDs in the future. (Fischer et al., 2010).

In terms of cancer survival, a recent retrospective analysis (Call et al., 2015) of perioperative care of patients undergoing pancreatic cancer resection yielded fascinating results: Patients who had received intraoperative dexamethasone had better long-term survival rates than did other patients. A 44% hazard ratio reduction with dexamethasone was observed. Dexamethasone increased median survival to 622 days compared with 479 days in other patients. The authors refer to an earlier hypothesis of an animal study (Rhim et al., 2012) that inflammation may be necessary for pancreatic cancer progression. On the other hand, more patients in the dexamethasone group received postoperative adjuvant treatment (Call et al., 2015).

1.6.5 Experimental trials on animals

Pancreatic inflammation and its prevention have been intensively studied with animal models and in cell cultures. Postoperative pancreatitis may be a significant mediator of postoperative complications related to PD (Chapter 1.4), so in the future therapeutic strategies to prevent postoperative complications may converge with upcoming treatments of acute pancreatitis.

Inflammatory mediator TNF-α is known to increase in serum during acute pancreatitis, and blocking the TNF-α inflammation with anti-TNF-α antibodies or pentoxifylline has been found to decrease mortality in animal models. Blocking TNF-α may therefore be a promising new method to decrease pancreatic inflammation in humans. (Bang et al., 2008). One trial has been made of treating acute alcohol hepatitis with infliximab, a TNF-α inhibitor. The study was arrested prematurely due to increased infection related mortality in the infliximab intervention group. (Naveau et al., 2004). Caution is still necessary when conducting clinical trials with new anti-inflammatory drugs.

A recently discovered, gaseous signaling molecule hydrogen sulfide, H(2)S, has been investigated as a possible mediator of acute pancreatitis. H(2)S has been found to have both pro- and anti-inflammatory roles. Sodium hydrosulfide NaHS on the other hand is an H(2)S donor. A cerulein induced acute experimental pancreatitis in mice was found to attenuate significantly when (NaHS) at the right dose (10mg/kg) was administered, but the benefits were lost if the dose was 5mg/kg or 15mg/kg. (Sidhapuriwala et al., 2009). This supports the idea that as a pharmacologic agent H(2)S is really dose-dependent, and future clinical trials should be conducted with good design and due caution.

Glucocorticoids are well-known anti-inflammatory drugs that are used in several different inflammatory diseases, and their use to suppress pancreatic inflammation is also a subject of great interest. Synthetic glucocorticoid dexamethasone has been

shown to suppress NF-κB and MCP-1 (Scheinman et al., 1995; Yubero et al., 2009) and it has successfully attenuated acute pancreatitis in animal models (Paszt et al., 2004; Yubero et al., 2009).

1.6.6 Can we reduce postoperative complications?

PD associated mortality and POPF rates have decreased especially in the past few decades, but no new revolutionary improvements to the surgical techniques or postoperative treatment have been developed during this time (Chapters 1.2.2, 1.2.3, 1.6.1 & 1.6.2). More importantly, it has been realized that hospital volume and surgeon's personal experience have radical effects on complication rates. Low-volume community hospitals have 3- to 4-fold higher in-hospital mortality than do high-volume centers (Birkmeyer et al., 1999). The long-term postoperative survival of pancreatic cancer patients has likewise improved in high-volume centers (Fong et al., 2005). Surgeons with less experience, fewer than 50 PDs performed, had increased morbidity (53 vs. 39%) and POPF rates (20 vs. 10%) compared with more experienced surgeons (Schmidt et al., 2010).

Attempts to reduce complications with variations of pancreaticojejunostomy or pancreaticogastrostomy have produced inconsistent results, as other groups have failed to repeat the excellent results of the original communication (Chapter 1.6.1). The use of somatostatin routinely for the treatment of POPF has been questioned, but the recent trial with pasireotide produced promising results. Antioxidant therapy did not demonstrate any clinical assets. Ulinastatin may have a role in preventing postoperative pancreatitis, but further studies are needed to approve its use (Chapter 1.6.2). Many PD patients suffer from obstructive jaundice and it has been a matter of debate whether these patients should receive preoperative biliary stenting. A recent meta-analysis concluded that preoperative stenting predisposes to increased morbidity and thus it doesn't recommend routine stenting to jaundice patients who are about to undergo surgery (Fang et al., 2013).

Some high-risk patients may be identified with the methods developed. However, even though soft pancreas, small pancreatic duct, ASA score, intraoperative blood

loss and ischemia all are associated with a higher risk for complications, several high-risk patients have still not been identified (Chapter 1.5). Ideally, all patients at risk of developing postoperative complications should be identified pre- or peroperatively, so that treatment – and possible prophylactic measures – can be targeted correctly. To achieve this goal, we first need to understand the pathophysiology of pancreas-related complications.

AIMS OF THE STUDY

The aim of this thesis was to investigate postoperative pancreatic inflammation: its predisposing factors, and the relation to and prevention of postoperative complications. This can be divided into four study aims:

I. To study which cells in the pancreatic parenchyma are associated with pancreatic inflammation and further to determine a method for identifying high-risk patients.

II. To investigate how fast the inflammatory process progresses after the surgical trauma.

III. To study the paracrine interplay between acinar and stellate cells.

IV. To investigate whether complications after PD among high-risk patients can be reduced with anti-inflammatory treatment.

MATERIALS AND METHODS

3.1 Cell cultures

Primary mouse pancreatic acinar cells were prepared from 6-7-week-old male mice using the long-term cell culture technique and cryopreserved according to methods previously described by our group (Bläuer et al., 2011; Bläuer et al., 2013). The culture medium for acinar cells involved DMEM/F12 (Invitrogen, Grand Island NY) with a 3-fold supplementation of DMEM amino acids (Invitrogen), 25 ng/ml EGF (Sigma St. Louis, MO), 5 µg/ml insulin (Sigma), 0.01% SBTI, 2 mM sodium pyruvate (Invitrogen), 0.2 mg/ml growth factor-reduced Matrigel (BD, Franklin Lakes NJ) and penicillin (50U/ml) / streptomycin (50 µg/ml) (Invitrogen).

Mouse PSCs were obtained by mincing the pancreas and transferring the mass to T75 culture bottles in 7 ml culture medium consisting of DMEM/F12 supplemented with 10% fetal calf serum (FCS), L-glutamine and penicillin (100 U/ml) / streptomycin (100 µg/ml). The outgrown PSCs were collected after 7-9 days from culture, then reseeded in T75 bottles and allowed to grow to near confluence. Finally, the cell cultures were detached and cryopreserved in liquid nitrogen. Mouse 3T3 fibroblasts obtained from ATCC (Manassas, VA) were defined as controls and reproduction was performed in the same medium as with PSCs.

Preliminary experiments were performed to evaluate PSC and 3T3 fibroblast growth in the FCS-free acinar cell-specific medium. Cells were inserted into 96-well plates at a density of 2500 cells per well in their normal FCS-containing medium and allowed to attach for 3 days. The cells were then incubated for 7 days in acinar cell-specific or in FCS-containing medium. The number of cells was evaluated on days 4 and 7 using the colorimetric crystal violet assay (Bläuer et al., 2009).

The co-culture was created in a 24-well with acinar cell-specific medium. 50 µl aliquots of suspension with approximately 10,000 acinar cells were inserted into the center of each well. On the next day 250 µl fresh culture medium was added. 10,000 PSCs were seeded onto cell culture inserts placed in a separate 12-well plate. On the following day the inserts were transferred into the acinar cell wells and co-cultures were maintained for 4 days. Half of the medium in each well was renewed every day. Controls consisted of monocultures with acinar cells or PSC. Co-culture control was set up with mouse 3T3 fibroblasts instead of PSCs.

Secretory capability of acinar cells was determined as amylase release in basal and cerulein-stimulated conditions. Acinar cells from monocultures and acinar-stellate or acinar-3T3 fibroblast co-cultures were rinsed with fresh culture medium, after which they were left in 100 µl of medium without (basal secretion) or with 0.1nM of cerulein (Sigma). After 1h of incubation the media were analyzed with a Cobas c111 autoanalyzer (Roche, Mannheim, Germany). A similar volume of medium containing 0.1% Triton X-100 was added in wells and incubated for another 1h and the cellular amylase content was analyzed. Amylase release was calculated as the percentage from the total amylase content.

Antibodies against fibronectin (Abcam, Cambridge, UK) and collagen I (Novus Biologicals, Cambridge, UK) were used by immunocytochemical means to evaluate ECM protein expression in PSCs cultured alone compared to those co-cultured with acinar cells. Antibodies against high mobility group box 1 (HMGB1) and nuclear factor-κB (NF-κB) p65 (both from Abcam, Cambridge, UK) were used to study the effect of co-culture on necrosis and inflammation, respectively. Prior to consecutive incubations with primary and secondary antibodies (Novex Histostain Plus kit, Invitrogen, Carlsbad, CA) the cells were fixed with 4% paraformaldehyde and permeabilized in 94% ethanol (Bläuer et al., 2009; Bläuer et al., 2011). Immunoreactive proteins were found with diaminobenzidine (DAB; Novex Liquid DAB Substrate Kit, Invitrogen, Carlsbad, CA). Hematoxylin counterstaining was used. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Promega, Madison, WI, USA) was conducted according to the protocol of the

manufacturer to find out the level of apoptosis in mono- and co-cultures. Acinar cells nursed with DNase I (Promega) were used as positive controls.

To rule out any potential direct effects of cryopreserved acinar cells on PSCs, conditioned media were collected from both acinar cell monocultures and acinar-PSC co-cultures. The conditioned media were diluted 1:3 with fresh medium and applied to the PSC monocultures for 4 days with daily medium renewal and finally collagen I expression in PSCs cultured in each conditioned medium was determined.

The morphology of acinar cells in mono- and co-cultures was examined under a phase-contrast microscope (Nikon Eclipse Ti-S, Tokyo, Japan). PSC number and migration were assessed by the amount of cells adhering to the top and bottom surfaces of the insert membrane in five randomly selected areas (200x magnification).

3.2 Human pancreatic samples

In Study I, the whole paraffin control section of frozen section was photographed into electronic form with a microscope using 10 times objective magnification. Each picture (medium 133 (range 38 – 280) per patient) was analyzed by 2 researchers blind to the patients' clinical data. The areas of different cell types were analyzed with a graphics tablet and Adobe Photoshop program as previously described (Laukkarinen et al., 2007). Areas of normal acini and damaged acini, fibrosis, intrapancreatic fat (normal and necrotic), and peripancreatic fat were calculated. Number of leukocytes (predominantly neutrophil granulocytes and lymphocytes) was also graded as 0 to 2 (0, none; 1, few/some; 2, many).

In Study II, at the time of the transection of the pancreas during PD, a tissue sample (size 2 mm thick, 10 mm in diameter) was harvested from the cut edge of the pancreas. The specimen was divided into five pieces which were immediately immersed in physiologic NaCl solution to avoid drying. The tissue was thus predisposed to surgical trauma followed by ischemia *ex vivo*. At 15 minutes, 2 – 2.5 hours, 4 hours, or 6 hours the NaCl solution was replaced with 4% paraformaldehyde to fix the samples at the different time points. The samples were allowed to fix overnight, then dehydrated and

embedded in paraffin. 5 μm thick sections were cut for immunohistochemical analysis, which was performed using anti-NF-κB p50 (1:200 dilution; AbD Serotec, Oxford, UK) and anti-MCP-1 (1:200 dilution; AbD Serotec) antibodies. Omission of the primary antibodies and the use of non-immunized mouse and rabbit IgG were used as controls. The staining was done with a broad-spectrum Histostain-Plus kit (Invitrogen, Camarillo, CA, USA). All sections received a light counterstaining with hematoxylin. The slides were analyzed under a microscope (Nikon Microphot-FXA). NF-κB staining was seen in the nuclei of the acinar cells, and MCP-1 activation was found in the cytoplasm of the acinar and ductal cells. The percentage of NF-κB-stained acini out of the total number of acini at 15-minute and 4-hour samples was determined from representative areas using a magnification of 250. This quantitative analysis was done by two independent researchers. The means (±SEM) of the three acinar cell-rich and the three fibrotic samples were subsequently calculated. The intensity of MCP-1 staining in samples was determined semi-quantitatively and expressed as low, moderate, or high.

In Study IV, during normal frozen section analysis for malignancies, the pathologist also estimated the number of acinar cells in the CEP: If there were more than 40% acini, the subject was considered a risk patient (Figure 1). This 40% cut-off was based on the results of Study I (Chapter 4.2).

3.3 Patients

In Study I, data from 43 consecutive PD patients treated in the period 2007 – 2009 in Tampere University Hospital were prospectively collected. Only patients with complete prospective day-by-day postoperative standard monitoring were included and thus 3 patients were excluded. Finally, the patient material consisted of 40 individuals. Tampere binding pancreaticojejunal anastomosis was used on every patient. 20 patients also received a biodegradable stent in the pancreaticojejunal anastomosis. The postoperative data with routine measurements of the 40 patients was collected retrospectively from a prospective database in Study I. The pancreatic duct diameter was measured and operation time and operative blood loss were registered. Follow-up consisted of routine measurements of urine trypsinogen (postoperative

days 1-6), drain amylase (postoperative day 3), registration of local complications: internationally defined (Chapter 1.3) DGE, PPH and POPF, length of hospital stay, 30- and 90-day mortality.

For Study II 6 individuals undergoing PD were chosen on the basis of the histopathology of the cut edge of the pancreas: 3 with acinar cell-rich pancreas (>40 % acini on the cut edge) and 3 with fibrotic pancreas (>60 % fibrosis on the cut edge).

In Study IV, 160 PD patients treated in the period February 2011 – May 2015 in Tampere University Hospital were assessed for eligibility. The patients were consecutive apart from summers 2013 and 2014, when the study was interrupted due to research staff logistics. Exclusion criteria were: ongoing cortisone treatment for any reason, ceftriaxone allergy or PD indication other than tumor. Preoperative data such as BMI, ASA class, coronary heart disease (CHD), chronic obstructive pulmonary disease (COPD), diabetes, smoking, preoperative ERCP or PTC were all prospectively recorded. Patients were randomized into two groups: hydrocortisone and placebo group. Tampere binding pancreaticojejunal anastomosis was used on every patient.

Patients received routine antibiotic prophylaxis (Rocephalin 2 g (ceftriaxone; Roche, Espoo, Finland; Metronidazole 500 mg (metronidazole; Braun, Melsungen, Germany) i.v. and, depending on the randomization, either 100 mg hydrocortisone or placebo i.v. in the morning before surgery. On the basis of the frozen section analysis, the pathologist informed the surgeon during the operation whether the subject was deemed a risk patient: Treatment with cortisone or placebo was discontinued if there were fewer than 40% acini in the CEP, and normal protocol postoperative treatment was resumed. If the patient belonged to the risk group, treatment was continued with 100mg of hydrocortisone (or placebo) i.v. every 8 hours until the evening of second postoperative day. Every patient also received ceftriaxone 2g i.v. once a day until the evening of the second postoperative day. (Figure 1).

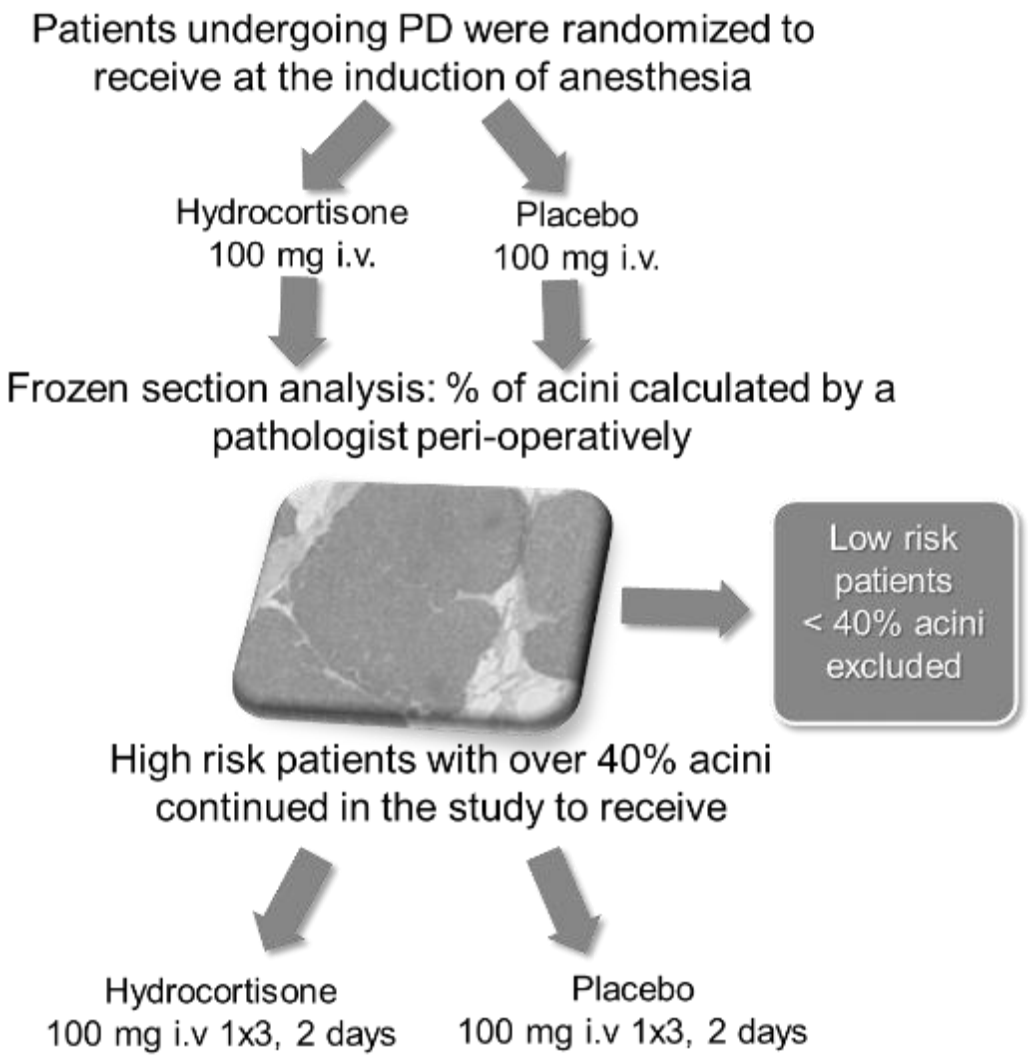


Figure 1. *Perioperative medication in Study IV. During frozen section analysis, patients with under 40% acini in the CEP were excluded. High-risk patients with over 40% acini continued to receive randomization-based medication until the evening of the second postoperative day.*

The randomization list was done by a biostatistician. On the morning of the surgery, the research nurse delivered the medicine or placebo bag (depending on the patient's number on the list) to the surgical ward. The cortisone solution consisted of hydrocortisone sodium succinate (Solu-Cortef; Pfizer Manufacturing, Puurs, Belgium) in 0.9% sodium chloride solution (Natriumklorid Braun, 9 mg/ml): 100mg per 2ml hydrocortisone sodium succinate was added into 100ml of 0.9% sodium chloride solution in polyethylene infusion bags. The placebo solution was prepared by adding 2ml of 0.9% sodium chloride solution into 100ml of 0.9% sodium chloride

solution in polyethylene infusion bags. The hydrocortisone and placebo bags were identical in color and other external features.

Study IV was randomized, double-blinded and the patients' postoperative data was collected prospectively. Randomization was decoded at the 2-year analysis and at the final analysis. During surgery, the pancreatic duct diameter was measured and operation time and operative blood loss were registered. Follow-up consisted of routine measurements of urine trypsinogen (postoperative days 1 – 6), CRP and leukocyte levels (postoperative days 1 – 3), drain amylase (postoperative day 3), registration of local complications: internationally defined (Chapter 1.3) DGE, PPH and POPF, biliary leakages, intra-abdominal abscesses, wound infections, thromboembolisms, length of hospital stay, 90-day mortality. Major complication was considered to be Clavien-Dindo III-IV (Chapter 1.3.4). Primary endpoints were overall major complications (Clavien-Dindo III-IV) and urine trypsinogen release (two or more positive days), and secondary endpoints were PD-specific complications (POPF, PPH, DGE), mortality and general infectious complications.

3.4 Statistics

In study IV, power calculations were originally performed according to two different hypotheses: Trypsinogen release ("chemical response") and overall complications ("clinical response") to reduce 50% in high-risk patients with hydrocortisone treatment. The trypsinogen hypothesis needed higher number of patients to be included and was thus used to designate the sample size (alpha 0.05, 80% power). Based on our previous studies we calculated that three times more patients should initially be recruited. The statistical analyses in Studies I and IV were performed using SPSS statistical software. χ^2 test for cross-tabulated qualitative variables and Mann-Whitney test for quantitative variables were used to evaluate the statistical significance between groups. Multivariate analysis was performed in Study I to identify variables associated with complications. Quantitative analysis of Study II was done by comparing the means (±SEM) of the two study groups. Two-way ANOVA was used to analyze statistical significance in Study III.

3.5 Ethics

Studies involving human subjects (I, II & IV) were conducted in accordance with the Helsinki Declaration. The study protocol of Study I was approved by the medical director of Tampere University Hospital, Finland. Studies II and IV were approved by Tampere University Hospital ethics committee and Study IV was registered in clinicaltrials.gov in 2011, identity code NCT01460615.

RESULTS

4.1 Association of acini and fibrosis with complications

Study I included 20 women and 20 men median age 66 (range 21 – 80) years. Final histological diagnosis was ductal adenocarcinoma in 24 patients, cancer of the biliary duct in 7 patients, periampullary cancer in 3 patients, chronic pancreatitis in 3 patients, benign mucinous tumor in 1 patient, endocrine adenoma in 1 patient, and metastases from breast lobular carcinoma in 1 patient.

As the results of Study I emerged, it was clear that fibrosis protected from and acinar cells predisposed to complications. The complications were substantially increased if there were over 40% acinar cells in the CEP and thus two subgroups were formed. The high-risk, acinar subgroup (12/40 patients): pancreatic acinar cells covering more than 40% of CEP. The low-risk, fibrosis subgroup (19/40 patients): fibrosis covering more than 60% of CEP.

Nearly every patient (92% (11/12)) in the acinar subgroup suffered from at least one complication. The difference is vast when compared to patients with less than 40% of acinar cells in CEP (32 %; 9/28, $p<0.001$). Only 17% of the patients in the acinar subgroup had normal gastric emptying compared to 89% among the rest of the patients. High frequency of acinar cells in CEP correlated significantly with large number of positive urine trypsinogen days ($r=0.516$; $p=0.001$) and high drain amylase ($r=0.532$; $p=0.001$). In the acinar subgroup, number of positive trypsinogen days (2.42 ± 0.71 vs. 0.43 ± 0.18; $p=0.02$) and amount of drain amylase on third postoperative day (149 ± 58 vs. 10 ± 2 U/l; $p=0.036$) were significantly higher than among the rest of the patients.

Prevalent fibrosis in turn correlated negatively with positive urine trypsinogen days ($r=-0.434$; $p=0.005$), drain amylase ($r=-0.405$; $p=0.011$), and wound infections

(r=-0.482; p=0.002). In the fibrosis subgroup, positive urine trypsinogen days (0.37 ± 0.22 vs. 1.62 ± 0.48; p=0.024) and drain amylase levels (12 ± 2 vs. 89 ± 35 U/l; p=0.047) were significantly lower than among patients with less than 60% of fibrosis in CEP. The areas of intrapancreatic, peripancreatic, normal or necrotic fat, did not correlate with trypsinogen, amylase or complications. Nor was any major difference found in the distributions of fat between the two subgroups.

The acinar subgroup had more extensive pancreatic inflammation and a higher complication rate than the fibrosis subgroup (Table 1). Thus a risk identification method for patients undergoing PD was developed: If an individual undergoing PD has over 40% acini in the CEP, he/she should be classified as a risk patient.

Complications	Acinar subgroup n=12	Fibrosis subgroup n=19
Postoperative pancreatic inflammation	50%	11%
POPF	17%	0%
PPH	8%	5%
DGE gr. A	42%	0%
gr. B	8%	5%
gr. C	33%	5%
Wound infections	42%	0%

Table 1. *Comparison of complication rates between acinar subgroup (acini over 40% of the CEP) and fibrosis subgroup (fibrosis over 60% of CEP) in Study I. POPF = Postoperative pancreatic fistula, PPH = Postpancreatectomy hemorrhage, DGE = Delayed Gastric Emptying. There were more complications in the acinar subgroup than in the fibrosis subgroup.*

4.2 Postoperative inflammation in acinar cell-rich and fibrotic pancreas

The acinar cell-rich group in Study II comprised: 50-year-old female with neuroendocrine carcinoma of the head of the pancreas, 55 and 57-year-old males with adenocarcinomas of the head of the pancreas. The fibrotic group consisted of a 78-year-old male with serous cystadenoma of the head of the pancreas, and 60 and 74-year-old males with adenocarcinomas of the head of the pancreas.

The results of Study II shed light on the molecular background of the differences between acinar cell-rich and fibrotic pancreas. Qualitative analysis revealed the progression of NF-κB activation in acinar cell-rich pancreata during the 6-hour period (Figure 2) such that the highest NF-κB expression was at 4 hours (Figures 2 & 3).

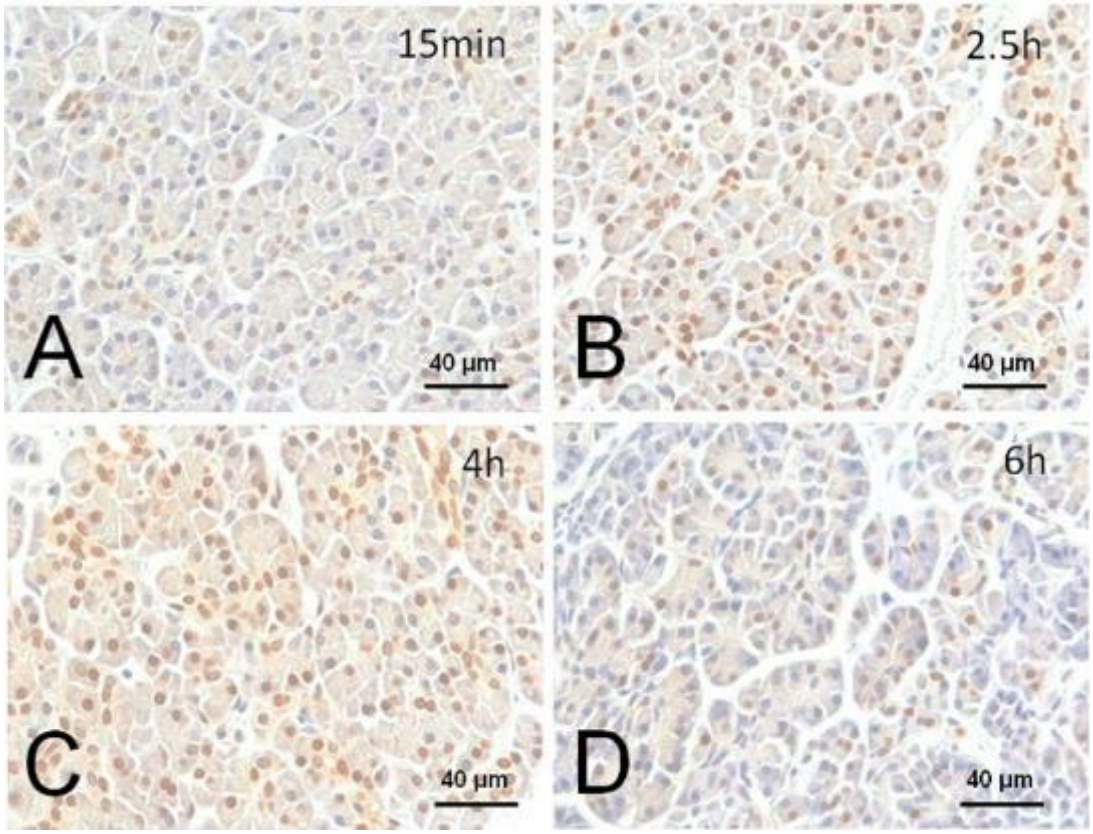


Figure 2. *Progression of NF-κB activation in acinar cell-rich pancreas. 15-minute (A), 2.5-hour (B), 4-hour (C) and 6-hour (D) samples. Some staining of acinar cell nuclei can be seen at 15 minutes, significant amplification is seen at 2.5 hours, and almost every acini is stained at 4 hours. Finally, the activation decreases at 6 hours.*

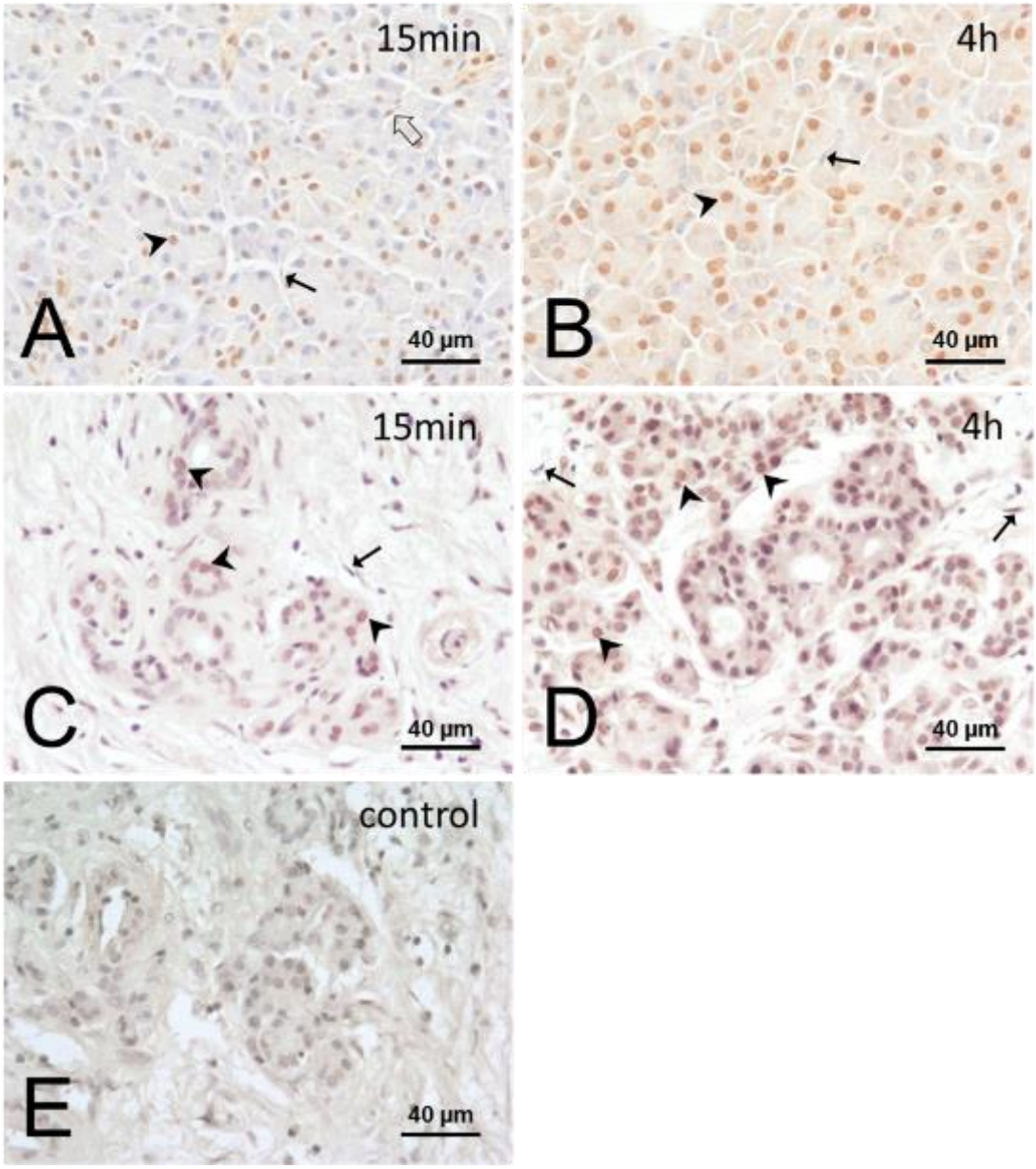


Figure 3. *NF-κB staining in acinar cell-rich (A, B) and fibrotic (C, D) pancreata. A, C are 15-minute samples and B, D are 4-hour samples. Arrowheads show NF-κB-expressing nuclei in acini. NF-κB-positive fibroblasts were rare (open arrow in A), the fibroblast being primarily negative (arrows). The increase in NF-κB activation is more distinct in acinar cell-rich pancreata than in fibrotic pancreata. Control stainings remained negative (E).*

In the fibrotic pancreata, acinar cell activation of NF-κB was also detected, but the tissue expression of NF-κB did not increase over time (Figure 3). NF-κB-positive fibroblasts were scarce, the fibroblast nuclei being predominantly unstained. In all

tissue sections the intensity of NF-κB staining appeared even and no gradient from outside to inside was detectable.

Quantitative analysis for the acinar cell-rich pancreata revealed that acinar cell NF-κB activation increased from mild at 15 minutes (35 % ± 7 %, mean ± SEM) to high (74 % ± 4 %) during the first 4 hours (Figure 4). NF-κB expression was 30% (± 6%) at 15 minutes and 35% (± 4%) at 4 hours in the fibrotic pancreata (Figure 4).

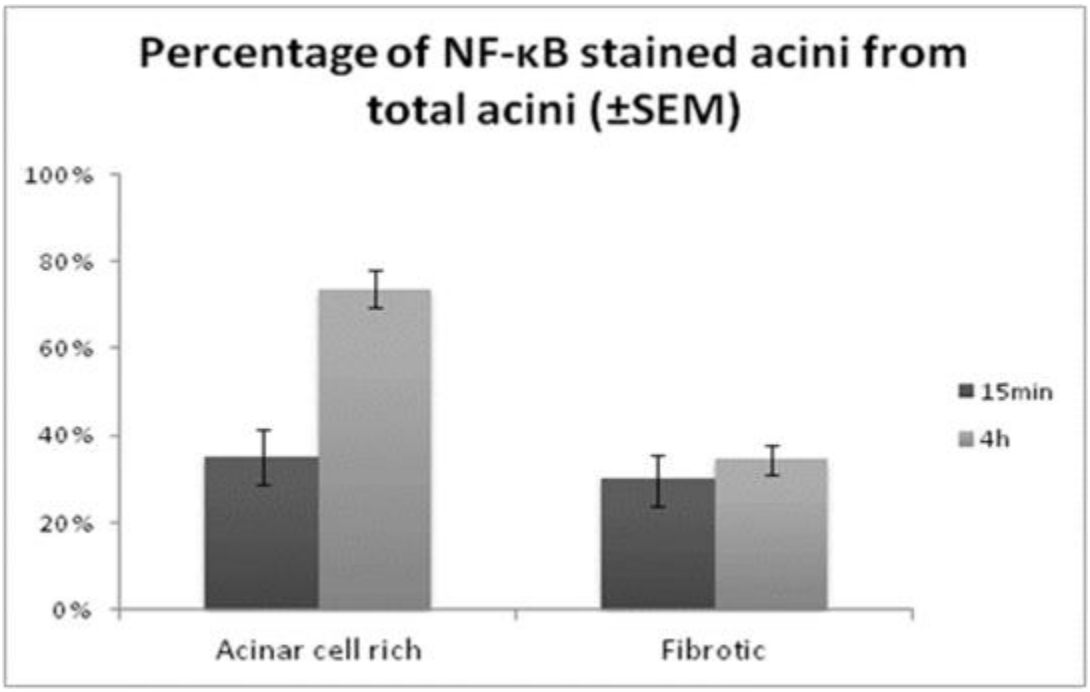


Figure 4. *Comparison of NF-κB activation in acinar cell-rich and fibrotic pancreata. The means (±SEM) of the three acinar cell-rich and the three fibrotic samples were calculated and then compared at 15 minutes and 4 hours. In acinar cell-rich pancreata, a significant increase in NF-κB expression occurs between 15 minutes (35%) and 4 hours (74%). In fibrotic pancreata, the change between 15 minutes (30%) and 4 hours (35%) is minor.*

Acinar cell MCP-1 activation increased from low at 15 minutes to moderate during the first 4 hours in acinar cell-rich pancreas, whereas in ductal cells MCP-1 staining was extremely intense at both time points (Figure 5). Acinar and ductal cells did not express MCP-1 at 15 minutes in fibrotic pancreas and only slight staining was seen at 4 hours (Figure 5).

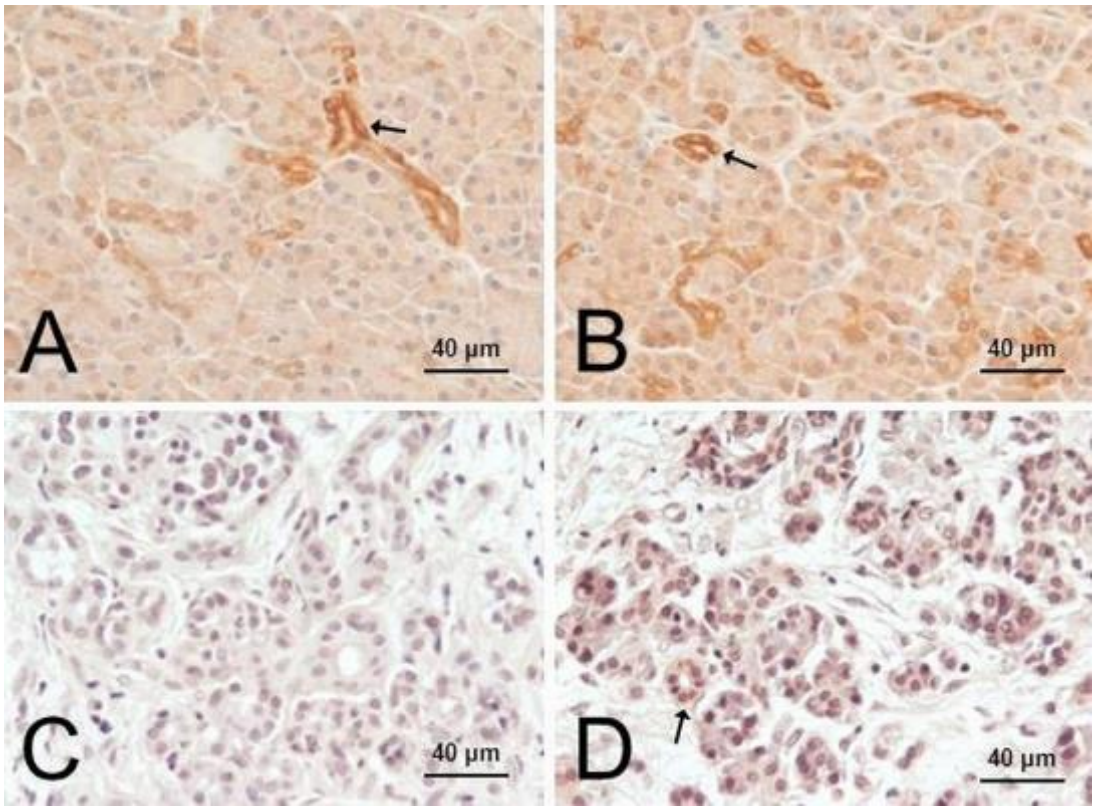


Figure 5. *MCP-1 expression in acinar cell-rich (A, B) and fibrotic (C, D) pancreata. 15-minute (A, C) or 4 hour (B, D) samples. MCP-1 staining was equally intense in the ductal cells of acinar cell-rich pancreata after 15 minutes and 4 hours (A, B, arrows), whereas intra-acinar MCP-1 expression was observed to increase slightly over time. MCP-1 remained absent in fibrotic pancreas in ductal and acinar cells at 15 minutes (C). At 4 hours, weak staining is seen in ductal cells (D, arrow).*

4.3 Paracrine interplay between acinar and stellate cells

Acini monocultures in Study III exhibited a healthy monolayer structure. Acinar cells cultured together with fibroblasts (controls) remained morphologically similar to monocultures, whereas deformed and vacuolized acinar cells with prominent cell boundaries dominated in the acinar-PSC co-cultures. (Figure 6).

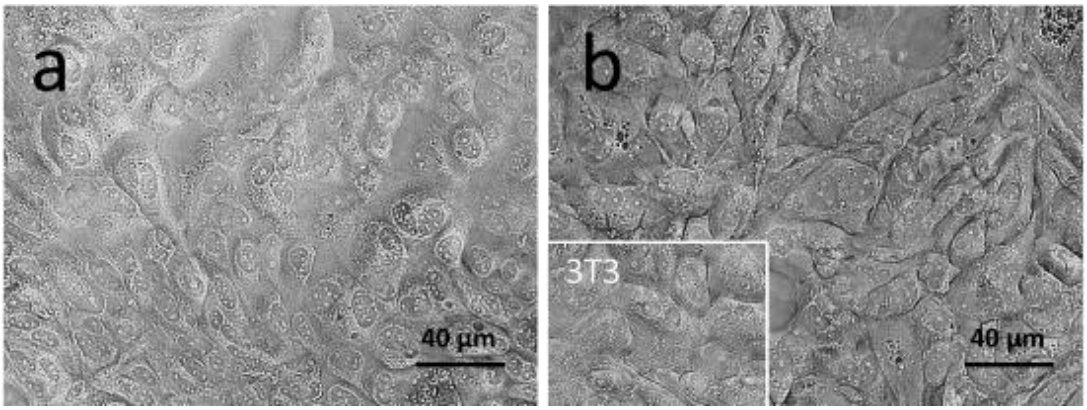


Figure 6. *Acinar cell morphology in monoculture (a), 3T3 fibroblast control co-culture (inset in b) and acinar-PSC co-culture (b). The acinar cell morphology was found to be remarkably healthier in mono- and control co-cultures than significant acinar cell deformity in acinar-PSC co-cultures.*

Acinar monocultures and control (fibroblast) co-culture cerulein stimulation produced a 2.2-fold increase in amylase secretion compared to basal secretion (p=0.0648 and 0.0318 respectively) whereas acinar-PSC co-cultures were insensitive to cerulein stimulation. In both PSC and control acinar co-cultures the mean basal levels of amylase release were about 1.5-fold higher than those in monocultures. (Figure 7).

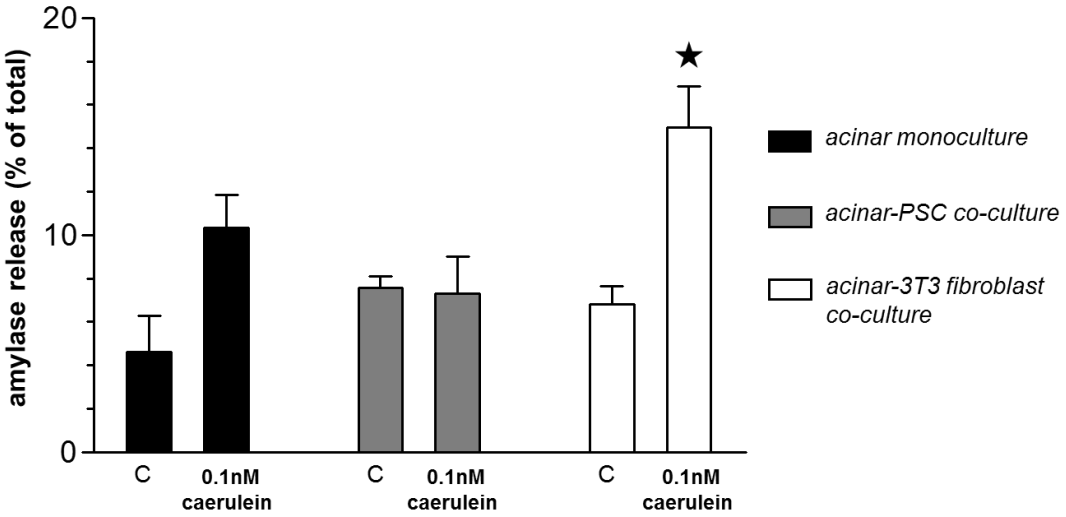


Figure 7. *Basal- and cerulein stimulated amylase secretions in acinar monoculture, PSC-acinar and control (3T3 fibroblast) co-cultures. The PSC-acinar co-cultures were found to be insensitive to cerulein stimulation.*

Acinar cells stimulated migration of PSCs to 1.8-fold in co-cultures compared to PSC monocultures (p=0.043). The expression of ECM proteins remained low in PSC monocultures as seen in immunocytochemical analyses of collagen I and fibronectin (Figure 8). Co-culture with acinar cells on the other hand markedly increased the amount of both proteins (Figure 8).

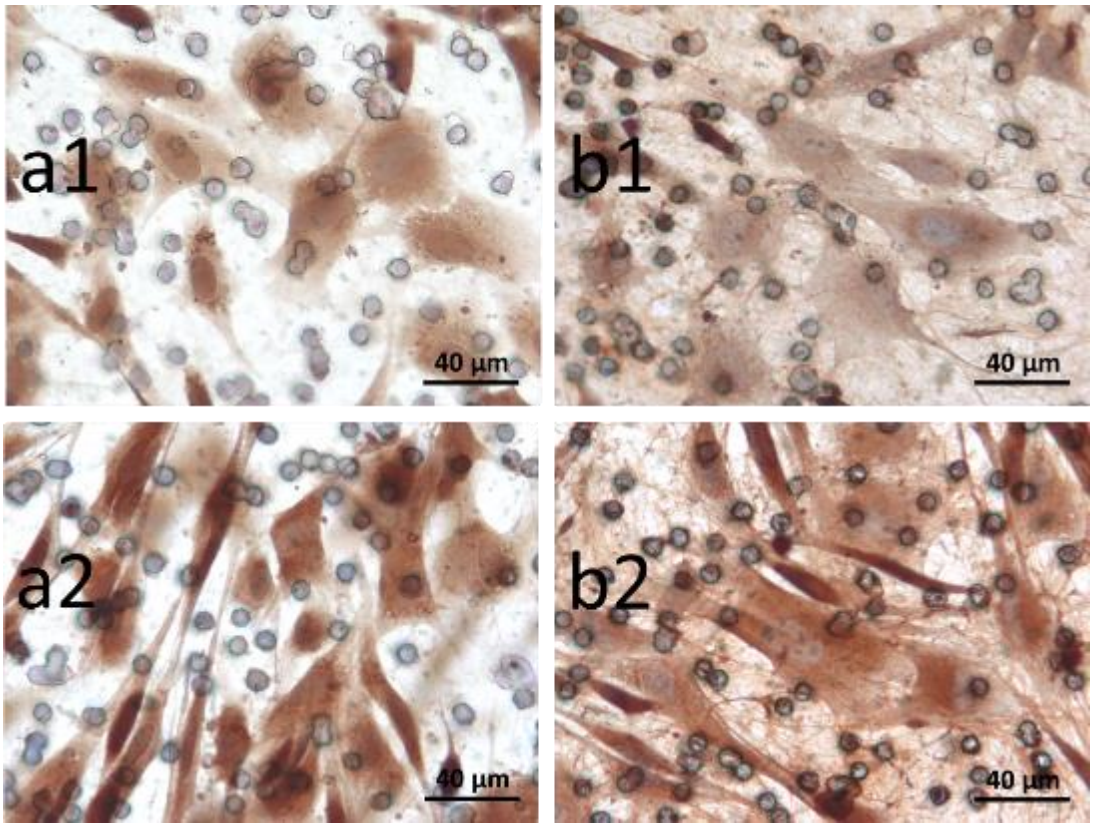


Figure 8. *Immunocytochemical analysis of ECM proteins. Collagen I (a1,a2) and fibronectin (b1,b2) in PSCs maintained alone (a1,b1) or in co-culture with acinar cells (a2,b2) for 4 days. Both proteins increased significantly in co-cultures compared to monocultures.*

PSC collagen I expression was slightly increased by conditioned medium from acinar cell monocultures (Figure 9) whereas a strong stimulation was evoked by conditioned medium from acinar-PSC co-cultures (Figure 9).

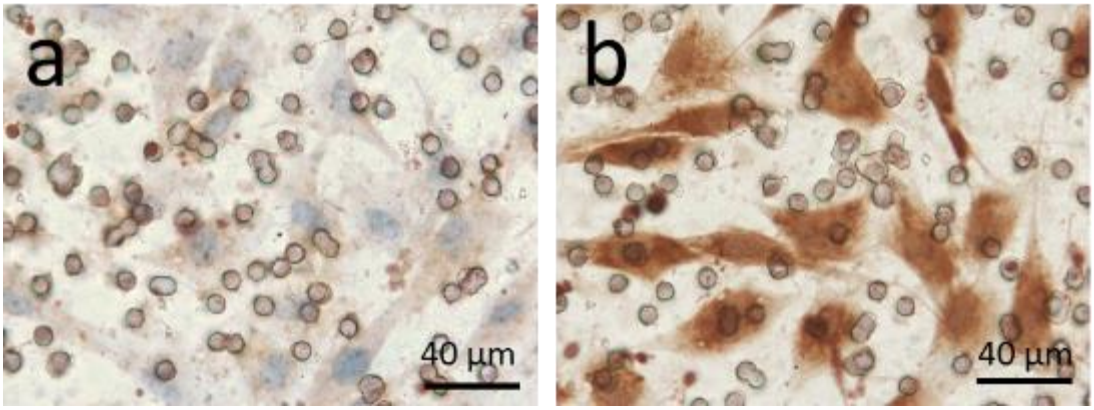


Figure 9. *Effect of conditioned media on PSC collagen I manifestation. The cells were maintained in cell culture inserts for 4 days in medium supplemented 1:3 with conditioned medium from acinar monocultures (a) or acinar-PSC co-cultures (b). Exposure to conditioned medium from co-cultures resulted in a remarkable increase in collagen I expression, whereas only a slight increase was evoked by acinar cell monocultures.*

The early necrosis marker HMGB1 was highly activated in acinar cells in monoculture (Figure 10A) whereas in co-cultures HMGB1 was less abundant or absent (Figure 10B). A similar loss of nuclear HMGB1 was observed in acinar cells monocultures exposed to 0.1nM cerulein (Figure 10G). No change on HMGB1 was observable in co-cultured cells stimulated with cerulein (Figure 10H). Apoptotic cells were rare in all cultures (Figures 10C-J). A light cytoplasmic staining of NF-κB was seen in monocultures (Figure 10E) while co-cultured cells displayed vast nuclear activation (Figure 10F). Few cells in monocultures treated with cerulein displayed nuclear immunoreactivity (Figure 10K). Additional changes with cerulein were not evoked in co-cultured cells (Figure 10L).

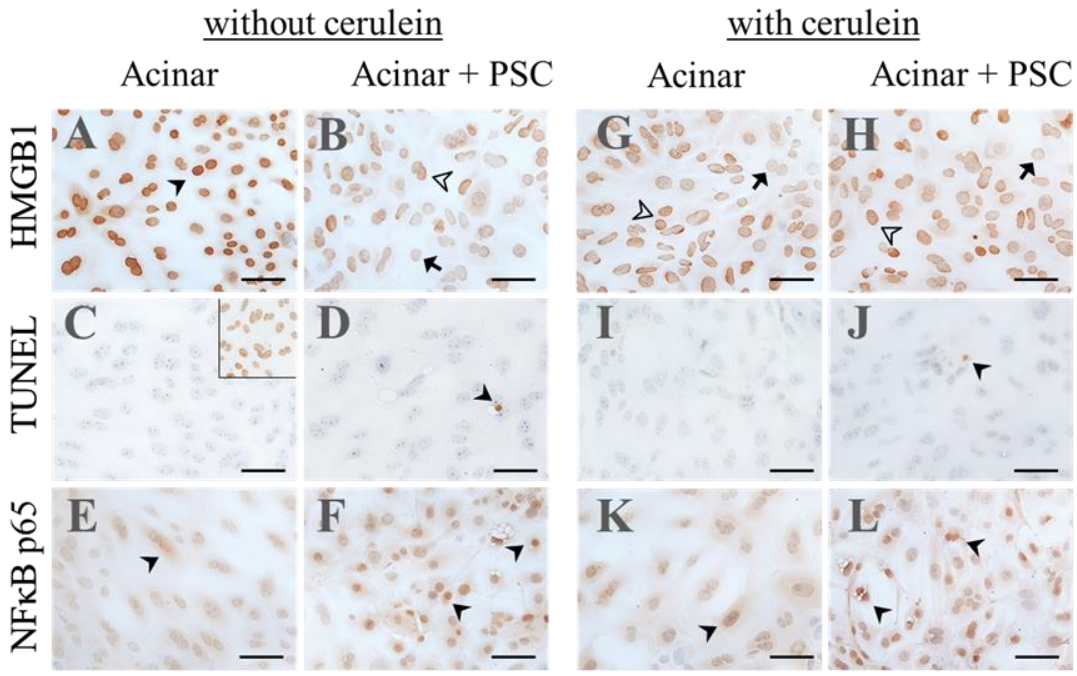
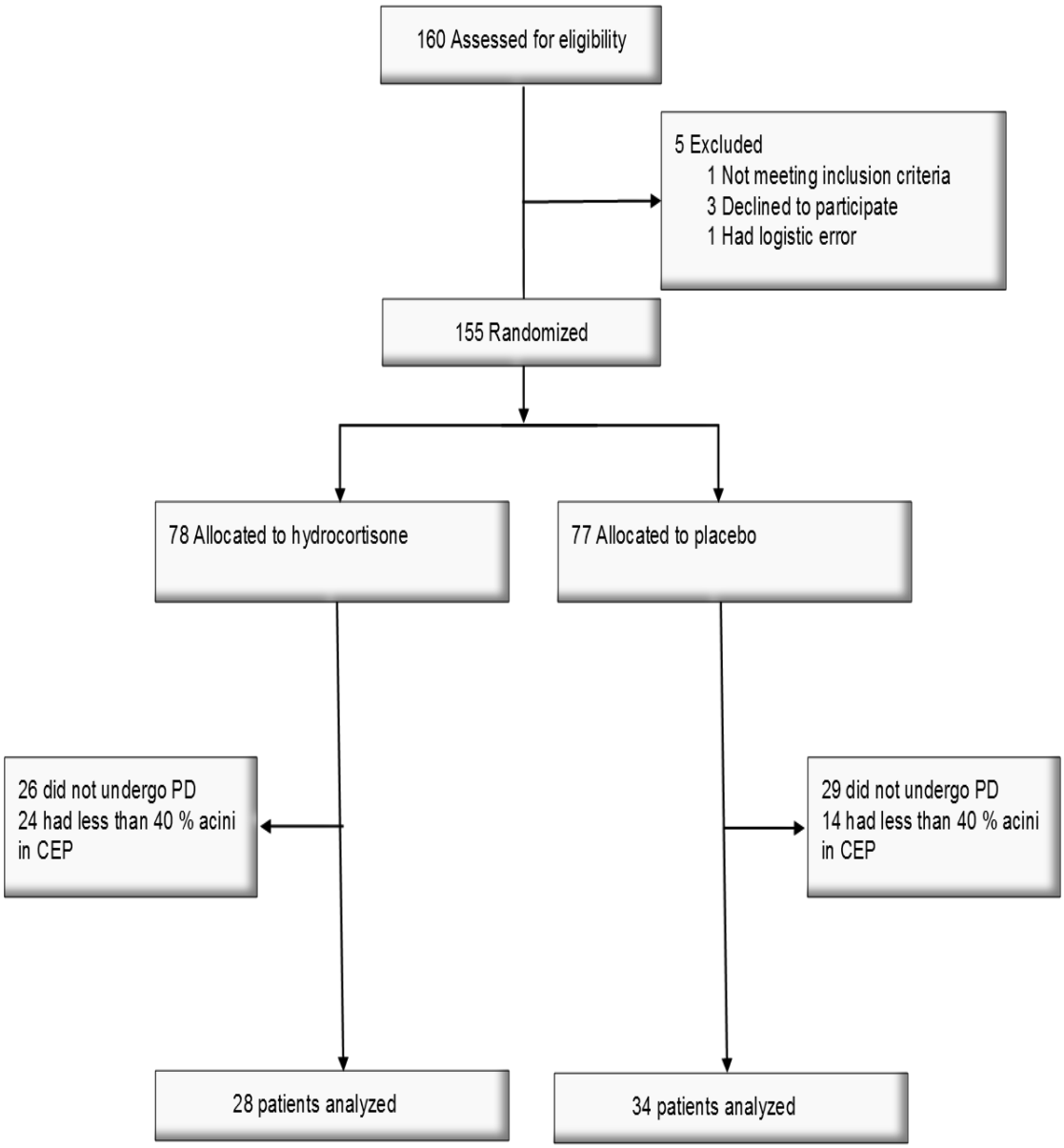


Figure 10. *Effect of co-culturing on acinar cell necrosis (HMGB1), apoptosis (TUNEL) and inflammation (NF-κB). Bars 40 µm. Acinar cells were nurtured 4 days alone or in co-culture with pancreatic stellate cells (PSCs) without (A-F) or with (G-L) exposure to 0.1nM cerulein for 1 hour. A vast activation of the necrosis marker HMGB1 predominated in monocultures (A; arrowhead). In co-culture (B) and cerulein-treatment of mono- or co-cultures (G, H) HMGB1-staining was less intense (open arrowheads) or negative (arrows). Apoptotic cells (TUNEL-staining in C-J) were rare (arrowheads in D-J) in all cultures. Inset in C shows TUNEL-positive acinar cells after exposure to DNase I. A light cytoplasmic staining of NF-κB was seen in monocultures (E; arrowhead). Increased nuclear translocation of NF-κB was detected in co-cultured acinar cells (F, L; arrowheads). Few immunopositive nuclei were found in cerulean stimulated monoculture (K; arrowhead).*

4.4 Reduction of complications through hydrocortisone

In Study IV, 55 patients did not undergo PD and 38 patients had less than 40% acini in CEP and were thus excluded. Altogether 62 patients were included in the final analysis (Flow chart of Study IV). There were 23 women and 39 men median age 65

(range 43 – 83) years. The final histopathological diagnoses were: 27 ductal adenocarcinomas, 14 biliary duct carcinomas, 7 duodenal adenocarcinomas, 5 IPMN, 3 GIST, 3 chronic pancreatitis, 2 metastases from kidney cancer and one inflammation and fibrosis of the biliary duct. There were no significant differences in pre- and peroperative characteristics between the hydrocortisone and placebo groups indicating a successful randomization (Table 2).



Flow chart of Study IV. *78 patients were randomized to the hydrocortisone group and 77 to the placebo group. 28 in the hydrocortisone group and 34 in the placebo group underwent PD and had over 40% acini in CEP.*

Characteristics	All patients (n=62)	Hydrocortisone (n=28)	Placebo (n=34)	P value
Male	39 (63%)	18 (64%)	21 (62%)	0.838
Age	65 (43-83)	63 (43-82)	66 (45-83)	0.128
BMI	26 (17-38)	25 (18-38)	26 (17-38)	0.707
Diabetes	14 (23%)	5 (18%)	9 (27%)	0.420
CHD	4 (7%)	1 (4%)	3 (9%)	0.620
COPD	1 (2%)	0 (0%)	1 (3%)	1.000
Prior abdominal surgery	28 (45%)	10 (36%)	18 (53%)	0.175
Smoking	14 (23%)	7 (25%)	7 (21%)	0.679
Preoperative ERCP/PTC:				0.197
No biliary stent	20 (32%)	10 (35%)	10 (29%)	
ERCP	29 (47%)	15 (54%)	14 (41%)	
PTC	13 (21%)	3 (11%)	10 (29%)	
ASA class:				0.644
I	2 (3%)	1 (4%)	1 (3%)	
II	28 (48%)	13 (50%)	15 (46%)	
III	27 (46%)	12 (46%)	15 (46%)	
IV	2 (3%)	0 (0%)	2 (6%)	
Operative time	285 (200-431)	279 (230-365)	303 (200-431)	0.126
Operative blood loss	800 (130-4700)	785 (130-2500)	900 (300-4700)	0.379
Pancreatic duct diameter	3 (1-15)	3 (2-8)	4 (1-15)	0.127

Table 2. *Pre- and peroperative characteristics of patients in Study IV. Randomization was successful as there were no differences between the two study groups.*

The risk identification method of 40% acinar cells was validated in Study IV: Patients with under 40% acini in CEP had very few PD-specific complications with a POPF rate of 0%, PPH (B & C) rate of 3% and DGE (B & C) rate of 26%.

Hydrocortisone treatment did not alter trypsinogen release (two or more positive days 46 vs. 50% (p=0.779), hydrocortisone vs. placebo. When comparing intervention-requiring or life-threatening complications (Clavien-Dindo III-IV), a significant difference between the hydrocortisone and placebo groups was seen: 18% vs. 41% ($p<0.05$) respectively (Tables 3 & 4). The risk ratio for the primary endpoint of Clavien-Dindo III-IV was 0.4337 (CI 95% 0.1780 to 1.0563, p=0.0659) for hydrocortisone when compared with placebo. This means that the number needed to treat (NNT) – or in this case to prevent - a Clavien-Dindo III-IV complication with a hydrocortisone is as low as 4.

There were no 90-day mortalities. In PD-specific complications (POPF, PPH, DGE), a trend for higher complication rates was observed in the placebo group. Regarding the secondary endpoint of infectious complications, the number of intra-abdominal abscesses was actually greater in the placebo group and there were no differences in wound infections. (Table 3). The CRP values (highest value from postoperative day two to three) were significantly reduced in the hydrocortisone group 98 (9-334) vs. 163 (52-317) (median, range; p=0.001) of the placebo group. On the other hand leukocyte levels did not differ between the two groups 14 (8-24) vs. 14 (7-32) (median, range; p=0.792).

One patient (3.6%) in the hydrocortisone group received intensive care: this was due to mesenteric vein thrombosis. Three patients (9.4%) in the placebo group needed intensive care; one due to ESBL sepsis, one due to severe postoperative pancreatitis followed by grade C hemorrhage, one due to grade C POPF with relaparotomy followed by transient hepatic failure. All of these patients healed eventually. Between hydrocortisone 9 (6-25) and placebo groups 11 (6-55) (median, range; p=0.638), there were no significant differences in the hospital stay. The readmission rates on the other hand were 0% for the hydrocortisone and 12% for the placebo group (p=0.06).

Complications	All risk patients (n=62)	Hydrocortisone (n=28)	Placebo (n=34)	P value
Clavien-Dindo III-IV	19 (31%)	5 (18%)	14 (41%)	<0.05
Trypsinogen release	30 (48%)	13 (46%)	17 (50%)	0.779
90-day mortality	0	0	0	
POPF (B & C)	12 (19%)	3 (11%)	9 (27%)	0.118
PPH (B & C)	12 (19%)	4 (14%)	8 (24%)	0.359
DGE (B & C)	23 (37%)	8 (29%)	15 (44%)	0.207
Biliary leakage	5 (8%)	1 (4%)	4 (12%)	0.238
Intra-abdominal abscess	10 (16%)	3 (11%)	7 (21%)	0.293
Wound infection	7 (11%)	3 (11%)	4 (12%)	0.897

Table 3. *All complications among high-risk patients. Values shown as frequencies (percentages). Statistical significance (p value) with Pearson Chi-Square (2-tailed). Clavien-Dindo III-IV = Patients requiring surgical, endoscopic or radiological intervention, or with a life-threatening complication (ICU treatment). Trypsinogen release (two or more positive urine trypsinogen days). POPF Postoperative Pancreatic Fistula. PPH = Postpancreatectomy hemorrhage. DGE = Delayed Gastric Emptying. There were significantly more Clavien-Dindo III-IV complications in the placebo group than in the hydrocortisone group.*

Clavien-Dindo III-IV	POPF	PPH	Other	Overall
Hydrocortisone n=28	1 (4%)	3 (11%)	1 (4%)	5 (18%)
Placebo n=34	4 (12%)	5 (15%)	5 (15%)	14 (41%)
				P<0.05

Table 4. *Etiology of Clavien-Dindo III-IV complications. POPF = Postoperative Pancreatic Fistula. PPH= Postpancreatectomy haemorrhage. Other = Biliary leakage, intra-abdominal abscess, thromboembolic, or sepsis, that required surgical/radiological intervention or intensive care. There were significantly more Clavien-Dindo III-IV complications in the placebo group than in the hydrocortisone group.*

DISCUSSION

Over the years, PD related mortality has decreased in high volume centers (1 - 5%), but morbidity remains high at 18 – 58% (Braga et al., 2011; DeOliveira et al., 2006; Halloran et al., 2002). POPF is often considered as the clinically most challenging complication and earlier it has been shown that postoperative pancreatitis predisposes to POPF (Raty et al., 2006; Uemura et al., 2012). Experimental animal studies of acute pancreatitis have found that acinar cells may behave as inflammatory cells (Sah & Saluja, 2011). This thesis endeavored to shed light on the postoperative inflammatory process of the pancreas. Does the proportion of acinar cells correlate with complications? How fast does the inflammatory process begin in pancreas after surgical trauma? Do inflammatory acinar cells have paracrine interplay with stellate cells? Is it possible to reduce postoperative complications with anti-inflammatory treatment?

The major finding of Study I was that high frequency of acinar cells increased postoperative complications whereas extensive fibrosis protected against complications. Nearly every complication was in the group of patients with over 40% acinar cells. Neither the amount of intra- nor extrapancreatic fat correlated with complications. This was somewhat surprising, as earlier studies (Mathur et al., 2007; Rosso et al., 2009) found a correlation between fat and POPF. Mathur and colleagues stated that POPF patients were more likely than other patients to have a “high pancreatic fat score” (50% vs. 13%). In the study by Rosso et al. (2009) the positive predictive value for POPF was 20.6%. Scrutinizing these results closely, one could state that fatty infiltration correlates with POPF but is not very accurate in predicting whether the patient would suffer from a POPF. For example, if we had applied Rosso’s method (over 10% fat infiltration = risk patient) to our Study I patient material, we would have had 26 out of 40 (= 65 %) risk patients. It must also be kept in mind that the anastomoses in these studies were different and this may have

influenced the results. Tampere binding anastomosis, for example, could intuitively protect against fat-related, labile suturing anastomoses.

The results obtained from Study I enabled the development of a new risk patient identification method. During every PD, a frozen section analysis of CEP is routinely performed. During this analysis the pathologist can also determine whether there are more than 40% acini in the CEP and notify the surgeon in the operating room. This analysis is extremely easy to execute and only takes the pathologist a few minutes. The earlier risk identification method (soft pancreas combined with pancreatic duct < 3mm) proposed by Ansorge and colleagues (Ansorge et al., 2012b) achieved 37% accuracy with POPF grades B & C, but unfortunately no other complications were reported in the study so it is not known whether this method can predict other PD related complications. In this study a duct-to-mucosa anastomosis was also used, thus intuitively making the anastomosis more sensitive to duct-related problems. Braga and colleagues combined ASA score, intraoperative blood loss, pancreatic duct diameter, and pancreatic consistency into a risk score. Patients with high scores had 36% ratio for Clavien-Dindo III-V complication but on the other hand 7% of patients in the low-risk group also had III-V complications. Both studies are interesting, but rather problematic when adapted to clinical use. In Study IV of this thesis, patients with under 40% acini had no POPF and only 3% PPH (B&C). In the high-risk group of patients receiving placebo there were 41% Clavien-Dindo III-IV. In light of these results, 40% acini is an excellent cut-off value to detect high-risk patients and this validates the findings of Study I.

In Study II we found that in acinar cell-rich pancreata, acini NF-κB and MCP-1 activation increased from mild at 15 minutes to high after the first 4 hours, and ductal MCP-1 expression was strong at both time points. Acinar cell NF-κB and MCP-1 activation, and also ductal cell expression of MCP-1, were detected in fibrotic pancreata during the 6-hour monitoring, but expression of these markers remained slight. These results corroborate the results of Study I that patients with acinar cell-rich pancreas develop clinically significant pancreatic inflammation. Although we can say that a vast amplification was seen during the first 4 hours in acinar cell-rich pancreas, this immunohistochemical study is hindered by the fact that in this setting

the tissues suffered from total ischemia. We can thus say that the postoperative pancreatic inflammation probably starts within the first hours of surgical trauma but the exact timetable of the inflammatory cascade remains obscure.

The role of acini in acute pancreatitis is under debate. Earlier it was thought that uncontrolled activation of acinar trypsinogen leads to autodigestion and so to local inflammation (Frossard et al., 2008; Koike et al., 1982; Steer et al., 1984) but in recent years the role of trypsinogen has been attenuated (Sah & Saluja, 2011). It has been even found in animal models that pancreatic inflammation is not related to trypsinogen activation (Dawra et al., 2011; Singh et al., 2009). Acinar cells have been shown to express NF-κB, MCP-1, IL-6, TNF- α, and other inflammatory mediators in animal models thereby demonstrating that acinar cells can act as inflammatory cells. Stellate cells have also been found to stimulate the inflammatory process in pancreas by secreting chemokines such as MCP-1 and IL-8. (Apte et al., 2012; Vonlaufen et al., 2007). In Study III, we wanted to study whether stellate and acinar cells also have inflammatory effects on each other. It was found that the presence of pancreatic stellate cells resulted in deterioration of acinar cells, rendering them insensitive to secretagogue stimulus. Vast inflammation and necrotic changes were identified in co-cultured acinar cells. Acinar cells for their part increased pancreatic stellate cell migration and protein expression. These results promote the speculation of the inflammatory roles of acini and pancreatic stellate cells but also compel us in the future to investigate more minutely the complex humoral interaction between different cells in the pancreas.

In Study IV, high-risk patients receiving hydrocortisone had an 11% incidence of clinically relevant POPF. If we also include the non-high-risk patients from the hydrocortisone group (with 0% POPF rate) we obtain a POPF rate of 3/52 = 6%. This low POPF rate is promising when compared with e.g. Allen and colleagues' results of 10% POPF rate with pasireotide after PD (Allen et al., 2014). There are two main differences between these two trials. Firstly, we targeted the medicine successfully at high-risk patients only, so that only 62% of the PD patients eventually needed to be treated with all doses. Secondly, hydrocortisone did not produce any significant side effects leading to failure of the treatment protocol, whereas a significant proportion

of patients were excluded during the pasireotide trial due to exclusion criteria related to ECG. Unfortunately, our study was underpowered to show statistically significant differences in the POPF rate between hydrocortisone and placebo groups. The "definitive" POPF rates will be registered when more data accumulate in our institution in the future.

A significant reduction in major complications was found in the hydrocortisone group. There was over twice the amount of Clavien-Dindo III-IV complications in the placebo group when compared with the hydrocortisone group. Regarding Clavien-Dindo III-IV complications the number needed to treat (NNT) with hydrocortisone is 4. This means that every fourth patient treated with hydrocortisone avoids a major complication. Furthermore, the recent findings of dexamethasone-related improved survival (Call et al., 2015) can now be re-examined from a new angle: One logical explanation is that patients who received dexamethasone in that study had fewer inflammation related postoperative complications and thus the adjuvant treatment was initiated earlier.

Corticosteroids could predispose to wound infections or stress ulcers, which caused us to be quite cautious with the dosage. A study of the prevention of atrial fibrillation in cardiac surgery by corticosteroids (Halonen et al., 2007), a similar dose, 100mg of hydrocortisone three times a day was used without increased adverse effects (such as increased infectious complications). We considered the usage of this same dose ethically acceptable in this first study of pancreatic surgery. Our hypothesis was that the first dose should be given before the surgical trauma to the high risk patients, so the first dose was given at the induction of anesthesia to all patients. The infectious complications did not increase with hydrocortisone in our study. It would be fascinating to compare the current dose with a larger dose of hydrocortisone in future studies, because in animal studies of corticosteroid treatment for experimental acute pancreatitis, the doses have been significantly larger, e.g. 10mg/kg (Yu et al., 2014).

Hydrocortisone is an old, inexpensive medicine with well-known effects on the human body. The main side-effect of susceptibility to infections did not manifest in this study. With an NNT number of 4, insignificant side-effects and excellent cost-

effectiveness, hydrocortisone appears a promising new treatment for postoperative complications after PD.

SUMMARY AND CONCLUSIONS

During the 20th and 21st centuries, vast advances have been seen in the field of surgery, but pancreatic surgery has struggled with high morbidity. Understanding postoperative pancreatic inflammation and its connection to complications has recently attracted more attention. The results of this thesis strongly support the idea of a significant association of postoperative pancreatic inflammation with complications. During this project, new methods for more precise risk patient identification and for the prevention of complications were developed. The conclusions of this thesis are:

I. 40% acini cut-off is easy to use and detects high-risk patients well.

II. Pancreatic inflammation begins immediately after surgical trauma in high-risk patients.

III. Fibrosis protects against complications. Inflamed acinar and stellate cells stimulate each other leading to fibrosis.

IV. Hydrocortisone treatment is effective in reducing complications among high-risk patients.

V. The following protocol for PD is recommended:

   a. Every PD patient receives 100mg of hydrocortisone at the induction of anesthesia.
   b. High risk patients are identified during the frozen section analysis using the 40% acini cut-off.
   c. Hydrocortisone 100mg x 3 is continued for two days among high-risk patients.

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ORIGINAL COMMUNICATIONS

The Risk for Immediate Postoperative Complications After Pancreaticoduodenectomy Is Increased by High Frequency of Acinar Cells and Decreased by Prevalent Fibrosis of the Cut Edge of Pancreas

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Objectives: Soft pancreas is considered as a factor for pancreatitis after pancreaticoduodenectomy, which in turn constitutes a high risk for local complications. The aim was to analyze the proportion of different cell types in the cut edge of pancreas (CEP) in relation to postoperative pancreatitis and other complications after pancreaticoduodenectomy.

Methods: Data from postoperative follow-up was collected on 40 patients who had undergone pancreaticoduodenectomy. Positive urine trypsinogen-2, an early detector of pancreatitis, was checked on days 1 to 6 after operation. Drain amylase was measured on postoperative day 3. Anastomotic leakages, delayed gastric emptying, and other complications were registered. The areas of different cell types were calculated from the entire hematoxylin-eosin–stained section of CEP.

Results: High frequency of acinar cells in the CEP significantly increased positive urine trypsinogen-2 days, drain amylase values, and delayed gastric emptying. In a subgroup of patients with more than 40% acini in the CEP, there were significantly more postoperative complications. Increased fibrosis correlated with a small number of positive urine trypsinogen-2 days and postoperative complications.

Conclusions: A large number of acinar cells in the CEP increases, whereas extensive fibrosis in the CEP decreases, the risk for postoperative complications after pancreaticoduodenectomy. These results emphasize the importance of acini in the development of postoperative complications.

Key Words: acinar cell, fibrosis, postoperative, complications, pancreaticoduodenectomy, pancreatitis

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Although postoperative mortality (0%–6%) after pancreaticoduodenectomy (Whipple) operation is low, postoperative complications are still extremely common (40%–60%). The most severe complications include pancreaticojejunal anastomotic leakage (2%–14%), wound infection (8%–10%), hemorrhage (5%–7%), and delayed gastric emptying (DGE, 15%–45%).[1–3] The wide range in the complication frequencies is partly explained by different surgical techniques, surgeon- and hospital-related volumes, and partly by discrepancy in the definitions of complications.[1–4] Recently, we have shown that local complications are often preceded by postoperative pancreatitis or by milder pancreatic irritation,[3] which can be diagnosed by measuring urine trypsinogen.[5]

It has been suggested that the consistency of the pancreas may have an effect on the risk for developing postoperative complications. The correlation between pancreaticojejunal anastomotic leakage and the amount of intrapancreatic fat has been investigated in 2 different studies.[6,7] Mathur et al[6] (2007) showed that fatty pancreas increases the risk for postoperative anastomotic leakages. This study reported that fat may be related to the softness of the pancreas. Hard pancreas in turn was associated with fewer pancreaticojejunal anastomotic leakages.[6] Rosso et al[7] (2009) also found a correlation between fat and leakages: if 10% or more of the patient's cut edge of pancreas (CEP) area consisted of fat, the risk for leakages increased. The amount of fat was found to increase with patient's age and body mass index. Palpable hardness of the pancreas, in turn, was related to the extent of fibrosis.[7]

The size of the pancreatic duct and its possible correlation with postoperative morbidity and mortality has also been under investigation. In a recent study from Sweden, small duct size and "normal texture" (not hard) of the remnant of the pancreas were associated with a high risk for pancreaticojejunostomy-associated morbidity.[8]

However, to the best of our knowledge, no study investigating the quantitative histology of the CEP in relation to postoperative pancreatitis (as diagnosed by urine trypsinogen test), and postoperative complications, has been reported; and this was the aim of the current study.

MATERIALS AND METHODS

Patients

Data from 43 consecutive patients who underwent pancreaticoduodenectomy (Whipple) in 2007-2009 in Tampere University Hospital were collected retrospectively from a prospective database. In all of them, a new technique for pancreaticojejunal anastomosis[9] had been used. In this technique, the cut pancreas is tucked inside the jejunal limb and secured in place with a purse string suture and 5 to 7 peripancreatic sutures, without any stitches placed through the pancreas itself.[9] The patients received routine antithrombotic (enoxaparine, 40 mg subcutaneous per day; Klexane, Sanofi-Aventis, France) and antibiotic (ceftriaxone, 1 g intravenous in anesthesia induction; Rocephalin, Roche, Germany) prophylaxis and were monitored and treated according to the standard Whipple protocol of Tampere University Hospital.

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For the present study, we aimed to include all the patients subjected to complete day-by-day postoperative standard monitoring of signs for complications and therefore excluded patients subsequently undergoing a second laparotomy for other than pancreas or pancreaticojejunal anastomosis-related reasons. With these criteria (no postoperative trypsinogen or amylase values available), 3 of the 43 patients, were excluded from the final patient material.

Thus, the final patient material consisted of 40 individuals, 20 men and 20 women; median age, 66 years (range, 21–80 years). Twenty patients had a biodegradable pancreatic stent inserted into the pancreaticojejunal anastomosis.[9] The final histological diagnosis was ductal adenocarcinoma in 24 patients, periampullary cancer in 3 patients, cancer of the biliary duct in 7 patients, chronic pancreatitis in 3 patients, benign mucinous tumor in 1 patient, endocrine adenoma in 1 patient, and metastases from breast lobular carcinoma in 1 patient.

Postoperative Follow-Up

Positive urine trypsinogen was used as an early detector of pancreatitis. The result was considered significant if positive urine trypsinogen was found on at least 2 postoperative days.[5] Trypsinogen was measured daily from the first to the sixth postoperative days.

The amount of amylase was measured on the third postoperative day from a drain left next to the pancreaticojejunal anastomosis in the operation. The drain was then removed, which is why later values could not be obtained in uncomplicated cases. Pancreaticojejunal anastomotic leakages were graded (A, B, or C) using the International Study Group of Pancreatic Fistula definition.[10]

Delayed gastric emptying (DGE) and postpancreatectomy hemorrhage were graded (A, B, or C) according to the International Study Group of Pancreatic Surgery definition.[11,12] The numbers of wound infections and all other complications were recorded.

The primary end points of this study were the following: (1) overall complication rate, (2) pancreaticojejunal anastomotic leakages, (3) DGE, (4) postpancreatectomy hemorrhage, (5) wound infections, (6) pancreatitis (≥2 positive urine trypsinogen days), and (7) overall number of positive trypsinogen days.

Histological Analysis

The whole paraffin control section of each patient's frozen section of CEP was photographed into an electronic form with a microscope (Leica DMD 108) using 10 times objective magnification (size of the magnified area, 1.271 mm^2) and avoiding any overlapping of the tissue and leaving no tissue behind. Each picture (medium, 133 [range, 38–280] per patient) was analyzed by 2 researchers blinded to the patients' clinical data, using a graphics tablet and Adobe Photoshop program. The following cell types were calculated: (1) area of normal acini and injured acini, (2) area of fibrosis, (3) areas of intrapancreatic fat (normal and necrotic), and peripancreatic fat, (4) number of leukocytes (predominantly neutrophil granulocytes and lymphocytes), number graded as 0 to 2 (0, none; 1, few/some; 2, many). Finally, each patient's clinical data were combined with the CEP histological analysis.

The data are shown as median and range. To calculate the statistical significance of the differences between the groups, the Fisher exact test was used for linear nonparametric variables and the χ^2 test for cross-tabulated variables. Differences of $P < 0.05$ were considered statistically significant.

The study was conducted in accordance with the Helsinki Declaration. The study protocol was approved by the medical director of Tampere University Hospital, Finland.

RESULTS

Distributions of different cell types in the 40 patients varied widely. As the connection between certain cell types and the extent of complications began to emerge, 2 further subgroups were formed: the acinar subgroup (more than 40% acini in the CEP; 12/40) and the fibrosis subgroup (more than 60% fibrosis in the CEP; 19/40; Fig. 1, Table 1).

Overall Complication Rate

Nearly every patient (92% [11/12]) in the subgroup with more than 40% acinar cells in CEP had at least one complication (Fig. 2). The difference is considerable compared to patients with less than 40% of acinar cells in CEP (32% [9/28]; $P < 0.001$). In the acinar subgroup, 50% were considered to have significant pancreatitis (≥2 days of positive urine trypsinogen),

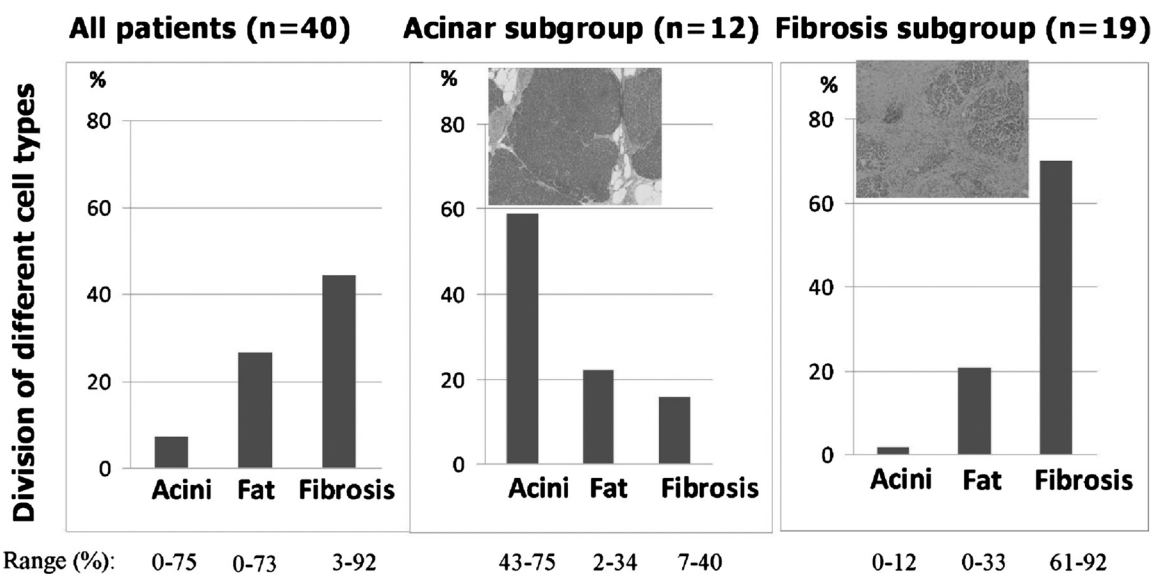

FIGURE 1. Division (median and range) of different cell types in all patients, acinar subgroup (patients with acini covering more than 40% of the CEP area), and fibrosis subgroup (patients with fibrosis covering more than 60% of the CEP area).

TABLE 1. Division (Median and Range) of Different Cell Types in all Patients, Acinar Subgroup (Patients With Acini Covering More Than 40% of the CEP Area) and Fibrosis Subgroup (Patients With Fibrosis Covering More Than 60% of the CEP Area)

Distribution of Cell Types, %; Median (Range)	Total Acini	Injured Acini	Fibrosis	Total Fat	Intrapancreatic Fat	Necrotic Intrapancreatic Fat	Peripancreatic Fat
All patients	6 (0–75)	1 (0–10)	44 (3–92)	24 (0–73)	24 (0–66)	2 (0–28)	1 (0–27)
Acinar subgroup	54 (43–75)	5 (2–10)	16 (7–40)	20 (2–34)	20 (2–27)	2 (0–12)	0 (0–5)
Fibrosis subgroup	1 (0–12)	1 (0–8)	70 (61–92)	21 (0–33)	18 (0–32)	2 (0–13)	0 (0–4)

17% had leakage of the pancreaticojejunal anastomosis, 83% had DGE (grades A–C), 8% had a postpancreatectomy hemorrhage, and 42% had a wound infection (Figs. 2, 3). Of the patients with more than 60% of fibrosis in the CEP, only 4 (21%) of the 19 patients, compared with 16 (76%) of 21 patients with less than 60% of fibrosis in CEP experienced at least one complication ($P < 0.001$). In the fibrosis subgroup, altogether, 11% had pancreatitis, 0% had leakage, 11% had DGE (grades A–C), 5% had hemorrhage, and 0% had wound infection (Figs. 2, 3).

Acinar Subgroup

Patients with acinar cells covering more than 40% of CEP had more DGE (grades A–C) than the patients with acinar cells covering less than 40% of CEP ($P = 0.002$). Only 17% of the patients in the acinar subgroup had normal gastric emptying, whereas the patients with less than 40% of acinar cells in CEP had 89% with normal gastric emptying. There were 42% grade A, 8% grade B, and 33% grade C DGEs in the acinar subgroup (Fig. 3); whereas the patients with less than 40% of acinar cells in CEP had 0% grade A, 7% grade B, and 4% grade C DGEs.

There were only 2 leakages in the pancreaticojejunal anastomoses in this study (overall 5%, NS; one grade A and one grade C), and both of these had acinar cells covering 40% or more of the CEP area (grade A patient, 55%, and grade C patient, 70%; Fig. 3). The patient with the grade A leakage had a biodegradable stent in the pancreaticojejunal anastomosis, whereas the patient with the grade C leakage had no stent in the anastomosis. There was also one postpancreatectomy hemorrhage (1/12 [8%]; grade A) in this subgroup.

The high frequency of acinar cells in CEP correlated significantly with the large number of positive urine trypsinogen days ($r = 0.516$; $P = 0.001$) and high drain amylase ($r = 0.532$; $P = 0.001$). In the acinar subgroup, the number of positive trypsinogen days (2.42 ± 0.71 vs 0.43 ± 0.18; $P = 0.02$) and the amount of drain amylase on postoperative day 3 (149 ± 58 vs 10 ± 2 U/L; $P = 0.036$) were significantly higher than in the rest of the patients with acinar cells covering less than 40% of the CEP area (28/40). Half of the patients in the acinar subgroup, compared to only 11% of the rest of the patients (6/12 [50%] vs 3/28 [11%]; $P = 0.03$), were considered to have pancreatitis, as they had positive urine trypsinogen values on a minimum of 2 postoperative days.

Fibrosis Subgroup

In the fibrosis subgroup, there were no wound infections, and only 3 (16%) of the 19 patients showed signs of any postoperative infection (all three had elevated temperature and CRP; Figs. 2, 3) compared to 8 (38%) of 21 wound infections ($P = 0.002$) and 8 (38%) of 21 signs of other infections in the patients with fibrosis covering less than 60% of the CEP area. There was one postpancreatectomy hemorrhage (1/19 [5%]; grade C) in the subgroup of patients with more than 60% fibrosis in CEP.

Extensive fibrosis correlated with small number of positive urine trypsinogen days ($r = -0.434$; $P = 0.005$), low drain amylase ($r = -0.405$; $P = 0.011$), and fewer wound infections ($r = -0.482$; $P = 0.002$). In the fibrosis subgroup, positive urine trypsinogen days (0.37 ± 0.22 vs 1.62 ± 0.48; $P = 0.024$) and drain amylase levels (12 ± 2 vs 89 ± 35 U/L; $P = 0.047$) were significantly lower than in the patients with less than 60% of fibrosis in CEP. The area of injured acini correlated positively with the extent of fibrosis ($r = 0.545$; $P < 0.001$).

The median value of leukocytes in CEP with all patients was 1.03 (range, 0.14–1.89). The number of leukocytes increased with fibrosis ($r = 0.741$; $P < 0.001$).

The areas of any type of fat, whether intrapancreatic, peripancreatic, normal or necrotic, did not correlate with trypsinogen or amylase. Nor was any major difference found in the distributions of fat between the 2 subgroups (Fig. 1, Table 1). The only connection of fat with other factors was that the total area of fat correlated negatively with fibrosis ($r = -0.348$; $P = 0.028$) and leukocytes ($r = -0.336$; $P = 0.034$). There were 7 patients with a body mass index (BMI) greater than 30 kg/m^2. Their histopathologic diagnoses were 4 pancreatic ductal adenocarcinomas, 2 bile duct adenocarcinomas, and 1 metastasis from lobular carcinoma of the breast. The group of patients with a BMI greater than 30 kg/m^2 had no significant differences in the amount of acinar cells and fibrosis in the CEP, or in the complication rates, compared with other patients.

The patients with a biodegradable pancreatic stent (20/40) did not differ from those without stent regarding different cell types in CEP or in the number of postoperative complications. No preoperative characteristic (such as dilatation of pancreatic duct, BMI, or age) correlated with histological results or postoperative complications in our study.

DISCUSSION

Complications are common after pancreaticoduodenectomy (Whipple). Soft pancreas is considered a risk factor for postoperative pancreatitis, which, in turn, may increase the risk for anastomotic leakage. The aim of our study was to analyze the number of different cell types in the cut edge of pancreas

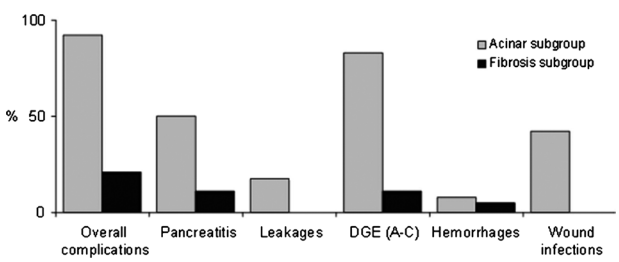

FIGURE 2. Comparison of complication rates between acinar (acini covering more than 40% of the CEP area) and fibrosis (fibrosis covering more than 60% of the CEP area) subgroups.

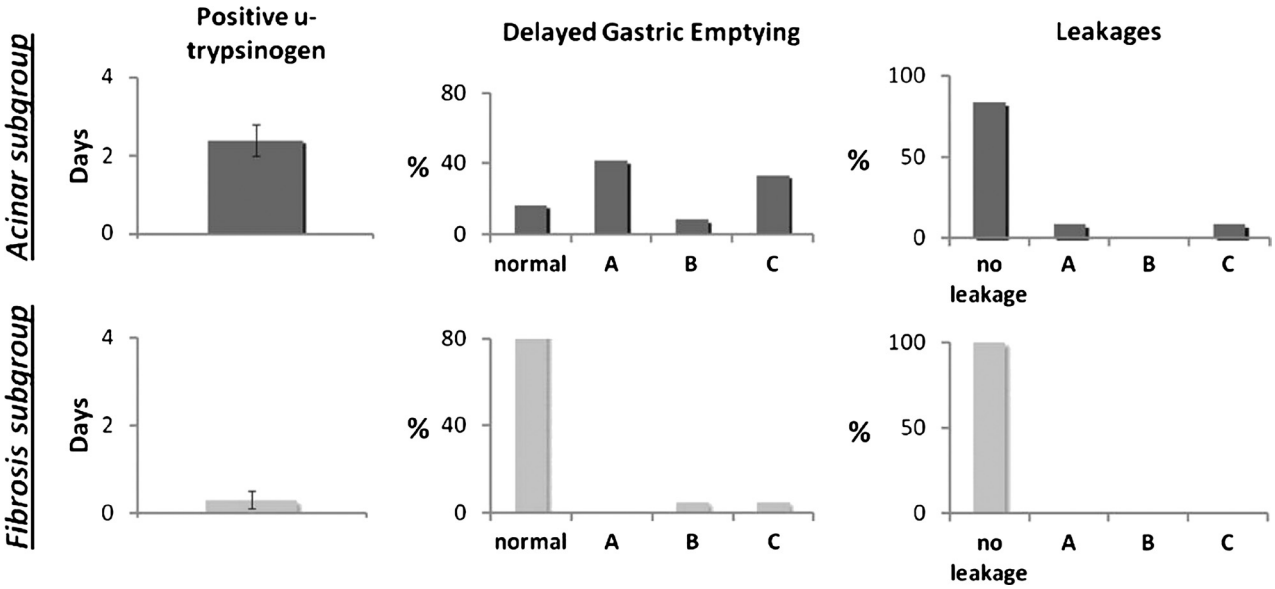

FIGURE 3. Comparison of acinar (acini covering more than 40% of the CEP area) and fibrosis (fibrosis covering more than 60% of the CEP area) subgroups. A large number of acinar cells predispose to complications after Whipple operation: it is associated with greater number of days with positive urine trypsinogen value ($P < 0.05$) and DGE ($P = 0.02$). Extensive fibrosis, in turn, protects against complications as it correlates with smaller number of days with positive urine trypsinogen value ($P < 0.05$).

(CEP) in relation to early postoperative complications after pancreaticoduodenectomy. It was found that the risk for immediate postoperative complications after pancreaticoduodenectomy was increased by high frequency of acinar cells and decreased by extensive fibrosis of the CEP.

The development of postoperative pancreatitis is a complex process, and the risk factors are not fully understood. The prevailing theory is that acute pancreatitis is caused by uncontrollable activation of trypsin leading to excitation of other digestive enzymes and, eventually, autodigestion and inflammation.[13] The most recent studies show that in addition to tissue ischemia, damage to the pancreas itself, such as resection of the pancreas and suturing of pancreaticojejunal anastomosis, can initiate a widespread inflammation. Cautious handling of the pancreas during surgery has attracted more attention in recent years.[14,15] Postoperative pancreatitis may predispose to leakages of the pancreaticojejunal anastomosis: 60% of the patients with computed tomography–diagnosed postoperative pancreatitis have been reported to develop an International Study Group of Pancreatic Fistula–defined leakage.[3]

The widely used term "soft pancreas" has not been characterized in detail. It usually means less fibrosis, but whether the pancreas consists of acinar cells or fat tissue cannot be determined without histological analysis. In earlier studies,[6–8] no inclusive quantitative histological analysis of the CEP was performed. Mathur et al[6] (2007) showed that fatty pancreas increases the risk for postoperative anastomotic leakages and claimed that fat may be related to the softness of the pancreas. Hard pancreas was, in turn, related to fewer pancreaticojejunal anastomotic leakages. Rosso et al[7] (2009) also found a correlation between fat and leakages: if 10% or more of the patient's CEP area consisted of fat, the risk for leakages increased.

Ansorge et al[8] (2010), in turn, highlighted the role of pancreatic duct diameter in postoperative morbidity. However, in their study, the technique for pancreaticojejunal anastomosis was a duct-to-mucosa end-to-side pancreaticojejunostomy.[8] A new pancreaticojejunal anastomosis technique[9] was used in our study, in which the pancreatic remnant is tucked into the jejunum with no sutures in the pancreatic duct, thus intuitively making it less vulnerable to complications associated with duct size. This hypothesis is substantiated by the finding that the number of postoperative complications in patients with a biodegradable pancreatic stent (20/40) did not differ from nonstented patients.

In a recent study of Balentine et al[16] (2011), it was stated that neither the amount of intra-abdominal fat (defined by preoperative computed tomography) nor BMI is associated with complications after pancreatic resection.

To the best of our knowledge, this study is the first to show that a large number of acinar cells may predispose the pancreas to develop pancreatitis postoperatively, which can be seen as a large number of positive urine trypsinogen days (has been shown to be an applicable marker for postoperative pancreatitis[5]). Postoperative pancreatitis itself is considered to be a result of perioperative tissue damage and/or ischemia. We have hypothesized that pancreatitis predisposes to other complications, such as International Study Group of Pancreatic Surgery grading–based DGE,[3] the extent of which was significantly greater in patients with acini covering more than 40% of CEP than in the other patients (83% vs 11%; $P = 0.002$), and possibly to leakages, the number of which was too small in our study to show statistical significance (2; both patients having more than 40% of CEP acinar cells).

Pancreatic fibrosis, in turn, protected patients against postoperative pancreatitis seen in fewer positive urine trypsinogen days and low drain amylase. Operation time increased with fibrosis, but the fibrosis subgroup still had fewer wound infections. This may be explained by the protective feature of fibrosis with the anastomosis: the fewer complications the patient has in the gastrointestinal tract, the less burden is placed on the immune system and the less likely the wound is to become infected. This hypothesis is supported by the fact that surgical and nonsurgical traumas have been shown to suppress cell-mediated immunity.[17] Overall, patients with fibrotic pancreas were most likely to undergo surgery without complications.

In this study, nearly every patient with a complication belonged in the subgroup of patients with more than 40% acini

in CEP. The considerable numbers of DGE, pancreatitis, and wound infections in this subgroup stress the significant role of acinar cells in the etiology of postoperative complications after pancreaticoduodenectomy.

Leukocytes and injured acinar cells increased in proportion to the extent of fibrosis, a phenomenon probably related to the ongoing chronic inflammation process. There were no significant differences in the number of leukocytes or injured acinar cells between patients with chronic pancreatitis (n = 3) or patients with tumor-related chronic pancreatitis (n = 18).

Surprisingly, the areas of any type of fat, whether intrapancreatic, peripancreatic, normal, or necrotic, did not correlate with postoperative pancreatitis as detected by positive urine trypsinogen or high drain amylase. Thus, according to our study, it seems that fatty pancreas does not play a significant role in the initiation of postoperative inflammation cascade leading to postoperative pancreatitis, whereas the number of acinar cells is indeed significant.

Both earlier studies of Mathur et al (2007) and Rosso et al (2009) concentrated on finding a connection between fat and pancreatic leakage. In the study of Mathur et al, patients with pancreatic leakage were more probable to have "high pancreatic fat score" (50% vs 13%) than other patients. In the study of Rosso et al (2009), the specificity of fatty infiltration in predicting pancreatic leakage was only 53.5% (positive predicting value, 20.6%). Taken together, fatty infiltration was not very accurate when predicting whether the patient would have a pancreatic leakage. For example, if we would have applied the method of Rosso et al to our study (more than 10% fat infiltration = risk patient), we would have had 26 of 40 (65%) risk patients.

There was no monitoring of postoperative pancreatitis in either one of the earlier studies,[6,7] so no correlation between fat and postoperative pancreatitis was shown. We believe that one of the most important factors in the development of pancreatic leakage (and other local complications) is postoperative pancreatitis.[6]

According to our data, it seems that intrapancreatic fat is distributed rather equally both in acinar (median, 20%) and fibrosis (median, 18%) subgroups. However, if our patient material would have been greater—and more leakages occurred—a slight connection between fat and leakages might have been seen because fat correlated negatively with fibrosis. It is understandable that if a patient has, for example, 80% to 100% fibrosis in the CEP, the percentage of fat stays low; and it seems that less fat means fewer complications.

It may be that fat itself is also associated with leakages because of surgical, mechanical reasons. Intuitively, fat might be a risk factor for a nonsolid leaking anastomosis where sutures cannot be tightened well enough. It might be that the anastomosis used in our study (no sutures through the pancreas) would protect from this fat-related leakage. There were only 2 pancreatic leakages in our study, and thus the possible role of pancreatic fat as a risk factor for postoperative pancreatic leakage cannot be excluded based on our study material.

We conclude that high frequency of acinar cells in CEP increases the risk for immediate postoperative complications, whereas extensive fibrosis in CEP protects against immediate postoperative complications after pancreaticoduodenectomy. Because manual evaluation of the pancreas or other preoperative or perioperative factors cannot reliably predict the consistency of the pancreas, the risk for postoperative complications can only be determined with histological analysis. Special emphasis can be given to the fact that nearly every patient with a complication belonged in the subgroup of patients with more than 40% acini in the CEP. We hypothesize that a pancreas rich in acinar cells may be more prone to the initiation postoperative inflammation cascade owing to any kind of perioperative tissue injury, and the inflammation leads to pancreatitis and subsequently the development of DGE and leakages. As the CEP frozen section is in most cases already analyzed already during the surgery to ensure a cancer cell–free cut margin, the number of acinar cells (more or less than 40%) could also be estimated perioperatively by the pathologist, giving the surgeon a better idea of those patients at increased risk of developing postoperative complications. The analysis takes the pathologist only few minutes, and thus this method can be easily adapted to clinical use. The identification of high-risk patients may also be important in future trials attempting to prevent complications.

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Research Article

Difference in Early Activation of NF-κB and MCP-1 in Acinar-Cell-Rich versus Fibrotic Human Pancreas Exposed to Surgical Trauma and Hypoxia

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Objectives. Previously we have shown that a pancreas with over 40% acinar cells is exposed to postoperative pancreatitis and other complications after pancreaticoduodenectomy (PD). Our aim was to analyze the expression of NF-κB and MCP-1 in the cut edge of human pancreas after PD in both acinar-cell-rich and fibrotic pancreata. *Methods.* Several pancreatic samples from six patients, three with acinar-cell-rich and three with fibrotic pancreata, were exposed to surgical trauma in PD, and thereafter to hypoxemia for 15 minutes, 2–2.5 hours, 4 hours, or 6 hours, to mimic postoperative conditions of the pancreatic remnant in a patient. Immunohistochemical analysis of inflammation markers (NF-κB, MCP-1) was performed. *Results.* In the acinar-cell-rich pancreata, intra-acinar NF-κB and MCP-1 expression increased from mild at 15 minutes to high during the first 4 hours, whereas in ductal cells MCP-1 staining was highly intense at both time points. Acinar cell NF-κB and MCP-1 expression and ductal cell MCP-1 expression were also observed in the fibrotic pancreata, but the activation remained low throughout the 6 hours. *Conclusions.* In acinar-cell-rich pancreas, an extensive inflammatory cascade begins almost immediately after surgical trauma. Fibrosis may limit the progression of inflammatory process in pancreas.

1. Introduction

Pancreaticoduodenectomy (PD) has become a standard operation with low mortality. However, at 42%–60% perioperative morbidity remains substantial. The most common complications include delayed gastric emptying, postoperative pancreatic fistula, wound infections, and postpancreatectomy hemorrhage [1–4].

A pancreas at high risk of severe complications can be predicted perioperatively. Acinar-cell-rich pancreas (defined as showing over 40% of acinar cells in the pancreatic transection line) is accompanied by an increased risk of postoperative pancreatitis or milder pancreatic irritation [3, 5, 6]. In our previous study [6], 92% of the patients with acinar-cell-rich pancreas developed postoperative complications. The complication rate decreased to 21% when there was more than 60% of fibrosis in the pancreatic transection line. We hypothesized that intraoperative pancreatic injury may immediately activate the inflammatory cascade in the remaining pancreas and that this activation may differ in acinar-cell-rich and fibrotic pancreas.

According to the prevailing theory, acute pancreatitis is set off by uncontrollable activation of trypsin leading to excitation of other digestive enzymes and, eventually, autodigestion and inflammation [7]. The inflammatory cascade, especially the signaling molecules involved, has been under intense scrutiny in recent years. Several signaling molecules have been shown to play important roles in the progression of the inflammation process in the pancreas. They include among others nuclear factor κB (NF-κB); monocyte chemoattractant protein 1 (MCP-1); interleukins IL-1, IL-2, and IL-6; platelet-activating factor (PAF); substance P; and tumor necrosis factor α (TNF-α) [8–10]. Both NF-κB and MCP-1 are shown to upregulate early in acute pancreatitis [9–13].

In animal models, NF-κB activates within 30 minutes and MCP-1 within 60 minutes in acinar cells after the induction of inflammation [11, 14] and this activation leads to exacerbation of acute pancreatitis [9, 15, 16].

The inflammatory cascade in human pancreas after surgical trauma has not been previously investigated. The aim of this study was to investigate postoperative inflammation in acinar-cell-rich and fibrotic human pancreas exposed to surgical trauma and hypoxia.

2. Materials and Methods

From among the patients undergoing PD in Tampere University Hospital, six individuals were chosen for the study based on the histopathology of the cut edge of the pancreas: three with acinar-cell-rich pancreas (>40% acini on the cut edge) and three with fibrotic pancreas (>60% fibrosis on the cut edge). In the acinar-cell-rich group, the patients and the final histopathological diagnoses were as follows: 50-year-old female with neuroendocrine carcinoma of the head of the pancreas, 55- and 57-year-old males with adenocarcinomas of the head of the pancreas. In the fibrotic group, the diagnoses were as follows: 78-year-old male with serous cystadenoma of the head of the pancreas, 60- and 74-year-old males with adenocarcinomas of the head of the pancreas.

During the operation, at the time of the transection, a tissue sample (size 2 mm thick, 10 mm in diameter) was harvested from the cut edge. The specimen was cut into five pieces which were immersed in physiologic NaCl solution to prevent them from drying. The tissue was thus exposed to surgical trauma followed by ischemia ex vivo, in an endeavor to mimic the conditions at the cut edge of the pancreatic remnant in the patient. At 15 minutes, 2–2.5 hours, 4 hours, or 6 hours, the NaCl solution was replaced by 4% paraformaldehyde and the samples were allowed to fix overnight. The samples were then dehydrated and embedded in paraffin. Sections (5 μm thick) were cut for immunohistochemical analysis.

Immunohistochemical analysis was performed using the following antibodies at the dilutions indicated: anti-NF-κB p50 (1 : 200; AbD Serotec, Oxford, UK) and anti-MCP-1 (1 : 200; AbD Serotec). Controls included omission of the primary antibodies and the use of nonimmunized mouse and rabbit IgG. The staining was performed with a broad-spectrum Histostain-Plus kit (Invitrogen, Camarillo, CA, USA) as previously described [17]. The sections were lightly counterstained with hematoxylin.

The slides were then subjected to microscopic analysis (Nikon Microphot-FXA). Quantitative analysis of NF-κB 15-minute and 4-hour samples was performed by two independent researchers (ML, MB). The percentage of activated acinar cells (stained nucleus) out of the total number of acini in each sample was determined from representative areas using a magnification of 250. The means (±SEM) of the three acinar-cell-rich and the three fibrotic samples were then calculated. Differences in the intensity of MCP-1 staining were determined semiquantitatively and expressed as low, moderate, or high.

The study protocol was approved by the ethics committee of Tampere University Hospital.

3. Results

NF-κB staining was seen in the nuclei of acinar cells, and MCP-1 activation was found in the cytoplasms of acini and ductal cells. Qualitative analysis revealed the progression of NF-κB activation in acinar-cell-rich pancreata during the 6-hour period (Figure 1) such that the highest NF-κB expression was at 4 hours (Figures 1 and 2). In the fibrotic pancreata, acinar cell activation of NF-κB was also detected, but the tissue expression of NF-κB did not increase over time (Figure 2). NF-κB-positive fibroblasts were scarce, with the fibroblast nuclei being predominantly unstained. In all tissue sections the intensity of NF-κB staining appeared even and no gradient from outside to inside was detectable.

Quantitative analysis for the acinar-cell-rich pancreata showed that acinar cell NF-κB activation increased from mild at 15 minutes (35% ± 7%, mean ± SEM) to high (74% ± 4%) during the first 4 hours (Figure 3). NF-κB activation was 30% (±6%) at 15 minutes and 35% (±4%) at 4 hours in the fibrotic pancreata (Figure 3).

Acinar cell expression of MCP-1 increased from low at 15 minutes to moderate during the first 4 hours in the acinar-cell-rich pancreata, whereas in ductal cells MCP-1 staining was highly intense at both time points (Figure 4). Acini and ductal cells did not express MCP-1 at 15 minutes in the fibrotic pancreata and only minor staining was observed at 4 hours (Figure 4).

4. Discussion

An acinar-cell-rich pancreas is at higher risk of post-PD complications than is a fibrotic pancreas. Intraoperative pancreatic injury may activate the inflammatory cascade differently in acinar-cell-rich pancreas and fibrotic pancreas. The role of inflammation markers in human pancreas following surgical trauma has not been previously studied and was the focus of this study. It was concluded that the intra-acinar cell inflammatory cascade may lead to pancreatitis almost immediately after induction of injury by surgical trauma and ischemia in acinar-cell-rich human pancreas, whereas fibrosis may limit the progression of inflammation in pancreas.

Several signaling molecules (such as IL-1, IL-2, IL-6, PAF, substance P, TNF-α, MCP-1, and NF-κB) have been shown to play important roles in the progression of experimental acute pancreatitis [8–10]. Studies have shown that both NF-κB and MCP-1 upregulate early in acute pancreatitis and may exacerbate its severity [9–16, 18, 19], which is why these markers were chosen for our study.

NF-κB has been shown to regulate the transcription of several genes involved in immunity and inflammation [9]. Numerous studies have demonstrated an early and significant activation of pancreatic NF-κB when acute experimental pancreatitis is induced in rats or mice using agents such as cerulein, taurocholate, and bile-pancreatic duct ligation [9, 11–13]. Acinar cells are considered to play a key role especially

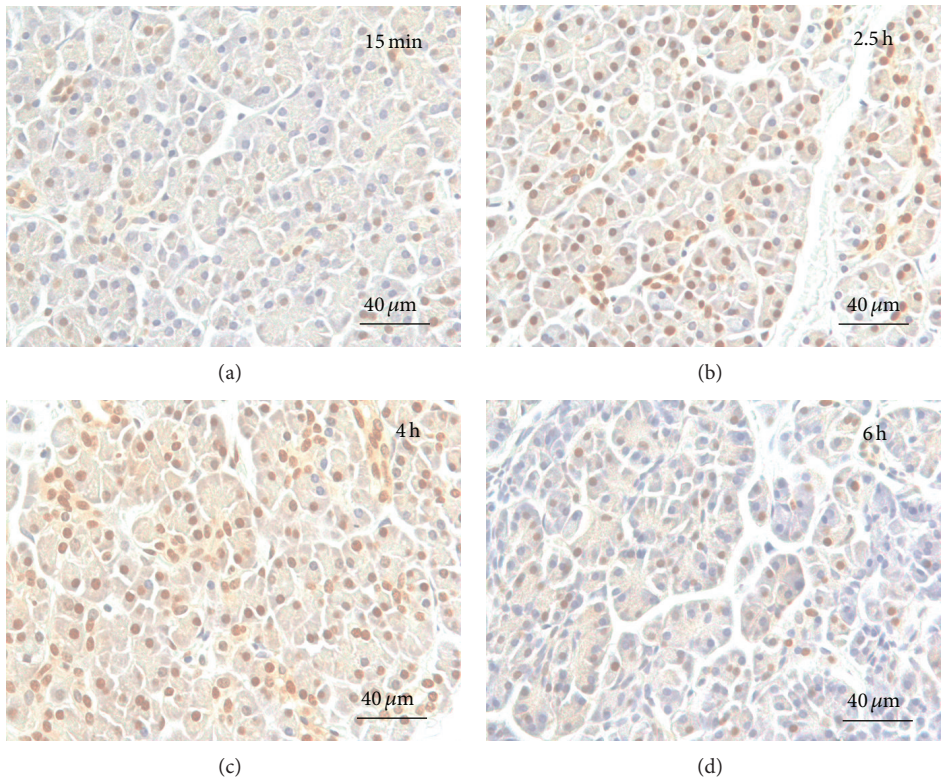

Figure 1: NF-κB activation in acinar-cell-rich pancreas. Immediately after sampling, parallel portions of each tissue specimen were immersed in saline and kept at room temperature for 15 minutes (a), 2.5 hours (b), 4 hours (c), or 6 hours (d), after which they were fixed and processed for immunohistochemical analysis. The slides were counterstained with hematoxylin. Slight staining of acinar cell nuclei can be seen at 15 minutes (a) and significant amplification is observed at 2.5 hours (b). Almost every acinar cell nucleus is stained at 4 hours (c) and activation decreases at 6 hours (d).

in early (within 30 minutes) pancreatic NF-κB activation in experimental acute pancreatitis [11]. Activation of NF-κB is followed by an increased number of proinflammatory cytokines and influx of inflammatory cells into the pancreas, leading to exacerbation of pancreatitis [9]. The importance of NF-κB in the inflammatory process is substantiated by the fact that inhibiting its activation using antioxidants (e.g., *N*-acetylcysteine) or anti-inflammatory agents (e.g., peroxisome proliferator-activated receptor γ, PPARγ) has been shown to reduce the severity of pancreatitis in animal models [9, 15, 16, 18].

MCP-1 has been associated with several inflammatory diseases, including pancreatitis. Monocytes, T-lymphocytes, acinar cells, and stellate cells have all been shown to express MCP-1, and MCP-1 has been seen to upregulate in acute and chronic pancreatitis [10]. Acini express MCP-1 as early as 60 minutes after induction of acute experimental pancreatitis [14]. The importance of MCP-1 in the pathogenesis of pancreatic inflammation was substantiated in a study by Zhao et al. [19], where pancreatic inflammation and fibrosis was significantly reduced in rats with experimental chronic pancreatitis by giving them antichemokine gene therapy. In a study by Ishibashi et al. [16] the severity of acute pancreatitis was attenuated by blocking MCP-1 activity in rat models.

Knowledge about the role of acinar cells in the pathogenesis of acute pancreatitis has progressed over recent years. It has been suggested that acinar cells can act in the same manner as inflammatory cells. The latest studies show that the acini may be promoters of the inflammatory cascade. They secrete cytokines, chemokines, and adhesion molecules, resulting in activation and recruitment of circulating leukocytes [20, 21].

The consistency of the pancreas has been shown to affect the risk of post-PD complications. A soft pancreas and a small pancreatic duct diameter are known to increase morbidity [22, 23]. Postoperative pancreatitis, or subclinical pancreatic irritation, has recently been noted as a precursor of postoperative complications such as delayed gastric emptying and postoperative pancreatic fistula [3, 6]. In animal models, any injury to pancreatic parenchyma with scalpel or sutures has been shown to initiate an inflammatory process in the parenchyma that spreads throughout the pancreas [24, 25]. In our previous study, patients with acinar-cell-rich pancreas developed massive postoperative inflammation that exposed them to clinically significant complications [6]. So as far as we know, molecular-level events related to post-PD pancreatitis in the remnant of pancreas after PD have not been studied before.

In the postoperative state the pancreatic remnant suffers from hypoxia to some extent but not from *total* ischemia as in our ex vivo study. We recognize that this study therefore does

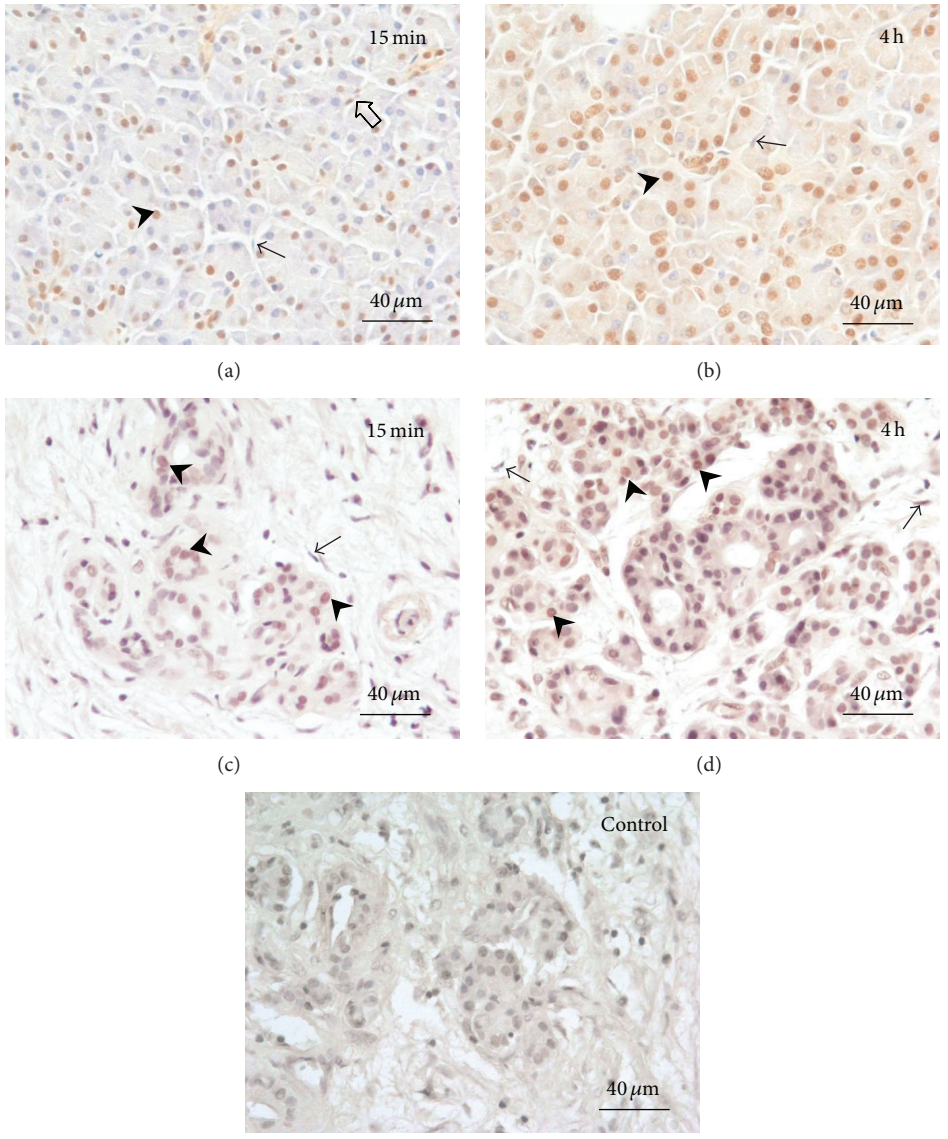

Figure 2: NF-κB expression in acinar-cell-rich ((a), (b)) and fibrotic ((c), (d)) pancreata. (a) and (c) are 15-minute sample and (b) and (d) represent 4-hour time points. Arrowheads indicate representative NF-κB-expressing nuclei in acinar cells. NF-κB-positive fibroblasts were rare (open arrow in (a)), the fibroblast nuclei being predominantly negative (arrows). The increase in NF-κB activation is more prominent in acinar-cell-rich pancreata ((a) and (b)) than in fibrotic pancreata ((c) and (d)). Control stainings were negative (e).

not perfectly mimic postoperative conditions in the patient. Hypoxia has been shown to be an independent inducer of acute pancreatitis [26] and presumably acts as an aggravating factor for surgically induced pancreatic inflammation. The intensity of acinar cell activation may therefore be magnified in this setting. Hypoxia-induced acinar cell necrosis may also explain the decreased activation of NF-κB at 6 h samples (Figure 1(d)), which is why we decided to use 4 h samples in our quantitative analyses.

In this study we found that in the acinar-cell-rich pancreata, acinar cell NF-κB and MCP-1 activation increased from mild at 15 minutes to high after the first 4 hours, and ductal MCP-1 expression was highly intense at both time points. In the fibrotic pancreata, acinar cell expression of NF-κB and MCP-1 and also ductal cell expression of MCP-1 were detected at the 6-hour monitoring, but the tissue expression of these markers remained lower. Our findings of the limiting role of fibrosis in pancreatic inflammation are also in line with a recent study of Acharya and colleagues [27], where fibrosis was seen to reduce acinar cell necrosis among patients with acute-on-chronic pancreatitis.

Whether and how fast the inflammation exacerbates into clinically relevant pancreatic inflammation or even pancreatitis are not known. However, in our previous study we showed that it is patients with acinar-cell-rich pancreas who develop clinically relevant pancreatic inflammation [6].

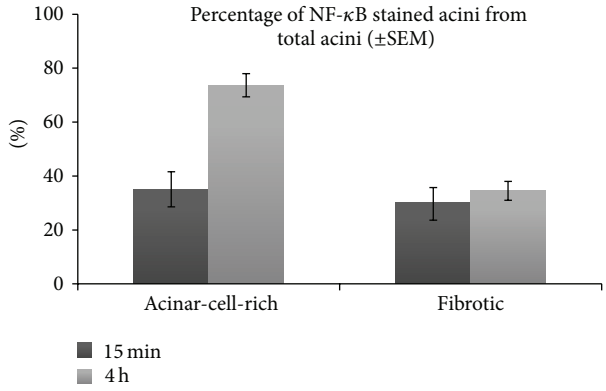

Figure 3: Comparison of NF-κB activation in acinar-cell-rich and fibrotic pancreata. The means (±SEM) of the three acinar-cell-rich and the three fibrotic samples were calculated and then compared at 15 minutes and 4 hours. In acinar-cell-rich pancreata, a significant increase in NF-κB expression occurs between 15 minutes (35%) and 4 hours (74%). In fibrotic pancreata, the change between 15 minutes (30%) and 4 hours (35%) is minor.

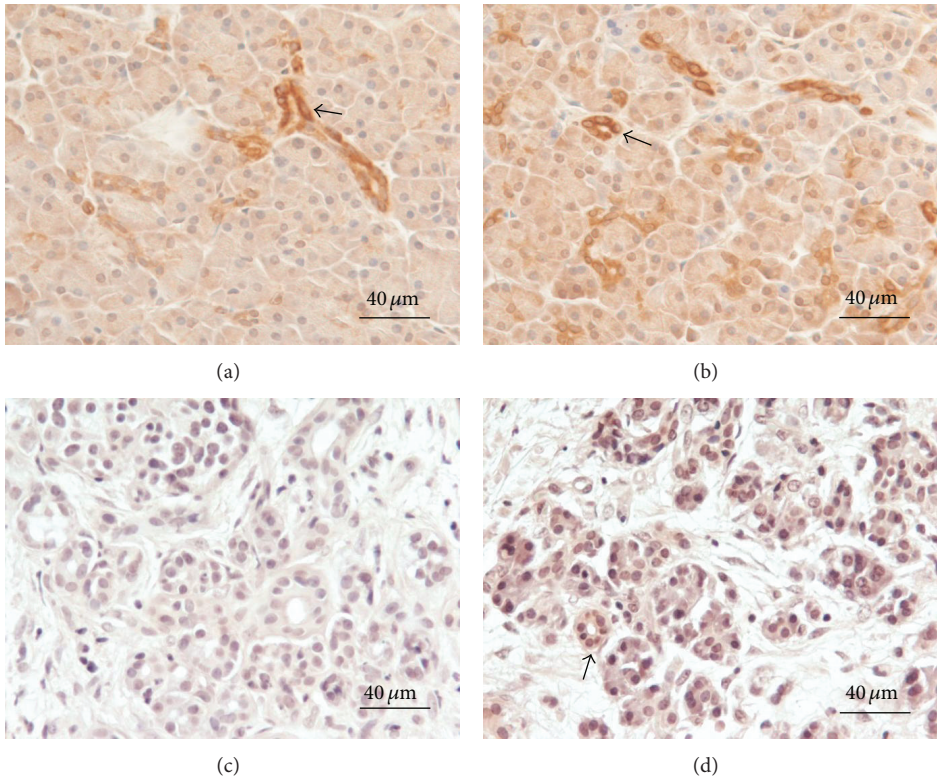

Figure 4: MCP-1 expression in acinar-cell-rich ((a), (b)) and fibrotic ((c), (d)) pancreata. Immediately after sampling, parallel portions of each tissue specimen were immersed in saline and kept at room temperature for 15 minutes ((a), (c)) or 4 hours ((b), (d)), after which they were fixed and processed for immunohistochemical analysis. MCP-1 staining was equally intense in the ductal cells of acinar-cell-rich pancreata after 15 minutes and 4 hours ((a), (b), arrows), whereas intra-acinar MCP-1 expression was observed to slightly increase with time. In fibrotic pancreata, MCP-1 in ductal and acinar cells remained undetectable at 15 minutes (c). At 4 hours, weak staining can be detected in ductal cells ((d), arrow). The slides were counterstained with hematoxylin.

5. Conclusions

We hypothesize that a patient undergoing PD who has a large amount of acinar cells in the transection line (i.e., in the pancreatic remnant) is at high risk of developing a massive postoperative inflammatory cascade in the pancreas. The first 4 hours after the induction of surgical trauma may play an important role in the patient's postoperative prognosis. As postoperative pancreatitis often precedes other complications after PD, future therapeutic strategies targeting

postoperative complications could consider anti-inflammatory treatments and could also focus them on perioperative—not just postoperative—treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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